
**FACULTAD DE FARMACIA**

DEPARTAMENTO DE QUÍMICA ANALÍTICA, NUTRICIÓN Y BROMATOLOGÍA

# Mushrooms as a source of high-value bioactive molecules: conversion of ergosterol into vitamin $D_2$, extraction, stabilisation, and a study using bakery products

## DOCTORAL THESIS

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Ana María González Paramás, Profesora Titular de Nutrición y Bromatología de la Universidad de Salamanca (España) y Lillian Bouçada de Barros, Investigadora del Centro de Investigação de Montanha del Instituto Politécnico de Bragança (Portugal), directoras del trabajo "Mushrooms as a source of high-value bioactive molecules: conversion of ergosterol into vitamin $D_2$, extraction, stabilisation, and a study using bakery products", realizado por Rossana Veviana Centeio Cardoso para optar al Grado de Doctor con Mención Doctorado Internacional, AUTORIZAN la presentación del mismo al considerar que se han alcanzado los objetivos inicialmente previstos.

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**ABSTRACT**

Many factors put Europe at risk of vitamin D deficiency. This deficiency causes the body to absorb less calcium and phosphate, and if there is not enough calcium and phosphate to keep the body healthy, it can cause several serious health disorders. Natural (unenriched) foods by themselves rarely provide sufficient vitamin D to prevent deficiency. Vitamin D-enriched foods and vitamin D supplements can help prevent deficiency when sun exposure is inadequate, but most of them are from synthetic sources. So, vitamin D from a natural source would be an alternative to overcome the problems of synthetic additives and help provide health benefits. The food industry is constantly searching for ingredients from natural sources due to their multifunctional properties, competitive efficacy, and lower toxicity effects, which can be used as a food fortifier. Mushrooms have been an essential part of our diet for many years due to their rich nutritional content and abundance in biomolecules, specifically ergosterol, a provitamin $D_2$. However, reaching a more significant number of individuals is necessary. Consequently, it has been necessary to choose a food matrix consumed by a large proportion of the world's population, such as cereals, mainly wheat, whose flour is used to develop many food products worldwide. Further, to obtain vitamin D from a natural source (mushrooms - vitamin $D_2$) is necessary to develop several methodologies, from producing mushrooms to obtaining vitamin $D_2$. After obtaining vitamin $D_2$, its stabilisation is also essential, so encapsulation methodologies are a choice to ensure the properties of vitamin D2 when incorporated into the final product. Vitamin D is essential for many functions in the body, but mainly for the bones, so validating the concept that vitamin $D_2$ promotes calcium absorption is needed. The following description shows that it is possible to obtain and stabilise natural vitamin $D_2$ from mushrooms and incorporate it into a flour matrix using innovative extraction, irradiation, and encapsulation technologies.

As a result, in order to maximize the bioactive compounds, present in mushrooms, a study has been done to supplement mushroom substrates with calcium silicate. This supplementation has been linked to increased resistance against insect pests. However, few studies show the impact of this supplementation on the different agronomic parameters of oyster mushrooms (*Pleurotus ostreatus* var. florida) or even their chemical and bioactive composition (antioxidant, antibacterial and antifungal activities).

Considering the increased production and consumption of mushrooms, the high perishability represents a significant commercial drawback. In addition to increasing the product's shelf life, preservation processes should be harmless to consumers. Therefore, the effects of gamma radiation and electron beam on the chemical and nutritional composition of fresh and dried samples of *Agaricus bisporus* Portobello was evaluated. A study illustrating the possibility of adding value to bioresidues left over from the mushroom cultivation industry was done. In essence, unused mushroom parts were subjected to extraction and UV-C irradiation to increase the vitamin $D_2$ content and validate its extraction. The concentration of vitamin $D_2$ in three different mushroom species (*Agaricus bisporus*, *A. bisporus* Portobello, and *Pleurotus ostreatus*) was determined by high-performance liquid chromatography (HPLC) using an ultraviolet (UV) detector. These results are good indicators of the viability of industrial mushroom surpluses as sustainable food sources of vitamin $D_2$, and also help to reinforce the circularity principles associated with the mushroom production chain.

Studies were continued with the best irradiation conditions and the mushroom that presented the best vitamin $D_2$ content (*A. bisporus* Portobello). In this sense, the Portobello mushroom was subjected to extraction (use of more environmentally friendly methodologies (UAE) and solvents (ethanol)) to obtain the extract rich in ergosterol, which was then irradiated to convert the ergosterol into vitamin $D_2$. New fortified foods open the opportunity to design new tailor-made products that not only feed and nourish but also provide bioactive compounds necessary for the proper functioning of the body, avoiding dependence on the intake of drugs or supplements with synthetic contents. In this context, vitamin $D_2$ is a suitable candidate to be ingested in a simple way in the daily diet, namely in bread. One of the strategies to ensure the protection of bioactivity during the production of fortified bread involves its encapsulation to be incorporated in the form of particles, facilitating a homogeneous distribution in the bread dough. In this proof of concept, stabilization studies were performed where particles containing vitamin $D_2$ contents were prepared using polymers, through solid dispersion technology. The qualitative and quantitative characterization of vitamin $D_2$ encapsulation allowed corroborating the effectiveness of the solid dispersion process for all formulations. Moreover, the methodology allowed the development of a final formulation with the Vit$D_2$-enriched extract using k-carrageenan polymer (KC-Bioactive) and the incorporation in the bread of specific amounts of vitamin $D_2$,

which were subsequently identified and quantified, validating the proof of concept of the developed fortified bread.

Furthermore, the promising extract (UAE) was subjected to proof-of-concept evaluation of increased calcium absorption by osteoblast bone cell line (h-FOB 1.19) by in vitro assay. The results indicated that the VitD$_2$-enriched extract increased calcium absorption by h-FOB 1.19 cell lines.

Finally, seven types of wheat and rye flour were studied for their physical and chemical properties as well as the presence of mycotoxins and microorganisms. In this sense, increasing the confidence of the European consumer, and the Portuguese in particular, in the products they buy and consume daily. To our knowledge, this is the first characterization of wheat and rye flour for Portuguese consumers. Studies on flour made from cereal by-products characterize them as sustainable ingredients for developing new bakery products and functional foods and contribute to better use of biological resources, efficiency, and circularity. Cereal by-products (wheat germ, corn bran-germ blend, rye bran, and wheat bran) from the flour milling industry were characterised for their nutritional value and chemical composition, as well as antioxidant and antibacterial activities. All the flour mentioned above can serve as a food matrix fortified with vitamin D$_2$. The final products (flour and bread) were developed using one of the flours mentioned above (wheat flour type 65). The sensory evaluation of the samples was examined by comparing the performance of flour and bread samples fortified with KC-Bioactive and free form (VitD$_2$-enriched extract) with the unfortified (control) samples.

## RESUMEN

Son numerosos los factores que contribuyen a la existencia de riesgo de déficit de vitamina D en la población europea. Este déficit favorece una menor absorción de calcio y fosfato, lo que puede causar varios trastornos de salud graves. Los alimentos naturales (no enriquecidos), por sí mismos, rara vez proporcionan suficiente vitamina D para prevenir el déficit, por lo que alimentos enriquecidos con vitamina D y/o suplementos de vitamina D pueden ayudar a prevenir la carencia cuando la exposición al sol es inadecuada. Sin embargo, la mayoría de los suplementos disponibles utilizan vitamina D que proviene de fuentes sintéticas, por lo que disponer de vitamina D de una fuente natural sería una alternativa para superar los problemas que conlleva el uso de aditivos sintéticos y contribuiría a proporcionar beneficios para la salud. En este sentido, la industria alimentaria está constantemente buscando ingredientes de fuentes naturales debido a sus propiedades multifuncionales, eficacia competitiva y efectos de menor toxicidad. Los hongos han sido una parte esencial de nuestra dieta durante muchos años debido a su rico contenido nutricional y abundancia en biomoléculas, específicamente ergosterol, una provitamina $D_2$. Sin embargo, es necesario llegar a un número más significativo de individuos, por lo que, a la hora de pensar en un grupo de alimentos candidatos a la fortificación, es necesario elegir una matriz alimentaria como los cereales, principalmente el trigo, consumida por un gran porcentaje de la población mundial, y cuya harina se utiliza para desarrollar muchos productos alimenticios en todo el mundo. A lo largo de esta tesis se han propuesto alternativas para solventar alguna de las cuestiones arriba planteadas. Así, con el objetivo de obtener vitamina D de una fuente natural (hongos - vitamina $D_2$) ha sido necesario desarrollar varias metodologías, desde la producción de hongos hasta la obtención de vitamina $D_2$. Después de obtener la vitamina $D_2$ su estabilización también es esencial, por lo que se han desarrollado metodologías de encapsulación para asegurar que se mantienen sus propiedades cuando se incorpora al producto final. Por otra parte, asumiendo que la vitamina D es esencial para muchas funciones en el organismo, pero principalmente para los huesos, fue necesario validar el concepto de que la vitamina $D_2$ promueve la absorción de calcio.

El trabajo llevado a cabo en la presente Tesis doctoral muestra que es posible obtener y estabilizar la vitamina $D_2$ natural de los hongos e incorporarla a una matriz de harina utilizando tecnologías innovadoras de extracción, irradiación y encapsulación. Por otra parte, con el fin de maximizar los compuestos bioactivos presentes en los hongos, se ha realizado un estudio

para complementar los sustratos donde se cultivan los hongos con silicato de calcio. Esta suplementación se ha relacionado con una mayor resistencia contra las plagas de insectos. Sin embargo, pocos estudios muestran el impacto de esta suplementación en los diferentes parámetros agronómicos de los hongos de ostra (*Pleurotus ostreatus* var. florida) o incluso su composición química y bioactividad (actividades antioxidantes, antibacterianas y antifúngicas).

Teniendo en cuenta el aumento de la producción y el consumo de hongos, el hecho de ser un alimento muy perecedero representa un inconveniente comercial significativo. Es necesario, por tanto, diseñar procesos de conservación que, además de aumentar la vida útil del producto, sean inofensivos para los consumidores. En este sentido, se evaluaron los efectos de la radiación gamma y de haz de electrones en la composición química y nutricional de muestras frescas y secas de *Agaricus bisporus* Portobello. Por otra parte, se evaluó la posibilidad de agregar valor a los biorresiduos sobrantes de la industria del cultivo de hongos. En esencia, las partes de hongos no utilizadas se sometieron a extracción e irradiación UV-C para aumentar el contenido de vitamina $D_2$ y validar su extracción. La concentración de vitamina $D_2$ en tres especies diferentes de hongos (*Agaricus bisporus, A. bisporus* Portobello y *Pleurotus ostreatus*) se obtuvo mediante cromatografía líquida de alta resolución (HPLC) utilizando un detector ultravioleta (UV). Estos resultados son buenos indicadores de la viabilidad de los excedentes de setas industriales como fuentes alimentarias sostenibles de vitamina $D_2$, y también ayudan a reforzar los principios de circularidad asociados a la cadena de producción de setas.

Se continuaron los estudios con las mejores condiciones de irradiación y el hongo que presentó el mejor contenido de vitamina $D_2$ (*A. bisporus* Portobello). En este sentido, el hongo Portobello se sometió a extracción (uso de metodologías más respetuosas con el medio ambiente (UAE) y disolventes (etanol)) para obtener el extracto rico en ergosterol, que luego se irradiaba para convertir el ergosterol en vitamina $D_2$. Una vez optimizados los métodos de obtención de vitamina $D_2$, se trabajó en el desarrollo de alimentos fortificados que abren la oportunidad de diseñar nuevos productos que no solo alimentan y nutren, sino que también proporcionan compuestos bioactivos necesarios para el buen funcionamiento del organismo, evitando la dependencia de la ingesta de fármacos o suplementos con contenidos sintéticos. En este contexto, la vitamina $D_2$ es un candidato adecuado para ser ingerido de forma sencilla en

la dieta diaria, concretamente utilizando como matriz el pan. Una de las estrategias para asegurar la protección de la bioactividad durante la producción de panes fortificados consiste en su encapsulación para ser incorporada en forma de partículas, facilitando una distribución homogénea en la masa de pan. En esta prueba de concepto, se realizaron estudios de estabilización donde se prepararon partículas que incluían contenidos de vitamina $D_2$ utilizando polímeros, mediante tecnología de dispersión sólida. La caracterización cualitativa y cuantitativa de la encapsulación de vitamina $D_2$ permitió corroborar la efectividad del proceso de dispersión de sólidos para todas las formulaciones. Además, la metodología permitió el desarrollo de una formulación final con el extracto enriquecido con $VitD_2$ utilizando polímero de k-carragenina (KC-Bioactive) y la incorporación en los panes de cantidades específicas de vitamina $D_2$, que posteriormente fueron identificadas y cuantificadas, validando la prueba de concepto de los panes fortificados desarrollados. Además, el extracto enriquecido en vitamina $D_2$ más prometedor se sometió a una evaluación de prueba de concepto del aumento de la absorción de calcio por la línea celular ósea de osteoblastos (h-FOB 1.19) mediante un ensayo in vitro, confirmándose la hipótesis de partida.

Para aumentar la confianza del consumidor europeo, y de los portugueses en particular, en los productos que compran y consumen diariamente, se estudiaron las propiedades físicas y químicas de siete tipos de harinas de trigo y centeno, así como la posible presencia de micotoxinas y microorganismos. Hasta donde sabemos, esta es la primera caracterización de harinas de trigo y centeno de origen portugués. Los estudios sobre harinas elaboradas a partir de subproductos de cereales las caracterizan como ingredientes sostenibles para el desarrollo de nuevos productos de panadería y alimentos funcionales y contribuyen a un mejor aprovechamiento de los recursos biológicos, la eficiencia y la circularidad. Los subproductos de cereales (germen de trigo, mezcla de salvado de maíz-germen, salvado de centeno y salvado de trigo) de la industria de molienda de harina se caracterizaron por su valor nutricional y composición química, así como por sus actividades antioxidantes y antibacterianas. Todas las harinas mencionadas anteriormente pueden servir como una matriz alimenticia fortificada con vitamina $D_2$. Los productos finales (harina y pan) se desarrollaron utilizando una de las harinas mencionadas anteriormente (harina de trigo tipo 65). Se examinaron las características sensoriales de las muestras de harina y pan fortificadas con KC-Bioactive y forma libre extracto enriquecido con $VitD_2$) y se comparó su rendimiento con las muestras no fortificadas (control).

## LIST OF PUBLICATIONS

**The studies developed within this Ph.D. thesis led to the following articles, derived from the work carried out, copies of which are included as annexes:**

1. Rossana V. C. Cardoso, Ângela Fernandes, João C.M. Barreira, Sandra Cabo Verde, Amilcar L. Antonio, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Effectiveness of gamma and electron beam irradiation as preserving technologies of fresh *Agaricus bisporus* Portobello: A comparative study. Food Chemistry, 2019, 278, 760–766. (Annex A)
2. Rossana V. C. Cardoso, Ângela Fernandes, Sandrina A. Heleno, Paula Rodrigues, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Physicochemical characterization and microbiology of wheat and rye flour. Food Chemistry, 2019, 280, 123–129. (Annex B)
3. Rossana V. C. Cardoso, Márcio Carocho, Ângela Fernandes, Diego Cunha Zied, Juan Valenzuela Cobos, Ana M. González-Paramás, Isabel C. F. R. Ferreira, Lillian Barros. Influence of Calcium Silicate on the Chemical Properties of *Pleurotus ostreatus* var. florida (Jacq.) P. Kumm. Journal of Fungi, 2020, 6, 299. (Annex C)
4. Rossana V. C. Cardoso, Márcio Carocho, Ângela Fernandes, José Pinela, Dejan Stojkovic´, Marina Sokovic´, Diego Cunha Zied, Juan Diego Valenzuela Cobos, Ana M. González-Paramás, Isabel C. F. R. Ferreira, Lillian Barros. Antioxidant and Antimicrobial Influence on Oyster Mushrooms (*Pleurotus ostreatus*) from Substrate Supplementation of Calcium Silicate. Sustainability, 2021, 13, 5019. (Annex D)
5. Rossana V. C. Cardoso, Marcio Carocho, Ângela Fernandes, João C. M. Barreira, Sandra Cabo Verde, Pedro M. P. Santos, Amilcar L. Antonio, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Combined effects of irradiation and storage time on the nutritional and chemical parameters of dried *Agaricus bisporus* Portobello mushroom flour. Journal of Food Science, 2021, 86, 2276-2287. (Annex E)
6. Rossana V. C. Cardoso, Ângela Fernandes, José Pinela, Maria Inês Dias, Carla Pereira, Tânia C. S. P. Pires, Márcio Carocho, Esteban Fernández Vasallo, Isabel C. F. R. Ferreira, Lillian Barros. Valorisation of Cereal By-Products from the Milling Industry

as a Source of Nutrients and Bioactive Compounds to Boost Resource-Use Efficiency. Agronomy, 2021, 11, 972. (Annex F)

7. Rossana V. C. Cardoso, Angela Fernandes, João C.M. Barreira, Rui M. V. Abreu; Filipa Mandim, Ana María González-Paramás, Isabel C.F.R. Ferreira, Lillian Barros. A Case Study on Surplus Mushrooms Production: Extraction and Recovery of Vitamin $D_2$. Agriculture, 2021, 11, 579. (Annex G)

**Published review article, book chapter and other articles in process:**

8. Rossana V. C. Cardoso, Ângela Fernandes, Ana M. Gonzaléz-Paramás, Lillian Barros, and Isabel C.F.R. Ferreira. Flour fortification for nutritional and health improvement: A review. Food Research International, 2019, 125, 108576. (Annex H)
9. Rossana V. C. Cardoso, Taofiq Oludemi, Ângela Fernandes, Isabel C.F.R. Ferreira and Lillian Barros. Chapter 5: Bioactive Properties of Mushrooms with Potential Health Benefits. I*n* Dejan Stojkovic and Lillian Barros - Edible Fungi: Chemical Composition, Nutrition and Health Effect: Royal Society of Chemistry, 2022. Submitted. (Annex I)

**Oral and poster communications in scientific conferences:**

1. Rossana V. C. Cardoso, Ângela Fernandes, Amilcar L. Antonio, Sandra Cabo Verde, Paramás, Ana M. G, Lillian Barros, Isabel C. F. R. Ferreira. Effects of gamma radiation on the chemical composition of *Agaricus bisporus* Portobello. V Encontro de Jovens Investigadores do IPB, 2017, Bragança, Portugal. Oral
2. Ângela Fernandes, Rossana V. C. Cardoso, Amilcar L. Antonio, Sandra Cabo Verde, Lillian Barros, Isabel C. F. R. Ferreira. Efeito da radiação gama e feixe de eletrões na concentração de ergosterol em *Agaricus bisporus* (J.E. Lange) Imbach. 10º Encontro Nacional de Cromatografia, 2017, IPB, Bragança, Portugal. Oral
3. Rossana V. C. Cardoso, Ângela Fernandes, João C. M. Barreira, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C.F.R. Ferreira. Irradiação UV-C de cogumelos para obtenção de vitamina $D_2$. XXV Encontro Galego-Portugués de Química, 2019, Santiago de Compostela, Galicia, España. Oral
4. Rossana V. C. Cardoso, Ângela Fernandes, João C.M. Barreira, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Irradiação ultravioleta de cogumelos

como fonte de Vitamina $D_2$. VI Encontro de Jovens Investigadores do IPB, 2019, Bragança, Portugal. Oral

5. Rossana V. C. Cardoso, Ângela Fernandes, João C. M. Barreira, Sandra Cabo Verde, Amílcar L. Antonio, Pedro M.P. Santos, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Gamma irradiation preserves nutritional and chemical composition of *Agaricus bisporus* Portobello. XX EuroFoodChem Congress, 2019, Porto, Portugal. Oral
6. Rossana V. C. Cardoso, Ângela Fernandes, José Pinela, Maria Inês Dias, Carla Pereira, Tânia C. S. P. Pires, Márcio Carocho, Esteban Fernández Vasallo, Isabel C. F. R. Ferreira, Lillian Barros. Cereal by-products from the milling industry: Source of Nutrients and Bioactive Compounds. 5th International Symposium on Phytochemicals in Medicine and Food - PhD Forum, 2021, China. Oral
7. Rossana V. C. Cardoso, Ângela Fernandes, José Pinela, Maria Inês Días, Carla Pereira, Tânia C. S. P. Pires, Márcio Carocho, Esteban Fernández Vasallo, Isabel C. F. R. Ferreira, Lillian Barros. Cereal milling by-products as sources of nutrients and antioxidant phenolic compounds. Trends in grain-based foods, 2022, IPB, Bragança, Portugal. Oral
8. Rossana V. C. Cardoso, Ângela Fernandes, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Physicochemical characterization of wheat and rye flour. XXIV Encontro Luso-Galego de Química, 2018, Porto, Portugal. Poster
9. Rossana V. C. Cardoso, Ângela Fernandes, João C. M. Barreira, Amilcar L. Antonio, Pedro M. P. Santos, Sandra Cabo Verde, Ana M. G. Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Electron-beam irradiation preserves nutritional profile of *Agaricus bisporus* Portobello. IFT-EFFoST 2018 International Nonthermal Processing Workshop and Short course, 2018, Sorrento-Salerno, Italy. Poster
10. Rossana V. C. Cardoso, Ângela Fernandes, João C. M. Barreira, Amilcar L. Antonio, Sandra Cabo Verde, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Electron beam irradiation preserves organic acids in *Agaricus bisporus* Portobello. XXIV Encontro Luso-Galego de Química, 2018, Porto, Portugal. Poster

11. Rossana V. C. Cardoso, Ângela Fernandes, Amilcar L. Antonio, Sandra Cabo Verde, Ana M. González-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Effects of irradiation on the ergosterol and organic acid of *Agaricus bisporus* Portobello. Encontro de Ciência e Tecnologia em Portugal, 2018, Lisbon, Portugal. Poster
12. Rossana V. C. Cardoso, Ângela Fernandes, Amilcar L.Antonio, Sandra Cabo Verde, Ana M.. González-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Irradiation and storage time effects on chemical parameters of *Agaricus bisporus* Portobello. 6$^{th}$ Portuguese Young Chemists Meeting, 2018, Setúbal, Portugal. Poster
13. Rossana V. C. Cardoso, Ângela Fernandes, João C. M. Barreira, Amilcar L. Antonio, Sandra Cabo Verde, Ana M. G. Paramás, Lillian Barros, Isabel C.F.R. Ferreira. Effects of electron-beam irradiation on fatty acids profile of *Agaricus bisporus* Portobello. UNIFood Conference, 2018, Belgrade, Serbia. Poster
14. Rossana V. C. Cardoso, Ângela Fernandes, João C.M. Barreira, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C.F.R. Ferreira. UV-C radiation increases Vitamin $D_2$ content in *Pleurotus ostreatus*. 11$^{th}$ Encontro Nacional de Cromatografia, 2019, Caparica, Portugal. Poster
15. Rossana V. C. Cardoso, Márcio Carocho, Ângela Fernandes, Diego Cunha Zied, João C.M. Barreira, Ana M. Gonzaléz-Paramás, Lillian Barros, Isabel C. F. R. Ferreira. Effects of calcium silicate on the chemical and bioactive composition of *Pleurotus ostreatus* var. Florida. 11$^{th}$ Encontro Nacional de Cromatografia, 2019, Caparica, Portugal. Poster
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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| AAPH | 2.20-Azobis(2-amidinopropane) dihydrochloride |
| Aβ | Amyloid beta |
| ABTS | 2,20-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid |
| AChE | Acetylcholinesterase |
| ACSO | S-Allyl-L-cysteine sulfoxide |
| AD | Alzheimer's disease |
| ADA | Adenosine deaminase |
| ADH | Alcohol dehydrogenase |
| AF | Aflatoxins |
| ALDH | Aldehyde dehydrogenase |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| α-MSH | Alpha-melanocyte-stimulating hormone |
| ANOVA | Analysis of variance |
| AOAC | Official Methods of Analysis |
| AP-1 | Activator protein 1 |
| AST | Aspartate aminotransferase |
| ATR | Attenuated total reflectance |
| BChE | Butyrylcholinesterase |
| BE | Biological efficiency |
| BHT | Butylated hydroxytoluene |
| BMDCs | Bone-marrow-derived dendritic cells |
| CaCo-2 | Colorectal adenocarcinoma |

| | |
|---|---|
| CAT | Catalase |
| CCl4 | Carbon tetrachloride |
| CECOG | Centro de Estudos em Cogumelos |
| CFU | Colony forming unit |
| CHROMagar | Chromogenic culture medium |
| CNS | Central nervous system |
| COX-2 | Cyclooxygenase-2 |
| CS | Calcium Silicate |
| CTL | Cytotoxic T-lymphocytes |
| CV | Coefficient of variation |
| CVD | Cardiovascular diseases |
| DAD | Diode array detector |
| DLS | Dynamic light scattering |
| DM | Diabetes mellitus |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMH | 1,2-Dimethylhydrazine |
| DMM | Dietary mixed micelles |
| DMSO | Dimethyl sulfoxide |
| DOX | Doxorubicin |
| DPPH | 2,20-Diphenyl-1-picrylhydrazyl |
| DRBC | Dichloran Rose Bengal Chloramphenicol |
| DSS | Dextran sulfate sodium |
| DTG | Derivative thermogravimetry |
| EB | Electron beam radiation |
| ECM | Extracellular matrix |

| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| EE | Encapsulation efficiency |
| EMM | Estimated marginal means |
| ESBL | Extended-spectrum b-lactamases |
| ESI-MS | Electrospray ionization mass spectrometry |
| ET | Exposure times |
| FAME | Fatty acids methyl ester |
| FAO | Food and Agriculture Organization |
| FBS | Foetal bovine serum |
| FFI | Food Fortification Initiative |
| FID | Flame ionization detector |
| FRAP | Ferric reducing antioxidant power |
| FSG | Fasting serum glucose |
| FSI | Fasting serum insulin |
| FTIR | Fourier-transform infrared spectroscopy |
| GAIN | Global Alliance for Improved Nutrition |
| GC | Gas chromatography |
| GCSF | Granulocyte colony stimulating factor |
| GFDx | Global Fortification Data Exchange |
| GI | Gamma irradiation |
| GSH | Glutathione |
| GSH-Px | Glutathione peroxidase |
| GST | Glutathione S-transferase |
| H22 | Hepatoma cells |
| $H_2O$ | Water |

| | |
|---|---|
| $H_2O^2$ | Hydrogen peroxide |
| HbA1 | Haemoglobin subunit alpha 1 |
| HbA1c | Glycosylated haemoglobin |
| HBSS | Hank's balanced salt solution |
| HEA | Hydroxyethyl adenosine |
| Hela | Cervical carcinoma |
| HepG2 | Hepatocellular carcinoma |
| H-FOB 1.19 | Osteoblast cell line |
| HN | Harvest Number |
| HOMA-IR | Homeostasis model assessment of insulin resistance |
| HPLC | High performance liquid chromatography |
| Humtyr | Human tyrosinase |
| IAEA | International Atomic Energy Agency |
| ID | Irradiation dose |
| IFN-γ | Interferon gamma |
| IL | Interleukins |
| IL-10 | Interleukin 10 |
| IL-1β | Interleukin 1 beta |
| iNOS | Inducible nitric oxide synthase |
| INT | *p*-Iodonitrotetrazolium chloride |
| ISA | Iron Sulfite Agar |
| ISO | International Organization for Standardization |
| IZD | Internal zone diameter |
| KC | k-Carrageenan |
| KCL | Potassium chloride |

| | |
|---|---|
| KH2PO4 | Potassium phosphate monobasic anhydrous |
| LC | Load capacity |
| LDA | Linear discriminant analysis |
| LDH | Lactate dehydrogenase |
| LDL-C | Low-density lipoprotein cholesterol |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LPS | Lipopolysaccharides |
| MAPKs | Mitogen-activated protein kinases |
| MBC | Minimum bactericidal concentration |
| MC | Mogi-das-Cruzes |
| MCF-7 | Breast adenocarcinoma) |
| MCP-1 | Monocyte chemoattractant protein 1 |
| MDA | Malondialdehyde |
| MDA-TBA | Malondialdehyde-thiobarbituric acid |
| MeOH | Methanol |
| MH | Mueller Hinton |
| MIC | Minimal inhibitory concentration |
| MIP-1α | Macrophage inflammatory protein-1 alpha |
| MIP-1β | Macrophage inflammatory protein-1 beta |
| MIP-2 | Macrophage inflammatory protein-2 |
| MITF | Microphthalmia-associated transcription factor |
| MMP | Matrix metalloproteinases |
| MMP-9 | Matrix metallopeptidase 9 |
| MPL | Maximum permissible levels |

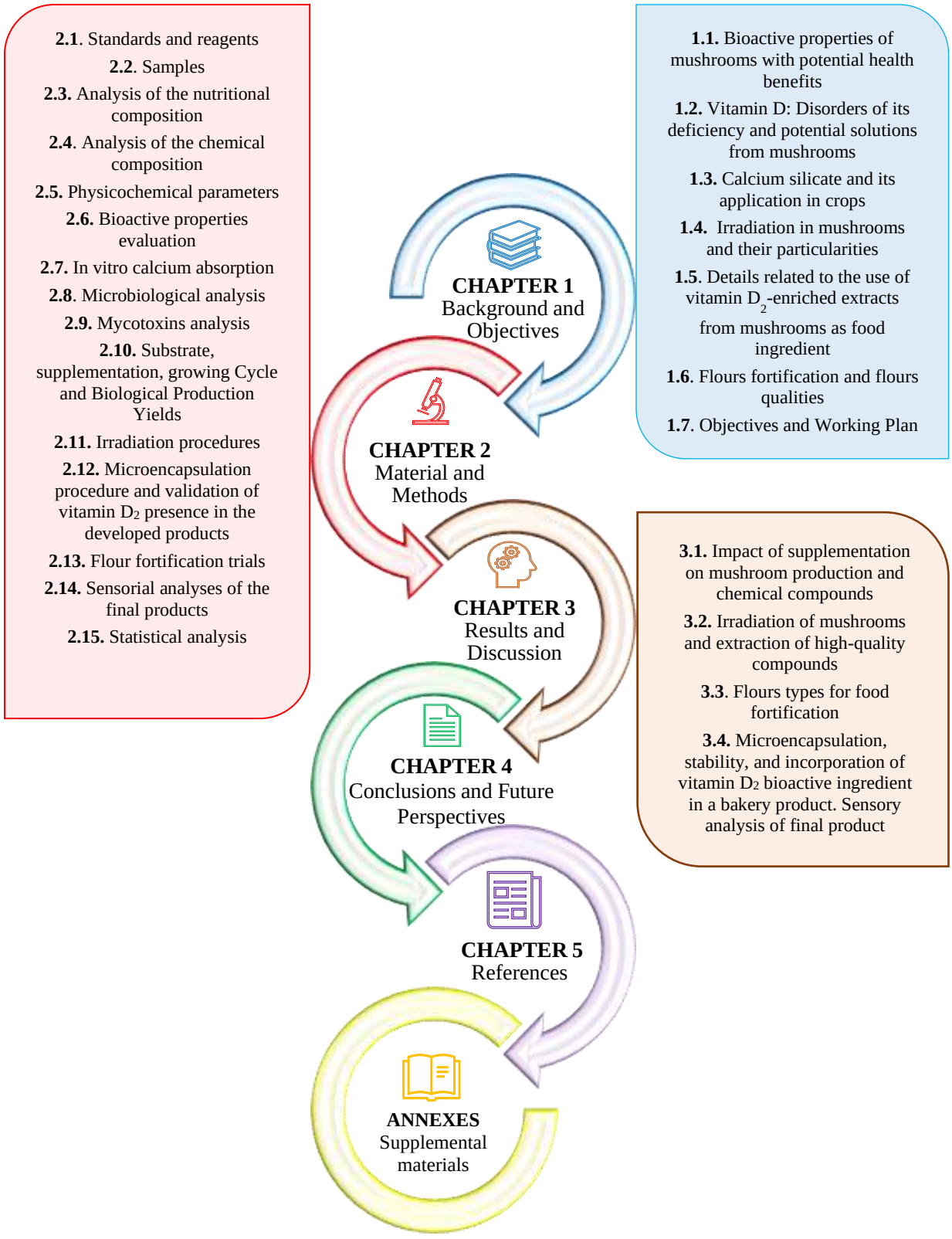
| | |
|---|---|
| MPO | Myeloperoxidase |
| mRNA | Messenger ribonucleic acid |
| MRSA | Methicillin resistant *Staphylococcus aureus* |
| MS | Mass spectrometry |
| MSSA | Meticillin-sensitive Staphylococcus aureus |
| MUFA | Monounsaturated fatty acids |
| MUG | 4-methylumbelliferyl-beta-D-glucuronide |
| Mushtyr | Mushroom tyrosinase |
| MYD88 | Myeloid differentiation 88 |
| MYP | Mannitol yolk polymyxin |
| Na2HPO4 | Disodium hydrogen phosphate |
| NaCL | Sodium chloride |
| NCI-H460 | Non-small cell lung cancer |
| NF-kβ | Nuclear factor kappa β |
| NGF | Nerve growth factor |
| NO | Nitric oxide |
| ORAC | Oxygen radical absorbance capacity |
| OTA | Ochratoxin A |
| OxHLIA | Oxidative hemolysis inhibition assay |
| PBS | Phosphate buffered saline |
| PCA | Plate Counting Agar |
| PCAs | Principal component analysis |
| PDA | Potato dextrose agar |
| PDB | Potato dextrose broth |
| PGE2 | Prostaglandin E2 |

| | |
|---|---|
| pH | Potential of Hydrogen |
| PI3K | Phosphatidylinositol-3-kinase |
| PIH | Post-inflammatory hyperpigmentation |
| PLP2 | Porcine liver primary cells |
| PP | Presidente Prudente |
| PUFA | Polyunsaturated fatty acids |
| PVP | Polyvinylpyrrolidone |
| RANTES | Regulated on activation, normal T-cell expressed and secreted |
| RDA | Recommended daily allowance |
| RI | Refractive index |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RP | Reducing power |
| RPMI | Roswell Park Memorial Institute |
| RSDr | Repeatability relative standard deviations |
| SD | Standard deviation |
| SDs | Solid dispersion |
| SEM | Scanning electron microscopy |
| SFA | Saturated fatty aci |
| SFE | Supercritical fluid extraction |
| SOD | Superoxide dismutase |
| SRB | Sulforhodamine B |
| SRC | Sulphite-reducing clostridia |
| SREBF2 | Sterol regulatory element-binding protein 2 |
| STAT3 | Signal transducer and activator of transcription 3 |

| | |
|---|---|
| TAC | Total antioxidant capacity |
| TBARS | Thiobarbituric acid reactive substances |
| TBT | Technical Barriers to Trade |
| TCA | Trichloroacetic acid |
| TG | Thermogravimetry |
| TGA | Thermogravimetric analysis |
| TGF-β | Transforming growth factor beta |
| TGs | Triglycerides |
| TIMP-1 | Tissue inhibitor of metalloproteinase 1 |
| TLR | Toll-like receptor |
| TLR4 | Toll-like receptor 4 |
| TNF-α | Tumour necrosis factor alpha |
| TOS | Total oxidant status |
| TPA | Texture profile analysis |
| TRIF | TIR-domain-containing adapter-inducing interferon-b |
| TRP | Tyrosinase-related proteins |
| UAE | Ultrasound-assisted extraction |
| UFLC | Ultra-fast liquid chromatography |
| UNICEF | United Nations International Children's Emergency Fund |
| UV | Ultraviolet |
| UVGI | Ultraviolet germicidal irradiation |
| VCAM-1 | Vascular cell adhesion molecule 1 |
| VEGF | Vascular endothelial growth factor |
| $VitD_2$ | Vitamin $D_2$ |
| VLDL-C | Very low-density lipoprotein cholesterol |

| | |
|---|---|
| VOC | Volatile organic compound |
| WFP | World Food Program |
| WHO | World Health Organization |

## SCOPE AND LAYOUT



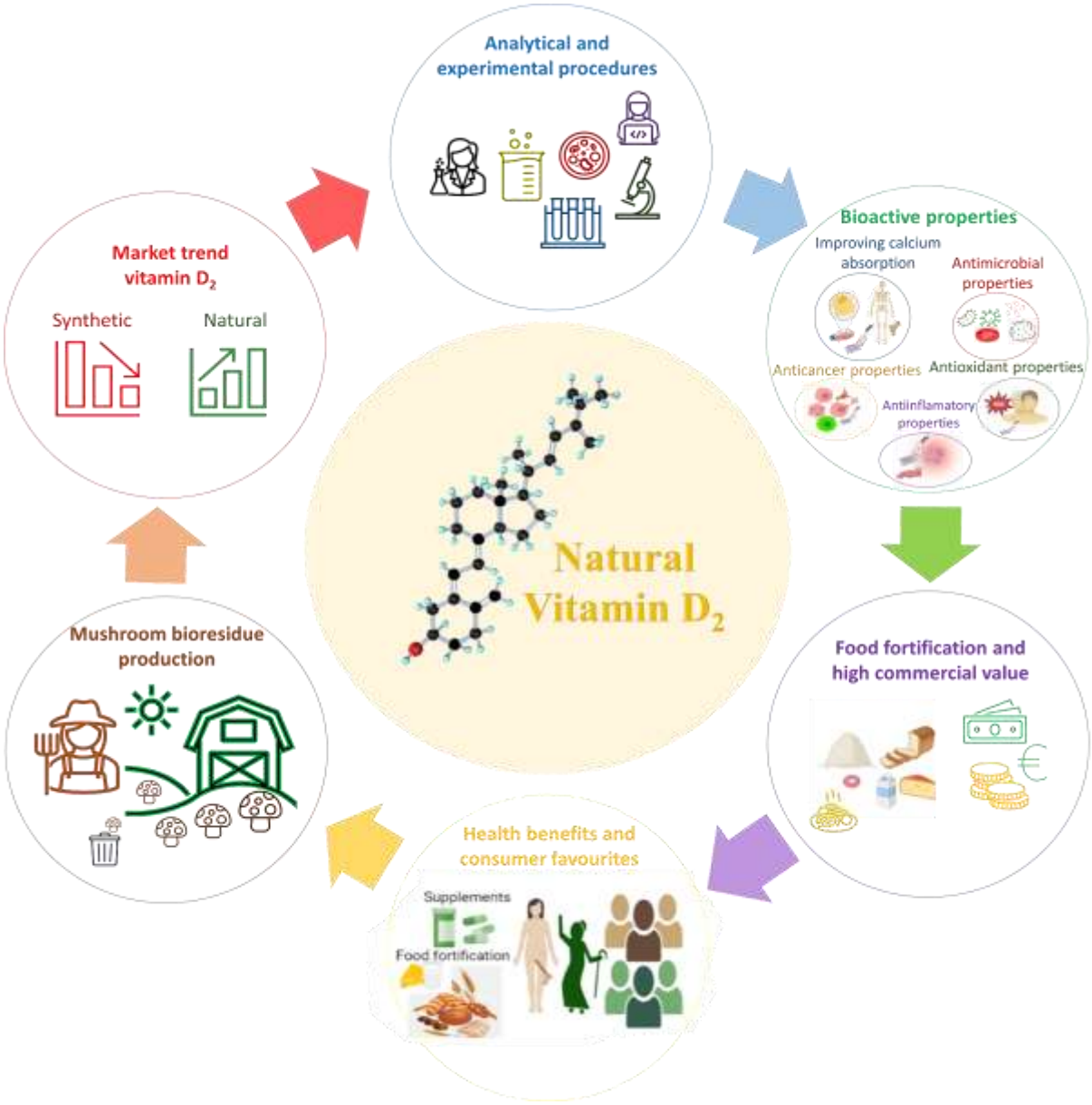
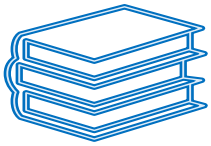



**Figure 1:** Flowchart of the valorisation of mushroom bioresidues and their respective evaluations and market perspective



# CHAPTER 1

# Background and Objectives

### 1.1. Bioactive properties of mushrooms with potential health benefits

Macrofungi are a group of organisms from the Fungi kingdom generally grouped in the families Ascomycota and Basidiomycota, which under special conditions develop a fruiting body of sufficient size to be seen with the naked eye and grow under the soil (Martinez-Medina et al., 2021a). In 2019, global production of mushrooms and truffles was around 11.9 million tons worldwide. China is by far the largest producer of mushrooms globally, producing around 8.93 million metric tons of mushrooms and truffles annually. For producing regions, Asia is the largest producer, and is responsible for 82.8% of total global production, followed by Europe, the Americas, Oceania, and Africa with 12%, 4.5%, 0.4%, and 0.3%, respectively. Poland is the largest European producer of mushrooms, with a total production of 3.62 million metric tons, representing 3.05% of the total global volume (Market Insight Reports, 2021).

For a long time, mushrooms have played an important role in several aspects of human activity. Particularly, edible mushrooms are used extensively in cooking and make up part of a low-calorie diet. In different world regions, the demand and consumption of mushrooms are mostly related to different characteristics such as organoleptic properties, attractive taste and flavour, and a very accessible and marketable product (Martinez-Medina et al., 2021a). Wild and cultivated mushrooms contain a huge diversity of biomolecules with nutritional (since they are rich in minerals, proteins, fibres, and carbohydrates, and are low caloric foods due to a low content in fat) and/or medicinal properties. Due to these properties, they have been recognized as functional foods and as a source for developing medicines and nutraceuticals (Mariajosé Alves et al., 2012; Gebreyohannes et al., 2019; Maity et al., 2021). The molecular structure, molecular weight, size, branching pattern, conformation, solubility, and intra- and intermolecular association of these compounds are important for showing biological responses, antioxidant potential, in mushrooms extracts (Maity et al., 2021). Their pharmacological action and therapeutic interest in promoting human health have been known for thousands of years. In the recent past, mushrooms have become increasingly important as potential natural agents to prevent and treat numerous diseases, such as cancer, cardiovascular diseases, diabetes mellitus and neurodegenerative diseases (Alves et al., 2012; Cruz et al., 2016; Martinez-Medina et al., 2021a).

In this respect, this topic aims to analyse and summarize current knowledge and trends regarding the bioactive properties of mushrooms. In addition, important biological properties are discussed with their potential health benefits, which could be useful for the full use of

mushrooms. A compilation of the published information from 2015 onwards has been explored.

### 1.1.1. Mushroom consumption and potential health benefits

The shift towards natural-based diets has been accelerating at varying rates due to the increased awareness of the health and environmental impacts of excessive and ultra-processed foods. Since ancient times, mushrooms have traditionally been collected in various geographical locations for human consumption; and this practice has increased in recent years, mainly due to the growing awareness of their therapeutic and beneficial effects (Mleczek et al., 2021; Reis et al., 2017). Edible, medicinal, and wild mushrooms are the major components of the global mushroom industry. Medicinal and edible mushrooms are mostly found in higher basidiomycetes with a saprophytic growth habit, which allows them to grow on various agricultural waste based substrates (Bellettini et al., 2019). Edible mushrooms are the leading component, accounting for approximately 54% of global mushroom production, while medicinal and wild mushrooms make up 38% and 8% of the total, respectively (Marçal et al., 2021; Royse et al., 2017). *Agaricus, Lentinula, Pleurotus, Auricularia*, and *Flammulina* represent the five main genera, constituting over 85% of the world's commercial mushroom production. Other mushroom genera with well documented nutritional and medicinal properties include *Ganoderma, Grifola, Trametes, Clitocybe, Antrodia, Cordyceps, Xerocomus, Calvatia, Hericium, Volvariella, Schizophyllum, Inonotus, Inocybe, Lactarius, Albatrellus, Russula, Boletus, Cantharellus, Lactarius, Morchella, Macrolepiota,* and *Fomes* (Taofiq et al., 2015; Taofiq et al., 2017a). *Agaricus bisporus* (white and brown button mushroom) represents about 40% of worldwide production, followed by *Lentinula edodes*, *Pleurotus ostreatus,* and *Flammulina velutipes* ( Yadav & Negi, 2021). The global mushroom market share is expected to grow significantly in the coming years due to these nutritional advantages and health-promoting benefits. In addition, mushroom production and processing generate a large volume of by-products in the form of caps, stipes, mushrooms that do not fit with commercial standards, and spent mushroom substrate (SMS), being predominantly discarded with a high environmental impact and treatment costs for the industry (Antunes et al., 2020; Silva et al., 2020). These mushroom by-products constitute a real economic loss due to the abundance of bioactive compounds, which can be extracted and utilized as value-added ingredients. Hence, some integral approaches for the sustainable valorisation of these by-products in developing innovative food and pharmaceutical formulations have been adopted

further to improve the economic performance of the mushroom processing industry and promote a circular economy in line with current consumer preferences towards sustainability.

The widespread utilizations of mushroom extracts and their associated metabolites in the development of nutraceutical and functional food formulations are due to the extensive range of nutritional, preventive, therapeutic, and structurally diverse bioactive compounds (Reis et al., 2017). These biomolecules include polysaccharides (chitosans, *β*-glucans, fucogalactan, and lentinan), proteins (lectin and cordymin), fatty acids (linoleic, oleic, and palmitic), terpenoids and steroids (ergosterol, betulin, zhankuic acid, and ganoderic acid), phenolic compounds (caffeic acid, gallic acid, cinnamic acid, inotilone, hispolon, *p*-hydroxybenzoic acid, *p*-coumaric acid, and protocatechuic acid), vitamins (thiamine, riboflavin, biotin, and tocopherols), and other unique molecules (ergothioneine, cordycepin, and hispidin) (Martinez-Medina et al., 2021b; Taofiq et al., 2017a). Mushrooms have also been well characterized in their mineral content and have been shown to be rich in elements such as potassium, phosphorus, calcium, magnesium, sodium, iron, zinc, and copper (Haro et al., 2020). Apart from their desirable status in the culinary realm, their health promoting properties are also significant, namely antioxidant, antiinflammatory, antimicrobial, immunomodulatory, cytotoxic, antimutagenic, cardioprotective, hepatoprotective, antidiabetic, and anti-ageing properties, validated via *in vitro* and *in vivo* studies (**Figure 2**) (Fernandeset al., 2016b; Ferreira et al., 2015; Reis et al., 2015; Taofiq et al., 2017a). Studies have indicated that mushrooms exposed to ultraviolet (UV), gamma, and electron beam irradiation under certain conditions convert ergosterol to vitamin $D_2$ (Cardoso et al., 2017). These irradiated mushrooms effectively maintain serum 25(OH)D levels and are currently being utilized as food fortification ingredients to reduce vitamin D deficiency (Cashman, 2015). *In vivo* studies in a mouse model of osteoporosis have shown that the consumption of vitamin $D_2$-enhanced mushrooms have also been associated with improved bone health (Chen et al., 2015).

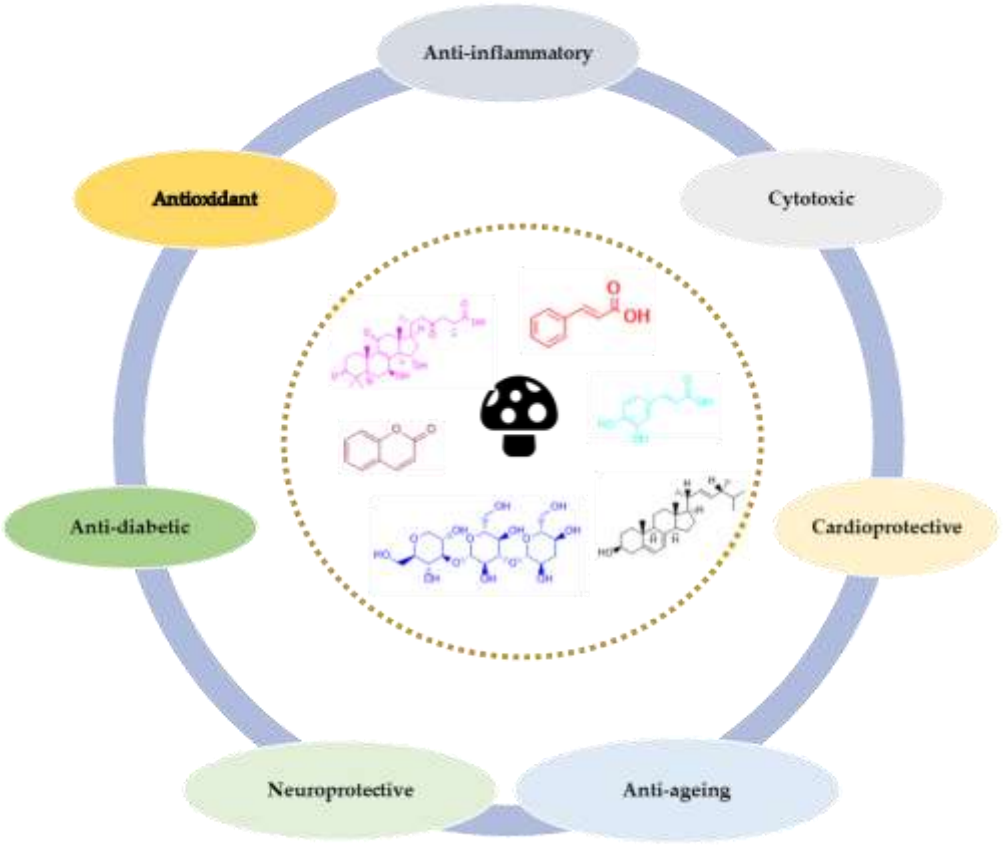



**Figure 2:** Bioactive properties of mushroom extracts

The use of mushroom extracts in the development of food formulations is widespread in baked goods (pasta, bread, and cookies), sausages, hamburgers, dairy products (cheese), beverages, and soups (Francisco et al., 2018; Patinho et al., 2021). Additionally, considering the need to reduce foods from animal origins to enable better global sustainability, there is a growing search for plant-based protein sources as an alternative to animal protein, and mushrooms represent a viable option for obtaining high-quality protein with a complete profile of essential amino acids, often covering the recommended dietary requirement (González et al., 2020). Several commercially available products containing blends of mushroom extracts, or their metabolites have been developed into patents and sold in the market as daily health supplements.

### 1.1.2. Extracts and individual compounds from mushrooms with bioactive properties

#### *1.1.2.1. Antioxidant Activity*

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) accompany several cellular metabolic processes. Oxygen is an essential component for the oxidation of organic

compounds and energy production ( Yadav & Negi, 2021), and when a small amount of oxygen is reduced, it produces highly reactive chemical substances called ROS. Thus, ROS represents the most important class of all free radicals generated in living organisms (Chou et al., 2021; Pessoa et al., 2020; Pisoschi et al., 2021). They are related to several physiological processes and are beneficial to cells when produced in small quantities. However, in situations in which the balance between the production of these reactive species and the antioxidant defences is destroyed, oxidative stress begins (Kozarski et al., 2015; Sánchez, 2017). ROS are highly reactive and when in excess they can cause irreversible damage to the structure of cells. The production of free radicals can be of endogenous or exogenous origin, referring to metabolic interactions or environmental factors, respectively (**Figure 3**). Free radicals are related to several human diseases and participate as essential elements in many of them, which shows how extensive is the oxidative damage caused by them (Goswami et al., 2021; Sakemi et al., 2021). Natural or synthetic antioxidants are chemical compounds that significantly delay or prevent oxidation even at low concentrations compared to an oxidizable substrate. Organisms have antioxidant defence and repair systems that eliminate free radicals and protect the body from oxidative damage. The harmful effect of free radicals occurs when they are in excessive amounts in the body, exceeding its capacity to neutralize them with its natural defence systems (**Figure 3**).

Antioxidants have the power to intercept free radicals formed by cell metabolism or by exogenous sources, preventing oxidative stress and consequent damage to cells. Thus, antioxidant supplements, or natural products with antioxidant capacity, can help reduce oxidative damage in the body, preventing hundreds of diseases, including many types of cancer, diabetes, obesity, and cardiovascular diseases (Carocho et al., 2018; Costa et al., 2016; Sharpe et al., 2021a). The appreciation of the relationship between food and health is growing every day, and healthy nutrition focused on natural products, mainly of vegetal origin, can help combat free radicals. Many natural products have bioactive molecules with various beneficial health claims (Goswami et al., 2021; Živković et al., 2019).

Mushrooms have been present in the human diet for many years and have long been valued as tasty, nutritious, healthy, and valuable food. They are rich in bioactive molecules with health benefits, and in recent years the amounts consumed have increased significantly, involving many species ( Bach et al., 2019; Živković et al., 2019). Mushrooms are rich in antioxidants, including polysaccharides, phenols, proteins, peptides, carotenoids, ergosterol (pre-vitamin

$D_2$), and vitamins C and E (Islam et al., 2019; Kozarski et al., 2015; Reis et al., 2017; Sánchez, 2017; Yadav & Negi, 2021). Several studies are available on the antioxidant properties of mushrooms, such as *Agaricus bisporus* samples (Cardoso et al., 2017; Krüzselyi et al., 2020), *Agaricus campestris* L. (Akata et al., 2019), *Pleurotus ostreatus* (Barbosa et al., 2020; Cardoso et al., 2017, 2021; Menaga et al., 2021), *Lentinula edodes* (Boonsong et al., 2016; Sharpe et al., 2021b), *Macrolepiota procera* (Akata et al., 2019), *Pleurotus pulmonarius* (Fr.) (Amirullah et al., 2021), among others (**Table 1**). Therefore, with the growing interest in the beneficial health effects of antioxidants, it is essential to study the antioxidant potential of the most consumed mushroom species worldwide through the varieties of existing methods to determine the antioxidant activity of extracts or substances in *in vitro* and *ex vivo* models (Bach et al., 2019; Kozarski et al., 2015; Nowacka-Jechalke et al., 2018). Several methods have been developed to evaluate the antioxidant capacity of edible and medicinal mushroom species (see **Table 1**).

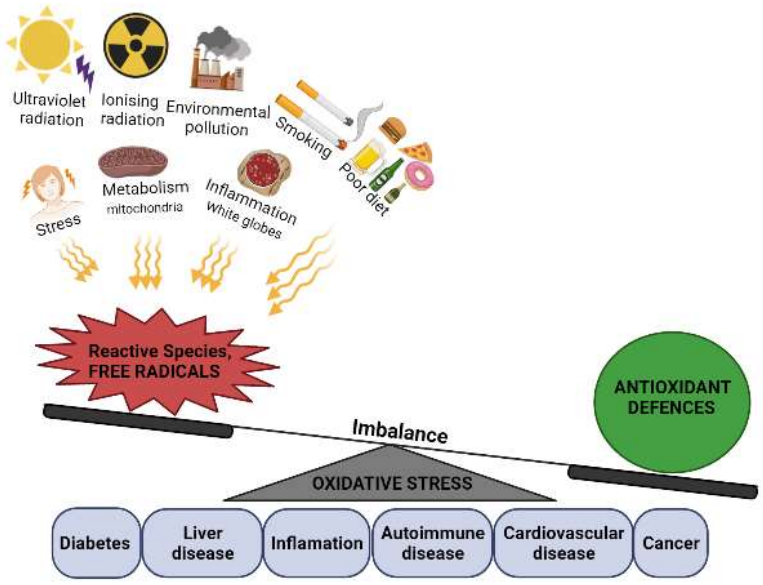



**Figure 3:** The results of the imbalance between reactive species and antioxidant defences

Given the *in vitro* condition, the most applied are the DPPH radical scavenging (2,2-diphenyl-1-picrylhydrazyl), ABTS•+ radical scavenging capacity (2,20-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid), reducing power (RP), and *β*-carotene bleaching. Polysaccharides such as heteroglycan, β-glucan, and lentinan obtained from different edible and medicinal mushrooms have presented high ferrous ion chelating, radical scavenging, and reducing power properties that have contributed to their multifunctional health-promoting function. Some of these polysaccharide-rich mushrooms include *Termitomyces clypeatus*

(Pattanayak et al., 2015), *Hohenbuehelia serotina* (Li & Wang, 2016), and *Meripilus giganteus* (Maity et al., 2017). An optimized extract of *Pleurotus ostreatus* rich in heteropolysaccharides, *β*-glucans, *α*-glucans, and oligosaccharides, besides presenting a solid radical scavenging activity, also showed a promising capacity to protect cells from oxidative damage induced by hydrogen peroxide ($H_2O_2$) (Barbosa et al., 2020). In addition to the well-reported *in vitro* antioxidant effects of these mushrooms and their related metabolites, some ex vivo and *in vivo* studies involving animal models have also shown promising results. Among these, Popović et al. (2010) evaluated antioxidative activity using an aqueous suspension of *Coprinus comatus*, with oxidative stress being induced in rats using alloxane and carbon tetrachloride. Jayakumar et al. (2011) conducted a review of the *in vivo* antioxidant activity of *Pleurotus ostreatus*. The antioxidant potential was evaluated under an acute oxidative stress in animal models induced by carbon tetrachloride ($CCl_4$) and a chronic state of stress (ageing). In addition to the significant reduction in lipid peroxidation, the levels of enzyme antioxidant defences such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), which are involved in the destruction of ROS, were significantly elevated. Recently, the *in vivo* antioxidant effect of *Coprinus comatus* was confirmed using an animal model of $CCl_4$-induced hepatotoxicity (Stilinović et al., 2020). The authors reported that oral ingestion of its methanolic extract suppressed $CCL_4$-induced liver damage, reduced lipid peroxidation, and increased levels of GSH-Px. The beneficial antioxidant and renoprotective effects of an acidic hydrolytic polysaccharide extract from *Lentinula edodes* extract were also conducted using an *in vivo* model. The extract significantly enhanced the levels of SOD, GSH-Px, and CAT enzymes, while also suppressing the level of malondialdehyde (MDA), a vital indicator of lipid peroxidation, responsible for cell membrane disruption and oxidative induced cell damage (Song et al., 2020). These findings imply that mushrooms extract contains various molecular lead compounds with potent antioxidant effects, and they are currently used as additives in nutraceutical formulations to manage diseases associated with oxidative stress.

**Table 1:** Antioxidant potential in mushrooms extracts

| Mushroom species | Extracts | Methods | References |
|---|---|---|---|
| *A. bisporus, F. velutipes, L. edodes,* and *A. brasiliensis* | Ethanolic | DPPH, ABTS and FRAP | (Bach et al., 2019) |
| *A. bisporus* and *P. ostreatus* | Water, methanolic and ethanolic | ABTS, and FRAP | (Wang et al., 2021) |
| *A. campestris* and *B. edulis* | Methanolic extract | DPPH and RP | (Kosanić et al., 2017) |
| *Agaricus sp., A. cylindracea, B. loyo, C. lebre, C. espinosae, F. velutipes, G. gargal, L. deliciosus, L. edodes, M. procera, M. cónica, Pleurotus sp., Ramaria sp, Suillus sp, T. terreum,* and *X. chrysenteron* | Dichloromethane and methanolic | DPPH | (Jacinto-Azevedo et al., 2021) |
| *A. silvaticus, H. rufescens,* and *M. giganteus* | Methanol and ethyl acetate extract | DPPH, ABTS, FRAP, and catalase | (Garrab et al., 2019) |
| *Amanita sp., L. volemus, Russula sp., Termitomyces sp., T. crissum, V. volvacea, A. hygrometricus, A. trappei, A. auricula, C. cibarius, C. Craterellus,* and *Lentinus sp.* | Methanolic extract | DPPH and FRAP | (Butkhup et al., 2018) |
| *A. lanipes* | Methanolic extract | ABTS and TOS | (Kaygusuz et al., 2017) |
| *B. edulis* | Ethanolic and water extract | DPPH, FRAP, hydroxyl oxide, nitric oxide, and superoxide anion | (Novakovic et al., 2017) |
| *B. edulis, B. pinophilus, B. aureus, A. mellea, T. aestivum, L. piperatus, L. deliciosus, P. eryngii, R. botrytis,* and *R. virescens* | Ethanolic extract | DPPH, Chelating, RP, and inhibition of lipid peroxidation | (Vamanu, 2018) |
| *B. griseipurpureus* | Dichloromethane and Methanolic extract | DPPH, ABTS, and ORAC, | (Sudjaroen & Thongkao, 2017) |
| *C. cinereus, C. pistillaris, C. nebularis* and *H. punicea* | Methanol, ethanol and water extract | DPPH, ABTS, and RP | (Dimitrijevic et al., 2019) |
| *C. cylindracea, F. velutipes, L. duriusculum, Pleurotus sp., Agaricus sp, and L. edodes* | Methanolic | DPPH | (Krüzselyi et al., 2020) |
| *C. cornucopioides* | Acetone | DPPH, superoxide anion, and RP | (Kosanić et al., 2019) |
| *G. lucidum* and *A. bisporus* | Water extract | DPPH | (Sriramulu & Sumathi, 2017) |
| *H. marmoreus, A. vaginata, A. auricular, G. lucidum, L. edodes, L. polychrous, L. squarrosulus, P. ostreatus, P. ostreatus, P. pulmonarius, P. sajar caju, A. cytindracea, T. fusiformis, F. velutipes,* and *V. volvacea* | Hexane, ethyl acetate, ethanol, methanol, and water extract | ABTS | (Chaiharn et al., 2018) |

| | | | |
|---|---|---|---|
| *I. obliquus, G. frondosa, Ganoderma sp, L. edodes, T. versicolor, and H. erinaceus* | ReishiHydroalcoholic and water | ORAC, FRAP, DPPH, and NanoCerac | (Sharpe et al., 2021b) |
| *L. leucothites* | Ethanolic extract | DPPH | (Sevindik et al., 2018) |
| *M. lobayensis* | Ethanolic extract | DPPH, ABTS superoxide radical, hydroxyl radical quenching, Chelating ability of metal ion, RP, and TAC | (Khatua et al., 2019) |
| | Methanolic extract | DPPH, ABTS, RP, and chelating ability of ferrous ion | (Khatua et al., 2017) |
| *M. giganteus* | Methanolic extract | DPPH, RP, β-carotene bleaching, and TBARS | (Stojković et al., 2017) |
| *M. cognata* and *M. stridula* | Ethyl acetate, methanolic, and water | DPPH, ABTS, and RP, phospho-molybdenum, and metal chelating | (Bahadori et al., 2019) |
| *P. eryngii* and *S. belinii* | Methanolic extract | DPPH, RP, TBARS, and β-carotene bleaching | (Souilem et al., 2017) |
| *P. florida* | Methanolic, ethanolic, water, ethyl acetate and hexane | DPPH and RP | (Menaga et al., 2021) |
| *P. ostreatus* | Water | DPPH, ABTS, RP, Metal Chelating, and Hydroxyl Radical | (Goswami et al., 2021) |
| | Ethanolic | DPPH and Hydroxyl radical scavenging | (Zhang et al., 2021) |
| | Water | DPPH | (Barbosa et al., 2020) |
| | ethyl acetate and n-Butanol extract | DPPH and ABTS | (Lu et al., 2017) |
| *P. ostreatus var. Florida* | Ethanolic | TBARS and OxHLIA | (Cardoso et al., 2021) |
| *P. ostreatus* and *A. bisporus* | Methanolic extract | DPPH, RP, β-carotene, TBARS | (Cardoso et al., 2017) |
| *Pleurotus sp.* | Hydroalcoholic extract | DPPH, ORAC, ABTS, and β-carotene bleaching | (Adebayo et al., 2018) |
| *P. pulmonarius* | Ethanolic extract | TBARS, Conjugated dienes formation, DPPH, and RP | (Amirullah et al., 2021) |
| *R. subalpina* | Methanolic extract | DPPH, ferrous ion chelating, and RP | (Acharya et al., 2017) |
| *Russula sp.* | Methanolic and ethanolic | OxHLIA and TBARS | (Kostić et al., 2020) |
| *T. indicum* | Methanol and ethanol extract | DPPH and ABTS | (Li et al., 2019) |
| *T. equestre* | water and methanol extract | DPPH | (Muszyńska, Kała, et al., 2018) |

TBARS - Thiobarbituric acid reactive substances; OxHLIA - Oxidative hemolysis inhibition assay; DPPH - 2,2′-diphenyl-1-picrylhydrazyl; RP - Reducing power; ORAC - Oxygen radical absorbance capacity, FRAP - Ferric reducing antioxidant power; ABTS - 2,2’-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid; TAC - Total antioxidant capacity; TOS - Total oxidant status

*1.1.2.2. Anti-inflammatory, neuroinflammatory, and neuroprotective activities*

Inflammation is a complex biological process triggered in response to external stimuli such as infections, injuries, irritants, ultraviolet radiations, and toxins (Taofiq et al., 2016a). However, persistent inflammation has been associated with diabetes, arthritis, obesity, metabolic syndrome, cancer, and several cardiovascular diseases et al., 2018). Pro-inflammatory cells (mainly macrophages and monocytes) play a key role in natural immunity by releasing specialized inflammatory mediators, which include vasoactive amines, eicosanoids, proinflammatory cytokines, and acute-phase proteins, which mediate the inflammatory process by preventing further tissue damage and ultimately resulting in healing and restoration of tissue function. However, over secretion of these inflammatory mediators causes accumulation of oxidative products and increased cytotoxicity, leading to tumour development, DNA damage, and programmed cell death et al., 2018). External stimuli such as bacterial lipopolysaccharides, viral proteins, toxins, or endogenous proteins activate cascades of various transcription factors, namely activator protein 1 (AP-1), signal transducer and activator of transcription 3 (STAT3), and nuclear factor kappa β (NF-kB). NF-kB comprises five structurally related proteins, NF-kB1 (p50), NF-kB2 (p52), RelA (p65), RelB, and c-Rel, responsible for the expression of a vast array of inflammatory mediators (Taofiq et al., 2016c). In the absence of stimuli, NF-kB complexes (usually in the forms of p60 and p65 subunits), are confined in the cytoplasm and attached to inhibitory proteins, including from the IkB family. Once the toll-like receptor (TLR) ligands are stimulated, IkB proteins are rapidly phosphorylated, which leads to the ubiquitination and degradation of IkBα (**Figure 4**). This frees the NF-kβ complex (mainly p65 and p50) to translocate from the cytosol into the nucleus to instantaneously provoke the release of pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and cytokines like interferon-gamma (IFN-g), interleukins (IL-1b and IL-6), and tumour necrosis factor-alpha (TNF-α) (Peng et al., 2015). The mechanism behind the inflammatory process is mainly controlled by the NF-kβ pathway and trying to inhibit some of these specific steps in the pathway leading to NF-kβ release is crucial to suppressing the inflammation process (**Figure 4**).

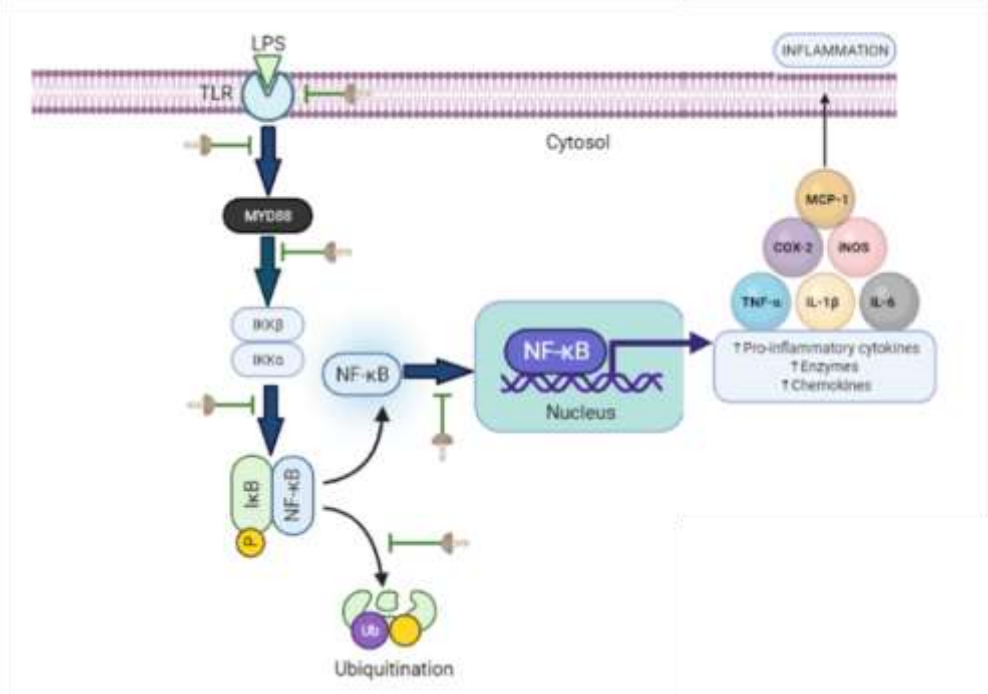



**Figure 4:** The nuclear factor-kappa beta (NF-kb) pathway. Abbreviations: toll-like receptors (TLR), lipopolysaccharides (LPS), myeloid differentiation protein 88 (MyD88), IL (interleukins), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumour necrosis factor (TNF-α), monocyte chemoattractant protein-1 (MCP-1), IkappaB kinase (IKK)

*In vitro* models such as lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells, mouse splenocytes, NIH/3T3 cell, HaCaT, RBL-2H3, and IFN-γ activated murine macrophages have been utilized as effective models to evaluate the anti-inflammatory activities of mushroom extracts and their related metabolites (**Table 2**) (Chan et al., 2015; Choe et al., 2015; Feng et al., 2020; Jeong et al., 2020). Additionally, numerous animal models such as a dextran sulfate sodium (DSS)-induced colitis model, carrageenan-induced mice models, formalin-induced mice models, and an acetic acid-induced mice model have been utilized to determine the anti-inflammatory properties of bioactive ingredients derived from mushroom (**Table 2**) (Lima et al., 2016).

Polysaccharide and sulfated polysaccharide-rich extracts obtained from *Antrodia cinnamomea* significantly reduced levels of TNF-α, IL-1β, and IL-6 in LPS-induced RAW 264.7 macrophage cells (Cheng et al., 2016, 2018). At the same time, a more distinct anti-inflammatory effect via downregulation of NF-kβ expression was reported for its oligosaccharide and ergostatrien-3β-ol rich extracts ( Tsai et al., 2015; Zheng et al., 2018). Polysaccharide rich fractions from *Grifola frondose* (S.-J. Wu et al., 2017), *Hericium erinaceus* (Ren et al., 2018a), *Lentinula edodes* (acidic polysaccharide and b-D-glucan) (Morales et al.,

2020; Song et al., 2020), *Phellinus baumii* (heteropolysaccharide) (Sun et al., 2021), and *Schizophyllum commune* (exopolysaccharide) (Du et al., 2017b; Lima et al., 2016) presented interesting anti-inflammatory effects in RAW 264.7 macrophage cells, human monocyte THP-1 cell line, carrageenan-induced paw oedema, and DSS-induced colitis models. Sterol-rich extract from *G. lucidum* shows anti-inflammatory effects via attenuation of the NF-kβ pathway by restraining degradation of IkB-α and phosphorylation of NF-kβ p65 (Xu et al., 2021). Its phenolic-rich extract significantly suppressed gene expression of COX-2, TNF-α, iNOS, IL-1β, IL-6, IL-10, and NF-kB in RAW 264.7 macrophage cells and a DSS-induced colitis model (Hasnat et al., 2015). The mechanism behind the anti-inflammatory effect of mushroom extracts and their bioactive metabolites has been associated with the downregulation and dephosphorylation of NF-kB, inhibition of mRNA expression of TLR4, inhibition of NF-kB translocation into the nucleus, and degradation of IkB-α (Xu et al., 2021). However, due to the current pharmaceutical practices involving highly purified compounds, the utilization of complex mixtures of bioactive compounds with unknown concentrations is difficult to reconcile. Moreover, the recovery of complex mushroom-derived compounds such as polysaccharides requires energy-consuming and costly extraction processes. Hence, low-molecular-weight compounds, such as N6-(2-hydroxyethyl) adenosine (HEA), cordycepin, methyl 9-oxo-(10E,12E)-octadecadienoate, polyozellin, and poricoic acid C, also have been studied (**Table 2**) and known to inhibit inflammatory mediator production, block steps leading to NF-kβ activation, and suppress *in vivo* induced inflammation. As shown in **Table 2**, different research studies have highlighted mushrooms' very promising anti-inflammatory effects, mainly based on their extracts and bioactive compounds. Nevertheless, intensive preclinical and clinical research into these bioactive molecules is still lacking.

Neuroinflammation is a critical defence mechanism against infectious agents and neuronal injuries in the central nervous system (CNS). When microglia cells are activated in response to brain injury or immunological stimuli, such as LPS, amyloid beta (Aβ), and lipoteichoic acid, pro-inflammatory cytokines and neurotoxic mediators are released (Bach et al., 2019). However, prolonged release of these inflammatory mediators may lead to aberrant phagocytosis, causing neuronal damage, thereby contributing to the pathogenesis of neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease (AD), Huntington's disease, motor neuron disease, prion disease, and multiple sclerosis (Cai et al., 2017; Geng et al., 2017). Presently, the use of non-steroidal antiinflammatory drugs against neurodegenerative disorders only slows down the disease progression transiently, and as such,

there is increasing attention to finding potent and safe neuroprotective agents from natural sources that are able to prevent or treat these disorders. Mushroom extracts have been reported to display neuroprotective effects mainly via *in vitro* studies conducted in LPS and Aβ induced BV2 microglial cells, whose mechanisms of action are largely associated with downregulation of the gene expression of different inflammatory mediators (Geng et al., 2017). Bioactive compounds such as cordycepin, polysaccharides, and phenolic acids have been reported to present a neuroprotective effect in different *in vitro* and *in vivo* models (Cai et al., 2017; Peng et al., 2015). Mushroom-derived triterpenes (**Table 2**) such as triterpenoid-enriched fractions from *Antrodia cinnamomea* (Y. T. Chen et al., 2016), polyoxygenated cyathane diterpenoids from *Cyathus africanus* (Y. T. Chen et al., 2016), erinacine C from *Hericium erinaceus* (Wang & Chuang, 2019), and lanostane-type triterpenoids from *Inonotus obliquus* (R. W. Kou et al., 2021) have been reported to display neuroprotective effects in BV2 microglial cells via downregulation of mRNA expression of IkB-α, NF-kβ, p65, iNOS, and COX-2. Studies have also evidenced the potential of medicinal and edible mushrooms as ‘‘brain food’’ being utilized to manage neurodegenerative disorders. The reported neuroprotective effects of these mushrooms and their bioactive secondary metabolites have been associated with their potential to reduce beta amyloid-induced neurotoxicity, and to promote nerve growth factor (NGF) synthesis, anti-acetylcholinesterase, and anti-neuroinflammatory activity (Phan et al., 2015). Inhibitors of acetylcholinesterase improve cholinergic function by preventing the breakdown of acetylcholine into choline and acetate. Extracts or compounds with strong inhibitory capacity against acetylcholinesterase have been widely utilized in the management of AD (Phan et al., 2015). The most common inhibitors of acetylcholinesterase utilized as treatment options for the management of AD include galantamine, rivastigmine, and donepezil that are ineffective in the long term with several associated side effects, including hepatotoxicity, nausea, diarrhoea, lack of appetite, gastrointestinal toxicity, and other side effects (Rahman et al., 2016; Takahashi et al., 2019). Hence, several mushroom extracts have been utilized as complementary and alternative medicines in the form of nutraceuticals and functional foods loaded with bioactive molecules that can potentially suppress the severity of AD. Submerged polysaccharide-rich extracts from two different *Schizophyllum commune* strains were examined for their acetylcholinesterase inhibitory capacity (Mišković et al., 2021). The extracts presented promising inhibitory capacity with $IC_{90}$ values of 79.73 ± 26.34 mg $mL^{-1}$ in comparison to donepezil (87.92%), the positive control. Water and hydroethanolic extract from *Pholiota adiposa*, among other studied mushrooms, also presented up to 30.9 and 35%

acetylcholinesterase (AChE) inhibitory activity, respectively (Kim et al., 2016). Butyrylcholinesterase (BChE) inhibitors have also been reported to be viable therapeutic options in managing AD by promoting normal cholinergic function and preventing the breakdown of acetylcholine. Six macrofungi, namely *Coprinus comatus, Macrolepiota mastoidea, Agaricus campestris, Lycoperdon utriforme, Macrolepiota procera*, and *Leucoagaricus leucothites* were reported to prevent both AChE and BChE inhibition, and the promising results were expressed as galantamine equivalent (Akata et al., 2019). A cholinesterase inhibitory effect of methanol extract rich in phenolic compounds from fruiting bodies of *Pleurotus pulmonarias* was reported to present a strong inhibitory effect against AChE and BChE, with the latter being more effective than galantamine (Nguyen et al., 2016). Bio-guided fractionation performed on the fruiting bodies of mushroom has presented very promising AChE inhibitory activity. *Pleurotus florida* yielded a resveratrol-rich extract that inhibits acetylcholinesterase activity and combat oxidative stress in an *in vivo* animal model (Randhawa et al., 2021), while a gallic acid-rich fraction obtained from *Ganoderma mediosinense* hydromethanolic extract inhibited AChE ($IC_{50}$, 0.10 ± 0.02 $mgmL^{-1}$) (A. Kaur et al., 2019). Regarding the contribution of terpenoids and related compounds from mushrooms on AChE inhibitory activity, five novel meroterpenoids, possessing a g-lactone motif obtained from *Ganoderma lucidum* presented AChE inhibitory activities with $IC_{50}$ values of 7.37–59.86 μM. Several lanostanoids obtained from the ethyl acetate extract of *Haddowia longipes* showed very low inhibitory capacity against AChE (<40%) at 100 μM (Zhang et al., 2015). Based on the beta-amyloid cascade hypothesis, several bioactive compounds obtained from mushrooms have shown promising inhibitory effects against Aβ aggregation, thereby reducing Aβ-induced neurotoxicity (Akiba et al., 2020). Ten monoterpenoids were isolated from the chloroform extracts of *Albatrellus yasudae*, and all the tested compounds presented potential Aβ40-aggregation inhibitory capacity with $IC_{50}$ between 12.3–221 μM, with the carboxyl and farnesyl groups exhibiting a more significant contribution to the bioactivity (Akiba et al., 2020). A growing body of scientific evidence has shown that dietary interventions involving mushroom extracts and their associated metabolites have shown promising potential to suppress the severity of neurodegenerative disorders due to their neuroprotective, antioxidant, and anti-inflammatory effects. However, studies involving clinical trials are significantly lacking and should be conducted to strengthen their utilization further in combatting these diseases.

**Table 2:** Anti-inflammatory and neuroinflammatory effects of mushroom extracts and their individual compounds

| Species | Bioactive form | Bioactive property | Model | Key findings and suggested mechanism of action | Reference |
|---|---|---|---|---|---|
| *A. blazei* | Polypeptide | Anti-inflammatory effect | NIH/3T3 cell | ↓downregulate the expression of NF-κBp65 and TLR4<br>↓ levels of TNF-α, IL-1β and IL-6 | (Feng et al., 2020) |
| *A. rugosum* | Ethanolic extract | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓production of TNF-α and NO | (Chan et al., 2015) |
| *A. cinnamomea* | Triterpenoid-enriched fractions | Neuroprotection | LPS induced BV2 microglia cells | ↓production of NO,<br>↓mRNA expression of iNOS and COX-2 | (Chen et al., 2016) |
| | Sulfated polysaccharide | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ levels of TNF-α, IL-1β and IL-6 | (Cheng et al., 2018) |
| | Polysaccharides and sulfated polysaccharides | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ levels of TNF-α and IL-6 | (Cheng et al., 2016) |
| | Oligosaccharides | Anti-inflammatory effect | RAW 264.7 macrophage cells and LPS-induced mouse model | ↓mRNA expression of IL-6, IL-8, IL-1, TNF-α and MCP-1<br>↓ activation of MAPK and Akt signalling pathways | (Zheng et al., 2018) |
| | Ergostatrien-3β-ol | Anti-inflammatory effect | Mouse skin ischemia model induced by skin flap surgery | ↓gene expression of iNOS, IL-6, TNF-α, IκB, and NF-κB | (Tsai et al., 2015) |
| *A. mellea* | Xylosyl 1,3-galactofucan | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ levels of TNF-α and cytokine monocyte chemotactic protein-1 (MCP-1) | (Chang et al., 2018) |
| | Ethyl acetate extract | Neuroprotection | LPS induced BV2 microglia cells | ↓production of NO, IL-6, TNF-α and IL-1β<br>↓ phosphorylation of IκB-α and NF-κB p65 | (Geng et al., 2017) |
| *C. cicadae* | N6-(2-Hydroxyethyl) adenosine (HEA) | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓gene expression of TLR4, IκB, p- IκB, and COX-2<br>↓production of TNF-α and PGE2 | (Lu et al., 2015) |
| *C. militaris* | Cordycepin | Neuroprotection | LPS induced BV2 microglia cells and hippocampal neurons from the brain of prenatal mouse | ↓ levels of TNF-α and IL-1β<br>↓ mRNA expression of iNOS and COX-2<br>↓ mRNA expression of p65, IκBα, and phosphorylated IκBα (p-IκBα) | (Peng et al., 2015) |
| *C. africanus* | Polyoxygenated cyathane diterpenoids | Neuroprotection | LPS induced BV2 microglia cells | ↓production of NO | (Wei et al., 2018) |

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|---|---|---|---|---|---|
| *D. indusiata* | Polysaccharide | Anti-inflammatory effect | DSS-induced colitis model | ↓ levels of TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-17, IL-4, and IL-10<br>↑ dephosphorylation of NF-κB and MAPK,<br>↓ mRNA expression of iNOS, COX-2, TNF-α, and IL-6 | (Kanwal et al., 2020) |
| *F. fomentarius* | Methyl 9-Oxo-(10E,12E) - octadecadienoate | Anti-inflammatory effect | Murine macrophage cells | ↓ secretion of NO and PG E2,<br>↓downregulation of iNOS and COX-2,<br>↓ levels of TNF-α and IL-6<br>↓ activation of STAT3 | (Choe et al., 2015) |
| *G. lucidum* | Polysaccharide | Neuroprotection | LPS- and Aβ-induced BV2 microglia and primary mouse microglia cells | ↓ mRNA expressions of IL-1β, IL-6, iNOS and MCP-1<br>↑ mRNA expressions of TGFβ | (Cai et al., 2017) |
| | Ganodermanontriol | Anti-inflammatory effect | Ana-1 macrophage cell line | ↓ levels of IL-1β, IL−6, TNF-α, and PEG2<br>↓ translocation of NF-κB<br>↓ mRNA expression of TLR4 | (Hu et al., 2020) |
| | Phenolic rich extract | Anti-inflammatory effect | RAW 264.7 macrophage cells and Dextran sulfate sodium (DSS)-induced colitis model | ↓ gene expression of COX-2, TNF-α, iNOS, IL-1β, IL-6, IL-10, NF-κB | (Hasnat et al., 2015) |
| *G. frondosa* | Polysaccharide rich extract | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓production of NO, PGE2, IL-6, TNF-α and IL-1β<br>↓ activation of NF-κB | (S.-J. Wu et al., 2017) |
| *H. erinaceus* | Polysaccharide | Anti-inflammatory effect | DSS-induced colitis model | ↓ Serum levels of NO, IL-6, IL-1β, and TNF-α<br>↓ mRNA expression of COX-2 and iNOS<br>↓ phosphorylation of NF-κB p65, IκB-α, MAPK and Akt | (Ren et al., 2018a) |
| | Erinacine C | Neuroprotection | BV-2 microglial cells | ↓ production of NO, IL-6, and TNF-α<br>↓ mRNA expression of iNOS<br>↓ mRNA expression of NF-κB and IκB-α | (Wang & Chuang, 2019) |
| *I. obliquus* | Lanostane-type triterpenoids | Neuroprotection | BV-2 microglial cells | ↓production of NO<br>↓ mRNA expression of iNOS | (Kou et al., 2021) |
| *L. edodes* | Acidic polysaccharides | Anti-inflammatory effect | LPS-induced mouse model | ↓ serum levels of TNF-α, IL-6, and IL-1β | (Song et al., 2020) |
| | Polysaccharides | Anti-inflammatory effect | LPS-induced mouse model | ↓ serum levels of TNF-α, IL-6, and IL-1β | (Ren et al., 2018b) |

| | | | | | |
|---|---|---|---|---|---|
| | $\alpha$- and $\beta$-D-glucans | Anti-inflammatory effect | Human monocyte THP-1 cell line | ↓ secretion of TNF-α, IL-6, and IL-1β | (Morales et al., 2020) |
| *L. rhinocerus* | Extract | Neuroprotection | HT22 Hippocampal neuronal cell model | ↓ number of apoptotic cells | (Kittimongkolsuk et al., 2021) |
| *P. baumii* | Heteropolysaccharide | Anti-inflammatory effect | RAW 264.7 macrophage cells and DSS-induced colitis model | ↓ phosphorylation of STAT-1<br>↓ mRNA expression of iNOS and TNF-α | (Sun et al., 2021) |
| *P. linteus* | Hispidin | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ translocation of NF-κB and IκB degradation | (Shao et al., 2015) |
| *P. eryngii* | Polysaccharide | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ production of IL-1β, IL-6, IL-10, and TNF-α | (Siqian Li & Shah, 2016) |
| *P. multiplex* | Polyozellin | Anti-inflammatory effect | *In vitro* cells (HaCaT and RBL-2H3) and DFE/DNCB-induced AD animal model | ↓ gene expression levels of TNF-α, IFN-γ, IL-4, IL-13, IL-31, IL-17A, STAT1, and NF-κB | (Jeong et al., 2020) |
| *P. cocos* | Poricoic acid C | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓production of NO<br>↓gene expression of iNOS and COX-2<br>↓ downregulation of NF-κB | (Lee et al., 2017a) |
| | Lanostane triterpenoids | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ production of NO and PGE2<br>↓ mRNA expression of iNOS and COX-2 | (Lee et al., 2017b) |
| *P. cubensis* | Psilocybin-rich extract | Anti-inflammatory effect | Human U937 macrophage cells | ↓ level of TNF-α, IL-1β, IL-6, IL-10, and COX-2 | (Nkadimeng et al., 2021) |
| *S. commune* | Exopolysaccharide | Anti-inflammatory effect | DSS-induced colitis model | ↓ serum levels of IFN-γ, IL-4, IL-10, and IL-17 | (Du et al., 2017a) |
| *X. nigripes* | Aqueous and hydroethanolic mycelial extract | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓production of NO, IL-6, TNF-α and COX-2 | (Divate & Chung, 2017) |

#### *1.1.2.3. Antimicrobial properties*

Multiple-drug resistance in human pathogenic microorganisms has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used to treat infectious diseases. This situation has forced scientists to search for new antimicrobial substances from various sources to be used as novel antimicrobial chemotherapeutic agents. The scientific community, while searching for new therapeutic alternatives, has studied many kinds of mushrooms, and has found several therapeutic activities (Alves et al., 2013; Alves et al., 2012; Shen et al., 2017).

Although many natural/synthetic antimicrobial agents have been isolated/developed to kill pathogenic microorganisms effectively, global antimicrobial resistance is an increasing public health problem (Jakubczyk & Dussart, 2020). Mushrooms need antibacterial and antifungal compounds to survive in their natural environment. Therefore, antimicrobial compounds could be isolated from many mushroom species and could be a benefit on humans (Garcia et al., 2021; Jakubczyk & Dussart, 2020; Maity et al., 2021; Shen et al., 2017).

The research into mushrooms is extensive, and hundreds of species have demonstrated a broad spectrum of pharmacological activities, including antimicrobial activity. The two most used methodologies to assess the antimicrobial activity of mushroom extracts include the microdilution method and the disk diffusion method. The microdilution method comprises microdilutions of the extract in a liquid medium using microplates to determine the minimum inhibitory concentration (MIC) values. In the disk diffusion method, the extract is incorporated in disks at different concentrations, and the halo of growth inhibition is determined and represented by internal zone diameter (IZD) values (Alves et al., 2012). Therefore, the results for antimicrobial activity may be expressed in different units.

Several mushroom extracts have been reported to have antimicrobial activity against Gram-positive and Gram-negative bacteria (**Table 3**). It is presented as a mean value and, in parentheses, the range of variability of the literature data. *A. blazei* commercial sample from Brazil should be highlighted, as its ethanolic extract revealed MIC mean = 0.144 mg $mL^{-1}$ and minimum bactericidal concentration (MBC) mean = 0.225 mg $mL^{-1}$ against Gram-positive bacteria, like *S. aureus, B. cereus, M. flavus*, and *L. monocytogenes*, even lower than the standard ampicillin, and MIC mean = 0.138 mg $mL^{-1}$ and MBC mean = 0.225 mg $mL^{-1}$ against Gram-negative bacteria, such as *E. coli, S. enteritides, S. typhimurium*, and *E. cloacae* (Corrêa

et al., 2018). *A. bisporus* ethanolic extracts from a commercial sample from Portugal showed an inhibitory effect upon all the tested Gram-positive bacteria (MIC = 10 mg $mL^{-1}$) (Taofiq et al., 2016b). *A. mellea* methanolic extract also revealed antimicrobial properties against Gram-positive and Gram-negative bacteria strains (MIC mean = 7.19 and 9.49 mg $mL^{-1}$, respectively) (Kostić et al., 2017). The methanolic extract of *H. tessulatus* showed activity against two Gram-positive bacteria (*S. aureus* and *B. subtilis*, MIC mean = 7.5 mg $mL^{-1}$ ) and Gram-negative bacteria (*E. coli, P. aeruginosa, S. typhi*, and *K. pneumonia*, MIC mean = 7.25 mg $mL^{-1}$ ) (Chowdhury et al., 2015). *L. edodes* hydroethanolic extract demonstrated good activity against two Gram-negative bacteria, *E. coli* and *S. enteritidis* (MIC mean = 3 mg $mL^{-1}$) (Bach et al., 2019). The methanolic extracts of *M. giganteus* wild samples from Serbia showed an antibacterial effect against Gram-positive and Gram-negative bacteria with MIC means of 0.23 and 0.819 mg $mL^{-1}$ , respectively (Stojković et al., 2017). *M. conica* methanolic extracts from wild samples from Serbia and Portugal also presented an inhibitory effect upon all the tested Gram-positive and Gram-negative bacteria (Vieira et al., 2016). *P. ostreatus* hydroethanolic extracts from commercial samples from Portugal showed an antibacterial effect against Gram-positive and Gram-negative bacteria with MIC means of 0.32 and 0.28 mg $mL^{-1}$, respectively (Cardoso et al., 2021).

Shameem et al. (2017) tested the antimicrobial activity of butanol extract from *M. esculenta* and revealed a significant antibacterial effect against *S. aureus* with MIC and MBC means of 0.25 and 0.75 mg $mL^{-1}$, respectively and, *E. coli* and *P. aeruginosa* presented MIC and MBC means of 0.75 and 1.25 mg $mL^{-1}$, respectively.

Data available from the literature indicate that mushroom species showed antimicrobial activity against Gram-positive and Gram-negative bacteria, particularly, against pathogenic microorganisms, including bacteria associated with nosocomial infections (*P. aeruginosa, L. monocytogenes, S. aureus, K. pneumoniae, M. morganii, P. mirabilis*, etc.) and multiresistance (MRSA).

**Table 3:** Mushroom extracts with reported antibacterial activity against gram-positive and gram-negative bacteria [a]

| Mushrooms | Origin | Samples | Extracts | Activity against Gram (+) and (-) bacteria | Method | References |
|---|---|---|---|---|---|---|
| *A. bisporus* | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus* | MIC = 100 (-) | (Bach et al., 2019) |
| | | | | Gram (-) bacteria: *E. coli* and *S. enteritidis* | MIC = 150 (100-200) | |
| | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus* | MIC = 59.52 (-)<br>MBC = 59.52 (-) | (Fogarasi et al., 2020) |
| | | | | Gram (-) bacteria: *S. typhimurium*, *P. aeruginosa*, *E. coli* | MIC = 59.52 (-)<br>MBC = 59.52 (-) | |
| | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus* | MIC = 125 (50-200) | (Bach et al., 2019) |
| | | | | Gram (-) bacteria: *E. coli* and *S. enteritidis* | MIC = 200 (-) | |
| | Portugal | Commercial | Ethanol | Gram (+) bacteria: Enterococcus faecalis, MSSA and MRSA | MIC = 10 (-) | (Taofiq et al., 2016a) |
| | | | | Gram (-) bacteria: *E. coli* and *P. aeruginosa* | MIC = >20 | |
| *A. brasiliensis* | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus* | MIC = 75 (50-100) | (Bach et al., 2019) |
| | | | | Gram (-) bacteria: *E. coli* and *S. enteritidis* | MIC = 200 (-) | |
| *A. blazei* | Brazil | Commercial | Ethanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus* and *L. monocytogenes* | MIC = 0.575 (0.075-0.20)<br>MBC = 0.225 (0.10-0.40) | (Corrêa et al., 2018) |
| | | | | Gram (-) bacteria: *E. coli*, *S. enteritides*, *S. typhimurium*, *E. cloacae* | MIC = 0.138 (0.025-0.40)<br>MBC = 0.225 (0.05-0.60) | |
| *A. mellea* | Serbia | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *L. monocytogenes* and *M. flavus* | MIC = 7.19 (3.75-12.50)<br>MBC = 13.61 (6.25-21.7) | (Kostić et al., 2017) |
| | | | | Gram (-) bacteria: *P. aeruginosa*, *E. coli*, *S. typhimurium* and *E. cloacae* | MIC = 9.49 (3.12-15)<br>MBC = 16.95 (6.25-25) | |
| *B. edulis* | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus* | MIC = 20.92 (13.49-28.34)<br>MBC = 28.34 | (Fogarasi et al., 2020) |
| | | | | Gram (-) bacteria: *S. typhimurium*, *P. aeruginosa*, *E. coli* | MIC = 20.92 (13.49-28.34)<br>MBC = 28.34 (-) | |
| *C. cibarius* | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus* | MIC = 59.52 (-)<br>MBC = 59.52 (-) | (Fogarasi et al., 2020) |
| | | | | Gram (-) bacteria: *S. typhimurium*, *P. aeruginosa*, *E. coli* | MIC = 59.52 (-)<br>MBC = 59.52 (-) | |
| *F. velutipes* | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus* | MIC = 37.5 (25-50) | (Bach et al., 2019) |
| | | | | Gram (-) bacteria: *E. coli* and *S. enteritidis* | MIC = (-) | |
| *G. lucidum* | Portugal | Commercial | Ethanol | Gram (+) bacteria: *E. faecalis*, *L. monocytogenes*, MSSA and MRSA | MIC = 10 (5-20) | (Taofiq et al., 2017a) |
| | | | | Gram (-) bacteria: *E. coli*, *E. coli* ESBL, *K. pneumoniae*, *K. pneumoniae* ESBL, *M. morganii*, *P. aeruginosa* and *P. mirabilis* | MIC = 16 (1-20) | |
| *H. tessulatus* | Bangladesh | Commercial | Methanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | MIC = 7.5 (7-8) | (Chowdhury et al., 2015) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhi* and *K. pneumonia* | MIC = 7.25 (6-9) | |
| *L. piperatus* | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus* | MIC = 41.83 (26.99-56.68)<br>MBC = 56.68 (-) | (Fogarasi et al., 2020) |
| | | | | Gram (-) bacteria: *S. typhimurium*, *P. aeruginosa*, *E. coli* | MIC = 41.83 (26.99-56.68)<br>MBC = 56.68 (-) | |
| *L. edodes* | Bangladesh | Commercial | Methanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | MIC = 14.06 (1.56-12.50) | (Chowdhury et al., 2015) |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhi* and *K. pneumonia* | MIC = 3 (-) | |
| | Portugal | Commercial | Ethanol | Gram (+) bacteria: Enterococcus faecalis, MSSA and MRSA | MIC = 3.33 (2.5-5) | (Taofiq et al., 2016a) |
| | | | | Gram (-) bacteria: *E. coli* and *P. aeruginosa* | MIC = >20 | |
| | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus* | MIC = 100 (-) | (Bach et al., 2019) |
| | | | | Gram (-) bacteria: *E. coli* and *S. enteritidis* | MIC = 3 (2-4) | |
| *M. giganteus* | Serbia | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus* and *L. monocytogenes* | MIC = 0.23 (0.0125-0.60)<br>MBC = 0.48 (0.035-0.60) | (Stojković et al., 2017) |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium* and *E. cloacae* | MIC = 0.819 (0.025-2.50)<br>MBC = 1.72 (0.035-5.00) | |
| *M. conica* | Portugal | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus* and *L. monocytogenes* | MIC = 2.05 (0.70-3.75)<br>MBC = 3.99 (0.95-7.5) | (Vieira et al., 2016) |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium* and *E. cloacae* | MIC = 3.16 (0.70-5.62)<br>MBC = 4.23 (0.95-7.50) | |
| | Serbia | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus* and *L. monocytogenes* | MIC = 3.99 (0.95-3.75)<br>MBC = 7.97 (1.87-15.00) | |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium* and *E. cloacae* | MIC = 6.56 (3.75-7.5)<br>MBC = 13.13 (7.50-15.00) | |
| *M. esculenta* | India | Wild | Butanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | IZD = 11.5 (8-15) | (Shameem et al., 2017) |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa* and *P. vulgaris* | IZD = 14 (11-19) | |
| | | | Ethyl acetate | Gram (+) bacteria: *S. aureus* and *B. subtilis* | IZD = 11 (8-14) | |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa* and *P. vulgaris* | IZD = 8 (-) | |
| | | | Butanol | Gram (+) bacteria: *S. aureus* | MIC = 0.25 (-)<br>MBC = 0.75 (-) | |
| | | | | Gram (-) bacteria: *E. coli* and *P. aeruginosa* | MIC = 0.5 (0.25-0.75)<br>MBC = 0.75 (0.5-1) | |
| | | | Ethyl acetate | Gram (+) bacteria: *S. aureus* | - | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | Gram (-) bacteria: *E. coli* and *P. aeruginosa* | MIC = 0.75 (-)<br>MBC = 1.25 (-) | |
| *P. ostreatus* | Bangladesh | Commercial | Methanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | MIC = 6.5 (6-7) | (Chowdhury et al., 2015) |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhi* and *K. pneumonia* | MIC = 6.5 (5-8) | |
| | Portugal | Commercial | Ethanol | Gram (+) bacteria: Enterococcus faecalis, MSSA and MRSA | MIC =5 (2.5-10) | (Taofiq et al., 2016a) |
| | | | | Gram (-) bacteria: *E. coli* and *P. aeruginosa* | MIC = >20 | |
| | Portugal | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus*, *B. cereus* and *L. monocytogenes* | MIC = 0.32 (0.25-0.35)<br>MBC = 0.58 (0.35-0.70) | (Cardoso et al., 2021) |
| | | | | Gram (-) bacteria: *E. coli*, *E. cloacae* and *S. typhimurium* | MIC = 0.28 (0.25-0.35)<br>MBC = 0.47 (0.35-0.70) | |
| | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus* and *B. cereus* | MIC = 59.52 (-)<br>MBC = 59.52 (-) | (Fogarasi et al., 2020) |
| | | | | Gram (-) bacteria: *S. typhimurium*, *P. aeruginosa* and *E. coli* | MIC = 59.52 (-)<br>MBC = 59.52 (-) | |
| *P. sajor-caju* | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *E. faecalis*, *L. monocytogenes*, MSSA and MRSA | MIC = 10 (-) | (Finimundy, Barros, et al., 2018) |
| | | | | Gram (-) bacteria: *P. aeruginosa*, *E. coli*, ESBL, *K. pneumoniae*, *K. pneumoniae* ESBL and *M. morganii* | MIC = (-) | |
| *P. squamosus* | Portugal | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus* and *L. monocytogenes* | MIC = 2.00 (0.20-3.13)<br>MBC = 4.00 (0.39-6.25) | (Fernandes, Petrović, et al., 2016) |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimuriu* and *E. cloacae* | MIC = 2.05 (0.39-3.13)<br>MBC = 4.10 (0.78-6.25) | |
| | Serbia | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus* and *L. monocytogenes* | MIC = 1.23 (0.40-1.50)<br>MBC = 2.44 (0.75-3.00) | |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium* and *E. cloacae* | MIC = 0.80 (0.40-1.50)<br>MBC = 1.79 (1.14-3.00) | |
| | Romania | Wild | Methanol | Gram (+) bacteria: *B. cereus*, *M. flavus* and *S. aureus* | MIC = 10.6 (1.2-20.4)<br>MBC = 21.2 (2.4-40.8) | (Mocan et al., 2018) |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium*, *L. monocytogenes* and *E. cloacae* | MIC = 16.44 (0.61-20.4)<br>MBC = 32.8 (1.2-40.8) | |
| *R. integra* | Serbia | Wild | Ethanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis* and *S. warneri* | MIC = 4.61 (0.78-12.50)<br>MBC = 7.42 (1.56- 12.50) | (Kostić et al., 2020) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | Gram (-) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 7.81 (3.12-12.50)<br>MBC = 9.38 (6.25-12.50) | |
| | | | Methanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis* and *S. warneri* | MIC = 3.74 (0.39- 12.50)<br>MBC = 5.40 (0.78-12.50) | |
| | | | | Gram (-) bacteria: *E. cloacae* and *S. maltophilia* | MIC = (-)<br>MBC = (-) | |
| *R. nigricans* | Serbia | Wild | Ethanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis* and *S. warneri* | MIC = 3.66 (1.56-7.50)<br>MBC = 6.73 (3.12-12.50) | |
| | | | | Gram (-) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 3.91 (1.56-6.25)<br>MBC = 7.81 (3.12-12.50) | |
| | | | Methanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis* and *S. warneri* | MIC = 3.27 (0.39-12.50)<br>MBC = 5.01 (0.78-12.50) | |
| | | | | Gram (-) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 3.91 (3.12-12.50)<br>MBC = 6.25 (-) | |
| *R. rosea* | Serbia | Wild | Ethanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis* and *S. warneri* | MIC = 2.93 (0.20-12.50)<br>MBC = 4.26 (0.39-12.50) | |
| | | | | Gram (-) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 9.38 (6.25-12.50)<br>MBC = 12.50 (-) | |
| | | | Methanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis* and *S. warneri* | MIC = 3.41 (0.20-12.50)<br>MBC = 5.30 (0.39-12.50) | |
| | | | | Gram (-) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 6.25 (-)<br>MBC = 12.50 (-) | |
| *T. aestivum* | Spain | Wild | Ethyl acetate | Gram (+) bacteria: *L. monocytogenes* | IZD = 8.5 | (Tejedor-Calvo et al., 2021) |
| *T. gennadii* | Spain | | Methanol | Gram (-) bacteria: *S. flexneri* | IZD = 8.1 | |
| *T. magnusii* | Italy | | Water | Gram (+) bacteria: *S. aureus* and *M. luteus* | IZD = 13.9 (8.1-19.7) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *T. melanosporum* | Spain | | Water | Gram (+) bacteria: *S. aureus* | IZD = 8.7 | |
| *V. bohemica* | India | Wild | Butanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | IZD = 10.5 (9-12) | (Shameem et al., 2017) |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa* and *P. vulgaris* | IZD = 11.7 (9-15) | |
| | | | Ethyl acetate | Gram (+) bacteria: *S. aureus* and *B. subtilis* | IZD = 13.5 (12-15) | |
| | | | | Gram (-) bacteria: *E. coli*, *P. aeruginosa* and *P. vulgaris* | IZD = 11 (9-13) | |
| | | | Butanol | Gram (+) bacteria: *S. aureus* | MIC = 0.25 (-)<br>MBC = 0.5 (-) | |
| | | | | Gram (-) bacteria: *E. coli* and *P. aeruginosa* | MIC = 0.25 (-)<br>MBC = 0.75 (-) | |
| | | | Ethyl acetate | Gram (+) bacteria: *S. aureus* | - | |
| | | | | Gram (-) bacteria: *E. coli* and *P. aeruginosa* | MIC = 0.75 (-)<br>MBC = 1 (-) | |

[a] (-) MIC and MBC not found for the concentrations tested; MSSA- methicillin-sensitive *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*; ESBL- Extended spectrum β-lactamases; MIC/MBC – mg/mL; IZD - inhibition zone diameters (mm).

**Table 4** shows, different extracts obtained from several mushroom species that have been described in the literature as possessing antifungal activity. Stojković et al. (2017) reported antifungal activity of *M. giganteus* methanolic extracts against *A. fumigatus, A. versicolor, A. ochraceus, A. niger, T. viride, P. funiculosum, P. ochrochloron*, and *P. aurantiogriseum* with MIC and MFC means of 0.138 and 0.31 mg $mL^{-1}$, respectively. Ethanolic extract of *A. blazei* showed activity against *A. fumigatus, A. versicolor, A. ochraceus, A. niger, C. crusei, P. funiculosum*, and *P. verrucosum* var. cyclopium with MIC and MFC means of 0.75 and 0.94 mg $mL^{-1}$ (Corrêa et al., 2018). *P. ostreatus* hydroethanolic extract presented promising activity against some of the above mentioned fungal strains with MIC and MFC means of 0.30 and 0.49 mg $mL^{-1}$, respectively (Cardoso et al., 2021).

Most studies about antibacterial and antifungal activities of mushrooms describe the action of the extracts focused on screening, without identifying the compounds responsible for this activity (Alves et al., 2012). Prebiotics are substances that induce the growth or action of microorganisms that contribute to the well-being of their host. Particularly, mushrooms are considered a potential source of prebiotics as they contain phenolic compounds and several active polysaccharides, such as chitin, hemicellulose, $\beta$- and $\alpha$-glucans, mannans, xylans, and galactans, which make them the right choice for prebiotics. Different bioactive compounds from mushrooms have been shown to alter gut microbiota and improve health status. The gut microbiota comprises trillions of bacteria that contribute to nutrient acquisition and energy regulation. Current research indicates that diet is the most important factor as it significantly affects the composition, diversity, and richness of gut microbiota. The microorganisms present in the gut play an important role in the health of the digestive system and influence the immune system. The medicinal metabolites from mushrooms can act as immunomodulatory agents to activate gut microbiota (Jayachandran et al., 2017; Li et al., 2021; Yin et al., 2020). These bioactive ingredients can modulate the gut microbiota and improve body health through various regulatory mechanisms, including enriching microbiota diversity, promoting beneficial bacteria, increasing the bacteroides/firmicutes ratio, decreasing harmful bacteria, and increasing short-chain fatty acid-producing bacteria (Li et al., 2021).

**Table 4:** Mushroom extracts with reported antifungal activity

| Mushroom | Origin | Samples | Extracts | Activity against | Method | References |
|---|---|---|---|---|---|---|
| *A. mellea* | Serbia | Wild | Methanol | *A. niger, A. ochraceus, A. versicolor, A. fumigatus, T. viride, P. funiculosum, P. ochrochloron, P. verrucosum* var. *cyclopium* and *C. albicans* | MIC = 7.28 (6.25-15)<br>MFC = 12.81 (6.25-25) | (Kostić et al., 2017) |
| *A. blazei* | Brazil | Commercial | Ethanol | *A. fumigatus, A. versicolor, A. ochraceus, A. niger, C. crusei, P. funiculosum* and *P. verrucosum* var. *cyclopium* | MIC = 0.57 (0.20-0.80)<br>MFC = 0.94 (0.40-1.60) | (Corrêa et al., 2018) |
| *G. lucidum* | Portugal | Commercial | Ethanol | *C. albicans* | MIC = >20 | (Taofiq, et al., 2017a) |
| *H. tessulatus* | Bangladesh | Commercial | Methanol | *C. albicans* and *S. cerevisiae* | MIC = 5.5. (5-6) | (Chowdhury et al., 2015) |
| *L. edodes* | Bangladesh | Commercial | Methanol | *C. albicans* and *S. cerevisiae* | MIC = 1 (-) | |
| *M. giganteus* | Serbia | Wild | Methanol | *A. fumigatus, A. versicolor, A. ochraceus, A. niger, T. viride, P. funiculosum, P. ochrochloron* and *P. aurantiogriseum* | MIC = 0.138 (0.025-0.30)<br>MFC = 0.31 (0.050-0.40) | (Stojković et al., 2017) |
| *M. conica* | Portugal | Wild | Methanol | *P. aurantiogriseum, A. versicolor, A. niger* and *T. viride* | MIC = 3.59 (0.78-6.25)<br>MFC = 5.94 (1.56-12.5) | (Vieira et al., 2016) |
| | Serbia | | | | MIC = 9.06 (1.56-12.5)<br>MFC = 13.12 (3.12-25.00) | |
| *M. esculenta* | India | Wild | Butanol | *A. niger, C. albicans, C. kruesie, C. paraloposis* and *A. fumigates* | IZD = 17.25 (11-18) | (Shameem et al., 2017) |
| | | | Ethyl acetate | *A. niger, C. albicans, C. kruesie, C. paraloposis* and *A. fumigates* | IZD = 18 (-) | |
| *P. squamosus* | Portugal | Wild | Methanol | *A. fumigatus, A. ochraceus, A. versicolor, A. niger, P. funiculosum, P. ochrochloron, P. aurantiogriseum* and *T. viride* | MIC = 1.46 (0.78-3.13)<br>MFC = 2.93 (1.56-6.25) | (Fernandes et al., 2016a) |
| | Serbia | Wild | Methanol | *A. fumigatus, A. ochraceus, A. versicolor, A. niger, P. funiculosum, P. ochrochloron, P. aurantiogriseum* and *T. viride* | MIC = 0.71 (0.40-1.50)<br>MFC = 1.41 (0.75-3.00) | |
| *P. ostreatus* | Bangladesh | Commercial | Methanol | *C. albicans* and *S. cerevisiae* | MIC = 4 (-) | (Chowdhury et al., 2015) |
| | Portugal | Commercial | Hydroethanol | *A. fumigatus, A. ochraceus, A. niger, P. funiculosum, P. ochrochloron* and *P.v. cyclopium* | MIC = 0.30 (0.08-0.75)<br>MFC = 0.49 (0.35-0.70) | (Cardoso et al., 2021) |
| *V. bohemica* | India | Wild | Butanol | *A. niger, C. albicans, C. kruesie, C. paraloposis* and *A. fumigates* | IZD = 14 (9-22) | (Shameem et al., 2017) |
| | | | Ethyl acetate | *A. niger, C. albicans, C. kruesie, C. paraloposis* and *A. fumigates* | IZD = 10 (7-14) | |

MIC/MFC – mg/mL; IZD - inhibition zone diameters (mm).

*1.1.2.4. Immunomodulatory and cytotoxic properties*

The immune system is an essential foundation for building the body and is responsible for recognizing, drowning, and fighting against diseases and toxic microorganisms. Through what is called immunity of two types, acquired or innate immune systems, the immune system acts in multifaceted immune responses to control and eliminate harmful organisms or substances (**Figure 5**) (Sindhu et al., 2021; Yin et al., 2021). Immunomodulators are substances that work efficiently with the immunologic system to increase or decrease the host response against certain microorganisms (Nicoletti, 2021). In the immune system, macrophages are the first cells to recognize pathogens. When macrophages are activated, they can fight infectious agents directly by phagocytosis or indirectly by synthesizing and secreting nitric oxide (NO), reactive oxygen species (ROS), interleukins (IL), and tumour necrosis factor-α (TNF-α), which act as regulators of the immune system.

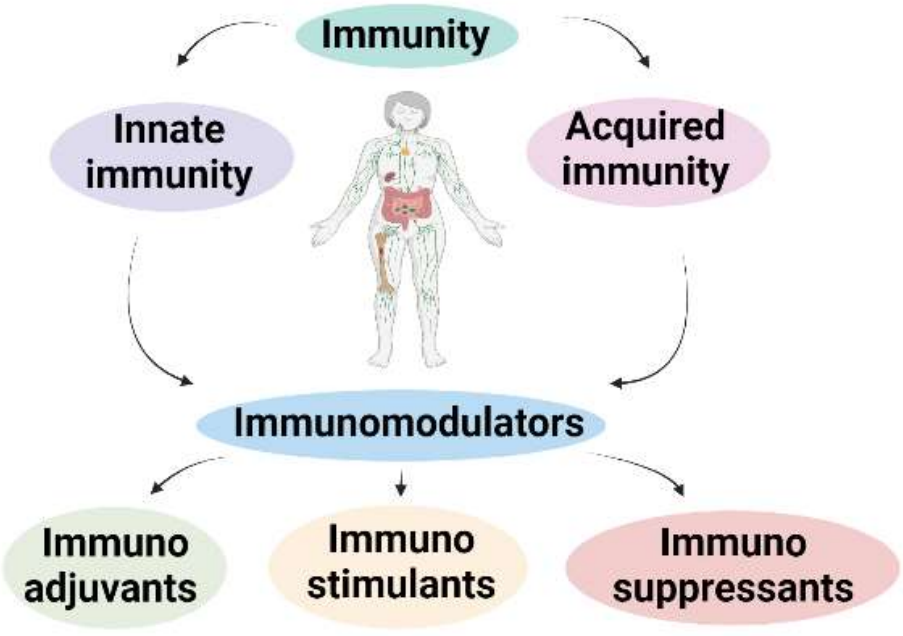



**Figure 5:** Immunity and Immunomodulators types

Mushrooms belonging to the Basidiomycota and Ascomycota phylum stand out for their anticancer and immunomodulatory properties. These include *Pleurotus ostreatus, Agaricus blazei Murrill, Polyporus umbellatus, Dictyophora indusiata, Lentinus edodes, Ganoderma lucidum, Inonotus obliquus, Boletus edulis, Grifola frondosa, Calocybe indica, Hericium erinaceus, Phellinus linteus, Ganoderma formosanum, Sparassis crispa,* and *Cordyceps militaris,* among others (**Table 5**) (Hyder & Dutta, 2021; Meng et al., 2016). The anticancer

activities of mushroom polysaccharides (**Table 5**) have been extensively studied in recent years. The activation mechanisms of these polysaccharides against cancer cells involve a complex sequence of responses, inducing both innate and adaptive immune systems (Kothari et al., 2018). Polysaccharides obtained from natural matrices possess immense beneficial properties, such as antitumour and immunomodulatory activities. They help the body resist diverse biological tensions and increase immunity against the progression of cancer cells, thus boosting immunity by stimulating the central biological systems (Dwi et al., 2022). Immunomodulatory polysaccharides have been reported to present beneficial effects on the regulation of macrophage immune function (Dwi et al., 2022). When activated by mushroom polysaccharides, the innate immune system stimulates the body's defence mechanisms by activating macrophages, T-lymphocytes, *β*-lymphocytes, cytotoxic T-lymphocytes (CTL), and natural killer cells to produce cytokines such as tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin 1 beta (IL-1β), and they have invariable antiproliferative activity (Dwi et al., 2022). Edible mushrooms have been much highlighted because of their immense benefits and bioactive compounds, and principally their immunomodulatory activity. Additionally, some species have been studied recently for their immunomodulatory and cytotoxic properties. Polysaccharides of mushrooms are found principally in the cell walls, in the fruiting bodies where they concentrate the most significant quantity (Yan et al., 2018; Zhao et al., 2020a), and glycogen, cellulose, and starch are the most commonly occurring polysaccharides (Navarro et al., 2019). *β*-Glucans are glucose polymers linked up together through 1,3 linear *β*-glycosidic chains and are essential active constituents derived from mushrooms. Furthermore, depending on the source of β-glucans, variations in chain size occur, and many of these variations will determine their bioactive properties. Therefore, because of their health benefits, there has been growing interest in their use in treating disease (R. Kaur et al., 2020; Wani et al., 2021). Edible mushrooms are more aptly considered nutraceutical foods, and nowadays, there is great interest in the medicinal use of these mushrooms that have bioactive compounds with anti-inflammatory, antidiabetic, anticancer, immunomodulatory properties, among others (Lin et al., 2021; Nowakowski et al., 2021; Patra et al., 2021). The polysaccharide from *Pleurotus citrinopileatus* revealed antitumor activity on H22 tumour-bearing mice (Lin et al., 2021; Nowakowski et al., 2021; Patra et al., 2021). *Coprinus comatus* and *Lactarius deliciosus* showed an inhibitory effect on U87MG and LN-18 glioblastoma cells viability (Nowakowski et al., 2021). *Grifola frondosa* contains promising immunomodulatory agents and effective antitumour polysaccharides (Zhao et al.,

2021). The polysaccharide of *Agaricus bitorquis* shows an immunomodulatory effect on RAW264.7 (Lin et al., 2021). *Pholiota nameko* induces apoptosis of human breast adenocarcinoma (Takahashi et al., 2019). Polysaccharides from *Lactarius volemus* were investigated for antiproliferative and immunomodulatory activities on RAW264.7 murine macrophages, human lung cancer cells H1299, and human breast cancer cells MCF-7 (Zhong et al., 2021). More studies involving mushrooms and their immunomodulatory and cytotoxic properties are presented in **Table 5**.

**Table 5:** Immunomodulatory and cytotoxic effect of mushrooms extracts and their compounds

| Species | Compounds | Experimental models | Immunomodulatory/cytotoxic Effects | References |
|---|---|---|---|---|
| *A. auricula-judae* | Polysaccharide | RAW 264.7 cells | Promote the release of NO and secretion of TNF-α, IL-6 and IL-10 | (Zhang et al., 2018a) |
| *A. bitorquis* | Polysaccharide | RAW 264.7 cells | Promoted viability and phagocytic ability, increasing levels of NO, ROS, TNF-α | (Lin et al., 2021) |
| *A. bisporus* | Polysaccharide | SW 620 cells | Cell growth inhibition | (Sriramulu et al., 2020) |
| *A. bisporus* and *L. edodes* | - | PC3 cells | Decreased nuclear and total NF-κB activity/decreased the proliferation of cells | (French et al., 2019) |
| *A. blazei* | - | MIAPaCa-2, PCI-35, and PK-8 cells | Inhibited cell proliferation and upregulated of pro-apoptotic | (Matsushita et al., 2018) |
| *A. blazei, H. erinaceus and G. frondosa* | - | KG1a, HL 60, Meg 01, INA-6, RPMI-8226 and U226 cells | Caused a cytotoxic and immunomodulatory effect in cells | (Tangen et al., 2017) |
| *A. camphorata* | - | Mice, PLC/PRF/5, HepG2, SMMC-7721, Huh7, MHCC 97, MHCC 97L, HCCLM3 and MIHA cells | Downregulated protein levels of phosphorylated and total STAT3 and JAK2 in cells/reduced cell viability, induced apoptosis, and retarded migration and invasion in cultured cells | (Zhu et al., 2018) |
| | - | SW620 and SW480 cells | Inhibitory effect on emigration and invasion of cancer cell | (Hseu et al., 2017) |
| | - | B16-F0 cells | Exhibited cytotoxic effect and inhibited the migration ability of cells. | (Wang et al., 2017a) |
| *A. cinnamomea* | - | Mice, LLC and CL1-5 cells | Inhibited tumour growth and metastasis | (Huang et al., 2019) |
| *A. cinnamomea* | - | Mice and T47D cells | Inhibited cells proliferation, induced autophagy, decreased tumour volume and inhibited tumour growth | (Chen et al., 2019) |
| | | | | |
| *A. mellea* | Polysaccharide | RAW264.7 cells | Increases phagocytosis of macrophages and induces secretion of NO, ROS, TNF-α, IL-1β and IL-6 | (Yan et al., 2018) |
| *B. edulis* | Phenolic and flavonoid | MCF-7 cells | Shoed antiproliferative effects | (Novakovic et al., 2017) |
| | - | MCF-7 cells | antiproliferative effects against the cell line | (Novakovic et al., 2017) |
| *C. cibarius, C. comatus, L. perlatum and L. delicious.* | - | SVGp12, U87MG and LN-18 cells | Inhibition of proliferation and induction of apoptosis | (Nowakowski et al., 2021) |

| | | | | |
|---|---|---|---|---|
| *C. militaris* | Polysaccharide | RAW 264.7 cells | Release of NO, TNF-α, IL-6, and IL-10, and mainly induced M1 polarization of macrophages | (Zhang et al., 2020a) |
| | Polysaccharide | Mice, splenic lymphocytes, RAW264.7 cells, YAC-1 lymphoma cell, and Natural killer (NK) cells | Promoted the proliferation of lymphocytes and promoted lymphocyte secretion of the NO, TNF-α, and IL-2, strengthened the phagocytosis of macrophages and induced M1 polarization/ enhanced cytotoxicity of NK cells | (Bi et al., 2018) |
| *C. versicolor* | Polysaccharide | RAW 264.7 cells | Induction of NO production and induced nitric oxide synthase (iNOS) and TNF-αmRNA expression level | (Zhang et al., 2021) |
| *C. radicata* | Polysaccharide | RAW 264.7 cells | Improved the proliferation and phagocytosis and induced the secretion of NO, iNOS, TNF-α, IFN-γ, IL-6, IL-1β and IL-10 | (Wang et al., 2018a) |
| *C. versicolor and L. edodes* | Polysaccharide | Peripheral blood mononuclear cells | Reduction of Th2 cytokines and IL-10 in cells cultures | (Sknepnek et al., 2021) |
| *F. velutipes* | Polysaccharide | RAW 264.7 cells | Regulate the expression of NO, TNF-α, IL-6 | (Ye et al., 2020) |
| *G. frondosa* | Polysaccharide | Mice and H22 hepatoma cells | Increasing the percentage of $CD4^+CD8^+$ T cells, B cells and Treg cells and the secretion of IL-2, IL-12p70, TNF-α and IFN-γ/inhibit liver tumours growth | (Zhao et al., 2021) |
| *G. lucidum* | Polysaccharide | Mice, MDA-MB-231 and 4T1 cells | Significantly suppressed the growth of tumours | (Jiao et al., 2020) |
| | Polysaccharide | Mice | Produced better protection of the spleen and thymus, promoting haematopoiesis, and improving IgA levels in serum | (Li et al., 2020) |
| | Polysaccharide | GBM8901, U87, MDA-MB-231 and 4T1 cells | Inhibited the growth of cancer cells, inducing apoptosis, inhibited cell migration and cell cycle arrest | ( Cheng et al., 2020; Jiao et al., 2020) |
| | Polysaccharide | Mice | Protect spleen and thymus and promoting haematopoiesis, and improve IgA levels | (Li et al., 2020) |
| *G. neo-japonicum* | Terpenoid and sterol | CCD-18Co, HCT 116 and HT 29 cells | Exerted a potent cytotoxic effect on colonic carcinoma cells | (Lau et al., 2020) |
| *G. applanatum* | - | SEC and Caco-2 cell | Decrease in the volume of the tumour mass and tumour growth | (Elkhateeb et al., 2018) |
| *G. atrum* | Polysaccharide | T lymphocytes | Increased the secretion of IL-2, IFN-γ, and IL-12 | (Xiang et al., 2017) |
| *G. leucocontextum* | Polysaccharide | RAW 264.7 cells | Activating mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K)/Akt and nuclear factor- kappa B (NF-κB) signalling pathways | (Gao et al., 2020) |
| *G. tsugae* | - | AN3 CA, HEC- 1-A and KLE cells | Inhibited cell proliferation, induced cell cycle arrest and inhibited the Akt signalling pathway | (Tsai et al., 2021) |

| | | | | |
|---|---|---|---|---|
| *G. frondosa* | Polysaccharide | Mice | Improve or reverse the CTX-induced immunosuppression, enhance the spleen and thymus indices, spleen lymphocyte proliferation and cytokines production | (Lia et al., 2018) |
| | - | SMMC-7721 cells | Reduction of cell viability and induction of cell apoptosis | (Zhao et al., 2017a) |
| | Polysaccharide | RAW 264.7 cells | Increased TNF-α, IL-6, IFN-γ, MIP-1β, MIP-1α and MIP-2 levels | ( Meng et al., 2017) |
| *G. lucidum* | Polysaccharides and triterpenes | Mice, SNU719 and MKN1-EBV cells | Induced lytic reactivation of the Epstein-Barr virus - risk factor of gastric cancer/ induced cancer cell apoptosis | (Huh et al., 2019) |
| | Polysaccharide | Rats/ RG2 glioma cells and human myelogenous leukaemia cells K562 | Increase the concentration of serum IL-2, TNF-α, and INF-γ/enhance the cytotoxic activity of NK cells and T cells in glioma-bearing rats. | (Wang et al., 2018b) |
| | - | Mice, HEK293T and HEK293FT Cells, MDA-MB-231 cell and 4T1 cell | Inhibited Wnt-induced hyper-proliferation of cancer cells | (Zhang, 2017a) |
| | Triterpenoids | Mice and HCT16 cells | Inhibited cell migration, proliferation and xenograft tumour growth | ( Li et al., 2017) |
| *H. erinaceus* | Polysaccharide | Human monocytic cell lines THP-1 | Enhance the levels of TNF-α, IL-1β and IL-6 and induce the proliferation of lymphocytes | (Wu et al., 2019) |
| *I. baumii* | - | SMMC-7721 cells | Induce apoptosis and autophagy in tumours cells, and inhibit the growth of tumours | (Yang et al., 2019) |
| *I. obliquus and P. linteus* | - | Mice, yeast, RAW264.7, A2780, HeLa, AML1, S18, and CL-81 cells | Enhanced the phagocytosis of macrophages/inhibitory effect on tumour development and progression | (Fang et al., 2020) |
| *L. edodes* | Polysaccharide | Mice and MCF-7 cells | Induction of apoptosis | (Weiyong Li et al., 2018) |
| | - | HEp-2 and MRC-5 cells | Inhibition of cell proliferation and induced depolarization of mitochondria | (Finimundy, Scola, et al., 2018) |
| *L. volemus* | Polysaccharide | RAW 264.7, H1299 and MCF-7 cells | Induced the secretion of NO, IL-6 and TNF-α/inhibit the proliferation of cells | (Zhong et al., 2021) |
| *L. tigris* | - | Mice and MCF-7, A549, PC3, and 184B5 cells | Show antiproliferative effect and inhibited the growth of cells | (Kong et al., 2020) |
| *M. importuna* | Polysaccharide | RAW 264.7 and 4T1 cells | Promote the secretion of TNF-α, IL-6 and NO and enhance the phagocytosis of macrophages/enhance the antitumour activity of doxorubicin (DOX) and inhibit the growth of tumours | (Peng et al., 2021; Y. Wen et al., 2021) |
| *M. oreades* | - | MCF-7, MDA-MB-231 and HT-29 cells | Inhibited all human cancer cell lines | (Shomali et al., 2019) |

| | | | | |
|---|---|---|---|---|
| *M. giganteus* | - | Jurkat and HL- 60 cells | Induced apoptosis in Jurkat and HL-60 cell and induced cell-cycle arrest | (Lenzi et al., 2018) |
| *M. esculenta* | Fatty acids and sterols | A549, H1264, H1299 and Calu-6 cells c | Induction of apoptosis | (Lee et al., 2018) |
| *P. highking* | - | MCF-7 cells | Reduced the proliferation and viability of cells and reduced size and number of the tumour spheres | (Haque & Islam, 2019) |
| *P. nameko* | Polysaccharide | Mice and MCF-7 cells | Activated the death receptor pathway and mitochondrial apoptosis pathway of /inhibit malignant proliferation of tumours | (Zhang et al., 2020b) |
| *P. eryngii* | Polysaccharide | THP-1 cells | Increasing the secretion of IL-1β, IL-10 and NO | (Abreu et al., 2021) |
| | Protein | Mice, CCD-18Co, HCT116 and MC38 cells | Suppressed tumour development and proliferation induced cell cycle arrest and led to cellular apoptosis | (Yuan et al., 2017) |
| | Polysaccharide | Mice | Increased concentrations of TNF-α, IFN-γ, IL-1, IL-2 and IL-6 | (Ma et al., 2017) |
| *P. highking* | - | MDA-MB-231 and HCC-1937 cells | Reduced the number and size of the tumour-spheres, suppressed the migratory ability of the cells, and reduced the mRNA expression of Ki-67, MMP-9, and vimentin in the treated tumour-sphere cells. | (Haque et al., 2020) |
| *P. ostreatus* | - | KG-1 and Jurkat cells | Inhibited cell proliferation, migration, expression and promoted apoptosis | (Ebrahimi et al., 2018) |
| *P. ostreatus* | - | KG-1 cells | Induced apoptosis and increased anticancer effects | (Ebrahimi et al., 2018) |
| *P. ferulae* | - | Mice, H22 and HepG2 cells | Inhibited the growth of cells, increased ROS generation and suppressed migration | (Yang et al., 2018a) |
| *P. sajor-caju* | - | HCT116 and MRC-5 cells | Induced apoptosis and cell cycle arrest | (Finimundy, Abreu, et al., 2018) |
| *P. grammocephalus* | Polysaccharide | Mice and RAW 264.7 cells | Activation of macrophage and splenocyte and thymocyte stimulatory properties | (Patra et al., 2021) |
| *P. rhinocerus* | Polysaccharide | Murine immature BMDCs | Induce the BMDC maturation, upregulated the expression of membrane phenotypic marker, stimulate the release of MIP-1α, MIP-2, and IL-2, and upregulation of the expression of IL-2, IL-6, MIP- 1α, MIP-2, RANTES, IL-12p40p70, IL-12p70, TIMP-1, IFN-γ, KC, MCP-1, and GCSF | (Marrow et al., 2019) |
| *S. imbricatus* | - | Mice and MDA-MB-231, MDA-MB-468, MCF-7, MT-1 and 4T1 cells | Inhibited the proliferation and invasive properties of breast cancer cells and decreased the tumour volume and weight in mice | (Tan et al., 2020) |

| | | | | |
|---|---|---|---|---|
| *S. commune* | Polysaccharide | RAW264.7 cells | Produced NO and cytokines by upregulating mRNA expression levels | (Yelithao et al., 2019) |
| *T. lobayense* | Polysaccharide | RAW264.7 cells | Enhances the phagocytic uptake capacity, increase NO production, Increases secretion of IL-6 and TNF-α | (Zhang et al., 2018b) |
| *T. versicolor* | Polysaccharide | Hela cells | Induction of cell death by apoptosis | (Wang et al., 2017a) |
| *T. heimii* | Polysaccharide | Mice, Vero and HCT cells | Diminished hyperplasia in 1, 2-dimethylhydrazine (DMH) induced colon cancer | (Singha et al., 2021) |

#### *1.1.2.5. Cardioprotective properties*

Cardiovascular diseases (CVD) are among the leading cause of death in the world, afflicting the heart and blood vessels. They include high blood pressure, coronary heart disease, cerebrovascular disease, atherosclerosis, heart failure, and peripheral vascular disease (Corrêa et al., 2017). Diets rich in functional ingredients such as antioxidants, dietary fibre, omega-3 polyunsaturated fatty acids (PUFAs), vitamins, minerals, and phenolic compounds have been associated with a lower risk of cardiovascular disease in numerous studies (Caz et al., 2016; Gil-Ramírez et al., 2016). Cholesterol has long been considered a significant risk factor for heart diseases. Plant sterols, also known as phytosterols, are found in several plant species and have been shown to compete with dietary cholesterol by inhibiting absorption and enabling its elimination from the body (Corrêa et al., 2017). Mycosterols including ergosterol (ergosta-5,7,22-trien-3β-ol), ergosta-5,8,22- trien-3-ol, ergosta-7,22-dien-3-ol, ergosta-5,7-dien-3-olm and fungisterol (ergosta-7-en-3-ol) either occurring in their free or esterified forms have gained prominence in recent years due to their well-reported beneficial effects on human health (Heleno et al., 2017; Taofiq et al., 2019a). Because of their structural similarities to the dietary mixed micelles (DMM) during *in vitro* digestion, these biomolecules have a cholesterol-lowering impact by competing with cholesterol. In simulated gastrointestinal digestion utilizing Caco-2 cells, ergosterol-rich extracts produced by supercritical fluid extraction (SFE) from *Agaricus bisporus* were found to successfully displace cholesterol (up to 67%) from the DMM (Gil-Ramírez et al., 2013). The extract mentioned above's precise mechanism of action was linked to a significant reduction in mRNA expression of sterol regulatory element-binding protein 2 (SREBF2), a protein-coding gene that regulates the expression of numerous genes involved in cholesterol homeostasis, absorption, and biosynthesis (Gil-Ramírez et al., 2016). A diet supplemented with an antioxidant-rich extract from *A. brasiliensis* was reported to significantly reduce the total cholesterol level *in vivo* and downregulate mRNA expression of cholesterol metabolism genes, including SREBP-2, HMG-CoA reductase, LDLR, and PPAR-α (de Miranda et al., 2017). The effect of ergosterol peroxide derived from *Ganoderma lucidum* on SREBP-1 mRNA expression in 3T3-L1 preadipocyte cells has also been described (Y. U. Jeong & Park, 2020). The cholesterol-lowering effect of lard functionalized with extracts from *P. ostreatus* and *L. edodes* rich in *β*-glucans, water-soluble polysaccharides, and ergosterol was analysed (Caz et al., 2016). A significant reduction (up to 22 - 42%) in plasma levels of total cholesterol was observed in the studied *in vivo* model. However, mRNA expressions of cholesterol-related genes (NPC1L1 and ABCG5) in the jejunum, cecum, and liver were not

suppressed. Although the precise molecular mechanism is still not completely defined, the cardioprotective effect was associated with hindering the bioavailability of cholesterol and inhibiting the expression of several genes responsible for cholesterol absorption and metabolism in intestinal models (de Miranda et al., 2017)·(Y. U. Jeong & Park, 2020).

Atherosclerosis is a multifaceted disease that contributes to the pathogenesis of CVDs, characterized by endothelial dysfunction, lipid deposition, inflammatory cell infiltration, smooth muscle cell proliferation, intravascular wall neoangiogenesis, and plaque development (Hsu et al., 2018). Although the exact cause of atherosclerosis is unknown, persistent inflammation of the artery walls triggered by procoagulant proteins, proinflammatory cytokines, metalloproteinases, and growth factors have all been identified as major contributions to the disease's aetiology (Hsu et al., 2018; Kim et al., 2019). Commonly consumed mushrooms, such as *A. bisporus*, *L. edodes*, and *P. ostreatus*, abundant in nutrients, soluble dietary fibres, and several health-promoting bioactive compounds, are beneficial in suppressing inflammation, improving lipid profiles, and ameliorating heart disease and atherosclerosis (Kim et al., 2019). The potential of *A. bisporus* (portobello) and *L. edodes* extracts, rich in ergothioneine, in ameliorating atherosclerosis was conducted *in vivo*. The results showed a significant decrease in plasma lipid concentrations (cholesterol and triglycerides), mRNA expression of vascular cell adhesion molecule 1 (VCAM-1) and mean aortic lesion area (Kim et al., 2019). *In vivo* studies involving the infusion of angiotensin II in a mouse model and subsequent supplementation with a diet rich in *Grifola gargal* extract presented a significant decrease in the weight of the heart, vessels, and atheromatous lesions. In addition, the extract also induces the secretion of transforming growth factor beta (TGF-β) vascular endothelial growth factor (VEGF), both vital signalling molecules responsible for promoting angiogenesis (Harada et al., 2015). Several studies have shown that mushroom extracts and their associated bioactive molecules possess antioxidant, anti-inflammatory, and lipid-lowering effects that can potentially slow down the progression of atherosclerosis, thereby potentiating a cardioprotective effect (Krittanawong et al., 2020). The mechanism behind these cardioprotective properties of mushroom extracts remains obscure but it has been attributed to suppression of mRNA expression of several genes related to cholesterol absorption and metabolism, decreased oxidative damage due to their potent radical scavenging and metal chelating effect, and downregulation of the expression of inflammatory mediators (de Miranda et al., 2017; S. H. Kim et al., 2019). To the best of our knowledge, no studies have been conducted in clinical settings to evidence the high therapeutic potential of these

mushroom metabolites against any cardiovascular diseases. Hence, more work still needs to be performed to determine these bioactive ingredients' clinical effectiveness and potential toxicity effect.

#### *1.1.2.6. Antidiabetic properties*

Diabetes mellitus (DM) is a chronic metabolic disease and increasing millions of people worldwide suffer from it and its complications. DM is one of the most common life-threatening endocrine diseases caused by defects in insulin secretion, insulin resistance, or both (Furman et al., 2020; Nweze et al., 2020). There are different types of DM: type 1, earlier recognized as insulin-dependent DM, where hormonal insulin is not produced due to damage to pancreatic $\beta$-cells, and people with this type of DM need daily insulin administration (Bharti et al., 2018; Ismail Iid et al., 2020); type 2 diabetes, previously known as non-insulin-dependent DM, is more predominant and is recognized by a progressive reduction in insulin secretion by pancreatic $\beta$-cells and a relative diminution in the sensitivity of target tissues to the action of this hormone. Type 2 DM can be prevented or delayed by lifestyle intervention (Khursheed et al., 2020). Other forms of DM include gestational DM, which occurs due to any degree of glucose intolerance with it beginning or first being recognized during pregnancy (Gulati et al., 2019); other types of DM can also be induced by genetic defects, endocrine disorders, other types of diseases, therapy, and malnutrition (American Diabetes Association, 2021).

DM is an irreversible disease with no cure, however glycaemic control is fundamental for both the prevention of diabetic complications and the reduction of mortality. In uncontrolled DM, hyperglycaemia is a common occurrence and eventually leads to infections, serious injuries, dysfunction, and multiple organ failure, particularly in the heart, kidneys, eye, blood vessels, and nerves (Asrafuzzaman et al., 2017; Ogunlana et al., 2021). Depending on the nature of DM, insulin and other synthetic medicines are widely used in its treatment. However, with prolonged medicine administration, some insulin resistance and side effects cases may develop, which leads to the search for more natural, safe, and efficient alternatives (Kazeem & Davies, 2016; Prabhakar, 2020).

The treatment of DM and its chronic and vascular complications have a significant economic impact on people's lives and the wider economy. However, the best weapon is the prevention of DM, focusing on avoiding or delaying its onset and complications with dietary control and exercise (Bharti et al., 2018). Thus, with concern to treating this disease, the key is in identifying the individually targeted factors that can lead to better glycaemic control. In

addition to regular oral and injectable medications, natural product-based therapy is highly indicated because of its efficiency, few adverse effects, and low cost. Thus, due to the benefits of current antidiabetic medicines, the acceptance and use of these natural products are increasing dramatically (Bharti et al., 2018).

Natural products, including mushrooms, are of great help in managing DM (Ismail Iid et al., 2020; Necyk & Zubach-Cassano, 2017) (**Figure 6**). They have been reported to potentially control DM by reducing oxidative stress, mimicking insulin action, increasing insulin secretion and activity, regulating glucose transport type 4, glucose uptake inhibiting a-amylase and a-glucosidase, and protecting pancreatic *β*-cells (Khursheed et al., 2020; Stojkovic et al., 2019). Over the years, several bioactive compounds have been isolated from mushrooms (**Table 6**), such as polysaccharides, dietary fibre, and other compounds of therapeutic interest such as lectins, lactones, terpenoids, alkaloids, metal chelating agents, and also several enzymes such as laccase, superoxide dismutase, glucose oxidase, and peroxidase, with favourable effects on glucose homeostasis (Deveci et al., 2021; Khursheed et al., 2020; Prabhakar, 2020) (**Figure 6**). In addition, mushrooms have been prestigious as a source of these compounds and have been highly valued as potent sources of antidiabetic and hypglycaemic compounds, promising in treating diabetes as biological antihyperglycaemic agents (**Table 6**) (Deveci et al., 2021; Khursheed et al., 2020; Prabhakar, 2020).

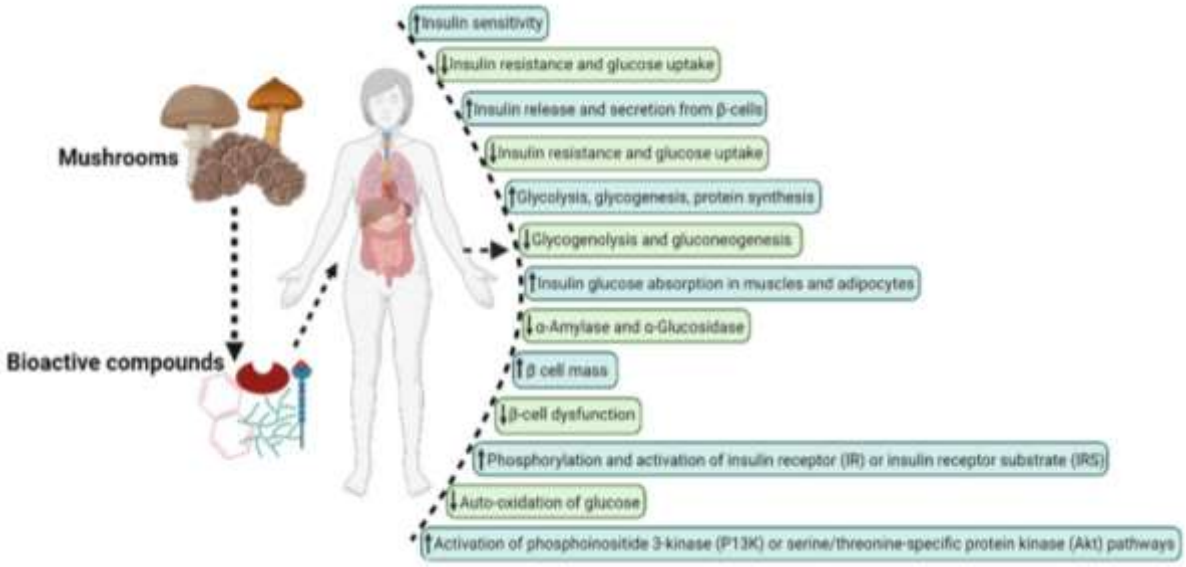



**Figure 6:** Favourable effects of mushroom bioactive compounds on DM

Several species of mushrooms are known for their hypoglycaemic or antidiabetic activities (**Table 6**), including *Agaricus bisporus* (Balakrishnan & Loganayagi, 2018), *Agaricus*

*subrufescens* (Henriques et al., 2016), *Auricularia auricular* (A. Lu et al., 2018; H. Xiang et al., 2021), *Antrodia cinnanomea* (Chung et al., 2018), *Coprinus comatus* (Ratnaningtyas et al., 2019), *Cordyceps militaris* (Kim et al., 2017), *Ganoderma lucidum* (Shaher et al., 2020), *Grifola frondosa* (Guo et al., 2020a), *Inonotus obliquus* (Wang et al., 2017c), *Lentinus edodes* (Afiati et al., 2019), *Pleurotus ostreatus* (Agunloye & Oboh, 2021), *Pleurotus eryngii* (Zhao et al., 2020b), *Pleurotus citrinopileatus* (Sheng et al., 2019), *Pleurotus pulmonarius* (Balaji et al., 2020), and *Wolfiporia extensa* (Wansen Li et al., 2020). The bioactive compounds in mushrooms have significantly contributed to regulating and managing various diseases, including DM. Thus, the various mushrooms presenting health benefits provide an opportunity to plan and develop new formulations and therapies for DM.153 However, extensive studies are needed to investigate the untapped mushrooms and extract and purify antidiabetic biomolecules that can be used to treat and manage DM.

**Table 6:** Antidiabetic effect of mushroom extracts on rodents

| Samples | Parts/components | Antidiabetic effect | References |
|---|---|---|---|
| *A. auricula* and *A. polytricha* | Polysaccharides | Reduction of fasting blood glucose, promotion of insulin synthesis and the reduction of the level of plasma glucose | (Xiang et al., 2021) |
| *A. auricular* | Polysaccharides | Reduced HbA1 serum levels | ( Hu et al., 2017) |
| | Dried fruiting body /polysaccharides | Reduction of blood glucose level | (Lu et al., 2018) |
| *A. bisponus and P. ostreatus* | Powder | Decrease in serum glucose level | (Nweze et al., 2020) |
| *A. bisporus* | Ethanol and methanol extract | Decrease in the levels of glucose | (Balakrishnan & Loganayagi, 2018) |
| *A. cinnamomea* | Mycelium powder | Decreased plasma glucose levels and improved insulin resistance | (Chung et al., 2018) |
| | | | |
| *C. militaris* | Acidic-extractable polysaccharides | Decreased blood glucose, improved glucose and insulin resistance, enhanced antioxidant enzyme activities and attenuated the injuries of the liver, kidney, and pancreas | (Zhao et al., 2018) |
| *C. comatus* | Ethanol extract | Reduced the blood glucose, glycosylated haemoglobin (HbA1c) levels and increased the plasma insulin level | (Ratnaningtyas et al., 2019) |
| *C. gigantea* | Methanol extract | Reduced the blood glucose level | (Ogbole et al., 2019) |
| *C. ventricosum* | Mycelial polysaccharides | Decrease blood glucose levels | (Yuntao Liu et al., 2017) |
| *C. rutilus* | Ethanol extract | Decrease blood glucose concentration | (Zhang et al., 2017b) |
| *G. frondosa* | Polysaccharide-chromium (III) | Regulated the mRNA expression related to glucose | (Guo et al., 2020a) |
| | Polysaccharides | Regulated mRNA expression levels of the genes responsible for hepatic glucose metabolism | (Guo et al., 2020b) |
| | Polysaccharides | Decrease in fasting blood glucose levels and reducing the inflammatory factor content, and preventing renal fibrosis | (Tao Jiang et al., 2020) |
| | Polysaccharides | Decreased fasting blood glucose levels and glucose tolerance | (Guo et al., 2019) |
| | Mycelium/polysaccharides | Reduced fasting blood glucose levels | (Kou et al., 2019) |
| | Extracts | Improved glucose intolerance | (Aoki et al., 2018) |
| | Heteropolysaccharide | Improved blood glucose levels and glucose intolerance | (Chen et al., 2018) |
| | Polysaccharides | Decreased the fasting blood glucose level, improved oral glucose tolerance, alleviated insulin resistance, and protected against liver and kidney injury | (Chen et al., 2019) |
| *G. lucidum* | Polysaccharides | Decreases in the levels of fasting blood glucose and insulin | (Chen et al., 2020) |
| | Spores | Reduced the blood glucose and oxidative stress | (Shaher et al., 2020) |

| | | | |
|---|---|---|---|
| | Beta-glucans | Decrease in blood glucose levels | (Bach et al., 2018) |
| | Ethanol extract | Decreases blood glucose level, increase insulin level, and decrease HbA1c level. | (Ratnaningtyas et al., 2018) |
| *G. pfeifferi* and *G. resinaceum* | Aqueous and ethanol extracts/polysaccharides | Prevented significant increase in glycaemia values | (Rašeta et al., 2020) |
| *I. obliquus* | Polysaccharides | Decreased fasting glucose level, an improvement on the liver, kidney, pancreas, glucose tolerance capacity, and increase of hepatic and muscle glycogen | (Wang et al., 2017c) |
| *I. obliquus* | Polysaccharides | Reduce fasting blood glucose levels, improve glucose tolerance ability, increase hepatic glycogen level and ameliorate insulin resistance | (Wang et al., 2017d) |
| *L. edodes* | Powder | Increase in maternal insulin levels | (Laurino et al., 2019) |
| *L. edodes* | β-glucans extract | Reduce blood glucose levels | (Afiati et al., 2019) |
| *L. edodes* | Freeze-dried powder | Improved the glucose tolerance, potentiating insulin secretion, and reduced insulin resistance | (Yang et al., 2018b) |
| *L. rhinocerotis* | Freeze-dried powder | Reducing elevated blood glucose concentrations | (Nyam et al., 2017) |
| *M. conica* | Methanol extract | Reducing elevated blood glucose and improved liver and kidney damages | (Begum et al., 2021) |
| *P. ostreatus* | Ergosterol | Improved insulin resistance, reducing fasting blood glucose levels and protecting pancreas and liver | (Xiong et al., 2017) |
| | Powder | Reduction of the serum glucose level | (Asrafuzzaman et al., 2018) |
| *P. ostreatus* and *L. subnudus* | Dried mushrooms | Reduction on fasting blood glucose, the activity of α-amylase, α-glucosidase, and angiotensin-1 converting enzyme | (Agunloye & Oboh, 2021) |
| *P. cocos* | Gold-nanoparticles | Reduced blood glucose status | (Wansen Li et al., 2020) |
| *P. linteus* | Extract/polysaccharides | Improves insulin resistance | (Yangyang Liu et al., 2020) |
| *P. pulmonarius* | Mycelia | Reducing blood glucose levels, decreasing injuries in the pancreas, liver, and kidney | (Balaji et al., 2020) |
| *P. eryngii* | Polysaccharides | Showed renoprotective effects, decreasing glucose levels and improving antioxidant status | (Zhang et al., 2018c) |
| *P. citrinopileatus* | Aqueous extract | Inhibitory activities on α-amylase, α-glucosidase | (Chen et al., 2017) |
| *P. tuberregium* | Aqueous extract | Reducing plasma sodium and raising plasma potassium levels and had no deleterious effect on the liver and kidney | (Ikewuchi et al., 2017) |
| *S. luridus* | Polysaccharides | Decreasing blood glucose levels and attenuating the kidney and liver injures | (Zhang et al., 2018d) |
| | | | |
| | Ethanol extract | Decrease blood glucose levels | (Ekowati et al., 2018) |
| *T. melanosporum* | Aqueous extract | Reduced glucose levels and attenuated oxidative stress | (Zhang et al., 2018e) |

#### *1.1.2.7. Anti-ageing properties*

Skin ageing is a complex biological process that is induced through two independent intrinsic and extrinsic routes, responsible for the progressive loss of structural integrity, elasticity, and physiological functions of the skin (Kammeyer & Luiten, 2015). Ultraviolet radiation is the main environmental mutagen responsible for the oxidative damage to cellular components such as cell walls, lipid membranes, mitochondria, and skin DNA by generating free radicals. These free radicals (reactive oxygen species) activate many skin disease-related enzymes, such as collagenase, elastase, hyaluronidase, tyrosinase, and xanthine oxidase, and increase the expression of matrix metalloproteinases (MMPs) responsible for degrading the different components of the extracellular matrix (collagen, elastin, fibronectin, and hyaluronic acid), thus resulting in skin damage and premature skin ageing (Taofiq et al., 2020). These ECM components are responsible for accelerating wound healing, contributing to mechanical resilience and maintaining the structural integrity of the skin (Yadav et al., 2015). Biomolecules with strong free radical scavenging and key enzyme inhibitory effects are vital ingredients in developing nutraceutical or cosmeceutical formulations to prevent skin ageing (Shangshang Li et al., 2018). Numerous extracts and compounds obtained from edible and medicinal mushrooms, such as *Antrodia camphorata, Auricularia auricula-judae, Ganoderma lucidum, Inonotus obliquus, Lentinula edodes, Phellinus vaninii, Pholiota nameko, Piptoporus betulinus, Pleurotus citrinopileatus, Russula capensis, Schizophyllum commune, Tricholoma magnivelare,* and *Tricholoma matsutake,* have been widely investigated and found to possess strong antioxidant, anticollagenase, antihyaluronidase, antielastase, wound healing, antiinflammatory, antimicrobial, antityrosinase, and photoprotective effects against UV-B and UV-A radiation as shown in **Table 7**. These biological activities are associated with major cell signal transduction pathways related to ageing, inflammation, hyperpigmentation, and extracellular matrix degradation (**Table 7**) (Park et al., 2015; Taofiq et al., 2016b).

Inhibition of enzymes such as collagenase, hyaluronidase, and elastase has been associated with maintaining the skin's structural integrity. Fractions prepared from *P. citrinopileatus* (n-butanol, aqueous, and methanol extract) showed up to 9.7%, 10.8%, and 25.4% hyaluronidase inhibition activity, respectively (T. X. Meng et al., 2011). In comparison, an aqueous and acetonic extract prepared from the mycelial suspension of *Trametes lactinea* presented 88% antihyaluronidase activity at 100 mg $mL^{-1}$ (Yahaya & Don, 2012). Aqueous extracts obtained from *P. ostreatus, G. lucidum,* and *Auricularia polytricha* presented over 50% inhibition of

hyaluronidase enzyme (Abd Razak et al., 2020). To the best of our knowledge, only these findings have been reported so far on the hyaluronidase inhibition activity of mushroom extracts or their individual compounds. Polysaccharide-rich extracts obtained from *Auricularia auricula-judae, G. lucidum*, and *Pholiota nameko* have been reported to significantly inhibit collagenase enzyme activity and promote collagen synthesis in a fibroblast cell model (Kozarski et al., 2019; Mapoung et al., 2021; Sung et al., 2020). Extracts obtained from different mushrooms have also been reported to present a potent elastase inhibitory effect, as shown in **Table 7**. MMPs are specialized enzymes produced by epithelial cells, fibroblasts, and inflammatory cells and are associated with degrading the ECM, collagen proteolysis, and contribute to other destructive processes such as tumour invasion and inflammation (S. Y. Kim et al., 2014). The studies presented in **Table 7** showed that mushroom extracts and their associated metabolites present potential anti-ageing effects by inhibiting collagenase, elastase, and hyaluronidase. The anti-elastase activity has been attributed to downregulation of mRNA expression of MMPs (MMP-1, MMP-3, and MMP-9) and increased expression of type 1 procollagen in UVA- and UVB-irradiated keratinocyte cells (S. Y. Kim et al., 2014; Lee et al., 2021). Hence, more work is still needed to determine the mechanism behind the anti-collagenase potential of these mushroom extracts or their individual compounds.

Tyrosinase is the key enzyme involved in the synthesis of melanin. Melanin production is crucial for preventing UV-induced skin damage, and the overproduction of this pigment has resulted in hyperpigmentation (Mukherjee et al., 2018). Bioactive ingredients with the capacity to bind to the active site of the tyrosinase enzyme and cause enzyme inactivation have become the most prominent and successful targets for melanogenesis inhibitors. Besides the competitive and non-competitive inhibition potential against tyrosinase, mushroom extracts have also been reported to decrease melanocyte metabolism, resulting in reduced melanin synthesis. Fractions from *Inonotus obliquus* containing betulin, trametenolic acid, inotodiol, and lanoserol are reported to present very little or no cytotoxic effect in B16 melanoma cells (Z. F. Yan et al., 2014). The extract reduced cellular tyrosinase activity and significantly lowered melanin content in α-MSH-stimulated B16 melanoma cells. Several extracts obtained from medicinal and edible mushrooms, as shown in **Table 7**, are potent inhibitors of tyrosinase enzyme, being also effective in suppressing melanin production in cultured melanocytes and other in vitro models. These findings make these extracts potential ingredients to develop formulations against diseases resulting from aberrant melanin production. However, most tyrosinase inhibition activities currently reported have also presented

incomparable/irreproducible $IC_{50}$ values due to varied assay conditions, including using different substrates (L-dihydroxyphenylalanine and tyrosine) and substrate concentrations, various incubation times, and the use of other assay ingredients. Cosmetics or skin-lightening formulations commercially available contain hydroquinone, kojic acid, arbutin, azelaic acid, ellagic acid, and resveratrol (Kashif et al., 2017). Despite being widely used, these compounds have been reported to alter melanosome formation, promote their destruction, and present unsatisfactory clinical efficacy (Arrowitz et al., 2019). The lack of efficacy of the available tyrosinase-targeting compounds might be associated with most studies being conducted exclusively using tyrosinase isolated from the mushroom *Agaricus bisporus* (mushTYR) (Ashooriha et al., 2020). In the past, mushTYR was the only commercially available enzyme, but recent studies have shown that its catalytic activities and substrate specificities are significantly different from human tyrosinase (humTYR). Very few studies have been published on humTYR inhibitors, with thiamidol being one of the most promising candidates (Mann et al., 2018). To the best of our knowledge, most tyrosinase inhibition properties of mushrooms or their associated metabolites heavily relied on enzymatic assays performed using mushroom tyrosinase (mushTYR) (Im et al., 2019; Kozarski et al., 2019; Satooka et al., 2017), mainly because there were no available humTYR-based assays and mushTYR assays are relatively inexpensive. Hence, with the recent advances in humTYR structural information (Choi et al., 2021; Mann et al., 2018), more work needs to be carried out to find bioactive ingredients from mushrooms with the best humTYR and melanin inhibition potential to develop effective and safe cosmeceutical and nutricosmetic formulations. All signalling pathways associated with melanogenesis are controlled by the master regulator, microphthalmia-associated transcription factor (MITF), a melanocyte-specific transcription factor that is responsible for differentiation of melanocyte cells that modulates the expression of tyrosinase and tyrosinase-related proteins (TRP-1 and TRP-2) (Y. M. Chen et al., 2019). Hence, most tyrosinase-inhibiting bioactives tend to target the downregulation of MITF expression to inhibit the whole process of melanogenesis, and as such more targeting of the expression of these melanogenesis-related proteins, including MITF, TRP-1, and TRP-2 should be conducted.

**Table 7:** Anti-ageing effect of mushroom extracts and their individual compounds

| Species | Bioactive form | Bioactive property | Key findings and suggested mechanism of action | Reference |
|---|---|---|---|---|
| *A. Camphorata* | Extract | Wound healing | - Cell viability of fibroblast cells was maintained at 100 µg/mL<br>- Closure of 60% was observed in the wound healing scratch assay | (Amin et al., 2015) |
| *A. auricula-judae* | Water-soluble polysaccharide-rich extract | Wound healing and collagen synthesis | - Fibroblast and keratinocyte cell migration and proliferation were promoted at 20 µg/mL<br>- Collagen synthesis increased up to 40% at 25 µg/mL<br>- Wound closure was accelerated in mice wound healing model | (Mapoung et al., 2021) |
| *G. lucidum* | Crude polysaccharide | Anti-tyrosinase, anti-elastase and anti-collagenase | - Extract effectively inhibited tyrosinase, elastase, and collagenase up to 50% at 0.37, 1.01, and 0.49 mg/mL, respectively | (Kozarski et al., 2019) |
| | Ethanolic extract | Wound healing | - Cell viability of keratinocyte cells was maintained at 640 µg/mL<br>- At 10 µg/mL, significant improvement in the cell migration capacity was observed in the scratch assay | (Abate et al., 2020) |
| | Coldwater and hot water extract | Anti-tyrosinase and anti-hyaluronidase | - Tyrosinase inhibition activity reached 45%<br>- Up to 72% inhibition of hyaluronidase was observed | (Abd Razak et al., 2020) |
| *I. obliquus* | Triterpenoid rich fractions | Anti-tyrosinase and melanin inhibition | - Petroleum ether fraction (3.81 µg/mL) showed a stronger tyrosinase inhibitory effect than kojic acid (5.23 µg/mL)<br>- Fractions presented little or no cytotoxicity on B16 Melanoma cells<br>- Cellular tyrosinase activity and melanin content were inhibited in α-MSH-stimulated B16 melanoma cells. | (Yan et al., 2014) |
| *L. edodes* | Hydroethanolic extract | MMP inhibition assay | - Cell viability was maintained in HaCaT keratinocyte cells at 50 µg/mL<br>-The expression of MMP-1 and MMP-9 were significantly inhibited<br>- The expression of typeIprocollagen in UVA and UVB-irradiated HaCaT keratinocytes was increased | (Lee et al., 2021) |
| *P. vaninii* | Methanolic extract | Anti-tyrosinase, anti-elastase and anti-collagenase | - The cell viability of B16-F10 murine melanoma cells was maintained up to 85% at 750 µg/mL<br>- The tyrosinase inhibitory activity ranged from 55.83% to 96.16% at 0.125–2.0 mg/mL<br>- Melanin synthesis was suppressed (71.18% to 27.61%) in B16-F10 melanoma cells at 25–500 µg/mL<br>- Elastase and collagenase were inhibited up to 71.24 and 63.02% at 2.0 mg/mL, respectively | (Im et al., 2019) |
| *P. nameko* | Polysaccharides | Anti-collagenase and wound healing | - Collagenase was inhibited up to 61% at 500 µg/mL<br>- Cell migration and proliferation in L929 mouse fibroblast cells were maintained | (Sung et al., 2020) |

| | | | | |
|---|---|---|---|---|
| | | | - A significant increase in closure rate (54.75%) was observed in the wound healing scratch assay | |
| *P. betulinus* | Water-soluble β-D-glucan | Wound healing | - Cell viability was maintained at 1000 µg/mL<br>- Cell migration was enhanced presenting a closure of 55% in the wound healing scratch assay | (de Jesus et al., 2018) |
| *P. citrinopileatus* | Methanol extract | Anti-tyrosinase, anti-hyaluronidase, and melanin inhibition | - Cell viability of B16 Melanoma cells was maintained at 100 µg/ml<br>- The tyrosinase inhibitory activity ranged from 2.5% to 10.5% at 0.125–2.0 mg/mL<br>- Melanin content was suppressed in B16 Melanoma cells<br>- Hyaluronidase enzyme was inhibited up to 25% at 100 µg /mL | (Meng et al., 2011) |
| *R. capensis* | Aqueous and methanolic extract | Anti-collagenase and wound healing | - Extract (100 µg/mL) mildly inhibits dermal fibroblast proliferation and significantly improved cell migration<br>- Extract inhibited collagenase up to 20% at 200 µg/mL<br>- Fibroblast migration in MRHF cells was enhanced and up to 40% wound closure rate was observed | (Pringle et al., 2021) |
| *S. commune* | Coldwater extract | Anti-tyrosinase and anti-hyaluronidase | - Tyrosinase inhibition activity reached 98% comparable to kojic acid (94.4%)<br>- Up to 40% inhibition of hyaluronidase was observed | (Abd Razak et al., 2020) |
| *T. fuciformis* | Polysaccharides | Collagen synthesis | Collagen levels were elevated in UVA and UVB exposed animal models | (Wen et al., 2016) |
| *T. magnivelare* | Methyl cinnamate | Anti-tyrosinase and melanin inhibition | - Cell viability of B16-F10 melanoma cells was maintained<br>- Total melanin production was significantly suppressed in a concentration-dependent manner | (Satooka et al., 2017) |
| *T. matsutake* | Mycelial extract | Anti-elastase | - Extract presented potent elastase inhibitory effect (81.4±3.92%) similar to phophoramidon (89.6±7.74%) used as positive control<br>- Cell viability of Human fibroblasts was maintained at 100 µg/ml<br>- mRNA expression of MMP-1 and MMP-3 in fibroblast treated cells were decreased in a dose-independent manner | (Kim et al., 2014) |

The wound-healing effect of mushrooms has been one of the most studied beneficial effects and is well described in different scientific findings (Abate et al., 2020; Pringle et al., 2021). The wound-healing process is characterized by a complex sequence of cellular and molecular events involving inflammation, fibroplasia, neovascularization, collagen deposition, epithelialization, and wound contraction (Mapoung et al., 2021). Most mushroom extracts and their compounds have been investigated (**Table 7**), targeting relevant wound repair mechanisms such as cells (fibroblasts and keratinocyte) proliferation and migration, inhibition of reactive oxygen species (ROS) production, modulation of inflammatory mediators secretion, and deposition of ECM components (collagen and hyaluronic acid) (Abate et al., 2020; Amin et al., 2015; de Jesus et al., 2018; Mapoung et al., 2021; Pringle et al., 2021). The wound-healing potential of most mushroom species has been associated with their rich content in polysaccharides such as water-soluble *β*-D-glucan from *P. betulinus*, a low-molecular-weight polysaccharide from *P. nameko*, and water-soluble polysaccharide from *A. auricula-judae* (de Jesus et al., 2018; Mapoung et al., 2021; Sung et al., 2020). However, their related mechanism of action has not been fully elucidated. Nonetheless, mushrooms' anti-ageing and wound healing potential have increasingly been reported, linked to their richness in triterpenes, polysaccharides, and phenolic compounds. These multifunctional bioactive properties have intensified the use of mushrooms and their related metabolites as components in cosmetic formulation development, a niche that has received increased attention in recent years.

#### *1.1.2.8. Hepatoprotective properties*

The liver is a vital and complex organ of the human body, maintaining homeostasis by regulating various physiological functions such as carbohydrate, protein, and fat metabolism, detoxification, secretion of bile, and storage of vitamins. The liver is also involved in most biochemical pathways related to growth, combatting diseases, nutrient supply, energy provision, and reproduction (Soares et al., 2013).

Liver damage is a widespread disease that, in most cases, involves oxidative stress. Normally, host cells are protected from oxygen-derived radical injury by naturally occurring free radical scavengers and antioxidants. When these natural defences are overwhelmed by excessive generation of prooxidants, a situation of oxidative stress evolves, and cellular macromolecules suffer oxidative damage (Nitha et al., 2013; Soares et al., 2013).

Both reactive oxygen and nitrogen species are the major initiators of oxidative stress, and they appear due to various pathogenic diseases, exposure to radiation, tissue injury, etc. Most of the hepatotoxins induce tissue injury after having been metabolized to free radicals and cause subsequent cell damage through a mechanism of covalent binding and lipid peroxidation. Oxidative stress has been implicated in the pathogenesis of acute and chronic liver injury in a variety of pathophysiological conditions such as hepatotoxin exposure, intrahepatic cholestasis, alcoholic liver injury, hepatocellular carcinoma, liver ischaemia and viral hepatitis, fibrosis, cirrhosis, steatohepatitis and biliary disease, inflammation, and necrosis of the liver, among others. Thus, maintaining a healthy liver is crucial for overall health and human well-being (Dandapat et al., 2015; Nitha et al., 2013; Soares et al., 2013).

Clinically, serum enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT), hepatic lipids (total cholesterol and triglycerides (TGs)), and enzymes involved in alcohol metabolism (alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)) are commonly used as biochemical markers for early diagnosis of hepatic injury. Serum ALT and AST activities are elevated when a hepatic injury occurs since ALT and AST could leach out of hepatocytes into the blood circulation (Zhao et al., 2017b).

Carbon tetrachloride ($CCl_4$), a selective hepatotoxic chemical agent, is one of the most widely used toxins for the experimental induction of liver fibrosis in laboratory animals. The principal causes of $CCl_4$-induced hepatic damage are lipid peroxidation, decreased activities of antioxidant enzymes, and the generation of free radicals leading to liver fibrosis and cirrhosis (Nitha et al., 2013; Osman & Toliba, 2019).

The hepatoprotective properties and the greatest contributions described by different authors in several mushroom species are summarized in **Table 8**.

The protective effects of *L. deliciosus* and *A. cylindracea* supplementation against $CCl_4$-induced oxidative stress were investigated by measuring the adenosine deaminase (ADA) and myeloperoxidase (MPO) levels and histopathological changes in liver and kidney tissues in an *in vivo* model (Dogan et al., 2021). The $CCl_4$-induced histopathology was increased significantly in the *A. cylindracea* and $CCl_4$ + *A. cylindracea* groups due to the toxic effects of *A. cylindracea*. On the other hand, *L. deliciosus*-treated rats exhibited significantly fewer degenerative and necrotic changes in liver and kidney in the $CCl_4$ + *L. deliciosus* group compared to rats in the $CCl_4$-treated group. Purified polysaccharide isolated from *L. deliciosus*

is a potential source of antioxidants and natural immunostimulants, which could explain the protective effect of this mushroom (Dogan et al., 2021).

In another study, $CCl_4$-induced oxidative stress was tested in an adult male Sprague–Dawley rat. The authors found that $CCl_4$ toxicity increased blood urea, uric acid, and creatinine levels compared to the control samples. Pretreatment of *G. lucidum* extracts significantly decreased urea, uric acid, and creatinine levels, proposing that it would counteract nephrotoxicity caused by $CCl_4$. Otherwise, treatment with *G. lucidum* extract restored malondialdehyde (MDA), hydrogen peroxide ($H_2O_2$) contents, superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) levels (Dabdoub et al., 2020). The restoration activities of oxidative stress parameters by *G. lucidum* can protect the enzymes; this species contains several antioxidant compounds and exhibits significant strong scavenging free radical activity (Dabdoub et al., 2020).

The combination of the garlic-derived amino acid, S-allyl-L-cysteine sulfoxide (ACSO), and ornithine or arginine from *H. marmoreus* on $CCl_4$-induced hepatic injury was examined by Yamaguchi et al. (2021). Some mushrooms have highly active arginase that catalyses the hydrolysis of arginine to ornithine and urea, accumulating a considerable amount of urea in the fruit bodies to regulate osmotic pressure. All extracts prepared from garlic and *H. marmoreus* with low and high contents of ACSO and arginine or ornithine significantly suppressed $CCl_4$-induced hepatic injury in rats. The co-administration of ornithine/arginine with ACSO suppressed $CCl_4$-induced hepatic injury in rats more potently than a single administration of ACSO, probably owing to synergistic effects. In another study, the hepatoprotective activity of the crude phenolic-rich extract (CPRE) isolated from *P. ostreatus* on $CCl_4$-induced oxidative stress was investigated in albino rats and several biochemical parameters were evaluated. Administration of $CCl_4$ for 28 days exhibited a significant increase in serum markers of liver damage (i.e., ALT, AST, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), urea, creatinine, total lipids, and TG) $CCl_4$ exposure significantly decreased the hepatic antioxidant enzyme activities such as SOD and glutathione peroxidase (GSH-Px). Contrarily, the CPRE treatments showed a significant increase in these hepatic activities (Osman & Toliba, 2019). The potential antioxidant activity of CPRE isolated from *P. ostreatus* against several oxidation systems in vivo contributed to its hepatoprotective effects in CCl4-induced liver injury in male albino rats (Osman & Toliba, 2019).

Ubhenin et al. (2019) evaluate the hepatoprotective, haematoprotective, and hypolipidaemic effects of *P. ostreatus* in $CCl_4$-induced liver injury in Wistar rats. The results revealed that $CCl_4$ caused a significant increase in lipid peroxidation due to the significantly elevated level of MDA in the hepatic tissues, whereas the levels or activities of reduced GSH, CAT, SOD, GPx, and glutathione S-transferase (GST) in the liver tissues were significantly reduced. Dyslipidaemia and haematotoxicity were manifested by a significant increase in the serum levels of TG, total cholesterol, very low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein cholesterol (LDL-C), and white blood cells count. The ability of the powdered *P. ostreatus* to mitigate against $CCl_4$-induced hepatotoxicity is probably due to its antioxidant and enzyme modulatory effects.

The hepatoprotective effects of modified polysaccharide from *C. comatus* on alcohol-induced liver injury were investigated. The mushroom extract significantly increased the SOD, GSHPx, and CAT activities, which may be because the extract activated nuclear factor erythroid 2-related factor-2 in mouse livers. These results indicated that the activities of antioxidant enzymes might be one of the main mechanisms for contributing to the protective effects of modified polysaccharides from *C. comatus* against alcohol-induced liver injury (Zhao et al., 2019).

Chemical screening and analysis of the hepatoprotective efficacy of *P. tuber-regium* extracts in $CCl_4$ hepatotoxic rat was evaluated by the authors (Dandapat et al., 2015). When *P. tuber-regium* extract was administered to hepatotoxic rats, AST, ALT, and ALP activity significantly decreased, and the concentration of serum albumin and protein significantly increased compared to the hepatotoxic group. *P. tuber-regium* extract possessed antioxidant activity in a dose-dependent manner, possibly due to a relatively high concentration of bioactive compounds like flavonoids, tannins, alkaloids, and phenolics (Dandapat et al., 2015).

The hepatoprotective effects and antioxidant activities of hot-water-extractable polysaccharides and enzymatic-extractable polysaccharides from *L. sulphureus* in acute alcohol-induced alcoholic liver disease mice were investigated by the authors (Zhao et al., 2017b). The elevation of AST and ALT activities could be attenuated by supplementation of both hot-water-extractable polysaccharides and enzymatic-extractable polysaccharides. Polysaccharides confer effective hepatoprotection against acute alcohol-induced alcoholic liver disease, possibly by reducing oxidative stress. Enzymatic-extractable polysaccharides showed

superior effects, indicating that enzymatic hydrolysis has a potential effect on enhancing bioactivities (Zhao et al., 2017b).

**Table 8:** Main mushrooms with reported hepatoprotective properties and the main contribution

| Mushroom species | Liver injury | Main role | Reference |
|---|---|---|---|
| *A. cylindracea* | $CCl_4$ | *A. cylindracea* supplementation causes severe pathology in liver and kidney tissues in rats. | (Dogan et al., 2021) |
| *C. comatus* | Alcohol | A modified polysaccharide from *C. comatus* significantly attenuated the hepatic and serum lipid levels, enhanced antioxidant enzyme activities, markedly improved alcohol metabolism system and inflammatory response, and mitigated alcohol-induced liver injury histopathologically | (Zhao et al., 2019) |
| *G. lucidum* | $CCl_4$ | *G. lucidum* extract can significantly prevent the $CCl_4$ induced liver and kidney damage, restored MDA, $H_2O_2$ contents, SOD, CAT and GSH levels. | (Dabdoub et al., 2020) |
| *H. marmoreus* | $CCl_4$ | All extracts prepared from garlic and buna-shimeji with low and high contents of S-allyl-L-cysteine sulfoxide and arginine or ornithine significantly suppressed $CCl_4$-induced hepatic injury in rats. | (Yamaguchi et al., 2021) |
| *L. deliciosus* | | Supplementation with *L. deliciosus* exhibits beneficial effects in liver and kidney tissues of $CCl_4$ treated rats. | (Dogan et al., 2021) |
| *L. sulphureus* | Acute Alcohol | Both hot-water-extractable polysaccharides and enzymatic-extractable polysaccharides confer effective hepatoprotection against acute alcohol-induced alcoholic liver disease. | (Zhao et al., 2017b) |
| *P. ostreatus* | $CCl_4$ | The crude phenolic rich extract treatments showed significant increases in SOD and GPx hepatic activities. | (Osman & Toliba, 2019) |
| | | Daily supplementation of powdered Oyster mushroom subsided dyslipidemia, hepatotoxicity and hematotoxicity. | (Ubhenin et al., 2019) |
| *P. tuber-regium* | $CCl_4$ | The activity of aspartate aminotransferase, alanine transaminase, alkaline phosphatase and concentration of bilirubin significantly increased in *P. tuber-regium* extract-treated hepatotoxic rats. | (Dandapat et al., 2015) |

## 1.2. Vitamin D: Disorders of its deficiency and potential solutions from mushrooms

Micronutrient deficiencies can lead to unfavourable health consequences, such as growth problems, immune competence, mental and physical development, and poor reproductive outcomes. It has also been related to an increased occurrence, severity, and mortality of infectious diseases such as malaria, diarrhoea, pneumonia, and other diseases. These deficiencies have numerous unfavourable results across all populations and age groups, with children and women of reproductive age being more vulnerable (Black et al., 2013; Gibson & Hotz, 2002; Looman et al., 2019).

Vitamins are essential for the growth, metabolism, and maintenance of a healthy body; deficiencies of these nutrients have resulted in irreversible physical and cognitive consequences. These compounds also play an essential role in the normal functioning of almost all organs (Looman et al., 2019). According to Global Fortification Data Exchange (GFDx) and the Food and Agriculture Organization (FAO), an estimated billion people are deficient in different types of vitamins (FAO et al., 2018; GFDx, 2021). In several countries, women and children suffer from severe deficiencies caused by inadequate amounts of vitamins. It has also been reported that several micronutrient deficiencies cohabit and are more prevalent in developing countries (Farhat et al., 2019; Hemery et al., 2018).

Vitamin D is a liposoluble vitamin mainly found in fish oils, liver, and egg yolks. The body also produces it when ultraviolet rays from the sun strike the skin. Vitamin D is essential for strong bones and teeth and helps the body absorb calcium and phosphorus from food (Papoutsis et al., 2020). Several disorders can be caused by a lack of vitamin D, including rickets, osteomalacia, osteoporosis, muscle weakness, cancer, autoimmune diseases, depression, infections, and diabetes which can occur in children and adults (Salemi et al., 2021).

There are several ways to fight against disorders caused by a lack of vitamin D. Many strategies have been used to avoid vitamin D deficiency, such as exclusive breastfeeding during babies first six months, food fortification, food diversification, and nutritional supplementation (Bains et al., 2021; Hemery et al., 2018; WHO & FAO, 2006) Sun exposure is also an excellent way to get vitamin D, and many foods are now being fortified with vitamin D (Bennett et al., 2013; S. V. S. Souza et al., 2022). Mainly, fortification is a method of incorporating nutrients or bioactive nutritional components into food products to improve the diet's nutritional quality. Food fortification is an effective strategy for increasing vitamin D intake in populations at risk for deficiency. By placing vitamin D in food products consumed daily, it reaches target populations, from which daily dietary requirements of micronutrients are scarcely satisfied (Dwyer et al., 2015; Moulas & Vaiou, 2018).

Some countries have added vitamin D to their food supply in vitamin D-fortified milk, yoghurts, margarine, flour, breakfast cereals, bread, and other food products. Vitamin D-fortified foods are available in the United States, Canada, many European countries (mainly Finland, United Kingdom, Ireland, and Sweden), and a few other countries (Moulas & Vaiou, 2018; Pilz et al., 2018; Ritu & Gupta, 2014).

Mushrooms are a rich source of bioactive compounds, including ergosterols (precursor of vitamin $D_2$), pigments, and bioactive peptides, which have health benefits. The photochemical conversion of ergosterol in mushrooms to vitamin $D_2$ occur in the presence of ultraviolet (UV) light. While all edible mushrooms contain some vitamin $D_2$, certain species are exceptionally high in this nutrient (Taofiq et al., 2017b). Some of the most vitamin $D_2$-rich mushrooms include Portobello, shiitake, and reishi mushrooms. Consuming vitamin $D_2$-rich mushrooms may help to improve vitamin D status and support health (Cardwell et al., 2018; Krishan Kumar et al., 2021; Martin et al., 2020; D. Yadav & Negi, 2021).

Mushrooms can supplement the diet or be used as functional food ingredients in processed foods. Mushrooms can improve public health by providing bioactive ingredients that can reduce the risk of several diseases. The cost-effectiveness of using mushrooms as a source of vitamin D ingredients for public health is impressive and has the advantage of being installed in the usual dietary patterns without a significant change in eating or health practices and is generally well accepted by the general public (Das et al., 2021; Valverde et al., 2015).

There is some evidence that vitamin $D_2$-rich mushrooms may help to improve vitamin D status. A study found that the mushroom-derived vitamin $D_2$ food ingredient effectively kept 25(OH)D-total in healthy, recreationally active volunteers (Pinto et al., 2020). Another study discovered that the vitamin $D_2$-fortified shiitake mushroom might help postmenopausal women increase vitamin $D_2$ bioavailability and retard trabecular bone loss (Won et al., 2019). Chen et al. (2015) suggest that pulsed irradiated mushrooms may increase bone density in osteoporotic mice. However, further human studies are needed to show their efficacy in preventing osteoporosis. Also, a study noticed that vitamin $D_2$-rich mushrooms increased serum 25-hydroxyvitamin D levels in healthy adults (Stepien et al., 2013).

### 1.3. Calcium silicate and its application in crops

Mushrooms have been a highly prized food since ancient times due to their value and medicinal properties. Thus, innovative methods are continually under research and development to maximize the production of these bioactive compounds.

Silicon (Si) is the second most abundant element in the earth's crust, and its effect on organisms can be different, serving as a cellular chemical element or providing a natural morphological element modification (Gonçalves et al., 2012; Kumara et al., 2016; P. Parthiban et al., 2018; Packirisamy Parthiban et al., 2019; Thongsook & Kongbangkerd, 2011). As a cellular chemical

element, it plays an essential role in the mineral nutrition of plants, aiding development by increasing the organism's biomass. In addition, it also increases resistance to biotic and abiotic stresses such as diseases and pests, excess toxic metals, saline stress, and water deficiency, among others (Gonçalves et al., 2012). Some studies indicate that Si affects the nutritional status of crops. Therefore, Si is believed to influence plant nutrient uptake and nutrient efficiency.

On the other hand, calcium silicate is also used to provide a natural morphological modification in insects, i.e. when ingested, damage occurs to the oral apparatus of pests that attack plants due to its crystalline physical structure (Korndorfer et al., 2010; Kumara et al., 2016; Phattharachindanuwong et al., 2018; Pozza et al., 2009; Saigusa et al., 2000; Tubana et al., 2016). Previous studies have reported reduced insect feeding on turfgrass treated with calcium silicate, as well as others, showing that calcium silicate increases the resistance of sugarcane to the African talker *Eldana saccharina* (Keeping & Meyer, 2002; Korndorfer et al., 2010). Studies that could indicate the feasibility of using calcium silicate in the agricultural sector have been widely discussed. However, to make its use possible, agronomic research is needed to indicate the positive effects on the soil-plant or mushroom system, both as a corrective material and as a source of nutrients (Prado & Natale, 2005). During the last 20 years, the benefits of Si supplementation in crops have been publicized. Nevertheless, much of this information has not been consolidated, and to the best of our knowledge, there are very few studies on Si supplementation in mushroom cultivation. A report on supplementation in oyster mushroom substrates was published by Thongsook & Kongbangkerd (2011) and described the influence of calcium and silicon on the biological and production yields of *P. ostreatus*.

### 1.4. Irradiation in mushrooms and their particularities

For centuries, mushrooms have been included in the human diet, mainly because of organoleptic and nutritional characteristics, such as low fat levels and large amounts of carbohydrates, proteins, amino acids, vitamins, phosphorus, and other minerals. As the consumption of fresh mushrooms grows, an increase in their production is necessary, demanding additional research to clarify the role of mushrooms in human diets and their consumption benefits (PR Newswire, 2022).

In recent years, the knowledge about edible mushrooms' chemical composition and nutritional value has been increasing. In the post-harvest stage, mushrooms quickly lose quality, as

indicated by moisture loss, discolouration, degradation of nutrients, and hyphae development (Â. Fernandes, Barreira, et al., 2014a). Nevertheless, their high perishability, which leads to immediate quality loss after harvesting, represents a drawback for their distribution and marketing as fresh products. This high perishability is a decisive disadvantage that reduces their economic value. Accordingly, there is a growing need to extend their shelf life, both in fresh and processed forms, improving yields and economic competitiveness for producers and traders and increasing their quality for consumers (Bernaś, 2018; Kic, 2018; Taofiq et al., 2017b; Zhang et al., 2018f).

There has been extensive research on the most suitable technology for mushroom preservation. Irradiation technology has proven to be scientifically feasible, practical, and worthwhile for several food products (fruits and vegetables, fresh or dried) (Akram et al., 2013; Fernandes et al., 2017). This technique mainly aims to destroy microorganisms or insects, eliminate toxins, and improve functional properties, with a minor impairment in sensory and nutritional quality (Akram & Kwon, 2010; Fernandes et al., 2015a).

Gamma radiation and electron-beam radiation are techniques that have been validated previously and constitute an alternative to other preservation technologies (Ferreira et al., 2018) while maintaining the chemical profile, freshness, and overall security, although the beneficial and harmful effects are always dose-dependent and case-specific; hence, the importance of testing doses within current legislation on different foods (Fernandeset al., 2014a, 2014b). Scientific reports have shown the benefits of this technology on the physical, chemical, nutritional, and bioactive properties of several edible mushroom species (Fernandes at al., 2013a, 2014a, 2014b, 2014c, 2016b, 2017).

The effects of ultraviolet (UV) light on food products, mainly in mushrooms, have been studied to understand the preservation and enhancement of bioactive compounds and the conversion of ergosterol into vitamin $D_2$ (Taofiq et al., 2017b). UV light effectively preserves mushrooms by reducing the number of the microorganisms present and the moisture content of the mushrooms (Cardwell et al., 2018; Olea, 2013). The bioactive compounds in mushrooms can also be enhanced by exposure to UV light.

The *Agaricus bisporus*, white mushrooms, are within the Agaricaceae family with the least growth time to have the white and soft body, which, when allowed to grow for more extended periods, starts to develop a brown tone that tends to intensify. These brown mushrooms are

called Portobello and have a more intense and textured taste than the white ones (Bernaś, 2018; Wang et al., 2018c; Wang et al., 2018a). Additionally, this variety is rich in bioactive compounds (Djekic et al., 2017; Teichmann et al., 2007).

Interaction of ionising radiation with natural matrices is multifactorial, where some molecules may protect others from radiation effects, requiring case-by-case studies (Antonio et al., 2018). UV-irradiated mushrooms (**Table 3**) present a high conversion rate from ergosterol to vitamin $D_2$. However, more study is needed to determine the bioavailability of vitamin $D_2$ from irradiated mushrooms and the long-term effects of this conversion (Taofiq et al., 2017b). In this way, validating all the processing technology and the processed product is a requisite to assure the feasibility of the preservation process under validation and the conversion of ergosterol to vitamin $D_2$.

### 1.4.1. Gamma radiation

Gamma rays are a type of electromagnetic radiation produced in nuclear decay processes. They are highly energetic due to their high frequency, low wavelength, and high penetrating capacity (Lima, 2015). Gamma irradiation (GI) is used on a full commercial scale to control food spoilage and extend shelf life. It is a safe and effective way to kill bacteria, viruses, and parasites in food. Irradiated food is just as safe as food treated with other methods, such as heat. The gamma irradiation technology used in food has been identified and studied over the years for potential consumer concerns, such as effects on the nutritional quality, organoleptic properties, and safety of the food, effects on the public's perception, environment, and workers who are involved in the processing of the food. The World Health Organization (WHO), International Atomic Energy Agency (IAEA), and Food and Agriculture Organization (FAO) have endorsed food irradiation as a safe processing method (Ravindran & Jaiswal, 2019).

As the year progresses, gamma irradiation has gained popularity in mushroom preservation. The influence of gamma irradiation on mushrooms' chemical and biochemical composition has been reported in numerous studies (Fernandes et al., 2013b; Fernandes et al., 2016b; Fernandes et al., 2016c; Fernandes et al., 2013a, 2014c; Tianjia Jiang et al., 2010; Kortei et al., 2016). However, its effect on mushrooms' bioactive composition, antioxidant activity, and physico-chemical parameters still needs to be well explored.

### 1.4.2. Electron beam radiation

Electron beam radiation (EB) is ionising radiation emitted from an electron gun. The radiation is used to treat thin and low-density products. Electron beam radiation's dose rate is high, allowing a quick treatment time and capable of penetrating deep into the product (Â. Fernandes, Barreira, et al., 2014a). Electron beam technology can be divided into low energy applications comprising in-line sterilisation of packaging materials and in-line disinfestation/sterilisation of food surfaces; medium energy applications that involve phytosanitary treatment of packaged foods; and high energy applications focused on pasteurisation of foods and food ingredients (Lung et al., 2015).

With the growth of the middle-class, high-quality food, free of synthetic additives, microbial pathogens, pesticides, and other chemicals, are expected. The most common use of electron beam radiation is to treat food products to kill harmful pathogens and improve the product's shelf life, appearance, flavour, and texture (Duan et al., 2010; Ravindran & Jaiswal, 2019).

The irradiation of mushrooms with electron beams is an effective means of controlling post-harvest decay, and this non-chemical method is a fast, reliable, and effective method for reducing the number of spoilage microorganisms on the surface of mushrooms. The electrons penetrate the surface of the mushrooms and kill the microorganisms without altering the mushrooms' appearance, taste, or nutritional value (Ravindran & Jaiswal, 2019). Irradiated mushrooms are effectively preserved and have a longer shelf life than non-irradiated mushrooms. However, mushrooms irradiated with electron beam radiation have been reported to alter their chemical and biochemical composition (Duan et al., 2010; Fernandes et al., 2014a, 2015b, 2016c).

### 1.4.3. UV radiation

UV radiation is electromagnetic rays with a wavelength shorter than visible light. It is mainly emitted from the sun and is used in the food industry to kill bacteria and other microorganisms and improve the appearance and taste of food. Ultraviolet radiation is also a powerful disinfectant used to disinfect food packaging and surfaces (Hinds et al., 2019; Koutchma, 2008; Ramesh et al., 2016).

Ultraviolet light is a part of the electromagnetic spectrum which ranges from 100 to 400 nm. It is the most common type of ultraviolet light, categorised as UV-A, with a wavelength range of

320 to 400 nm. With a wavelength range of 280 to 320 nm, UV-B is a shorter and more energetic type of ultraviolet light (Ramesh et al., 2016; Sadeghifar & Ragauskas, 2020). With a wavelength range of 100 to 280 nm, UV-C is the most active, potentially harmful type of UV light (Ploydaeng et al., 2021; Ramesh et al., 2016).

Mushrooms can have significant concentrations of steroid alcohol, ergosterol, which upon exposure to UV irradiation, is converted to bioavailable and bioactive vitamin $D_2$. Mushrooms exposed to UV radiation were found to have increased levels of vitamin $D_2$ (**Table 9**) (Martin et al., 2020).

**Table 9:** Contents of vitamin $D_2$ in *Agaricus bisporus* sample after UV irradiation

| Radiation source | Vitamin $D_2$ content (µg/g dw) | | References |
|---|---|---|---|
| | Before radiation | After radiation | |
| UV-A | - | 22.9 | (Jasinghe & Perera, 2006) |
| UV-A | 0.07 | 0.15 | (Teichmann et al., 2007) |
| UV-B | <6 | 16.7 µg/g dw | (Ko et al., 2008) |
| UV-B | 0.01 | 7.28 | (Roberts et al., 2008) |
| UV-B | 0.5 | 4.10 | (Simon et al., 2011) |
| UV-B | 0.005 | 27 | (Kalaras et al., 2012) |
| UV-B | 4.475 | 157.3 | (Sapozhnikova et al., 2014) |
| UV-B | 0.1 | 3.9 | (Urbain & Jakobsen, 2015) |
| UV-B | - | 42.08 | (Sławińska et al., 2016) |
| UV-B | - | 67.1 | (Urbain et al., 2016) |
| UV-B | - | 91.5 | (Bilbao-sainz et al., 2017) |
| UV-B | - | 406 | (Nölle et al., 2017) |
| UV-B | - | 14.43 | (Salemi et al., 2021) |
| UV-C | - | 34.4 | (Jasinghe & Perera, 2006) |
| UV-C | 0.07 | 10.14 | (Teichmann et al., 2007) |
| UV-C | - | 23.1 | (Koyyalamudi et al., 2009) |
| UV-C | - | 0.03 | (Bennett et al., 2013) |
| UV-C | 4.3 | 9.4 | (Guan et al., 2016) |
| UV-C | 7.9 | 13.4 | (Guan et al., 2016) |
| UV-C | - | 7.75 | (Salemi et al., 2021) |
| UV-C | - | 262 | (Nzekoue et al., 2022) |
| UV-C | - | 204 | (Nzekoue et al., 2022) |

Among the types of UV radiation, UV-C is used in several settings, including food processing plants and laboratories. Ultraviolet germicidal irradiation (UVGI) is a disinfection method that uses short-wavelength ultraviolet (UV-C) light to kill or inactivate microorganisms. UV-C is effective against many microorganisms, including bacteria, viruses, and mould. Mushrooms are food products that are often grown in dark, moist environments, and to disinfect the surface

of the mushrooms and prevent the growth of bacteria, UV-C can be used (Delorme et al., 2020; Martin et al., 2020; Papoutsis et al., 2020; Ploydaeng et al., 2021). Some studies reported that, in mushrooms, UV-C is also used to convert ergosterol to vitamin $D_2$, which is harmless to humans (Cardwell et al., 2018; Guan et al., 2016; Kamweru & Tindibale, 2016; Koyyalamudi et al., 2009; Salemi et al., 2021; Taofiq et al., 2017b), but irradiation conditions may differ from study to study. Additionally, some studies have shown that UV-C successfully increases the amount of vitamin $D_2$ in mushrooms compared to other UV radiations (D. Hu et al., 2020; Nzekoue et al., 2022; Papoutsis et al., 2020; Teichmann et al., 2007). Nevertheless, UV-C irradiation has been less often investigated for the bioconversion of ergosterol to vitamin $D_2$ compared to UV-A and UV-B irradiation.

## 1.5. Details related to the use of vitamin $D_2$-enriched extracts from mushrooms as food ingredients

### 1.5.1. Extraction methods

The health benefits of vitamin $D_2$ and the increasing demand for bioactive compounds as value-added ingredients with potential applications in the food and nutraceutical industries have stimulated its investigation of the most consumed mushrooms, including *Agaricus bisporus* and *Pleurotus ostreatus* (Heleno et al., 2016; Q. Jiang et al., 2020).

Considering all the health benefits of vitamin D and the increasing demand for foods rich in this compound, the analysis requires optimising a fast and efficient extraction and recovery method. Among various extraction techniques, conventional extraction methods require long extraction times, high solvent and energy consumption, and sometimes lower extraction yield. Given these limitations, some unconventional extraction and conversion techniques have been used to maximise the compound's extraction (Heleno et al., 2016; Kshitiz Kumar et al., 2021; Picot-allain et al., 2021). Among the extraction methods, ultrasound-assisted extraction (UAE) offers the benefits of short extraction time, lower solvent consumption and high extraction yield (Kshitiz Kumar et al., 2021; Medina-torres et al., 2017).

The UAE method is founded on the principle of acoustic cavitation, which is capable of disrupting cell walls, thus leading to the liberation of bioactive compounds, and has been widely used recently in the extraction of ergosterol and vitamin $D_2$ (Heleno et al., 2016; Medina-torres et al., 2017; Nzekoue et al., 2022). However, to obtain an improved vitamin $D_2$-rich extract, the extraction method and experimental domain must be chosen carefully so that

different extraction variables do not interfere with the extraction yield and maximise the response according to the desired objectives.

Taofiq et al. (2019b) and Heleno et al. (2016) evaluated parameters such as the effect of time, power, and solvent of the UAE on ergosterol extraction, but in both studies, the effect of these parameters on vitamin $D_2$ extraction was not considered. Therefore, further studies are needed to evaluate the influence of different parameters of the UAE to optimise the ergosterol and vitamin $D_2$ extraction yields. Heleno et al. (2016) optimised ergosterol extraction in *Agaricus bisporus* using UAE, and the best values were obtained with ethanol as solvent, at a power of 375 W, for 15 min. Nzekoue et al. (2022) also evaluated the best UAE conditions for ergosterol and vitamin $D_2$ extraction in mushrooms, and the highest extraction yields were obtained with ethanol as solvent, at a power of 240 W, for 30 min.

### 1.5.2. Efficiency and safety

The benefits of fortification of flour or derivative flour products positively affect the entire life cycle of the population, especially in children and pregnant women, preventing the birth of children with intellectual disabilities or malformations or deficiencies. It is one of the most efficient ways of combating malnutrition and controlling various diseases linked to vitamin deficiency (Vlaic, Mureșan, Muste, Mureșan, Mureșan, et al., 2019). If consumed regularly, many health benefits are gained from fortified flour, mainly because it can help maintain body nutrients more efficiently than supplements. Since fortified flour or derived products provide nutrients that are similar to those provided by an adequate and balanced diet, fortified foods will contain "natural" amounts of nutrients, and this does not happen with supplements on their own (Dwyer et al., 2015; V. R. Preedy & Watson, 2019; Whiting et al., 2016).

Flour fortification can improve the nutritional status of a large portion of the population, regardless of social class, as they are a staple food widely distributed and consumed worldwide. This fortification does not require changes in existing food patterns of populations, is a very cost-effective method, and is also more efficient in reducing the risk of multiple deficiencies resulting from deficits in food supply or a poor diet. It is a significant benefit primarily for women of childbearing age, during periods of pregnancy and lactation (increasing the rate of vitamins in breast milk and reducing the use of supplements), as well as for growing children who need nutrients daily for growth and development (Berner et al., 2014; Datta & Vitolins, 2016; Dwyer et al., 2015; FFI, 2018; V. R. Preedy & Watson, 2019).

The main intentions of food law are to protect consumers' health, facilitate trade, and protect against consumer fraud. Food fraud is committed to deceive consumers, and it often arises from the need for competition between manufacturers, businesses, food establishments and large food retailers (Esteki et al., 2019; Spink, 2019). Legislations require appropriate control in the fortification process to ensure that micronutrient levels are adequately within acceptable limits. The legislation also serves to prevent fortification with nutrients from unsafe or nutritionally unnecessary products, and the constant vigilance of the flour fortification industry, and food fraud vulnerability assessments, brings benefits to the industry and ensures the safety and integrity of the supply chain of fortified flour or derived products (Marks et al., 2018).

The wide variety in each country's particularities and the public health goals worldwide have resulted in many different approaches to regulating fortified foods or derived products. In most countries, law or cooperative arrangements establish fortification standards (flour). In some countries, the fortification of food is achieved without any form of management guidance or quality control. The increased distance between marketing food, whether fortified or not, from its place of production to the final consumer by Global Trade has made it difficult to track the source of unintentional contamination, quality control concerns, and food safety (Esteki et al., 2019; Johnson et al., 2004). Quality control is performed to evaluate whether the fortified product follows the established technical standards, using objective and measurable indicators. It typically consists of collecting samples of fortified food, depending on the production system, and determining its nutrient content. It is essential to routinely collect and analyse the samples to verify and control whether the technical standards are being met. Quality control focuses on purely public health optics and, in this case, concentrates mainly on indicators and criteria that are relevant to the food fortification process (Esteki et al., 2019; Johnson et al., 2004; WHO & FAO, 2006)

The management tools available to establish an appropriate control over food fortification are food laws, related measures and a broader food control system. This management has the function of protecting public health, and it is generally recommended that all forms of fortified foods be adequately regulated to ensure that food fortification is safe and effective for specific population groups, mainly those with nutrient deficiency risks (Marques et al., 2012; WHO & FAO, 2006). Food fortification techniques follow the principles established by the Codex Alimentarius to ensure food security (FAO & WHO, 2015). Any legislation on food fortification should also include the World Trade Organization (WTO) Agreement on the

Application of Sanitary and Phytosanitary Measures (SPS) and the WTO Agreement on Technical Barriers to Trade (TBT), which have added new values to standards, guidelines, Codex codes and recommendations (FAO & WTO, 2017; Orriss, 1998; WHO & FAO, 2006).

In the case of fortified foods, the population should be protected from consuming toxic levels of micronutrients or nutritionally ineffective. Evaluating the toxicity of food ingredients is a controlling requirement since vitamin D is essential for general health, especially for bone. It is also necessary to conduct studies on bone cell lines, specifically with osteoblasts. Determining cytotoxicity is crucial to identify the toxic effects caused by this food ingredient before they are available for consumption (Rampersad, 2012).

Determining the efficacy of vitamin $D_2$-enriched extracts is complex as the necessary clinical studies ideally have to go through several steps. In this case, the development of vitamin $D_2$-enriched extracts involves several steps, ranging from extraction to identify the bioactive compound, irradiation to increase the values of the compound, studies to ensure the stability of the compound, *in vitro* studies to assess the bioactive properties, and *ex vivo* studies to understand the mechanism behind calcium absorption through vitamin $D_2$-enriched extract supplementation (Aloia et al., 2014; Cardwell et al., 2018; Taofiq et al., 2019a). Furthermore, many food ingredients developed are tested only on the active ingredient with minimal testing on the final product.

### 1.5.3. Stabilisation and Degradation

Bioactive compounds from natural sources are chemical compounds that occur naturally and biologically and affect the body. Bioactive compounds from mushrooms (vitamin $D_2$) can be used for health purposes. Stabilisation of this bioactive compound is essential because its activity might be lost during processing and storage. Degradation might also occur during the development and shelf life of the product. There is a need to develop methods to stabilise bioactive compounds. There are many ways to stabilise bioactive compounds, including encapsulation technologies, that would help preserve the bioactive compound's activity and extend the shelf life of the developed products (Francisco et al., 2018).

The use of encapsulation technologies is not a recent invention, as various forms of this technology have been employed for centuries. Encapsulation technology has been employed in various industries in more recent times, including food. Encapsulation technology in the food industry has many benefits, including protecting the food from oxidation (Ribeiro et al.,

2015). Encapsulation can also protect the food from bacteria and other contaminants and help improve its stability. Another essential benefit of encapsulation technology is that it can help improve food ingredients' bioavailability. That means the food will be more accessible for the body to absorb and digest. The encapsulation can also help to protect the nutrients in the food. Finally, encapsulation technology can help control the release of food ingredients, which is essential for people looking for specific health benefits (e.g., improving vitamin D intake). It can also help ensure that the food is digested slowly, which can help improve the person's overall health. Additionally, encapsulation technology can improve the taste and texture of developed food products (Dias et al., 2015; Leimann et al., 2019; Samara et al., 2019).

The choice of the encapsulation process will depend on the material to be encapsulated, the desired final product and the processing equipment and facilities available. The most common encapsulation methods are coacervation, spray-based processes, extrusion-based processes, emulsion-based processes, liposomes, and supercritical fluid-based processes (Dias et al., 2015; Vincekovic et al., 2017).

Many different goals can be achieved through encapsulation, including modulating the release phenomenon (rate, duration, and extent of release), protecting the active ingredient from degradation or from interacting with its environment, enhancing the stability of the active ingredient, facilitating the manufacturing process, and enhancing the aesthetics of the final product (Aguiar et al., 2016; Dias et al., 2015; Vincekovic et al., 2017). In general, the release of an active ingredient from a controlled-release formulation is a function of the wall material's dissolution rate and the active ingredient's diffusion coefficient. The dissolution rate of wall material depends on its chemical composition and the environmental conditions it is exposed. The dissolution rate of wall material is also a function of the type of active ingredient (Vincekovic et al., 2017).

Using safe and environmentally friendly raw materials to develop food ingredients using microencapsulation processes is essential. Microencapsulated ingredients can offer several advantages over encapsulated ingredients. However, it is essential to evaluate the performance of the microencapsulated ingredients after incorporation into the final products. This evaluation can help ensure that the benefits of the encapsulated ingredients are realised in the final product. Nevertheless, it may take some time for this evaluation process. The microencapsulation process should also follow legal regulations.

Among the available technologies, spray drying is one of the most common and versatile methods to encapsulate food ingredients. It can produce particles with a homogenous size distribution, high efficiency, high loading capacity, and the ability to ensure a release (Vincekovic et al., 2017). Francisco et al. (2018) incorporated *Agaricus bisporus* extract in microencapsulated forms in yoghurts using the spray drying technique. The results showed promising potential in preserving bioactive properties throughout storage. Samara et al. (2019) used spray drying *Spirulina platensis* as an effective ingredient to improve yoghurt formulations. In a study, spray drying was applied to curcumin-based using different polymers to produce more stable natural colourants that can be used as effective curcumin-based colouring systems (Leimann et al., 2019). Ribeiro et al. (2015) microencapsulated mushroom extracts by spray drying using maltodextrin as encapsulating material. The results show that the microencapsulated extracts were effective, and the antioxidant activity was preserved over time. These studies used the spray drying technique but with different encapsulation materials. Thus, it is essential to conduct further studies to understand better the encapsulation materials chosen. Furthermore, the behaviour of the microcapsules needs to be further studied to understand the mechanism of diffusion and interaction with the body to understand if microencapsulated vitamin D maintain their stability and bioavailability after ingestion.

### 1.6. Flour fortification and flour qualities

The development of new products has a strategic role in the food industry because consumers increasingly demand products with high nutritional value that provide health benefits (Páramo-Calderón et al., 2019).

Cereals are common staple foods since they are versatile, tasty, always available on the market, accessible and culturally acceptable, and consumed every day by all age groups, so they can be considered an excellent fortification vehicle (Pachón et al., 2015). Since the early days, cereals and cereal products have been the main components of the human diet worldwide. Major cereal crops include wheat, rice, maize, and barley. Maize (or corn) is the most produced, but it is less important than wheat and rice, the most important cereals for human nutrition (Garg et al., 2021; V. R. Preedy & Watson, 2019).

Cereals are commonly consumed after being processed by milling industries through crushing using different mechanical actions of force, such as compression, impact, and shear, resulting in reduced particle sizes according to the desired end-use products. At the industrial level, the

complex process of crushing, successive sieving, and refining leads to separating the husk from the endosperm and germ, resulting in several types of flour (Koletta et al., 2014). When flour is fortified at the industrial level (flour production), the food products produced in the food preparation industries (e.g., bakeries) are easier to prepare. This makes the cereal industry unit much more potent in this type of fortification than a bakery or even supplements (Johnson et al., 2004; Pachón et al., 2015).

The first cereal product to be largely fortified was wheat flour, and the first recommendations about cereals fortification from the World Health Organization (WHO) referred to wheat and maize flour. At the beginning of 2015, 83 countries demanded the fortification of wheat flour, of which 14 required the fortification of maize flour simultaneously (Pachón et al., 2015; WHO et al., 2009). Ninety-one countries have legislation requiring fortifying at least one grain of industrially milled cereal. The flour is fortified with vitamins, minerals, amino acids, and other micronutrients, depending on the country. Within these, 90 countries fortify wheat flour alone or other grains, taking away the country Papua New Guinea mandated only for rice flour fortification (**Figure 7**). Each country adopts its fortification standard depending on the geographic region, income status, food vehicle(s) and nutrient(s). With the focus on vitamin D, the total number of flour fortified with this nutrient according to the fortification standard is presented in **Table 10** (GFDx, 2021).

**Figure 7:** Mandatory fortification of different types of flour around the world

Wheat flour (64 countries) Wheat flour and maize flour (17 countries) Wheat flour and rice (5 countries: Nicaragua, Panama, Peru, Philippines, Solomon Islands) Wheat flour, maize flour, and rice (2 countries: Costa Rica and the United States) Rice (1country: Papua New Guinea) (FFI, 2021)

Worldwide, millions of tons of flour are used for human consumption each year, and they are consumed as noodles, bread, pasta, and other flour products (Pachón et al., 2015). According to the Food Fortification Initiative, out of the 250 metric tons of industrially milled wheat flour,

26 metric tons of maize flour, and 171 metric tons of rice, 34%, 57%, and 1%, respectively for each one of the flour was fortified (FFI, 2018; Marks et al., 2018).

As a staple food common in many countries, flour is often considered one of the most suitable vehicles for multimicronutrient fortification (Hemery et al., 2018). In addition, fortified flour can be important source of bioactive compounds since flour can be fortified with vitamins, other nutrients, and biocompounds, including those present in mushrooms, thereby reducing the risk of multiple deficiencies and improving health benefits (Oghbaei & Prakash, 2012; V. R. Preedy & Watson, 2019; WHO et al., 2009). Efforts made by some countries to adopt mandatory fortification of flour with vitamin D help combat this deficiency (**Table 10**). These initiatives are an excellent example for other countries, considering their programs. However, appropriate legislation is needed to ensure the impact, safety, and intended benefits to the health of the fortified food (Luthringer et al., 2015; Marks et al., 2018; Serdula, 2010). Therefore, it is necessary to explore how nutrient fortification contributes to the context of the current nutrient intakes.

**Table 10:** Food vehicles fortification with vitamin D in some countries (GFDx, 2021)

| Countries | Food Vehicles | Legislation status |
|---|---|---|
| Belize | Rice | Voluntary fortification |
| Jordan | Wheat Flour | Mandatory fortification |
| Kuwait | Wheat Flour | Voluntary fortification |
| Mongolia | Wheat Flour | Mandatory fortification |
| Palestine | Wheat Flour | Mandatory fortification |
| Peru | Rice | Mandatory fortification |
| Qatar | Wheat Flour | Voluntary fortification |
| Saudi Arabia | Wheat Flour | Voluntary fortification |
| United Arab Emirates | Wheat Flour | Voluntary fortification |
| United States of America | Rice<br>Maize flour | Mandatory fortification |

Nutrition International directs and supports flour fortification efforts in developing countries through various programs and partners such as Global Alliance for Improved Nutrition (GAIN), United Nations International Children's Emergency Fund (UNICEF), World Food Program (WFP), Food Fortification Initiative (FFI), Unite State Centres for Disease Control and other organizations, working within different countries (South Africa, Yemen, Iran, India, Pakistan, Nepal, Bolivia, Central and South America and the Middle East, Indonesia, Nigeria, among others countries). They support and expand fortification programs of flour with different micronutrients to combat the deficiency of these nutrients and improve health disorders (Nutrition International, 2022).

Many flours can be fortified or used to fortify other flour in the market, and the following topics highlight the main produced and consumed cereals flour worldwide.

### 1.1.1. Wheat flour

Wheat is originally from the Levant region of the Near East and Ethiopia (Ihsan et al., 2015). The third largest cereal production in the world is wheat, after maize and rice, and it is the second most consumed globally after rice. Wheat flour is considered one of the most appropriate vehicles for multinutrient fortification (including from mushrooms) (**Table 11**) because of its global consumption and the high consumption of bread and pasta worldwide (Awika, 2011; Field et al., 2021; J. P. Peña-Rosas et al., 2014). Standard Portuguese wheat flour range from type 55–65 (white wheat flour), used in pastry, to type 150 (wholemeal flour) used in pasta and whole-grain bread (Weekend Bakery, 2022). Wheat flour was the first cereal grain product to be extensively fortified, the World Health Organization (WHO) recommends that the fortification of wheat and other flour is an opportunity to improve health (WHO et al., 2009).

Processed industrial fortification of wheat flour, when properly implemented, is an effective, inexpensive, and straightforward strategy to provide vitamins and other nutrients to the world population, thereby improving the nutritional quality of food supply and providing a public health benefit (FFI, 2022a; J. P. Peña-Rosas et al., 2014).

Globally the effort to begin fortifying wheat flour was launched during the 1940s to improve the health of populations (Bishai & Nalubola, 2002). Wheat flour has been fortified with different micronutrients, including Vitamin D, in different parts of the world (**Table 10**) (FFI, 2022c; GFDx, 2021; Marks et al., 2018; Pachón et al., 2015; J. P. Peña-Rosas et al., 2014; Serdula, 2010). A simulation model was used to compare the cost-effectiveness of the combined strategy of wheat flour fortification and targeted vitamin D supplementation with the current strategy of wheat flour fortification alone. The simulation results showed that the combined strategy is more cost-effective than the current strategy (M. Aguiar et al., 2020). Another study showed that fortifying wheat flour with vitamin D increased *per capita* vitamin D supply in low/lower-middle income countries (Cashman & Dea, 2019).

### 1.1.2. Rye flour

Rye is a European cereal with extensive production centred in Europe. In the pastry/baking industry, standard Portuguese rye flour range from type 70 - 85 (semi-integral flour), used in

the bakery, to type 130–170 (wholemeal flour) and give bread increasing solid darkness, and it is widely used cereal worldwide. In the Middle East, rye is developed as a secondary crop, and it has an excellent wintering capacity and high tolerance to drought and cold and develops well on low-fertility soils. Because of these characteristics, it is becoming a low-risk and economical crop (Heiniö et al., 2016; Kaminski et al., 2011; Redant et al., 2017; Weekend Bakery, 2022).

There are several reasons for the increased interest in rye, a whole grain which contains all three parts of the grain: the bran, the germ, and the endosperm. Whole grains benefit our health because of their nutritional profile and high levels of dietary fibre. Besides its excellent nutritional quality, it also has many health benefits: decreasing the absorption of triglycerides and blood cholesterol levels, reducing blood glucose, prevention of constipation, and prebiotic effects. These characteristics allow the classification of rye products as functional foods, proving a relationship between diet and health (Benítez et al., 2018; Grossmann & Koehler, 2016; Kaminski et al., 2011; V. R. Preedy & Watson, 2019; Redant et al., 2017). There are few studies on rye fortification, but some studies have presented the fortification of rye flour with cellulose fibre (Fuckerer et al., 2015, 2016) to increase its health benefits. In another study, the authors looked at high-fibre sourdough rye bread fortified with vitamin D in healthy women aged 25–45. They found that fortification with vitamin D provided approximately 10 µg/d, which effectively increased serum 25(OH)D concentration (Natri et al., 2006).

### 1.1.3. Maize flour

Maize is grown worldwide, millions of tonnes are effectively produced today, and they can come in various colours. The main maize producing countries are the United States, China, India, France, Brazil, Argentina, and Indonesia. It is also the leading food preferred by billions of consumers in sub-Saharan Africa and Latin America. Tons of maize flour are milled annually, and its consumption is performed in many forms. After rice and wheat , maize is the third most frequently consumed cereal globally (Agricultural Market Intelligence Centre, 2021; WHO et al., 2009).

About 65% of industrially processed maize in the world is fortified (FFI, 2018). In many countries, maize fortification has been practised for several years, and this ingredient is used to prepare many everyday dishes. About 17 countries have mandatory legislation to fortify maize flour across the African and American continent (Enzama et al., 2017; FFI, 2022a; WHO, 2016).

Maize flour can be fortified with several nutrients and compounds from different sources, including mushrooms (**Table 11**), some of them are used to replace nutritional contents, and others are used to prevent deficiencies of specific micronutrients relevant to health (GFDx, 2021; WHO, 2016). Maize flour is typically fortified with nutrients, such as vitamin D, thiamin, riboflavin, niacin, and iron. It also has a high starch content, making it a good choice for baking (Dichi & Miglioranza, 2013; Khamila et al., 2020; Páramo-Calderón et al., 2019).

### 1.1.4. Rice flour

Rice is the staple food for more than half the world's population and is the principal cereal in many developing countries. In most Asian countries, rice provides between 50% and 80% of the caloric intake. In South and Southeast Asia, most women and children are anaemic, and rice's nutritional value significantly impacts their health (FFI, 2022b; V. R. Preedy & Watson, 2019; WHO, 2018). Such as wheat and maize flour fortification, rice fortification with vitamins, minerals, and other micronutrients is a public health opportunity in order to prevent deficiencies in these compounds and severe diseases, and it is often used in baking and cooking as a way to add nutrients to recipes (de Pee, 2014; FFI, 2022b; Forsman et al., 2014).

According to GFDx (2021), 5 countries of the Asian continent, 8 of the American continent and 2 of Oceania fortify rice with different minerals and vitamins. Only Peru and the United States of America (mandatory fortification) and Belize (voluntary fortification) fortify rice with vitamin D. Rice is tough to fortify because most grains are not processed. Therefore, the alternative is to fortify rice flour instead of the rice grain, which can be fortified with the same methods used for the other flour. Rice flour has been fortified with minerals and vitamins, so it is a good source of nutrients for people who do not eat meat or other animal-based products (J. Peña-Rosas et al., 2019).

In Sri Lanka, rice flour is processed, and its cost is approximately equal to wheat flour, leading to a growing interest by the population (WFP et al., 2017). Rice flour has been fortified in countries such as the Philippines and Guyana, which have a very significant consumption of this product (Johnson et al., 2004; Marks et al., 2018).

**Table 11:** Some mushrooms used as flour fortifiers[a]

| Mushrooms | Nutrients and class of bioactive compounds[b] | References |
|---|---|---|
| *Agaricus bisporus* | Minerals<br>Proteins<br>Vitamins<br>Fatty acid<br>Carotenoids<br>Flavonoids<br>Phenolic acid<br>Phytosterols<br>Tocopherols | (Mahamud et al., 2012), (A. Sulieman et al., 2017), (Sulieman et al., 2019), (Caro et al., 2017), (Ishara et al., 2018), and (Hasan & Aunsary, 2020) |
| *Boletus edulis* | | (Vlaic, Mureșan, Muste, Mureșan, Pop, et al., 2019) |
| *Calocybe indica* | | (Oyetayo & Oyedeji, 2017) and (Rathore et al., 2019) |
| *Cordyceps militaris* | | (C. Chen et al., 2021) |
| *Lactarius deliciosus* | | (Süfer & Bozok, 2021) |
| *Pleurotus eryngii* | | (Biao et al., 2020) |
| *Pleurotus ostreatus* | | (Azeez et al., 2018), (Farzana et al., 2019), (Irakiza et al., 2021), (Ishara et al., 2018), (Morris-quevedo et al., 2021), (Oyetayo & Oyedeji, 2017), (Parvin et al., 2020), (Siyame et al., 2021), and (Sonkar & Singh, 2015) |
| *Pleurotus Sajorcaju* | | (Adeoye et al., 2019), (Chaudhari et al., 2018), (Condé et al., 2017), and (Prodhan et al., 2015) |

[a] The natural sources mentioned are already used to fortify flour or can be used for the same purpose.
[b] Nutrients and classes of bioactive compounds that may be related to mushrooms.

### 1.1.5. Other flour from cereal by-products: nutritional properties and bioactive compounds

Cereal grains are rich in phytochemicals and nutrients, such as phenolic acids, flavonoids, carbohydrates, dietary fibres, proteins, and tocopherols, among other constituents, which have a vital role in preventing cardiovascular and digestive system diseases, overweight and obesity, inflammation, type 2 diabetes, and some types of cancer (Monnet et al., 2019). Some studies have shown that consumers are increasingly aware of the relationship between diet and disease, and there is a trend towards a gradual decrease in the consumption of animal-derived protein and demand for plant-based diets, which have well-known physical and environmental health benefits (H. Kim et al., 2019; Lynch et al., 2018).

The food industry has focused on producing functional foods based on different cereals due to the growing consumer demand for healthier foods (Bigliardi & Galati, 2013; Klopčič et al., 2020). For this purpose, cereals and their subproducts offer unlimited potential and are excellent raw materials for producing functional foods and ingredients, particularly for designing novel food products based on cereals or their by-products (Saini et al., 2019). They include rice, wheat, rye, maize, barley, sorghum, millet, and oats, among others, and their

global production is pervasive as they are the basis of many human diets worldwide. Therefore, the sector considers the sustainability and the efficient use of the by-products generated by the crops or during cereal processing (Tapia-Hernández et al., 2019). These result in valuable by-products during millings, such as bran, germ, coat, husk, or endosperm, which could be a good source of potentially marketable ingredients and bioactive compounds (Saini et al., 2019). The milling industries commonly release these by-products in the field or direct them to animal feed, bioethanol production, cosmetics, meat substitutes, and nutraceutical/pharmaceutical products, among other applications (Tsadik & Emire, 2015).

The endosperm is broken down into fine particles (flour) while the germ and bran are removed during milling. The germ is a good source of vitamins (B and tocopherol), minerals, proteins, dietary fibre, carbohydrates, fatty acids, flavonoids, glutathione, and sterols (Tsadik & Emire, 2015; Yun et al., 2018). The bran contains fibres and antioxidants, heteroxylans, cellulose, proteins, starch, phenolic acids, fats, and minerals (Anderson & Simsek, 2019; Holguín-acuña et al., 2008).

With the growing world population and the limited resources our planet can provide, it is essential to produce enough food to meet the growing demands and needs of the human population and ensure food security. However, with restricted arable land, the agri-food industry by-products should become recycled within the food chain and, thus, be valorised as a sustainable source of food and food ingredients (Arte et al., 2019; Saini et al., 2019), also promoting the circular economy.

## 1.7. Objectives and Working Plan

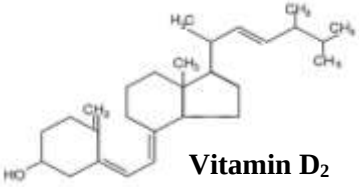
A **deficient intake** of **vitamin D** has been **linked** to the development of **several chronic diseases**, including cancer and osteoporosis.

This **deficiency** is **directly caused** by the **lack** of **foods rich** or **fortified** with **this vitamin** and **indirectly** by the **inexistence** of **sustainable methods** for its **obtaining** from **natural sources**.

**Synthetic vitamin D** is highly **used** in **pharmaceutical supplements** and **fortified foods**, but it is **expensive** and **difficult** to **obtain**; on the other hand, **mushrooms** are a **rich** source of **ergosterol** that can be **converted** into **vitamin $D_2$** by **irradiation.**

The **present thesis** used **mushroom bioresidues** as a **sustainable** starting **material** to **obtain vitamin $D_2$**, avoiding the need to use mushroom samples to be commercialised. These **mushroom bioresidues** were used and **optimised** using **sustainable** and **efficient technological strategies** appropriate for **extraction**, **irradiation**, and **stabilization** of **vitamin $D_2$**, and in a later stage, proceeded its **incorporation** into new **bakery**/**pastry products**.

**Several activities were proposed to accomplish this thesis aims, each one providing new insights to respond to the problem of the deficient daily intake of vitamin D:**



**Vitamin $D_2$**

Supplementation of mushroom production substrate with calcium silicate to maximise bioactive compounds was carried out. Evaluation of nutritional, chemical and bioactive properties was conducted. **Results ➞ CHAPTER 3 – 3.1**

Extraction to maximize the recovery of ergosterol compounds from mushrooms, comparing conventional technique (heated ultrasonic baths) with a more sustainable method (ultrasound-assisted extraction). **Results ➞ CHAPTER 3 – 3.2**

Study of methodologies to obtain fractions rich in vitamin $D_2$. Irradiation of extracts using gamma radiation, electron beam, and UV technologies and their effect on the physicochemical and nutritional composition were carried out. In addition, the effect of vitamin $D_2$-enriched extract on calcium absorption was conducted using a human osteoblast cell line model. **Results ➞ CHAPTER 3 – 3.2**

Evaluation of the quality of wheat and rye flours used to configure the final product by determining the physicochemical and the occurrence of mycotoxins and microbial contaminations. Furthermore, some cereal milling subproducts were evaluated as an alternative food to be used as a fortification matrix. **Results ➞ CHAPTER 3 – 3.3**

The stabilization/encapsulation procedures were applied in the most promising extracts to allow safer and more effective use of vitamin $D_2$ in functional foods. Furthermore, characterization of the particles was conducted, including size distribution, thermal stability, loading capacity (LC), and encapsulation efficiency (EE). **Results ➞ CHAPTER 3 – 3.4**

Fortification of flour with vitamin $D_2$-enriched extracts and comparing their performance between encapsulated and free forms through evaluating their nutritional and physicochemical properties. **Results ➞ CHAPTER 3 – 3.4**

Validation of the final application and utilization of the flours in bakery products (bread) confection. **Results ➞ CHAPTER 3 – 3.4**

# CHAPTER 2

# Material and Methods

## 2.1. Laboratory Supplies

HPLC grade acetonitrile (99.9%), *n*-hexane (95%), and ethyl acetate (99.8%) were purchased from Fisher Scientific (Lisbon, Portugal); methanol (99.9%), ethanol (99.8%) Dimethyl sulfoxide (99.9%) and 2,2,4-Trimethylpentane (Iso-octane, 99.5%), were purchased from Fisher Scientific (Loughborough, UK). The fatty acid methyl ester standard mixture 47885-U, formic acid, 6-hydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid (Trolox), L-ascorbic acid, 2.20-Azobis(2-amidinopropane) dihydrochloride (AAPH), the tocopherol (α, β, γ, and δ isoforms), free sugars, organic acids standards, lipopolysaccharide (LPS), *α*-Amylase, ellipticine, phosphate buffered saline (PBS), acetic acid, sulforhodamine B (SRB), trichloroacetic acid (TCA), polymers polyvinylpyrrolidone (PVP) and Tris were obtained from Sigma-Aldrich (St. Louis, MO, USA). Butylated hydroxytoluene (BHT) was purchased from Merck (Darmstadt, Germany). CHROMagar Salmonella Plus medium was purchased from Frilabo (Milheirós, Maia Portugal), and tryptic soy broth (TSB) and Mueller-Hinton (MH) from Biolab® (Budapest, Hungary). The standards of ergosterol, vitamin $D_2$, and k-carrageenan (KC) were purchased from Acrōs Organics (New Jersey, USA). Phenolic compounds standards were acquired from Extrasynthèse (Genay, France). *p*-Iodonitrotetrazolium chloride (INT), calcium chloride, thiamine, casamino acids, malt extract, and agar were obtained from Panreac AppliChem (Barcelona, Spain). Potato dextrose agar (PDA) and potato dextrose broth (PDB) mediums were acquired from Oxoid microbiology products (Hampshire, UK). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 µg/mL, respectively), RPMI-1640 (Roswell Park Memorial Institute), and DMEM (Dulbecco's Modified Eagle Medium) were from Hyclone (Logan, Utah, USA). Arsenazo III, surfactant, and Tween 80 was from Alfa Aesar by Thermo Fisher Scientific (Haverhill, Massachusetts, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). The ingredients for producing the bread were acquired from common sources.

## 2.2. Samples

The strains of *Pleurotus ostreatus* var. florida (Jacq.) P. Kumm used in this study were collected from producers in the city of Mogi-das-Cruzes (coordinates: –23.52327, –46.2659766), henceforth described as MC (Funghi & Flora company, Valinhos City, Sao Paulo State, Brazil, codified as FF 20), and another in the city of Presidente Prudente (–21.9629448,

–51.6337427), described as PP (Brasmicel company, Suzano City, Sao Paulo State, Brazil, codified as PF 14), both in the state of São Paulo in Brazil. The samples had been identified, and their strains were deposited with the following codifications (POS 16/01 and POS 16/02) at the "Centro de Estudos em Cogumelos (CECOG)", Faculty of Agrarian and Technologic Sciences of the São Paulo State University (Universidade Estadual Paulista–UNESP, Sao Paulo State, Brazil), at Campus Dracena in the city of Dracena (Zied et al., 2019a). The inoculum was prepared by subculturing the mushroom, following the steps of production of subculture (petri dish with potato dextrose agar (PDA)), production of mother spawn, and production of grain spawn (sorghum with lime and gypsum) for compost inoculation, as described by Andrade et al. (2007).

*Agaricus bisporus* Portobello fresh and dry samples were acquired in a local market in Bragança, northeast Portugal, in June 2017. Fresh samples were divided into four groups: control (non-irradiated, 0 kGy), sample 1 (1 kGy), sample 2 (2 kGy) and sample 3 (5 kGy) with eighteen specimens (approximately 200 g) per group (72 mushrooms in total). The dose has been limited to 5 kGy because higher doses could compromise mushroom integrity (particularly its texture). Each group was further divided into equal parts (9 specimens each), corresponding to each irradiation methodology. On the other hand, for dry samples, mushrooms were divided into two groups of 15 mushrooms each and further submitted to a drying process. Samples were dried at 30 ºC in an oven for 4 days; each group was further subdivided into five subgroups (200 g per group): Control (non-irradiated, 0 kGy); sample 1, irradiated at 1 kGy, sample 2 (2 kGy), sample 3 (5 kGy) and sample 4 (10 kGy).

The bioresidues from *P. ostreatus* and *A. bisporus* production (Portobello and white mushroom) were supplied by Ponto Agrícola, Baião, north of Portugal. Subsequently, the fresh samples were lyophilised, reduced to a fine, dried powder, and mixed to obtain homogenised samples.

The Milling Company ("Moagem do Loreto", Bragança, Portugal) kindly donated seven flour representatives of commercial diversity in December 2017, namely T55, T65, T85, T130, T150 and T170. The wheat and rye flour were analysed and divided considering the degree of refinement, namely T55 and T65 (wheat flour – refined samples), T150 (whole wheat flour), T70 and T85 (rye flour – refined samples) and T130 and T170 (whole rye flour).

Cereal by-products (wheat germ, maize bran–germ mixture, rye bran, and wheat bran) were kindly supplied by the "Dacsa Group", a food ingredients industrial group from Almàssera-Valencia, Spain, in March 2018. The dry samples were reduced to fine powder, packaged in sealed plastic bags, and stored at −20 ºC until further analysis.

## 2.3. Analysis of the nutritional composition

### 2.3.1. Proximate composition

For proximate composition, the samples were analysed for moisture, energy value and macronutrients (proteins, ash, fat, and carbohydrates) by the Official Methods of Analysis (AOAC, 2019). Moisture (AOAC 925.09) was assessed using a moisture meter (PMB 163 Moisture Analyzer, Adam Equipment, Oxford). The crude protein (AOAC 920.87) was assessed using the macro-Kjeldahl method (N × 4.38 for mushrooms, 5.7 for wheat flour, cereal by-products flour and bread, and 6.25 for rye flour) (Mariotti, Tomé, & Mirand, 2008) using an automatic distillation and titration unit (Model Pro-Nitro-A, JP Selecta, Barcelona). The ash content (AOAC 923.03) was determined by incineration ≈ 600 °C, and the crude fat (AOAC 920.85) was determined using a Soxhlet apparatus extracting a known weight of the powdered sample with petroleum ether. Total carbohydrates and energy value were determined according to the formulae: Total carbohydrates (g/100 g) = 100 - ($g_{moisture}$ + $g_{fat}$ + $g_{ash}$+ $g_{proteins}$); Energy (kcal/100 g) = 4 × ($g_{proteins}$ + $g_{carbohydrate}$s) + 9 × ($g_{fat}$).

### 2.3.2. Free Sugars

Free sugars were analysed in an HPLC system coupled to a refraction index (RI) detector as previously described by Spréa et al. (2020). Briefly, the samples (≈ 1 g) were spiked with 1mL of internal standard (raffinose for mushrooms and melezitose for wheat and rye flour, cereal by-products flour and bread, 25 mg/mL) and extracted with 80% ethanol at 80 °C. The mixture was centrifuged, and the supernatant was concentrated and defatted with ethyl ether. After concentration at 40 °C, the residues were dissolved in 5 mL of water and filtered through 0.2-µm nylon filters. Identification was achieved by comparing the sample retention times with those of the authentic standards, while quantification was based on the internal standard method, with calibration curves constructed with commercial standards of fructose (y = 1.04x; $r^2$ = 0.999), glucose (y = 0.935x; $r^2$ = 0.999), mannitol (y = 0.892x; $r^2$=0.999), sucrose (y = 0.977x; $r^2$ = 0.999), trehalose (y = 0.991x; $r^2$ = 0.999), and raffinose (y = 0.891x, $r^2$ = 0.9999). The results were expressed in g/100 g dw.

### 2.3.3. Fatty acids

Samples (≈ 1.5 g) were extracted using the Soxhlet apparatus with petroleum ether as recycling solvent. After transesterification of the fat fraction obtained by Soxhlet extraction (Spréa et al., 2020), the fatty acid methyl ester (FAME) mixture was analysed by gas chromatography (GC) with flame ionization detection using a YOUNG IN Chromass 6500 GC System apparatus equipped with a split/splitless injector, a flame ionization detector (FID), and a Zebron-Fame column. Identifications were made by chromatographic comparison of the retention times of the sample FAME peaks with the standard. The results were recorded and processed using Clarity DataApex 4.0 Software (Prague, Czech Republic) and expressed in relative percentage (%) of each fatty acid.

### 2.3.4. Tocopherols

Tocopherols were analysed accordingly to a procedure described by Spréa et al. (2020), using the HPLC system coupled to a fluorescence detector (FP-2020; Jasco), programmed for excitation at 290 nm and emission at 330 nm. Briefly, the samples (500 mg) were spiked with a 100 µL of BHT solution (10 mg/mL) and tocol (IT, 250 µL - 2 µg/mL for mushrooms and 400 µL - 50 µg/mL for wheat, rye, and cereal by-products flour), and homogenised first with 4 mL of methanol and then with 4 mL of hexane. Then, 2 mL of saturated NaCl aqueous solution were added, the mixture was homogenised, centrifuged, and the upper layer was collected. The extraction was repeated twice with hexane. The extracts were dried under a nitrogen stream, redissolved in n-hexane (1mL for mushrooms and 2 mL for wheat, rye, and cereal by-products flour), dehydrated, and filtered through 0.22-µm syringe filters. Chromatographic separation was performed in the normal phase on a Polyamide II column (5 µm particle size, 250 × 4.6 mm; YMC, Kyoto, Japan). Identification was made by chromatographic comparison with authentic standards and quantification was based on the fluorescence signal response of each standard, using the internal standard (tocol) method and calibration curves constructed from commercial standards of α-tocopherol ($y = 1.295x$; $r^2 = 0.991$), β- tocopherol ($y = 0.396x$; $r^2$), γ-tocopherol ($y = 0.567x$; $r^2$), and δ-tocopherol ($y = 0.678x$; $r^2 = 0.992$). The results were expressed in mg/100 g dw.

### 2.3.5. Organic acids

The organic acids profile was analysed by ultra-fast liquid chromatography (UFLC; Shimadzu 20A series, Kyoto, Japan) following a previously described and optimised procedure by Pereira

et al. (2013). Briefly, the samples (≈ 1 g) were stirred with 25 mL of meta-phosphoric acid for 25 min, at room temperature, and filtered, first through Whatman Nº 4 filter paper and then through 0.2 µm nylon filters. Chromatographic separation was achieved in the reverse phase on a C18 column (5 µm particle size, 250 × 4.6 mm; Phenomenex, Torrance, CA, USA). Detection was performed in a diode array detector (DAD) (at 215 and 245 nm (for ascorbic acid). The detected compounds were identified and quantified by chromatographic comparison of the peak area with calibration curves obtained from commercial standards of oxalic ($y = 1 \times 107x + 231{,}891$; $r^2 = 0.9999$), quinic ($y = 671557x + 14583$; $r^2 = 0.9998$), malic ($y = 950{,}041x + 6255.6$; $r^2 = 0.9999$), ascorbic ($y = 4 \times 107x + 1 \times 106$; $r^2 = 0.9909$), shikimic ($7 \times 107x + 175{,}156$; $r^2 = 0.9999$), citric ($y = 1 \times 106x - 10{,}277$; $r^2 = 0.9997$), and fumaric ($y = 185{,}062x + 117{,}588$; $r^2 = 1$) acids. The results were expressed in g/100 g dw.

#### 2.3.6. Gluten determination

A known weight of wheat and rye flour (10 g) was placed in a mortar, and 5.5 mL of NaCl solution (2%) was added dropwise. Afterwards, the sample was stirred with the pestle, and the mixture was compressed and shaped into a ball. After kneading, it was allowed to stand for 25 – 30 min. After this process, the samples were washed with water to remove all the starch, until the washing liquids did not turn blue with the iodine solution (Iodine (I - 0.64 g), potassium iodide (KI - 2 g) 0.4% w/v). The obtained gluten was drained and extended in a watch glass, weighed (wet gluten) and placed in a drying oven at 50 °C to obtain the dry gluten (Panreac Quimica, 1977). The results were expressed in g/100 g mb and dw.

### 2.4. Analysis of the chemical composition

#### 2.4.1. The hydroethanolic extracts preparation

The sample was placed in a beaker (~1.5 g) and was extracted by magnetic stirring with ethanol: water (30 mL, 80:20, v/v) at room temperature and 150 rpm for 1h. The extract was separated from the residue by filtration through Whatman Nº. 4 paper in a round flask. The residue was re-extracted under the same conditions, and the filtrates were evaporated at 40 °C to remove ethanol (rotary evaporator, Büchi, Flawil Switzerland]). Subsequently, the aqueous phase of each sample was frozen and lyophilised (freeze 4.5 FreeZone model 7750031, Labconco, Kansas, USA) to obtain the respective extracts.

### 2.4.2. Phenolic compounds

To analyse phenolic compounds, the extracts were dissolved in ethanol: water (20:80, v/v at 10 mg/mL) and filtered through a 0.22-µm disposable LC filter disk. The analysis was performed in an HPLC system (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) coupled with a diode array detector (DAD, using 280, 330 and 370 nm as preferred wavelengths) and a Linear Ion Trap LTQ XL mass spectrometer (MS, Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Chromatographic separation was performed on a Waters Spherisorb S3 ODS-2 column (3 µm, 4.6 mm × 150 mm; Waters, Milford, MA, USA). The operating conditions were previously described by Bessada et al. (2016), as well as the identification and quantification procedures. The results were given in µg/100g dw for mushrooms and mg/g of extract for cereal by-product flour.

### 2.4.3. Ergosterol and vitamin $D_2$

#### *2.4.3.1. Ergosterol direct extraction method*

Samples were vortex-extracted (1 min; LBX V05 series, Barcelona, Spain) with n-hexane and further centrifuged (4000 rpm, 10 min; K24OR refrigerated centrifuge; Centurion Scientific Limited, Chichester, UK) twice (the supernatant was removed between each step). The combined supernatants were dried under a nitrogen stream and dissolved in MeOH (1 mL) (adapted from Guan et al., 2016).

#### *2.5.3.2. Ergosterol extract by Ultrasound-assisted extraction (UAE)*

The UAE was carried out, and ergosterol-enriched extracts were obtained according to the previously optimised conditions with 3 g of sample and 100 mL of ethanol, 375 W, and 15 min (Heleno et al., 2016). The obtained extraction solutions were filtered through a Whatman paper Nº 4 and then evaporated under reduced pressure to remove the solvent, and the dry weight was obtained to deduce the extraction yield, according to **Equation 1**.

$$(\mathbf{1})\ Extraction\ yield\ (\%) = \frac{dry\ weight\ of\ mushrooms}{extract\ weight\ of\ mushrooms} \times 100$$

#### *2.5.3.3. Ergosterol and vitamin $D_2$ Ultrasound bath extractions*

With some modifications, ergosterol and vitamin D2 were determined after an extraction procedure previously described by Tsai et al. (2014). Each sample (≈ 2 g) was extracted with 10 mL of dimethyl sulfoxide (DMSO) using an ultrasound bath (30 min at 45 °C, series LBX

V05, Barcelona, Spain), followed by the addition of 10 mL of methanol/water (1:1, v/v) and 20 mL of hexane with re-extraction in an ultrasound bath. The samples were then centrifuged thrice (3000 rpm, 10 min, Centurion K24OR refrigerated centrifuge, West Sussex, UK), adding 20 mL of hexane and removing the supernatant between each step. At the end of the extraction, the supernatants were pooled and dried using a rotary evaporator (40 °C, Büchi, Flawil Switzerland) and the dry weight was obtained to deduce the extraction yield. Finally, the extract was re-dissolved in 1 mL of methanol.

*2.5.3.4. Identification and quantification of ergosterol and vitamin $D_2$*

The identification and quantification were performed according to the procedure descried by Barreira et al. (2014), using an HPLC (Knauer, Smartline System 1000, Berlin, Germany) coupled to a UV detector (Knauer Smartline 2500, working at 280 nm as preferred wavelength). The chromatographic separation was performed through an Inertsil 100A ODS-3 reverse-phase column (5 µm, 250 × 4.6 mm; BGB Analytik AG, Boeckten, Switzerland), at 35 °C. The mobile phase was a mixture of methanol: acetonitrile (70:30, v/v), fed at 1 mL/min. Data were analysed using Clarity 2.4 Software (DataApex, Podohradska, Czech Republic), ergosterol, and vitamin $D_2$ (Sigma-Aldrich, St. Louis, MO) were quantified using a calibration curve obtained with commercial standards. The results were expressed in mg/100 g, µg/g of dw and mg/g of extract.

## 2.5. Physicochemical parameters

### 2.5.1. Colour

Colour was measured at three different points on the samples. This test was performed with a Konica Minolta CR400 colourimeter (Chiyoda, Tokyo, Japan) with the illuminant D65, a standard illuminant defined by the International Commission on Illumination (CIE) (8 mm aperture and observation at 10°). Thus, *L** represents lightness (L = 0 black, L = 100 white), *a** represents redness (-a = 0 green, +a = redness) and *b** represents yellowness (-b = blue, +b = no yellowness). Colour space values were registered using the data software "Spectra Magic Nx" (version CM-S100W 2.03.0006).

### 2.5.2. Texture

For texture analysis, the samples were subjected to analysis on a TA.XT Plus Texturometer (Stable Micro Systems, Vienna Court, Godalming, UK). The analysis performed was a texture

profile analysis (TPA) that mimics human mastication by making more than one compression on the same food, extracting several parameters (hardness, adhesion, resilience, cohesion, elasticity, gumminess, and chewiness) through the use of macros. The handling conditions were the same as described previously (Fernandes et al., 2022). The results were studied using the Exponent program (Stable Micro Systems).

#### 2.5.3. pH measurement

One gram of each sample was macerated in 2 mL of distilled water. The pH was measured using Hanna Instruments models FC2022/HALO and HI 99161 (RI, USA).

#### 2.5.4. Water activity

Water activity was measured using a Dew Point Water Activity Meter 4TE (Aqua Lab, Cromer, Australia).

### 2.6. Bioactive properties evaluation

#### 2.6.1. Antioxidant activity

The thiobarbituric acid reactive substances (TBARS) and oxidative haemolysis inhibition (OxHLIA) assays were performed for antioxidant activity evaluation. Trolox was used as a positive control in both *in vitro* antioxidant assays. For the TBARS assay, the hydroethanolic extracts were re-dissolved in ethanol/water (80:20, v/v), and subjected to dilutions between 20 and 0.078 mg/mL. The inhibition of lipid peroxidation in porcine brain homogenates (*Sus scrofa*) was evaluated by the decrease in TBARS; the colour intensity of malondialdehyde-thiobarbituric acid (MDA-TBA) was measured at 515 nm. The inhibition rate (%) was calculated according to Spréa et al. (2020), and the results were expressed in $IC_{50}$ values (mg/mL, extract concentration providing 50% of antioxidant activity).

The antihemolytic activity used the OxHLIA assay described and optimised by Lockowandt et al. (2019). An erythrocyte solution (2.8%, *v/v*; 200 µL) prepared in PBS (pH 7.4) was mixed with 400 µL of: (i) extract solution (6–500 µg/mL in PBS); (ii) PBS (control); (iii) water (for complete haemolysis); or (iv) Trolox (7.81–250 µg/mL PBS). After pre-incubation at 37 °C for 10 min with shaking, 200 µL of AAPH (160 mM in PBS) was added, and the optical density was measured at 690 nm every ~10 min in a microplate reader (Bio-Tek Instruments, ELX800)

until complete haemolysis. The results were expressed in $IC_{50}$ values (µg/mL), meaning the concentration of extract capable of promoting a Δt delay in haemolysis of 60 and 120 min.

### 2.6.2. Antimicrobial activity

The antimicrobial activity for mushroom extracts was analysed according to the previously described procedure (Glamočlija et al., 2015). The following Gram (+) bacteria, *Staphylococcus aureus* (AtacTCC 11632), *Bacillus cereus* (food isolate) and *Listeria monocytogenes* (NCTC 7973), as well as Gram (-) bacteria *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 35030) and *Salmonella Typhimurium* (ATCC 13311), were tested; as for the tested micromycetes, the following were used: *Aspergillus fumigatus* (ATCC 2011), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ni*ger (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* from the American Type Culture Collection (ATCC 9112), *Penicillium aurantiogriseum* (food isolate), *Penicillium verrucosum* var. cyclopium (food isolate), and *Trichoderma viride* (IAM 5061). The microorganisms are deposited at the Mycological laboratory, Department of Plant Physiology, Institute for Biological research "Siniša Stanković", University of Belgrade, Serbia. A microdilution method was implemented to perform the antimicrobial assay (Tsukatani et al., 2012). Bacterial/fungal suspensions were adjusted with sterile saline until the concentration of $1.0 \times 10^6$,$1.0 \times 10^8$, or $1.0 \times 10^5$ CFU/mL. The mushroom extracts were re-dissolved in 30% ethanol, mixed with nutrient media for bacteria (Tryptic Soy Broth) or micromycetes (Malt medium) containing bacterial/fungal inoculum ($1.0 \times 10^5$ or $1.0 \times 10^6$ CFU per well) in a final volume of 100 µL. Minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) were defined as described previously (Glamočlija et al., 2015). Ampicillin and streptomycin (Panfarma, Belgrade, Serbia) were used as positive controls for the antibacterial activity test, while the commercial antifungals bifonazole and ketoconazole (Srbolek, Belgrade, Serbia) were used as positive controls for antifungal assay. Thirty per cent ethanol was used as a negative control. Furthermore, sodium sulphite (E221) and potassium metabisulphite (E224) food additives were used as positive controls and the results were expressed in mg/mL.

For flour samples, the antibacterial activity was evaluated by the broth microdilution method coupled to the rapid *p*-iodonitrotetrazolium chloride (INT) colourimetric assay (Alves et al., 2014). The tested microorganisms were clinical isolates from patients hospitalised in various

*Bacillus cereus* (ISO 7932:2004): By the spread plate technique, 0.2 mL of each dilution was inoculated into MYP medium (mannitol yolk polymyxin). Incubation was performed at 30 °C for 24-48 h in the inverted position, and counting was performed on plates containing 10 to 150 colonies (LOQ = 1.7 log CFU/g).

Yeasts and moulds (ISO 21527-1/2:2008): 0.2 mL of each suspension was spread on Petri plates containing 15 mL of Dichloran Rose Bengal Chloramphenicol (DRBC) base (Lyophilchem, Italy) in duplicate. The plates were further incubated in the upright position at 25 °C for 72 h for yeast counts and 120 h for mould counts, counting plates that contained less than 150 colonies (LOQ = 1.7 log CFU/g).

Sulphite-reducing clostridia (SRC) (ISO 15213:2003): 5 mL of each suspension were transferred to a 50 mL falcon tube and further heat-treated in a water bath at 80 °C for 10 min. The suspension was immediately cooled in ice, and 25 mL of Iron Sulfite Agar (ISA) (Liofilchem, Italy) were added. The mixture was homogenised and allowed to solidify. Afterwards, 5 mL of ISA medium was added to create anaerobiosis. The falcon tubes were incubated at 30 °C for 24–48 h, and black spots were counted (LOQ = 2 CFU/g).

*Salmonella spp* (ISO 6579-1: 2017): To analyse the presence of *Salmonella spp*. flour samples were homogenised in BPW and incubated for 18 h-24 h at 37 °C ± 1 °C. Afterwards, 0.1 mL of this suspension was transferred to 10 mL of Ramba QUICK Salmonella enrichment broth (Frilabo, Portugal). The mixture was incubated for 7 h ± 1 h at 41.5 °C ± 0.5 °C. Afterwards, 10 µL were spread onto a Petri dish containing CHROMagar Salmonella Plus medium, which also detects lactose-positive Salmonella, meeting the requirements. Serological or biochemical tests must confirm any presumptive positive result (purple colonies).

## 2.9. Mycotoxins analysis

### 2.9.1. Safety aspects

For AFs and OTA handling, the security rules were carefully followed due to the high toxicity of these substances. Protective equipment was used when managing these solutions, and all the materials were cleansed by autoclaving before discarding. The reusable materials were disinfected for 12 h, submerged in a 10% bleach solution and washed with distilled water (E. Pereira et al., 2017).

### 2.9.2. Aflatoxins determination

Aflatoxins were extracted and purified using the method recommended by VICAM for determining AF in corn, raw peanuts and peanut butter (AOAC, 2019), with slight modifications. Briefly, 25 g of flour was extracted by stirring with sodium chloride (5 g) and methanol/water (125 mL, 70:30, *v/v*) for 20 min (25 °C at 150 rpm). The mixture was filtered through a Whatman No. 4 filter paper (SigmaAldrich Co., St. Louis, MO, USA) and an aliquot of the filtrate (15 mL) was taken and diluted with 30 mL of ultra-pure water. The extract was homogenised and filtered through a Whatman glass microfiber filter (934-AH). Subsequently, the filtered extract (15 mL) was purified through an immunoaffinity column (AflaTest WB, VICAM, Watertown, MA, USA) by gravity at a rate of approximately 1–2 drops/s. The column was then washed twice with 10 mL of ultra-pure water. AF was eluted from the column with 1 mL of HPLC grade methanol and collected in a glass vial, filtered through 0.2 µm nylon filters (Whatman) and analysed by HPLC (Smartline, nauer, Berlin, Germany) coupled to a photochemical post-column derivatization reactor (PHRED unit, Aura Industries, New York, NY, USA), a fluorescence detector (FP-2020, Jasco, Easton, MD, USA), set to λex 365 nm and λem 435 nm, using the Clarity 2.4 Software (DataApex, Prague, Czech Republic). The compounds were separated using isocratic elution with a reverse-phase C18 column (100 mm × 4.6 mm, Merck Chromolith Performance, Darmstadt, Germany) at 35 °C (7971 R Grace oven). The mobile phase consisted of a mixture of water/acetonitrile/methanol (3:1:1, *v/v/v*) with a flow rate of 0.8 mL/min and the injection volume was 10 µL. AFs were identified by chromatographic comparison with the standard (Aflatoxin B + G mixture, Sigma-Aldrich Co. St. Louis, MO, EUA), and quantification was based on the fluorescence signal response (E. Pereira et al., 2017).

### 2.9.3. Ochratoxin A determination

A standard method for determining OTA in wheat (AOAC, 2019), as described by VICAM, was used to analyse flour. Briefly, 50 g of each sample was extracted by stirring (25 °C at 150 rpm) with 200 mL of acetonitrile: water (6:4, *v/v*) for 20 min and subsequently filtered through Whatman No. 4 filter paper. Afterwards, the extract (10 mL) was diluted with phosphate-buffered saline pH 7.0 (40 mL; PBS: NaCl (8 g), $Na_2HPO_4$ (1.2 g), $KH_2PO_4$ (0.2 g), KCl (0.2 g) in distilled water to a total volume of 1 L), and further filtered through a Whatman glass microfiber filter (934-AH). The filtered extract (10 mL) was purified through an Ochratest WB immunoaffinity column (VICAM, Watertown, MA, USA), and the column was washed first

with PBS (10 mL) and then with ultra-pure water (10 mL). Then OTA was eluted with HPLC-grade methanol (1.5 mL), collected in a glass vial, filtered through 0.2 µm nylon filters (Whatman) and analysed by HPLC as described above for AFs, but without the derivatization process. The fluorescence detector was set to λex 330 nm and λem 465 nm, the mobile phase consisted of a mixture with water/acetonitrile/acetic acid (29.5:70:0.5, *v/v/v*), with a flow rate of 0.8 mL/min, and the injection volume was 10 µL. OTA was identified by chromatographic comparison with the standard (OTA standard solution (Sigma Aldrich Co. St. Louis, MO, EUA) and quantification were based on the fluorescence signal response (E. Pereira et al., 2017).

#### 2.9.4. In-house method validation

AF mix (5 µg/mL for AFB1 and AFG1 and 1.5 µg/mL for AFB2 and AFG2) and OTA (10 µg/mL) standard stock solutions were prepared and stored at -20 °C. Working standard solutions of AF (100 ng/mL for AFB1 and AFG1, 30 ng/mL for AFB2 and AFG2) and ochratoxin A (100 ng/mL) were prepared from stock solutions daily. Precision and recovery were performed by spiking the blank sample with 10 µg/kg of AFB1, AFG1 and OTA, and 3 µg/kg of AFB2 and AFG2. One set of the unspiked sample was used as blank. Each set was composed of three replicates (E. Pereira et al., 2017). Instrumentation calibration parameters were determined following the methodology previously described by the authors (Arita et al., 2014) and the recovery rates were calculated based on the three spiked replicates (flour was artificially contaminated), by calculation of the ratio of recovered AFs and OTA concentration to the known spiked concentration. Linearity, the limit of detection (LOD), and limit of quantification (LOQ) were determined by three series of analyses using six standard solutions with concentrations ranging from 0.5 to 50 ng/mL for AFB1 and AFG1, 0.15 to 15 ng/mL for AFG1 and AFG2, and 0.1 to 20 ng/mL for OTA. LOD and LOQ were calculated according to the following equations (Arita et al., 2014): LOD = 3 × (SD/M) and LOQ = 10 × (SD/M), where SD is the standard deviation of the intercept of the regression line obtained from the calibration curve, and M is the slope of the line (E. Pereira et al., 2017).

### 2.10. Substrate, supplementation, growing Cycle and Biological Production Yields

The substrate was made using a mixture of sugarcane bagasse (500 kg), *Brachiaria dictyoneura* (1000 kg), rice bran (100 kg) and wheat bran (100 kg), calcitic limestone (50 kg), and gypsum (50 kg), as described by Zied et al. (2019b). During phase I of the process, *B. dictyoneura* and

sugarcane bagasse (bulk material) were moistened for 2 days. On the 3rd day, the pile was assembled, and on the 4th day, the pile was turned, and the additional materials (rice and wheat bran, calcitic limestone, and gypsum) were added. Afterwards, two more turns were performed, and on the 7th day, the substrate was transferred to a pasteurization chamber (phase II). The substrate was pasteurised between 65 and 72 °C for 20 h and subsequently conditioned between 55 and 48 °C for 1 day. After pasteurization and conditioning, with the substrate at ambient temperature, the strains (PP and MC) were inoculated (2% of the substrate's wet weight) together with the addition of calcium silicate (concentration of 0.5, 1, 2, and 4% substrate's wet weight), in plastic bags containing 2 kg of the wet substrate, following supplementation methodologies during spawning (Carrasco et al., 2018; Pardo-Giménez et al., 2016; D. J. Royse et al., 2004). A control treatment was used as a reference (without applying calcium silicate) to verify the technique's viability. Each treatment had 5 repetitions, represented by bags of 2 kg of the substrate. The chemical-physical composition of the substrate used was N content—1.03%, C/N ratio—63/1, pH—6.7, and moisture—67%.

During the spawn run, the air temperature and relative humidity were maintained at 26 ± 1.5 °C and 80%, respectively. For the induction of primordia and harvest, the temperature was reduced to 22 ± 1.5 °C while the relative humidity was increased up to 90%. The mushrooms were harvested twice (1st flush started 27 days after inoculation and 2nd flush started 39 days after inoculation) during the crop cycle, totalling 45 days of the growing cycle. The interval between flushes varied from 3 to 7 days, depending on the strain and the calcium silicate percentages. After harvesting, the number of mushrooms per harvest and per supplementation was recorded, as was their weight. Prior to the chemical analysis, all the fruiting bodies (samples) were lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas, MO, USA) and reduced to a fine powder (20 mesh).

Biological efficiency (BE) is essential to understanding the substrate's capacity to produce mushrooms. Thus, it was calculated with the equation published by Thongsook and Kongbangkerd (2011) for each flush: **Equation 2**.

$$(\mathbf{2})\ BE(\%) = \frac{fresh\ weight\ of\ mushrooms}{dry\ weight\ of\ substrate} \times 100$$

The yield is an analysed variable widely adopted in commercial crops and was calculated using **Equation 3.**

$$(\mathbf{3})\ Y(\%) = \frac{fresh\ weight\ of\ mushrooms}{fresh\ weight\ of\ substrate} \times 100$$

The productivity rate indicates how well the crop reacts to input by providing outputs, thus, being a reason between BE and the precocity, namely the time it took for the mushrooms to be ready for harvest: **Equation 4**. Daily productivity rate of the mushrooms.

$$(\mathbf{4})\ PR(\%\ per\ day) = \frac{biological\ efficiency\ (\%)}{precocity\ (days)}$$

## 2.11. Irradiation procedures

### 2.11.1. Gamma and electron bean irradiations in fresh and dry mushrooms

The irradiations were performed in Centro de Ciências e Tecnologias Nucleares (Instituto Superior Técnico, Universidade de Lisboa, Portugal). For fresh mushroom samples, the irradiation doses were indicated as 1, 2 and 5 kGy. Groups corresponding to each irradiation dose were divided into three subgroups (three mushrooms: subgroup 1 was promptly analysed (0 days), subgroup 2 was stored 4 days at 5 °C and subgroup 3 was stored 8 days under the same conditions. Considering the typical shelf lifetime of fresh mushrooms, there was no need to assay at longer time intervals. While for dry mushrooms, samples were dried at 30 °C in an oven for 4 days; each group was further subdivided into five subgroups (200 g per group): Control (non-irradiated, 0 kGy); sample 1, irradiated at 1 kGy, sample 2 (2 kGy), sample 3 (5 kGy) and sample 4 (10 kGy), after irradiation and prior to the laboratory analysis, the samples were ground to a fine dried powder (20 mesh) through an automated mill and mixed to obtain homogenised samples, and then kept in the dark at room temperature in airtight flasks until further analysis. One batch of the samples was analysed immediately (0 months), while the other two were stored for 6 and 12 months, respectively, at room temperature in the dark in airtight containers.

Gamma irradiation was conducted in a Co-60 experimental four sources chamber (Precisa 22; Graviner Manufacturing Company Ltd, Gosport, UK), reaching a total activity of 105 TBq (2.84 kCi). The absorbed doses were measured by standard dosimeters (Batch X; Amber Perspex Harwell, Didcot, UK). For fresh mushrooms samples irradiated, doses after irradiation, dose rates and dose uniformity ratios (Dmax/Dmin) were: 1.1 ± 0.1 kGy, 2.4 ± 0.2 kGy and 5.4 ± 0.2 kGy; 1.4 kGy/h and 1.3, respectively. For dry mushrooms, the estimated absorbed doses,

### 2.12.2. Bake trials

The bread were prepared as “mini-bijou”, following a basic recipe with some modifications: 25g of wheat flour, 0.5g of salt and sugar, 0.75g of yeast, 0.5 mL of oil and 15 mL of water. In the first step, the flour was fortified with 0.05g of particles according to the load capacity of developed particle. After fortification of the flour, the bread was made and baked in the preheated oven at ≈ 150 ºC for 15-20 min. Bake trials were carried out under laboratory conditions. Dough mixing, processing, fortification with vitamin $D_2$ and baking were performed on laboratory-scale equipment. The bread produced were lyophilised and stored until analysis.

### 2.12.3. Characterization

The samples were characterised by scanning electron microscopy (SEM), dynamic light scattering (DLS), Fourier-transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), and high-performance liquid chromatography (HPLC). The presence of the particles in the bread was analysed by SEM and quantified by HPLC.

The yield (%) of the spray-drying process to produce particles system was calculated following **Equation 5,** where have the mass (g) of the produced particles is designated $w_p$ and the sum of the mass (g) of the starting compounds (polymer, Tween80, $VitD_2$ /$VitD_2$-enriched extract, citric acid and sodium citrate) are $w_i$.

$$(\mathbf{5})\ Yield = \frac{w_p}{w_i} \cdot 100$$

The particles' morphology and the bread were analysed by SEM using a Phenon Pro microscope from Phenom-World (Eindhoven, Netherlands). Samples were deposited on carbon sheets for analysis.

The particle size and distribution of the particles were analysed by DLS using a Mastersizer 3000 equipped with a Hydro MV unit, Malvern Instruments (Worcestershire, United Kingdom). Results were determined by averaging five measurements for each sample at room temperature, using distilled water as dispersing medium. The sample's particle size corresponding to the particle percentages (10, 50 and 90%, D10, D50 and D90, respectively) were determined in volume and number.

The functional groups and interactions of the samples were analysed by FTIR using an ABB Inc. FTIR, model MB3000 (Montreal QC, Canada), equipped with a diamond attenuated total reflectance (ATR) accessory. The spectra were collected from 4000 to 550 $cm^{-1}$ by averaging 32 scans at a resolution of 4 $cm^{-1}$. The spectra were acquired and normalised using the Horizon MB v.3.4 software.

The thermal stability of the particles was analysed by TGA using a NETZSCH TG 209F3 Tarsus (Selb, Germany). Between 5 and 10 mg of sample was placed in alumina crucibles and heated from 30 to 800 °C at a heating rate of 30 °C/min under a nitrogen atmosphere (40 mL/min). The TG profile and the corresponding derivative thermogravimetry curves (DTG) were acquired using Netzsch Proteus thermal analysis (v.5.2.1) software.

The vitamin $D_2$ loading capacity (LC) and encapsulation efficiency (EE) of the particles and the amount of Vit$D_2$ inside de flour and bread fortified with the particle was quantified by HPLC. For all samples, ≈ 30 mg of particles added in 5 mL of DMSO:$H_2O$ (9:1) were incubated in a water bath at 60 °C for 10 min. The solution was cooled in an ice bath, and 10 mL of iso-octane were added, maintaining the resultant solution under agitation over night at ambient temperature. Then, 2 mL of deionised water were added and left to stand for 15 min to separate the DMSO from the isooctane. The samples were centrifugated at 2000 x *g* for 10 min (refrigerated centrifuge, Centurion K24OR, West Sussex, United Kingdom). The samples' supernatant was removed and evaporated (≈ 40 °C, Hei-VAP Advantage, Heidolph, Germane). The dried extracts were redissolved in 1 mL of methanol, filtered (0.1 μm *nylon* Whatman filters) to be analysed by HPLC (Lamsen et al., 2020) using a Knauer system, Smartline 1000 HPLC (Berlin, Germany) coupled to an ultraviolet detector (UV, Knauer Smartline 2500), according to the operating conditions described by Barreira et al. (2014). The results were analysed using the software Clarity 2.4 (DataApex, Pod ohradska, Czech Republic). Vitamin $D_2$ was quantified based on a calibration curve obtained from a commercial standard vitamin $D_2$.

Furthermore, to extract vitamin $D_2$ in the bread and quantify by HPLC, the same method as described above for LC and EE was used, with some modifications in the amounts of reagents to accommodate the weights of the bread produced (on average, 30 g). Thus, 100 mL of DMSO: $H_2O$ (9:1), 200 mL of iso-octane, and 40 mL of deionised water were used. Furthermore, to degrade the bread's starch and facilitate homogenisation and extraction, 1 mL of $\alpha$-amylase was added at the beginning of the extraction.

The loading capacity (LC) of the particles, mg of vitamin $D_2$ per g of particles, was determined according to **Equation 6**. where $w_{enc}$ corresponds to the vitamin $D_2$ quantity encapsulated per gram of particles determined by HPLC and $w_{part}$ reflects the mass of the particles.

$$(\mathbf{6})\ Loading\ capacity = \frac{w_{enc}}{w_{part}}$$

The encapsulation efficiency of the particles was calculated using **Equation 7**. where $w_{enc}$ corresponds to the vitamin $D_2$ quantity encapsulated per gram of particles determined by HPLC and $w_{teor}$ reflects the theoretical quantity of vitamin $D_2$ encapsulated per gram of particles according to the formulation (e.g., vitamin $D_2$ mass/total initial compounds mass including citric acid, sodium citrate, polymers, Tween80 and vitamin $D_2$).

$$(\mathbf{7})\ Encapsulation\ efficiency = \frac{w_{enc}}{w_{teor}} \times 100$$

### 2.13. Flour fortification trials

Tests were performed, where the KC-Bioactive particles and the Vit$D_2$-enriched extract obtained by UAE were used to evaluate the viability of incorporating the particles and free extract into flour and the preservation of Vit$D_2$ after fortification. Two flours were prepared separately, and subsequently, these fortified samples were submitted to extraction (by the same method for LC/EE and ergosterol/vitamin $D_2$) and quantification (HPLC) processes of vitamin $D_2$ as described above.

### 2.14. Sensorial analyses of the final products

For sensorial analyses, bread was made in the local bakery Pão de Gimonde, located in Northeast Transmontano. For the formulation of a batch of 5 commercial bread (control) of approximately 600 g baked were used 2 kg of wheat flour type-65, 200 g of rye bread dough (bakery secret), 30 g of salt, and 1.4 L of water. The dough formulation process consists of putting the ingredients mentioned above into a container and stirring the dough until all the ingredients are mixed as homogeneously as possible. Let it rest for 85 min, then put it in moulds and store it in a cold and humid environment until the next day to be baked. For the formulation of samples 1 and 2, the same procedure as for the control formulation was carried out, but using flour previously fortified with KC-Bioactive and Vit$D_2$-enriched extract (guaranteeing 100% of the recommended daily allowance (RDA)).

After the bread confection procedure, the products were submitted for sensorial analysis. The sensory analysis was conducted with a random public, with variations in age between 18 and 65 years old, both genders being students, collaborators, and academics from the Instituto Politécnico de Bragança, Bragança, Portugal. For the execution of the analyses, three bread samples were served: a control sample containing the commercial formulation, sample 1 made with flour fortified with KC-Bioactive and sample 2 containing flour fortified with free $VitD_2$-enriched extract. The bread samples were served to the participants in small squares wrapped in aluminium foil and demarcated with the name control and the numbers of each sample.

The same flour used to make the bread were served in small paper cups equally identified for the flour samples sensorial analysis. For the evaluation intervals, a glass of water and napkins were provided to clean the palate and hands. The acceptance of the bread was evaluated according to a hedonic scale (like it extremely to dislike it extremely) compared to the control. The criteria are colour, appearance, smell, aroma, taste, texture, mouthfeel, and overall product acceptance. For the flour samples were evaluated with the same scale and criteria except for mouthfeel.

### 2.15. Statistical analysis

Overall, all assays were performed with three replicates, and data are expressed as mean ± standard deviation (SD). Some comparison methodologies were used while carrying out this thesis, namely one-way ANOVA, two-way ANOVA (particularly generalised linear model ANOVA), Wilks' λ test, Student's t-test, Levene's test, Tukey's HSD test, post-hoc test, (unpair *t*-test, and Tamhane's T2, also advanced classification tools like principal component analysis (PCAs), Pearson's correlations, linear discriminant analysis (LDA), and visualization techniques. These methodologies were performed using IBM SPSS statistics for Windows, Version 22.0., Version 23.0., Version 24.0 and Version 25.0 (IBM Corp., Armonk, New York, USA), Prisma (9.0.1), Numpy (version 1.19.2), Pandas (version 1.1.3), Matplotlib (version 3.5.1) and Seaborn (version 0.11.1). The other tool used for assessing and evaluating some data collected was the open-source integrated development environment (IDE) R studio 2022.07.1. To carry out some different analysis, libraries such as tidyverse, reshape2, ggplot2, plyr, grid, ggcorrplot and factoextra were extensively employed. As a parallel, Scipy package (release 1.6.1) and Python 3.8.12 using the opensource IDE Spyder 5.1.5 was used.

# CHAPTER 3

# Results and Discussion

## 3.1. Impact of supplementation on mushroom production and chemical compounds

### 3.1.1. Calcium silicate supplementation effect on chemical properties of *Pleurotus ostreatus*

#### *3.1.1.1. Biological Efficiency and Crop Yield Ratio Table*

**Table 12** shows five parameters of the mushroom crop with the supplementation of CS during the two harvest periods (1st and 2nd flushes), namely mushroom weight, number of mushrooms per harvest, biological efficiency, yield ratio, and productivity rate. **Table 12** is divided into two sections, each one pertaining to the locations of the strains, namely "Presidente Prudente" (PP) and "Mogi-das-Cruzes" (MC). Each section is further divided into upper and lower sections, referring to the two factors: Harvest Number (HN) and Calcium Silicate supplementation (CS). This sectioning of the tables derives from the two-way ANOVA employed to understand the influence of each factor individually, and thus, in the upper section of **Table 12**, within the ranges of the means are all the values from the different CS, namely 0, 0.5, 1, 2, and 4%, and in the lower sections are all the values from harvests. This type of representation of the results allows for a much more trustworthy interpretation of the results, for each factor that caused change can be analysed independently of the influence of the other. The existence of an interaction between both factors is demonstrated by HN × CS $p < 0.05$, and thus, only general conclusions can be extracted, whilst HN × CS $p > 0.05$ shows that each factor can be classified independently.

Analysing the strains from Presidente Prudente, there was no interaction among the two factors for any of the analysed parameters. Still, no significant difference was recorded among the different supplementation concentrations ($p > 0.05$), and while there is a significant difference between the two harvest periods ($p < 0.05$), being clear that all parameters are reduced in the second harvest when compared to the quantities of the first. The same tendencies can be observed for the strain from Mogi-das-Cruzes, with no statistical differences among the supplementation quantity of CS but with a significant reduction from the first harvest to the second (classified with an *). One interesting conclusion that can be immediately extracted from the agronomical parameters investigated in this study is that calcium silicate supplementation of the substrate does not influence the total weight of the mushrooms or the number of mushrooms per harvest. Furthermore, the biological efficiency is also untouched, as

is the yield ratio and the productivity rate in either harvest (1st and 2nd flushes). Previous studies report similar findings, namely that incorporating calcium sources did not affect production yield (Thongsook & Kongbangkerd, 2011). This is a positive effect, provided that supplementation would not be successful if it reduced any type of yield or productivity. For instance, the first harvest of Presidente Prudente saw a reduction from 167 to 110 g and a reduction of mushrooms from 50 in the first harvest and 35 in the second. The biological efficiency for Mogi-das-Cruzes was reduced from 108% in the first harvest to 84% in the second. However, supplementation did not have a positive effect on yields by increasing them either on the first or second harvest. Still, and as expected, the yields of the first harvest are almost always higher than the ones of the second, and the treatment of calcium silicate did not change this. The decrease in yield in the second harvest of *Pleurotus ostreatus* var. florida was reported by Thongsook & Kongbangkerd (2011), and it is natural in mushroom production once the substrate begins to be spent.

#### *3.1.1.2. Effect of Supplementation on Individual Compounds*

**Table 13** shows the compounds detected in the mushrooms' chemical characterisation, namely ergosterol and its derivative, vitamin $D_2$, along with various soluble sugars like fructose, mannitol, and trehalose, and also three tocopherols $\alpha$-, $\beta$-, and $\gamma$-. Regarding ergosterol, the main sterol in mushrooms, it was found in much higher amounts than its derivative, vitamin $D_2$. The amounts of these two compounds were similar in both mushroom strains. Trehalose was the highest sugar in both strains, with a maximum of 19 g/100 g, while mannitol and fructose did not score over 2.8 and 0.2 g/100 g, respectively. Of the four tocopherol isoforms, only δ-tocopherol was not identified. This is not uncommon, provided that in *Pleurotus ostreatus* var. florida mushrooms, not all isoforms are usually present (Bouzgarrou et al., 2018; Fernandes et al., 2015b). Still, in both mushrooms, $\beta$-tocopherol was the most abundant.

Regarding PP mushrooms, there was a significant interaction among the two factors, HN and CS, allowing for some general tendencies from the EMM plots (**Figure 8**). Still, regarding strains from MC, $\alpha$-tocopherol showed that the mushrooms from the first harvest presented a statistically higher amount of this isoform than the second harvest, while all other compounds displayed a significant interaction. Some tendencies were drawn from the EMM plots, allowing

further analysis. These results show that, once again, supplementation of the substrate with CS does not seem to alter the chemical composition of these mushrooms.

In **Figure 8a** the EMM plots of vitamin $D_2$ of Presidente Prudente mushrooms are shown, and it is clear that there was a reduction from the first to the second harvest in terms of this vitamin. Still, the control sample showed a lower reduction but was also the sample with a lower amount of this compound to start with. Thus, supplementation of calcium silicate showed higher amounts of this vitamin, although the highest results were found at 2%. Furthermore, even at the second harvest, there was a higher amount of vitamin $D_2$ than in the control samples. The fact that supplementation of calcium silicate increases the quantity of vitamin $D_2$ in mushrooms is quite interesting, provided this vitamin is a beneficial compound for human health. Vitamin D comprises other vitamers beyond $D_2$, although $D_2$ and $D_3$ are the most important ones. While $D_3$ is responsible for a higher saturation of vitamin D in the human blood, it can only be produced in our skin with the help of UV light, and thus, in countries with low sun incidence, most of this vitamin enters the human body through the diet. Still, animals mainly produce $D_3$, and with the tendency of the human diet to become more vegetarian based, $D_2$ becomes a promising alternative, being found mainly in mushrooms and plants (Bikle, 2014; Kamweru & Tindibale, 2016).

All vitamers of vitamin D are essential for the human body, especially by reducing the risk of osteoporosis and other ailments of the skeleton, beyond the adjuvant effects in cancer, and cardiovascular diseases, among others (Bikle, 2014). Interestingly, the highest amount was recorded at a 2% concentration of calcium silicate, with the 4% resulting in a reduction in vitamin $D_2$. The results for the Mogi-das-Cruzes strain show similar tendencies, with the highest amount of vitamin $D_2$ found at supplementation of 2%. Thus, supplementing the mushroom substrate with calcium silicate (2%) increases the conversion of ergosterol to vitamin $D_2$ (ergocalciferol), which can be an essential manner of increasing the natural production of this vitamer, becoming more available in the diet.

**Figure 8b** shows the behaviour of $\gamma$-tocopherol in PP during the two harvest periods, and once again, an interesting pattern can be found, higher supplementation of calcium silicate seems to induce the production of this compound, while lower amount (up to 1%) and the control sample, although increasing from the first harvest to the second does not have as much as the

2 and 4% supplementation. The highest quantity found was for 2% supplementation at the first harvest, namely 0.13 mg/100 g, while the highest quantity in the control sample did not go over 0.035 mg/100 g, even at the second harvest. **Figure 8c** represents the total tocopherols of the Mogi-das-Cruzes strain for the several supplementation percentages, and once again, lower supplementation of calcium silicate (1%) seems to reduce the quantity of total tocopherols, while a higher amount seems to promote their production, especially 4%. Still, 0.5% CS promotes the production of tocopherols during the second harvest, which overall showed a higher value of total tocopherols in all tested percentages of CS, especially the lowers ones. This higher amount in the second harvest can be due to stress induced by calcium silicate over a more extended period, provided that tocopherols are synthesised in response to stress situations as a product of the secondary or defensive metabolism of plants and mushrooms (Barros et al., 2008; Kozarski et al., 2015).

**Table 14** shows the different individual fatty acids found in the two strains, represented as relative percentages of total fatty acids. The table only shows the most abundant fatty acids, namely the ones with a relative percentage above 1%. Other fatty acids like C10:0 (decanoic acid), C11:0 (undecanoic acid), C12:0 (dodecanoic acid), C14:0 (tetradecanoic acid), C16:1 (hexadecanoic acid), C17:0 (heptadecanoic acid), C18:3n3 ((9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid), C20:0 (eicosanoic acid), C20:1 (eicosenoic acid), C20:2 (Eicosadienoic acid), C20:5n3 ((5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid), C22:2 (docosanoic acid), C23:0 (tricosanoic acid), C24:0 (tetracosanoic acid) and C24:1 ((15Z)-tetracosenoic acid) were identified and quantified, but due to their very low amounts were not tabled and further discussed. Mushrooms have a very low amount of fat, and *Pleurotus ostreatus* var. florida is not an exception, the most abundant one is polyunsaturated, and thus the profile found in the studied mushrooms are consistent with the ones from literature, with C18:2n6c ((9Z,12Z)-octadeca-9,12-dienoic acid) being the most quantified, followed by C18:1n9c (octadec-9-enoic acid) and C16:0 (hexadecanoic acid) (Fernandes et al., 2015b).

As expected, the PUFAs (polyunsaturated fatty acids) accounted for over 66% of all fatty acids, which makes these mushrooms very healthy foods due to the beneficial effects of unsaturated fatty acids on human health. Saturated and monounsaturated fatty acids showed approximately 13 to 14 and 18 to 19%, respectively, making saturated fat very low. Overall, unsaturated fatty acids accounted for approximately 80% of the total. There was a significant interaction among

both factors, HN and CS, and thus no individual classifications could be made, so, where possible, some general conclusions were extracted from the EMM plots, shown in **Figure 9**, although the overall fatty acid profile of the mushrooms did not change much due to the supplementation of CS. Still, by analysing **Figure 9**, section **(a)** in the PP mushrooms, an interesting pattern can be found for oleic acid (common name, C18:1n9c), where despite variations in the second harvest, the amounts of this molecule were never as much as the ones found in the first harvest, indicating that the best mushrooms in terms of this unsaturated fatty acid are found in the first harvest. This supplementation only affects the second harvest, although ever so slightly. Inversely, all saturated fatty acids are found in higher amounts during the second harvest, and thus, the first harvest shows lower amounts for all CS concentrations. Still, at 1% of silicate supplementation, there seems to be a very drastic reduction in SFAs (saturated fatty acids) (**Figure 9b**). Finally, regarding section **(c)** of **Figure 9**, pertaining to the Mogi-das-Cruzes strain, a similar pattern is found, with higher amounts of SFAs in the second harvest. Unfortunately, no patterns could be found in the EMM for unsaturated fatty acids. Overall, it seems that the variations in fatty acids are more related to the passage of time than with supplementation of CS, with SFAs being higher in the second harvests and unsaturated ones in the first harvest. This pattern could be due to the freshness of the substrate and higher amounts of compounds the mushrooms can absorb, and thus have higher unsaturated fatty acids, whilst the second harvest, with a lower amount of available nutrients in the substrate, shows a higher amount of saturated fatty acids (Nieto & Chegwin A, 2013). There seems to be minimal effect of CS supplementation on the overall profile of fatty acids, with time and available nutrients being the main reason for the changes in the profile.

**Table 12:** Biological parameters of the two strains during the two harvest periods and different supplementation concentrations

| | | Mushroom Weight (g) | Number of Mushrooms | Biological Efficiency (%) | Yield Ratio (%) | Productivity Rate (% per Day) |
|---|---|---|---|---|---|---|
| **Presidente Prudente (PP)** | | | | | | |
| Harvest number (HN) | First flush | 167 ± 52 * | 50 ± 21* | 104 ± 32 * | 8 ± 2 * | 4 ± 1 * |
| | Second flush | 110 ± 41 | 35 ± 16 | 69 ± 25 | 5 ± 2 | 2.0 ± 0.8 |
| p-value (n = 10) | Student T test | <0.001 | 0.009 | <0.001 | <0.001 | <0.001 |
| Calcium silicate supplementation (CS) | Control | 164 ± 65 | 40 ± 22 | 103 ± 41 | 8 ± 3 | 3 ± 2 |
| | 0.5% | 147 ± 59 | 43 ± 16 | 92 ± 36 | 7 ± 3 | 3 ± 1 |
| | 1% | 129 ± 45 | 47 ± 27 | 81 ± 28 | 6 ± 2 | 3 ± 1 |
| | 2% | 124 ± 52 | 39 ± 21 | 77 ± 32 | 6 ± 2 | 3 ± 1 |
| | 4% | 129 ± 48 | 43 ± 16 | 80 ± 30 | 6 ± 2 | 3 ± 1 |
| p-value (n = 25) | Tukey's HSD test | 0.296 | 0.897 | 0.296 | 0.296 | 0.571 |
| HN CS (n = 50) | p-value | 0.826 | 0.332 | 0.826 | 0.826 | 0.697 |
| **Mogi-das-Cruzes (MC)** | | | | | | |
| Harvest number (HN) | First flush | 172 ± 27 * | 46 ± 6* | 108 ± 13 * | 9 ± 1 * | 4 ± 1 * |
| | Second flush | 135 ± 68 | 33 ± 4 | 84 ± 42 | 7 ± 2 | 2 ± 1 |
| p-value (n = 10) | Student T test | 0.044 | 0.031 | 0.044 | 0.044 | <0.001 |
| Calcium silicate supplementation (CS) | Control | 174 ± 79 | 48 ± 30 | 108 ± 49 | 9 ± 4 | 4 ± 2 |
| | 0.5% | 156 ± 43 | 35 ± 18 | 97 ± 27 | 8 ± 2 | 3 ± 1 |
| | 1% | 159 ± 50 | 41 ± 12 | 100 ± 31 | 8 ± 2 | 3 ± 1 |
| | 2% | 156 ± 44 | 43 ± 26 | 97 ± 27 | 8 ± 2 | 3 ± 1 |
| | 4% | 124 ± 94 | 30 ± 19 | 78 ± 59 | 6 ± 5 | 3 ± 2 |
| p-value (n = 25) | Tukey's HSD test | 0.529 | 0.350 | 0.529 | 0.529 | 0.708 |
| HN CS (n = 50) | p-value | 0.455 | 0.264 | 0.455 | 0.455 | 0.430 |

In each row, for the Harvest number (HN), the asterisk (*) means different statistical differences between the two harvests, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision but rather as the recorded values' range.

**Table 13:** Different bioactive compounds and soluble sugars detected in the two mushroom strains along the two harvests and CS supplementation concentrations

| | | Ergosterol (mg/100 g) | Vitamin $D_2$ (µg/100 g) | Fructose (g/100 g) | Mannitol (g/100 g) | Trehalose (g/100 g) | Soluble Sugars (g/100 g) | α-Tocopherol (mg/100 g) | β-Tocopherol (mg/100 g) | γ-Tocopherol (mg/100 g) | Total Tocopherols (mg/100 g) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Presidente Prudente (PP)** | | | | | | | | | | | |
| Harvest number (HN) | First flush | 107 ± 6 | 743 ± 133 | 0.16 ±0.03 | 2.2±0.2 | 19 ± 2 | 22 ± 2 | 0.002±0.001 | 0.26±0.03 | 0.05±0.06 | 0.31±0.07 |
| | Second flush | 107 ± 8 | 593 ± 73 | 0.2±0.1 | 2.2±0.6 | 15 ± 4 | 17 ± 4 | 0.002±0.001 | 0.3 ± 0.07 | 0.06±0.04 | 0.36±0.05 |
| *p*-value (n = 10) | Student T test | 0.288 | 0.031 | 0.007 | 0.811 | <0.001 | <0.001 | 0.580 | <0.001 | <0.001 | <0.001 |
| Calcium silicate supplementation (CS) | Control | 101 ± 8 | 496 ± 27 | 0.20±0.03 | 2.8±0.5 | 15 ± 3 | 18 ± 3 | 0.002±0.001 | 0.32±0.03 | 0.02±0.01 | 0.34±0.04 |
| | 0.5% | 109 ± 6 | 658 ± 71 | 0.13±0.03 | 1.8±0.4 | 18 ± 3 | 20 ± 3 | 0.002±0.001 | 0.24±0.04 | 0.009±0.004 | 0.25±0.04 |
| | 1% | 110 ± 1 | 734 ± 144 | 0.19±0.08 | 1.91±0.06 | 16 ± 8 | 18 ± 8 | 0.002±0.001 | 0.34±0.08 | 0.02±0.01 | 0.36±0.09 |
| | 2% | 113 ± 1 | 779 ± 90 | 0.16±0.03 | 2.0±0.2 | 19 ± 1 | 21 ± 2 | 0.001 ±0.0001 | 0.26±0.01 | 0.13±0.01 | 0.39±0.01 |
| | 4% | 101 ± 4 | 671 ± 89 | 0.2±0.1 | 2.3±0.2 | 18 ± 2 | 21 ± 2 | 0.001 ± 0.0001 | 0.23±0.03 | 0.108±0.004 | 0.34±0.03 |
| *p*-value (n = 25) | Tukey's HSD test | <0.001 | <0.001 | 0.006 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| HN × CS (n = 50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Mogi-das-Cruzes (MC)** | | | | | | | | | | | |
| Harvest number (HN) | First flush | 111 ± 6 | 899 ± 167 | 0.14±0.03 | 1.9 ±0.2 | 19 ± 2 | 21 ± 2 | 0.003 ±0.001 * | 0.2 ±0.1 | 0.05±0.03 | 0.25±0.09 |
| | Second flush | 116 ± 10 | 829 ± 175 | 0.18±0.08 | 1.8 ±0.4 | 17 ± 4 | 19 ± 4 | 0.0018±0.0001 | 0.28 ±0.06 | 0.04 ±0.04 | 0.32±0.05 |
| *p*-value (n = 10) | Student T test | <0.001 | <0.001 | <0.001 | 0.062 | <0.001 | <0.001 | 0.009 | <0.001 | <0.001 | <0.001 |
| Calcium silicate supplementation (CS) | Control | 113 ± 4 | 555 ± 51 | 0.2± 0.1 | 2.3±0.3 | 18 ± 2 | 20 ± 2 | 0.0025 ±0.0009 | 0.261 ±0.005 | 0.013 ±0.003 | 0.28±0.05 |
| | 0.5% | 123 ± 1 | 951 ± 90 | 0.16± 0.03 | 1.8 ±0.4 | 17 ± 5 | 19 ± 6 | 0.0022 ±0.0003 | 0.32 ±0.08 | 0.011 ±0.003 | 0.33±0.08 |
| | 1% | 100 ± 3 | 880 ± 50 | 0.15 ±0.01 | 1.6 ±0.2 | 16 ± 3 | 18 ± 3 | 0.002 ± 0.001 | 0.1 ±0.1 | 0.04 ±0.03 | 0.2 ±0.1 |
| | 2% | 117 ± 7 | 1022 ± 31 | 0.10 ±0.02 | 1.63±0.05 | 18 ± 2 | 19 ± 2 | 0.003 ±0.001 | 0.23 ±0.02 | 0.08 ±0.02 | 0.31±0.01 |
| | 4% | 114 ± 6 | 913 ± 30 | 0.19 ±0.06 | 2.0 ±0.2 | 20 ± 2 | 22 ± 2 | 0.0014 ±0.0007 | 0.25 ±0.03 | 0.087±0.006 | 0.34±0.03 |
| *p*-value (n = 25) | Tukey's HSD test | <0.001 | <0.001 | 0.006 | <0.001 | <0.001 | <0.001 | 0.130 | <0.001 | <0.001 | <0.001 |
| HN × CS (n = 50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.058 | <0.001 | <0.001 | <0.001 |

In each row, for the Harvest number (HN), the asterisk (*) means different statistical differences among the two harvests, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

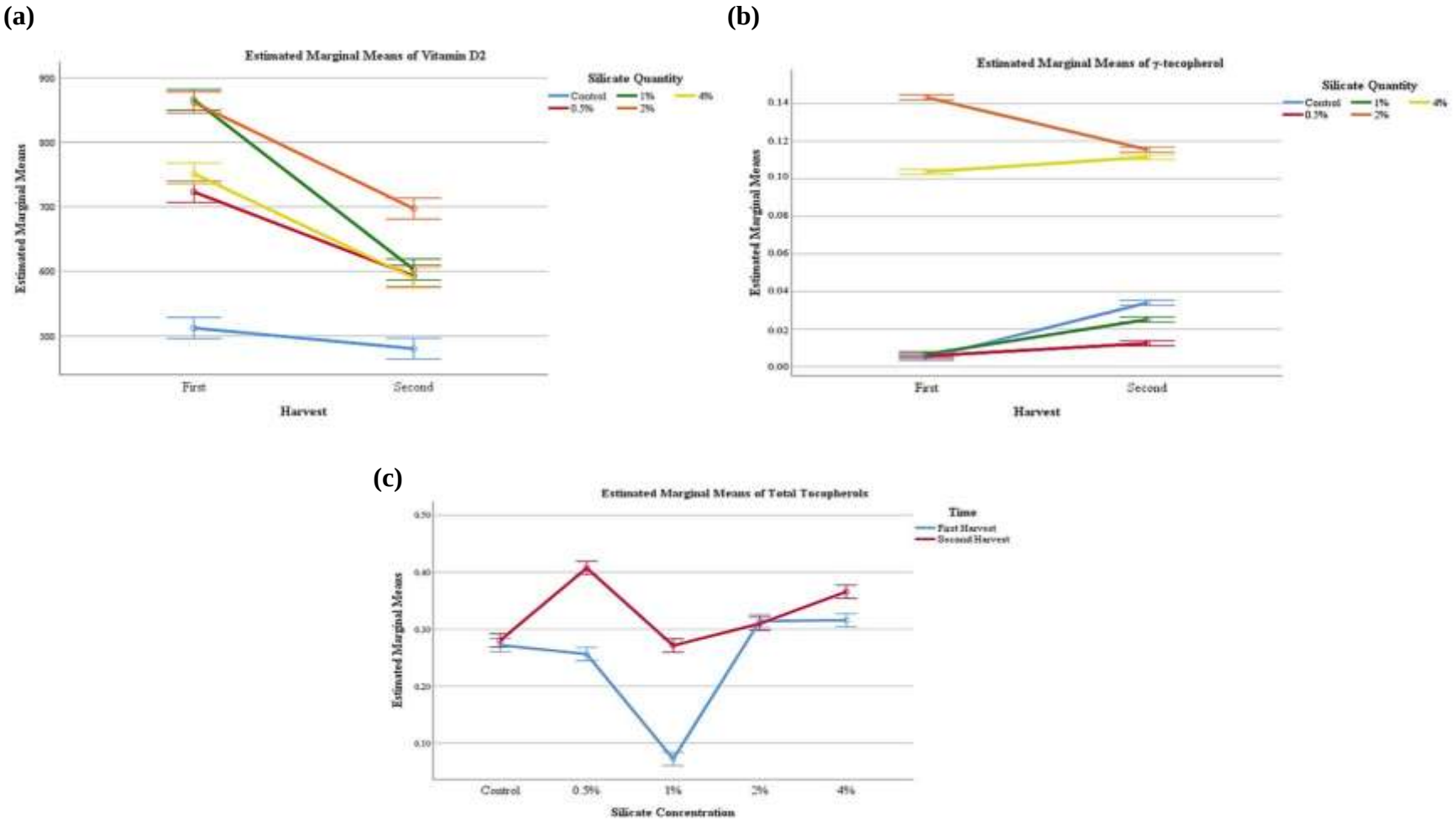



**Figure 8:** EMM plots of the Presidente Prudente mushrooms during the two harvest periods: (a) vitamin $D_2$, (b) γ-tocopherol. EMM plots of Mogi-das-Cruzes at different CS concentrations: (c) total tocopherols

**Table 14:** Fatty acids detected through GC-FID from the two mushroom strains across the two harvest periods and CS supplementation, expressed in relative percentage of themselves. Only fatty acids with a relative above 1% are tabled, although others were identified

| | | C15:0 | C16:0 | C18:0 | C18:1n9C | C18:2n6c | SFAs | MUFAs | PUFAs |
|---|---|---|---|---|---|---|---|---|---|
| **Presidente Prudente (PP)** | | | | | | | | | |
| Harvest number (HN) | First flush | 1.4±0.1 | 11.7 ± 0.4 | 2.8 ±0.2 | 14.1±0.3 | 65.8±0.7 | 18.5±0.6 | 14.6±0.3 | 66.8±0.7 |
| | Second flush | 1.7±0.2 | 11.9 ± 0.5 | 2.7 ±0.2 | 12.5±0.9 | 66±1 | 19±1 | 13.0±0.9 | 67±1 |
| *p*-value (n = 10) | Student T test | <0.001 | 0.025 | <0.001 | <0.001 | 0.002 | <0.001 | <0.001 | 0.006 |
| Calcium silicate supplementation (CS) | Control | 1.7±0.3 | 11.7 ± 0.2 | 2.79 ±0.06 | 13±1 | 66.6±0.7 | 18.7±0.8 | 14±1 | 67.6±0.6 |
| | 0.5% | 1.5±0.4 | 11.8 ± 0.2 | 3.0 ±0.1 | 13±1 | 66.4±0.4 | 19.2±0.7 | 13±1 | 67.3±0.4 |
| | 1% | 1.3 ± 0.07 | 11.5 ± 0.2 | 2.7 ±0.2 | 13.8±0.4 | 66.9±0.3 | 17.8±0.1 | 14.3±0.4 | 67.9±0.4 |
| | 2% | 1.59±0.07 | 12.1 ± 0.8 | 2.80 ±0.07 | 13.9±0.5 | 64.3±0.8 | 20±1 | 14.5±0.4 | 65.4±0.8 |
| | 4% | 1.6±0.2 | 12.0 ± 0.6 | 2.65 ±0.09 | 13.9±0.5 | 66±1 | 19.4±0.5 | 13±1 | 67 ± 1 |
| *p*-value (n = 25) | Tukey's HSD test | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| HN CS (n = 50) | *p*-value | <0.001 | <0.001 | 0.001 | <0.001 | 0.002 | 0.001 | <0.001 | 0.001 |
| **Mogi-das-Cruzes (MC)** | | | | | | | | | |
| Harvest number (HN) | First flush | 1.29±0.06 | 11.6 ± 0.3 | 2.7 ± 0.1 | 13.6±0.6 | 66.4±0.6 | 18.5±0.6 | 14.1±0.6 | 67.4±0.6 |
| | Second flush | 1.6±0.1 | 12.4 ± 0.3 | 2.8 ± 0.2 | 13±1 | 66±1 | 19.4±0.5 | 13±1 | 67±1 |
| *p*-value (n = 10) | Student T test | <0.001 | <0.001 | 0.543 | <0.001 | 0.045 | <0.001 | <0.001 | 0.146 |
| Calcium silicate supplementation (CS) | Control | 1.5±0.1 | 12.2 ± 0.4 | 2.7 ± 0.1 | 13.9±0.2 | 65±1 | 19±1 | 14.3±0.2 | 66±1 |
| | 0.5% | 1.4±0.2 | 11.9 ± 0.6 | 2.7 ± 0.3 | 13±1 | 66.8±0.5 | 18±1 | 13±1 | 67.7±0.5 |
| | 1% | 1.5±0.3 | 11.9 ± 0.2 | 0.287 ± 0.03 | 13±1 | 67.2±0.8 | 18.6±0.3 | 13.1±0.1 | 68.3±0.9 |
| | 2% | 1.39±0.07 | 12.3 ± 0.4 | 2.7 ± 0.1 | 12.5±0.1 | 66.6±0.3 | 19.2±0.2 | 13.1±0.1 | 67.7±0.3 |
| | 4% | 1.4±0.1 | 11.9 ± 0.8 | 2.8 ± 0.1 | 14.0±0.3 | 65.2±0.8 | 19.2±0.4 | 14.6±0.4 | 66.1±0.8 |
| *p*-value (n = 25) | Tukey's HSD test | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | 0.001 | <0.001 | <0.001 |
| HN × CS (n = 50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

C15:0—Pentadecanoic acid, C16:0—Hexadecanoic acid, C18:0—Octadecanoic acid, C18:1n9C—Octadec-9-enoic acid, C18:2n6c-(9Z,12Z)-octadeca-9,12-dienoic acid (IUPAC nomenclature). SFAs—saturated fatty acids, MUFAs—monounsaturated fatty acids, PUFAs—polyunsaturated fatty acids. In each row, for the Harvest number (HN). The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

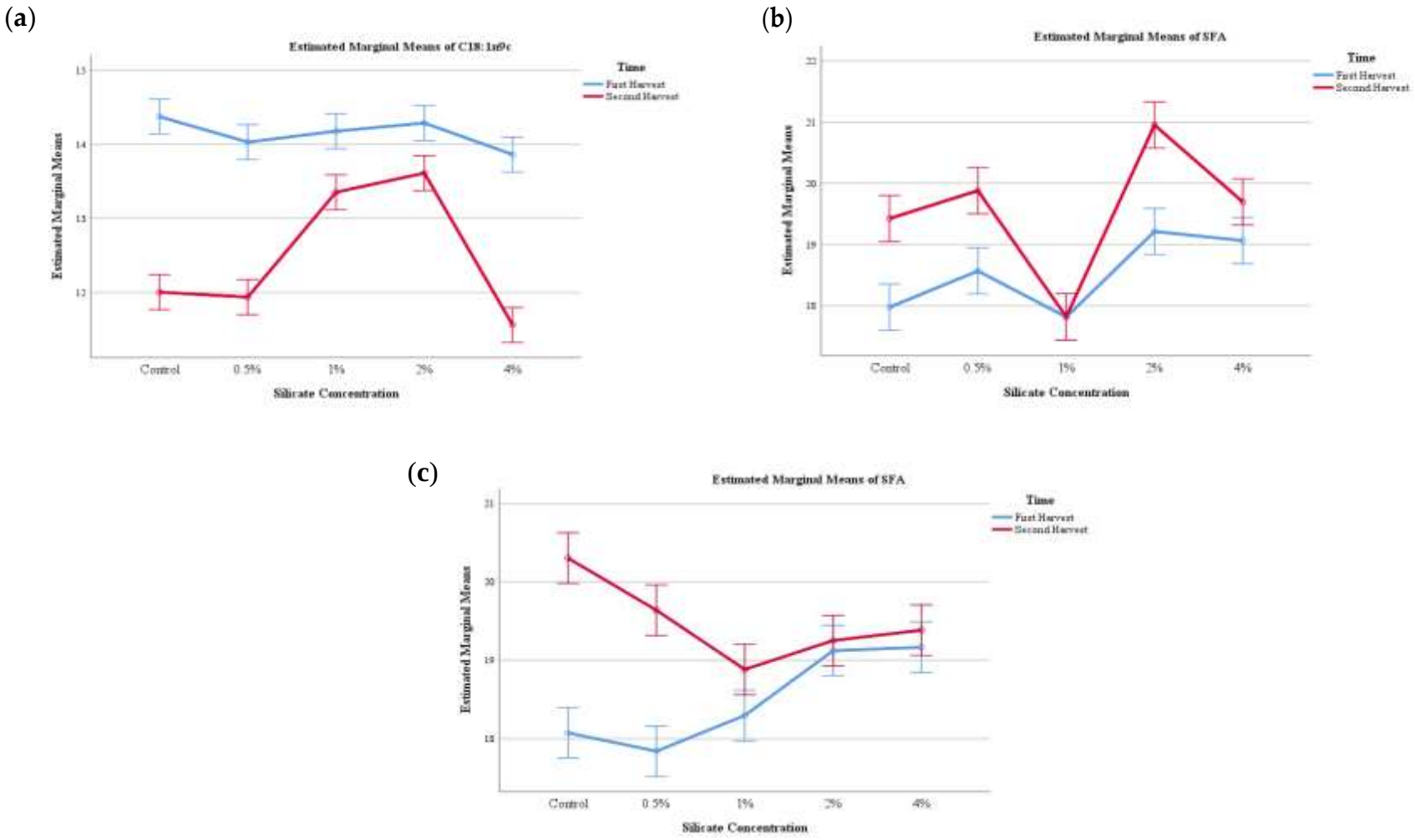



**Figure 9:** EMM plots of the Presidente Prudente mushrooms at different CS concentrations: (a) C18:1n9c (Octadec-9-enoic acid), (b) Saturated Fatty Acids (SFAs). EMM plots for Mogi-das-Cruzes: (c) SFAs

### 3.1.2. Antioxidant and antimicrobial influence in *Pleurotus ostreatus* from substrate supplemented with calcium silicate

#### *3.1.2.1. Organic Acids and Phenolic Compounds*

**Table 15** shows the profile in organic acids and phenolic compounds of both mushrooms with CS supplementing during the two harvest periods (1st and 2nd flushes).

**Table 15** is divided into two sections, each pertaining to the strains' locations, namely MC and PP. As same as commented before, each section is further divided into an upper and lower section, referring to the two factors, namely HN and CS, for the two-way ANOVA employed to understand the influence of each factor individually.

Regarding organic acids, three were detected, namely oxalic acid, malic acid and fumaric acids, with malic acid being the most abundant one. Overall, a significant interaction was sought for all three organic acids for both mushroom provenances and their total amount, and thus only some general conclusions could be extracted from the EMM plots present in **Figure 10**.

The plots showed that malic acid from MC mushrooms supplemented with calcium silicate (**Figure 10a**) showed higher values of this organic acid and a further increase from the first to the second flush, which was positively correlated with the increasing percentage of calcium silicate. Malic acid is an essential carboxylic acid with beneficial effects on health (Pozza et al., 2009; S.-Y. Tsai et al., 2014). Supplementation with 0.5% calcium silicate seems to favour malic acid production, especially in the first flush. Still, supplementation of 4% does not seem to increase the amount of malic acid in the first flush compared to supplementation with 0.5%. Still, it greatly stimulates its production in the second flush.

With regards to the phenolic compounds, once again, a significant interaction was detected for all, both for MC and PP mushrooms. Of the main compounds detected, protocatechuic acid was the most abundant, followed by *p*-coumaric acid and cinnamic acid, which was the least abundant. The EMM plots in **Figure 10** allow for some general tendencies, namely that for MC, supplementation with 4% showed higher amounts of malic acid, while the control sample showed the least (**Figure 10a**). In contrast, the first flush showed higher values of *p*-coumaric acid, with an increase related to the highest supplementation percentages. For the second harvest, this tendency was inverted (**Figure 10b**). Still, for PP, the second harvest showed higher *p*-coumaric acid values, which correlated with higher supplementation of calcium

silicate (**Figure 10c**). Cinnamic acid is the least abundant phenolic compound shown, and in the EMM plot for PP where the highest amount was obtained for the second crop, which showed a maximum of this phenolic compound at 1% calcium silicate supplementation. (**Figure 10d**).

**Table 15:** Organic and phenolic acids detected through ultrafast liquid chromatograph (UFLC)-diode array detector (DAD) and HPLC-DAD-ESI/MS (electrospray ionization coupled to a mass spectrometer), respectively, of the mushrooms provenances across the two harvest periods in DW

| | | Oxalic Acid (g/100 g) | Malic Acid (g/100 g) | Fumaric Acid (g/100 g) | Total Organic Acids (g/100 g) | Protocatechuic Acid (g/100 g) | *p*-coumaric Acid (g/100 g) | Cinnamic Acid (g/100 g) | Total Phenolic Acids (g/100 g) |
|---|---|---|---|---|---|---|---|---|---|
| **Mogi-das Cruzes (MC)** | | | | | | | | | |
| Harvest Number (HN) | First | 0.19±0.04 | 2.7±0.3 | 0.250 ±0.008 | 3.2 ±0.3 | 116±27 | 56±17 | 36 ± 11 | 208 ±48 |
| | Second | 0.22±0.05 | 3.0±0.3 | 0.257 ±0.009 | 3.5 ±0.3 | 177±46 | 32±12 | 30±4 | 239 ±56 |
| *p*-value (n = 15) | *t*-test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Calcium Silicate Concentration (CS) | Control | 0.22 ±0.08 | 2.51±0.3 | 0.251 ±0.002 | 2.9 ±0.3 | 137±53 | 52±1 | 26±5 | 216±57 |
| | 0.5% | 0.21 ±0.03 | 3.1±0.1 | 0.25 ±0.02 | 3.5 ±0.2 | 167±74 | 33±8 | 37±2 | 237±64 |
| | 1% | 0.16 ±0.01 | 2.7±0.2 | 0.250 ±0.003 | 3.1 ±0.2 | 146±46 | 37±6 | 27±1 | 211±41 |
| | 2% | 0.17±0.02 | 2.87±0.02 | 0.253±0.005 | 3.30±0.04 | 112±16 | 41±28 | 35±12 | 187±56 |
| | 4% | 0.24 ±0.03 | 3.2±0.3 | 0.259 ±0.002 | 3.7 ±0.2 | 170±10 | 56±21 | 40 ±9 | 266±21 |
| *p*-value (n = 6) | THSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| HN × CS (n = 30) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Presidente Prudente (PP)** | | | | | | | | | |
| Harvest Number (HN) | First | 0.20 ±0.08 | 3.5±0.8 | 0.28 ±0.02 | 4.0 ±0.8 | 140±62 | 23±8 | 21±2 | 185 ±67 |
| | Second | 0.3 ±0.2 | 2.8±0.4 | 0.27 ±0.02 | 3.4 ±0.3 | 193±29 | 60±19 | 32±5 | 285 ±38 |
| *p*-value (n = 15) | *t*-test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Calcium Silicate Concentration (CS) | Control | 0.3 ±0.2 | 2.6±0.2 | 0.27 ±0.02 | 3.20 ±0.04 | 113±58 | 42±31 | 24±6 | 179 ±94 |
| | 0.5% | 0.208±0.008 | 4±1 | 0.27± 0.04 | 4 ±1 | 198±41 | 51±33 | 26±6 | 274 ±81 |
| | 1% | 0.3 ±0.2 | 3.0± 0.6 | 0.278 ±0.004 | 3.5 ±0.4 | 190±16 | 23±5 | 31±12 | 245 ±33 |
| | 2% | 0.25 ±0.03 | 3.5±0.6 | 0.29 ±0.01 | 4.1 ±0.7 | 211±13 | 51±21 | 27±2 | 290 ±11 |
| | 4% | 0.25 ±0.05 | 3.0±0.3 | 0.252 ±0.009 | 3.5 ±0.3 | 122±41 | 41±10 | 25±4 | 188 ±55 |
| *p*-value (n = 6) | THSD test | <0.001 | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| HN × CS (n = 30) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. *t*-test represents a student's *t*-test, while THSD test means Tukey's honest significance test.

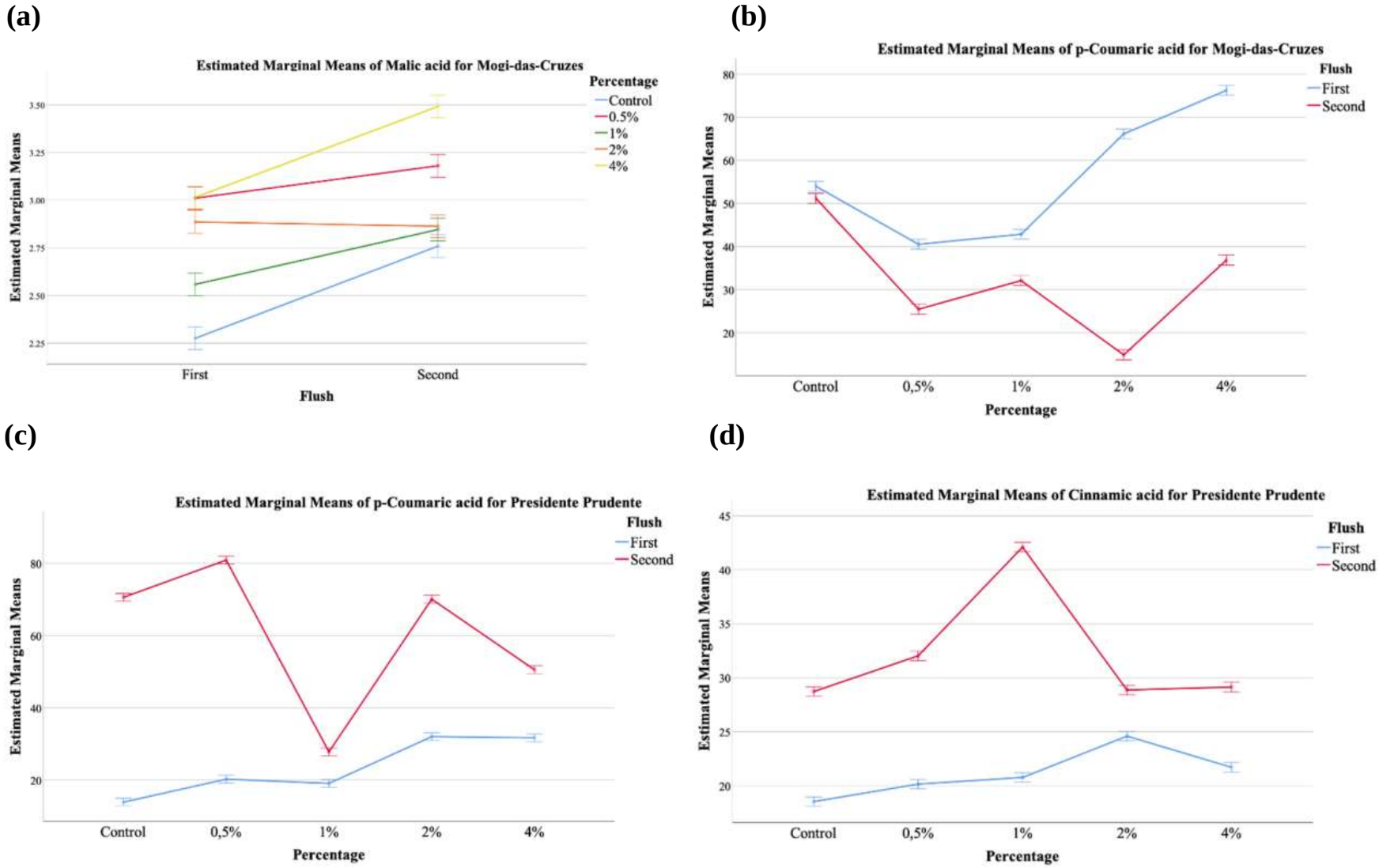



**Figure 10:** EMM plots of mushrooms from Mogi das Cruzes (a,b), (a) malic acid, (b) *p*-coumaric acid, and Presidente Prudente (c,d), (c) *p*-coumaric acid, (d) cinnamic acid, mushrooms at the two different harvest periods

### *3.1.1.1. Antioxidant Activity and Cytotoxicity in Non-Tumour Cell Line*

**Table 16** shows the results of the two antioxidant activity assays carried out for the mushroom extracts, namely TBARS and OxHLIA, and once again, a significant interaction was detected for both. The EMM plots show that for MC, in the OxHLIA assay, only the 0.5% supplementation of calcium silicate did not show activity, compared to the other supplementation percentages (**Table 16**). However, in the 4%, the activity reduced from the first to the second flush (**Figure 11**). For PP mushrooms, the supplementation with calcium silicate showed better activity in the second flush, only showing activity in the second flush. In terms of the TBARS assay, no EMM plots could be shown, although **Table 16** shows a significant interaction between the flushes and the calcium silicate concentration, implying that the slight changes found for this assay were due to both factors. Both samples were tested against a porcine liver primary cell line, showing no cytotoxicity at the tested concentrations, which indicates that the supplementation of calcium silicate in the mushroom substrate does not induce any cytotoxic effect. This result highlights the use of calcium silicate as a stimulant for bioactive molecules in certain crops without the drawbacks of other substrate supplementations.

**Table 16:** Antioxidant activity of the mushrooms provenances across the two harvest periods

| | | **TBARS ($IC_{50}$ mg/mL)** | **OxHLIA ($IC_{50}$ µg/mL)** |
|---|---|---|---|
| **Presidente Prudente (PP)** | | | |
| Harvest Number (HN) | First | 0.8±0.3 | 41±23 |
| | Second | 0.5±02 | 56±38 |
| *p*-value (n = 15) | *t*-test | <0.001 | <0.001 |
| Calcium Silicate Concentration (CS) | Control | 0.7±0.3 | 67±8 |
| | 0.5% | 0.8±0.1 | 80±30 |
| | 1% | 0.6±0.1 | 41±12 |
| | 2% | 0.8±0.6 | 55±16 |
| | 4% | 0.4±0.1 | n.a. |
| *p*-value (n = 6) | THSD test | <0.001 | <0.001 |
| HN × CS (n = 30) | *p*-value | <0.001 | <0.001 |
| **Mogi-das Cruzes (MC)** | | | |
| Harvest Number (HN) | First | 0.7±0.1 | 16±32 |
| | Second | 0.5±0.2 | 56±51 |
| *p*-value (n = 15) | *t*-test | <0.001 | <0.001 |
| Calcium Silicate Concentration (CS) | Control | 0.4±0.2 | n.a. |
| | 0.5% | 0.44±0.05 | n.a. |
| | 1% | 0.6±0.2 | 30±33 |
| | 2% | 0.75±0.02 | 54±60 |
| | 4% | 0.69±0.05 | 96±20 |
| *p*-value (n = 6) | THSD test | <0.001 | <0.001 |
| HN × CS (n = 30) | *p*-value | <0.001 | <0.001 |

The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision but rather as the range of the recorded values. na: no activity. t-test represents a student's *t*-test, while THSD test means Tukey's honest significance test.

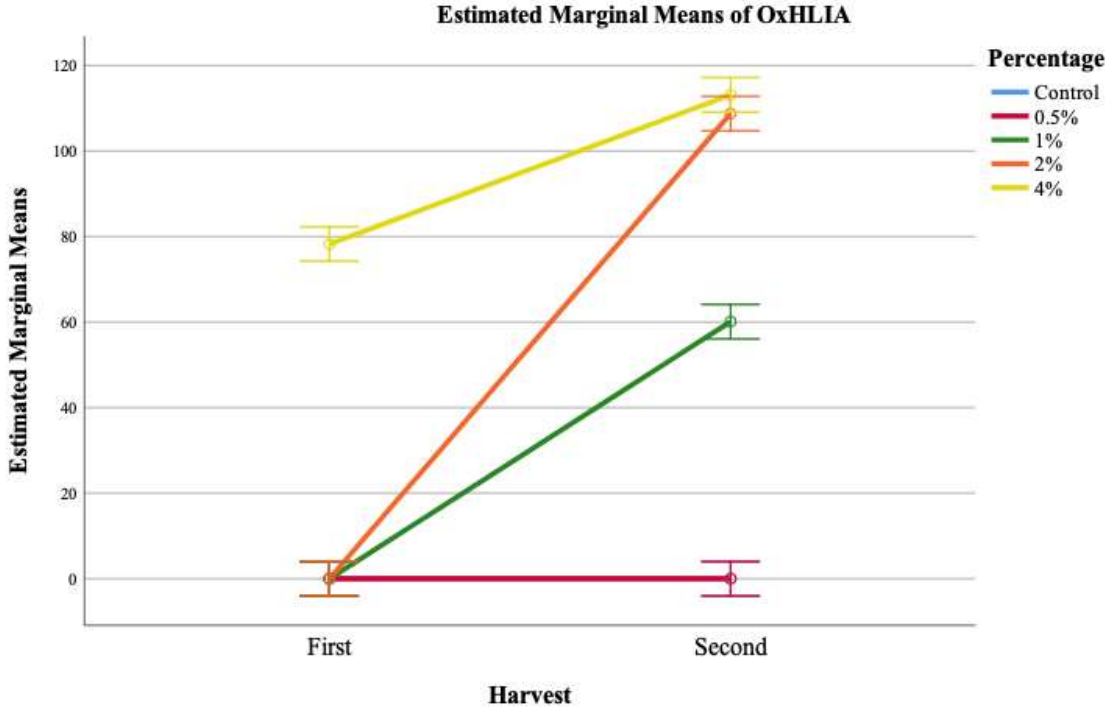



**Figure 11:** Estimated marginal means (EMM) plot of the oxidative haemolysis inhibition (OxHLIA) analysis of the Mogi-das- Cruzes mushrooms

### *3.1.1.2. Antimicrobial Activities*

The minimum inhibition concentration (MIC) for the antimicrobial activity of the different mushrooms is represented in **Figure 12**, where each microorganism corresponds to a colour, and all the different supplementation results are expressed within that same colour. Furthermore, the minimum inhibition concentrations of the positive controls are represented in each case through the red and blue horizontal lines.

The red line represents ketoconazole for the antifungal activity and streptomycin for the antibacterial activity, while the blue line represents bifonazole for the antifungal activity and ampicillin for the antibacterial activity. The mushrooms from PP showed uniform values and low variations from the first flush to the second (**Figure 12a**). The best result was sought against *B. cereus*, in which some percentages of supplementation displayed better activity than both positive controls.

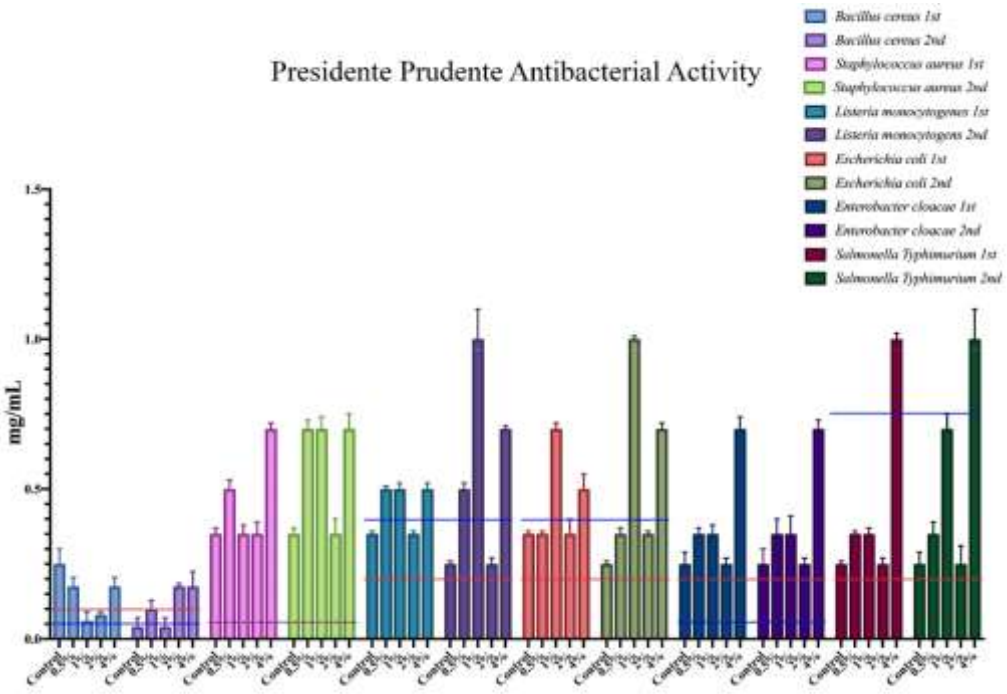



**Figure 12(a)** cont.

Furthermore, most samples of MC showed better results against *S. typhimurium* when compared to ampicillin. Interestingly, the antifungal activity for the mushrooms of PP (**Figure 12b**) showed different results, in which the higher supplementation showed better results than the positive controls in both flushes for the same fungi, *A. ochraceus* and *P. ochlorochloron* in the second flush. The antibacterial activity for mushrooms from MC, represented in **Figure 12c**, showed that the mushrooms in the second flush had lower antibacterial activity and that supplementation did not have much influence on the activity, although, for *B. cereus*, *L. monocytogenes*, and *E. coli*, the values of the mushrooms were very close to the activity of one of the positive controls. Regarding **Figure 12d**, the antifungal activity for the mushrooms from MC, the supplementation did not seem to influence the antifungal activity, and while there seemed to be a decrease from the first to the second flush, excellent results were sought against *P. ochrochloron*, in which the mushroom seemed to have higher activity in the first flush than

both positive controls. Furthermore, the first flush was also between both positive controls for *A. ochraceus* and *P. funiculosum*.

Overall, *P. ostreatus* mushrooms seem to have a higher antibacterial than antifungal activity and, in some cases, supplementation with calcium silicate increase this activity, especially in PP against fungi and MC against bacteria.

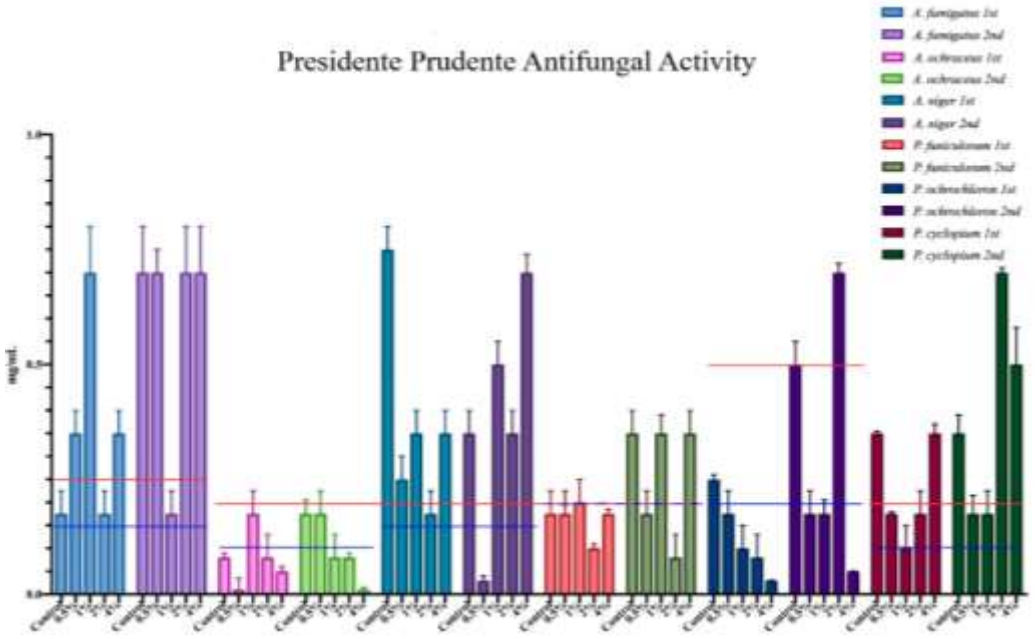



**Figure 12(b)** cont.

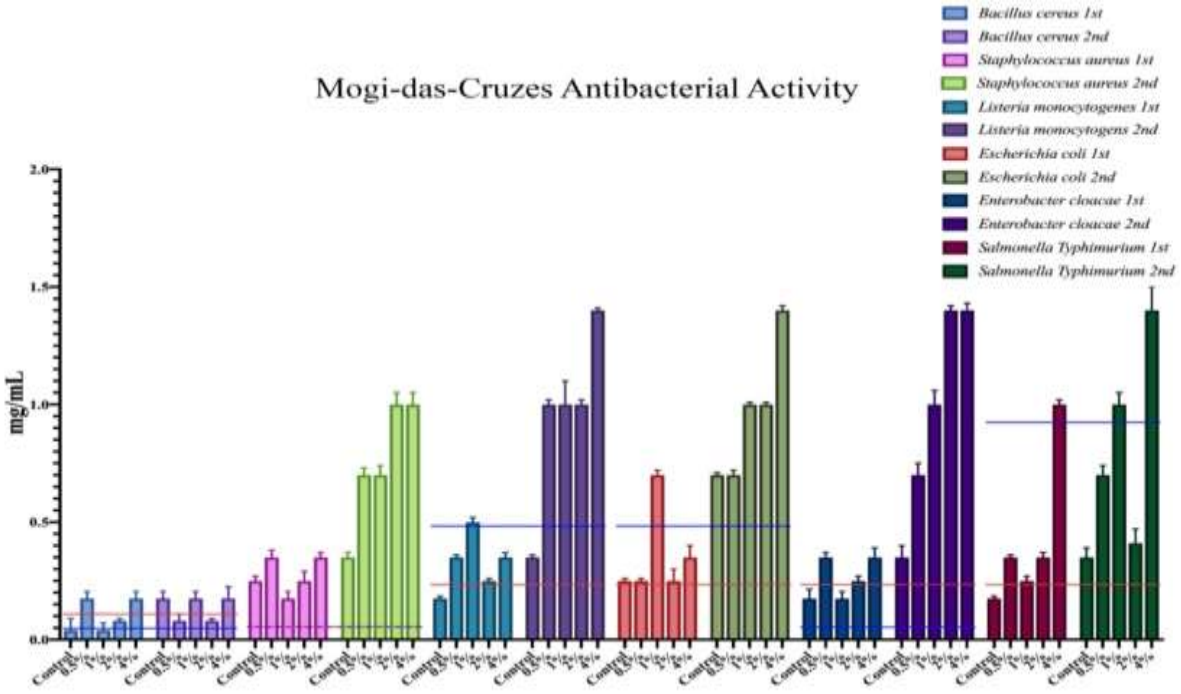
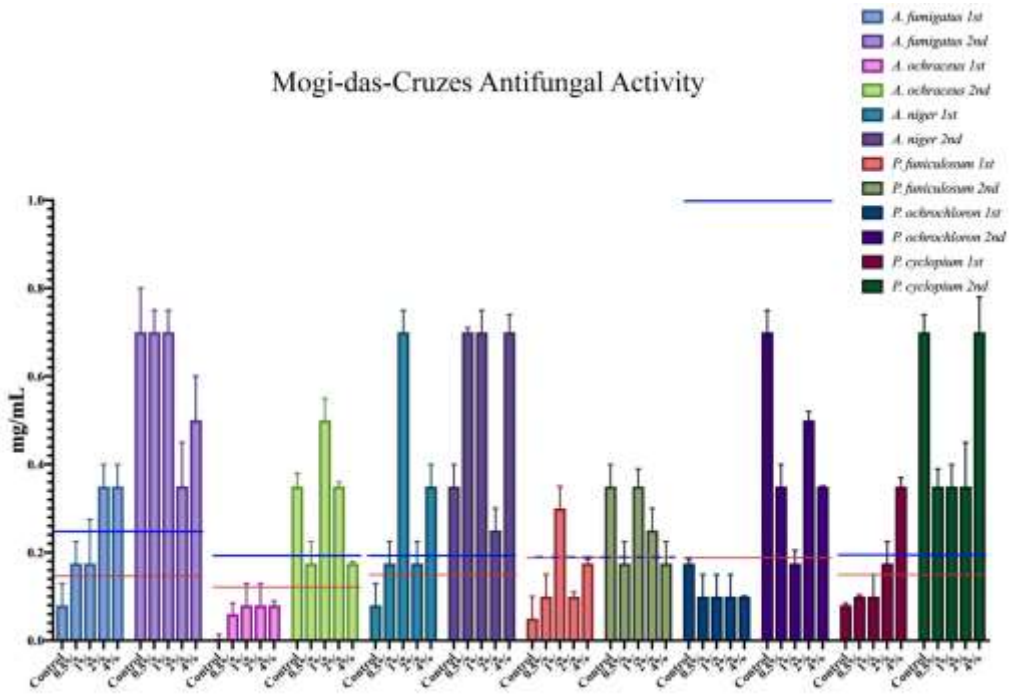



**Figure 12(c)** cont.




**(d)**

**Figure 12:** Antimicrobial activity of the mushrooms from both flushes and different CS supplementation percentages compared to positive controls. For the antibacterial activity, the blue line represents the ampicillin minimum inhibitory concentrations (MIC) while the red line represents the MIC of streptomycin. In the antifungal charts, the red lines represent the MIC of ketoconazole and the blue the MIC of bifonazole. (a) antifungal activity of the mushrooms from Presidente Prudente; (b) antibacterial activity of the mushrooms from Presidente Prudente; (c) antifungal activity of the mushrooms from Mogi-das-Cruzes; (d) antibacterial activity of the mushrooms from Mogi-das-Cruzes

## 3.2. Irradiation of mushrooms, extraction of high-quality compounds

### 3.2.1. Effectiveness of gamma and electron beam radiation as preserving technologies of fresh *Agaricus bisporus* Portobello

Results obtained in all analytical assays were divided into samples treated by gamma radiation (first half of each table) and samples treated by electron beam radiation (second half of each table) to understand their effects throughout time better. In each case, the variability of the result resulted from combining two distinct factors: radiation dose, with the levels 0, 1, 2 and 5 kGy and storage time (ST), with the levels 0, 4 and 8 days. In such cases, the interaction among factors (GI/EB×ST) was also analysed to verify if changes potentially induced by one factor are dissimilar within each level of the other.

In the present topic, and independently of the parameter, the interaction was significant in all cases, not allowing to present the statistical classification that resulted from the performed multiple comparison test (Tukey's HSD test). Accordingly, the identifiable tendencies observed for each parameter were obtained from the estimated marginal mean (EMM) plots that were generated in the GLM analysis.

In what concerns nutritional composition (**Table 17**), gamma irradiation (GI) caused a higher number of significant changes than ST, which had a significant effect only on protein and carbohydrate content. No unequivocal trends could be obtained from the EMM plots in each case. In fact, the values quantified in each case are highly similar, with moisture as the major component (89%). This high-water percentage could increase the release of primary free radicals (hydroxyl, hydrogen atoms and hydrated electrons) as a result of irradiation, thereby justifying the need to study several different chemical parameters, as performed in this work. On a dry weight (dw) basis, carbohydrates were the main component (64–65 g/100 g dw), followed by protein (23.2–24.5 g/100 g dw), ash (9.2–9.9 g/100 g dw) and fat (1.7–1.8 g/100 g dw). The results obtained in electron beam (EB) irradiated samples were similar. At least one EB dose caused a significant change in all nutritional parameters, while ST only affected protein and carbohydrate content. Despite the detected significant differences, the EMM plots showed that the only observed overall tendency was the higher protein content in samples irradiated with 5 kGy. All in all, it seems obvious that neither GR, nor EB, exert any remarkably negative effect over the nutritional parameters of stored (up to 8 days) Portobello samples, which is in agreement with the results obtained in other mushroom species (Fernandes et al., 2016b; Fernandes et al., 2014b, 2015a).

Moving on to the polar compound profiles (**Table 18**), herein represented by organic acids and sugars, which are important indicators of reliable preservation conditions (Barreira et al., 2010), several significant differences were detected. Nearly all parameters (except for mannitol and grouped sugars) showed a significantly different value for at least one GI dose or a specific ST. However, these differences corresponded to overall trends only in the case of malic acid (higher in samples irradiated with GI at 5 kGy and lower in non-stored samples) and grouped organic acids (higher in samples stored for 8 days).

In the case of Portobello treated with EB, the significant differences were detected in a higher number of parameters since this effect was observed in all cases except trehalose content

($p$=0.051). Furthermore, several tendencies could be obtained from the corresponding EMM plots: non-irradiated samples showed lower contents in malic acid (0.5 g/100 g dw) and grouped organic acids (2.7 g/100 g dw), but higher concentration of mannitol (38 g/100 g dw) and grouped sugars (41 g/100 g dw); samples irradiated with 2 kGy gave the highest value in quinic acid (1.0 g/100 g dw), which showed the lowest value (0.8 g/100 g dw) in non-stored samples, similarly to malic acid (1.6 g/ 100 g dw) and grouped organic acids (3.0 g/100 g dw). The low-extent changes detected in sugars, and organic acids also agree with previous reports describing the effects of irradiation in related mushroom species (Fernandes et al., 2016b; Fernandes et al., 2015a).

Regarding lipophilic compounds, the studied molecules were fatty acids, tocopherols and ergosterol. Fatty acids are also considered good indicators of suitable shelf-life conditions (Barreira et al., 2014; E. Pereira et al., 2016), while tocopherols and ergosterol are well known for their bioactivity, mainly antioxidant and hypocholesterolemic effects, respectively. In addition to those presented in **Table 19**, other fatty acids were quantified, specifically C6:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:1, C17:0, cis-C18:1n−9, *trans*-C18:2n−6, C18:3n−3, C20:1, C20:2, C21:0 and C23:0, but in percentages below 0.5% (however, all were used in the linear discriminant analysis discussed in the next section).

Gamma-irradiated samples presented statistical differences in MUFA and *β*-tocopherol results for ID and *α*-tocopherol and ergosterol regarding ST effect. Some of these differences corresponded to overall trends observable in the EMM plots, namely higher percentages of C16:0 (8.6%) in samples irradiated with 5 kGy, lower C20:0 (1.6%) in the same samples and lower C18:0 (3.4%) in non-irradiated ones. Non-stored samples, in turn, showed lower percentages of C20:0 (1.6%) and lower *β*-tocopherol content (9.9 μg/100 g dw), while the lowest percentages of *cis*-C18:2n−6 (78.6%) and PUFA (79%) were measured in samples stored for 8 days, which, on the other hand, gave the highest SFA percentage (20.1%).

In the case of EB-irradiated Portobello (**Table 19**), most parameters also presented significant differences, except C18:0 and *β*-tocopherol regarding EB effect, and MUFA, *α*-tocopherol and *β*-tocopherol, in ST. From the corresponding EMM plots, it was possible to conclude that samples irradiated with 1 kGy presented higher percentages of *cis*-C18:2n−6 (78.9%) and PUFA (79.4%) and lower percentages of SFA (19.5%), while non-irradiated ones showed the

lowest content (1.8%) of C20:0. In ST, it was only possible to verify that 8 days stored samples showed the lowest percentage of *cis*-C18:2n−6 (78.1%).

The slight differences in lipophilic compounds (which are prone to be oxidised) were previously reported in mushrooms (Fernandes, et al., 2016c) and may result from autoxidation processes since Portobello samples were not stored in oxygen-free conditions. Since the occurrence of this important phenomenon might affect the sensorial quality of mushrooms, it is worth mentioning, however, that the results obtained herein seem to indicate that lipid oxidation occurred to a minor extent (as indicated by the maintenance of percentages of fatty acids more prone to be oxidised).

**Table 17:** Proximate composition and energy value of fresh *Agaricus bisporus* Portobello submitted to different irradiation conditions and storage times. The results are presented as mean ± SD.[1]

| | | Moisture (g/100 g fw) | Fat (g/100 g dw) | Proteins (g/100 g dw) | Ash (g/100 g dw) | Carbohydrates (g/100 g dw) | Energy (kcal/100 g dw) |
|---|---|---|---|---|---|---|---|
| | | | | Gamma irradiation (GI) | | | |
| GI | 0 kGy | 89±2 | 1.8±0.1 | 24.2±0.5 | 9.2±0.5 | 65±1 | 372±2 |
| | 1 kGy | 89±1 | 1.7±0.1 | 23.2±0.4 | 9.9±0.5 | 65±1 | 369±2 |
| | 2 kGy | 89±1 | 1.7±0.1 | 23.2±0.4 | 9.9±0.5 | 65±1 | 369±3 |
| | 5 kGy | 89±1 | 1.8±0.1 | 24.5±0.4 | 9.2±0.4 | 64±1 | 372±2 |
| | ANOVA *p*-value (n = 27)[2] | 0.090 | <0.001 | <0.001 | <0.001 | 0.003 | <0.001 |
| ST | 0 days | 89±1 | 1.8±0.1 | 24.3±0.5 | 9.6±0.5 | 64±1 | 370±4 |
| | 4 days | 89±1 | 1.8±0.1 | 23.5±0.5 | 9.5±0.5 | 65±1 | 371±3 |
| | 8 days | 89±1 | 1.7±0.1 | 23.5±0.5 | 9.6±0.3 | 65±1 | 370±1 |
| | ANOVA *p*-value (n = 36)[3] | 0.074 | 0.105 | <0.001 | 0.425 | <0.001 | 0.464 |
| | GI×ST *p*-value (n = 108)[4] | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | | | | Electron beam irradiation (EB) | | | |
| EB | 0 kGy | 90±1 | 1.8±0.1 | 24.7±0.5 | 9.2±0.4 | 64±1 | 372±2 |
| | 1 kGy | 89±1 | 1.7±0.1 | 23.6±0.4 | 9.8±0.4 | 65±1 | 369±2 |
| | 2 kGy | 90±1 | 1.7±0.1 | 23.6±0.4 | 9.8±0.5 | 65±1 | 369±3 |
| | 5 kGy | 89±1 | 1.8±0.1 | 24.9±0.5 | 9.2±0.4 | 64±1 | 372±2 |
| | ANOVA *p*-value (n = 27)[2] | <0.001 | <0.001 | <0.001 | <0.001 | 0.001 | <0.001 |
| ST | 0 days | 89±1 | 1.8±0.1 | 24.9±0.5 | 9.6±0.5 | 64±1 | 370±3 |
| | 4 days | 90±1 | 1.8±0.1 | 23.7±0.4 | 9.4±0.5 | 65±1 | 371±3 |
| | 8 days | 89±1 | 1.7±0.1 | 24.0±0.4 | 9.6±0.2 | 65±1 | 370±1 |
| | ANOVA *p*-value (n = 36)[3] | 0.518 | 0.237 | <0.001 | 0.176 | <0.001 | 0.217 |
| | EB×ST *p*-value (n = 108)[4] | 0.003 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

[1] Results are reported as mean values of each irradiation dose (GI or EB), aggregating results from 0, 4 and 8 days, and mean values of ST, combining all irradiation doses (from GI or EB). [2] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one GI or EB. [3] If $p < 0.05$, the corresponding parameter had a significant difference for at least one of the time intervals. [4] The interaction among factors was significant in all cases; thereby, the statistical classification could not be indicated.

**Table 18:** Polar compounds (organic acids and sugars) of fresh *A. bisporus* Portobello submitted to different irradiation conditions and storage times. The results are presented as mean ± SD.[1]

| | | Sugars (g/100 g dw) | | | | Organic acids (g/100 g dw) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Fructose** | **Mannitol** | **Trehalose** | **Total** | **Oxalic acid** | **Quinic acid** | **Malic acid** | **Total** |
| | | | | | Gamma irradiation (GI) | | | | |
| GI | 0 kGy | 0.6±0.1 | 36±2 | 1.6±0.4 | 38±3 | 0.7±0.1 | 0.8±0.1 | 1.5±0.1 | 3.0±0.3 |
| | 1 kGy | 0.7±0.2 | 36±3 | 1.4±0.3 | 38±2 | 0.7±0.1 | 0.9±0.1 | 1.6±0.1 | 3.2±0.3 |
| | 2 kGy | 0.6±0.1 | 36±3 | 1.2±0.2 | 37±3 | 0.6±0.1 | 0.9±0.1 | 1.5±0.1 | 3.1±0.1 |
| | 5 kGy | 0.6±0.1 | 35±3 | 1.5±0.3 | 37±2 | 0.6±0.1 | 0.8±0.1 | 1.7±0.1 | 3.2±0.2 |
| | ANOVA *p*-value (n = 27)[2] | 0.007 | 0.712 | 0.001 | 0.559 | 0.025 | <0.001 | <0.001 | 0.012 |
| ST | 0 days | 0.5±0.1 | 36±3 | 1.5±0.2 | 38±3 | 0.6±0.1 | 0.8±0.1 | 1.5±0.1 | 2.9±0.2 |
| | 4 days | 0.6±0.1 | 36±2 | 1.2±0.2 | 37±2 | 0.6±0.1 | 0.9±0.1 | 1.6±0.1 | 3.1±0.1 |
| | 8 days | 0.7±0.2 | 36±3 | 1.5±0.4 | 38±3 | 0.8±0.1 | 0.9±0.1 | 1.6±0.1 | 3.4±0.1 |
| | ANOVA *p*-value (n = 36)[3] | <0.001 | 0.976 | <0.001 | 0.700 | <0.001 | <0.001 | <0.001 | <0.001 |
| | GI×ST *p*-value (n = 108)[4] | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | | | | | Electron beam irradiation (EB) | | | | |
| EB | 0 kGy | 0.8±0.2 | 38±2 | 1.8±0.5 | 41±3 | 0.5±0.1 | 0.8±0.1 | 1.3±0.1 | 2.7±0.1 |
| | 1 kGy | 0.7±0.2 | 33±2 | 1.5±0.4 | 35±2 | 0.6±0.1 | 0.9±0.1 | 1.7±0.1 | 3.2±0.1 |
| | 2 kGy | 0.6±0.1 | 31±7 | 1.5±0.2 | 33±7 | 0.6±0.1 | 1.0±0.1 | 1.8±0.1 | 3.4±0.2 |
| | 5 kGy | 0.7±0.1 | 34±2 | 1.7±0.2 | 36±2 | 0.6±0.1 | 0.9±0.1 | 1.9±0.1 | 3.4±0.1 |
| | ANOVA *p*-value (n = 27)[2] | <0.001 | <0.001 | 0.051 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST | 0 days | 0.7±0.1 | 36±3 | 2.0±0.5 | 39±4 | 0.6±0.1 | 0.8±0.1 | 1.6±0.2 | 3.0±0.3 |
| | 4 days | 0.8±0.1 | 34±1 | 1.4±0.1 | 37±1 | 0.6±0.1 | 0.9±0.1 | 1.7±0.2 | 3.2±0.3 |
| | 8 days | 0.5±0.1 | 32±7 | 1.5±0.3 | 34±7 | 0.6±0.1 | 1.0±0.1 | 1.7±0.2 | 3.3±0.3 |
| | ANOVA *p*-value (n = 36)[3] | <0.001 | <0.001 | <0.001 | <0.001 | 0.022 | <0.001 | 0.063 | 0.001 |
| | EB×ST *p*-value (n = 108)[4] | 0.003 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.011 | 0.005 |

[1] Results are reported as mean values of each irradiation dose (GI or EB), aggregating results from 0, 4 and 8 days, and mean values of ST, combining all irradiation doses (from GI or EB). [2] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one GI or EB. [3] If $p < 0.05$, the corresponding parameter had a significant difference for at least one of the time intervals. [4] The interaction among factors was significant in all cases; thereby, the statistical classification could not be indicated.

All in all, irradiation seems to be a suitable conservation technique, owing to its capacity to maintain the chemical profiles of Portobello fresh samples (**Figure 13)** for extended shelf-life periods. The effective cost should be considered concerning its application at the industrial level. The high price of the irradiation equipment might be considered a strong constraint, but it should also be borne in mind that the operational costs (*e.g*., product transportation) are much less than other presently available conservation technologies.

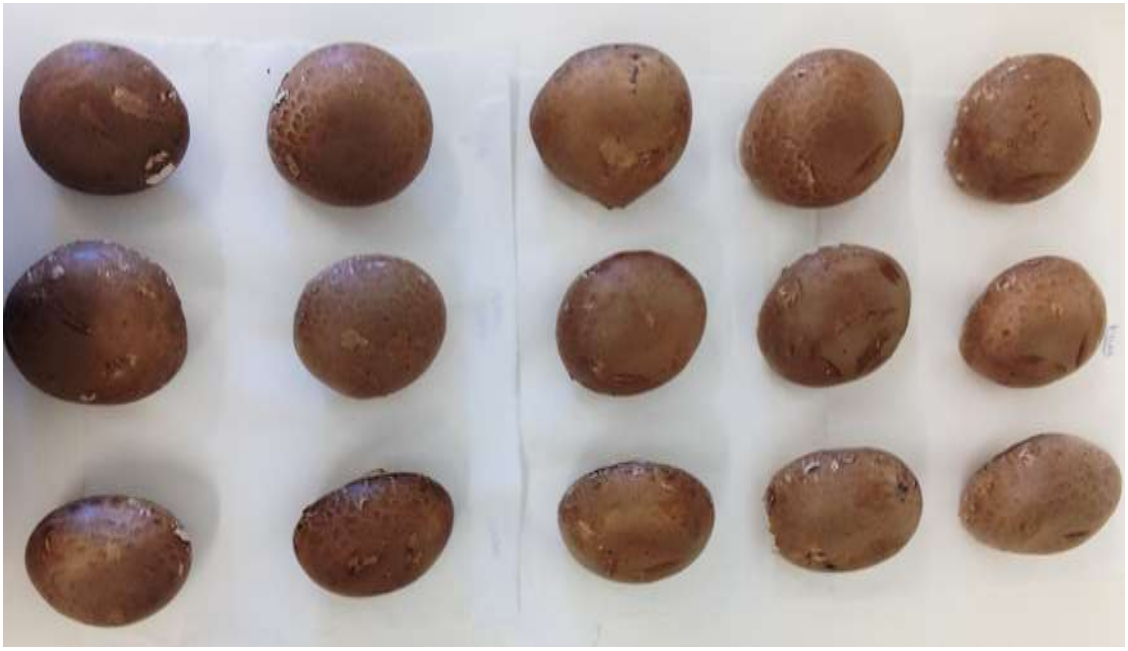


**Figure 13:** Fresh *A. bisporus* Portobello samples

According to the analysis of results described in the previous section, GI and EB seemed to have dissimilar effects on the chemical composition of stored Portobello mushrooms. Therefore, we hypothesised that the effects produced by each irradiation type could be different enough to discriminate them. To verify this hypothesis, a linear discriminant analysis (LDA) was applied to the complete set of results without separating those obtained with each irradiation type (as presented in **Tables 17–19**). The first three discriminant functions of the obtained model included 98.8% (first function: 96.5%; second function: 1.5%; third function: 0.8%) of the observed variance (**Figure 14**). Among the 42 variables (corresponding to each analysed parameter) included in the LDA, only 12 (moisture, protein, mannitol, grouped sugars, quinic acid, grouped organic acids, C16:1, C20:0, C20:2, SFA, PUFA and ergosterol) were considered as not having the discriminant ability, therefore indicating a high dissimilarity among samples treated with GI or EB. The most obvious separation effect observed in **Figure**

**14** is the location of markers corresponding to EB at the positive end of the corresponding axis, while GI markers were placed at the opposite end of the same axis. This separation is more relevant if we consider the percentage of variability explained by function 1 (96.5%); indeed, this is a clear indicator of the high dissimilarity among GI-irradiated and EB- irradiated samples of Portobello. Taking into account the correlations among functions and variables coefficients, the parameters with highest contribution to the separation resulting from function 1, were grouped sugars (37.8 g/100 g dw in GI-irradiated samples and 36.4 g/100 g dw in EB-irradiated samples) and ergosterol (237 mg/100 g dw in GI-irradiated samples and 228 mg/100 g dw in EB-irradiated samples), indicated that these are the two variables with highest changes between GI and EB. Another interesting observation results from the fact that, in addition to the complete individualization of markers corresponding to each irradiation technology, it was also possible to discriminate the irradiation doses assayed within GI and EB. In fact, function 2 divided GI doses in two groups: a first one integrating 0 and 5 kGy markers and a second one including 1 and 2 kGy markers. The greatest differences among these two groups were related with protein (higher in non-irradiated samples and those irradiated with 5 kGy) and C20:2 (higher in samples irradiated with 1 or 2 kGy). In the case of EB-irradiated samples, the four dose levels were also divided in a similar way (0 and 5 kGy on the positive side, 1 and 2 kGy on the negative side), mainly due to the higher percentages of C20:0 in samples irradiated with 1 or 2 kGy, and also their lower protein content. Function 3, in turn, was mostly correlated with moisture and C16:1, contributing to separate markers from non-irradiated samples and those of samples irradiated with 5 kGy, concerning GI treatment, and samples irradiated with 1 and 2 kGy for EB-irradiated ones. In addition, to verify the parameters with the highest changes within each irradiation type, we also intended to check which parameters were more affected by storage. In this second LDA, the two defined discriminant functions included 100.0% (first function: 76.8%; second function: 23.2%) of the observed variance (**Figure 15**). Among the 42 variables included in the LDA, 16 (moisture, fat, protein, ash, energy, oxalic acid, malic acid, ergosterol, fructose, grouped sugars, $\alpha$-tocopherol, $\beta$-tocopherol, grouped tocopherols, C12:0, C17:0, *trans*-C18:2n−6, C20:0, C20:2, C22:0, C23:0, MUFA and PUFA) were not selected as being discriminant, indicating that those were the least affected by storage.

As it might be easily observed in **Figure 15**, function 1 separates mainly markers corresponding to non-stored samples from those belonging to samples stored for 8 days. The variables more

correlated with function 1 were quinic acid, organic acids, and C24:0 (all higher in 8 days-stored samples). Function 2, on the other hand, projected the markers corresponding to 4-days-stored samples away from those belonging to non-stored samples and those stored for 8 days. The variables more correlated with this second function were carbohydrates (higher after 4 days of storage) and C16:0 (lower after 4 days of storage). In both performed LDAs, the classification performance was 100% accurate, both for original grouped cases and for the cross-validated grouped cases.

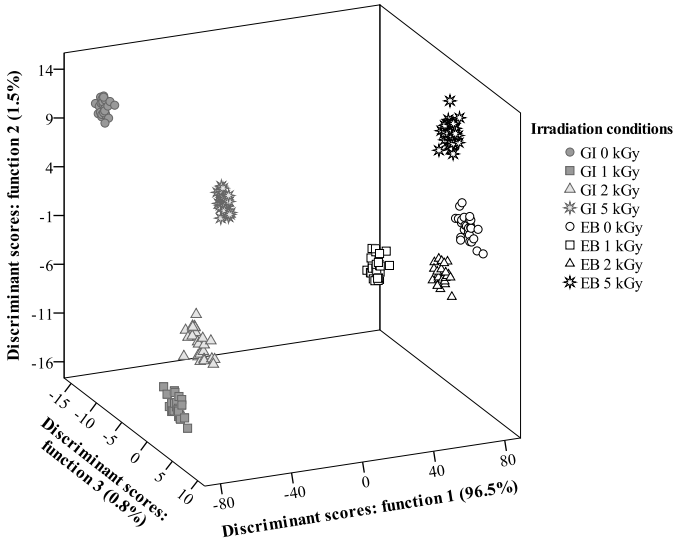



**Figure 14:** Three-dimensional distribution of gamma and electron beam irradiation markers according to the canonical discriminant functions coefficients defined from all parameters analysed in *A. bisporus* Portobello

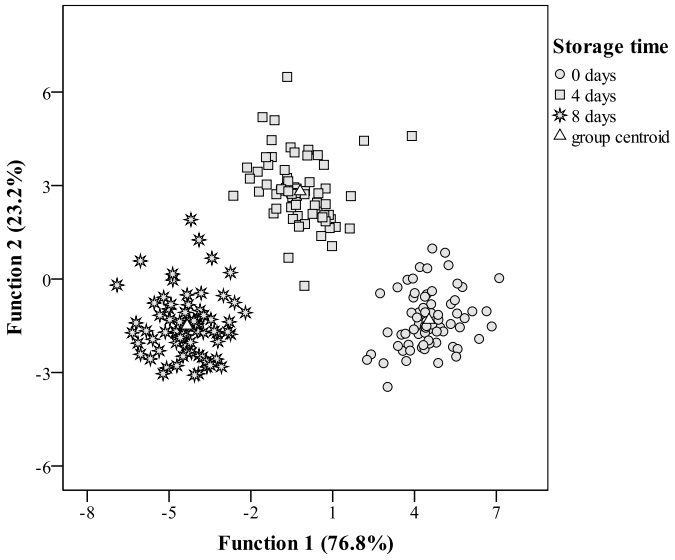



**Figure 15:** Two-dimensional storage time markers according to the canonical discriminant functions coefficients defined from all parameters analysed in *Agaricus bisporus* Portobello

**Table 19:** Lipophilic compounds (fatty acids, tocopherols and ergosterol) of fresh *A. bisporus* Portobello submitted to different irradiation conditions and storage times. The results are presented as mean±SD.[1]

| | | Fatty acids (relative percentage) | | | | | | | | | Tocopherols (µg/100 g dw) | | Ergosterol |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **C16:0** | **C18:0** | **C18:2n6c** | **C20:0** | **C22:0** | **C24:0** | **SFA** | **MUFA** | **PUFA** | ***α*-tocopherol** | ***β*-tocopherol** | **(mg/100 g dw)** |
| | | Gamma irradiation (GI) | | | | | | | | | | | |
| GI | 0 kGy | 8.1±0.1 | 3.4±0.1 | 79.7±0.4 | 1.8±0.2 | 1.4±0.1 | 1.2±0.1 | 19.2±0.5 | 0.8±0.1 | 80.1±0.4 | 0.50±0.05 | 10.1±0.4 | 232±9 |
| | 1 kGy | 7.8±0.3 | 4.1±0.2 | 79.6±0.5 | 1.9±0.3 | 1.4±0.2 | 1.2±0.1 | 19.3±0.5 | 0.9±0.2 | 80.0±0.4 | 0.50±0.03 | 10.3±0.5 | 236±15 |
| | 2 kGy | 7.8±0.4 | 4.2±0.1 | 78.7±0.5 | 1.9±0.1 | 1.5±0.1 | 1.3±0.2 | 20.0±0.5 | 0.9±0.1 | 79.1±0.5 | 0.50±0.04 | 10.3±0.4 | 250±22 |
| | 5 kGy | 8.6±0.2 | 4.1±0.5 | 78.6±0.3 | 1.6±0.1 | 1.2±0.1 | 1.2±0.1 | 20.1±0.2 | 0.9±0.1 | 79.0±0.3 | 0.47±0.04 | 10.3±0.3 | 231±22 |
| | ANOVA *p*-value (n = 27)[2] | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.360 | <0.001 | 0.010 | 0.328 | <0.001 |
| ST | 0 days | 8.4±0.2 | 3.8±0.3 | 79.4±0.5 | 1.6±0.1 | 1.3±0.1 | 1.2±0.1 | 19.2±0.5 | 0.9±0.2 | 79.8±0.5 | 0.50±0.04 | 9.9±0.5 | 239±17 |
| | 4 days | 7.7±0.5 | 3.9±0.3 | 79.5±0.5 | 1.9±0.2 | 1.4±0.1 | 1.2±0.1 | 19.3±0.5 | 0.8±0.1 | 79.8±0.5 | 0.49±0.04 | 10.5±0.4 | 236±19 |
| | 8 days | 8.1±0.2 | 4.2±0.5 | 78.6±0.5 | 1.9±0.2 | 1.4±0.1 | 1.3±0.1 | 20.1±0.4 | 0.8±0.1 | 79.0±0.5 | 0.50±0.04 | 10.4±0.4 | 237±22 |
| | ANOVA *p*-value (n = 36)[3] | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.543 | <0.001 | 0.743 |
| | GI×ST *p*-value (n = 108)[4] | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | | Electron beam irradiation (EB) | | | | | | | | | | | |
| EB | 0 kGy | 8.2±0.5 | 4.1±0.2 | 78.6±0.2 | 1.8±0.1 | 1.5±0.2 | 1.1±0.1 | 20.0±0.3 | 1.0±0.2 | 79.0±0.2 | 0.51±0.03 | 10.2±0.3 | 216±11 |
| | 1 kGy | 7.5±0.3 | 4.2±0.1 | 78.9±0.4 | 2.1±0.1 | 1.5±0.1 | 1.2±0.1 | 19.5±0.3 | 1.0±0.2 | 79.4±0.4 | 0.50±0.04 | 10.0±0.3 | 226±17 |
| | 2 kGy | 7.7±0.1 | 4.1±0.2 | 78.0±0.5 | 2.2±0.1 | 1.6±0.1 | 1.3±0.1 | 20.6±0.5 | 0.9±0.1 | 78.5±0.5 | 0.47±0.04 | 10.1±0.4 | 233±15 |
| | 5 kGy | 8.1±0.1 | 4.1±0.1 | 78.2±0.2 | 2.0±0.1 | 1.5±0.1 | 1.2±0.1 | 20.2±0.1 | 0.9±0.1 | 78.6±0.2 | 0.51±0.04 | 10.1±0.5 | 238±10 |
| | ANOVA *p*-value (n = 27)[2] | <0.001 | 0.082 | <0.001 | <0.001 | 0.001 | <0.001 | <0.001 | 0.026 | <0.001 | 0.002 | 0.119 | <0.001 |
| ST | 0 days | 8.1±0.5 | 4.1±0.2 | 78.5±0.2 | 1.9±0.2 | 1.4±0.1 | 1.1±0.1 | 20.1±0.3 | 0.9±0.2 | 79.0±0.2 | 0.50±0.04 | 10.2±0.3 | 222±18 |
| | 4 days | 7.8±0.2 | 4.1±0.1 | 78.6±0.5 | 1.9±0.1 | 1.5±0.1 | 1.1±0.1 | 19.9±0.5 | 1.0±0.1 | 79.1±0.5 | 0.50±0.04 | 10.0±0.4 | 234±12 |
| | 8 days | 7.7±0.3 | 4.2±0.1 | 78.1±0.5 | 2.1±0.2 | 1.6±0.1 | 1.3±0.1 | 20.4±0.5 | 1.0±0.1 | 78.6±0.5 | 0.49±0.04 | 10.1±0.3 | 229±14 |
| | ANOVA *p*-value (n = 36)[3] | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.428 | <0.001 | 0.317 | 0.145 | 0.004 |
| | EB×ST *p*-value (n = 108)[4] | 0.003 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.087 | <0.001 |

[1]Results are reported as mean values of each irradiation dose (GI or EB), aggregating results from 0, 4 and 8 days, and mean values of ST, combining all irradiation doses (from GI or EB). [2]If $p<0.05$, the corresponding parameter presented a significantly different value for at least one GI or EB. [3]If $p<0.05$, the corresponding parameter had a significant difference for at least one of the time intervals. [4]The interaction among factors was significant in all cases; thereby the statistical classification could not be indicated.

### 3.2.2. Combined effects of irradiation and storage time on the nutritional and chemical parameters of dried *Agaricus bisporus* Portobello mushroom

Regarding the dry *A. bisporus* Portobello (**Figure 16**), the tabled results include nutritional, soluble sugars, and ergosterol profiles (**Table 20**), tocopherols and organic acids (**Table 21**), and fatty acids (**Table 22**) and are divided into two sections, the top one belonging to gamma-irradiated samples and the bottom one to those treated with the electron beam. Each section is further divided to elucidate ST (upper part) and ID (lower part) effects with the values presented for each ID, including all ST periods and vice-versa.

In dw basis, the most abundant nutrients were carbohydrates, followed by proteins (**Table 20**). The fat content was very low (<1.4 g/100 g (dw), which was similar to the values presented above by fresh mushroom samples. For gamma-irradiated samples, there was a significant ($p < 0.05$) interaction (ST × ID) for moisture, crude fat, proteins, and carbohydrates, meaning that both ID and ST had a significant impact on the changes registered for these nutrients. Inversely, ash and energy were classified individually and did not show a significant interaction. Ash content decreased over ST, although they presented significantly higher values in irradiated samples, as previously observed in irradiated foods (Khan et al., 2018).

Regarding the energy values of gamma-irradiated samples, this parameter tended to increase with ST but decreased for higher ID. Considering the EMM plots, it was possible to observe that the longer the ST, the lower the fat content (**Figure 17(a)**), but with IDs of 5 and 10 kGy, this nutrient seems to be preserved, which is a beneficial aspect, since the loss of fat content is related to rancidification of food. Similar results were reported by Fernandes et al. (2017). Storage had a role in reducing fat over time, but the treatment at 5 kGy seems to preserve the integrity of these molecules during storage.

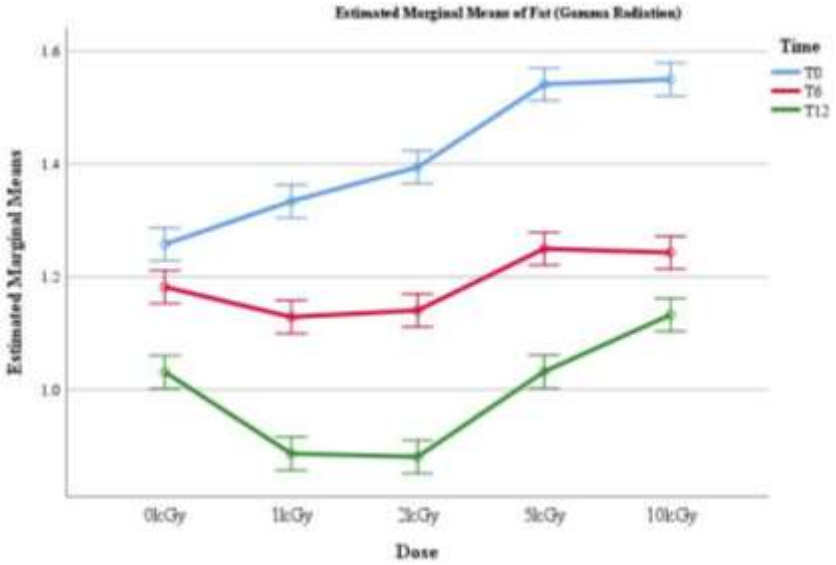
In terms of the nutritional profile of electron-beam irradiated samples, a significant interaction was also found for ash and energy, showing that electron-beam had similar effects to those of gamma radiation, with a significant decrease of ash and an increase of energy values over ST. At the same time, a reduction of these parameters was detected with the increase of ID. For nutrients with a significant interaction among factors, the EMM plots (**Figure 17(b)**), show a reduction of protein content for the stored samples, while a lower dose of irradiation (1 kGy) seems to reduce the loss of protein content in stored samples slightly. This behaviour was also

reported by (Fernandes et al., 2014b). **Table 20** shows the soluble sugars and ergosterol content for both irradiation technologies. The detected sugars were fructose, mannitol, and trehalose, and mannitol was the most abundant one, followed by trehalose. The same sugar profile had been identified previously in various instances for irradiated sugars. Regarding gamma radiation, a non-significant interaction was found for mannitol and trehalose. Mannitol was classified independently, showing that while this sugar decreased over ST, the increase of ID preserved it. Trehalose did not show significant differences among ST or ID, but it can be observed that trehalose increased over the 12 months with no influence from ID. The EMM plot for total sugars, **Figure 17(c)**, shows that the amount of these sugars reduces over time, although stored samples showed that gamma radiation had increasing preserving capacity from 1 to 5 kGy, while the 10 kGy dose showed a better preserving potential in non-stored samples. Electron-beam-irradiated samples showed a significant interaction for all sugars with no observable tendency in the EMM.

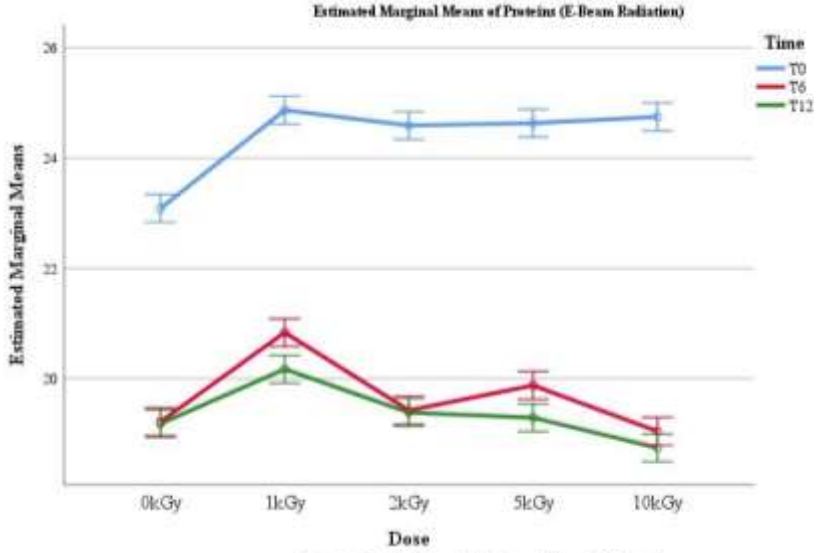
Ergosterol was detected in samples irradiated by both technologies and showed a significant interaction with values around 239 mg/100 g for gamma radiation and varying from 234 to 347 mg/100 g for electron beam. In a previous work by Cardoso et al. (2019a), an increase in ergosterol was also recorded for irradiated samples. Overall, slight changes were detected in the nutritional and sugar profile of dried Portobello samples treated with gamma radiation or electron beam, clearly showing that ST had a higher impact than ID, as shown in **Figure 17**.

**Figure 16:** Dried *A. bisporus* Portobello samples

**a)**




**b)**




**c)**

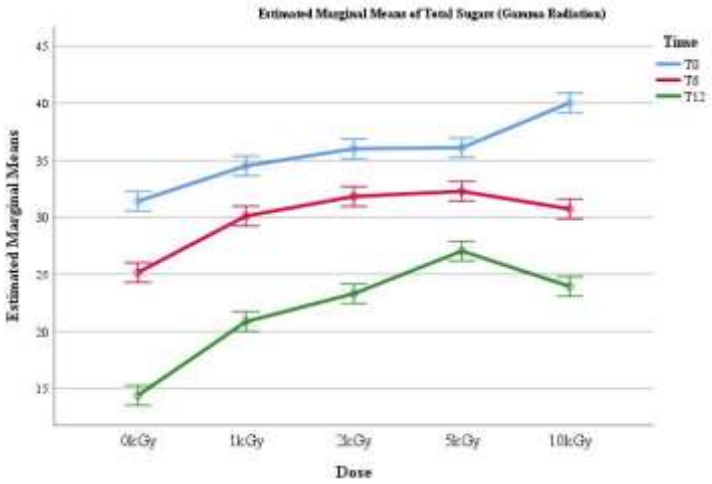



**Figure 17:** Estimated marginal means (EMM) plots of (a) fat (gamma radiation), (b) proteins (electron-beam radiation), and (c) total sugars (gamma radiation)

**Table 21** shows two classes of molecules detected in the mushrooms, namely, tocopherols and organic acids. Regarding tocopherols, all four isoforms were detected in gamma and electron-beam-irradiated mushrooms, with $\beta$-tocopherol showing the highest amounts and $\alpha$-tocopherol the lowest. A significant interaction was found for all isoforms, not allowing for the presence of individual classifications. Considering the EMM plots, and starting by gamma radiation, a general tendency for the preservation of tocopherols was found for doses of 1 kGy with a much deeper impact resulting from ST, which tended to reduce the amount of these bioactive molecules (**Figure 18(a)**). This same tendency, a decrease in the bioactive molecules over time, was reported previously by Fernandes et al. (2017). In the case of electron-beam-irradiated mushroom samples, ST also showed a higher impact than ID. All IDs showed preserving capabilities with an increase of about 50 mg/100 g (**Figure 18(b)**), while ST showed a high impact as seen in the gap between non-stored samples (blue line) and stored samples (red: 6 months, green: 12 months).

Organic acids are a group of simple organic molecules with acidic properties present in all living organisms. Three organic acids were detected in the irradiated mushrooms: Oxalic, quinic, and malic acid. Quinic and malic acids were the most abundant, practically in *ex aequo*, while oxalic acid was not detected over 1 g/100 g. For both irradiation processes, a significant interaction was found for all organic acids, and thus general conclusions were sought from the EMM plots. In **Figure 18c)** (oxalic acid content in gamma-irradiated Portobello), the effect of ST had a higher influence than ID, although in this case, the dose of 2 kGy seems to have protecting capabilities (for the non-stored samples and 6 months stored ones, while samples stored for 12 months showed a better response at 5 kGy). This was also verified for quinic acid (results not shown). Regarding electron-beam-irradiated mushroom samples, and in line with the observed for the gamma-irradiated samples, organic acids showed a significant interaction in all cases. Irradiated samples (independently of ID) tend to present higher organic acid contents, while a significant decrease was observed over ST. Independently of ID, electron-beam preserved organic acids in Portobello mushroom, while long STs reduce their amounts. These are essential effects to take into account for these small molecules due to their relevance in taste, aroma, and overall appreciated properties of mushrooms. Fernandes et al. (2014d) also mentioned that 1 kGy of gamma radiation was effective in preserving organic acids in

*Macrolepiota procera* mushrooms, pointing out that irradiation could be a complementary preserving technique.

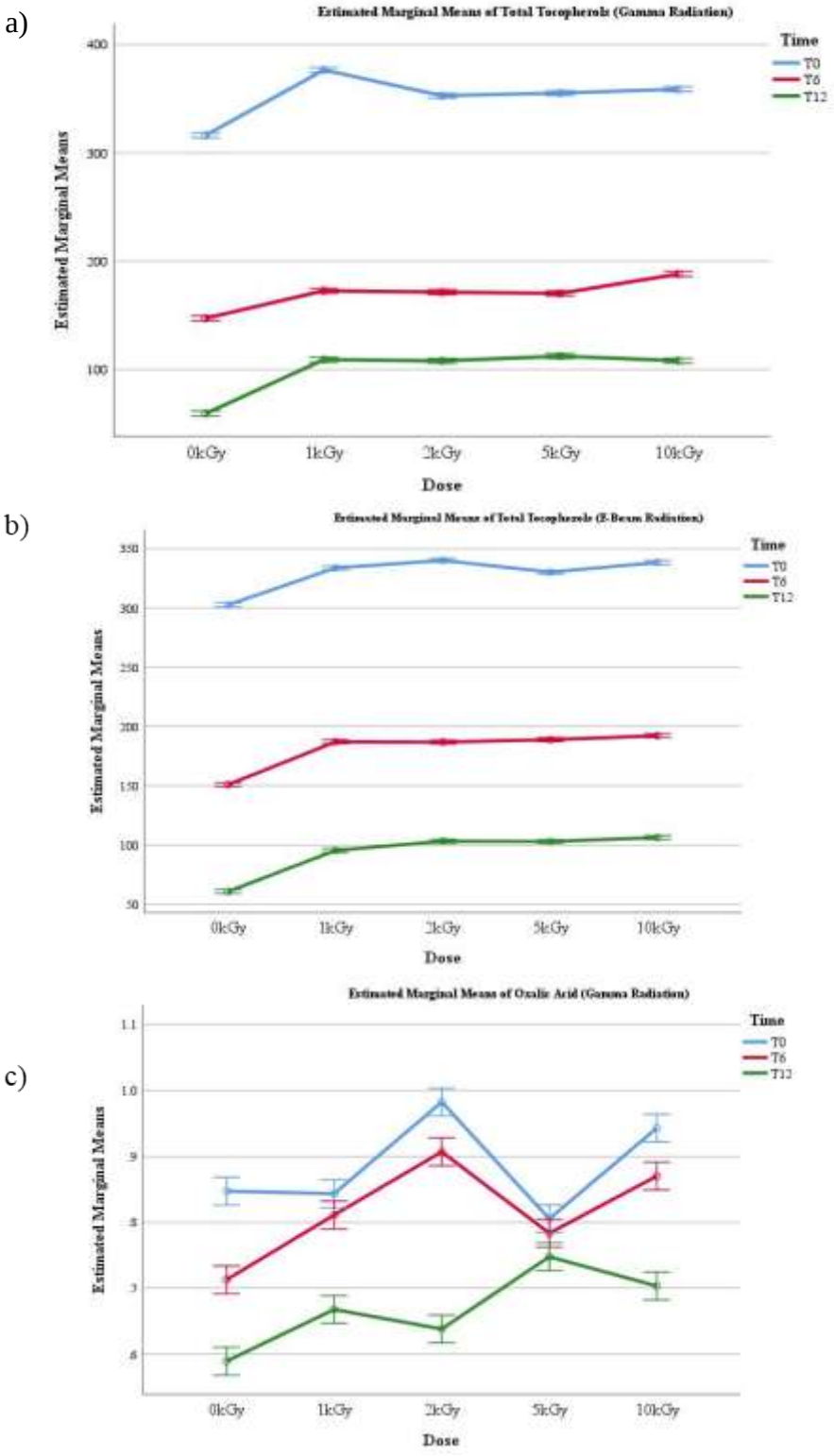



**Figure 18:** EMM plots of (a) total tocopherols (gamma radiation), (b) total tocopherols (electron-beam radiation), and (c) oxalic acid (gamma radiation)

**Table 22** shows the individual fatty acids found in both mushrooms, as well as the groups of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA), expressed in relative percentage. Only the eight most abundant individual fatty acids are shown, from a total of 16 identified. In both cases, C18:2 was the most abundant fatty acid with a range of 72% to 81%, followed by C16:0. Overall, PUFA showed the highest amounts, also ranging from 81% to 82%, while SFA only registered a percentage of 12% to 34%, and MUFA being the lowest group, under 2%. This high incidence of PUFA shows the health beneficial unsaturated fat found in mushroom samples, which also helps their acceptance as healthy foods. Regarding the individual fatty acids, all samples showed a significant interaction between ST and ID, meaning that even though there were very low variations in the fatty acids, both ST and ID influenced the results. No tendencies could be extracted from the EMM plots, thus, concluding that due to the very low moisture, lipid peroxidation was very low, and the influence of the ST would not be drastic. Concomitantly, the influence of the different IDs did not greatly affect the individual fatty acids.

**Table 20:** Centesimal composition, soluble sugars, and ergosterol quantities in gamma and electron-beam irradiated Portobello mushrooms

| | | Moisture (g/100g fw) | Crude Fat (g/100g fw) | Proteins (g/100g fw) | Ash (g/100g fw) | Carbohydrates (g/100g fw) | Energy (g/100g fw) | Fructose (g/100g dw) | Mannitol (g/100g dw) | Trehalose (g/100g dw) | Total Sugars (g/100g dw) | Ergosterol (mg/100g dw) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gamma Radiation | | | | | | | | | | | | |
| Storage Time (ST) | 0 Months | 11±2 | 1.4±0.1 | 24±1 | 9.6±0.1$^{c}$ | 65±1 | 369±1$^{a}$ | 0.43±0.05 | 34±3$^{c}$ | 0.8±0.1 | 36±3 | 293±39 |
| | 6 Months | 11±2 | 1.19±0.1 | 21±1 | 9.4±0.1$^{b}$ | 68±1 | 368±2$^{a, b}$ | 0.34±0.04 | 29±3$^{b}$ | 0.6±0.1 | 30±3 | 275±14 |
| | 12 Months | 12±1 | 1.0±0.1 | 21±1 | 9.0±0.3$^{a}$ | 96±2 | 372±2$^{b}$ | 0.28±0.05 | 22±2$^{a}$ | 2.0±0.8 | 21±4 | 238±30 |
| *p*-value (n=25) | Tukey's HSD test | 0.038 | <0.001 | <0.001 | <0.001 | <0.001 | 0.040 | <0.001 | <0.001 | 0.545 | <0.001 | <0.001 |
| Irradiation Dose (ID) | Control | 11±3 | 1.2±0.1 | 21±2 | 8.5±0.2$^{a}$ | 69±2 | 372±2$^{b}$ | 0.29±0.07 | 24.8±1.9$^{a}$ | 0.4±0.1 | 24±7 | 248±51 |
| | 1 kGy | 12±1 | 1.1±0.2 | 21±2 | 9.6±0.3$^{b}$ | 68±3 | 367±1$^{a}$ | 0.33±0.07 | 27.6±1.7$^{a, b}$ | 0.6±0.2 | 28±6 | 283±42 |
| | 2 kGy | 12±2 | 1.1±0.2 | 22±2 | 9.5±0.3$^{b}$ | 67±2 | 367±2$^{a}$ | 0.32±0.08 | 29.5±2.2$^{b}$ | 0.5±0.3 | 30±6 | 272±26 |
| | 5 kGy | 10±2 | 1.3±0.2 | 22±1 | 9.6±0.2$^{b}$ | 67±1 | 368±1$^{a}$ | 0.39±0.06 | 30.8±1.7$^{b}$ | 0.6±0.3 | 32±4 | 282±25 |
| | 10 kGy | 10±1 | 1.3±0.2 | 23±1 | 9.6±0.3$^{b}$ | 66±1 | 368±1$^{a}$ | 0.40±0.06 | 30.3±1.9$^{b}$ | 0.7±0.3 | 31±7 | 258±27 |
| *p*-value (n=5) | Tukey's HSD test | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.001 | 0.452 | <0.001 | <0.001 |
| ST×ID (n=50) | *p*-value | <0.001 | <0.001 | <0.001 | 0.390 | <0.001 | 0.176 | <0.001 | 0.326 | 0.443 | <0.001 | <0.001 |
| Electron Beam Radiation | | | | | | | | | | | | |
| Storage Time (ST) | 0 Months | 11±2 | 1.29±0.03 | 24.4±0.7 | 9.3±0.1$^{c}$ | 65.0±0.9 | 369±1$^{a}$ | 0.43±0.06 | 23±3 | 0.88±0.05 | 34±3 | 347±27 |
| | 6 Months | 10±2 | 1.21±0.03 | 19.7±0.7 | 9.1±0.1$^{b}$ | 69.9±0.9 | 369±1$^{a, b}$ | 0.38±0.06 | 30±4 | 0.5±0.1 | 31±4 | 300±23 |
| | 12 Months | 12±1 | 1.14±0.05 | 19.3±0.5 | 8.9±0.1$^{a}$ | 70.5±0.7 | 370±1$^{b}$ | 0.30±0.05 | 20±5 | 0.4±0.1 | 21±5 | 234±28 |
| *p*-value (n=25) | Tukey's HSD test | 0.007 | <0.001 | <0.001 | <0.001 | <0.001 | 0.045 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Irradiation Dose (ID) | Control | 10±2 | 1.17±0.09 | 20±2 | 8.5±0.2$^{a}$ | 70±2 | 372±1$^{b}$ | 0.30±0.06 | 21±6 | 0.5±0.2 | 22±6 | 250±48 |
| | 1 kGy | 12±2 | 1.23±0.07 | 22±2 | 9.2±0.3$^{b}$ | 67±2 | 369±1$^{a}$ | 0.40±0.06 | 36±7 | 0.5±0.3 | 27±8 | 304±66 |
| | 2 kGy | 10±1 | 1.21±0.06 | 21±2 | 9.2±0.2$^{b}$ | 68±3 | 369±1$^{a}$ | 0.35±0.07 | 27±6 | 0.6±0.2 | 28±6 | 308±54 |
| | 5 kGy | 12±1 | 1.22±0.06 | 21±2 | 9.3±0.2$^{b}$ | 68±3 | 368±2$^{a}$ | 0.37±0.03 | 29±5 | 0.6±0.2 | 30±6 | 298±33 |
| | 10 kGy | 13±1 | 1.24±0.06 | 21±3 | 9.2±0.2$^{b}$ | 69±3 | 369±1$^{a}$ | 0.4±0.1 | 33±4 | 0.6±0.2 | 34±4 | 309±46 |
| *p*-value (n=5) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST×ID (n=50) | *p*-value | 0.003 | 0.008 | <0.001 | 0.750 | <0.001 | 0.526 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

In each row, different letters mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision but rather as the range of the recorded values.

**Table 21:** Tocopherol isoforms and organic acids quantities in gamma and electron-beam-irradiated Portobello mushrooms

| | | **α-tocopherol (mg/100g dw)** | **β-tocopherol (mg/100g dw)** | **γ-tocopherol (mg/100g dw)** | **δ-tocopherol (mg/100g dw)** | **Total Tocopherols (mg/100g dw)** | **Oxalic Acid (g/100g dw)** | **Quinic Acid (g/100g dw)** | **Malic Acid (g/100g dw)** | **Total Organic Acids (g/100g dw)** |
|---|---|---|---|---|---|---|---|---|---|---|
| Gamma Radiation | | | | | | | | | | |
| Storage Time (ST) | 0 Months | 7.1±0.2 | 149±12 | 130±6 | 65±3 | 352±20 | 0.88±0.07 | 4.4±0.3 | 4.9±0.1 | 10.2±0.4 |
| | 6 Months | 6.7±0.3 | 67±9 | 47±2 | 51±5 | 170±14 | 0.82±0.07 | 4.0±0.4 | 4.5±0.5 | 9.3±0.9 |
| | 12 Months | 3.2±0.6 | 52±15 | 25±2 | 19±4 | 99±21 | 0.67±0.06 | 3.2±0.2 | 3.5±0.1 | 7.3±0.4 |
| *p*-value (n=25) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Irradiation Dose (ID) | Control | 4±2 | 69±46 | 63±44 | 38±21 | 174±113 | 0.7±0.1 | 3.5±0.5 | 4.0±0.6 | 8±1 |
| | 1 kGy | 5±2 | 95±50 | 73±52 | 46±20 | 219±120 | 0.78±0.08 | 3.9±0.4 | 4.5±0.7 | 9±1 |
| | 2 kGy | 5±2 | 93±44 | 66±47 | 46±20 | 211±110 | 0.8±0.1 | 4.2±0.9 | 4.5±0.7 | 9±2 |
| | 5 kGy | 5±1 | 93±45 | 67±47 | 47±20 | 213±110 | 0.8±0.1 | 3.8±0.5 | 4.5±0.7 | 9±1 |
| | 10 kGy | 5±2 | 98±43 | 67±48 | 48±20 | 218±111 | 0.8±0.1 | 3.7±0.5 | 4.0±0.7 | 9±1 |
| *p*-value (n=5) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST×ID (n=50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Electron Beam Radiation | | | | | | | | | | |
| Storage Time (ST) | 0 Months | 8.3±0.9 | 140±7 | 119±4 | 61±3 | 329±14 | 0.91±0.04 | 4.6±0.2 | 4.9±0.2 | 10.4±0.5$^{c}$ |
| | 6 Months | 5.1±0.4 | 70±7 | 58±6 | 48±3 | 181±16 | 0.78±0.06 | 3.4±0.2 | 3.6±0.4 | 7.8±0.3$^{b}$ |
| | 12 Months | 3.7±0.9 | 49±14 | 24±1 | 17±3 | 93±18 | 0.71±0.09 | 3.2±0.2 | 3.5±0.1 | 7.4±0.2$^{a}$ |
| *p*-value (n=25) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Irradiation Dose (ID) | Control | 4±2 | 69±47 | 60±39 | 37±20 | 171±106 | 0.7±0.1 | 3.5±0.6 | 3.6±0.8 | 7.7±0.3$^{a}$ |
| | 1 kGy | 6±2 | 88±40 | 68±42 | 43±21 | 205±104 | 0.7±0.1 | 3.9±0.6 | 4.0±0.8 | 8.6±0.6$^{b}$ |
| | 2 kGy | 6±2 | 91±41 | 69±43 | 43±20 | 210±104 | 0.84±0.1 | 3.7±0.7 | 4.0±0.7 | 8.6±0.5$^{b}$ |
| | 5 kGy | 6±2 | 91±39 | 68±43 | 42±18 | 207±100 | 0.84±0.1 | 3.7±0.7 | 4.1±0.6 | 8.7±0.5$^{b}$ |
| | 10 kGy | 6±2 | 92±40 | 69±42 | 44±20 | 212±102 | 0.86±0.1 | 3.8±0.7 | 4.1±0.6 | 9.0±0.2$^{b}$ |
| *p*-value (n=5) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST×ID (n=50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.164 |

In each row, different letters mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision but rather as the recorded values' range.

**Table 22:** Individual fatty acids, saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) quantities in gamma and electron-beam irradiated Portobello mushrooms, expressed in relative percentage

| | | **C15:0** | **C16:0** | **C18:0** | **C18:1** | **C18:2** | **C20:0** | **C22:0** | **C24:0** | **SFA** | **MUFA** | **PUFA** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gamma Radiation | | | | | | | | | | | | |
| Storage Time (ST) | 0 Months | 1.4±0.1 | 9±1 | 4.0±0.3 | 1.1±0.1 | 72±1 | 1.6±0.3 | 1.6±0.4 | 3.1±0.2 | 25±1 | 1.6±0.1 | 72±1 |
| | 6 Months | 1.9±0.2 | 10±1 | 4.5±0.5 | 1.1±0.2 | 74±3 | 1.6±0.1 | 1.3±0.1 | 1.1±0.1 | 24±2 | 1.5±0.6 | 74±3 |
| | 12 Months | 1.7±0.1 | 9±1 | 4.0±0.3 | 0.9±0.1 | 75±1 | 1.7±0.1 | 1.33±0.09 | 1.1±0.1 | 24±1 | 1.1±0.1 | 75±1 |
| *p*-value (n=25) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Irradiation Dose (ID) | Control | 1.6±0.2 | 9.4±0.4 | 4.0±0.2 | 1.1±0.1 | 74±1 | 1.7±0.1 | 1.6±0.4 | 1±1 | 24±1 | 1.5±0.3 | 74±1 |
| | 1 kGy | 1.7±0.4 | 10.2±1.2 | 4.2±0.6 | 1.1±0.1 | 74±1 | 1.5±0.3 | 1.3±0.1 | 2±1 | 25±1 | 1.4±0.3 | 74±1 |
| | 2 kGy | 1.6±0.2 | 8.7±0.5 | 4.0±0.4 | 0.9±0.1 | 75±2 | 1.7±0.1 | 1.5±0.4 | 2±1 | 23±2 | 1.2±0.3 | 76±2 |
| | 5 kGy | 1.8±0.2 | 10.1±0.9 | 4.6±0.6 | 1.1±0.1 | 72±2 | 1.6±0.1 | 1.2±0.2 | 2±1 | 26±1 | 1.7±0.6 | 73±2 |
| | 10 kGy | 1.6±0.1 | 8.9±0.5 | 4.1±0.1 | 0.9±0.1 | 75±2 | 1.7±0.1 | 1.4±0.1 | 1±1 | 24±2 | 1.2±0.2 | 75±2 |
| *p*-value (n=5) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST×ID (n=50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Electron Beam Radiation | | | | | | | | | | | | |
| Storage Time (ST) | 0 Months | 1.2±0.1 | 8.1±0.3 | 4.2±0.2 | 1.3±0.3 | 75±1 | 1.8±0.1 | 1.5±0.2 | 1.2±0.2 | 12±1 | 1.7±0.3 | 75±1 |
| | 6 Months | 1.6±0.1 | 9.8±0.4 | 4.2±0.1 | 0.4±0.4 | 81±1 | 1.0±0.7 | 1.2±0.1 | 1.0±0.1 | 25±19 | 0.5±0.4 | 81±1 |
| | 12 Months | 1.7±0.2 | 9.7±0.3 | 4.0±0.3 | 0.7±0.1 | 76±1 | 1.6±0.2 | 1.3±0.1 | 1.0±0.1 | 23±1 | 0.9±0.1 | 76±1 |
| *p*-value (n=25) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.810 | <0.001 | <0.001 |
| Irradiation Dose (ID) | Control | 1.5±0.1 | 9±1 | 3±2 | 0.9±0.2 | 78±2 | 1.5±0.1 | 1.2±0.2 | 1.0±0.1 | 21±2 | 1.2±0.4 | 78±2 |
| | 1 kGy | 1.5±0.2 | 9±1 | 3±2 | 0.9±0.2 | 78±3 | 1.6±0.1 | 1.3±0.1 | 0.9±0.1 | 21±2 | 1.2±0.4 | 78±2 |
| | 2 kGy | 1.5±0.2 | 9±1 | 3±2 | 0.6±0.4 | 77±2 | 1.7±0.1 | 1.3±0.1 | 1.0±0.1 | 22±2 | 0.9±0.6 | 77±2 |
| | 5 kGy | 1.6±0.3 | 9±1 | 3±2 | 0.6±0.4 | 77±4 | 1.2±0.8 | 1.4±0.2 | 1.3±0.3 | 34±22 | 0.9±0.6 | 77±4 |
| | 10 kGy | 1.4±0.3 | 9±1 | 3±2 | 0.9±0.8 | 77±5 | 1.2±0.8 | 1.5±0.3 | 1.2±0.1 | 22±4 | 1.1±0.9 | 77±5 |
| *p*-value (n=5) | Tukey's HSD test | <0.001 | 0.011 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.017 | <0.001 | <0.001 |
| ST×ID (n=50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.016 | <0.001 | <0.001 |

Presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision but rather as the recorded values' range.

### 3.2.3. Extraction and Recovery of Vitamin $D_2$ from mushrooms bioresidues irradiated with UV-C

#### *3.2.3.1. Method Validation*

For this case study, before the mushroom bioresidues extract analysis, the correlation coefficient ($R^2$), linearity range, and limits of detection and quantification (LOD and LOQ, respectively) of the methodology employed to determine vitamin $D_2$, were fully validated (**Table 23**). After the linearity check (linearity range: 0.78–50 µg/mL), a seven-level calibration curve (y = 11.909x + 6.9688) was made, using the peak/area ratio versus concentration of the standard concentration (µg/mL), reaching a correlation coefficient of 0.9992. The average of the double determinations for each level was used.

The LOD, calculated as the concentration corresponding to three times the standard error of the calibration curve, divided by the slope, was 1.67 µg/mL, while the LOQ, i.e., the concentration corresponding to ten times the calibration error, divided by the inclination, was 5.07 µg/mL. In order to evaluate the instrumental precision, the mushroom sample (*A. bisporus* Portobello, irradiated for 6 min at 3200 mJ/cm$^2$) was injected six times, and the chromatographic method proved to be precise, according to the coefficient of variation (CV) of 0.82%. Repeatability was evaluated by applying the whole extraction procedure six times to the same sample, and the CV value obtained was low (1.35%). The method accuracy was evaluated by the standard addition procedure (% of recovery). The standard mixture was added to the samples in three concentration levels (25%, 50% and 100% of the peak/area concentration, each in duplicate) before the extraction. The method showed good recovery results, averaging 94% (**Table 23**).

**Table 23:** Calibration parameters of the method for vitamin $D_2$ detection and quantification, and method validation parameters using *A. bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm$^2$)

| **Calibration curve** | **Correlation coefficient ($R^2$)** | **Linearity range (µg/mL)** | **Limit** | |
|---|---|---|---|---|
| | | | **LOD[1] (µg/mL)** | **LOQ[2] (µg/mL)** |
| y = 11.909x + 6.9688 | 0.9992 | 0.78 - 50 | 1.67 | 5.07 |
| **Precision CV, % (*n*=6)** | **Accuracy (recovery, %)** | | **Precision CV, % (*n*=6)** | |
| 0.82 | 1.35 | | 94 | |

[1] LOD: limit of detection of the chromatographic method; [2] LOQ: limit of quantification of the chromatographic method; CV: coefficient of variation.

#### *3.2.3.2. Conversion Conditions*

The starting point is using mushroom bioresidues as a sustainable material to obtain vitamin $D_2$, avoiding the use of mushrooms suitable for commercialization. In this sense, **Table 24** presents the vitamin $D_2$-enriched extracts content in different mushroom species exposed to different UV-C radiation doses and exposure times. As it is mandatory in any two-way ANOVA, the possible interaction among the assayed factors was verified (ET × UV-C). Since the interaction proved significant ($p < 0.050$) in all the cases, it became evident that one factor's effect depends on the second level. Therefore, the variation induced by every single factor could not be classified.

Nonetheless, it was possible to observe some evident trends, as confirmed by the individual *p*-values of each factor. A significant increase (from less than 4 µg/g dw to more than 100 µg/g dw in all the cases) in vitamin $D_2$ concentration was observed with the application of this irradiation type, most likely due to the conversion of ergosterol naturally present in the assayed mushroom. Furthermore, there were no significant differences in using 200, 800 or 3200 mJ/cm$^2$, which indicates that the vitamin $D_2$ increase may be achieved with the least energetic consumption, making this processing approach more competitive and with minimal environmental impact.

Regarding exposure time, there were no significant differences upon irradiating mushrooms for 6 or 10 min, but the intermediate assayed time was better than the 2 min. Hence, the optimal exposure time, considering the results obtained with the bioresidues of assayed mushroom species, turned out to be 6 min. The origin of the mushroom, applied dose, time after harvest, positioning of the mushrooms to the light source, fresh or dried samples, whole or sliced samples, and the method by which vitamin $D_2$ has been extracted, among others, influence the results obtained (Cardwell et al., 2018).

#### *3.2.3.3. Vitamin $D_2$-Enriched Extracts*

As for the mushrooms evaluated in this work (*e.g.*, for 6 min at 3200 mJ/cm$^2$), vitamin $D_2$-enriched extract levels in the *A. bisporus* Portobello samples reached a maximum concentration of 124 µg/g dw, and in the white *A. bisporus and P. ostreatus* samples they reached values of 125 µg/g dw (**Table 24**, **Figure 19**). In UV-C-irradiated *P. ostreatus* samples, Hu et al. (2020) reported a maximum concentration of approximately 24 µg/g dw of vitamin $D_2$ content.

Teichmann et al. (2007) reported 10.14 µg/g dw in white *A. bisporus* samples, Guan et al. (2016) reported 13.4 and 9.5 µg/g dw in white and Portobello *A. bisporus* samples, respectively, and Jasinghe & Perera (2006) reported 34.4 µg/g dw in white button mushrooms. Similarly, for UV-C-irradiated shiitake (*Lentinula edodes*) mushroom, Xu et al. (2020) obtained an increase in vitamin $D_2$ content until 20.11 µg/g dw. Concerning UV-B irradiation, Urbain et al. (2011) and Urbain et al. (2016) obtained 56.8 and 67.1 µg/g dw of vitamin $D_2$, respectively, in button mushrooms; Nölle et al. (2017) reported that fresh whole *A. bisporus*, followed by freeze-drying, obtained 45 µg/g dw of vitamin $D_2$, and slicing before UV-B irradiation led to a ten-fold increase.

**Table 24:** Vitamin $D_2$ content in different mushroom species exposed to different UV-C radiation doses and exposure times (ET). The results are presented as mean ± SD. [1]

| | | **Vitamin $D_2$-enriched extracts (µg/g dw)** | | |
|---|---|---|---|---|
| | | *A. bisporus* | *A. bisporus* Portobello | *P. ostreatus* |
| Exposure time (ET)/min | 0 | 3.77±0.02 | 3.7±0.2 | 2.38±0.04 |
| | 2 | 84±7 | 109±6 | 97±11 |
| | 6 | 125±8 | 124±11 | 125±11 |
| | 10 | 122±1 | 127±5 | 119±7 |
| ANOVA *p*-value[2] | | <0.001 | <0.001 | <0.001 |
| UV-C (mJ/cm$^2$) | 0 | 3.77±0.02 | 3.7±0.2 | 2.38±0.04 |
| | 200 | 104±22 | 113±9 | 107±20 |
| | 800 | 111±21 | 119±9 | 114±12 |
| | 3200 | 116±19 | 128±10 | 119±14 |
| ANOVA *p*-value[2] | | <0.001 | <0.001 | <0.001 |
| ET×UV-C *p*-value[3] | | <0.001 | 0.035 | <0.001 |

[1] Results are reported as mean values of each parameter (ET or UV-C), combining all exposure times and irradiation doses (from ET or UV-C). [2] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one ET or UV-C. [3] The interaction among factors was significant in all cases; thereby, the statistical classification could not be indicated.

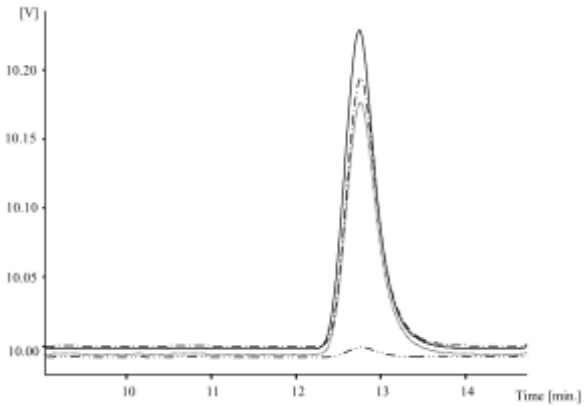



**Figure 19:** Vitamin $D_2$-enriched extracts chromatogram profile of *Agaricus bisporus* Portobello (-), white *A. bisporus* (.-.-) and *Pleurotus ostreatus* (...) irradiated with UV-C (6 min at 3200 mJ/cm2), and *A. bis*porus Portobello control samples (..-..-)

There are dissimilarities in the irradiation process and conditions to maximize the photoconversion of ergosterol into vitamin $D_2$ in mushrooms. Most of these cited studies were performed with the whole intact mushroom, with a longer irradiation time (20 min) and higher irradiation dose. Based on the case study considered in this work, we make the first attempt to establish the irradiation conditions and extraction procedure needed to maximize ergosterol conversion to vitamin $D_2$ from mushroom bioresidues production, avoiding the need to use mushroom samples that are suitable to be commercialised.

#### *3.2.3.4. Bioactivity of the Vitamin $D_2$-Enriched Extract*

The *in vitro* cytotoxicity of the vitamin $D_2$-enriched extract and standard vitamin $D_2$ was analysed. The effect of the vitamin $D_2$-enriched extract and standard vitamin $D_2$ in human tumoral cell lines (MCF-7, NCI-H460, AGS and CaCo-2) and non-tumoral bone cell line (h-FOB 1.19) growth are presented in **Table 25**. The $GI_{50}$ values represent the extract concentrations that cause a 50% inhibition of cell growth.

The sample of standard vitamin $D_2$ tested did not reveal cytotoxicity at the evaluated concentrations ($GI_{50}$ values > 400 µg/mL) for all the cell lines tested (tumoral and non-tumoral). However, the vitamin $D_2$-enriched extract presented effective activity in the AGS (82 µg/mL) tumoral cell line, and moderate activity in the NCI-H460 (293 µg/mL) and CaCo-2 (377 µg/mL) tumoral cell lines. The results obtained indicate that these effects may be related to the compounds (including ergosterol, phenolic compounds, organic acids, etc.) present in the mushroom extract, since the mushrooms are a rich source of bioactive compounds (Cardoso et al., 2017). It is noteworthy that neither vitamin $D_2$-enriched extracts or standard vitamin $D_2$ presented cytotoxicity against the non-tumoral bone cell, h-FOB 1.19 ($G_{I50}$ > 400 µg/mL).

**Table 25:** Antiproliferative and cytotoxicity activities of vitamin $D_2$-enriched extracts of *A. bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm$^2$) and standard vitamin $D_2$ (mean ± SD, n = 9)

| | **MCF-7** | **NCI-H460** | **AGS** | **CaCo-2** | **h-FOB 1.19** |
|---|---|---|---|---|---|
| **Vitamin $D_2$-enriched extracts** ($GI_{50}$ µg/mL) | >400 | 293±17$^b$ | 82±9$^c$ | 377±24$^a$ | >400 |
| **Vitamin $D_2$ standard** ($GI_{50}$ µg/mL) | >400 | >400 | >400 | >400 | >400 |

The cytotoxicity results were expressed as $GI_{50}$ values, corresponding to the sample concentration that inhibited 50% of the net cell growth. In row, different letters mean significant differences ($p < 0.05$).

## 3.3. Flour types for food fortification

### 3.3.1. Physicochemical characterization and microbiology of wheat and rye flour

#### *3.3.1.1. Macronutrients and energetic value*

In general, the quality of the flour is attributed to its moisture, gluten, fat, acidity, mineral, and protein contents. These properties reflect the effect of the processing and can be used to evaluate the technological or nutritional quality of the product (Hădărugă et al., 2016).

The results of the physicochemical parameters of the flour (**Figure 22**) are presented in **Table 29**. The highest pH value was found in whole rye flour T 130 (6.44) and the lowest in wheat flour T 65 (6.04). Similar results were found in wheat flour from Alegre, Brazil (5.28) Vieira et al., 2015) and in samples from Tocantins, Brazil, in a range from 6.0 to 6.1 (Macedo et al., 2017). Few studies are reporting the determination of pH in wheat or rye flour. The pH value (hydrogenation potential) is essential for detecting the treatments applied to the flour if it shows excessive alteration, as in the case of bleaching with chlorine. It is also a significant factor in the capacity of microorganisms' development in food. Contaminated flour contains some live yeast or other bacteria and may have a lower pH (less than 5.5) due to extra biological activity. According to this parameter, foods can be classified as low acidity (pH > 4.5), acidity (4.5–4.0) and high acidity (< 4.0) ( de Souza et al., 2008).

Regarding moisture content, the results range from 9.3% to 14.7% in whole rye flour T 130 and whole wheat flour T 150, respectively. These results are within the recommended maximum values for rye flour (14.5%) (Portaria n.$^{o}$254/2003 de 19 de Março, 2003). Similar results were reported in rye and wheat flour from Greece (9.76% and 15.94%, respectively) (Drakos et al., 2017). Moisture is an essential parameter in the storage of flour; high levels can increase microorganisms' growth and is a critical factor for fungi growth and mycotoxins production (Hădărugă et al., 2016). Thus, low levels are beneficial for a longer shelf-life of the product.

Protein content ranged between 6.93% and 14.6% in rye flour T 70 and whole wheat flour T 150, respectively. Puppo et al. (2005) reported protein values of 10.9% for wheat flour from Buenos Aires, Argentina, and Drakos et al. (2017) reported 9.68% in rye flour from Greece.

Regarding the ash content, wheat flour presented similar values, namely T 55 (0.61 g/100 g) and T 65 (0.69 g/100 g), to those reported by Frakolaki et al. (2018) in Greek samples (0.63 g/100 g). In rye flour samples, T 70 (0.85%) and T 85 (0.99%) revealed lower contents than those presented by Drakos et al. (2017) (1.55 g/100 g). These flours are extremely white due to their high degree of refining, which results from a higher grinding with the absence of husk or germ. The percentage of ash defines the commercial type of flour; the typical values for ash in wheat flour range between 0.49% and 2% and in rye flour from 0.79% to 2.5%, according to the regulation N° 254/2003 (SPCNA, 2003). Otherwise, whole wheat and whole rye flour presented higher ash contents (T 150–1.48%, T 130–1.27% and T 170–1.75%) since these samples contain a higher content of minerals, which do not incinerate at 550 °C (Guerreiro & Mata, 2006).

Regarding the fats content, the wheat flour T 55 presented the lowest amount (0.76 g/100 g), while the rye flour T 170 presented the highest amount (1.78 g/100 g). Fats are a parameter that has nutritional and physiological relevance in food because they are a source of essential fatty acids and energy. In addition, they play a vital role in food quality and can cause unpleasant tastes and smells in stored flour. It followed the same trend of ash content, but in the present study, the observed contents are lower than those reported by Bucsella et al. (2016), Molnáret al. (2016) from Budapest, Hungary (whole wheat flour: 2.36%, rye flour: 1.15% and whole rye flour: 3.07%).

The different types of flour (wheat and rye) contained comparable amounts of total carbohydrates ranging between 82.4 and 91.3 g/100 g. Similar results in wheat flour were reported by Kaminski et al. (2011) from Santa Maria/RS – Brazil (85.52 g/100 g), but, in the same study, these authors reported lower values for rye samples (59.88 g/100 g). In wheat samples from Greece, the authors reported 67.78 g/100 g of carbohydrates (Frakolaki et al., 2018).

Concerning dry and wet gluten, these were only detected in wheat flour. Dry and wet gluten ranged from 7.2% to 9.5% and 19.9% and 25.0%, respectively, according to the recommended minimum values by the Portuguese legislation (between 7 and 8% of dry gluten) (Portaria n.º254/2003 de 19 de Março, 2003). According to the same regulation, rye flour does not have minimum gluten values. Similar results were described in wheat flour samples from Greece, with 10.90% dry gluten and 28.24% wet gluten (Frakolaki et al., 2018). Wheat flour has a

medium to high protein content (10–16%); higher contents are helpful in industrial baking due to its higher gluten concentration, giving it more excellent elasticity and resistance to mechanical processing and influencing the hydration properties present in the flour. The flour with the lowest protein content is sold as flour for household use, which is the specific characteristic that differentiates wheat from rye flour. Wheat flour presents gluten-forming proteins with the capacity for mass-building, on the other hand, rye flour have less gluten-forming proteins, and the high soluble fibre content impairs the formation of this protein network (Kaminski et al., 2011).

Gluten consists of a viscous and elastic mass that provides the physical and rheological characteristics such as plasticity, viscosity and elasticity essential for mass modulation. Flours need to have a considerable amount of gluten so that the dough can absorb the water. Furthermore, the amount and quality of gluten determine a strong water absorption and a high elasticity of the dough, which is very favourable for carbon dioxide during the fermentation process of the bakery and pastry products (Hădărugă et al., 2016).

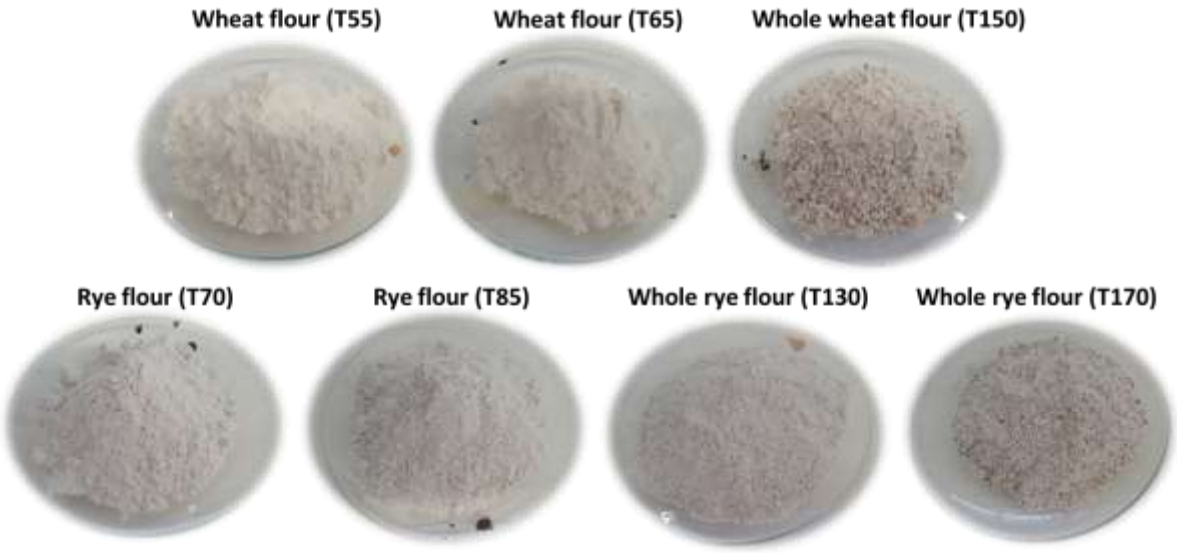



**Figure 22:** Type of wheat and rye flour

**Table 29:** Physicochemical analysis of different types of flour

| Parameter | Wheat refined flour | | Whole wheat flour | Rye refined flour | | Whole rye flour | |
|---|---|---|---|---|---|---|---|
| | T 55 | T 65 | T 150 | T 70 | T 85 | T 130 | T 170 |
| pH | 6.05± 0.01e | 6.04± 0.01e | 6.18± 0.01d | 6.35± 0.01c | 6.41± 0.01b | 6.44± 0.01a | 6.18± 0.01d |
| Moisture (g/100 g dw) | 14.1± 0.7a | 13.6± 0.3a | 14.7± 0.2a | 10.1± 0.1bc | 9.9 ± 0.1bc | 9.3 ± 1.3c | 11.5± 0.3b |
| Proteins (g/100 g dw) | 13.2± 0.8b | 13.4± 0.3b | 14.6± 0.3a | 6.93± 0.07d | 7.7 ± 0.1 cd | 8.2 ± 0.2c | 13.8± 0.1b |
| Ash (g/100 g dw) | 0.61± 0.02 g | 0.69± 0.01f | 1.47± 0.08b | 0.85± 0.01e | 0.99± 0.03d | 1.27± 0.03c | 1.75± 0.02a |
| Fats (g/100 g dw) | 0.92± 0.02d | 0.76± 0.02e | 1.51± 0.02b | 0.96± 0.05d | 1.29± 0.02c | 1.31± 0.01c | 1.73± 0.02a |
| Carbohydrates (g/100 g dw) | 85.2± 0.8c | 85.5± 0.3c | 82.4± 0.2d | 91.3± 0.1a | 90.0± 0.1b | 89.2± 0.1b | 82.7± 0.1d |
| **Energy (kcal/100 g dw)** | 402.2 ± 0.2a | 401.0 ± 0.1b | 401.7 ± 0.2b | 401.4 ± 0.2b | 402.5 ± 0.1a | 401.4 ± 0.1b | 401.6 ± 0.2b |
| **Gluten** | | | | | | | |
| **Wet (g/100 g mb)** | 25.0± 0.5a | 25.6± 0.1a | 19.9± 0.5b | nd | nd | nd | nd |
| **Dried (g/100 g dw)** | 9.5 ± 0.4a | 9.4 ± 0.2a | 7.2 ± 0.1b | nd | nd | nd | nd |

nd – not detect. Values are expressed in dry weight (dw) and wet gluten contents in mass (mb) as mean ± SD. In each row different letters represent significant differences ($p < 0.05$).

### *3.3.1.2. Microorganisms' analysis*

The results regarding the microorganisms analysed in the wheat and rye flour are presented in **Table 30**. Aerobic mesophiles, coliforms, yeasts, moulds, *E. coli* and sulphite-reducing clostridia were the microorganisms analysed and the presence of *Salmonella spp*. Regarding the rye flour, the samples T 130 and T 170 (whole flour) presented the highest contents in APC compared with the respective refined flour T 70 and T 85. In the case of coliforms, yeasts and moulds, the whole sample T 170 presented increased counts of these microorganisms compared with the other samples. Sulphite-reducing clostridia, *E. coli* and *Salmonella* were not detected in any samples. As far as we know, this is the first comparative report on the microbial analysis of different rye flour. Among the wheat samples, the analysed microorganisms were present in similar amounts between wheat and whole flour, except for moulds, where the whole sample (T 150) presented a significant increase in these microorganisms. The APC microorganisms obtained in the present work for the wheat flour T 55 and T 65 (4.33 and 4.20 LOG10 CFU/g, respectively) agree with the ones obtained by Eglezos (2010) that reported a value of 4.2 LOG10 CFU/g. The same authors reported a yeast content in the order of 3.0 LOG10 CFU/g, while in the present work, the counting was slightly lower (T 55 = 2.24, T65 = 2.35 LOG10 CFU/g). Also, according to Eglezos (2010), Salmonella was not detected in 25 g of sample. Khanom et al. (2016) also reported the microorganisms' content in packed and unpacked flour

samples and described the presence of 5.60 LOG10 CFU/g of total coliforms and 5.33 LOG10 CFU/g for yeasts and moulds in unpacked flour. The content in coliforms was higher than the ones obtained in the present study (T 55 = 2.00, T65 = 3.04 LOG10 CFU/g), and the content on yeasts and moulds (T 55 = 2.65, T65 = 2.54 LOG10 CFU/g). Berghofer et al. (2003) also reported the microbiological analysis of Australian wheat flour and reported the sample contamination with up to 102 CFU/g of APC and up to 1 CFU/g of coliforms, yeasts and moulds, with counting of 102 CFU/g. Salmonella spp. was absent in all different flour (25 g of sample). E. coli was below the LOQ as well as SRC.

Comparing the processed rye and wheat flour, the counting of the analysed microorganisms was not significantly different. Regarding the whole samples, it is possible to state that the rye whole flour T 170 presented a higher counting in APC microorganisms (5.42 LOG10 CFU/g) and Yeast's counting (4.20 LOG10 CFU/g). On the other hand, whole wheat flour (T 150) presented higher mould content (5.46 LOG10 CFU/g). Although the moisture contents significantly differ between rye and wheat samples, this parameter did not affect the microorganisms' contents.

The cereal grains are susceptible to contamination during ripening, harvesting, processing and storage. Microorganisms are constant contaminants of grain flour because they originate from the cereal’s vegetation period and are an integral part of the grain mass. Under unfavourable conditions, they are inactive and do not present a potential hazard (Plavsic et al., 2017).

*3.3.1.3. Mycotoxins analysis*

The calibration parameters of instrumentation (linear range, correlation coefficient (R2), equations of linear regression, limits of detection (LOD) and limits of quantification (LOQ) for AF and OTA are shown in **Table 31.** The result for what concerns the linearity in the reference and the calibration curves was adequate and satisfactory, with a coefficient of determination always more outstanding than 0.999. The performance was moderately sensitive, with detection limits up to 1.2 µg/kg for AFs and 0.7 µg/kg for OTA.

The recovery and within-laboratory reproducibility are the principal influence on the uncertainty measurement. Other causes, including mass, volume, purity of standards and calibration curve, offer a small contribution to the uncertainty values, not significantly affecting

the final value (Golge & Kabak, 2016). For that reason, the recovery and repeatability of the method were determined for the matrix under analysis.

**Table 32** shows the accuracy and precision of the AF and OTA analysis methods. The recovery was ascertained by spiking non-contaminated samples with known concentrations of each mycotoxin and comparing the response obtained for pure AF and OTA standard solutions at the same concentration levels. The recoveries of AF were in the range of 70–110% regulated by the Commission Regulation EC No 401/2006 (European Union, 2006b), except for AFB1, which presented 64.1% of the recovery. This result, which other authors have also reported for wheat (Torović, 2018), can be justified by the presence in the matrix of impurities such as lipids that are the main interferences with the purification step and the chromatographic separation (Manetta, 2002).

The repeatability relative standard deviations (RSDr) were 1.9–4.5%. These values agree with the regulated performance criteria for AF, which states RSDr < 21% for AFB1 and AFG1, and RSDr < 27% for AFB2 and AFG2 (European Union, 2006b).

The recovery rate of OTA was 103%, with an RSDr of 9.2%. These results agree with the regulated performance criteria for OTA that defines the recovery rate of 70–110% and repeatability RSDr < 21% (European Union, 2006b).

According to the European Commission Regulation 1881/2006, the maximum permissible levels (MPL) of AFB1 and total AF for all cereals and products derived from cereals, including processed cereal products, are 2 and 4 µg/kg, respectively. The MPL for OTA is 3µg/kg for all products derived from unprocessed cereals, including processed cereals and cereals intended for direct human consumption (European Union, 2006a). AFs and OTA were not detected in our study samples (< LOD). These results show that the levels of mycotoxin contamination of the samples are clearly below the regulated limits, even in the case of whole flour, which retain the most contaminated parts of the grains. Wheat flour showed higher moisture content (between 13.6% and 14.7%) than rye flour (9.3%–11.5%). Thus, a moisture content lower than 14.5% (on a wet weight basis) is necessary to ensure no mould spoilage or mycotoxin contamination (Magan et al., 2010). Contamination of wheat flour from markets in Rio de Janeiro, Brazil, with AFs has been reported earlier by Trombete et al. (2014). In whole wheat flour samples (n = 26) and refined wheat flour (n = 15), 7.7% and 6.7%, respectively, were positive for at least one aflatoxin (3.4 and 1.2 µg/kg, respectively), although at levels lower

than the limit established by Brazilian legislation (5 µg/kg). Ghali et al. (2008) evaluated samples from markets and traditional family reserves in Tunisia. They reported that 31.9% of cereals samples, including wheat and derived products, were contaminated with AFs (6.7 µg/kg) and AFB1 (2.2 µg/kg), with concentrations higher than the levels established by the EU. In Bulgaria, AFs were the predominant mycotoxins in wheat (69%), with an average level of 17 µg/kg. On the other hand, OTA was found in 16 out of 60 (26.7%) wheat flour samples from Turkey at concentrations levels of 0.247 µg/kg (Kara et al., 2015) and in Germany samples, values of 5.49 µg/kg were detected in rye meal bread (Zinedine et al., 2007).

**Table 30:** Analysis of microorganisms identified in the different types of flour

| Microorganisms analysed | Wheat refined flour | | Whole wheat flour | Rye refined flour | | Whole rye flour | |
|---|---|---|---|---|---|---|---|
| | T 55 | T 65 | T 150 | T 70 | T 85 | T 130 | T 170 |
| APC - $LOG_{10}$ CFU/g | 4.33 | 4.20 | 4.44 | 3.43 | 3.89 | 4.31 | 5.42 |
| Coliforms - $LOG_{10}$ CFU/g | 2.00 | 3.04 | 2.00 | 1.70 | < LOQ | 2.03 | 2.70 |
| Yeasts - $LOG_{10}$ CFU/g | 2.24 | 2.35 | < LOQ | 2.98 | 2.76 | 2.78 | 4.20 |
| Moulds - $LOG_{10}$ CFU/g | 2.46 | 2.10 | 5.46 | 2.60 | 2.44 | 1.88 | 3.00 |
| *Escherichia coli* - CFU/g | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| SRC - CFU/g | < 2 | < 2 | < 2 | < 2 | < 2 | < 2 | < 2 |
| *Salmonella* spp. | Absent | Absent | Absent | Absent | Absent | Absent | Absent |

APC – aerobic plate count; SRC – sulphite-reducing clostridia; CFU – colony forming units

**Table 31:** Calibration parameters of instrumentation for aflatoxins and ochratoxin A detection and quantification

| Standard | | $AFB_1$ | $AFB_2$ | $AFG_1$ | $AFG_2$ | OTA |
|---|---|---|---|---|---|---|
| Calibration curve | | y = 317.17x + 106.7 | y = 905.73x + 111.38 | y = 92.47x + 33.979 | y = 157.79x + 17.951 | y = 110.14x + 3.9186 |
| Correlation coefficient ($R^2$) | | 0.9996 | 0.9995 | 0.9996 | 0.9992 | 0.9992 |
| Linearity range (ng/mL) | | 0.5–50 | 0.15–15 | 0.5–50 | 0.15–15 | 0.1–20 |
| Limits | LOD[a] (µg/kg) | 1.2 | 0.4 | 1.1 | 0.5 | 0.7 |
| | LOQ[b] (µg/kg) | 3.5 | 1.2 | 3.2 | 1.5 | 2.0 |

$R^2$: Correlation coefficient; [a] LOD: limit of detection of the chromatographic method; [b] LOQ: limit of quantification of the chromatographic method.

**Table 32:** Accuracy and precision of the analytical methods for aflatoxins and ochratoxin A

| | **$AFB_1$** | **$AFB_2$** | **$AFG_1$** | **$AFG_2$** | **OTA** |
|---|---|---|---|---|---|
| Spiking level (µg/kg) | 10 | 3 | 10 | 3 | 10 |
| Mean Recovery (%) | 64.1 | 72.8 | 78.0 | 87.3 | 103 |
| RSDr (%)[a] | 4.5 | 2.0 | 4.4 | 1.9 | 9.2 |

[a] RSDr: Repeatability relative standard deviation.

### 3.3.2. Cereal by-products flour from the milling industry as a source of nutrients and bioactive compounds

Studying cereal by-products flour from the milling industry (**Figure 23**) as a source of nutrients and bioactive compounds can boost resource efficiency and contribute to the valorisation of cereal by-products generated worldwide. The results described here provide valid arguments to support the use of germ and bran by-products from the cereal industry as underexploited alternative sources of nutrients and bioactive compounds with potential health benefits for consumers. These by-products could be targeted to human nutrition as a sustainable way to promote the development of novel and functional foods and be fortified with other natural nutrient sources, such as vitamin $D_2$.


**Figure 23:** Cereal by-products flour from the milling industry

#### *3.3.2.1. Compositional Features of the Cereal By-Products*

The results in **Table 33** show the proximate composition of the cereal by-products, including fat, protein, ash, and carbohydrate content and energy value. Carbohydrates were the most prevalent constituents, ranging from 56.35 g/100 g dw in wheat germ to 78.12 g/100 g dw in maize bran–germ mixture. The results are slightly higher than the 46.07 g/100 g dw previously reported by Mahmoud et al. (2015) for wheat germ samples from Egypt. Proteins were the

second most abundant nutrients, and the contents differed significantly between cereal by-products. Interestingly, the samples with the lowest carbohydrate content had the highest protein level. As shown in **Table 33**, 30.0 g/100 g dw were quantified in wheat germ, whose 100-g portions can provide 53.6 and 65.2% of the recommended dietary allowances of protein for adult male and female individuals, respectively (National Academy of Sciences, 2006). The two by-products with germ, namely wheat germ and the maize bran–germ mixture, presented higher quantities of crude fat (9.64 and 8.08 g/100 g dw, respectively) than the bran samples. Navarro et al. (2016) and Mahmoud et al. (2015) reported a higher fat content in maize bran–germ (10.74 g/100 g dw) and wheat germ (10.29 g/100 g dw) samples from Brazil and Egypt, respectively. Fats are essential nutritional constituents of cereal by-products, a source of essential fatty acids and energy, and play an essential role in their quality (Cardoso et al., 2019b). Overall, in terms of energetic contribution, wheat germ presented the highest value (432.3 kcal/100 g dw) in accordance with the higher fat and protein contents, followed by the maize bran–germ mixture (429.8 kcal/100 g dw) and wheat bran (412.4 kcal/100 g dw). The rye bran samples showed the lowest energy value, reaching only 405.4 kcal/100 g dw. Mahmoud et al. (2015) reported similar energy values for wheat germ (430.3 kcal/100 g dw), while the National Food Institute (2021) presents lower values for wheat germ (379 kcal/100 g), wheat bran (291 kcal/100 g), and standard rye flour (322 kcal/100 g). The INRAE CIRAD AFZ (2021) composition tables present high energy values for maize germ (690 kcal/100 g) and bran (457 kcal/100 g). However, to the best of the authors' knowledge, this is the first study describing the energy value of the maize bran–germ mixture and rye bran.

**Table 33:** Proximate composition and energy value of the cereal by-products

| | **Wheat germ** | **Maize bran-germ** | **Rye bran** | **Wheat bran** |
|---|---|---|---|---|
| Fat (g/100 g dw) | $9.64\pm0.1^{d}$ | $8.08\pm0.01^{c}$ | $3.9\pm0.1^{a}$ | $5.05\pm0.02^{b}$ |
| Proteins (g/100 g dw) | $30.0\pm0.1^{d}$ | $11.2\pm0.2^{a}$ | $15.3\pm0.1^{b}$ | $16.4\pm0.1^{c}$ |
| Ash (g/100 g dw) | $3.97\pm0.05^{d}$ | $2.66\pm0.04^{a}$ | $3.54\pm0.04^{c}$ | $3.12\pm0.04^{b}$ |
| Carbohydrates (g/100 g dw) | $56.35\pm0.02^{a}$ | $78.12\pm0.04^{d}$ | $77.2\pm0.1^{c}$ | $75.42\pm0.01^{b}$ |
| Energy (kcal/100 g dw) | $432.3\pm0.1^{d}$ | $429.8\pm0.2^{c}$ | $405.4\pm0.1^{a}$ | $412.8\pm0.1^{b}$ |

Different letters indicate significant differences ($p < 0.05$) between samples in each line. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure.

**Table 34** shows the free sugars and organic acids detected in the cereal by-products, namely, five sugars and six organic acids characterised by HPLC-RI and UFLC-DAD, respectively. Regarding free sugars, sucrose was the most abundant in all cereal by-products, reaching 10.4 g/100 g dw in wheat germ, 3.84 g/100 g dw in the maize bran–germ mixture, and approximately 2.9 g/100 g dw in the bran samples. These results agree with those of Rizzello et al. (2010), who also reported sucrose as the significant soluble sugar in the wheat germ (7.2 g/100 g dw). **Table 34** also shows that the free sugar profile of the different samples is different, as some of these water-soluble molecules were not detected in some of the studied cereal by-products. Trehalose and fructose were detected only in the wheat germ (0.25 g/100 g dw) and maize bran–germ mixture (0.15 g/100 g dw), respectively, while rye bran did not contain glucose. Overall, the total amount of free sugars ranged from 3.51 g/100 g dw to 15.2 g/100 g dw in wheat germ, this last sample with a considerable difference from the other cereal by-products. The National Food Institute (2021) reported a similar value for a wheat germ sample from Denmark, namely 15.7 g/100 g dw of total free sugars. It is also interesting to note that free sugars correspond to 27% of the carbohydrate fraction of wheat germ and less than 6% in the remaining samples.

**Table 34:** Composition in sugars and organic acids of the cereal by-products

| | **Wheat germ** | **Maize bran-germ** | **Rye bran** | **Wheat bran** |
|---|---|---|---|---|
| **Free sugars (g/100 g dw)** | | | | |
| Fructose | nd | 0.15±0.02 | nd | nd |
| Glucose | $0.18\pm0.01^{c}$ | $0.16\pm0.01^{b}$ | nd | $0.14\pm0.01^{a}$ |
| Sucrose | $10.4\pm0.1^{c}$ | $3.84\pm0.01^{b}$ | $2.92\pm0.03^{a}$ | $2.9\pm0.1^{a}$ |
| Trehalose | 0.25±0.01 | nd | nd | nd |
| Raffinose | $4.65\pm0.03^{d}$ | $0.4\pm0.1^{a}$ | $0.59\pm0.01^{b}$ | $1.69\pm0.01^{c}$ |
| Total of free sugars | $15.2\pm0.1^{d}$ | $4.4\pm0.1^{b}$ | $3.51\pm0.02^{a}$ | $4.7\pm0.1^{c}$ |
| **Organic acid (g/100 g dw)** | | | | |
| Oxalic acid | $0.090\pm0.001^{c}$ | $0.105\pm0.001^{d}$ | $0.0471\pm0.0001^{a}$ | $0.0593\pm0.0001^{b}$ |
| Malic acid | nd | nd | tr | tr |
| Ascorbic acid | tr | tr | nd | nd |
| Shikimic acid | nd | nd | nd | tr |
| Citric acid | $0.857\pm0.002^{d}$ | $0.204\pm0.001^{a}$ | $0.424\pm0.001^{b}$ | $0.539\pm0.001^{c}$ |
| Fumaric acid | tr | tr | tr | tr |
| Total of organic acids | $0.946\pm0.002^{d}$ | $0.309\pm0.001^{a}$ | $0.471\pm0.001^{b}$ | $0.598\pm0.001^{c}$ |

Different letters indicate significant differences ($p < 0.05$) between samples in each line. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure. Nd-not detected; tr-traces (below LOQ).

Regarding organic acids, malic, ascorbic, shikimic, and fumaric acids were detected in trace amounts and just in some samples (**Table 34**). Citric acid was the main compound identified in the cereal by-products, and its levels contribute significantly to the total amounts of organic acids recorded in the wheat germ (0.946 g/100 g dw), followed by wheat bran (0.598 g/100 g dw), rye bran (0.471 g/100 g dw), and maize bran-germ mixture with the lowest levels (0.309 g/g 100 g dw). Oxalic acid was also detected in all samples, especially in the maize bran–germ mixture (0.105 g/100 g dw). To the best of the author's knowledge, this is the first report describing the organic acids composition of these by-products. Furthermore, wheat is recognised as a complete cereal because it has much higher soluble sugars and organic acids than other cereals, regardless of the cereal part under analysis (bran or germ). Sugars and organic acids are essential quality indicators related to preservation and storage conditions (Barreira et al., 2010; Cardoso et al., 2019a).

**Table 35** shows the lipophilic compounds detected in the cereal by-products, namely fatty acids, and tocopherols. The GC-FID analysis allowed the detection of 13 fatty acids, whose contents are presented in relative percentages. Linoleic acid (C18:2n6c) was the most abundant fatty acid, with levels up to 53% in all samples. Similar C18:2n6c contents (55.1 and 54.4%) were reported by Mahmoud et al. (2015) for a wheat germ oil sample from Egypt and by Abdelghany et al. (2020) for soybean seed accessions collected in China, respectively. This essential fatty acid is the precursor of eicosanoids, which take part in many biological processes and are required for normal human health. Oleic acid (C18:1n9c) ranked second and prevailed in the maize bran–germ mixture (29%). This omega-9 fatty acid was also previously reported as the second most abundant in soybean in a slightly lower relative percentage (24.6%) (Abdelghany et al., 2020). Regarding saturated fatty acids (SFA), palmitic acid (C16:0) was the major contributor and prevailed in wheat bran and germ samples (with approximately 18%), while stearic acid (C18:0) predominated in the maize bran–germ mixture (2.96%). In turn, the higher amounts of monounsaturated fatty acids (MUFA) were quantified in the maize bran–germ mixture (29.2%). In general, polyunsaturated fatty acids (PUFA) predominated in all samples, with levels ranging from 55.9 to 65.4%. The fatty acid profiles observed in the characterised cereal by-products are in agreement with previous reports (Armanino et al., 2002; Kan, 2015; Nikolić et al., 2008; Becker, 2007). Fatty acids are good food quality indicators

since lipid peroxidation affects cereals' overall quality and odour when stored for an extended period (Cardoso et al., 2019b; Thomas et al., 2015).

**Table 35:** Composition in fatty acids and tocopherols of the cereal by-products

| | Wheat germ | Maize bran-germ | Rye bran | Wheat bran |
|---|---|---|---|---|
| **Fatty acids (relative %)** | | | | |
| C14:0 | 0.12±0.01$^{a}$ | nd | 0.14±0.01$^{b}$ | 0.126±0.001$^{a}$ |
| C15:0 | 0.073±0.003$^{a}$ | nd | 0.15±0.01$^{c}$ | 0.104±0.001$^{b}$ |
| C16:0 | 18.02±0.02$^{c}$ | 10.6±0.3$^{a}$ | 15.59±0.04$^{b}$ | 18.3±0.2$^{c}$ |
| C16:1 | 0.17±0.02$^{b}$ | 0.101±0.004$^{a}$ | 0.27±0.02$^{d}$ | 0.20±0.01$^{c}$ |
| C18:0 | 0.86±0.01$^{a}$ | 2.955±0.001$^{c}$ | 1.3±0.1$^{b}$ | 1.32±0.01$^{b}$ |
| C18:1n9c | 13.4±0.1$^{a}$ | 29.0±0.1$^{d}$ | 17.31±0.04$^{c}$ | 16.07±0.04$^{b}$ |
| C18:2n6c | 57.1±0.1$^{d}$ | 54.8±0.2$^{b}$ | 53.9±0.1$^{a}$ | 56.3±0.2$^{c}$ |
| C18:3n3 | 8.0±0.1$^{c}$ | 1.04±0.01$^{a}$ | 8.6±0.1$^{d}$ | 5.17±0.05$^{b}$ |
| C20:0 | 0.205±0.001$^{a}$ | 0.48±0.01$^{b}$ | nd | 0.24±0.01$^{a}$ |
| C20:1 | 1.35±0.03$^{c}$ | 0.168±0.005$^{a}$ | 1.456±0.003$^{d}$ | 1.0±0.1$^{b}$ |
| C22:0 | 0.25±0.02$^{a}$ | 0.36±0.03$^{b}$ | 0.42±0.01$^{c}$ | 0.41±0.01$^{c}$ |
| C20:5n3 | 0.30±0.02$^{a}$ | nd | 0.47±0.01$^{c}$ | 0.40±0.02$^{b}$ |
| C24:0 | 0.20±0.01$^{a}$ | 0.46±0.01$^{c}$ | 0.49±0.03$^{d}$ | 0.29±0.01$^{b}$ |
| SFAs | 19.73±0.02$^{c}$ | 14.9±0.3$^{a}$ | 18.1±0.1$^{b}$ | 20.8±0.2$^{d}$ |
| MUFAs | 14.9±0.1$^{a}$ | 29.2±0.1$^{d}$ | 19.03±0.02$^{c}$ | 17.3±0.1$^{b}$ |
| PUFAs | 65.4±0.1$^{d}$ | 55.9±0.2$^{a}$ | 62.9±0.1$^{c}$ | 61.9±0.3$^{b}$ |
| **Tocopherols (mg/100 g dw)** | | | | |
| $\alpha$-Tocopherol | 13.46±0.01$^{d}$ | 3.38±0.02$^{a}$ | 4.1±0.1$^{b}$ | 5.23±0.04$^{c}$ |
| $\beta$-Tocopherol | 9.27±0.04$^{d}$ | 0.12±0.03$^{a}$ | 1.27±0.01$^{b}$ | 2.99±0.01$^{c}$ |
| $\gamma$-Tocopherol | nd | 1.61±0.03$^{b}$ | nd | 0.140±0.001$^{a}$ |
| $\delta$-Tocopherol | 0.046±0.001 | nd | nd | nd |
| Total of tocopherols | 22.8±0.1$^{d}$ | 5.10±0.02$^{a}$ | 5.4±0.1$^{b}$ | 8.35±0.04$^{c}$ |

Different letters indicate significant differences ($p < 0.05$) between samples in each line. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure. SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; nd - not detected.

The results of the tocopherols composition are also shown in **Table 35**. $\alpha$-Tocopherol was found in all cereal by-products and as the predominant isoform, especially in the wheat germ (13.46 mg/100 g dw), where it was possible to detect $\delta$-tocopherol. Górnaś et al. (2016) report lower $\alpha$-tocopherol values for wheat and rye bran (1.9 and 1.4 mg/100 g dw, respectively) than those achieved in the present study. In turn, higher levels were obtained by Ansolin et al. (2015)

in wheat germ oil (28.5 mg/100 g) and by Navarro et al. (2016) in maize bran–germ oil (13 mg/100 g dw). Wheat bran and germ samples stand out with the highest amounts of β-tocopherol (2.99 and 9.27 mg/100 g dw, respectively). Only two samples contained γ-tocopherol, namely the maize bran–germ mixture and wheat bran (1.61 and 0.14 mg/100 g dw, respectively). The tocopherol profiles herein agree with those previously reported (Ghafoor et al., 2017; Górnaś et al., 2016; Navarro et al., 2016). Overall, wheat by-products showed the highest concentration of tocopherols, with 22.8 mg/100 g dw quantified in the germ and 8.35 ± 0.04 mg/100 g dw in the bran. Based on the *α*-tocopherol values in **Table 35** and the recommended dietary allowances for vitamin E of 15 mg/day for healthy adults (National Academy of Sciences, 2006), it could be concluded that a 100 g portion of wheat germ contributes 61.8% to the intake of this liposoluble vitamin.

#### *3.3.2.2. Phenolic Composition of the Cereal By-Products*

Cereal by-products are good sources of phytochemicals such as phenolic compounds, which have gained significant attention since they exhibit a wide range of biological activities, including antioxidant and antimicrobial effects (Călinoiu & Vodnar, 2018; Hung, 2016). The chromatographic information (retention time, wavelengths of maximum absorption in the visible region (λmax), and mass spectral data) used in the tentative identification of the phenolic compounds detected in the hydroethanolic extracts prepared with the cereals by-products are shown in **Table 36**. Eleven phenolic compounds were tentatively identified in the extracts, including 4 phenolic acids (*p*-coumaroyl, caffeic, and chlorogenic acid derivatives), 6 flavonoids (luteolin and apigenin C-glycosylated derivatives) and one unknown compound that was found in maize bran–germ and rye bran samples. Regarding phenolic acids, peak 1 presented a pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 487 and a unique MS2 fragment at *m/z* 163 (loss of two hexosyl moieties), coupled to a characteristic UV spectrum of *p*-coumaric acid at 301 nm, the peak was tentatively identified as *p*-coumaric acid dihexoside. Peaks 2 and 3 were tentatively identified as caffeic acid hexoside, presenting a pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 341 and $MS^2$ fragments at *m/z* 179, 161, and 135, that correspond to the break of the caffeic acid unit and the loss of one hexosyl moiety (341−179 = 162 u). Finally, peak 9, with [M-H]− at *m/z* 515 and λmax 311 nm (characteristic of chlorogenic acid derivatives), was tentatively identified as 3,5-*O*-dicaffeoylquinic acid using the hierarchical fragmentation pattern previously reported by Clifford et al. (2003, 2005).

**Table 36:** Phenolic compounds tentatively identified in the cereal by-products' hydroethanolic extracts. It is presented the retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), and mass spectral data

| Peak | Rt (min) | *λmax* (nm) | $[M-H]^-$ (*m/z*) | $MS^2$ (*m/z*) | Tentative identification |
|---|---|---|---|---|---|
| **1** | 4.7 | 301 | 487 | 162 (100) | *p*-Coumaric acid dihexoside |
| **2** | 4.8 | 311 | 341 | 179(100), 161(15), 132(5) | Caffeic acid hexoside |
| **3** | 5.43 | 311 | 341 | 179(100), 161(15), 132(5) | Caffeic acid hexoside |
| **4** | 11.45 | 345 | 579 | 459(35), 429(10), 357(5), 327(10), 309(5) | Luteolin-*O*-pentoside-*C*-hexoside |
| **5** | 12.11 | 287 | 385 | 267 (100), 249(20) | Unknown compound |
| **6** | 12.86 | 336 | 563 | 545(43), 473(100), 443(7), 383(31), 353(28), 311(5) | Apigenin-*C*-pentoside-*C*-hexoside |
| **7** | 13.21 | 326 | 563 | 545(20), 473(92), 443(100), 383(28), 353(25), 311(5) | Apigenin-*C*-hexoside-*C*-pentoside |
| **8** | 14.21 | 336 | 563 | 545(32), 473(100), 443(98), 383(38), 353(31), 311(5) | Apigenin-*C*-pentoside-*C*-hexoside |
| **9** | 17.11 | 324 | 515 | 353(60), 191(100), 179(30), 173(5), 161(5), 135(5) | 3,5-*O*-Dicaffeoylquinic acid |
| **10** | 19.65 | 331 | 769 | 563(11), 545(81), 425(100), 335(31) | Sinapic acid ester of apigenin-*C*-diglycoside |
| **11** | 20.53 | 331 | 769 | 563(10), 545(89), 425(100), 335(12) | Sinapic acid ester of apigenin-*C*-diglycoside |

The flavonoids group represented most of the compounds identified in the cereal by-products, and apigenin derivatives were the ones with higher expression in all samples (except in maize bran). Peaks 6, 7, and 8 all presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 563 and characteristics $MS^2$ fragment at *m/z* 473 (90 u), 383 (90 u), 353 (30 u), that correspond to the loss of units in multiples of 30, leading to the identification of *C*-glycosylated derivatives. The differentiation between the three peaks takes into account the abundance of 100% in $MS^2$ fragment at *m/z* 473 in peaks 6 and 8 and the $MS^2$ fragment at *m/z* 443 in peak 7, which leads to the tentative identification of apigenin-*C*-pentoside-*C*-hexoside (peaks 6 and 8) and apigenin-*C*-hexoside-*C*-pentoside (peak 7) (Ferreres et al., 2018). Peaks 10 and 11 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 769 and an $MS^2$ fragmentation pattern coherent with that previously described by Hirawan & Beta (2011) in whole-wheat Spaghetti, being both tentatively identified as sinapic acid esters of apigenin-*C*-diglycoside. Finally, the last flavonoid found was peak 4, tentatively identified as luteolin-*O*-pentoside-*C*-hexoside ($[M-H]^-$ at *m/z* 579), using the chromatographic profile previously described by Roriz et al. (2014). As

presented in **Table 37** the flavonoids group represents the majority (in content) of the compounds found in all samples (except for maize bran), more specifically, 81% (4.7 mg/g extract) in wheat germ and 77% (3.4 mg/g extract) in wheat bran of the total phenolic compounds. These concentrations are mainly due to apigenin-*C*-pentoside-*C*-hexoside, with 2.95 mg/g of extract in the wheat germ and 2.09 mg/g of extract in wheat bran. In maize bran and rye bran, phenolic acids were detected in higher concentrations, 0.046 mg/g extract in maize bran (peak 2 corresponded to the only phenolic compound found in this sample) and 0.93 mg/g extract in rye bran (mainly due to the presence of *p*-coumaric acid dihexoside, in agreement with previous reports found in the literature (Andreasen et al., 2000; Kulichová et al., 2019; Skrajda-Brdak et al., 2019).

**Table 37:** Phenolic compounds content in the hydroethanolic extracts of the cereal by-products

| Peak | Tentative identification | Content (mg/g extract) | | | |
|---|---|---|---|---|---|
| | | **Wheat germ** | **Maize bran-germ** | **Rye bran** | **Wheat bran** |
| **1** | *p*-Coumaric acid dihexoside | nd | nd | 0.57±0.02 | nd |
| **2** | Caffeic acid hexoside | 0.088±0.001$^{b}$ | 0.046±0.001$^{c}$ | nd | 0.115±0.006$^{a}$ |
| **3** | Caffeic acid hexoside | nd | nd | nd | nd |
| **4** | Luteolin-*O*-pentoside-*C*-hexoside | 0.121±0.004* | nd | nd | 0.084±0.001* |
| **5** | Unknown compound | nd | nq | nq | nd |
| **6** | Apigenin-*C*-pentoside-*C*-hexoside | 0.602±0.01* | nd | nd | 0.39±0.01* |
| **7** | Apigenin-*C*-hexoside-*C*-pentoside | 1.004±0.058* | nd | nd | 0.80±0.05* |
| **8** | Apigenin-*C*-pentoside-*C*-hexoside | 2.9±0.1$^{a}$ | nd | 0.067±0.002$^{c}$ | 2.1±0.1$^{b}$ |
| **9** | 3,5-*O*-Dicaffeoylquinic acid | 0.34±0.01$^{a}$ | nd | 0.293±0.001$^{b}$ | 0.35±0.01$^{a}$ |
| **10** | Sinapic acid ester of apigenin-*C*-diglycoside | 0.16±0.01* | nd | nd | 0.140±0.001* |
| **11** | Sinapic acid ester of apigenin-*C*-diglycoside | 0.470±0.001$^{c}$ | nd | 0.057±0.001$^{b}$ | 0.413±0.007$^{a}$ |
| | Σ Phenolic acids | 1.066±0.002$^{a}$ | 0.046±0.001$^{d}$ | 0.93±0.02$^{c}$ | 1.017±0.001$^{b}$ |
| | Σ Flavonoids | 4.7±0.1$^{a}$ | nd | 0.067±0.002$^{c}$ | 3.4±0.1$^{b}$ |
| | Σ Phenolic compounds | 5.7±0.1$^{a}$ | 0.046±0.001$^{d}$ | 0.418±0.003$^{c}$ | 4.3±0.1$^{b}$ |

Calibration curves: apigenin-6-C-glucoside (y = 107025x + 61531, r2 = 0.9989, LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL; peak 6, 7, and 8); caffeic acid (y = 388345x + 406369, r2 = 0.9939, LOD = 0.78 µg/mL, and LOQ = 1.97 µg/mL; peaks 2 and 3); chlorogenic acid (y = 168823x − 161172, r2 = 0.9999, LOD = 0.20 µg/mL, and LOQ = 0.68 µg/mL; peak 9); luteolin-6-C-glucoside (y = 4087.1x + 72589, r2 = 0.9988, LOD = 0.20 µg/mL, and LOQ = 0.45 µg/mL; peak 4); *p*-coumaric acid (y = 301950x + 6966.7, r2 = 0.9999, LOD = 0.68 µg/mL, and LOQ = 1.61 µg/mL; peak 1); sinapic acid (y = 197337x + 30036, r2 = 0.9997, LOD = 0.17 µg/mL, and LOQ = 1.22 µg/mL; peaks 10 and 11). Statistically significant differences ($p$< 0.05) between more than two samples were assessed by a one-way ANOVA, using Tukey's significant difference (HSD), and are indicated by different letters; a Student's *t*-test assessed significant differences (p < 0.001) between two samples *. nd - not detected; nq - not quantified.

Wheat germ and bran were the samples presenting the highest concentration of phenolic compounds (5.7 and 4.4 mg/g extract, respectively), and a higher concentration of total phenolic acids (1.066 and 1.017 mg/g extract, respectively). The phenolic compounds of wheat were previously studied by different authors, with different phenolic acids and results obtained. Zou et al. (2015) reported ferulic acid as the major compound found in wheat germ (250 µg/g of extract) and 554 µg/g extract of total phenolic acids. Vaher et al. (2010), in bran spring wheat, also reported ferulic acid was the main one (268.9 µg/g dw) and the amount of total phenolic acids was 569 µg/g dw. López-Perea et al. (2019) reported caffeic acid as the major compound (0.86 µg/g dw), and the total phenolic compound was 100 µg/g dw in wheat bran. Skrajda-Brdak et al. (2019) reported ferulic acid (222.1 µg/g dw) as the main compound, and the total phenolic acids content was 273.2 µg/g dw for common wheat.

*3.3.2.3. Bioactivity of the Cereal By-Products*

Whole cereal grains have been described as having excellent bioactive properties due to their fractions, bran and germ, that comprise health-promoting bioactive compounds (Călinoiu & Vodnar, 2018). Therefore, the antioxidant activity of the cereal by-product hydroethanolic extracts was evaluated *in vitro* by the thiobarbituric acid reactive substances (TBARS) formation inhibition, using porcine brain tissue homogenates, and the oxidative hemolysis inhibition assay (OxHLIA), using sheep erythrocytes. The results are presented in **Table 38** and were expressed in $IC_{50}$ values, translating the extract concentration providing 50% of antioxidant activity in the TBARS assay or the concentration able to protect 50% of the erythrocyte population in OxHLIA. The cereal by-product with the best TBARS formation inhibition capacity was the wheat bran (with the lowest $IC_{50}$ value of 0.26 mg/mL), followed by the maize bran–germ mixture and rye bran. The lowest activity was shown by the wheat germ extract, not agreeing, therefore, with the highest content of phenolic compounds quantified in this sample (**Table 38**) but coinciding with the highest levels of citric acids (**Table 35**) and tocopherols (**Table 36**). For OxHLIA, only rye bran presented the capacity to protect the erythrocyte membrane from the free radicals generated in the system by the thermal decomposition of AAPH. The other extracts did not delay hemolysis compared to the negative control (PBS). It should be noted that the lower $IC_{50}$ values of Trolox are because this positive control is a pure antioxidant compound, while the natural extracts tested are complex mixtures of different constituents, some of which have no activity. Previous studies have demonstrated

the antioxidant activity of the studied cereals, but most of them used chemical methods, such as reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and Folin–Ciocalteu assays, among others (Korycinska et al., 2009; Samyor et al., 2016; Smuda et al., 2018). However, cell-based assays have been described as more suitable for measuring the antioxidant activity of natural products than chemical methods (Shahidi & Zhong, 2015). It is also worth noting that, according to some studies, cereal phenolic acids display antioxidant properties *in vitro* due to the presence of an aromatic phenolic ring (Călinoiu & Vodnar, 2018).

The antibacterial activity of the cereal by-products was also tested against Gram-positive and Gram-negative bacteria. The results are presented in **Table 39** as minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively). In general, the extracts were not very effective against the selected foodborne microorganisms. The best results were achieved against methicillin-resistant *Staphylococcus aureus* with the maize bran–germ mixture extract (MIC of 2.5 mg/mL) and rye bran and wheat bran extracts (MIC of 5 mg/mL). MBC values above 20 mg/mL were obtained in all cases. Among the tested microorganisms, the Gram-positive bacteria appear more susceptible to the extracts than the Gram-negative bacteria. The positive controls ampicillin, imipenem, and vancomycin yielded much lower MIC and MBC values, as expected for commercial antibiotics. Despite the low efficacy of the tested extracts, isolated compounds such as phenolic acids can display remarkable antimicrobial effects (Călinoiu & Vodnar, 2018), highlighting their potential for application as preservatives in the food and food-packing materials, among others. Regarding previous studies, Călinoiu & Vodnar (2020) reported better results for heat-processed wheat bran, with a MIC of 1.875 mg/mL against *Enterococcus faecalis*. In turn, a moderate antibacterial effect toward *Pseudomonas aeruginosa* was achieved with heat-processed wheat bran and fresh wheat bran extracts, with MIC values of 3.75 mg/mL and 7.5 mg/mL, respectively. The same authors attributed the moderate antibacterial activity to heat-processed wheat bran against *Escherichia coli* (MIC of 3.75 mg/mL) and *Salmonella typhimurium* (MIC of 7.5 mg/mL).

**Table 38:** Antioxidant activity ($IC_{50}$ values, mg/mL) of the cereal by-product hydroethanolic extracts

| | Wheat germ | Maize bran-germ | Rye bran | Wheat bran | Trolox |
|---|---|---|---|---|---|
| TBARS | 4.8±0.1[d] | 0.62±0.01[b] | 0.98±0.01[c] | 0.26±0.01[a] | 0.023±0.001 |
| OxHLIA, $\Delta t$ 60 min | na | na | 0.58±0.02 | na | 0.020±0.001 |
| OxHLIA, $\Delta t$ 120 min | na | na | 1.02±0.04 | na | 0.041±0.001 |

For TBARS, statistically significant differences ($p < 0.05$) between samples were assessed by a one-way ANOVA, using Tukey's significant difference (HSD), and are indicated by different letters; for OxHLIA, significant differences ($p < 0.001$) between the two samples * were assessed by a student's t-test. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure. na - no activity.

**Table 39:** Antibacterial activity (mg/mL) of the cereal by-product hydroethanolic extracts

| | Wheat germ | | Maize bran-germ | | Rye bran | | Wheat bran | | Ampicillin | | Imipenem | | Vancomycin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| **Gram-negative bacteria** | | | | | | | | | | | | | | |
| *Escherichia coli* | 10 | >20 | 10 | >20 | 20 | >20 | 20 | >20 | <0.15 | <0.15 | <0.0078 | <0.0078 | nt | nt |
| *Klebsiella pneumoniae* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | 10 | 20 | <0.0078 | <0.0078 | nt | nt |
| *Morganella morganii* | 20 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | <0.0078 | <0.0078 | nt | nt |
| *Proteus mirabilis* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | <015 | <0.15 | <0.0078 | <0.0078 | nt | nt |
| *Pseudomonas aeruginosa* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | 0.5 | 1 | nt | nt |
| **Gram-positive bacteria** | | | | | | | | | | | | | | |
| *Enterococcus faecalis* | 10 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | <0.15 | <0.15 | nt | nt | <0.0078 | <0.0078 |
| *Listeria monocytogenes* | 10 | >20 | 20 | >20 | 20 | >20 | 10 | >20 | <0.15 | <0.15 | <0.0078 | <0.0078 | nt | nt |
| MRSA | 10 | >20 | 2.5 | >20 | 5 | >20 | 5 | >20 | <0.15 | <0.15 | nt | nt | 0.25 | 0.5 |

MRSA - Methicillin-resistant Staphylococcus aureus; MIC - minimum inhibitory concentration; MBC - minimal bactericidal concentration; nt - not tested

## 3.4. Microencapsulation, stability and incorporation of vitamin $D_2$ bioactive ingredient in a bakery product. Sensory analysis of final product

### 3.4.1. Microencapsulation of Vit$D_2$-enriched extract

This step focuses on developing Vit$D_2$-enriched extract by SDs technique, with spray drying based operation, obtaining dry particles to incorporated into flour to make bread or other bakery and pastry products. In this context using stability studies, Vit$D_2$-enriched extract, was encapsulated by SDs using KC polymer. Thus, the particles obtained were characterised and incorporated into flour and made bread.

The yield value of the formulation process showed that the formulation preparation process presented a value of approximately 60%. The prepared particles (**Figure 24**) appear like a fine white powder comparable to flour.

The KC-Bioactive sample shows particles size in volume and number corresponding to 10, 50 and 90%. The particles showed an average size in number the $D_{50}$ value of 0.493 µm, indicating their nano/micro scale, while the 90% of the sample, $D_{90}$ showed values of 0.691 µm. Additionally, the narrow size distribution, which can be observed in **Figure 25**, denoted the homogeneity obtained during particle formation in terms of size.

**Figure 24:** Appearance of the KC-Bioactive particles produced by the DS method with spray-drying

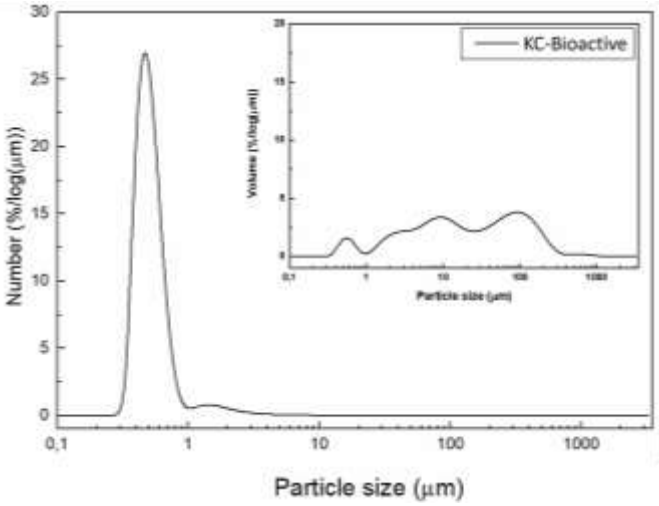



**Figure 25:** Particle size distribution of KC-Bioactive in number and in volume (in the corresponding insets)

The physicochemical properties and the effective encapsulation of $VitD_2$ were analysed/proofed by FTIR (**Figure 26**). The starting compounds (KC, Tween 80, and the $VitD_2$-enriched extract) and KC-Bioactive particles were analysed. The $VitD_2$-enriched extract showed the $VitD_2$ peaks (e.g., a 'shoulder' at 3260 cm-1) combined with additional peaks, where it is worth noting the peaks at 3332 and 3200 $cm^{-1}$ for the stretching vibration of hydroxyl groups, 2920 and 2852 $cm^{-1}$ for C-H groups, and 1619 $cm^{-1}$ for the stretching vibration of C=O groups. These peaks evidence the presence of added compounds present in the composition of the $VitD_2$-enriched extract. Compared with the spectrum of the corresponding KC-Bioactive particles, it was observed that the characteristic peaks of the $VitD_2$-enriched extract were masked, proving the encapsulation of the $VitD_2$-enriched extract that remained inside the KC-Bioactive particles (in a manner analogous to $VitD_2$).

The KC-Bioactive particle morphology was analysed by SEM (**Figure 27**). The particles presented a non-regular spherical shape, and the distribution was uniform.

The weight loss percentages and degradation temperature peaks for $VitD_2$-enriched extract particles are similar to $VitD_2$ standards around 340 ºC and KC-Bioactive round about 285 °C.

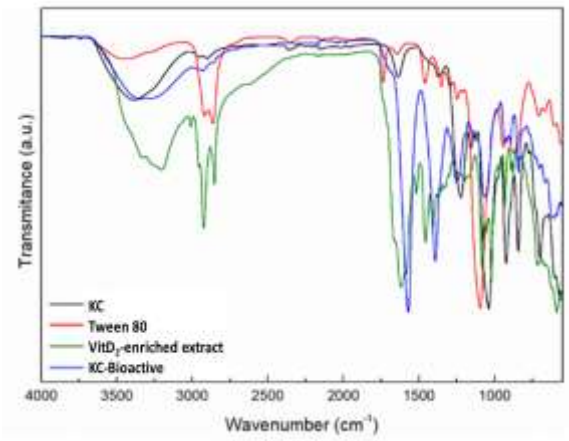



**Figure 26:** FTIR spectra of the reagents, physical mixtures and KC-Bioactive particles

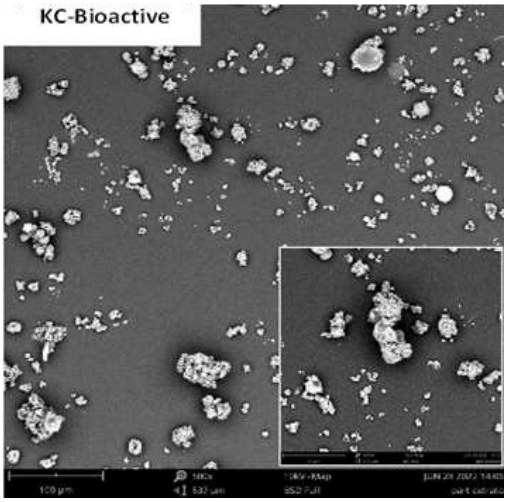



**Figure 27:** SEM image (500x magnifications) of KC-Bioactive. In the insets, its respective images at 1500x magnifications

For the KC-Bioactive sample made using spray drying, the LC was 40 µg $VitD_2$/g of particle, which is enough for flour fortification to ensure an adequate percentage of the RDA (15µg/day) (EFSA, 2016) of the final product. The EE was namely 69.9 %. It has been found that $VitD_2$ was effectively encapsulated and that the particles produced conducted to attractive EE for the further development of industrial food applications. Still, the fact that the KC polymer presents advantages, as it is naturally based. Polymers from natural sources (Biopolymers) are considered a widely used sustainable source (Fadilah et al., 2021; Hosseini et al., 2017). The result for EE of $VitD_2$ shows a good encapsulation efficiency. As has already been said, the question of stability can be improved through the proper selection of encapsulation materials and by linking them to appropriate microencapsulation techniques.

An initial screening was carried out to verify the bread production process. SEM analysed the morphology of the bread, including the control and that containing the KC-Bioactive particles, in this context (**Figure 29**). Overall, the breads showed homogenous morphology.


**Figure 28:** The appearance of control bread and bread made with KC-Bioactive particle prepared by solid dispersion technology and bread mad with $VitD_2$-enriched extract by UAE

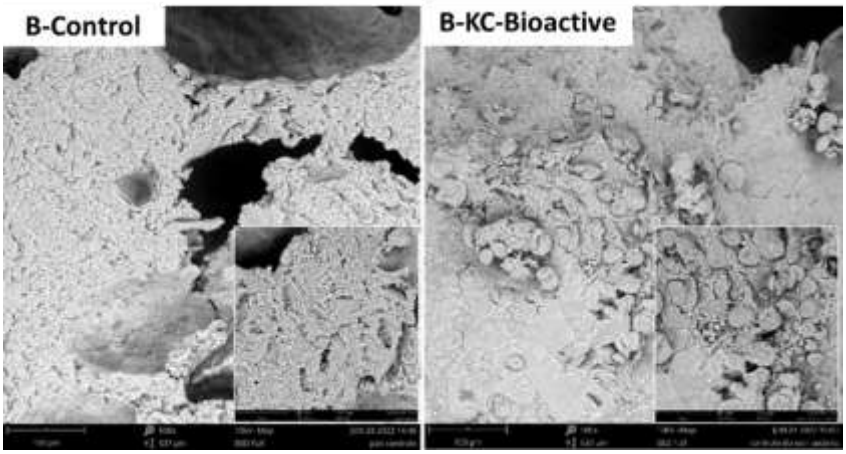



**Figure 29:** SEM images (500x magnifications) of the control bread and the bread containing KC-Bioactive particles. In the insets, their respective images at 1500x magnifications

The bioavailability of $VitD_2$ in the prepared fortified bread may be affected during bread making and storage, resulting in some loss, but according to the results, there was practically no loss, demonstrating the stability provided by microencapsulation (Maurya et al., 2020). The presented results allow knowing the fortification level to set the RDA and the physicochemical stability with the required characteristics for food fortification.

### 3.4.3. Characterisation of the used fortified flour and bread and sensorial analyses

**Table 40** shows several characteristics of the physicochemical analysis performed on the flour and bread fortified with $VitD_2$ (KC-Bioactive particles and free $VitD_2$-enriched extract), all compared to respective control samples. This table is divided into three parts, the first part with the analysis of the flour, the second part with the analysis of the crust of the bread and the third and last part with the analysis of the crumb of the bread.

Considering the first part of the **Table 40**, referring to the analyses performed on flour, the average pH of the fortified flour did not change much compared to the control (**Table 40**). Considering the interaction between the control and the fortified flour, the addition of $VitD_2$ only influenced the pH of the KC-Bioactive fortified flour (**Figure 30**). The addition of $VitD_2$ to the flour did not influence the water activity (**Table 40**).

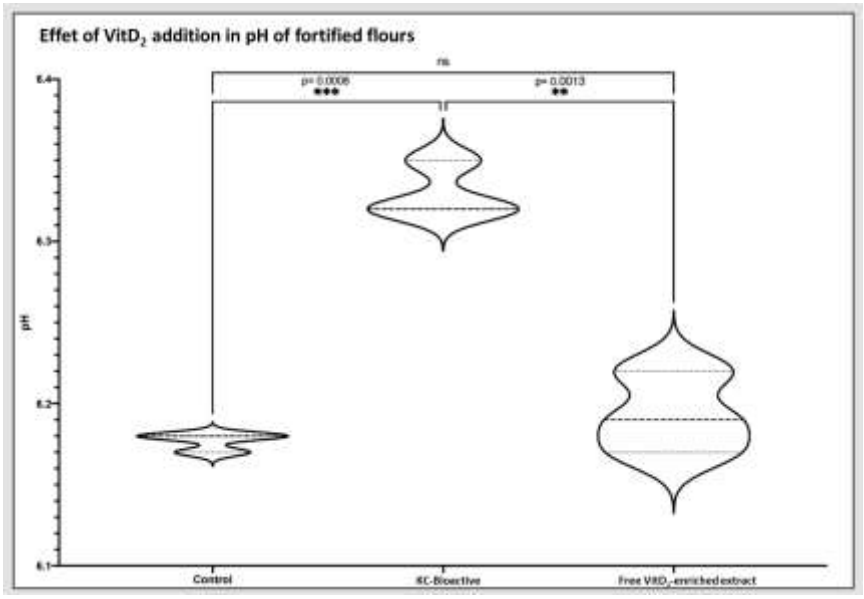



**Figure 30:** Effect on pH of flour fortified with $VitD_2$ (KC-Bioactive and free $VitD_2$-enriched extract)

By looking at **Table 40**, it seems that for the colour coordinates *L** (lightness) and *a** (redness), there were no statistical differences among the parameters compared (control, KC-Bioactive and free $VitD_2$-enriched extract), while *b** (yellowness) only showed a slight statistical difference between control and KC-Bioactive.

The second part of the table referring to the analysis of pH and water activity in the crust of the bread (**Table 40**), the pH of the crust of the bread fortified with KC-Bioactive showed significant differences when compared with the control, while for the crust of the bread fortified

with the extract enriched with $VitD_2$ there were no significant differences compared with the control (**Figure 31**). The water activity also followed the same trend as the pH, but with a slightly lower significance level (**Table 40**). This result is very satisfactory for the free $VitD_2$-enriched extract because when looking for new food ingredients, changes in food pH should be avoided because they are usually linked to changes in texture and organoleptic characteristics (Ueda et al., 2021).

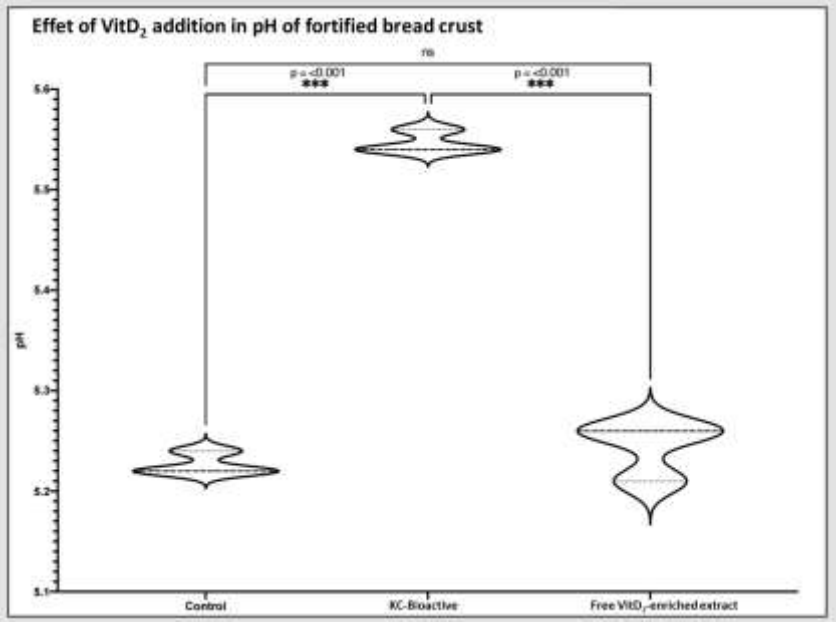



**Figure 31:** Effect on pH of bread crust fortified with $VitD_2$ (KC-Bioactive and free $VitD_2$-enriched extract)

The colourimetric analysis of the crust bread fortified with KC-Bioactive showed that this fortification did not significantly affect the colour of this bread compared with the control. Only the coordinate *b** (yellowness) showed a significant difference compared to the control. In contrast, the bread fortified with the free $VitD_2$-enriched extract significantly influenced the colouration of the bread produced with this extract compared to the control (**Table 40**). The results for the colour parameters for the crust of the bread showed that with the addition of the free $VitD_2$-enriched extract differed from the control, as expected from a food additive (Ueda et al., 2021). The KC-Bioactive bread did not differ much from the control, so the addition of $VitD_2$ in the encapsulated form (KC-Bioactive) to the bread made in this work justifies the no difference in the result of the colour parameters obtained, which proves that the encapsulation can protect the colour of the food ingredient from being transmitted to the incorporated matrix (Bajaj et al., 2021; Dias et al., 2017). For the texture of the crust of the bread it was only

measured once on one bread for each sample, so no statistical differences are mentioned here, but the values of this single reading are in **Table 40**.

The third and last part of **Table 40** refers to the crumb of bread fortified with $VitD_2$. The bread crumb pH (**Figure 32**) showed the same behaviour as the crusts (mentioned above) compared to the control. Further, the water activity was not influenced by the addition of $VitD_2$ (KC-Bioactive and free $VitD_2$-enriched extract) in comparison with the control. Adding to these changes the addition of $VitD_2$ also induced some changes in the interior colour of the fortified bread compared to the control, namely the crumb of the bread fortified with KC-Bioactive showed a slight change in *a** when compared to the control bread. *L** and *b** showed no significant differences. The crumb of bread fortified with free $VitD_2$-enriched extract also showed statistically relevant changes in terms of *a** and *b** when looked for interaction with the control bread. Thus, bread (crust and crumb) fortified with KC-Bioactive continues to show differences in only one colour coordinate, while bread fortified with extract enriched with $VitD_2$ shows alteration in more than one coordinate, always for a* (redness) and/or b* (yellowness).

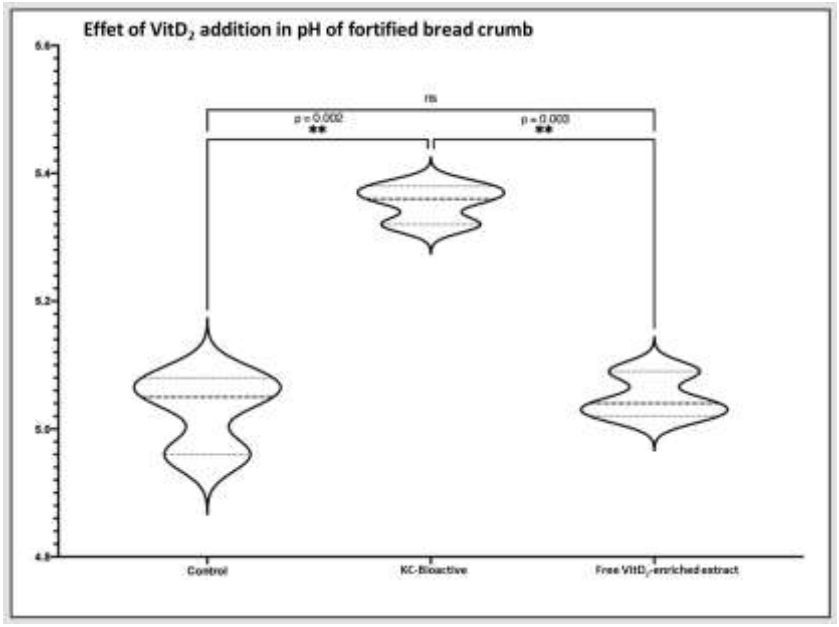



**Figure 32:** Effect on pH of bread crumb fortified with $VitD_2$ (KC-Bioactive and free $VitD_2$-enriched extract)

For texture analysis of the bread crumb the results are presented in **Table 40**, and most of the TPA parameters showed significant interaction, such as hardness, adhesiveness, gumminess and Chewiness (**Table 40**), showing that both KC-Bioactive and $VitD_2$-enriched extract had

influence on the result. As for the other parameters, resilience showed interaction only in the bread crumb fortified with KC-Bioactive when compared with the control. For cohesion and springiness, none of the bread fortified with $VitD_2$ showed significant interaction with the control.

**Table 40:** Physicochemical of flour and bread, control samples and fortified samples with KC-Bioactive particles and free VitD2-enriched extract (mean ± SD, n=3)

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Flour fortified with Vitamin $D_2$** | | | | | | | | | | |
| | | **Control** | | | **KC-Bioactive** | | | **Free $VitD_2$-enriched extract** | | |
| **pH** | | 6.177±0.005 | | | 6.33±0.01 | | | 6.19±0.02 | | |
| | Control × KC-Bioactive (*p*-value) | | | | 0.0008 | | | | | |
| | Control × free $VitD_2$-enriched extract (*p*-value) | | | | >0.05 | | | | | |
| | KC-Bioactive × free $VitD_2$-enriched extract (*p*-value) | | | | 0.0013 | | | | | |
| **Water activity**[1] | | 0,500± 0,007 | | | 0.495±0.002 | | | 0.5082±0.0007 | | |
| | | ***L**** | ***a**** | ***b**** | ***L**** | ***a**** | ***b**** | ***L**** | ***a**** | ***b**** |
| | | 91.9±0.3 | 0.82±0.07 | 10.5±0.3 | 91.9±0,8 | 0.81±0,02 | 9.8±0,2 | 90.2±0,8 | 0.87±0,07 | 9.9±0,1 |
| | | | ***L**** | | | ***a**** | | | ***b**** | |
| **Colour** | Control × KC-Bioactive (*p*-value) | | >0.9999 | | | 0.9564 | | | 0.0415 | |
| | Control × free $VitD_2$-enriched extract (*p*-value) | | 0.0733 | | | 0.4101 | | | 0.0879 | |
| | KC-Bioactive × free $VitD_2$-enriched extract (*p*-value) | | 0.0737 | | | 0.3059 | | | 0.7034 | |
| **Bread fortified with Vitamin $D_2$ (Crust)** | | | | | | | | | | |
| | | **Control** | | | **KC-Bioactive** | | | **Free $VitD_2$-enriched extract** | | |
| **pH** | | 5.227±0.009 | | | 5.547±0.009 | | | 5.24±0.02 | | |
| | Control × KC-Bioactive (*p*-value) | | | | <0.001 | | | | | |
| | Control × free $VitD_2$-enriched extract (*p*-value) | | | | >0.05 | | | | | |
| | KC-Bioactive × free $VitD_2$-enriched extract (*p*-value) | | | | <0.001 | | | | | |
| **Water activity** | | 0.89±0,02 | | | 0.8135±0.0006 | | | 0.9235±0.0003 | | |
| | Control × KC-Bioactive (*p*-value) | | | | 0.0055 | | | | | |
| | Control × free $VitD_2$-enriched extract (*p*-value) | | | | >0.05 | | | | | |
| | KC-Bioactive × free $VitD_2$-enriched extract (*p*-value) | | | | 0.0012 | | | | | |
| **Colour** | | ***L**** | ***a**** | ***b**** | ***L**** | ***a**** | ***b**** | ***L**** | ***a**** | ***b**** |
| | | 65±1 | 7.27±0.07 | 29.6±0.9 | 68±2 | 6.7±0,3 | 21.4±0,7 | 56±2 | 9.1±0,5 | 21±2 |
| | | | ***L**** | | | ***a**** | | | ***b**** | |
| | Control × KC-Bioactive (*p*-value) | | 0.3506 | | | 0.1997 | | | 0.002 | |
| | Control × free $VitD_2$-enriched extract (*p*-value) | | 0.0109 | | | 0.0049 | | | 0.0016 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| KC-Bioactive × free VitD$_2$-enriched extract (*p*-value) | | 0.0044 | | | 0.0018 | | 0.8659 |

| **Texture** [2] | **Hardness (g)** | **Adhesiveness (g s$^{-1}$)** | **Resilence (%)** | **Cohesiveness** | **Springiness (%)** | **Gumminess** | **Chewiness** |
|---|---|---|---|---|---|---|---|
| Control | 2486 | -0,5 | 33 | 0,7 | 97 | 1721 | 1662 |
| KC-Bioactive | 4413 | -1,0 | 30 | 0,7 | 93 | 2973 | 2751 |
| Free VitD$_2$-enriched extract | 4205 | -0,2 | 35 | 0,7 | 95 | 3147 | 2993 |

**Bread fortified with Vitamin D$_2$ (Crumb)**

| | **Control** | **KC-Bioactive** | **Free VitD$_2$-enriched extract** |
|---|---|---|---|
| **pH** | 5.03±0.05 | 5.35±0.02 | 5.05±0.03 |
| Control × KC-Bioactive (*p*-value) | | 0.002 | |
| Control × free VitD$_2$-enriched extract (*p*-value) | | >0.05 | |
| KC-Bioactive × free VitD$_2$-enriched extract (*p*-value) | | 0.003 | |
| **Water activity**[1] | 0,963±0,002 | 0.965±0.002 | 0.964±0.004 |

| **Colour** | ***L**** | ***a**** | ***b**** | ***L**** | ***a**** | ***b**** | ***L**** | ***a**** | ***b**** |
|---|---|---|---|---|---|---|---|---|---|
| | 61±1 | 0.43±0.03 | 13.1±0.1 | 62±1 | 0.63±0,07 | 14.1±0,5 | 64.6±0.9 | 0.65±0.02 | 11.6±0.5 |

| | ***L**** | ***a**** | ***b**** |
|---|---|---|---|
| Control × KC-Bioactive (*p*-value) | 0.5022 | 0.0026 | 0.0689 |
| Control × free VitD$_2$-enriched extract (*p*-value) | 0.0651 | 0.0017 | 0.0208 |
| KC-Bioactive × free VitD$_2$-enriched extract (*p*-value) | 0.2174 | 0.6451 | 0.0031 |

| **Texture** | **Hardness (g)** | **Adhesiveness (g s$^{-1}$)** | **Resilence (%)** | **Cohesiveness** | **Springiness (%)** | **Gumminess** | **Chewiness** |
|---|---|---|---|---|---|---|---|
| Control | 664±6 | 0.797±0.006 | 57.0±0.6 | 0.869±0.004 | 98.4±0.8 | 548±5 | 549±3 |
| KC-Bioactive | 407±6 | 0.53±0.01 | 52.99±0.98 | 0.880±0.007 | 99.5±0.6 | 658±8 | 351±4 |
| Free VitD2-enriched extract | 741±6 | 0.179±0.009 | 56±1 | 0.88±0.02 | 98±1 | 700±8 | 627±3 |
| Control × KC-Bioactive (*p*-value) | <0.0001 | <0.0001 | 0.0192 | 0.3455 | 0.2727 | 0.0002 | <0.0001 |
| Control × free VitD$_2$-enriched extract (*p*-value) | <0.0001 | <0.0001 | 0.3996 | 0.3839 | 0.4169 | <0.0001 | <0.0001 |
| KC-Bioactive × free VitD$_2$-enriched extract (*p*-value) | <0.0001 | <0.0001 | 0.0624 | 0.9942 | 0.0667 | 0.0059 | <0.0001 |

ns: not significantly; All assays were performed using a significance of 0.05; [1] did not present significance between the factors (Control × KC-Bioactive, Control × free VitD$_2$-enriched extract, and KC-Bioactive × free VitD$_2$-enriched extract) in *p*-value =0,05. [2] for the texture of the crust of the bread was measured only once on one bread for each sample.

**Table 41** shows the value of moisture, carbohydrates, proteins, ashes, sugars, fatty acids, and other components, comparing the control and fortified samples (flour and bread).

For all samples, the most abundant nutrients were carbohydrates, followed by crude protein. Overall, looking at the absolute values, there were no relevant differences in the nutritional assessment between the fortified samples and the respective controls. Some values even point to maintaining the nutritional characteristics after incorporating KC-Bioactive particles or free $VitD_2$-enriched extract. As regards sugars, there were also no pertinent differences to be reported. Previous studies have also reported no significant difference in the nutritional assessment between vitamin D fortified bread and non-fortified bread (Nikooyeh et al., 2016). Other authors have reported no significant effect of added encapsulated mushroom extract, but in cottage cheese samples, on nutritional properties (Ribeiro et al., 2015).

Overall, the addition of KC-Bioactive particles and free $VitD_2$-enriched extract did not affect the nutritional value of the bread when compared to the control samples.

**Table 42** shows the individual fatty acid profiles. Regarding the fatty acid of the flour, it shows higher amounts of PUFA, which ranged from 22.3% for the control to 23.5% for the fortified flour, followed by SFA and, lastly, MUFA. All the flour samples observed that linoleic acid (C18:2n6c) was present with higher content, followed by palmitic acid (C16:0). While in the bread (controls and fortified), oleic acid (C18:1n9c) had the highest content, followed by palmitic acid (C16:0). These two fatty acids were also reported in previous work being the majority found in fortified bread but with other ingredients (Melilli et al., 2020). Considering the bread-making ingredients, the most abundant fatty acids were SFA, followed by MUFA and PUFA. The other authors have also reported this sequence in normal "económicos" cakes (Fernandes et al., 2022). However, no comparable studies have published results for fatty acids in $VitD_2$-fortified bread.

The quality and safety of the flour and bread must be ensured when fortifying with other ingredients. The tests were carried out immediately after flour fortification and bread making (T0) and after 3 days (T3 - bread) and 30 days (T30 - flour) storage at room temperature (**Table 43**). Incorporating KC-Bioactive particles and free $VitD_2$-enriched extract were found to maintain microbial growth in the flour compared to the control, except for a slight yeast increase. While in the bread, incorporating KC-Bioactive particles and free $VitD_2$-enriched

extract does not influence microbial growth. Thus, the growth of the tested microorganisms (total aerobic mesophiles, coliforms, *Bacillus cereus*, yeasts and moulds) was not detected during the 30 days of analysis, which means that the baking process can eliminate possible microorganisms present in the initial ingredients (e.g. flour) as previously found in other products (Fernandes et al., 2022).

**Table 41:** Nutritional value, energy value and free sugars of flour and bread control samples and fortified samples with KC-Bioactive particles and free $VitD_2$-enriched extract (mean±SD, n=3)

| | Flour fortified with Vitamin $D_2$ | | | Bread fortified with Vitamin $D_2$ | | |
|---|---|---|---|---|---|---|
| | **Control** | **KC-Bioactive** | **Free $VitD_2$-enriched extract** | **Control** | **KC-Bioactive** | **Free $VitD_2$-enriched extract** |
| **Nutritional value (g/100 g dw)** | | | | | | |
| Moisture (%) | 12.9±0.3a | 12.4±0.3b | 12.7±0.3a | 43±1b | 42.9±0.1b | 44.5±0.6a |
| Fat | 1.3±0.1b | 2.1±0.2a | 1.31±0.08b | 1.14±0.02a | 0.60±0.04b | 1.1±0.1a |
| Proteins | 6.7±0.7c | 7.3±0.6b | 8.1±0.5a | 8.2±0.3b | 8±1b | 10.1±0.9a |
| Ash (mg/100 g dw) | 0.83±0.05b | 1.10±0.08a | 0.83±0.05b | 2.7±0.4a | 2.4±0.4b | 2.59±0.07a, b |
| Carbohydrates | 79.4±0.6a | 78.2±0.4b | 77.9±0.5b | 47.7±0.9b | 49±1a | 44.3±0.2c |
| **Energy (Kcal/100 g dw)** | 355±1b | 361±1a | 356±1b | 234±4a | 231.3±0.3b | 228±3c |
| **Free sugars (g/100 g dw)** | | | | | | |
| Fructose | nd | nd | nd | 0.24±0.04a | 0.24±0.03a | 0.20±0.02b |
| Glucose | nd | nd | nd | 0.41±0.05a | 0.310±0.003b | 0.42±0.03a |
| Maltose | 0.240±0.009a | 0.240±0.002a | 0.230±0.002b | 8.8±0.8a | 8.9±0.2a | 8.7±0.3a |
| Sum | - | - | - | 9.5±0.8a | 9.4±0.3a | 9.4±0.4a |

dw – dry weight; nd – not detected

**Table 42:** Fatty acids of the flour and bread control samples and fortified samples with KC-Bioactive particles and free $VitD_2$-enriched extract (mean±SD, n=3)

| | Flour fortified with Vitamin $D_2$ | | | Bread fortified with Vitamin $D_2$ | | |
|---|---|---|---|---|---|---|
| **Fatty acids (%)** | **Control** | **KC-Bioactive** | **Free $VitD_2$-enriched extract** | **Control** | **KC-Bioactive** | **Free $VitD_2$-enriched extract** |
| C8:0 | nd | nd | nd | 1.35±0.03 | 0.59±0.02 | 1.067±0.004 |
| C10:0 | nd | nd | nd | 0.177±0.007 | 0.179±0.006 | 0.207±0.009 |
| C11:0 | nd | nd | nd | 0.343±0.009 | 0.29±0.02 | 0.49±0.03 |
| C12:0 | nd | nd | nd | 0.35±0.01 | 0.30±0.02 | 0.321±0.006 |
| C14:0 | 0.121±0.007 | 0.1505±0.0007 | 0.148±0.005 | 0.57±0.02 | 0.900±0.006 | 0.90±0.08 |
| C15:0 | 0.127±0.001 | 0.1635±0.0007 | 0.160±0.007 | 1.58±0.07 | 1.13±0.07 | 1.20±0.06 |
| C16:0 | 17.9±0.4 | 19.1±0.2 | 19.2±0.4 | 31.2±0.1 | 36.0±0.6 | 33.1±0.7 |
| C16:1 | 0.153±0.004 | 0.158±0.005 | 0.17±0.01 | 0.27±0.02 | 0.333±0.002 | 0.393±0.009 |
| C17:0 | 0.16±0.01 | 0.188±0.004 | 0.17±0.01 | 0.355±0.004 | 0.836±0.006 | 0.91±0.02 |
| C18:0 | 1.54±0.08 | 1.68±0.09 | 1.60±0.06 | 10.4±0.3 | 10.06±0.01 | 10.7±0.3 |
| C18:1n9c | 16±1 | 14.1±0.8 | 13.8±0.3 | 41.17±0.09 | 36.5±0.7 | 37.5±0.6 |
| C18:2n6c | 56.1±0.4 | 56.7±0.2 | 57.7±0.5 | 5.97±0.05 | 4.98±0.04 | 4.89±0.01 |
| C18:3n3 | 4.2±0.2 | 4.1±0.1 | 4.1±0.2 | nd | nd | nd |
| C20:0 | 0.289±0.006 | 0.32±0.02 | 0.27±0.02 | 1.51±0.06 | 2.083±0.006 | 2.64±0.03 |
| C20:1 | 0.82±0.07 | 0.87±0.05 | 0.82±0.03 | 0.73±0.03 | 1.07±0.004 | 0.84±0.07 |
| C22:0 | 0.70±0.02 | 0.67±0.05 | 0.45±0.04 | 2.65±0.06 | 2.70±0.02 | 2.71±0.07 |
| C22:1 | 0.160±0.007 | 0.275±0.001 | 0.164±0.002 | nd | nd | nd |
| C22:2 | 0.13±0.01 | 0.162±0.008 | 0.124±0.009 | nd | nd | nd |
| C23:0 | 0.3035±0.0007 | 0.24±0.02 | 0.180±0.001 | nd | nd | nd |
| C24:0 | 1.16±0.03 | 0.96±0.06 | 0.84±0.02 | 1.41±0.08 | 2.06±0.04 | 2.17±0.02 |
| C24:1 | 0.213±0.03 | 0.15±0.01 | 0.15±0.01 | nd | nd | nd |
| **SFA** | 22.3±0.4c | 23.5±0.4a | 23.0±0.4b | 51.88±0.08c | 57.1±0.7a | 56.4±0.7b |
| **MUFA** | 17.3±0.9a | 15.6±0.7b | 15.1±0.3c | 42.16±0.04a | 37.9±0.7c | 38.7±0.7b |
| **PUFA** | 60.4±0.6c | 60.9±0.3b | 61.9±0.7a | 5.97±0.05a | 4.98±0.04b | 4.89±0.01c |

nd – not detected; SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids

**Table 43:** Microbial load (CFU/g) of the flour and bread samples and fortified samples with KC-Bioactive particles and free $VitD_2$-enriched extract (mean ±SD, n=3)

| | **Flour fortified with Vitamin $D_2$** | | | **Bread fortified with Vitamin $D_2$** | | |
|---|---|---|---|---|---|---|
| | **Control** | **KC-Bioactive** | **Free $VitD_2$-enriched extract** | **Control** | **KC-Bioactive** | **Free $VitD_2$-enriched extract** |
| **(CFU/g)** | ***0 days*** | | | ***0 days*** | | |
| Total aerobic mesophiles | 4.2±0.1c | 4.30±0.09a, b | 4.363±0.097a | 2.5±0.2a | 1.2±0.3c | 1.7±0.3b |
| *Bacillus cereus* | nd | nd | nd | nd | nd | nd |
| coliforms | 3.21±0.05b | 3.9±0.3a | 3.31±0.08b | nd | nd | nd |
| Yeasts | 2.4±0.1a | 2.3±0.2a | 1.6±0.3b | 3.0±0.2 | nd | nd |
| Moulds | 3.11±0.04a | 3.05±0.02b | 3.06±0.05b | nd | nd | nd |
| **(CFU/g)** | ***30 days*** | | | ***3 days*** | | |
| Total aerobic mesophiles | 3.8±0.4a | 3.5±0.3b | 3.7±0.4a | nd | nd | nd |
| *Bacillus cereus* | nd | nd | nd | nd | nd | nd |
| coliforms | 3.0±0.3a | 2.73±0.09c | 2.80±0.04b | nd | nd | nd |
| Yeasts | 2.14±0.06c | 2.3±0.2b | 2.49±0.07a | nd | nd | nd |
| Moulds | 3.04±0.06a | 2.89±0.03b | 2.89±0.03b | 2.1 ±0.2a | 2.0±0.2a, b | 1.9±0.2b |

nd – not detected; CFU - Colony Forming Unit.

According to the results of the sensory analysis, for panel characterization, the first approach taken was to describe the population of the testing panel for both samples (flour and bread), in the bread tasting panel shown in **Figure 33** A), a clusterisation of age groups was made according to specifications, separating in 7 groups ranging from under 18 years old to over 65 years old, with an unbalanced panel composed majoritarian of the female number of tester with a higher spike in the range age of 25-35 years old either for the female and male sex, the last one showing a second spike on the 55-64 years old. On the other hand, at the flour distribution (**Figure 33** B), the panel compositions were more homogeneous, with a female composition of 52% with the most considerable density around the groups ranges 19-34, while for males, the observation was almost similar for 2 group ranges, one between 19-34 and the other composed of panellist with higher than 65 years old.

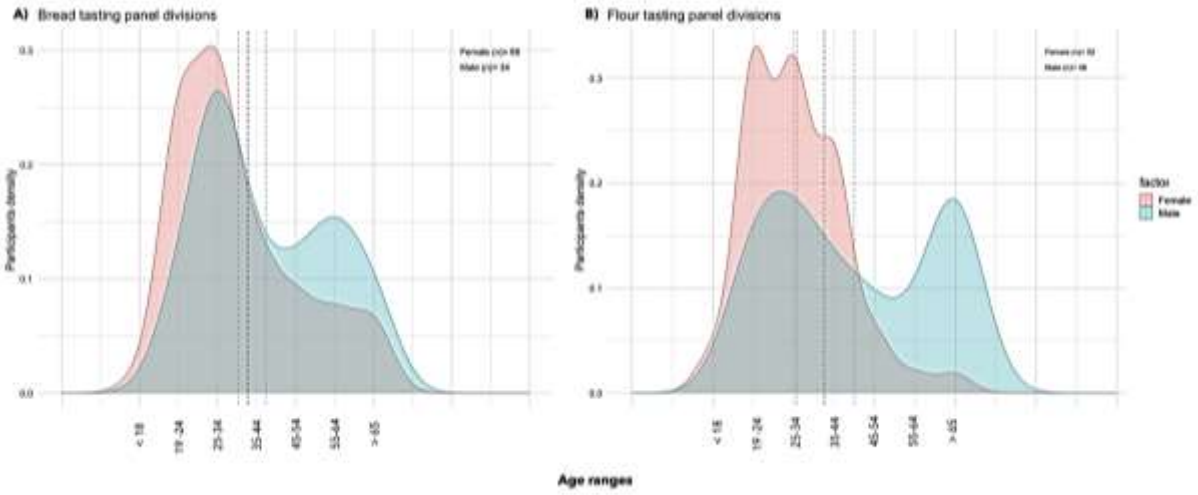



**Figure 33:** A) Bread tasting panel representation of group ages *vs*. participation density and B) flour tasting panel

As for the hedonic attributes correlation, the selected attributes to evaluate for the panellist include all the presented in **Figure 34**, dividing the figure into two complete attribute correlations, adding the columns as well Total 1 and Total 2, which amounts for the average response of every panellist considering all the evaluated attributes in either bread fortified with KC-Bioactive (B1) or fortified with free $VitD_2$-enriched extract (B2), or flour fortified with KC-Bioactive or with free $VitD_2$-enriched extract (F1 or F2 respectively). In both parts of the figure, we can observe how the panellist evaluated all the attributes in a positive correlation manner, meaning that no contrary effect was detected on the attributes evaluated.

It is interesting to observe how higher correlations were attributed in a unique product of each bread or flour, suggesting that all the parameters are evaluated with the same preferences. We cannot observe a single attribute acting as an antagonistic attribute to the total score. Although differences between similar products (bread or flour) follow the same trend, they do not correlate with the same strength, suggesting some differences between them. Therefore, the following analysis will highlight the expected differentiation in attributes. Finally, the correlation calculated values between Total B1 and B2 of 0.6 and total F1 and F2 of 0.8 suggest that higher differentiation will be visible more broadly on bread than the differences observed on the evaluated flour.

Overall, the organoleptic profile obtained from the selected panels is displayed in **Figure 35** for a comparison of the two different breads added with $VitD_2$ (B1: fortified with KC-Bioactive and B2: fortified with free $VitD_2$-enriched extract). In the figure, each attribute is represented with every single rating from the panel where a score value of 1 means extremely liked, 5 same as the control, and 9 extremely disliked. Additionally, the whole range, $2^{nd}$ and $3^{rd}$ quartile, and median was plot on top in a boxplot representation. Interestingly, the median values of all the B1 matches around the same score of 2 (Liked a lot), whereas B2 matches also between them but with a median score of 3 (liked moderately). For statistic evaluation the *t*-Student result of Total B1 *vs.* Total B2 was statistically significant with a *p*-values of 0.0007. This difference, although difficult to observe at first glance, is explained due to the wide range of values given to B2.

In **Figure 36** as well as **Figure 35**, the same methodology, visual characterisation and statistical evaluation were performed but this time for differentiation of flour. The total evaluation of the attributes did not show statistical differences, which is contrary to the case of bread. In the flour only 3 attributes of the F1 outperformed the F2 (colour, appearance, and acceptance), the other two were evaluated as equal (smell and texture), and curiously only F2 outperformed F1 on the aroma attribute. It is also important to highlight how the responses' ranges this time are spread almost evenly between attributes and samples of F1 and F2.

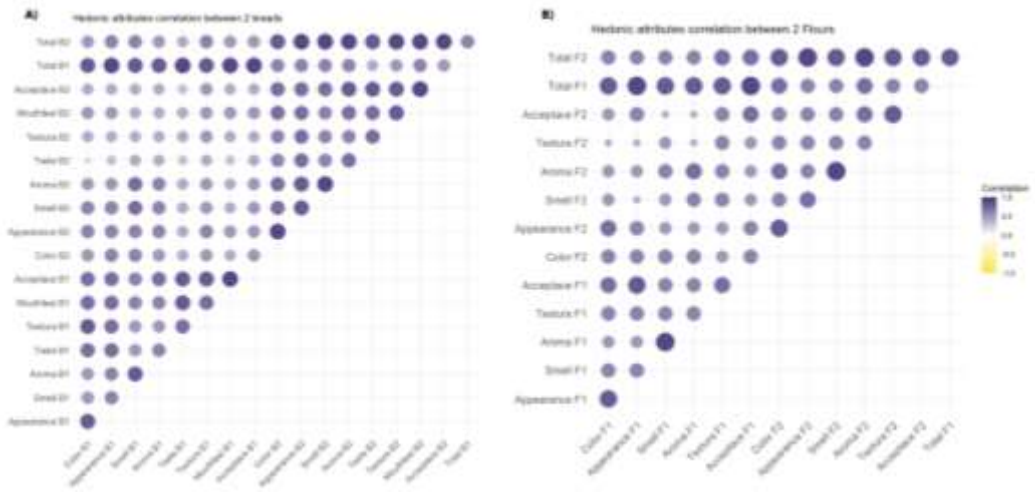


**Figure 34:** Correlation of evaluated organoleptic attributes in A) bread and B) flour with added vitamin $D_2$ either in the encapsulated form (B1 and F1) or the free form (B2 and F2)

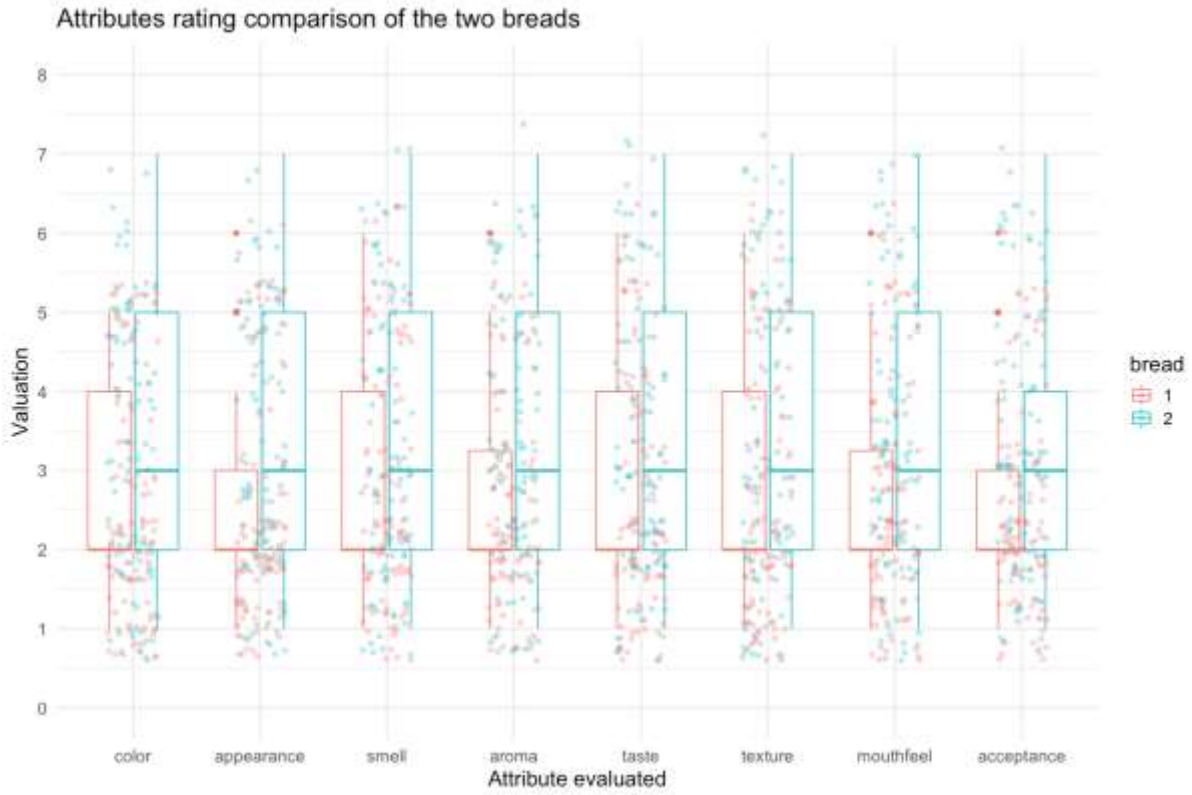



**Figure 35:** Boxplot with jitter points representation of bread attributes evaluation from the selected panel, were valuation axis goes from 1: "I like it extremely", 2: "I like it a lot", 3: "I like it moderately", 4: "I like it slightly", 5: "Like the control", 6: "Dislikes slightly", 7: "Dislikes moderately", 8: "Dislikes it a lot", 9: "Dislikes it extremely"

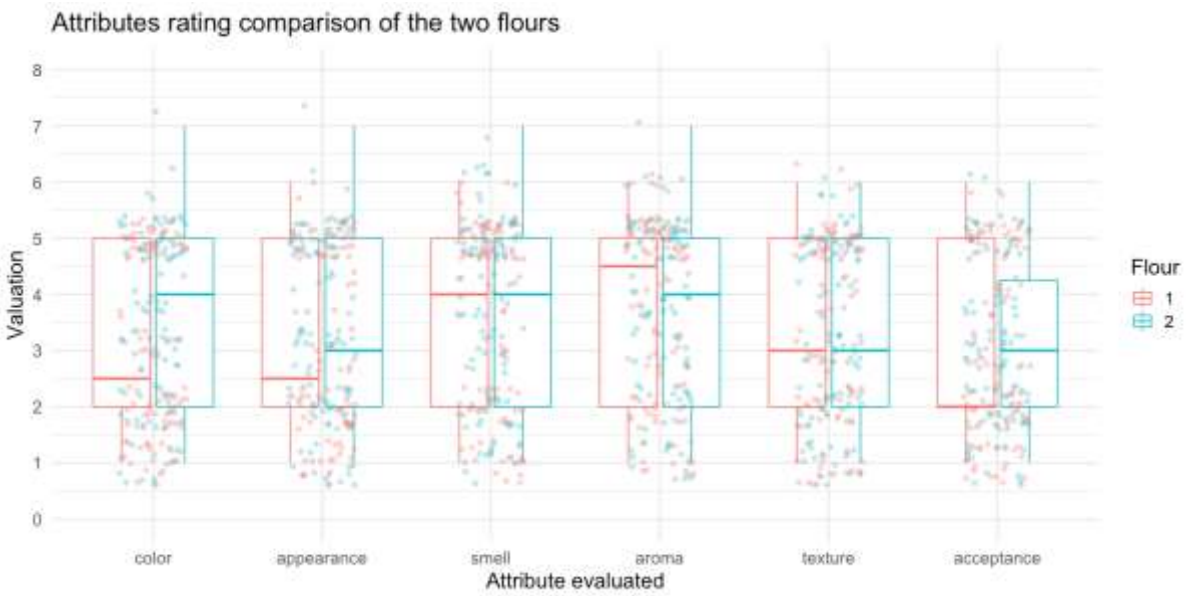



**Figure 36:** Boxplot with jitter points representation of flour attributes evaluation from the selected panel, were valuation axis goes from 1: "I like it extremely", 2: "I like it a lot", 3: "I like it moderately", 4: "I like it slightly", 5: "Like the control", 6: "Dislikes slightly", 7: "Dislikes moderately", 8: "Dislikes it a lot", 9: "Dislikes it extremely"

Finally, for attributes directions, the PCA plots of **Figure 37**, confirmed that the direction in which the attributes move is unidirectional, confirming the results presented on the correlation analysis, also shown in the part A) how these attributes are clearly separated between samples of bread, providing the statistical differences mention in the previous section. While part B) show a clear overlapping of samples of F2 with samples of F1 with a very narrow movement along the Y axis for the aroma F1 and colour F2. As described in the previews section, that approach between attributes between F1 and F2 was the determinant for not displaying significant differences between the flour samples tested.

Therefore, with all the performed analysis we can conclude that the encapsulation of vitamin $D_2$ would only be noticeable on bread incorporations but not in flour incorporations.

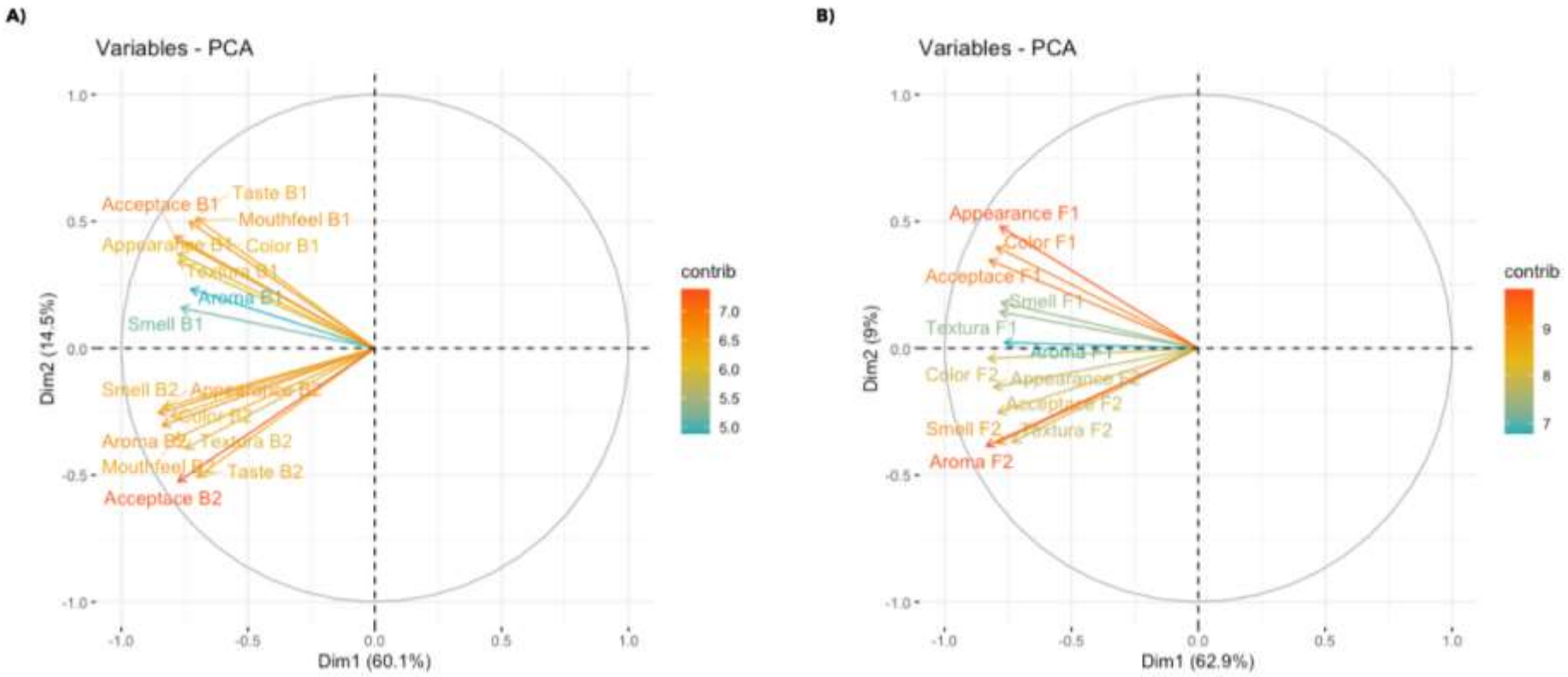



**Figure 37:** Principal Components Analysis representing same direction of evaluation and spread of the attributes as well as their contribute magnitude in A) bread and B) flour

# CHAPTER 4

# Conclusions and Future Perspectives

Mushrooms are a source of bioactive molecules that present an attractive potential to be used as food ingredients. The present study aimed to optimise sustainable and efficient technological processes appropriate for the extraction, irradiation, and stabilisation of vitamin $D_2$ to be used as potential ingredients for food fortification. Optimisation studies successfully obtained Vit$D_2$-enriched extracts from *A. bisporus* Portobello within more sustainable approaches. The development of a natural ingredient based on vitamin $D_2$ (from mushrooms) that can be used in food fortification is one of the focuses of this study, so the search for better parameters to obtain this ingredient is a continuous investigation. Mushrooms are sources of bioactive molecules such as ergosterol, which in this study was constantly looked for ways to increase its amount and conversion into vitamin $D_2$ since it is the precursor of this vitamin.

Therefore, studies were carried out to validate the effect of calcium silicate inside the mushroom cells. However, little is known about these effects on the mushrooms' chemical composition and biological efficiency. In this sense, calcium silicate supplementation seems to positively affect vitamin $D_2$ production. From a sustainability point of view, calcium silicate is moderately used in agriculture to help increase resistance to certain pests without the drawbacks of pesticides. In addition, it is a silicon source for mushrooms, and its use as a stimulant for bioactive compounds (ergosterol) in mushrooms is a valuable tool for their production. Thus, using calcium silicate can remain an essential supplement in mushroom production, although more studies on other mushrooms are needed to broaden knowledge.

On the other hand, the effect of gamma irradiation and electron beam irradiation on the different aspects of *Agaricus bisporus* Portobello was evaluated. Overall, in the fresh mushrooms, it was possible to conclude that gamma irradiation allowed higher ergosterol contents. Concerning the effect of storage time, it was possible to verify that the irradiations effectively maintained the chemical profiles of the Portobello samples. In the dried mushrooms, it was possible to validate the use of both technologies with minimal effects on their main characteristics, allowing their preservation for longer periods (up to 12 months), making them available to the food industry, allowing their incorporation in other food formulations, without compromising their main nutritional and bioactive characteristics. Ionising radiation did not induce severe changes in the tested parameters, and the slight changes benefit the preservation of some specific nutrients. Consequently, these technologies may represent practical preservation approaches for Portobello mushrooms and their bioactive compounds. Thus, in order to convert the ergosterol present in the mushrooms into vitamin $D_2$, a study was developed, where from the tested mushrooms bioresidues, *A. bisporus* Portobello was the species with the highest total

content of this vitamin under the specific UV irradiation conditions applied. Considering the attempts to establish better conditions to obtain the final extract ($VitD_2$-enriched extract), a methodology using "green solvents" for extracting an ergosterol-rich extract (Heleno et al., 2016) already optimised by our research group was adopted, and the irradiated with the conditions defined according to the preliminary results was used. The $VitD_2$-enriched extract effect on calcium absorption by bone cells was tested, and the results were promising, but it is necessary to explore longer incubation times or other $VitD_2$ concentrations to confirm the favourable behaviour of cells in the presence of $VitD_2$.

In order to stabilise the $VitD_2$-enriched extract, particles were prepared by solid dispersion technique to encapsulate this extract in KC polymer. The applicability of the particles was validated by flour fortification and the production of fortified bread. Supplementary TG results showed the non-degradation of $VitD_2$ during bread baking, and SEM images of the bread denoted the proper distribution of the particles in the bread dough. The flours are an excellent food matrix to be fortified with naturally vitamin D. With the results obtained in the characterization of wheat and rye flour, it was concluded that the physicochemical analyses of the flour were within limits established by the legislation, in this sense improving the consumer's confidence in the products they buy and consume daily. Wheat types are generally suitable for producing baked and pastry products, as they have a higher amount of protein that provides a higher concentration of gluten. On the other hand, rye flour contains traces of gluten, making it suitable for anyone trying to reduce the inflammatory reaction caused by gluten in the diet and contains complex carbohydrates, which are slower to digest and maintain satiety for longer. However, it should be noted that this study is a preliminary survey focusing on a set of samples. Sensory analysis using flour and bread made with KC-Bioactive particles and extracts enriched with $VitD_2$. The study revealed that bread samples made with KC-Bioactive were generally preferred, while flour fortified with KC-Bioactive was better in attributes such as colour, appearance and acceptance, but taking into account all attributes can be stated that there was no preferred flour.

In summary, using $VitD_2$-enriched extracts from mushroom bioresidues production could benefit several bio-based industries since applications of $VitD_2$ from this sustainable material are lacking. Consequently, the development of food applications of $VitD_2$-enriched extract from mushroom bioresidues production can be considered and valued, promoting the agricultural sector and the food industries. Overall, it can be concluded that the studied

mushroom bioresidues represent an essential source of vitamin $D_2$, which can be used as an ingredient for food fortification.

Focusing on the main interest of this work, the studies performed here used the extraction technique already optimised by our group to maximise the recovery of ergosterol in mushroom bioresidues; irradiation technology was used to maximise the conversion of ergosterol into $VitD_2$; the microencapsulation technique, with a natural polymer, of the extracts enriched in $VitD_2$ was successfully optimised to stabilise this vitamin and provide protection against degradation factors. However, the use of $VitD_2$-enriched extract, encapsulated or not, may depend on the industry that will use it, and the concentration to be incorporated changes; the $VitD_2$-enriched extract and KC-bioactive particles were validated in final applications in food matrices (flour and bread). Furthermore, the nutritional, physicochemical and sensorial composition of the developed final products was also determined; and studies of increased calcium absorption in the presence of $VitD_2$ in bone cells were carried out.

Beyond everything that has already been done for this thesis, something more can still be done: From a sustainable point of view, emphasis should be given to the bioresidues generated during the mushroom production processes in order to suppress the negative impact of these residues on the environment, while effectively contributing as sources of bioactive ingredients for the food industry; UV irradiation has proven to be effective in converting ergosterol into $VitD_2$ and therefore conduct a thorough investigation of irradiation doses and exposure times in order to better maximise this conversion would be important.

$VitD_2$ from natural sources needs to be stable for incorporation into food matrices, so spray-drying microencapsulation has been shown to be effective, but other methods of stabilising this vitamin could be explored; studies related to the in vivo effects of $VitD_2$-enriched extracts and their corresponding food formulations should be conducted. Volunteers could be asked about their perception of fortified products based on improvement of high blood pressure, the immune system, etc. It would be interesting to continue studies examining all the flour mentioned here, fortified with vitamin $D_2$, for example, the quality and safety parameters over storage time.

# CHAPTER 5

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# A

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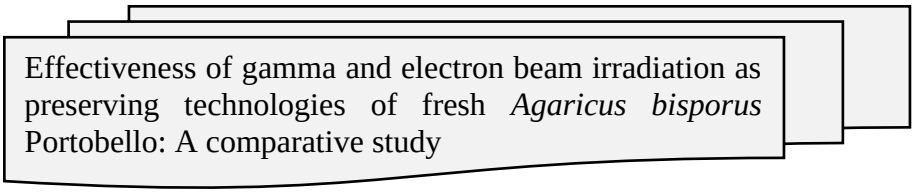
# ANNEXES

## Supplemental materials

**Annex A**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# Effectiveness of gamma and electron beam irradiation as preserving technologies of fresh *Agaricus bisporus* Portobello: A comparative study

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ABSTRACT

Mushroom production and consumption is increasing, but high perishability still represents a major commercial drawback. Besides increasing the product shelf-life, conservation processes should be innocuous to consumers. Therefore, the effects of gamma and electron beam radiation on chemical and nutritional composition of fresh samples of *Agaricus bisporus* Portobello (a highly commercialized species) were assessed. Mushrooms were irradiated at 1, 2 or 5 kGy, and analyzed at different times (0, 4 and 8 days). In general, irradiation type had higher effect than irradiation dose. Gamma irradiation was associated with higher contents in sugars and ergosterol, while the 5 kGy dose, independently of irradiation source, was linked with higher protein levels. Nonetheless, irradiation might represent an effective preservation methodology for Portobello mushrooms, as it was effective in maintaining their chemical profiles (except for minor organic acids and saturated fatty acids) throughout the assayed time intervals.

## 1. Introduction

Mushrooms have been included in the human diet for centuries, mainly because of organoleptic and nutritional characteristics, such as low lipid levels and large amounts of carbohydrates, proteins, amino acids, vitamins, phosphorus and other minerals (Reis, Martins, Vasconcelos, Morales, & Ferreira, 2017). Besides their gastronomic relevance, mushrooms have also been reported for their pharmaceutical application, which is mainly supported by their antioxidant, antimicrobial, immunomodulating and antitumor effects (Reis et al., 2017). In recent years, the knowledge about the chemical composition and nutritional value of edible mushrooms has been increasing. Nevertheless, their high perishability, which leads to immediate quality loss after harvesting, represents a drawback for their distribution and marketing as fresh products. Therefore, it is necessary to extend the shelf life of fresh mushrooms, as this is a key factor in making any food product profitable for longer periods of time, bringing benefits to the producer and distributors (Fernandes et al., 2014a).

Besides the need of extended storage periods, there is also a general trend to develop less severe, and therefore less harmful, food preservation techniques (Fernandes et al., 2015). In this sense, there has been extensive research on finding the most suitable technology for mushroom preservation. Among the available methods to preserve food, such as sun drying, hot air and oven drying (Ma, Chen, Zhu, & Wang, 2013), irradiation has also been applied as a decontamination technique, increasing shelf life and improving food safety. This technique is mainly intended to destroy microorganisms or insects, eliminate toxins and improve functional properties, with the least impairment in sensory and nutritional quality (Akram & Kwon, 2010). Gamma rays are a type of electromagnetic radiation produced in nuclear decay processes. They are highly energetic, due to their high frequency and consequently low wavelength, having high penetrating capacity (Lima, 2014). Electron beam radiation, in turn, is a type of ionizing energy that is usually characterized by its low penetration and high dose rates, being generally used for thin and low-density products (Fernandes et al., 2014a). However, both technologies are suitable for post-harvest treatment, ensuring hygienic and sensory quality of mushrooms (Fernandes et al., 2014a, 2014b).

The three main mushroom species in which the effects of ionizing radiations have been studied are *Agaricus bisporus* (Wani, Hussain, Meena, Dar, & Mir, 2009), *Lentinus edodes* (Jiang, Luo, Chen, Shen, & Ying, 2010), and *Pleurotus ostreatus* (Jasinghe & Perera, 2006), which

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might be easily understood if we consider their high production worldwide. *Agaricus bisporus* reach an annual production around 4.4 billion kg, considering white and brown (Portobello) types (Royse, Baars, & Tan, 2016). *A. bisporus* Portobello stands out for its nutritional characteristics, exceptional texture, stiff pulp, easy digestibility, characteristic taste and pleasant aroma, and culinary versatility (Raimundo & Beraldo, 2015). This species is also a rich source of minerals, vitamins (A, C and D), ergosterol, beta-carotene, phenolic compounds, and terpenes, among other molecules with antioxidant effects and potentially positive implications in several diseases, such as cancer, rheumatoid arthritis, atherosclerosis, as well as the degenerative processes associated with aging (Teichmann, Dutta, Staffas, & Jägerstad, 2007).

Accordingly, this work was designed to evaluate the effects of gamma and electron beam radiation on the chemical and nutritional composition of fresh Portobello commercial samples. Different radiation doses and storage times were tested, in order to verify the most suitable conditions to be applied to this particular type of food product.

## 2. Materials and methods

### 2.1. Sampling and irradiation processes

*Agaricus bisporus* Portobello samples (acquired in a local market in Bragança, northeast Portugal, in June 2017) were divided into four groups: control (non-irradiated, 0 kGy), sample 1 (1 kGy), sample 2 (2 kGy) and sample 3 (5 kGy) with eighteen specimens (approximately 200 g) per group (72 mushrooms in total). The dose has been limited to 5 kGy because higher doses could compromise mushroom integrity (particularly its texture). Each group was further divided into equal parts (9 specimens each), corresponding to each irradiation methodology.

Gamma irradiation was performed in Centro de Ciências e Tecnologias Nucleares (Instituto Superior Técnico, Universidade de Lisboa, Portugal) in a Co-60 experimental four sources chamber (Precisa 22; Graviner Manufacturing Company Ltd, Gosport, UK), reaching a total activity of 105 TBq (2.84 kCi). The absorbed doses were measured by standard dosimeters (Batch X; Amber Perspex Harwell, Didcot, UK). Doses after irradiation, dose rates and dose uniformity ratios ($D_{max}/D_{min}$) were: 1.1 ± 0.1 kGy, 2.4 ± 0.2 kGy and 5.4 ± 0.2 kGy; 1.4 kGy/h and 1.3, respectively.

Electron bean irradiation was carried out in the same facility indicated above, using LINAC equipment (GE Saturne 41) with an electron beam of 10 MeV (pulse duration: 4 μs; pulse frequency: 20–60 Hz), in a steel metal tray with four layers of acrylic (1 cm) 60 cm away from the beam exit, at an average dose rate of 0.5 kGy/min. The absorbed doses were 0.9 ± 0.1 kGy, 2.0 ± 0.1 kGy and 4.6 ± 0.2 kGy, with an uncertainty of 7% for the first dose and 10% for the other two doses (Amber FWT-60 dosimeters; Far West Technology, Inc., Goleta, CA). For simplicity, the irradiation doses were indicated in the text, tables and graphs as 1, 2 and 5 kGy.

Groups corresponding to each irradiation dose were divided in three subgroups (three mushrooms: subgroup 1 was promptly analyzed (0 days), subgroup 2 was stored 4 days at 5 °C and subgroup 3 was stored 8 days under the same conditions. Considering the typical shelf-life time of mushrooms, there was no need to assay at longer time intervals. Before being analyzed, all samples were lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO), powdered (20 mesh) and mixed to obtain homogeneous samples.

### 2.2. Nutritional value

Carbohydrates, fat, protein, ash and moisture were determined according to AOAC procedures (AOAC, 2016); a conversion factor of 4.38 was used in the macro-Kjeldahl determination of proteins; the Soxhlet extraction of fat was performed using petroleum ether; a temperature of 600 ± 15 °C was set in the muffle for ash determination; total carbohydrates were calculated as $100 - (g_{moisture} + g_{protein} + g_{fat} + g_{ash})$. The energy value was calculated as: energy (kcal) = $4 \times (g_{protein} + g_{carbohydrates}) + 9 \times (g_{fat})$.

### 2.3. Chemical composition

*Free sugars:* Free sugars were analyzed by HPLC (Knauer, Smartline System 1000) using a refraction index detector (RI, Knauer, Berlin, Germany) and an Eurospher 100-5 $NH_2$ column (5 μm, 250 × 4.6 mm; Knauer, Berlin, Germany), at 35 °C. The mobile phase consisted of a mixture of acetonitrile/water (70:30, *v/v*), maintaining a 1 mL/min flow. Sugars were identified by comparing retention times of standard compounds and quantified using the RI signal based on the internal standard (IS, raffinose). Data were analyzed using Clarity 2.4 software (DataApex, Podohradska, Czech Republic); results were given as g/100 g dry weight (dw).

*Organic acids:* Organic acids were analyzed in an ultrafast liquid chromatograph (UFLC; Shimadzu 20A series, Shimadzu Cooperation, Kyoto, Japan), using a photodiode array detector (PDA) with 215 nm as the preferred wavelength (Barros, Pereira, & Ferreira, 2013). Compounds were separated in a SphereClone (Phenomenex, Torrance, CA) reverse-phase $C_{18}$ column (5 μm, 250 × 4.6 mm i.d.), at 35 °C. A sulfuric acid solution (3.6 mM) running at 0.8 mL/min was used as mobile phase. Organic acids were quantified, comparing their peaks areas with commercial standards. Results were processed using LabSolutions Multi LC-PDA software (Shimadzu Corporation, Kyoto, Japan); results were expressed in g/100 g dw.

*Fatty acids:* Fatty acids were characterized (after Soxhlet extraction and derivatization) by gas chromatography with flame ionization detection (GC-FID) at 260 °C, using a DANI model GC 1000 instrument equipped with a split/splitless injector and a Zebron Kame column (30 m × 0.25 mm ID × 0.20 μm $d_f$, Phenomenex, Torrance, CA). The oven was set at an initial temperature of 100 °C, held for 2 min, followed by a 10 °C/min ramp to 140 °C, 3 °C/min ramp to 190 °C, 30 °C/min ramp to 260 °C and held for 2 min. The carrier gas (hydrogen) was maintained at a 1.1 mL/min flow, and samples were injected in split injection (1:50) mode at 250 °C. Fatty acids were identified by comparing the relative retention times of fatty acid methyl esters (FAME) peaks from samples with commercial standards. Results were processed using Clarity DataApex 4.0 Software (DataApex, Prague, Czech Republic) and expressed as a relative percentage of each fatty acid.

*Tocopherols:* Tocopherols were extracted after adding tocol (Matreya, Pleasant Gap, PA) to mushroom samples (Reis, Barros, Martins, & Ferreira, 2012). The analysis was carried out by HPLC (Knauer, Smartline System 1000, Berlin, Germany) using a fluorescence detector (FP-2020; Jasco, Easton, MD), programmed for excitation at 290 nm and emission at 330 nm. A Polyamide II normal-phase column (250 × 4.6 mm; YMC Waters) operating at 35 °C was used to resolve the compounds. The mobile phase consisted of an *n*-hexane/ethyl acetate mixture (70:30, *v/v*) at 1 mL/min. Tocopherols were identified by comparing chromatographic data with commercial standards and quantified based on the IS (tocol). Data were analyzed by Clarity 2.4 software (DataApex, Prague, Czech Republic) and results were expressed in μg/100 g dw.

*Ergosterol:* Samples were vortex-extracted (1 min; LBX V05 series, Barcelona, Spain) with *n*-hexane (using a 1:30 solid:liquid ratio) and further centrifuged (4000 rpm, 10 min; K24OR refrigerated centrifuge; Centurion Scientific Limited, Chichester, UK) twice (the supernatant was removed between each step). The combined supernatants were dried under a nitrogen stream and dissolved in MeOH (1 mL) (Guan et al., 2016). The identification and quantification of ergosterol was performed according to the procedure descried by Barreira, Oliveira, and Ferreira (2014), using an HPLC (Knauer, Smartline System 1000, Berlin, Germany), coupled to a UV (280 nm as preferred wavelength) detector (Knauer Smartline 2500). The chromatographic separation was performed through an Inertsil 100A ODS-3 reverse-phase column

(5 μm, 250 × 4.6 mm; BGB Analytik AG, Boeckten, Switzerland), at 35 °C. The mobile phase was a mixture of methanol:acetonitrile (70:30, *v/v*), fed at 1 mL/min. Data were analyzed using Clarity 2.4 Software (DataApex, Podohradska, Czech Republic) and ergosterol (Sigma-Aldrich, St. Louis, MO) was quantified using a calibration curve obtained with a commercial standard. The results were expressed in mg/100 g of mushroom (dw).

### 2.4. Statistical analysis

All statistical tests were applied considering a 5% significance level (SPSS, v. 22.0; IBM Corp., Armonk, NY). Data were expressed as mean ± standard deviation, presenting the significant numbers in agreement with the magnitude of the corresponding standard deviation.

The results were compared through analysis of variance (ANOVA) with type III sums of squares, performed using the general linear model (GLM) procedure. The parameters measured in *A. bisporus* Portobello samples (dependent variables) were analyzed using 2-way ANOVA, with "irradiation dose" (ID) and "storage time" (ST) as the statistical factors. Each table was divided in two sections: the top part corresponds to the gamma irradiation study, while the bottom section corresponds to the electron beam irradiation study. The statistical interaction among the two factors was also verified in both cases.

In addition, linear discriminant analysis (LDA) was used to compare gamma and electron beam irradiation as a whole, as well as to check the main parametric changes occurring throughout time. The stepwise technique was selected and the Wilks' λ test was applied, considering an *F*-value of 3.84 for entering a variable, and 2.71 for variable removal. A leaving-one-out cross validation procedure was carried out to verify the model performance.

## 3. Results and discussion

Results obtained in all analytical assays were divided in samples treated by gamma irradiation (first half of each table) and samples treated by electron beam irradiation (second half of each table), to better understand their effects throughout time. In each case, the variability of results resulted from combining two distinct factors: irradiation dose (ID), with the levels 0, 1, 2 and 5 kGy and storage time (ST), with the levels 0, 4 and 8 days. In such cases, the interaction among factors (ID × ST) was also analyzed, in order to verify if changes potentially induced by one factor are dissimilar within each level of the other.

In the present study, and independently of the analyzed parameter, the interaction was significant in all cases, not allowing to present the statistical classification that resulted from the performed multiple comparison test (Tukey's HSD test). Accordingly, the identifiable tendencies observed for each parameter were obtained from the estimated marginal mean (EMM) plots that were generated in the GLM analysis.

In what concerns nutritional composition (Table 1), gamma irradiation (GI) caused a higher number of significant changes than ST, which had a significant effect only on protein and carbohydrate content. In each case, no unequivocal trends could be obtained from the EMM plots. In fact, the values quantified in each case are highly similar, with moisture as the major component (89%). This high water percentage could increase the release of primary free radicals (hydroxyl, hydrogen atoms and hydrated electrons) as a result of irradiation, thereby justifying the need to study several different chemical parameters, as performed in this work.

On a dry weight (dw) basis, carbohydrates were the main (64–65 g/100 g dw) component, followed by protein (23.2–24.5 g/100 g dw), ash (9.2–9.9 g/100 g dw) and fat (1.7–1.8 g/100 g dw).

The results obtained in electron beam (EB) irradiated samples were similar. At least one EB dose caused a significant change in all nutritional parameters, while ST only affected protein and carbohydrate content. Despite the detected significant differences, the EMM plots showed that the only observed overall tendency was the higher protein content in samples irradiated with 5 kGy. All in all, it seems obvious that neither GI, nor EB, exert any remarkably negative effect over the nutritional parameters of stored (up to 8 days) Portobello samples, which is in agreement with the results obtained in other mushroom species (Fernandes et al., 2014b, 2015, 2016).

Moving on to the polar compound profiles (Table 2), herein represented by organic acids and sugars, which are important indicators of reliable preservation conditions (Barreira, Pereira, Oliveira, & Ferreira, 2010), several significant differences were detected. In fact, nearly all parameters (except for mannitol and grouped sugars) showed a significantly different value for at least one GI dose or a specific ST. However, these differences corresponded to overall trends only in the case of malic acid (higher in samples irradiated with GI at 5 kGy and lower in non-stored samples) and grouped organic acids (higher in samples stored for 8 days).

In the case of Portobello treated with EB irradiation, the significant differences were detected in a higher number of parameters, since this effect was observed in all cases except trehalose content ($p = 0.051$). Furthermore, several tendencies could be obtained from the corresponding EMM plots: non-irradiated samples showed lower contents in malic acid (0.5 g/100 g dw) and grouped organic acids (2.7 g/100 g dw), but higher concentration of mannitol (38 g/100 g dw) and grouped sugars (41 g/100 g dw); samples irradiated with 2 kGy gave the highest value in quinic acid (1.0 g/100 g dw), which showed the lowest value (0.8 g/100 g dw) in non-stored samples, similarly to malic acid (1.6 g/100 g dw) and grouped organic acids (3.0 g/100 g dw). The low-extent changes detected in sugars and organic acids are also in agreement with previous reports describing the effects of irradiation in related mushroom species (Fernandes et al., 2015, 2016).

In what concerns lipophilic compounds, the studied molecules were fatty acids, tocopherols and ergosterol. Fatty acids are also considered as good indicators of suitable shelf-life conditions (Barreira et al., 2014; Pereira et al., 2016), while tocopherols and ergosterol are well known for their bioactivity, particularly antioxidant and hypocholesterolemic effects, respectively. In addition to those presented in Table 3, other fatty acids were quantified, specifically C6:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:1, C17:0, *cis*-C18:1*n* − 9, *trans*-C18:2*n* − 6, C18:3*n* − 3, C20:1, C20:2, C21:0 and C23:0, but in percentages below 0.5% (however, all were used in the linear discriminant analysis discussed in the next section).

Gamma-irradiated samples presented statistical differences in MUFA and β-tocopherol in result of GI dose and in α-tocopherol and ergosterol, regarding ST effect. Some of these differences corresponded to overall trends observable in the EMM plots, namely higher percentages of C16:0 (8.6%) in samples irradiated with 5 kGy, lower C20:0 (1.6%) in the same samples and lower C18:0 (3.4%) in non-irradiated ones. Non-stored samples, in turn, showed lower percentages of C20:0 (1.6%) and lower β-tocopherol content (9.9 μg/100 g dw), while the lowest percentages of *cis*-C18:2*n* − 6 (78.6%) and PUFA (79%) were measured in samples stored for 8 days, which, on the other hand, gave the highest SFA percentage (20.1%).

In the case of EB-irradiated Portobello (Table 3), most parameters presented also significant differences, except C18:0 and β-tocopherol regarding EB effect, and MUFA, α-tocopherol and β-tocopherol, in ST. From the corresponding EMM plots, it was possible to conclude that samples irradiated with 1 kGy presented higher percentages of *cis*-C18:2*n* − 6 (78.9%) and PUFA (79.4%) and lower percentages of SFA (19.5%), while non-irradiated ones showed the lowest content (1.8%) of C20:0. In ST, it was only possible to verify that 8 days stored samples showed the lowest percentage of *cis*-C18:2*n* − 6 (78.1%).

The slight differences in lipophilic compounds (which are prone to be oxidized) were previously reported in mushrooms (Fernandes et al., 2016) and may result from autoxidation processes, since Portobello samples were not stored in oxygen-free conditions. Since the occurrence of this important phenomenon might affect the sensorial quality of

**Table 1**
Proximate composition and energy value of *Agaricus bisporus* Portobello submitted to different irradiation conditions and storage times. The results are presented as mean ± SD.[1]

| | | Moisture (g/100 g fw) | Fat (g/100 g dw) | Proteins (g/100 g dw) | Ash (g/100 g dw) | Carbohydrates (g/100 g dw) | Energy (kcal/100 g dw) |
|---|---|---|---|---|---|---|---|
| | | Gamma irradiation (GI) | | | | | |
| GI | 0 kGy | 89 ± 2 | 1.8 ± 0.1 | 24.2 ± 0.5 | 9.2 ± 0.5 | 65 ± 1 | 372 ± 2 |
| | 1 kGy | 89 ± 1 | 1.7 ± 0.1 | 23.2 ± 0.4 | 9.9 ± 0.5 | 65 ± 1 | 369 ± 2 |
| | 2 kGy | 89 ± 1 | 1.7 ± 0.1 | 23.2 ± 0.4 | 9.9 ± 0.5 | 65 ± 1 | 369 ± 3 |
| | 5 kGy | 89 ± 1 | 1.8 ± 0.1 | 24.5 ± 0.4 | 9.2 ± 0.4 | 64 ± 1 | 372 ± 2 |
| | ANOVA *p*-value ($n$ = 27)[2] | 0.090 | < 0.001 | < 0.001 | < 0.001 | 0.003 | < 0.001 |
| ST | 0 days | 89 ± 1 | 1.8 ± 0.1 | 24.3 ± 0.5 | 9.6 ± 0.5 | 64 ± 1 | 370 ± 4 |
| | 4 days | 89 ± 1 | 1.8 ± 0.1 | 23.5 ± 0.5 | 9.5 ± 0.5 | 65 ± 1 | 371 ± 3 |
| | 8 days | 89 ± 1 | 1.7 ± 0.1 | 23.5 ± 0.5 | 9.6 ± 0.3 | 65 ± 1 | 370 ± 1 |
| | ANOVA *p*-value ($n$ = 36)[3] | 0.074 | 0.105 | < 0.001 | 0.425 | < 0.001 | 0.464 |
| GI × ST *p*-value ($n$ = 108)[4] | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | | Electron beam irradiation (EB) | | | | | |
| EB | 0 kGy | 90 ± 1 | 1.8 ± 0.1 | 24.7 ± 0.5 | 9.2 ± 0.4 | 64 ± 1 | 372 ± 2 |
| | 1 kGy | 89 ± 1 | 1.7 ± 0.1 | 23.6 ± 0.4 | 9.8 ± 0.4 | 65 ± 1 | 369 ± 2 |
| | 2 kGy | 90 ± 1 | 1.7 ± 0.1 | 23.6 ± 0.4 | 9.8 ± 0.5 | 65 ± 1 | 369 ± 3 |
| | 5 kGy | 89 ± 1 | 1.8 ± 0.1 | 24.9 ± 0.5 | 9.2 ± 0.4 | 64 ± 1 | 372 ± 2 |
| | ANOVA *p*-value ($n$ = 27)[2] | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.001 | < 0.001 |
| ST | 0 days | 89 ± 1 | 1.8 ± 0.1 | 24.9 ± 0.5 | 9.6 ± 0.5 | 64 ± 1 | 370 ± 3 |
| | 4 days | 90 ± 1 | 1.8 ± 0.1 | 23.7 ± 0.4 | 9.4 ± 0.5 | 65 ± 1 | 371 ± 3 |
| | 8 days | 89 ± 1 | 1.7 ± 0.1 | 24.0 ± 0.4 | 9.6 ± 0.2 | 65 ± 1 | 370 ± 1 |
| | ANOVA *p*-value ($n$ = 36)[3] | 0.518 | 0.237 | < 0.001 | 0.176 | < 0.001 | 0.217 |
| EB × ST *p*-value ($n$ = 108)[4] | | 0.003 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

[1] Results are reported as mean values of each irradiation dose (GI or EB), aggregating results from 0, 4 and 8 days, and mean values of ST, combining all irradiation doses (from GI or EB).
[2] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one GI or EB.
[3] If $p < 0.05$, the corresponding parameter had a significant difference for at least one of the time intervals.
[4] The interaction among factors was significant in all cases; thereby the statistical classification could not be indicated.

**Table 2**
Polar compounds (organic acids and sugars) of *Agaricus bisporus* Portobello submitted to different irradiation conditions and storage times. The results are presented as mean ± SD.[1]

| | | Sugars (g/100 g dw) | | | | Organic acids (g/100 g dw) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Fructose | Mannitol | Trehalose | Total | Oxalic acid | Quinic acid | Malic acid | Total |
| | | Gamma irradiation (GI) | | | | | | | |
| GI | 0 kGy | 0.6 ± 0.1 | 36 ± 2 | 1.6 ± 0.4 | 38 ± 3 | 0.7 ± 0.1 | 0.8 ± 0.1 | 1.5 ± 0.1 | 3.0 ± 0.3 |
| | 1 kGy | 0.7 ± 0.2 | 36 ± 3 | 1.4 ± 0.3 | 38 ± 2 | 0.7 ± 0.1 | 0.9 ± 0.1 | 1.6 ± 0.1 | 3.2 ± 0.3 |
| | 2 kGy | 0.6 ± 0.1 | 36 ± 3 | 1.2 ± 0.2 | 37 ± 3 | 0.6 ± 0.1 | 0.9 ± 0.1 | 1.5 ± 0.1 | 3.1 ± 0.1 |
| | 5 kGy | 0.6 ± 0.1 | 35 ± 3 | 1.5 ± 0.3 | 37 ± 2 | 0.6 ± 0.1 | 0.8 ± 0.1 | 1.7 ± 0.1 | 3.2 ± 0.2 |
| | ANOVA *p*-value ($n$ = 27)[2] | 0.007 | 0.712 | 0.001 | 0.559 | 0.025 | < 0.001 | < 0.001 | 0.012 |
| ST | 0 days | 0.5 ± 0.1 | 36 ± 3 | 1.5 ± 0.2 | 38 ± 3 | 0.6 ± 0.1 | 0.8 ± 0.1 | 1.5 ± 0.1 | 2.9 ± 0.2 |
| | 4 days | 0.6 ± 0.1 | 36 ± 2 | 1.2 ± 0.2 | 37 ± 2 | 0.6 ± 0.1 | 0.9 ± 0.1 | 1.6 ± 0.1 | 3.1 ± 0.1 |
| | 8 days | 0.7 ± 0.2 | 36 ± 3 | 1.5 ± 0.4 | 38 ± 3 | 0.8 ± 0.1 | 0.9 ± 0.1 | 1.6 ± 0.1 | 3.4 ± 0.1 |
| | ANOVA *p*-value ($n$ = 36)[3] | < 0.001 | 0.976 | < 0.001 | 0.700 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| GI × ST *p*-value ($n$ = 108)[4] | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | | Electron beam irradiation (EB) | | | | | | | |
| EB | 0 kGy | 0.8 ± 0.2 | 38 ± 2 | 1.8 ± 0.5 | 41 ± 3 | 0.5 ± 0.1 | 0.8 ± 0.1 | 1.3 ± 0.1 | 2.7 ± 0.1 |
| | 1 kGy | 0.7 ± 0.2 | 33 ± 2 | 1.5 ± 0.4 | 35 ± 2 | 0.6 ± 0.1 | 0.9 ± 0.1 | 1.7 ± 0.1 | 3.2 ± 0.1 |
| | 2 kGy | 0.6 ± 0.1 | 31 ± 7 | 1.5 ± 0.2 | 33 ± 7 | 0.6 ± 0.1 | 1.0 ± 0.1 | 1.8 ± 0.1 | 3.4 ± 0.2 |
| | 5 kGy | 0.7 ± 0.1 | 34 ± 2 | 1.7 ± 0.2 | 36 ± 2 | 0.6 ± 0.1 | 0.9 ± 0.1 | 1.9 ± 0.1 | 3.4 ± 0.1 |
| | ANOVA *p*-value ($n$ = 27)[2] | < 0.001 | < 0.001 | 0.051 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| ST | 0 days | 0.7 ± 0.1 | 36 ± 3 | 2.0 ± 0.5 | 39 ± 4 | 0.6 ± 0.1 | 0.8 ± 0.1 | 1.6 ± 0.2 | 3.0 ± 0.3 |
| | 4 days | 0.8 ± 0.1 | 34 ± 1 | 1.4 ± 0.1 | 37 ± 1 | 0.6 ± 0.1 | 0.9 ± 0.1 | 1.7 ± 0.2 | 3.2 ± 0.3 |
| | 8 days | 0.5 ± 0.1 | 32 ± 7 | 1.5 ± 0.3 | 34 ± 7 | 0.6 ± 0.1 | 1.0 ± 0.1 | 1.7 ± 0.2 | 3.3 ± 0.3 |
| | ANOVA *p*-value ($n$ = 36)[3] | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.022 | < 0.001 | 0.063 | 0.001 |
| EB × ST *p*-value ($n$ = 108)[4] | | 0.003 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.011 | 0.005 |

[1] Results are reported as mean values of each irradiation dose (GI or EB), aggregating results from 0, 4 and 8 days, and mean values of ST, combining all irradiation doses (from GI or EB).
[2] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one GI or EB.
[3] If $p < 0.05$, the corresponding parameter had a significant difference for at least one of the time intervals.
[4] The interaction among factors was significant in all cases; thereby the statistical classification could not be indicated.

**Table 3**
Lipophilic compounds (fatty acids, tocopherols and ergosterol) of *Agaricus bisporus* Portobello submitted to different irradiation conditions and storage times. The results are presented as mean ± SD.[1]

| | | Fatty acids (relative percentage) | | | | | | | | | Tocopherols (μg/100 g dw) | | Ergosterol (mg/100 g dw) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C16:0 | C18:0 | C18:2n6c | C20:0 | C22:0 | C24:0 | SFA | MUFA | PUFA | α-Tocopherol | β-Tocopherol | |
| | | Gamma irradiation (GI) | | | | | | | | | | | |
| GI | 0 kGy | 8.1 ± 0.1 | 3.4 ± 0.1 | 79.7 ± 0.4 | 1.8 ± 0.2 | 1.4 ± 0.1 | 1.2 ± 0.1 | 19.2 ± 0.5 | 0.8 ± 0.1 | 80.1 ± 0.4 | 0.50 ± 0.05 | 10.1 ± 0.4 | 232 ± 9 |
| | 1 kGy | 7.8 ± 0.3 | 4.1 ± 0.2 | 79.6 ± 0.5 | 1.9 ± 0.3 | 1.4 ± 0.2 | 1.2 ± 0.1 | 19.3 ± 0.5 | 0.9 ± 0.2 | 80.0 ± 0.4 | 0.50 ± 0.03 | 10.3 ± 0.5 | 236 ± 15 |
| | 2 kGy | 7.8 ± 0.4 | 4.2 ± 0.1 | 78.7 ± 0.5 | 1.9 ± 0.1 | 1.5 ± 0.1 | 1.3 ± 0.2 | 20.0 ± 0.5 | 0.9 ± 0.1 | 79.1 ± 0.5 | 0.50 ± 0.04 | 10.3 ± 0.4 | 250 ± 22 |
| | 5 kGy | 8.6 ± 0.2 | 4.1 ± 0.5 | 78.6 ± 0.3 | 1.6 ± 0.1 | 1.2 ± 0.1 | 1.2 ± 0.1 | 20.1 ± 0.2 | 0.9 ± 0.1 | 79.0 ± 0.3 | 0.47 ± 0.04 | 10.3 ± 0.3 | 231 ± 22 |
| | ANOVA *p*-value ($n = 27$)[2] | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.360 | < 0.001 | 0.010 | 0.328 | < 0.001 |
| ST | 0 days | 8.4 ± 0.2 | 3.8 ± 0.3 | 79.4 ± 0.5 | 1.6 ± 0.1 | 1.3 ± 0.1 | 1.2 ± 0.1 | 19.2 ± 0.5 | 0.9 ± 0.2 | 79.8 ± 0.5 | 0.50 ± 0.04 | 9.9 ± 0.5 | 239 ± 17 |
| | 4 days | 7.7 ± 0.5 | 3.9 ± 0.3 | 79.5 ± 0.5 | 1.9 ± 0.2 | 1.4 ± 0.1 | 1.2 ± 0.1 | 19.3 ± 0.5 | 0.8 ± 0.1 | 79.8 ± 0.5 | 0.49 ± 0.04 | 10.5 ± 0.4 | 236 ± 19 |
| | 8 days | 8.1 ± 0.2 | 4.2 ± 0.5 | 78.6 ± 0.5 | 1.9 ± 0.2 | 1.4 ± 0.1 | 1.3 ± 0.1 | 20.1 ± 0.4 | 0.8 ± 0.1 | 79.0 ± 0.5 | 0.50 ± 0.04 | 10.4 ± 0.4 | 237 ± 22 |
| | ANOVA *p*-value ($n = 36$)[3] | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.543 | < 0.001 | 0.743 |
| GI × ST *p*-value ($n = 108$)[4] | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | | Electron beam irradiation (EB) | | | | | | | | | | | |
| EB | 0 kGy | 8.2 ± 0.5 | 4.1 ± 0.2 | 78.6 ± 0.2 | 1.8 ± 0.1 | 1.5 ± 0.2 | 1.1 ± 0.1 | 20.0 ± 0.3 | 1.0 ± 0.2 | 79.0 ± 0.2 | 0.51 ± 0.03 | 10.2 ± 0.3 | 216 ± 11 |
| | 1 kGy | 7.5 ± 0.3 | 4.2 ± 0.1 | 78.9 ± 0.4 | 2.1 ± 0.1 | 1.5 ± 0.1 | 1.2 ± 0.1 | 19.5 ± 0.3 | 1.0 ± 0.2 | 79.4 ± 0.4 | 0.50 ± 0.04 | 10.0 ± 0.3 | 226 ± 17 |
| | 2 kGy | 7.7 ± 0.1 | 4.1 ± 0.2 | 78.0 ± 0.5 | 2.2 ± 0.1 | 1.6 ± 0.1 | 1.3 ± 0.1 | 20.6 ± 0.5 | 0.9 ± 0.1 | 78.5 ± 0.5 | 0.47 ± 0.04 | 10.1 ± 0.4 | 233 ± 15 |
| | 5 kGy | 8.1 ± 0.1 | 4.1 ± 0.1 | 78.2 ± 0.2 | 2.0 ± 0.1 | 1.5 ± 0.1 | 1.2 ± 0.1 | 20.2 ± 0.1 | 0.9 ± 0.1 | 78.6 ± 0.2 | 0.51 ± 0.04 | 10.1 ± 0.5 | 238 ± 10 |
| | ANOVA *p*-value ($n = 27$)[2] | < 0.001 | 0.082 | < 0.001 | < 0.001 | 0.001 | < 0.001 | < 0.001 | 0.026 | < 0.001 | 0.002 | 0.119 | < 0.001 |
| ST | 0 days | 8.1 ± 0.5 | 4.1 ± 0.2 | 78.5 ± 0.2 | 1.9 ± 0.2 | 1.4 ± 0.1 | 1.1 ± 0.1 | 20.1 ± 0.3 | 0.9 ± 0.2 | 79.0 ± 0.2 | 0.50 ± 0.04 | 10.2 ± 0.3 | 222 ± 18 |
| | 4 days | 7.8 ± 0.2 | 4.1 ± 0.1 | 78.6 ± 0.5 | 1.9 ± 0.1 | 1.5 ± 0.1 | 1.1 ± 0.1 | 19.9 ± 0.5 | 1.0 ± 0.1 | 79.1 ± 0.5 | 0.50 ± 0.04 | 10.0 ± 0.4 | 234 ± 12 |
| | 8 days | 7.7 ± 0.3 | 4.2 ± 0.1 | 78.1 ± 0.5 | 2.1 ± 0.2 | 1.6 ± 0.1 | 1.3 ± 0.1 | 20.4 ± 0.5 | 1.0 ± 0.1 | 78.6 ± 0.5 | 0.49 ± 0.04 | 10.1 ± 0.3 | 229 ± 14 |
| | ANOVA *p*-value ($n = 36$)[3] | < 0.001 | 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.428 | < 0.001 | 0.317 | 0.145 | 0.004 |
| EB × ST *p*-value ($n = 108$)[4] | | 0.003 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.087 | < 0.001 |

[1] Results are reported as mean values of each irradiation dose (GI or EB), aggregating results from 0, 4 and 8 days, and mean values of ST, combining all irradiation doses (from GI or EB).
[2] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one GI or EB.
[3] If $p < 0.05$, the corresponding parameter had a significant difference for at least one of the time intervals.
[4] The interaction among factors was significant in all cases; thereby the statistical classification could not be indicated.

mushrooms, it is worth mentioning, however, that the results obtained herein seem to indicate that lipid oxidation occurred to a minor extent (as indicated by the maintenance of percentages of fatty acids more prone to be oxidized).

All in all, irradiation seem to be a suitable conservation technique, owing to its capacity to maintain the chemical profiles of this mushroom species for extended shelf-life periods. In what concerns its application at industrial level, the effective cost should be considered. The high price of the irradiation equipment might be considered as a strong constraint, but it should also be borne in mind that the operational costs (e.g., product transportation) are much less than other presently available conservation technologies.

## 4. Linear discriminant analysis

According to the analysis of results described in the previous section, GI and EB seemed to have dissimilar effects over the chemical composition of stored Portobello mushrooms. Therefore, we hypothesized that the effects produced by each irradiation type could be different enough to discriminate them. To verify this hypothesis, a linear discriminant analysis (LDA) was applied to the complete set of results, without separating those obtained with each irradiation type (as presented in Tables 1–3).

The first three discriminant functions of the obtained model included 98.8% (first function: 96.5%; second function: 1.5%; third function: 0.8%) of the observed variance (Fig. 1). Among the 42 variables (corresponding to each analyzed parameter) included in the LDA, only 12 (moisture, protein, mannitol, grouped sugars, quinic acid, grouped organic acids, C16:1, C20:0, C20:2, SFA, PUFA and ergosterol) were considered as not having discriminant ability, therefore indicating a high dissimilarity among samples treated with GI or EB.

The most obvious separation effect observed in Fig. 1 is the location of markers corresponding to EB at the positive end of the corresponding axis, while GI markers were placed at the opposite end of the same axis. This separation is more relevant if we consider the percentage of variability that was explained by function 1 (96.5%); indeed, this is a clear indicator of the high dissimilarity among GI-irradiated and EB-irradiated samples of Portobello. Taking into account the correlations among functions and variables coefficients, the parameters with highest contribution to the separation resulting from function 1 were grouped sugars (37.8 g/100 g dw in GI-irradiated samples and 36.4 g/100 g dw in EB-irradiated samples) and ergosterol (237 mg/100 g dw in GI-irradiated samples and 228 mg/100 g dw in EB-irradiated samples), indicating that these are the two variables with highest changes between GI and EB.

Another interesting observation results from the fact that, in addition to the complete individualization of markers corresponding to each irradiation technology, it was also possible to discriminate the irradiation doses assayed within GI and EB. In fact, function 2 divided GI doses in two groups: a first one integrating 0 and 5 kGy markers, and a second one including 1 and 2 kGy markers. The greatest differences among these two groups were related with protein (higher in non-irradiated samples and those irradiated with 5 kGy) and C20:2 (higher in samples irradiated with 1 or 2 kGy). In the case of EB-irradiated samples, the four dose levels were also divided in a similar way (0 and 5 kGy on the positive side, 1 and 2 kGy on the negative side), mainly due to the higher percentages of C20:0 in samples irradiated with 1 or 2 kGy, and also their lower protein content.

Function 3, in turn, was mostly correlated with moisture and C16:1, contributing to separate markers from non-irradiated samples and those of samples irradiated with 5 kGy, concerning GI treatment, and samples irradiated with 1 and 2 kGy for EB-irradiated ones.

In addition to verify the parameters with highest changes within each irradiation type, we also intended to check which parameters were more affected by storage. In this second LDA, the two defined discriminant functions included 100.0% (first function: 76.8%; second function: 23.2%) of the observed variance (Fig. 2). Among the 42 variables included in the LDA, 16 (moisture, fat, protein, ash, energy, oxalic acid, malic acid, ergosterol, fructose, grouped sugars, α-tocopherol, β-tocopherol, grouped tocopherols, C12:0, C17:0, *trans*-C18:2*n* − 6, C20:0, C20:2, C22:0, C23:0, MUFA and PUFA) were not selected as being discriminant, indicating that those were the least affected by storage.

As it might be easily observed in Fig. 2, function 1 separates mainly markers corresponding to non-stored samples from those belonging to samples stored for 8 days. The variables more correlated with function 1 were quinic acid, organic acids, and C24:0 (all higher in 8 days-stored samples). Function 2, on the other hand, projected the markers corresponding to 4-days-stored samples away from those belonging to non-stored samples and those stored for 8 days. The variables more correlated with this second function were carbohydrates (higher after 4 days of storage) and C16:0 (lower after 4 days of storage).

In both performed LDAs, the classification performance was 100%

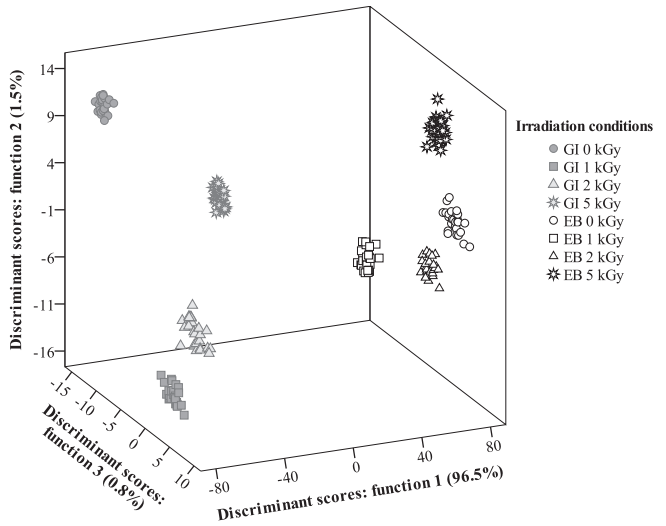


**Fig. 1.** Three-dimensional distribution of gamma and electron beam irradiation markers according to the canonical discriminant functions coefficients defined from all parameters analyzed in *Agaricus bisporus* Portobello.

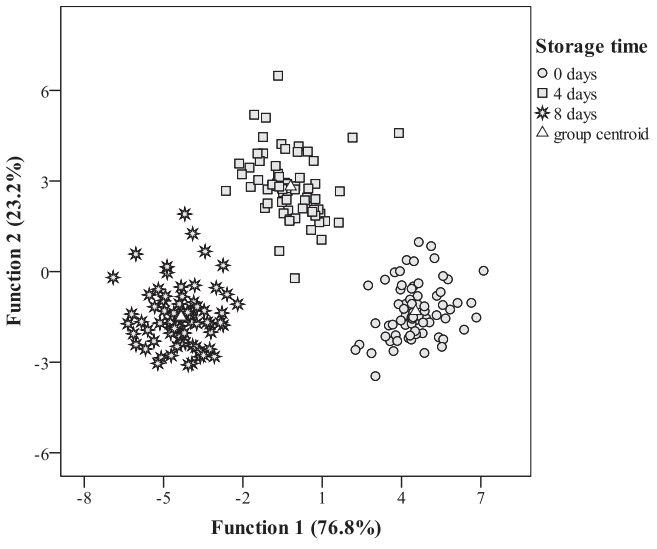


**Fig. 2.** Two-dimensional storage time markers according to the canonical discriminant functions coefficients defined from all parameters analyzed in *Agaricus bisporus* Portobello.

accurate, both for original grouped cases, as well as for the cross-validated grouped cases.

## 5. Conclusion

Overall, it was possible to conclude that the effects of each irradiation technology are more distinctive than those caused by different doses of the same irradiation type. When directly comparing gamma irradiation and electron beam irradiations, independently of the used dose, it could be concluded that gamma irradiation allowed higher contents in sugars and ergosterol. In addition, the 5 kGy dose, independently of irradiation type, tended to be associated with higher levels of protein. On the other hand, in what concerns the effect of storage time, it could be verified that GI and EB were effective in maintaining the chemical profiles of Portobello samples, except for quinic acid, grouped organic acids and some particular SFA. Accordingly, these technologies might represent effective preservation approaches for Portobello mushrooms.

## Declaration of interests

None.

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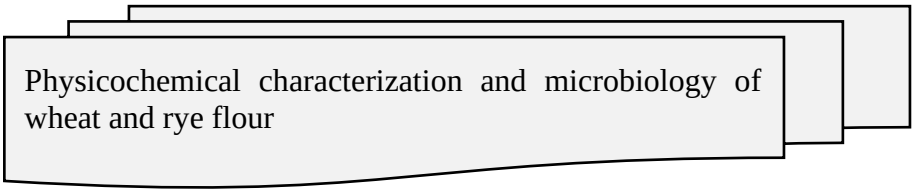
**Annex B**


Physicochemical characterization and microbiology of wheat and rye flour

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# Physicochemical characterization and microbiology of wheat and rye flours

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ABSTRACT

Seven types of wheat and rye flours were studied regarding their physical and chemical properties, as well as the presence of mycotoxins and microorganisms. The results revealed that flours presented moisture and ash contents below the recommended maximum limit. They also presented a low lipid content, which helps avoiding changes in the flours' smell and taste. From the microbiological analysis, comparing the refined rye and wheat flours, the counting in the analysed microorganisms was not significantly different. The whole flours presented high contents in almost all the tested microorganisms, highlighting the molds counting for the whole wheat flour and the aerobic plate counting for the whole rye flour. None of the samples presented *Salmonella* spp. Aflatoxins and ochratoxin A were not detected in any of the flours. To the best of our knowledge, this is the first characterization of wheat and rye flours for Portuguese consumers.

## 1. Introduction

Rye (*Secale cereale* L.) is a widely grown cereal consumed as bread in northern Europe and the main producers are Russia, Poland, Germany, Belarus and Ukraine. On the other hand, wheat (*Triticum aestivum* L.) is originally from the Levant region of the near East and Ethiopia (Ihsan, El-nakhlawy, & Ismail, 2015). It is the most cultivated cereal in the world, about one third of total cereals, while rye is grown at 2% worldwide. In general, rye and wheat flours are composed mainly by macronutrients such as of starch, water, proteins and other micronutrients, such as non-starch polysaccharides, lipids and ashes. Among the differences found between wheat and rye flours, the protein composition could be highlighted, being the content of prolamin and glutenin (as well as gluten) higher in wheat flours, while the content in albumins has been revealed to be higher in rye flours (Hadaruga, Costescu, Corpaş, Hədərugə, & Isengard, 2016). The consumption of wheat, rye, and related cereals can be harmful to susceptible individuals, due to specific proteins that are responsible for triggering hypersensitivity, such as wheat allergy, celiac disease (CD), and non-celiac gluten sensitivity (NCGS) (Schalk, Lexhaller, Koehler, & Scherf, 2017).

The chemical composition of cereal grains affects their functional and technological characteristics. Cereals are processed by crushing using different types of mills. Nevertheless, the force applied for cereals grinding, which can be implemented through compression, impact or shear, allows reducing the particle sizes according to the desired end use products. At the industrial level, complex process of crushing, successive sieving, and refining leads to the separation of the husk from the endosperm and germ resulting in several types of flours (Koletta, Irakli, Papageorgiou, & Skendi, 2014).

In Portugal, flour type numbers indicate the degree of cereal grinding. The flour types 55, 65 or 85 are characterized by being white flours due to fine grinding. This kind of flour contains only the endosperm of the grain, because the refining process removes the husk, also destroying the grain's vitamin content. The milling process separates fibre-rich bran from the rest of the grain and the fibre content is typically lower, but contains a little more starch and gluten. The flours type 130, 150 or 170 are made from the bran of the grain and give rise to a darker bread, preserving all the dietary and nutritious characteristics, containing several vitamins, high fibre content and unsaturated fat (Koletta et al., 2014).

Standard Portuguese wheat flours range from type 55–65 (white wheat flour) used in pastry, to type 150 (wholemeal flour) used in pastas and whole grain bread. Standards rye flours range from type 70 – 85 (semi-integral flour) used in bakery, to type 130–170 (wholemeal flours) and give bread strong increasing darkness (Weekendbakery, ,

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0308-8146/ 

2018).

According to the Portuguese Society of Nutrition and Food Sciences (SPCNA, 2003), flour for the baking and pastry industry, cookies and biscuits must be conform with the analytical characteristics of wheat flour (T 55–T 150: with maximum moisture of 14.5%, ash of 0.49–2%, respectively, and dry gluten, 8–7% respectively) and rye flour (T 70–T 170: maximum moisture of 14.5% and ash of 0.79–2.5%, respectively).

In Italy, for example, the commercial description applied to flours is based on ash content and degree of milling and the nomenclature ranges from 0 to 2; flours type 00 present an ash content of 0.4% and 9% of protein and flours type 2 may have 1% of ash and 15% of protein. In Germany, the nomenclature ranges between 405 and 1700, and in France between 45 and 150; and follows the same trend as Italy, which in relation to the percentage content of ash and proteins (Weekendbakery, 2018).

The type of flour, defined on the basis of the ash content, only guarantees the production of white or darker crumb bread. In the USA and United Kingdom, no numbered standardized flour types are defined, and the ash content is only rarely given in the flour label by the manufacturers. However, the legal required standard nutrition label of flours indicates the protein content and its advisable use: multipurpose, pastry, cakes, biscuits, and bread (Mata, 2006; Weekendbakery, 2018).

Although the growth of microorganisms is not sustained under such low water activity, foodborne bacteria and fungi can easily contaminate flour and survive for long time periods. Moreover, low moisture is known to increase heat resistance of foodborne pathogens. Several studies from Australia, Europe and North America report the presence of *Salmonella* spp., *Escherichia coli*, *Bacillus cereus*, and other deteriorating microorganisms in flour. Outbreaks of salmonellosis have also been associated with consumption of low-moisture foods, including wheat flour (Condón-Abanto, Condón, Raso, Lyng, & Álvarez, 2016).

Besides foodborne pathogens, contamination with mycotoxins is among the most serious problems affecting the safety and quality of cereals and cereal products. The most important groups of mycotoxins frequently identified in such products are aflatoxins (AFs) and ochratoxin A (OTA), produced by several species of the genera *Aspergillus* and *Penicillium*. Cereals are considered the main contributors to mycotoxins exposure in Europe and, being generally stable compounds, mycotoxins can be transferred from the cereal grains to the processed cereal products. Several studies have confirmed the contamination of cereal flours with these mycotoxins (Torović, 2018). Due to the importance of cereals in the European diet, the European Union has set stringent regulations on AF and OTA contamination of products derived from cereals: 2 μg/kg for AFB1, 4 μg/kg for total AF, and 3 μg/kg for OTA (European Union, 2006a).

In this perspective, the objective of this study was to evaluate the quality of wheat and rye flours according to the degree of refinement, by determining the physicochemical as well as the occurrence of mycotoxins and microbial contaminations. The degree of refinement is coded by the used number, a higher figure represents smaller grinding and the texture obtained is denser, as lower figure refers to higher grinding, resulting in whiter and thinner flours.

## 2. Materials and methods

### 2.1. Flours samples

Seven flours representative of the commercial diversity were kindly donated by the Milling Company “Moagem do Loreto”, Bragança, Portugal, in December 2017, namely T 55, T 65, T 85, T 130, T 150 and T 170. The wheat and rye flours were divided considering the degree of refinement, namely T 55 and T 65 (wheat flours – refined samples), T 150 (whole wheat flour), T 70 and T 85 (rye flours – refined samples) and T 130 and T 170 (whole rye flours).

### 2.2. Physicochemical analysis

#### 2.2.1. Macronutrients and energetic value

The samples were analysed for moisture, energetic value and macronutrients (fat, ash, proteins and carbohydrates) by AOAC methods (AOAC, 2016). The crude protein was evaluated by macro-Kjeldahl method ($N \times 5.7$ for wheat flour and 6.25 for rye flour) (Mariotti, Tomé, & Mirand, 2008) using an automatic distillation and titration unit (model Pro-Nitro-A, JP Selecta, Barcelona), ash content was determined by incineration at 550 ± 15 °C, and the crude fat was determined using a Soxhlet apparatus by extracting a known weight of powdered sample with petroleum ether. Total carbohydrates and energetic value were determined following the formulas: Total carbohydrates (g/100 g) = $100 - (m_{fat} + m_{ash} + m_{proteins})$ and Energy (kcal/100 g) = $4 \times (m_{proteins} + m_{carbohydrates}) + 9 \times (m_{fat})$.

#### 2.2.2. Gluten determination

A known weight of sample (10 g) was placed in a mortar and 5.5 mL of NaCl solution (2%) was added dropwise. Afterwards, the sample was stirred with the pestle, the mixture was compressed and shaped into a ball. After kneading, it was allowed to stand for 25–30 min. After this process, the samples were washed with water to remove all the starch, until the washing liquids did not turn blue with the iodine solution (I (0.64 g), KI (2 g) 0.4% *w/v*). The obtained gluten was drained and extended in a watch glass, weighed (wet gluten) and placed in a drying oven at 50 °C to obtain the dry gluten (Panreac Quimica, 1977).

#### 2.2.3. pH determination

One gram of each flour sample was macerated in 2 mL of distilled water. The pH was measured using a calibrated digital pH meter (portable food and dairy pH meter HI 99161, Hanna Instruments, Woonsocket, RI, USA).

### 2.3. Microbiological analysis

#### 2.3.1. General sample preparation

The preparation of samples for microbiological analysis followed the procedure described in ISO 6887-1:2003 (ISO, 2003). Flour samples (25 g) were mixed with 225 mL of buffered peptone water (BPW; Himedia, Italy) in stomacher bags and further homogenized in a stomacker equipment (ECN 710-0873, Italy) for 1 min at 300 units. The obtained suspensions were further diluted to obtain dilutions from $10^{-1}$ to $10^{-5}$. Each dilution was analysed in triplicate.

#### 2.3.2. Microorganisms analysis

*Aerobic plate count (APC)*: 1 mL of each prepared suspension was mixed with 20 mL of Plate Count Agar (PCA; Liofilchem, Italy) by the pour plate method, in triplicate (LOQ = 1 log UFC/g). The plates were further incubated in reversed position at 30 °C for 72 h and counted according to ISO 4833-2:2013 (ISO, 2013).

*Coliforms* and *E. coli:* For the coliforms counting, 1 mL of each suspension was mixed with 20 mL of Violet Red Bile Lactose Agar (VRBLA; Liofilchem, Italy), by the plate method, in duplicate (LOQ = 1 log UFC/g). For *E. coli* determination the medium was supplemented with 4-methylumbelliferyl-beta-D-glucuronide (MUG). The plates were further incubated in reversed position at 30 °C for 48 h and counted according to ISO 4832:2006 (ISO, 2006).

*Yeasts and molds*: 0.2 mL of each suspension was spread in petri dishes containing 20 mL of Dichloran Glycerol Agar Base (DG18; Liofilchem, Italy), in duplicate (LOQ = 1.7 log UFC/g). The plates were further incubated in upright position at 25 °C for: 72 h for yeast counting and 120 h for mould counting, according to ISO 21527-2:2008 (ISO, 2008).

*Sulphite-reducing clostridia (SRC)*: 5 mL of each suspension were transferred to a 50 mL falcon tube and further heat-treated in a water bath at 80 °C for 10 min. The suspension was immediately cooled in ice

and 25 mL of Iron Sulfite Agar (ISA; Liofilchem, Italy) were added (LOQ = 2 UFC/g). The mixture was homogenized and allowed to solidify. Afterwards, 5 mL of ISA medium was added to create anaerobiosis. The falcon tubes were incubated at 30 °C for 24–48 h and black spots were counted, according to ISO 15213:2003 (ISO, 2003).

*Salmonella* spp*:* To analyse the presence of *Salmonella* spp. 25 g of flour samples were homogenized in 225 mL of BPW and incubated for 18 h-24 h at 37 °C ± 1 °C. Afterwards, 0.1 mL of this suspension was transferred to 10 mL of Ramba QUICK *Salmonella* enrichment broth (Frilabo, Portugal). The mixture was incubated for 7 h ± 1 h at 41.5 °C ± 0.5 °C. Afterwards, 10 μL were spread onto a Petri dish containing CHRO Magar Salmonella Plus medium (Frilabo, Portugal), which also detects lactose-positive *Salmonella*, meeting the requirements of ISO 6579-1: 2017 (ISO, 2017). Any presumptive positive result (purple colonies) must then be confirmed by serological or biochemical tests according to ISO 6579-1: 2017.

### 2.4. Mycotoxins analysis

*Safety Considerations.* For AFs and OTA handling, the security rules were carefully followed due to the high toxicity of these substances. Protective equipment was used when managing these solutions and all the materials were cleansed by autoclaving before discarding.

The reusable materials were disinfected throughout 12 h, submerged in a 10% bleach solution and washed with distilled water (Pereira et al., 2017).

#### 2.4.1. Aflatoxins determination

Aflatoxins were extracted and purified using the method recommended by VICAM for the determination of AF in corn, raw peanuts and peanut butter (AOAC, 2008a), with slight modifications. Briefly, 25 g of flour were extracted by stirring with sodium chloride (5 g) and methanol/water (125 mL, 70:30, *v/v*) for 20 min (25 °C at 150 rpm). The mixture was filtered through a Whatman No. 4 filter paper (Sigma-Aldrich Co., St. Louis, MO, USA) and an aliquot of the filtrate (15 mL) was taken and diluted with 30 mL of ultra-pure water. The extract was homogenized and further filtered through a Whatman glass microfiber filter (934-AH). Subsequently, the filtered extract (15 mL) was purified through an immunoaffinity column (AflaTest WB, VICAM, Watertown, MA, USA) by gravity, at a rate of approximately 1–2 drops/s. The column was then washed twice with 10 mL of ultra-pure water. AF were eluted from the column with 1 mL of HPLC-grade methanol, collected in a glass *vial*, filtered through 0.2 μm nylon filters (Whatman) and analysed by HPLC (Smartline, Knauer, Berlin, Germany) coupled to a photochemical post-column derivatization reactor (PHRED unit, Aura Industries, New York, NY, USA), a fluorescence detector (FP-2020, Jasco, Easton, MD, USA) set to λex 365 nm and λem 435 nm and using the Clarity 2.4 Software (DataApex, Prague, Czech Republic). The compounds were separated using an isocratic elution with a reverse-phase C18 column (100 mm × 4.6 mm, Merck Chromolith Performance, Darmstadt, Germany) at 35 °C (7971 R Grace oven). The mobile phase consisted of a mixture with water/acetonitrile/methanol (3:1:1, *v/v/v*) with a flow rate of 0.8 mL/min and the injection volume was 10 μL. AFs was identified by chromatographic comparison with the standard (Aflatoxin B + G mixture, Sigma-Aldrich Co. St. Louis, MO, EUA) and quantification was based on the fluorescence signal response (Pereira et al., 2017).

#### 2.4.2. Ochratoxin A determination

A standard method for the determination of OTA in wheat (AOAC, 2008b), as described by VICAM, was used for the analysis of flours. Briefly, 50 g of each samples were extracted by stirring (25 °C at 150 rpm) with 200 mL of acetonitrile:water (6:4, *v/v*) for 20 min and subsequently filtered through Whatman No. 4 filter paper. Afterwards, the extract (10 mL) was diluted with phosphate-buffered saline pH 7.0 (40 mL; PBS: NaCl (8 g), $Na_2HPO_4$ (1.2 g), $KH_2PO_4$ (0.2 g), KCl (0.2 g) in distilled water to a total volume of 1 L), and further filtered through a Whatman glass microfiber filter (934-AH). The filtered extract (10 mL) was purified through an Ochratest WB immunoaffinity column (VICAM, Watertown, MA, USA) and the column was washed first with PBS (10 mL) and then with ultra-pure water (10 mL). Then OTA was eluted with HPLC-grade methanol (1.5 mL), collected in a glass *vial*, filtered through 0.2 μm nylon filters (Whatman) and analysed by HPLC as described above for AFs, but without the derivatization process. The fluorescence detector was set to λex 330 nm and λem 465 nm, mobile phase consisted of a mixture with water/acetonitrile/acetic acid (29.5:70:0.5, *v/v/v*), with a flow rate of 0.8 mL/min, and the injection volume was 10 μL. OTA was identified by chromatographic comparison with the standard (OTA standard solution (Sigma Aldrich Co. St. Louis, MO, EUA) and quantification was based on the fluorescence signal response (Pereira et al., 2017).

#### 2.4.3. In-house method validation

AF mix (5 μg/mL for AFB1 and AFG1, and 1.5 μg/mL for AFB2 and AFG2) and OTA (10 μg/mL) standard stock solutions were prepared and stored at −20 °C. Working standard solutions of AF (100 ng/mL for AFB1 and AFG1, and 30 ng/mL for AFB2 and AFG2) and ochratoxin A (100 ng/mL) were prepared from stock solutions daily. Precision and recovery were performed by spiking the blank sample with 10 μg/kg of AFB1, AFG1 and OTA, and 3 μg/kg of AFB2 and AFG2. One set of unspiked sample was used as blank. Each set was composed of three replicates (Pereira et al., 2017).

Instrumentation calibration parameters were determined following the methodology previously described by the authors (Arita, Calado, Venâncio, Lima, & Rodrigues, 2014) and the recovery rates were calculated based on the three spiked replicates (flour was artificially contaminated), by calculation of the ratio of recovered AFs and OTA concentration to the known spiked concentration. Linearity, limit of detection (LOD), and limit of quantification (LOQ) were determined by three series of analyses using 6 standard solutions with concentrations ranging from 0.5 to 50 ng/mL for AFB1 and AFG1, 0.15 to 15 ng/mL for AFG1 and AFG2, and 0.1 to 20 ng/mL for OTA. LOD and LOQ were calculated according to the following equations (Arita et al., 2014): $LOD = 3 \times (SD/M)$ and $LOQ = 10 \times (SD/M)$, where *SD* is the standard deviation of the intercept of the regression line obtained from the calibration curve, and *M* is the slope of the line (Pereira et al., 2017).

### 2.5. Statistical analysis

In all the assays, three samples were used and the analyses were performed triplicate. The results were expressed as mean ± standard deviation (SD), and the statistical parameters applied was analysis of variance (ANOVA) followed by HSD test of Tukey's with $\alpha = 0.05$ (SPSS v. 23.0).

## 3. Results and discussion

### 3.1. Macronutrients and energetic value

In general, the quality of the flour is attributed to its moisture, gluten, lipid, acidity, mineral, and protein contents. These properties reflect the effect of the processing and can be used to evaluate the technological or nutritional quality of the product (Hadaruga et al., 2016).

The results of physicochemical parameters are given in Table 1. The highest pH value was found in whole rye flour T 130 (6.44) and the lowest in wheat flour T 65 (6.04). Similar results were found in wheat flours from Alegre, Brazil (5.28) (Vieira, Freitas, Silva, Barbosa, & Silva, 2015) and in samples from Tocantins, Brazil in a range from 6.0 to 6.1 (dos Macedo, Soares, Souza, & Morais, 2017). There are few studies reporting the determination of pH in wheat or rye flours. The pH value (hydrogenation potential) is important for detecting the treatments

**Table 1**
Physicochemical analysis of different types of flour.

| Parameter | Wheat refined flours | | Whole wheat flour | Rye refined flours | | Whole rye flours | |
|---|---|---|---|---|---|---|---|
| | T 55 | T 65 | T 150 | T 70 | T 85 | T 130 | T 170 |
| pH | 6.05 ± 0.01e | 6.04 ± 0.01e | 6.18 ± 0.01d | 6.35 ± 0.01c | 6.41 ± 0.01b | 6.44 ± 0.01a | 6.18 ± 0.01d |
| Moisture (g/100 g dw) | 14.1 ± 0.7a | 13.6 ± 0.3a | 14.7 ± 0.2a | 10.1 ± 0.1bc | 9.9 ± 0.1bc | 9.3 ± 1.3c | 11.5 ± 0.3b |
| Proteins (g/100 g dw) | 13.2 ± 0.8b | 13.4 ± 0.3b | 14.6 ± 0.3a | 6.93 ± 0.07d | 7.7 ± 0.1 cd | 8.2 ± 0.2c | 13.8 ± 0.1b |
| Ash (g/100 g dw) | 0.61 ± 0.02 g | 0.69 ± 0.01f | 1.47 ± 0.08b | 0.85 ± 0.01e | 0.99 ± 0.03d | 1.27 ± 0.03c | 1.75 ± 0.02a |
| Lipids (g/100 g dw) | 0.92 ± 0.02d | 0.76 ± 0.02e | 1.51 ± 0.02b | 0.96 ± 0.05d | 1.29 ± 0.02c | 1.31 ± 0.01c | 1.73 ± 0.02a |
| Carbohydrates (g/100 g dw) | 85.2 ± 0.8c | 85.5 ± 0.3c | 82.4 ± 0.2d | 91.3 ± 0.1a | 90.0 ± 0.1b | 89.2 ± 0.1b | 82.7 ± 0.1d |
| Energy (kcal/100 g dw) | 402.2 ± 0.2a | 401.0 ± 0.1b | 401.7 ± 0.2b | 401.4 ± 0.2b | 402.5 ± 0.1a | 401.4 ± 0.1b | 401.6 ± 0.2b |
| *Gluten* | | | | | | | |
| Wet (g/100 g mb) | 25.0 ± 0.5a | 25.6 ± 0.1a | 19.9 ± 0.5b | nd | nd | nd | nd |
| Dried (g/100 g dw) | 9.5 ± 0.4a | 9.4 ± 0.2a | 7.2 ± 0.1b | nd | nd | nd | nd |

nd – not detect. Values are expressed in dry weight (dw) and wet gluten contents are expressed in mass basis (mb) as mean ± SD. In each row different letters represent significant differences ($p < 0.05$).

applied to the flour if it shows excessive alteration as in the case of bleaching with chlorine. It is also a significant factor for the capacity of microorganisms' development in the food. Contaminated flour contains some live yeast or other bacteria and may have a lower pH (less than 5.5) due to extra biological activity. According to this parameter, foods can be classified as: low acidity (pH > 4.5), acidity (4.5–4.0) and high acidity (< 4.0) (Souza, Álvares, Leite, Reis, & Felisberto, 2008).

Regarding moisture content, the results range from 9.3% to 14.7% in whole rye flour T 130 and whole wheat flour T 150, respectively. These results are within the recommended maximum values for rye flour (14.5%) (SPCNA, 2003). Similar results were reported in rye and wheat flours from Greece (9.76% and 15.94%, respectively) (Drakos et al., 2017). Moisture is an important parameter in the storage of flours; high levels can provide the growth of microorganisms and is a critical factor for fungi growth and mycotoxins production (Hadaruga et al., 2016). Thus, low levels are beneficial for a longer shelf-life of the product.

Protein content ranged between 6.93% and 14.6% in rye flour T 70 and in whole wheat flour T 150, respectively. Puppo, Calvelo, and Añón (2005) reported protein values of 10.9% for wheat flour from Buenos Aires, Argentina, and Drakos et al. (2017) report 9.68% in rye flour from Greece.

Regarding the ash content, wheat flour presented similar values, namely T 55 (0.61 g/100 g) and T 65 (0.69 g/100 g) to those reported by Frakolaki, Giannou, Topakas, and Tzia (2018) in Greek samples (0.63 g/100 g). In rye flour samples T 70 (0.85%) and T 85 (0.99%) revealed lower contents than those presented by Drakos et al. (2017) (1.55 g/100 g). These kinds of flours are extremely white due to their high degree of refining, which result from a higher grinding with the absence of husk or germ. It is the percentage of ash that defines the commercial type of flour; the standard values for ash in wheat flour range between 0.49% and 2% and in rye flour from 0.79% to 2.5% according to the regulation N° 254/2003 (SPCNA, 2003). Otherwise, whole wheat and whole rye flours presented higher ash contents (T 150–1.48%, T 130–1.27% and T 170–1.75%), since these samples contain a higher content of minerals, which do not incinerate at 550 °C (Mata, 2006).

Regarding the lipids content, the wheat flours T 55 presented the lowest amount (0.76 g/100 g) while the rye flours T 170 presented the highest one (1.78 g/100 g). Lipids are a parameter that has nutritional and physiological relevance in food because they are a source of essential fatty acids and energy. In addition, they play a key role in the quality of food and can cause unpleasant tastes and smells in stored flours. It followed the same trend of ash content, but in the present study the observed contents are lower than those reported by Bucsella, Molnár, Harasztos, and Tömösközi (2016) from Budapest, Hungary (whole wheat flour: 2.36%, rye flour: 1.15% and whole rye flour: 3.07%).

The different types of flour (wheat and rye) contained comparable amounts of total carbohydrates ranging between 82.4 and 91.3 g/100 g. Similar results in wheat flour were reported by Kaminski, da Silva, Nascimento Júnior, and Ferrão (2011) from Santa Maria/RS – Brazil (85.52 g/100 g), but, in the same study, these authors reported lower values for rye samples (59.88 g/100 g). In wheat samples from Greece the authors reported 67.78 g/100 g of carbohydrates (Frakolaki et al., 2018).

Concerning dry and wet gluten, these were only detected in wheat flours. Dry and wet gluten ranged from 7.2% to 9.5% and 19.9% and 25.0%, respectively; being in accordance with the recommended minimum values by the Portuguese legislation (between 7 and 8% of dry gluten) (SPCNA, 2003). According to the same regulation, rye flour does not have minimum gluten values. Similar results were described in wheat flour samples from Greece, 10.90% of dry gluten and 28.24 of wet gluten (Frakolaki et al., 2018). Wheat flour has a medium to high protein content (10–16%); higher contents are useful in industrial baking due to its higher concentration of gluten, which gives it greater elasticity and resistance to mechanical processing and influence the hydration properties present in the flours. The flours with the lowest protein content are sold as flour for household use and this is the specific characteristic that differentiates wheat from rye flours. Wheat flour presents gluten-forming proteins with capacity for mass-building, on the other hand, rye flours have less gluten-forming proteins and the high soluble fibre content impairs the formation of this protein network (Kaminski et al., 2011).

Gluten consists in a viscous and elastic mass that provides the physical and rheological characteristics such as plasticity, viscosity and elasticity important for the mass modulation. Flours need to have a considerable amount of gluten so that the dough can absorb the water. The amount and quality of gluten determine a strong water absorption and a high elasticity of the dough, which is very favourable for carbon dioxide during the fermentation process of the bakery and pastry products (Hadaruga et al., 2016).

### 3.2. Microorganisms analysis

The results regarding the microorganisms analysed in the wheat and rye flours are presented in Table 2. Aerobic mesophiles, coliforms, yeasts, molds, *E. coli* and sulphite-reducing clostridia were the microorganisms analysed, as well as the presence of *Salmonella* spp. Regarding the rye flours, the samples T 130 and T 170 (whole flours) presented the highest contents in APC when compared with the respective refined flours T 70 and T 85. In the case of coliforms, yeasts and molds, the whole sample T 170 presented increased counts of these microorganisms when compared with the other samples. Sulphite-reducing clostridia, *E. coli* and *Salmonella* were not detected in any of the samples. As far as we know, this is the first comparative report on the

**Table 2**
Analysis of microorganisms identified in the different types of flours.

| Microorganisms analysed | Wheat refined flours | | Whole wheat flour | Rye refined Flours | | Whole rye flours | |
|---|---|---|---|---|---|---|---|
| | T 55 | T 65 | T 150 | T 70 | T 85 | T 130 | T 170 |
| APC $LOG_{10}$ CFU/g | 4.33 | 4.20 | 4.44 | 3.43 | 3.89 | 4.31 | 5.42 |
| Coliforms $LOG_{10}$ CFU/g | 2.00 | 3.04 | 2.00 | 1.70 | < LOQ | 2.03 | 2.70 |
| Yeasts $LOG_{10}$ CFU/g | 2.24 | 2.35 | < LOQ | 2.98 | 2.76 | 2.78 | 4.20 |
| Molds $LOG_{10}$ CFU/g | 2.46 | 2.10 | 5.46 | 2.60 | 2.44 | 1.88 | 3.00 |
| *Escherichia coli* CFU/g | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| SRC CFU/g | < 2 | < 2 | < 2 | < 2 | < 2 | < 2 | < 2 |
| *Salmonella* spp. | Absent | Absent | Absent | Absent | Absent | Absent | Absent |

APC – aerobic plate count; SRC – sulphite-reducing clostridia; CFU – colony forming units.

microbial analysis of different rye flours.

Among the wheat samples, the analysed microorganisms were present in similar amounts between wheat and whole flours, except for molds where the whole sample (T 150) presented a significant increase in these microorganisms. The APC microorganisms obtained in the present work for the wheat flours T 55 and T 65 (4.33 and 4.20 $LOG_{10}$ CFU/g, respectively) are in agreement with the ones obtained by Eglezos (2010) that reported a value of 4.2 $LOG_{10}$ CFU/g. The same authors reported a yeast content in the order of 3.0 $LOG_{10}$ CFU/g, while in the present work the counting was slightly lower (T 55 = 2.24, T65 = 2.35 $LOG_{10}$ CFU/g). Also in accordance with Eglezos (2010), *Salmonella* was not detected in 25 g of sample. Khanom, Shammi, and Kabir, (2016) also reported the microorganisms' content in packed and unpacked flour samples and described the presence of 5.60 $LOG_{10}$ CFU/g of total coliforms and 5.33 $LOG_{10}$ CFU/g for yeasts and molds in unpacked flours. The results regarding the content in coliforms were higher than the ones obtained in the present study (T 55 = 2.00, T65 = 3.04 $LOG_{10}$ CFU/g); as well as the content on yeasts and molds (T 55 = 2.65, T65 = 2.54 $LOG_{10}$ CFU/g). Berghofer, Hocking, Miskelly, and Jansson (2003) also reported the microbiological analysis of Australian wheat flour and reported the sample contamination with up to $10^2$ CFU/g of APC, and up to 1 CFU/g of coliforms, yeasts and molds, with countings of $10^2$ CFU/g. *Salmonella* spp. was absent in all different flours (25 g of sample). *E. coli* was below the LOQ as well as SRC.

Comparing the refined rye and wheat flours, the counting in the analysed microorganisms was not significantly different. Regarding the whole samples, it is possible to state that the rye whole flour T 170 presented a higher counting in APC microorganisms (5.42 $LOG_{10}$ CFU/g), and in Yeasts counting (4.20 $LOG_{10}$ CFU/g). On the other hand, the wheat whole flour (T 150) presented higher content in molds (5.46 $LOG_{10}$ CFU/g). Although the moisture contents are significantly different between rye and wheat samples, the microorganisms' contents were not affected by this parameter.

The cereal grains are susceptible to contamination during the ripening, harvesting, processing and storage. Microorganisms are constant contaminants of grain flours, because they originate from the cereals vegetation period, and they are an integral part of the grain mass. Under unfavourable conditions they are inactive and do not present a potential hazard (Plavšić et al., 2017).

### 3.3. Mycotoxins analysis

The calibration parameters of instrumentation (linear range, correlation coefficient ($R^2$), equations of linear regression, limits of detection (LOD) and limits of quantification (LOQ) for AF and OTA are shown in Table 3. The result for what concerns the linearity in the reference and in the calibration curves was adequate and satisfactory, with a coefficient of determination always greater than 0.999. The performance was moderately sensitive, with detection limits up to 1.2 μg/kg for AFs and 0.7 μg/kg for OTA.

The recovery and within-laboratory reproducibility are reported as the main influence to the uncertainty measurement. Other causes including mass, volume, purity of standards and calibration curve offer small contribution in the uncertainty values, not exerting significant effect on the final value (Golge & Kabak, 2016). For that reason, recovery and repeatability of the method were determined for the matrix under analysis.

Table 4 shows the accuracy and precision of the AF and OTA analysis methods. The recovery was ascertained by spiking non-contaminated samples with known concentrations of each mycotoxin and comparison to the response obtained for pure AF and OTA standard solutions at the same concentration levels. The recoveries of AF were in the range of 70–110% regulated by the Commission Regulation (EC) No 401/2006 (European Union, 2006a), except for AFB1 which presented 64.1% of recovery. This result, which has also been reported by other authors for wheat (Torović, 2018), can be justified by the presence in the matrix of impurities such as lipids that are the main interferences with the purification step and with the chromatographic separation (Manetta, 2002). The repeatability relative standard deviations ($RSD_r$) were 1.9–4.5%. These values are in good agreement with the regulated performance criteria for AF, which states RSDr < 21% for AFB1 and AFG1, and RSDr < 27% for AFB2 and AFG2 (European Union, 2006a).

The recovery rate of OTA was 103%, with an $RSD_r$ of 9.2%. These results are in agreement with the regulated performance criteria for OTA that defines the recovery rate of 70–110%, and repeatability RSDr < 21% (European Union, 2006a).

According to the European Commission Regulation 1881/2006, the maximum permissible levels (MPL) of AFB1 and total AF for all cereals and all products derived from cereals, including processed cereal products, are 2 and 4 μg/kg respectively. The MPL for OTA is 3 μg/kg for all products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption (European Union, 2006b). In our study, AFs and OTA were not detected in any of the samples (< LOD). These results show that the levels of mycotoxin contamination of the samples are clearly below the regulated limits, even in the case of whole flours, which retain the most contaminated parts of the grains, and wheat flours, which showed higher moisture content (between 13.6% and 14.7%) than the rye flours (9.3%–11.5%) In fact, a moisture content lower than 14.5% (on a wet weight basis) is necessary to ensure that no mould spoilage or mycotoxin contamination occurs (Magan, Aldred, Mylona, & Lambert, 2010).

Contamination of wheat flour from markets in the metropolitan

**Table 3**
Calibration parameters of instrumentation for aflatoxins and ochratoxin A detection and quantification.

| Standard | | $AFB_1$ | $AFB_2$ | $AFG_1$ | $AFG_2$ | OTA |
|---|---|---|---|---|---|---|
| Calibration curve | | y = 317.17x + 106.7 | y = 905.73x + 111.38 | y = 92.47x + 33.979 | y = 157.79x + 17.951 | y = 110.14x + 3.9186 |
| Correlation coefficient ($R^2$) | | 0.9996 | 0.9995 | 0.9996 | 0.9992 | 0.9992 |
| Linearity range (ng/mL) | | 0.5–50 | 0.15–15 | 0.5–50 | 0.15–15 | 0.1–20 |
| Limits | LOD[a] (μg/kg) | 1.2 | 0.4 | 1.1 | 0.5 | 0.7 |
| | LOQ[b] (μg/kg) | 3.5 | 1.2 | 3.2 | 1.5 | 2.0 |

$R^2$: Correlation coefficient.
[a] LOD: limit of detection of the chromatographic method.
[b] LOQ: limit of quantification of the chromatographic method.

**Table 4**
Accuracy and precision of the analytical methods for aflatoxins and ochratoxin A.

| | $AFB_1$ | $AFB_2$ | $AFG_1$ | $AFG_2$ | OTA |
|---|---|---|---|---|---|
| Spiking level (μg/kg) | 10 | 3 | 10 | 3 | 10 |
| Mean Recovery (%) | 64.1 | 72.8 | 78.0 | 87.3 | 103 |
| RSDr (%)[a] | 4.5 | 2.0 | 4.4 | 1.9 | 9.2 |

[a] $RSD_r$: Repeatability relative standard deviation.

region of Rio de Janeiro, Brazil, with AFs has been reported earlier by Trombete et al. (2014). In whole wheat flour samples ($n$ = 26) and refined wheat flour ($n$ = 15) 7.7% and 6.7%, respectively were positive for at least one aflatoxin (3.4 and 1.2 μg/kg, respectively), although at levels lower than the limit established by Brazilian legislation (5 μg/kg).

Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, and Hedili (2008) evaluated samples from markets and traditional family reserves in Tunisia and reported that 31.9% of cereals samples, including wheat and derived products, were contaminated with AFs (6.7 μg/kg) and AFB1 (2.2 μg/kg), with concentrations higher than the levels established by the EU. In Bulgaria, AFs were found to be the predominant mycotoxins in wheat (69%) with an average level of 17 μg/kg. On the other hand, OTA was found in 16 out of 60 (26.7%) wheat flour samples from Turkey, at concentrations levels of 0.247 μg/kg (Kara, Ozbey, & Kabak, 2015) and in Germany samples values of 5.49 μg/kg were detected in rye meal bread (Zinedine, Juan, Idrissi, & Mañes, 2007).

## 4. Conclusion

Considering the results obtained from this study, it was concluded that the physicochemical analyses of wheat and rye flours were within the limits established by the Portuguese legislation and in accordance with the information procedure in the field of technical standards and rules previewed by the European Parliament and Council, in order to safeguard the competitive capacity of the national food industries concerning the European market (European Commission, 2016). In this sense, increasing the European consumer's confidence, and the Portuguese ones in particular, in the products that they buy and consume daily. Wheat and whole wheat types (T 55, T 65 and T 150) are appropriate for the manufacture of baked and pastry products since they had a superior amount of protein that provides a higher concentration of gluten.

On the other hand, the rye flour contains trace amounts of gluten, which makes it suitable for anyone who is trying to reduce the amount of inflammatory reaction caused by gluten in the diet; and contains complex carbohydrates, which have slower digestion and maintain satiety for longer time. Regarding the microbiological analysis, in general the whole rye samples presented higher contents in microorganisms when compared with the whole wheat flours. The wheat flours presented no significant differences between refined and whole samples, except in the molds counting (the whole samples exhibited higher content in these microorganisms). From the point of view of mycotoxins, contamination was not detected, which guarantees the safety of this product.

However, it should be stressed that the present study is a preliminary survey focused on one set of samples. It would be interesting to continue this study examining, for example, quality and safety parameters along the storage time.

## Declaration of interests

None declared.

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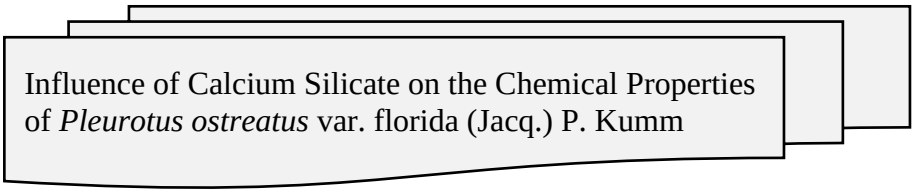
**Annex C**


Influence of Calcium Silicate on the Chemical Properties
of *Pleurotus ostreatus* var. florida (Jacq.) P. Kumm

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# Influence of Calcium Silicate on the Chemical Properties of *Pleurotus ostreatus* var. florida (Jacq.) P. Kumm

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**Abstract:** Supplementation of mushroom substrates has been linked to a higher resistance against insect pests, although few studies show the impact of this supplementation on the different agronomical parameters of mushrooms or even their chemical composition. In this work, the variation in the biological and chemical composition of oyster mushroom (*Pleurotus ostreatus* var. florida (Jacq.) P. Kumm) was analysed after varying the substrate supplementation of calcium silicate (0, 0.5, 1, 2, and 4% (*w*/*w*%)) during two harvest flushes. Overall, supplementation did not change the weight, the number of fruiting bodies, biological efficiency, yield ratio, and productivity rate of the mushrooms, although the harvest flushes did show significant differences. Furthermore, slight changes were found in the chemical composition with an increase in vitamin $D_2$ and tocopherols for the mushrooms with higher amounts of calcium silicate. Overall, the substrate supplementation did not seem to induce expressive changes or decrease production yields, and can, therefore, continue to be researched as a potential application to fight agronomical pests.

**Keywords:** *Pleurotus ostreatus* var. florida (Jacq.) P. Kumm; substrate supplementation; harvest; biological production; chemical composition

## 1. Introduction

Mushrooms have been a highly appreciated food since ancient times due to their nutritional value and medicinal properties. They are rich sources of compounds, namely, ergosterol (precursor of vitamin $D_2$), phenolic compounds, tocopherols, ascorbic acid, and carotenoids, responsible for the bioactive properties attributed to mushrooms [1].

An edible mushroom that has attracted quite a lot of interest in recent years is *Pleurotus ostreatus* (Jacq.) P. Kumm. (pearl oyster mushroom, tree oyster mushroom) due to the ease of its cultivation, great economic potential, and flavor [2,3]. There is, therefore, the need to further study its composition in bioactive compounds to better understand their health benefits, but also increase crop yields, thus increasing its production.

One variety of the oyster mushroom, the florida variety, also known as "Hiratake" is among the most consumed in the world, with its benefits being widely described [4,5]. Royse et al. [6] considered these species the second most popular cultivated mushroom of the last decades behind shiitake mushroom (*Lentinula edodes*), due to the easiness of its cultivation which can be achieved using lignocellulosic wastes.

Silicon (Si) is the second most abundant element in the earth's crust and its effect on organisms may be different, serving as a cellular chemical element or providing a natural morphological modification [7–11]. As a cellular chemical element, it plays an important role in the mineral nutrition of plants, aiding development by increasing the organism's biomass. Furthermore, it also increases resistance to biotic and abiotic stresses, such as diseases and pests, excess of toxic metals, saline stress, water deficiency, among others [7]. Some studies indicate that Si affects the nutritional status of crops, and thus, it is believed that Si influences nutrient absorption and nutritional efficiency of plants.

On the other hand, calcium silicate is also used to provide a natural morphological modification in insects, that is, when ingested occurs damage to the oral apparatus of pests that attack plants, due to its crystalline physical structure [9,12–16]. Previous studies have reported reduced insect feeding on turf treated with calcium silicate, as well as others that show that calcium silicate enhances resistance of sugarcane to the African talk borer *Eldana saccharina* [13,17].

Over the past 20 years, the benefits of Si supplementation in crops have been disseminated, however, much of this information has not been consolidated, and to the authors' best knowledge, there are very few studies on the supplementation of Si in oyster mushroom cultivation. One report on the use of Si on oyster mushrooms was published by Thongsook and Kongbangkerd [8] and described the influence of calcium and silicon on the biological and production yields of *P. ostreatus*.

In this work, the aim was to verify the effect of the crystalline structure on the cell expansion of the fungus, providing an increase for ergosterol inside the cells of the harvested mushrooms. Another concern was to assess the harmful effect on mushroom yield. Therefore, two strains of *Pleurotus ostreatus* var. florida were cultivated in varying concentrations of calcium silicate supplementation (0, 0.5%, 1%, 2%, and 4% (*w*/*w*)) and later harvested in two flushes, ten days apart. The mushrooms were analysed in terms of the total weight of harvested mushrooms, the number of mushrooms per flush, biological efficiency, yield ratio, and productivity rate. Furthermore, the chemical composition was analysed for the following groups of compounds: soluble sugars, tocopherols, individual fatty acids, ergosterol, and its derivative, vitamin $D_2$. The main goal was to verify the influence of substrate supplementation with Si on the viability of the mushrooms, but also the chemical profile.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Acetonitrile 99.9%, n-hexane 95%, and ethyl acetate 99.8% of HPLC grade were purchased from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sugar (D(-)-fructose, D(-)-mannitol, and D(+)-trehalose), tocopherol (α-, β-, and γ-isoforms), while Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). Dimethylsulfoxide (DMSO) (Fisher Scientific, Loughborough, UK) was used as a solvent in ergosterol and vitamin $D_2$ assays. Methanol, ethanol, hexane, and all other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

### 2.2. Mushroom Strains

The strains of *Pleurotus ostreatus* var. florida (Jacq.) P. Kumm used in this study were collected from producers in the city of Mogi-das-Cruzes (coordinates: –23.52327, –46.2659766) henceforth

described as MC (Funghi & Flora company, Valinhos City, Sao Paulo State, Brazil, codified as FF 20), and another in the city of Presidente Prudente (–21.9629448, –51.6337427), described as PP (Brasmicel company, Suzano City, Sao Paulo State, Brazil, codified as PF 14), both in the state of São Paulo in Brazil. The samples had been identified and their strains were deposited with the following codifications (POS 16/01 and POS 16/02) at the "Centro de Estudos em Cogumelos (CECOG)", Faculty of Agrarian and Technologic Sciences of the São Paulo State University (Universidade Estadual Paulista–UNESP, Sao Paulo State, Brazil), at Campus Dracena in the city of Dracena [18]. The inoculum was prepared by subculturing the mushroom, following the steps of production of subculture (petri dish with potato dextrose agar (PDA)), production of mother spawn, and production of grain spawn (sorghum with lime and gypsum) for compost inoculation, as described by Andrade et al. [19].

### 2.3. Substrate and Supplementation

The substrate was made using a mixture of sugarcane bagasse (500 kg), *Brachiaria dictyoneura* (1000 kg), rice bran (100 kg) and wheat bran (100 kg), calcitic limestone (50 kg), and gypsum (50 kg), as described by Zied et al. [20]. During phase I of the process, *B. dictyoneura* and sugarcane bagasse (bulk material) were moistened for 2 days. On the 3rd day, the pile was assembled, and on the 4th day, the pile was turned and, then, the additional materials (rice and wheat bran, calcitic limestone, and gypsum) were added. Afterward, two more turns were performed and on the 7th day, the substrate was transferred to a pasteurization chamber (phase II). The substrate was pasteurized between 65 and 72 °C for 20 h and subsequently conditioned between 55 and 48 °C for 1 day.

After pasteurization and conditioning, with the substrate at ambient temperature, the strains (PP and MC) were inoculated (2% of the substrate's wet weight) together with the addition of calcium silicate (concentration of 0.5, 1, 2, and 4% substrate's wet weight), in plastic bags containing 2 kg of wet substrate, following supplementation methodologies during spawning [21–23]. A control treatment was used as a reference (without the application of calcium silicate) to verify the viability of the technique. Each treatment had 5 repetitions, represented by bags of 2 kg of the substrate. The chemical-physical composition of the substrate used was N content—1.03%, C/N ratio—63/1, pH—6.7, and moisture—67%.

### 2.4. Growing Cycle

During the spawn run the air temperature and relative humidity were maintained at 26 ± 1.5 °C and 80%, respectively. For the induction of primordia and harvest, the temperature was reduced to 22 ± 1.5 °C while the relative humidity was increased up to 90%. The mushrooms were harvested twice (1st flush started 27 days after inoculation and 2nd flush started 39 days after inoculation) during the crop cycle, totalling 45 days of the growing cycle. The interval between flushes varied from 3 to 7 days depending on the strain and the calcium silicate percentages. After harvesting, the number of mushrooms per harvest and per supplementation was recorded, as was their weight. Prior to the chemical analysis, all the fruiting bodies (samples) were lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas, MO, USA) and reduced to a fine powder (20 mesh).

### 2.5. Influence of Calcium Silicate in Biological Production Yields

#### 2.5.1. Biological Efficiency

The biological efficiency (BE) is an important parameter to understand the capacity of the substrate to produce mushrooms, and thus, was calculated with the equation published by Thongsook and Kongbangkerd [8], for each flush:

$$BE(\%) = \frac{fresh\ weight\ of\ mushrooms\ (g)}{weight\ of\ dry\ substrate\ (g)} \times 100 \quad (1)$$

Equation (1). Biological efficiency of the mushrooms.

#### 2.5.2. Yield Ratio

The yield ratio is an analysed variable widely adopted in commercial crops, and was calculated following the equation:

$$Y(\%) = \frac{fresh\ weight\ of\ mushrooms\ (g)}{fresh\ weight\ of\ substrate\ (g)} \times 100 \quad (2)$$

Equation (2). Yield ratio of the mushrooms.

#### 2.5.3. Productivity Rate

The productivity rate provides an indication of how well the crop reacts to an input by providing outputs, thus, being a reason between BE and the precocity, namely days between inoculation and harvest:

$$PR(\%\ per\ day) = \frac{biological\ efficience\ (\%)}{precocity\ (days)} \quad (3)$$

Equation (3). Daily productivity rate of the mushrooms.

### *2.6. Chemical Composition*

#### 2.6.1. Soluble Sugars

For the analysis of soluble sugars, an extraction was performed with ethanol and water, followed by a filtration, previously described by Barros et al. [24]. Furthermore, the identification was performed on a Knauer High Performance Liquid Chromatograph (HPLC) (Knauer, Smartline system 1000) coupled with a refractive index detector (RI, Knauer, Berlin, Germany). The compounds were identified by comparing their retention times to the ones of commercial standards. The quantification was based on the RI signal response of each standard using the internal standard (IS) methodology, using raffinose as the IS. The HPLC conditions were as following: the mobile phase was a mixture of acetonitrile/water (70:30, *v/v*) with a flow rate of 1 mL/min. The chromatographic separation was achieved using a Eurospher 100-5 $NH_2$ column (5 µm, 250 × 4.6 mm, Knauer, Berlin, Germany) at 35 °C. Data were analysed using Clarity 2.4 software (DataApex, Podohradska, Czech Republic) and the results were reported in g per 100 g of dry weight (dw).

#### 2.6.2. Tocopherols

All four isoforms of tocopherols were screened and, if detected, quantified through HPLC after extraction with hexane, methanol, and water, as previously described by Barros et al. [24] using tocol as an IS. Chromatographic separation was performed with a Polyamide II normal-phase column (250 × 4.6 mm; YMC Waters) operating at 35 °C, and the mobile phase employed was a mixture of n-hexane and ethyl acetate (70:30, *v/v*) at a flow rate of 1 mL/min. This analysis was done using the equipment described for the soluble sugars, although coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA), designed for excitation at 290 nm and emission at 330 nm. Quantification was based on IS methodology, and the compounds were identified by chromatographic comparisons with commercial standards. Data were evaluated using Clarity 2.4 software and the results were expressed in mg per 100 g dry weight (dw).

#### 2.6.3. Ergosterol and Vitamin $D_2$

Ergosterol and vitamin $D_2$ were determined after an extraction procedure previously described by Tsai at al. [25] with some modifications. Each sample (1 g) was extracted with 10 mL of dimethyl sulfoxide (DMSO) using an ultrasound bath (30 min at 45 °C, series LBX V05, Barcelona, Spain), followed by the addition of 10 mL of methanol/water (1:1, *v/v*) and 20 mL of hexane with re-extraction in an ultrasound bath. The samples were then centrifuged thrice (3000 rpm, 10 min, Centurion K24OR refrigerated centrifuge, West Sussex, UK), adding 20 mL of hexane, and removing the supernatant

between each step. At the end of the extraction, the supernatants were pooled and dried using a rotary evaporator (40 °C, Büchi, Flawil Switzerland) and finally re-dissolved in 1 mL of methanol.

Using the HPLC described previously, and a UV detector (Knauer Smartline 2500), the identification and quantification of ergosterol and vitamin $D_2$ was performed according to the procedure validated by Barreira et al. [26] using 280 nm as the preferred wavelength. Chromatographic separation was performed with an Inertsil 100A ODS-3 reverse phase column (5 µm, 250 × 4.6 mm, BGB Analytik AG, Boeckten, Switzerland) at 35 °C. The mobile phase used was a mixture of methanol:acetonitrile (70:30, *v/v*), with a flow rate of 1 mL/min. Data were analysed using Clarity 2.4 software. Ergosterol (Sigma-Aldrich, St. Louis, MO, USA) was quantified based on a calibration curve obtained with a commercial standard. Vitamin $D_2$ was expressed in µg/100 g and ergosterol in mg per 100 g of mushroom, both in dw.

#### 2.6.4. Fatty Acids

Fatty acids were determined by gas chromatography (GC) coupled with a flame ionization detector (FID), after a transesterification procedure [24]. The analysis was carried out on a DANI model GC 1000 equipped with a split/splitless injector, the FID at 260 °C, and a Zebron-Kame column (30 m × 0.25 mm ID × 0.20 µm *df*, Phenomenex, Lisbon, Portugal). The initial column temperature was 100 °C, maintained for 2 min, then 10 °C/min until 140 °C, 3 °C/min until 190 °C, 30 °C/min until 260 °C and maintained for 2 min. Carrier gas flow (hydrogen) was 1.1 mL/min, measured at 100 °C. The split injection (1:50) was done at 250 °C. By comparing the relative retention times of FAME (*Fatty Acid* Methyl Esters) peaks of the samples with the standards, the fatty acid was identified. The results were recorded and processed using Clarity software (DataApex, Petrzilkova, Czechia) and fatty acids were expressed as a relative percentage.

### *2.7. Statistical Analysis*

Throughout the manuscript, all data are expressed as mean ± standard deviation. Samples were analysed through two-way analysis of variance (ANOVA) with type III sums of squares, after verifying homoscedasticity through a Levene's test. The post-hoc test used was either a Tukey's multiple test (homoscedastic sample) or Tamhane T2 test (non-homoscedastic samples) for the different Calcium Silicate supplementation and a Student's T test for the two harvest periods. Using a two-way ANOVA, it is possible to verify the influence of the two factors; Harvest number (HN) and calcium silicate supplementation (CS) independently from each other. If a significant interaction is detected among the two factors (HN × CS $p < 0.05$), they were evaluated simultaneously, and some tendencies can be extracted from the Estimated Marginal Means Plot (EMM). Inversely, if there is no significant interaction recorded among the two factors (HN × CS $p > 0.05$), they were analysed and classified independently using the post-hoc tests described above. Thus, the standard deviations were calculated from results obtained under different operational conditions, and should, therefore not be regarded as a measure of precision, rather as the range of the recorded values. All statistical analysis was performed using a significance of 0.05 and the SPSS software, version 25.

## 3. Results and Discussion

The focus of this work was to determine the influence of calcium silicate addition to the substrate of *Pleurotus ostreatus* var. florida mushrooms and assess its influence on biological efficiency, crop yield ratio, and chemical composition.

### *3.1. Biological Efficiency and Crop Yield Ratio*

Table 1 shows five parameters of the mushroom crop with the supplementation of CS during the two harvest periods (1st and 2nd flushes), namely mushroom weight, number of mushrooms per harvest, biological efficiency, yield ratio, and productivity rate. Table 1 is divided into two sections, each one pertaining to the locations of the strains, namely "Presidente Prudente" (PP)

and "Mogi-das-Cruzes" (MC). Each section is further divided into an upper and lower section, referring to the two factors, namely Harvest Number (HN) and Calcium Silicate supplementation (CS). This sectioning of the tables derives from the two-way ANOVA employed to understand the influence of each factor individually, and thus, in the upper section of Table 1, within the ranges of the means are all the values from the different CS, namely 0, 0.5, 1, 2, and 4%, and in the lower sections are all the values from harvests. This type of representation of the results allows for a much more trustworthy interpretation of the results, for each factor that caused change can be analysed independently of the influence of the other. The existence of an interaction between both factors is demonstrated by HN × CS $p < 0.05$ and thus only general conclusions can be extracted, whilst HN × CS $p > 0.05$ shows that each factor can be classified independently.

Analysing the strains from Presidente Prudente, it is clear that there was no interaction among the two factors for any of the analysed parameters. Still, no significant difference was recorded among the different supplementation concentrations ($p > 0.05$), while there is a significant difference amongst the two harvest periods ($p < 0.05$), being clear that all parameters are reduced in the second harvest when compared to the quantities of the first. The same tendencies can be observed for the strain from Mogi-das-Cruzes, with no statistical differences among the supplementation quantity of CS, but with a significant reduction from the first harvest to the second (classified with an *). One interesting conclusion that can be immediately extracted from the agronomical parameters investigated in this study is that calcium silicate supplementation of the substrate does not influence the total weight of the mushrooms or the number of mushrooms per harvest. Furthermore, the biological efficiency is also untouched as is the yield ratio and the productivity rate in either harvests (1st and 2nd flushes). Previous studies report similar findings, namely that the yield of production was not affected by the incorporation of calcium sources [8]. This is a positive effect, provided that supplementation would not be successful if it reduced any type of yield or productivity. For instance, the first harvest of Presidente Prudente saw a reduction from 167 to 110 g and a reduction of mushroom from 50 in the first harvest and 35 in the second.

The biological efficiency for Mogi-das-Cruzes was reduced from 108% in the first harvest to 84% in the second. Supplementation did not, however, excerpt a positive effect on yields, by increasing them either on the first or second harvest. Still, and as expected, the yields of the first harvest are almost always higher than the ones of the second, and the treatment of calcium silicate did not change this. The decrease in yield in the second harvest of *Pleurotus ostreatus* var. florida was reported by Thongsook et al. [8] and it's natural in mushroom production once the substrate begins to be spent.

### 3.2. Effect of Supplementation on Individual Compounds

Table 2 shows the compounds that were detected in the chemical characterization of the mushrooms, namely ergosterol and its derivative, vitamin $D_2$, along with various soluble sugars like fructose, mannitol, and trehalose, and also three tocopherols $\alpha$-, $\beta$-, and $\gamma$-. Regarding ergosterol, the main sterol in mushrooms, it was found in much higher amounts than its derivative, vitamin $D_2$. The amounts of these two compounds were similar in both mushroom strains. Trehalose was the highest sugar in both strains, with a maximum of 19 g/100 g while mannitol and fructose did not score over 2.8 and 0.2 g/100 g, respectively.

Of the four tocopherol isoforms, only $\delta$-tocopherol was not identified. This is not very uncommon, provided that in *Pleurotus ostreatus* var. florida mushrooms, not all isoforms are usually present [27,28]. Still, in both mushrooms, $\beta$-tocopherol was the most abundant. Regarding PP mushrooms, there was a significant interaction among the two factors, HN and CS, allowing for some general tendencies from the EMM plots (Figure 1). Still, regarding strains from MC, $\alpha$-tocopherol showed that the mushrooms from the first harvest presented a statistically higher amount of this isoform than the second harvest, while all other compounds displayed a significant interaction. Some tendencies were drawn from the EMM plots that allowed for some further analysis. These results show that, once again, supplementation of the substrate with CS does not seem to alter the chemical composition of these mushrooms.

**Table 1.** Biological parameters of the two strains during the two harvest periods and different supplementation concentrations.

| | | Mushroom Weight (g) | Number of Mushrooms | Biological Efficiency (%) | Yield Ratio (%) | Productivity Rate (% per Day) |
|---|---|---|---|---|---|---|
| **Presidente Prudente (PP)** | | | | | | |
| Harvest number (HN) | First flush | 167 ± 52 * | 50 ± 21 * | 104 ± 32 * | 8 ± 2 * | 4 ± 1 * |
| | Second flush | 110 ± 41 | 35 ± 16 | 69 ± 25 | 5 ± 2 | 2.0 ± 0.8 |
| *p*-value ($n$ = 10) | Student T test | <0.001 | 0.009 | <0.001 | <0.001 | <0.001 |
| Calcium silicate supplementation (CS) | Control | 164 ± 65 | 40 ± 22 | 103 ± 41 | 8 ± 3 | 3 ± 2 |
| | 0.5% | 147 ± 59 | 43 ± 16 | 92 ± 36 | 7 ± 3 | 3 ± 1 |
| | 1% | 129 ± 45 | 47 ± 27 | 81 ± 28 | 6 ± 2 | 3 ± 1 |
| | 2% | 124 ± 52 | 39 ± 21 | 77 ± 32 | 6 ± 2 | 3 ± 1 |
| | 4% | 129 ± 48 | 43 ± 16 | 80 ± 30 | 6 ± 2 | 3 ± 1 |
| *p*-value ($n$ = 25) | Tukey's HSD test | 0.296 | 0.897 | 0.296 | 0.296 | 0.571 |
| HN×CS ($n$ = 50) | *p*-value | 0.826 | 0.332 | 0.826 | 0.826 | 0.697 |
| **Mogi-das-Cruzes (MC)** | | | | | | |
| Harvest number (HN) | First flush | 172 ± 27 * | 46 ± 6 * | 108 ± 13 * | 9 ± 1 * | 4 ± 1 * |
| | Second flush | 135 ± 68 | 33 ± 4 | 84 ± 42 | 7 ± 2 | 2 ± 1 |
| *p*-value ($n$ = 10) | Student T test | 0.044 | 0.031 | 0.044 | 0.044 | <0.001 |
| Calcium silicate supplementation (CS) | Control | 174 ± 79 | 48 ± 30 | 108 ± 49 | 9 ± 4 | 4 ± 2 |
| | 0.5% | 156 ± 43 | 35 ± 18 | 97 ± 27 | 8 ± 2 | 3 ± 1 |
| | 1% | 159 ± 50 | 41 ± 12 | 100 ± 31 | 8 ± 2 | 3 ± 1 |
| | 2% | 156 ± 44 | 43 ± 26 | 97 ± 27 | 8 ± 2 | 3 ± 1 |
| | 4% | 124 ± 94 | 30 ± 19 | 78 ± 59 | 6 ± 5 | 3 ± 2 |
| *p*-value ($n$ = 25) | Tukey's HSD test | 0.529 | 0.350 | 0.529 | 0.529 | 0.708 |
| HN×CS ($n$ = 50) | *p*-value | 0.455 | 0.264 | 0.455 | 0.455 | 0.430 |

In each row, for the Harvest number (HN), the asterisk (*) means different statistical differences among the two harvests, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

**Table 2.** Different bioactive compounds and soluble sugars detected in the two mushroom strains along the two harvests and CS supplementation concentrations.

| | | Ergosterol (mg/100 g) | Vitamin $D_2$ (µg/100 g) | Fructose (g/100 g) | Mannitol (g/100 g) | Trehalose (g/100 g) | Soluble Sugars (g/100 g) | $\alpha$-Tocopherol (mg/100 g) | $\beta$-Tocopherol (mg/100 g) | $\gamma$-Tocopherol (mg/100 g) | Total Tocopherols (mg/100 g) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Presidente Prudente (PP)** | | | | | | | | | | | |
| Harvest number (HN) | First flush | 107 ± 6 | 743 ± 133 | 0.16 ± 0.03 | 2.2 ± 0.2 | 19 ± 2 | 22 ± 2 | 0.002 ± 0.001 | 0.26 ± 0.03 | 0.05 ± 0.06 | 0.31 ± 0.07 |
| | Second flush | 107 ± 8 | 593 ± 73 | 0.2 ± 0.1 | 2.2 ± 0.6 | 15 ± 4 | 17 ± 4 | 0.002 ± 0.001 | 0.3 ± 0.07 | 0.06 ± 0.04 | 0.36 ± 0.05 |
| *p*-value ($n$ = 10) | Student T test | 0.288 | 0.031 | 0.007 | 0.811 | <0.001 | <0.001 | 0.580 | <0.001 | <0.001 | <0.001 |
| Calcium silicate supplementation (CS) | Control | 101 ± 8 | 496 ± 27 | 0.20 ± 0.03 | 2.8 ± 0.5 | 15 ± 3 | 18 ± 3 | 0.002 ± 0.001 | 0.32 ± 0.03 | 0.02 ± 0.01 | 0.34 ± 0.04 |
| | 0.5% | 109 ± 6 | 658 ± 71 | 0.13 ± 0.03 | 1.8 ± 0.4 | 18 ± 3 | 20 ± 3 | 0.002 ± 0.001 | 0.24 ± 0.04 | 0.009 ± 0.004 | 0.25 ± 0.04 |
| | 1% | 110 ± 1 | 734 ± 144 | 0.19 ± 0.08 | 1.91 ± 0.06 | 16 ± 8 | 18 ± 8 | 0.002 ± 0.001 | 0.34 ± 0.08 | 0.02 ± 0.01 | 0.36 ± 0.09 |
| | 2% | 113 ± 1 | 779 ± 90 | 0.16 ± 0.03 | 2.0 ± 0.2 | 19 ± 1 | 21 ± 2 | 0.001 ± 0.0001 | 0.26 ± 0.01 | 0.13 ± 0.01 | 0.39 ± 0.01 |
| | 4% | 101 ± 4 | 671 ± 89 | 0.2 ± 0.1 | 2.3 ± 0.2 | 18 ± 2 | 21 ± 2 | 0.001 ± 0.0001 | 0.23 ± 0.03 | 0.108 ± 0.004 | 0.34 ± 0.03 |
| *p*-value ($n$ = 25) | Tukey's HSD test | <0.001 | <0.001 | 0.006 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| HN×CS ($n$ = 50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Mogi-das-Cruzes (MC)** | | | | | | | | | | | |
| Harvest number (HN) | First flush | 111 ± 6 | 899 ± 167 | 0.14 ± 0.03 | 1.9 ± 0.2 | 19±2 | 21 ± 2 | 0.003±0.001 * | 0.2 ± 0.1 | 0.05 ± 0.03 | 0.25 ± 0.09 |
| | Second flush | 116 ±10 | 829 ± 175 | 0.18 ± 0.08 | 1.8 ± 0.4 | 17 ± 4 | 19 ± 4 | 0.0018 ± 0.0001 | 0.28 ± 0.06 | 0.04 ± 0.04 | 0.32 ± 0.05 |
| *p*-value ($n$ = 10) | Student T test | <0.001 | <0.001 | <0.001 | 0.062 | <0.001 | <0.001 | 0.009 | <0.001 | <0.001 | <0.001 |
| Calcium silicate supplementation (CS) | Control | 113 ± 4 | 555 ± 51 | 0.2 ± 0.1 | 2.3 ± 0.3 | 18 ± 2 | 20 ± 2 | 0.0025 ± 0.0009 | 0.261 ± 0.005 | 0.013 ± 0.003 | 0.28 ± 0.05 |
| | 0.5% | 123 ± 1 | 951 ± 90 | 0.16 ± 0.03 | 1.8 ± 0.4 | 17 ± 5 | 19 ± 6 | 0.0022 ± 0.0003 | 0.32 ± 0.08 | 0.011 ± 0.003 | 0.33 ± 0.08 |
| | 1% | 100 ± 3 | 880 ± 50 | 0.15 ± 0.01 | 1.6 ± 0.2 | 16 ± 3 | 18 ± 3 | 0.002 ± 0.001 | 0.1 ± 0.1 | 0.04 ± 0.03 | 0.2 ± 0.1 |
| | 2% | 117 ± 7 | 1022 ± 31 | 0.10 ± 0.02 | 1.63 ± 0.05 | 18 ± 2 | 19 ± 2 | 0.003 ± 0.001 | 0.23 ± 0.02 | 0.08 ± 0.02 | 0.31 ± 0.01 |
| | 4% | 114 ± 6 | 913 ± 30 | 0.19 ± 0.06 | 2.0 ± 0.2 | 20 ± 2 | 22 ± 2 | 0.0014 ± 0.0007 | 0.25 ± 0.03 | 0.087 ± 0.006 | 0.34 ± 0.03 |
| *p*-value ($n$ = 25) | Tukey's HSD test | <0.001 | <0.001 | 0.006 | <0.001 | <0.001 | <0.001 | 0.130 | <0.001 | <0.001 | <0.001 |
| HN×CS ($n$ = 50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.058 | <0.001 | <0.001 | <0.001 |

In each row, for the Harvest number (HN), the asterisk (*) means different statistical differences among the two harvests, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

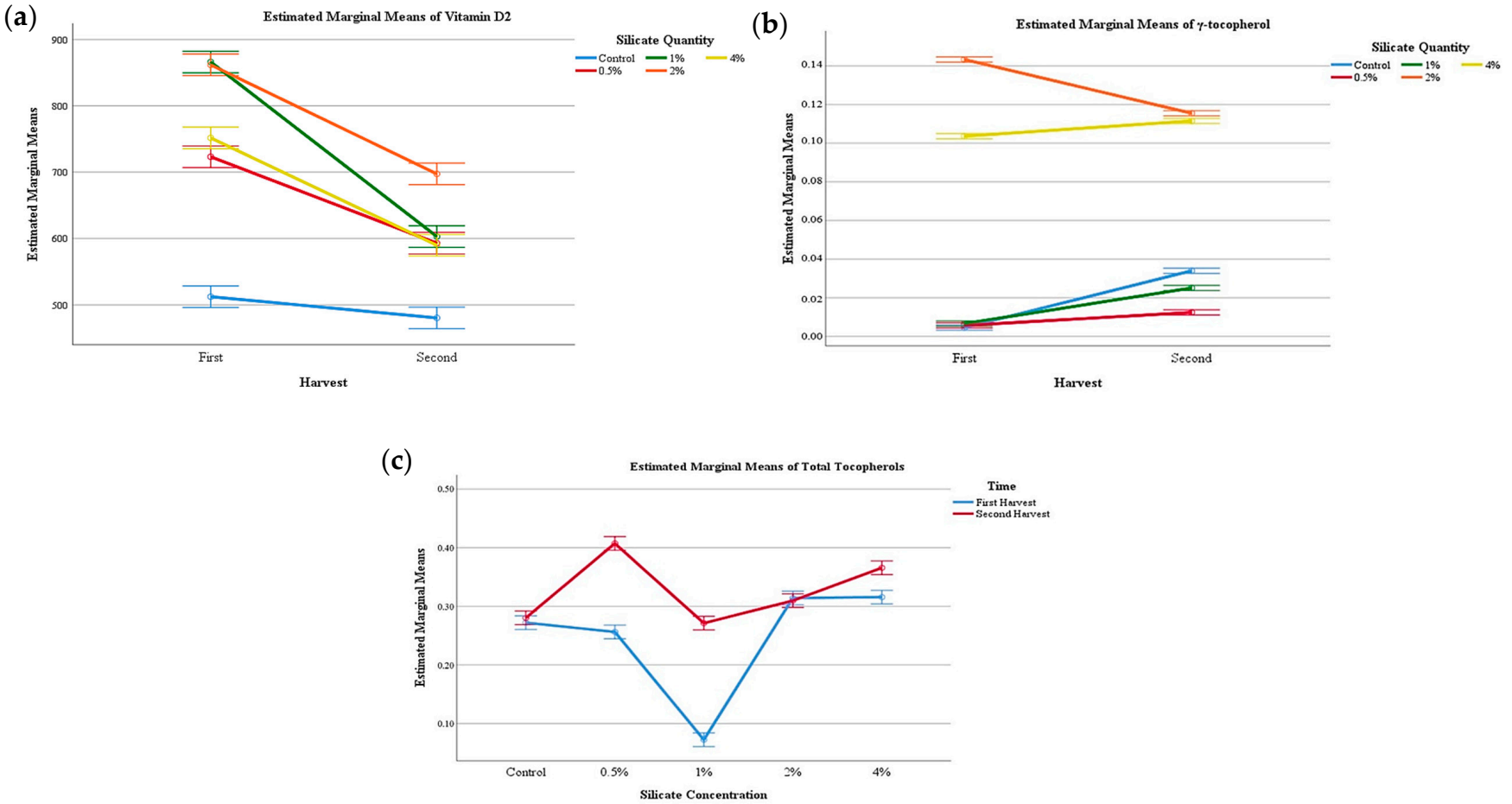


**Figure 1.** EMM plots of the Presidente Prudente mushrooms during the two harvest periods: (**a**) vitamin $D_2$, (**b**) $\gamma$-tocopherol. EMM plots of Mogi-das-Cruzes at different Si concentrations: (**c**) total tocopherols.

In Figure 1a the EMM plots of vitamin $D_2$ of Presidente Prudente mushrooms are shown, and it is clear that there was a reduction from the first to the second harvest in terms of this vitamin. Still, the control sample showed a lower reduction but was also the sample with a lower amount of this compound to start with. Thus, supplementation of calcium silicate showed higher amounts of this vitamin, although the highest results were found at 2%. Furthermore, even at the second harvest, there was a higher amount of vitamin $D_2$ than in the control samples. The fact that supplementation of calcium silicate increases the quantity of vitamin $D_2$ in mushrooms is quite interesting, provided this vitamin is a beneficial compound for human health. Vitamin D comprises other vitamers beyond $D_2$, although $D_2$ and $D_3$ are the most important ones. While $D_3$ is responsible for a higher saturation of vitamin D in the human blood, it can only be produced in our skin with the help of UV light, and thus, in countries with low sun incidence, most of this vitamin enters the human body through the diet. Still, animals mainly produce $D_3$, and with the tendency of the human diet to become more vegetarian based, $D_2$ becomes a promising alternative, being found mainly in mushrooms and plants.

All vitamers of vitamin D are important for the human body, especially by reducing the risk of osteoporosis and other ailments of the skeleton, beyond the adjuvant effects in cancer, cardiovascular diseases, among others [29]. Interestingly, the highest amount was recorded at a 2% concentration of calcium silicate, with the 4% resulting in a reduction in vitamin $D_2$. The results found for the Mogi-das-Cruzes strain show similar tendencies, with the highest amount of vitamin $D_2$ found at supplementation of 2%. Thus, supplementation of the mushroom substrate with calcium silicate (2%), increases the conversion of ergosterol to vitamin $D_2$ (ergocalciferol), which can be an important manner of increasing the natural production of this vitamer, becoming more available in the diet. Figure 1b shows the behaviour of $\gamma$-tocopherol in PP during the two harvest periods, and once again, an interesting pattern can be found, higher supplementation of calcium silicate seems to induce the production of this compound, while lower amount (up to 1%) and the control sample, although increasing from the first harvest to the second does not have as much as the 2 and 4% supplementation. The highest quantity found was for 2% supplementation at the first harvest, namely 0.13 mg/100 g, while the highest quantity in the control sample did not go over 0.035 mg/100 g, even at the second harvest. Figure 1c represents the total tocopherols of the Mogi-das-Cruzes strain for the several supplementation percentages, and once again lower supplementation of calcium silicate (1%) seems to reduce the quantity of total tocopherols, while higher amount seems to promote their production, especially 4%. Still, 0.5% CS promotes the production of tocopherols during the second harvest, which overall showed a higher value of total tocopherols in all tested percentage of CS, especially the lowers ones. This higher amount in the second harvest can be due to a stress induced by calcium silicate over the longer period of time, provided that tocopherols are synthesized in response to stress situation as a product of the secondary or defensive metabolism of plants and mushrooms [30,31].

Table 3 shows the different individual fatty acids found in the two different strains, represented as relative percentages of total fatty acids. The table only shows the most abundant fatty acids, namely the ones with a relative percentage above 1%. Other fatty acids like C10:0 (decanoic acid), C11:0 (undecanoic acid), C12:0 (dodecanoic acid), C14:0 (tetradecanoic acid), C16:1 (hexadecanoic acid), C17:0 (heptadecanoic acid), C18:3n3 ((9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid), C20:0 (eicosanoic acid), C20:1 (eicosenoic acid), C20:2 (Eicosadienoic acid), C20:5n3 ((5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid), C22:2 (docosanoic acid), C23:0 (tricosanoic acid), C24:0 (tetracosanoic acid) and C24:1 ((15Z)-tetracosenoic acid) were identified and quantified, but due to their very low amounts were not tabled and further discussed. Mushrooms have a very low amount of fat, and *Pleurotus ostreatus* var. florida is not an exception, the most abundant one is polyunsaturated, and thus the profile found in the studied mushrooms are consistent with the ones from literature, with C18:2n6c ((9Z,12Z)-octadeca-9,12-dienoic acid) being the most quantified, followed by C18:1n9c (octadec-9-enoic acid) and C16:0 (hexadecanoic acid) [27].

**Table 3.** Fatty acids detected through GC-FID from the two mushroom strains across the two harvest periods and CS supplementation, expressed in relative percentage of themselves. Only fatty acids with a relative above 1% are tabled, although others were identified and quantified.

| | | **C15:0** | **C16:0** | **C18:0** | **C18:1n9C** | **C18:2n6c** | **SFAs** | **MUFAs** | **PUFAs** |
|---|---|---|---|---|---|---|---|---|---|
| **Presidente Prudente (PP)** | | | | | | | | | |
| Harvest number (HN) | First flush | 1.4 ± 0.1 | 11.7 ± 0.4 | 2.8 ± 0.2 | 14.1 ± 0.3 | 65.8 ± 0.7 | 18.5 ± 0.6 | 14.6 ± 0.3 | 66.8 ± 0.7 |
| | Second flush | 1.7 ± 0.2 | 11.9 ± 0.5 | 2.7 ± 0.2 | 12.5 ± 0.9 | 66 ± 1 | 19 ± 1 | 13.0 ± 0.9 | 67 ± 1 |
| *p*-value ($n$ = 10) | Student T test | <0.001 | 0.025 | <0.001 | <0.001 | 0.002 | <0.001 | <0.001 | 0.006 |
| Calcium silicate supplementation (CS) | Control | 1.7 ± 0.3 | 11.7 ± 0.2 | 2.79 ± 0.06 | 13 ± 1 | 66.6 ± 0.7 | 18.7 ± 0.8 | 14 ± 1 | 67.6 ± 0.6 |
| | 0.5% | 1.5 ± 0.4 | 11.8 ± 0.2 | 3.0 ± 0.1 | 13 ± 1 | 66.4 ± 0.4 | 19.2 ± 0.7 | 13 ±1 | 67.3 ± 0.4 |
| | 1% | 1.3 ± 0.07 | 11.5 ± 0.2 | 2.7 ± 0.2 | 13.8 ± 0.4 | 66.9 ± 0.3 | 17.8 ± 0.1 | 14.3 ± 0.4 | 67.9 ± 0.4 |
| | 2% | 1.59 ± 0.07 | 12.1 ± 0.8 | 2.80 ± 0.07 | 13.9 ± 0.5 | 64.3 ± 0.8 | 20 ± 1 | 14.5 ± 0.4 | 65.4 ± 0.8 |
| | 4% | 1.6 ± 0.2 | 12.0 ± 0.6 | 2.65 ± 0.09 | 13.9 ± 0.5 | 66 ± 1 | 19.4 ± 0.5 | 13±1 | 67±1 |
| *p*-value ($n$ = 25) | Tukey's HSD test | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| HN×CS ($n$ = 50) | *p*-value | <0.001 | <0.001 | 0.001 | <0.001 | 0.002 | 0.001 | <0.001 | 0.001 |
| **Mogi-das-Cruzes (MC)** | | | | | | | | | |
| Harvest number (HN) | First flush | 1.29 ± 0.06 | 11.6 ± 0.3 | 2.7 ± 0.1 | 13.6 ± 0.6 | 66.4 ± 0.6 | 18.5 ± 0.6 | 14.1 ± 0.6 | 67.4 ± 0.6 |
| | Second flush | 1.6 ± 0.1 | 12.4 ± 0.3 | 2.8 ± 0.2 | 13 ± 1 | 66 ± 1 | 19.4 ± 0.5 | 13 ± 1 | 67 ± 1 |
| *p*-value ($n$ = 10) | Student T test | <0.001 | <0.001 | 0.543 | <0.001 | 0.045 | <0.001 | <0.001 | 0.146 |
| Calcium silicate supplementation (CS) | Control | 1.5 ± 0.1 | 12.2 ± 0.4 | 2.7 ± 0.1 | 13.9 ± 0.2 | 65 ± 1 | 19 ± 1 | 14.3 ± 0.2 | 66 ± 1 |
| | 0.5% | 1.4 ± 0.2 | 11.9 ± 0.6 | 2.7 ± 0.3 | 13 ± 1 | 66.8 ± 0.5 | 18 ± 1 | 13 ± 1 | 67.7 ± 0.5 |
| | 1% | 1.5 ± 0.3 | 11.9 ± 0.2 | 0.287 ± 0.03 | 13 ± 1 | 67.2 ± 0.8 | 18.6 ± 0.3 | 13.1 ± 0.1 | 68.3 ± 0.9 |
| | 2% | 1.39 ± 0.07 | 12.3 ± 0.4 | 2.7 ± 0.1 | 12.5 ± 0.1 | 66.6 ± 0.3 | 19.2 ± 0.2 | 13.1 ± 0.1 | 67.7 ± 0.3 |
| | 4% | 1.4 ± 0.1 | 11.9 ± 0.8 | 2.8 ± 0.1 | 14.0 ± 0.3 | 65.2 ± 0.8 | 19.2 ± 0.4 | 14.6 ± 0.4 | 66.1 ± 0.8 |
| *p*-value ($n$ = 25) | Tukey's HSD test | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | 0.001 | <0.001 | <0.001 |
| HN×CS ($n$ = 50) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

C15:0—Pentadecanoic acid, C16:0—Hexadecanoic acid, C18:0—Octadecanoic acid, C18:1n9C—Octadec-9-enoic acid, C18:2n6c-(9Z,12Z)-octadeca-9,12-dienoic acid (IUPAC nomenclature). SFAs—saturated fatty acids, MUFAs—monounsaturated fatty acids, PUFAs—polyunsaturated fatty acids. In each row, for the Harvest number (HN). The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

As expected, the PUFAs (polyunsaturated fatty acids) accounted for over 66% of all fatty acids, which makes these mushrooms very healthy foods due to the beneficial effects of unsaturated fatty acids on human health. Saturated and monounsaturated fatty acids showed approximately 13 to 14 and 18 to 19%, respectively, which makes the saturated fat very low. Overall, the unsaturated fatty acids accounted for approximately 80% of the total. There was a significant interaction among both factors, HN and CS, and thus no individual classifications could be made, so, where possible some general conclusions were extracted from the EMM plots, shown in Figure 2, although the overall fatty acid profile of the mushrooms did not change much due to the supplementation of CS. Still, by analysing Figure 2, section (a) in the PP mushrooms, an interesting pattern can be found for oleic acid (common name, C18:1n9c), where despite variations in the second harvest, the amounts of this molecule was never as much as the ones found in the first harvest, indicating that the best mushrooms in terms of this unsaturated fatty acid are found in the first harvest, and this supplementation only affects the second harvest, although ever so slightly. Inversely, all saturated fatty acids are found in higher amounts during the second harvest, and thus, the first harvest shows lower amounts for all CS concentrations. Still, at 1% of silicate supplementation, there seems to be a very drastic reduction in SFAs (saturated fatty acids) (Figure 2b). Finally, regarding section (c) of Figure 2, pertaining to Mogi-das-Cruzes strain, a similar pattern is found, with higher amounts of SFAs in the second harvest. Unfortunately, no patterns could be found in the EMM for the unsaturated fatty acids.

Overall, it seems that the variations in fatty acids are more related to the passage of time than with supplementation of CS, with SFAs being higher in the second harvests and unsaturated ones in the first harvest. This pattern could be due to the freshness of the substrate and higher amounts of compounds the mushrooms can absorb, and thus have higher unsaturated fatty acids, whilst the second harvest, with a lower amount of available nutrients in the substrate, shows a higher amount of saturated fatty acids [32]. There seems to be very little effect of CS supplementation on the overall profile of fatty acids, with time and available nutrients being the main reason for the changes in the profile.

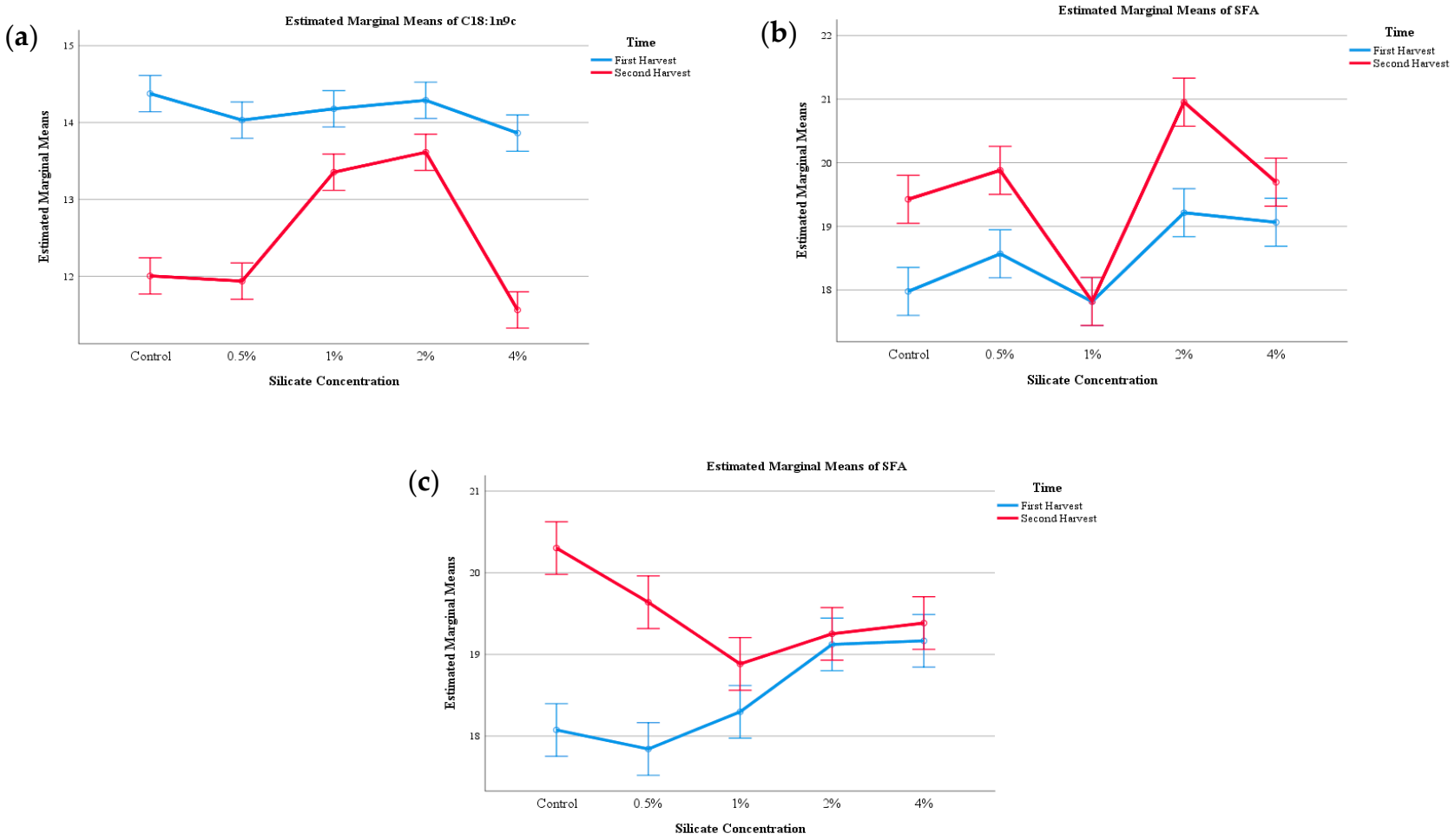


**Figure 2.** EMM plots of the Presidente Prudente mushrooms at different Si concentrations: (**a**) C18:1n9c (Octadec-9-enoic acid), (**b**) Saturated Fatty Acids (SFAs). EMM plots for Mogi-das-Cruzes: (**c**) SFAs.

## 4. Conclusions

Very little is known in terms of the effects of CS on the chemical composition and biological efficiency of mushrooms. In this work, the impact of CS on the biological and chemical composition of oyster mushrooms was studied. CS supplementation did not lower production yields and seems to have very light positive effects in terms of the production of vitamin $D_2$ and tocopherols. Interestingly, the harvest period, linked to the passage of time, seems to have a much deeper impact on the production yields (higher in the first harvest) and saturation of fatty acids.

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**Conflicts of Interest:** The authors state no conflict of interest.

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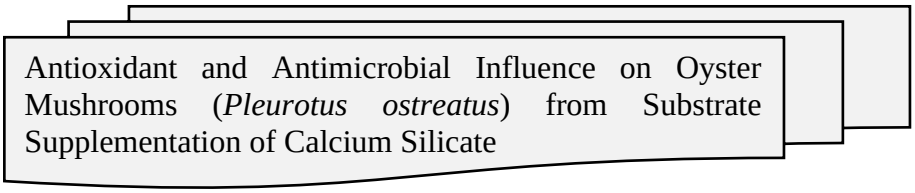
**Annex D**


Antioxidant and Antimicrobial Influence on Oyster Mushrooms (*Pleurotus ostreatus*) from Substrate Supplementation of Calcium Silicate

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# Antioxidant and Antimicrobial Influence on Oyster Mushrooms (*Pleurotus ostreatus*) from Substrate Supplementation of Calcium Silicate

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**Abstract:** Supplementation of the substrate of mushrooms with calcium silicate and other minerals is usually used as a preventive measure against pests and other contaminants during the production of oyster mushrooms. Little is known of the effects of this supplementation on the quality of the mushrooms produced. In the work described herein, the supplementation of oyster mushrooms was performed with 5 supplementation levels (0%, 0.5%, 1%, 2% and 4%) on mushrooms from two different locations in Brazil, the two flushes of mushrooms produced were analysed in terms of phenolic compounds, organic acids, and the antioxidant, antibacterial and antifungal activities, and finally the data was subjected to a linear discriminant analysis to understand the discrimination of the supplementation percentages. Overall, intermediate supplementation until 1% seemed to have a positive effect on the mushrooms from Mogi-das-Cruzes region, while high supplementation favoured the mushrooms from the region of Presidente Prudente. Supplementation showed positive effects on the mushrooms by increasing the production of some secondary metabolites while not showing any negative cytotoxic effects.

**Keywords:** oyster mushrooms; polyphenols; calcium silicate; antioxidant; antimicrobial



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## 1. Introduction

Bioactive compounds are found in many foods and are compounds capable of modulating metabolic processes and promoting health improvements, and have a biological value that goes beyond the nutritional value of the food. Examples of these compounds are polyphenols, carotenoids, tocopherols, organic acids, phytosterols, carbohydrates, fibres, vitamins C, A and E, and minerals, among others, which have antioxidant, antibacterial, and other health improving effects [1].

Mushrooms are very appreciated for having excellent nutritional and medicinal properties, being widely appreciated for their taste. Of all edible species of mushrooms, *Pleurotus ostreatus* (Jacq.) P. Kumm (oyster pearl mushroom) is one of the most cultivated and consumed, while also showing beneficial health properties [2,3]. Due to its broad importance to the human diet, there is a need to deepen the study of its composition in

bioactive compounds to better understand its health benefits, but also understand how to increase these bioactive compounds through sustainable methods.

Silicon and calcium are beneficial for plants and mushroom growth, they contribute towards the wall of the cells, thus helping to maintain textural integrity, while also stimulating the development of mushroom fruiting bodies. Supplementation with calcium in the form of calcium silicate improves post-harvest storage conditions, thus improving the quality of mushrooms and increasing their shelf life [4–7].

Calcium silicate bases have many applications and are used in different areas. They are used in the biomedical field, where they have shown great biocompatibility, antibacterial activity, and bioactivity; the latter plays a fundamental role in the regeneration and healing of tissues, and the performance of calcium silicate materials is largely attributable to their bioactivity [8–10]. It has also been widely used in the production and cultivation of different plant crops [11–15]. The high compatibility of calcium silicate and mushrooms when compared to other supplementation materials has attracted attention towards its supplementation in several crops.

There is currently no information on the possible effects of calcium silicate supplementation during cultivation on bioactive molecules of oyster mushrooms. A previous study showed the impact of calcium silicate on the agronomical yields and chemical composition of oyster mushrooms, pointing out that very slight changes were sought in terms of nutritional quality, but an increase in bioactive compounds like tocopherols and vitamin $D_2$ [3]. Following the results of that study, the effects of varying concentrations of calcium on the substrates of *P. ostreatus* were sought for the antioxidant, cytotoxic and antimicrobial activity, as well as the effects in polyphenols and organic acids.

## 2. Materials and Methods

### *2.1. Standards and Reagents*

High Performance Liquid Chromatography (HPLC) grade acetonitrile (99.9%), *n*-hexane (95%) and ethyl acetate (99.8%) were purchased from Fisher Scientific (Lisbon, Portugal). Organic acid standards and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and phenolic standards were acquired from Extrasynthèse (Genay, France). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were acquired from Oxoid microbiology products (Hampshire, United Kingdom). *p*-Iodonitrotetrazolium chloride (INT) from Panreac Applichem (Barcelona, Spain), Tryptic soy broth (TSB), and Mueller-Hinton (MH) from Biolab® (Hungary). All other reagents and solvents were of analytical grade and obtained from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

### *2.2. Samples*

One strain of *P. ostreatus* var. florida (Jacq.) P. Kumm was bought from producers of Mogi-das-Cruzes (coordinates: −23.52327, −46.2659766, company—Funghi & Flora) henceforth described as "MC", and another in Presidente Prudente (−21.9629448, −51.6337427, company—Brasmicel), described as PP, both in the state of São Paulo, in Brazil. The inoculum was prepared by subculturing the mushroom, following the steps of production of subculture (petri dish with potato dextrose agar), production of mother spawn, and production of grain spawn (sorghum with lime and gypsum) for compost inoculation, as described by [16]. Two flushes of mushrooms were harvested for each inoculum.

### *2.3. Bioactive Molecules*

#### 2.3.1. Organic Acids

Organic acids were identified and quantified following the procedures described and optimized previously by Barros, et al. (2013) [17] after extraction with metaphosphoric acid. The analysis was done by means of ultrafast liquid chromatograph (UFLC, Shimadzu 20A series, Shimadzu Corporation, Kyoto, Japan) coupled to photodiode array detector (PDA),

using 215 nm as the preferred wavelength. 3.6 mM of sulphuric acid was used as the mobile phase, at a flow rate of 0.8 mL/min. Quantification of organic acids was done by comparing the area of their peaks with calibration curves obtained from the commercial standards of each compound, using LabSolutions Multi LC-PDA software (Shimadzu Corporation, Kyoto, Japan), with the results being displayed in g per 100 g of dry weight (DW).

#### 2.3.2. Phenolic Compounds

For the analysis of phenolic compounds, each sample was placed in a beaker (~1 g) and was extracted by magnetic stirring with ethanol:water (30 mL, 80:20, *v*/*v*) at room temperature and 150 rpm, for 1h. The extract was separated from the residue by filtration through Whatman No. 4 paper in a round flask. The residue was re-extracted again under the same conditions and the filtrates were evaporated at 40 °C to remove ethanol (rotary evaporator, Büchi, Flawil Switzerland). Subsequently, the aqueous phase of each sample was frozen and lyophilized (freeze 4.5 FreeZone model 7750031, Labconco, Kansas, USA) to obtain the respective extracts. The hydroethanolic extracts obtained were dissolved in ethanol: water (80:20, *v*/*v* at 10 mg/mL) and filtered through a 0.22-µm disposable LC filter disk.

Phenolic compounds were determined in a Dionex Ultimate 3000 HPLC (Thermo Scientific, San Jose, CA, USA), coupled to a diode array detector (DAD, set at 280, 330, and 370 nm) and a mass detector (MS), and also equipped with a quaternary pump, an automated injector (5 °C), a degasser, and a temperature adjusted column chamber (35 °C) [18]. The detection of compounds was achieved through a diode array detector (DAD) set at 280 nm of wavelength and coupled to a mass detector. The separation of the compounds relied on a C18 columns (Waters Spherisorb S3 ODS-2 (3 µm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) operating at 35 °C and the mobile phase consisted of 0,1% of formic acid in water and acetonitrile, functioning in gradient mode. The gradient varied from 15% of acetonitrile for 5 min to 20% during another 5 min, then 10 min at 25%, another 10 min to 35% and finally another 10 min from 35% to 50%, being the column rebalanced from 10 min at a flux of 0.5 mL/min. The detection of masses was achieved using a Liner LTQ XL ion trap mass spectrometer (Thermo Finnigan, San Jose, Can, USA) equipped with an electrospray ionization source using nitrogen as the carrier gas [18]. The data was analyzed using Xcalibur software and the results were in µg per 100 g of DW.

### *2.4. Bioactivities*

#### 2.4.1. Antioxidant Activity

The thiobarbituric acid reactive substances (TBARS) inhibition and the oxidative hemolysis inhibition (OxHLIA) assays were performed for antioxidant activity evaluation. Trolox was used as a positive control in both in vitro assays. For the TBARS assay, the lyophilized extracts were dissolved in ethanol/water (80:20, *v*/*v*), and subjected to dilutions between 20 and 0.078 mg/mL. The inhibition of lipid peroxidation in porcine brain homogenates (*Sus scrofa*) was evaluated by the decrease in TBARS; the colour intensity of malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 515 nm. The inhibition rate (%) was calculated according to Spréa, et al. (2020) [19], and the results were expressed in $EC_{50}$ values (mg/mL, sample concentration providing 50% of antioxidant activity).

The antihemolytic activity used the OxHLIA assay, as described and optimized by Lockowandt, et al. (2019) [20]. An erythrocyte solution (2.8%, *v*/*v*; 200 µL) prepared in PBS (pH 7.4) was mixed with 400 µL of: (i) extract solution (6–500 µg/mL in PBS); (ii) PBS (control); (iii) water (for complete haemolysis); or (iv) trolox (7.81–250 µg/mL PBS). After pre-incubation at 37 °C for 10 min with shaking, 200 µL of AAPH (160 mM in PBS) were added and the optical density was measured at 690 nm every ~10 min in a microplate reader (Bio-Tek Instruments, ELX800) until complete hemolysis. The results were expressed in $IC_{50}$ values (µg/mL), meaning the concentration of extract capable of promoting a Δt delay in hemolysis of 60 and 120 min.

#### 2.4.2. Antimicrobial Activity

The antimicrobial activity was analysed according to the procedure described previously [21]. The following Gram (+) bacteria, *Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (food isolate) and *Listeria monocytogenes* (NCTC 7973), as well as Gram (-) bacteria *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 35030) and *Salmonella Typhimurium* (ATCC 13311), were tested; as for the tested micromycetes, the following were used: *Aspergillus fumigatus* (ATCC 2011), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* from the American Type Culture Collection (ATCC 9112) and *Penicillium verrucosum* var. cyclopium (food isolate). The microorganisms are deposited at Mycological laboratory, Department of Plant Physiology, Institute for Biological research "Sinisa Stanković", University of Belgrade, Serbia.

A microdilution method was implemented to perform the antimicrobial assay [22]. Bacterial/fungal suspensions were adjusted with sterile saline to a concentration of $1.0 \times 10^6$ CFU/mL. The mushroom extracts were dissolved in 30% ethanol, mixed with nutrient media for bacteria (Tryptic Soy Broth) or micromycetes (Malt medium) containing bacterial/fungal inoculum ($1.0 \times 10^5$ CFU per well) in a final volume of 100 µL.

Minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) were defined as described previously [21]. Ampicillin and streptomycin (Panfarma, Belgrade, Serbia) were used as positive controls for the antibacterial activity test, while the commercial antifungals bifonazole and ketoconazole (Srbolek, Belgrade, Serbia) were used as positive controls for antifungal assay. Thirty percent ethanol was used as a negative control.

#### 2.4.3. Cytotoxicity

The cytotoxicity of the mushrooms was analysed by means of the sulforhodamine B assay, as previously described by Barros, et al. (2013) [17]. Methanol:water extracts (80:20, *v*/*v*) were prepared for the cytotoxicity analyses, after that, they were redissolved in water to obtain stock solutions of 8 mg/mL and then subjected to further dilutions. Four human tumor cell lines were tested: NCI-H460 (non-small cell lung carcinoma), MCF-7 (breast adenocarcinoma), HepG2 (hepatocellular carcinoma), and HeLa (cervical carcinoma) of DSMZ (Leibniz-Institut DSMZ- Deutsche SammLung von Mikroorganismen und Zellkulturen GmbH). Each cell line was plated at a suitable density ($7.5 \times 10^3$ cells/well for MCF-7 and NCI-H460 or 1.010 cells/well for HeLa and HepG2) in 96-well plates and allowed to bind for 24 h.

To evaluate cytotoxicity in non-tumor cells, a culture of cells (called PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established by Tsukatani et al. 2011 [23]. The absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) [24]. Ellipticine was used as a positive control and the results were displayed at $GI_{50}$ values (µg/mL) (concentration which inhibited 50% of net cell growth).

### *2.5. Statistical Analysis*

Throughout the manuscript, all data are expressed as mean ± standard deviation. Samples were analysed through two-way analysis of variance (ANOVA) with type III sums of squares, after verifying homoscedasticity through a Levene's test. The post-hoc test used was either a Tukey's multiple test (homoscedastic sample) or Tamhane T2 test (non-homoscedastic samples) for the different Calcium Silicate supplementation and a Student's T test for the two harvest periods. Using a two-way ANOVA, it is possible to verify the influence of the two factors, Harvest number (HN) and calcium silicate supplementation (CS), independently from each other. If a significant interaction was detected among the two factors (HN × CS $p < 0.05$), they were evaluated simultaneously, and some tendencies can be extracted from the estimated marginal means (EMM) plot. Inversely, if there was no significant interaction recorded among the two factors (HN × CS $p > 0.05$), they were analysed and classified independently using the post-hoc tests described above. Thus,

the standard deviations were calculated from results obtained under different operational conditions, and should, therefore not be regarded as a measure of precision, rather as the range of the recorded values. All statistical analysis was performed using a *p*-value of 0.05 and using SPSS (version 25). A linear discriminant analysis (LDA) was performed to discriminate the different two harvests and percentage of supplementation. The LDA used the Wilk's λ test with and F-value of 3.84 for entering and 2.71 for removal, using the leave-one-out cross validation procedure.

## 3. Results and Discussion

This work focused on the effects of different percentages of calcium silicate supplementation on the different bioactivities and individual bioactive molecules of *P. ostreatus* mushrooms.

### *3.1. Organic Acids and Phenolic Compounds*

Table 1 shows the profile in organic acids and phenolic compounds of both mushrooms with the supplementation of CS during the two harvest periods (1st and 2nd flushes).

**Table 1.** Organic and phenolic acids detected through ultrafast liquid chromatograph (UFLC)-diode array detector (DAD) and HPLC-DAD-ESI/MS (electro-spray ionization coupled to a mass spectrometer), respectively, of the different mushroom provenances across the two harvest periods in DW.

| | | **Oxalic Acid (g/100 g)** | **Malic Acid (g/100 g)** | **Fumaric Acid (g/100 g)** | **Total Organic Acids (g/100 g)** | **Protoca techuic Acid (µg/100 g)** | ***p*-coumaric Acid (µg/100 g)** | **Cinnamic Acid (µg/100 g)** | **Total Phenolic Acids (µg/100 g)** |
|---|---|---|---|---|---|---|---|---|---|
| **Mogi-das Cruzes (MC)** | | | | | | | | | |
| Harvest Number (HN) | First | 0.19 ± 0.04 | 2.7 ± 0.3 | 0.250 ± 0.008 | 3.2 ± 0.3 | 116 ± 27 | 56 ± 17 | 36 ± 11 | 208 ± 48 |
| | Second | 0.22 ± 0.05 | 3.0 ± 0.3 | 0.257 ± 0.009 | 3.5 ± 0.3 | 177 ± 46 | 32 ± 12 | 30 ± 4 | 239 ± 56 |
| *p*-value (n = 15) | *t*-test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Calcium Silicate Concentration (CS) | Control | 0.22 ± 0.08 | 2.51 ± 0.3 | 0.251 ± 0.002 | 2.9 ± 0.3 | 137 ± 53 | 52 ± 1 | 26 ± 5 | 216 ± 57 |
| | 0.5% | 0.21 ± 0.03 | 3.1 ± 0.1 | 0.25 ± 0.02 | 3.5 ± 0.2 | 167 ± 74 | 33 ± 8 | 37 ± 2 | 237 ± 64 |
| | 1% | 0.16 ± 0.01 | 2.7 ± 0.2 | 0.250 ± 0.003 | 3.1 ± 0.2 | 146 ± 46 | 37 ± 6 | 27 ± 1 | 211 ± 41 |
| | 2% | 0.17 ± 0.02 | 2.87 ± 0.02 | 0.253 ± 0.005 | 3.30 ± 0.04 | 112 ± 16 | 41 ± 28 | 35 ± 12 | 187 ± 56 |
| | 4% | 0.24 ± 0.03 | 3.2 ± 0.3 | 0.259 ± 0.002 | 3.7 ± 0.2 | 170 ± 10 | 56 ± 21 | 40 ± 9 | 266 ± 21 |
| *p*-value (n = 6) | THSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| HN × CS (n = 30) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Presidente Prudente (PP)** | | | | | | | | | |
| Harvest Number (HN) | First | 0.20 ± 0.08 | 3.5 ± 0.8 | 0.28 ± 0.02 | 4.0 ± 0.8 | 140 ± 62 | 23 ± 8 | 21 ± 2 | 185 ± 67 |
| | Second | 0.3 ± 0.2 | 2.8 ± 0.4 | 0.27 ± 0.02 | 3.4 ± 0.3 | 193 ± 29 | 60 ± 19 | 32 ± 5 | 285 ± 38 |
| *p*-value (n = 15) | *t*-test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Calcium Silicate Concentration (CS) | Control | 0.3 ± 0.2 | 2.6 ± 0.2 | 0.27 ± 0.02 | 3.20 ± 0.04 | 113 ± 58 | 42 ± 31 | 24 ± 6 | 179 ± 94 |
| | 0.5% | 0.208 ± 0.008 | 4 ± 1 | 0.27 ± 0.04 | 4 ± 1 | 198 ± 41 | 51 ± 33 | 26 ± 6 | 274 ± 81 |
| | 1% | 0.3 ± 0.2 | 3.0 ± 0.6 | 0.278 ± 0.004 | 3.5 ± 0.4 | 190 ± 16 | 23 ± 5 | 31 ± 12 | 245 ± 33 |
| | 2% | 0.25 ± 0.03 | 3.5 ± 0.6 | 0.29 ± 0.01 | 4.1 ± 0.7 | 211 ± 13 | 51 ± 21 | 27 ± 2 | 290 ± 11 |
| | 4% | 0.25 ± 0.05 | 3.0 ± 0.3 | 0.252 ± 0.009 | 3.5 ± 0.3 | 122 ± 41 | 41 ± 10 | 25 ± 4 | 188 ± 55 |
| *p*-value (n = 6) | THSD test | <0.001 | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| HN × CS (n = 30) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. *t*-test represents a student's *t*-test, while THSD test means Tukey's honest significance test.

Table 1 is divided into two sections, each one pertaining to the locations of the strains, namely MC and PP. Each section is further divided into an upper and lower section,

referring to the two factors, namely HN and CS. This sectioning of the tables derives from the two-way ANOVA employed to understand the influence of each factor individually, and thus, in the upper section of Table 1, within the ranges of the means are all both the values of HN, and in the lower sections are all the values from CS, namely 0%, 0.5%, 1%, 2% and 4%. This type of representation of the results allows for a much more trustworthy interpretation of the results, for each factor that caused change can be analysed independently of the influence of the other. The existence of an interaction between both factors is demonstrated by HN × CS $p < 0.05$, and thus only allows for general conclusions which can be extracted from the estimated marginal means (EMM) plots, whilst HN × CS $p > 0.05$ shows that each factor can be classified independently using Tukey's HSD test to classify each factor individually.

Regarding organic acids, three were detected, namely oxalic acid, malic acid and fumaric acids, with malic acid being the most abundant one. Overall, a significant interaction was sought for all three organic acids for both mushroom provenances, as well as their total amount, and thus only some general conclusions could be extracted from the EMM plots, present in Figure 1.

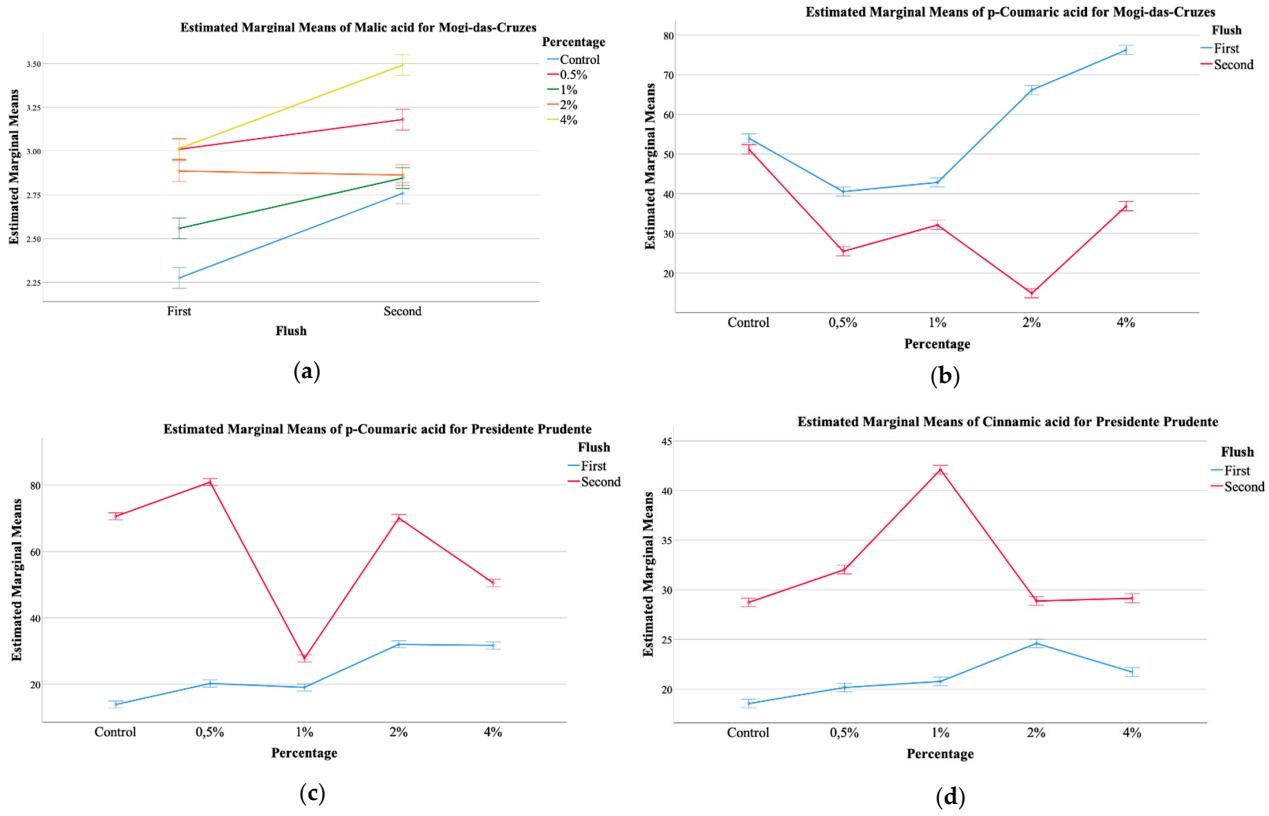



**Figure 1.** EMM plots of mushrooms from Mogi das Cruzes (**a**,**b**), (**a**) malic acid, (**b**) p-coumaric acid, and Presidente Prudente (**c**,**d**), (**c**) *p*-coumaric acid, (**d**) cinnamic acid, mushrooms at the two different harvest periods.

The plots showed that malic acid from MC mushrooms supplemented with calcium silicate (Figure 1a) showed higher values of this organic acid, and a further increase from the first to the second flush, which was positively correlated with the increasing percentage of calcium silicate. Malic acid is an important carboxylic acid with beneficial effects on health [12,25]. Its values in the mushrooms increased with the increase of calcium silicate supplementation. It seems that a supplementation with 0.5% of calcium silicate favors the production of malic acid, especially in the first flush. Still, supplementation of 4% does not seem to increase the amount of malic acid in the first flush when compared to supplementation with 0.5%, but greatly stimulates its production in the second flush. With regards to the phenolic compounds, once again, a significant interaction was detected for

all, both for MC and PP mushrooms. Of the main compounds detected, protocatechuic acid was the most abundant, followed by *p*-coumaric acid and finally cinnamic acid, which was the least abundant. The EMM plots in Figure 1 allow for some general tendencies, namely that for MC, supplementation with 4% shoed higher amounts of malic acid, while the control sample showed the least (Figure 1a), while the first flush showed higher values of *p*-coumaric acid with an increase related to the highest supplementation percentages, while for the second harvest this tendency was inverted (Figure 1b). Still, for PP, the second harvest showed higher values of *p*-coumaric acid, being this increase correlated with higher supplementation of calcium silicate (Figure 1c). Cinnamic acid, the least abundant phenolic compound showed and EMM plot for PP in which the highest amount was sought for the second harvest, that showed a maximum of this phenolic compound at 1% of supplementation with calcium silicate (Figure 1d).

### *3.2. Antioxidant Activity and Cytotoxicity in Non-Tumour Cell Line*

Table 2 shows the results of the two antioxidant activity assays carried out for the mushroom extracts, namely TBARS and OxHLIA, and once again a significant interaction was detected for both. The EMM plots show that for MC, in the OxHLIA assay, only the 4% supplementation of calcium silicate showed activity, although it reduced from the first to the second flush (Figure 2). For PP mushrooms, the supplementation with calcium silicate did not show any activity in the first flush, only showing activity in the second flush. In terms of TBARS assay, no EMM plots could be shown, although Table 2 shows a significant interaction between the flushes and the calcium silicate concentration implying that the slight changes found for this assay was due to both of the factors.

Both samples were tested against a porcine liver primary cell line, showing no cytotoxicity at the tested concentrations, which indicates that the supplementation of calcium silicate in the mushroom substrate does not induce any cytotoxic effect. This result highlights the use of calcium silicate as a stimulant for bioactive molecules in certain crops without the drawbacks of other substrate supplementations.

**Table 2.** Antioxidant activity of the different mushroom provenances across the two harvest periods.

| | | | |
|---|---|---|---|
| **Presidente Prudente (PP)** | | | |
| | | **TBARS ($EC_{50}$ mg/mL)** | **OxHLIA ($IC_{50}$ μg/mL)** |
| Harvest Number (HN) | First | 0.8 ± 0.3 | 41 ± 23 |
| | Second | 0.5 ± 0.2 | 56 ± 38 |
| *p*-value (n = 15) | *t*-test | <0.001 | <0.001 |
| Calcium Silicate Concentration (CS) | Control | 0.7 ± 0.3 | 67 ± 8 |
| | 0.5% | 0.8 ± 0.1 | 80 ± 30 |
| | 1% | 0.6 ± 0.1 | 41 ± 12 |
| | 2% | 0.8 ± 0.6 | 55 ± 16 |
| | 4% | 0.4 ± 0.1 | n.a. |
| *p*-value (n = 6) | THSD test | <0.001 | <0.001 |
| HN × CS (n = 30) | *p*-value | <0.001 | <0.001 |
| **Mogi-das Cruzes (MC)** | | | |
| Harvest Number (HN) | First | 0.7 ± 0.1 | 16 ± 32 |
| | Second | 0.5 ± 0.2 | 56 ± 51 |
| *p*-value (n = 15) | *t*-test | <0.001 | <0.001 |
| Calcium Silicate Concentration (CS) | Control | 0.4 ± 0.2 | n.a. |
| | 0.5% | 0.44 ± 0.05 | n.a. |
| | 1% | 0.6 ± 0.2 | 30 ± 33 |
| | 2% | 0.75 ± 0.02 | 54 ± 60 |
| | 4% | 0.69 ± 0.05 | 96 ± 20 |
| *p*-value (n = 6) | THSD test | <0.001 | <0.001 |
| HN × CS (n = 30) | *p*-value | <0.001 | <0.001 |

The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. na: no activity. *t*-test represents a student's *t*-test, while THSD test means Tukey's honest significance test.

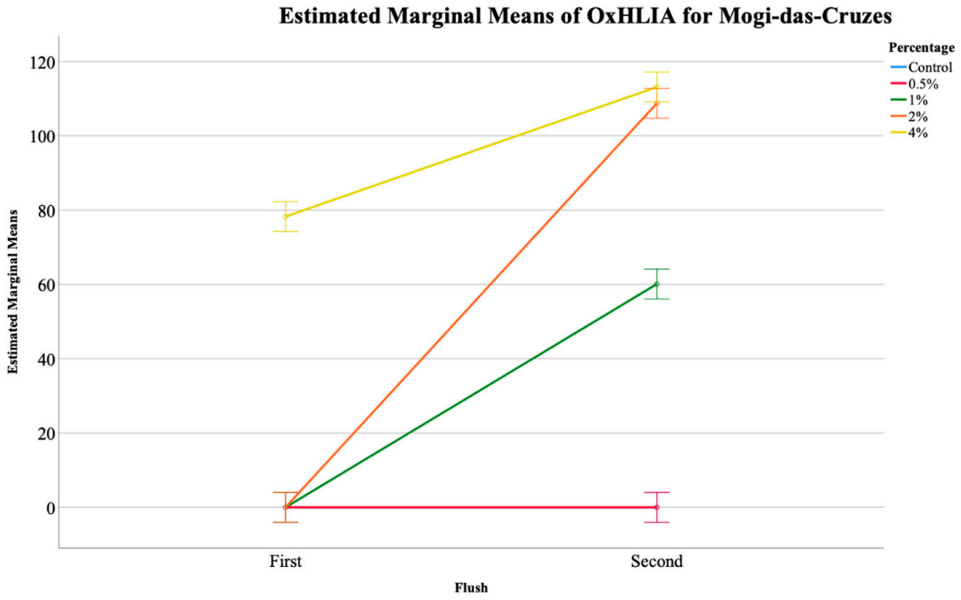
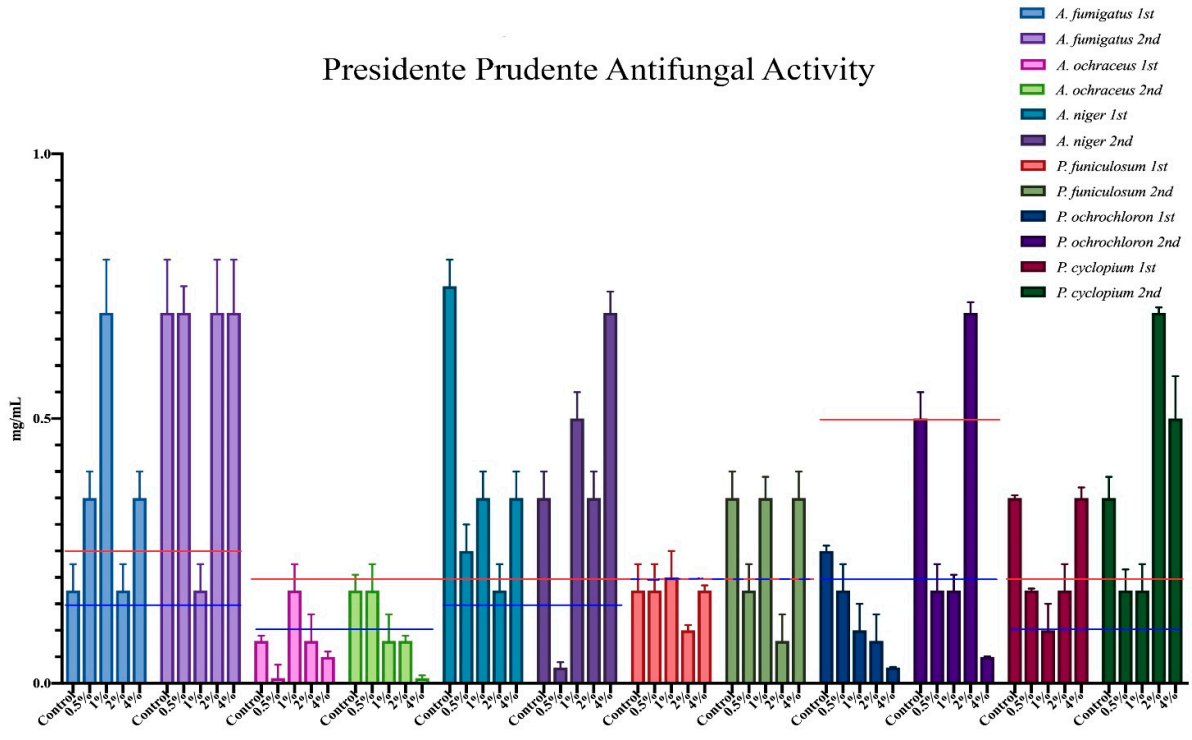



**Figure 2.** Estimated marginal means (EMM) plot of the oxidative hemolysis inhibition (OxHLIA) analysis of the Mogi-das-Cruzes mushrooms.

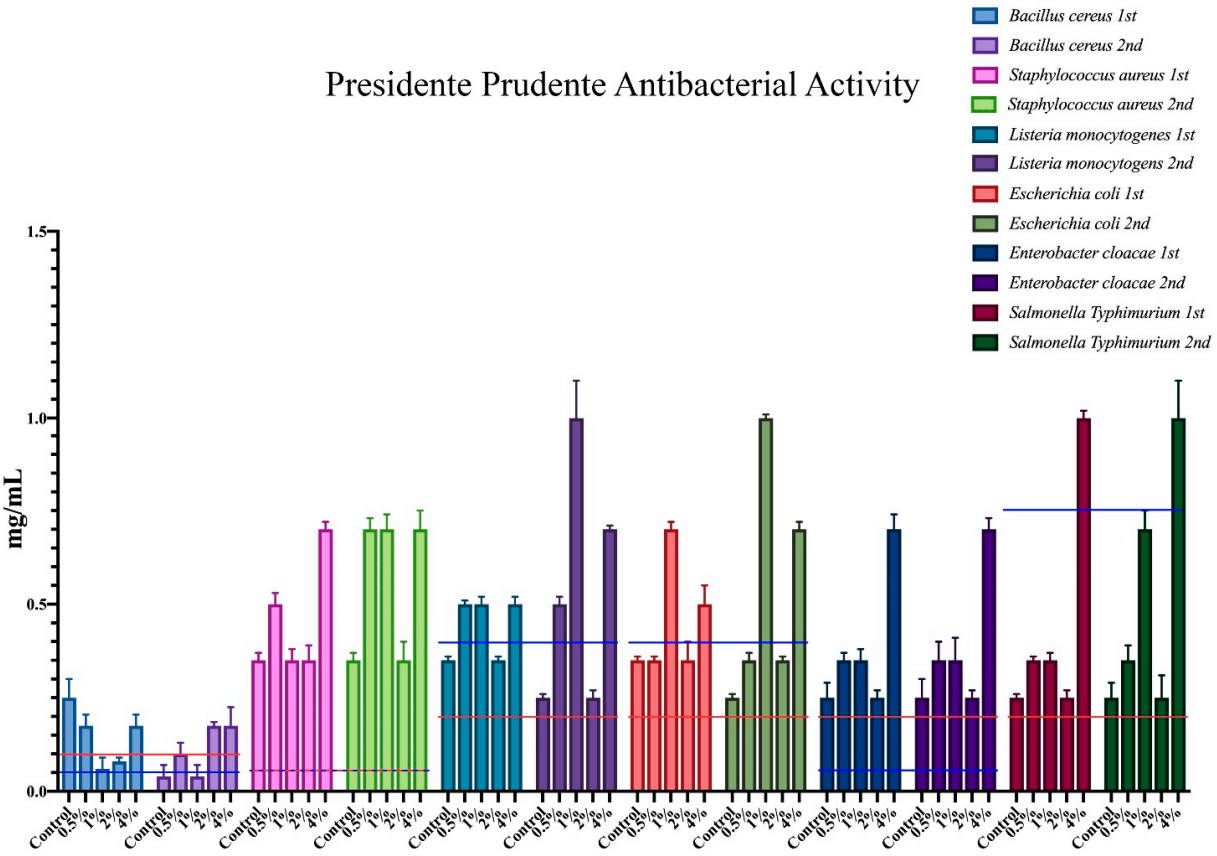
### *3.3. Antimicrobial Activities*

The minimum inhibition concentration (MIC) for the antimicrobial activity of the different mushrooms is represented in Figure 3, where each microorganism corresponds to a colour, and all of the different supplementation results are expressed within that same colour. Furthermore, the minimum inhibition concentrations of the positive controls are represented in each case through the red and blue horizontal lines.

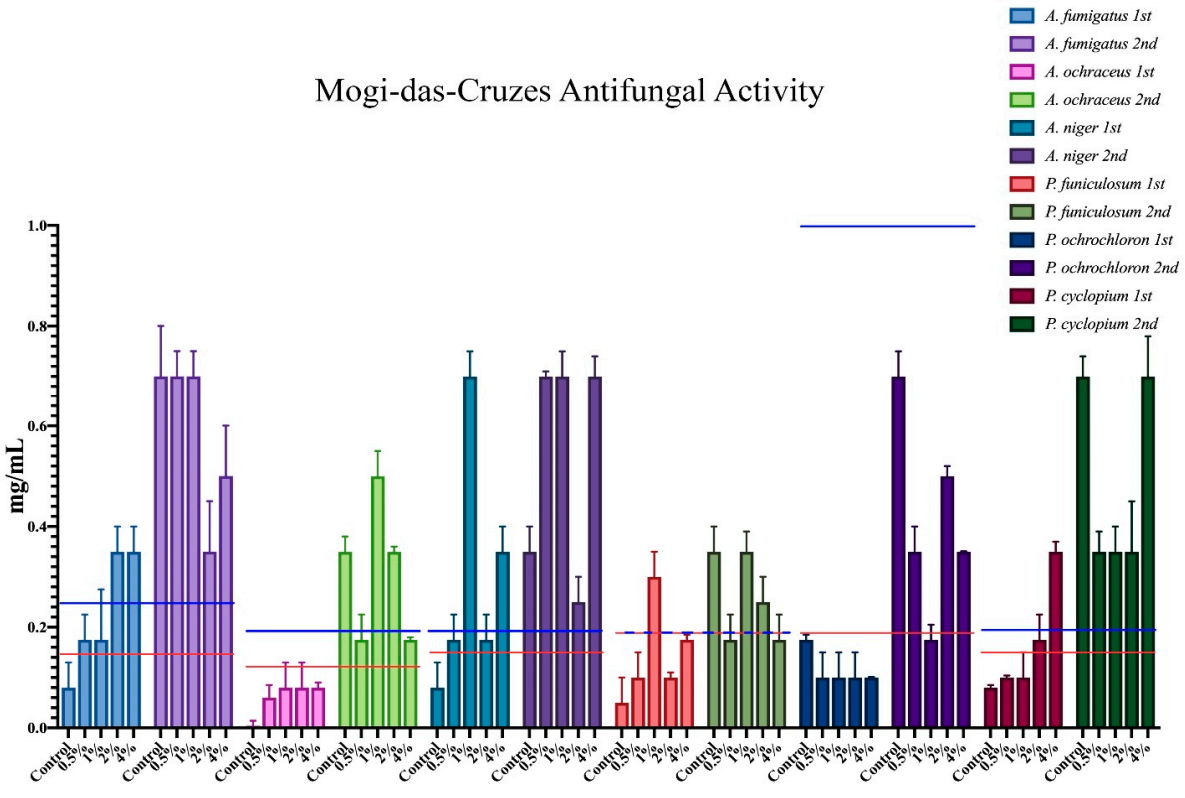



**(a)**

**Figure 3.** *Cont.*




**(b)**




**(c)**

**Figure 3.** *Cont*.

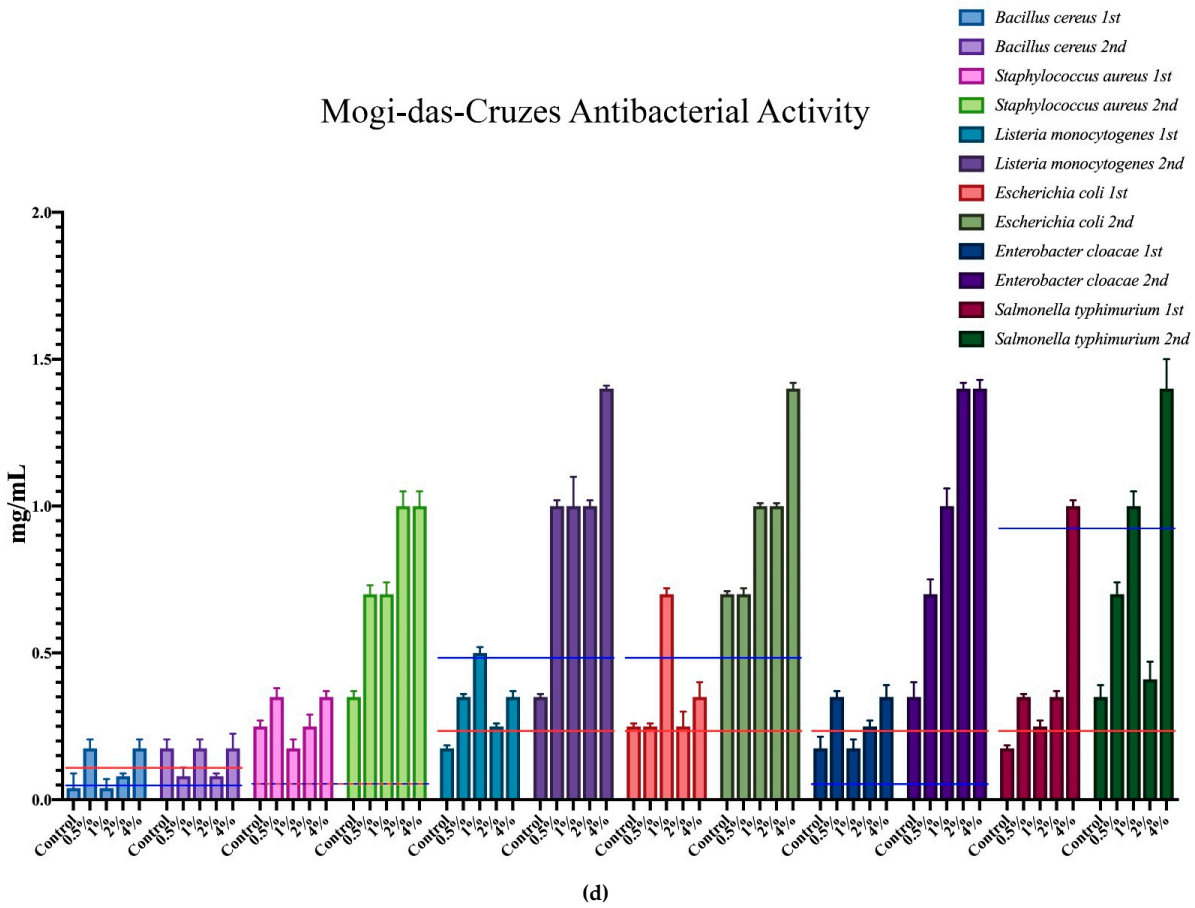



**Figure 3.** Antimicrobial activity of the mushrooms from both flushes and different supplementation percentages compared to positive controls. For the antibacterial activity, the blue line represents the ampicillin minimum inhibitory concentrations (MIC) while the red line represents the MIC of streptomycin. In the antifungal charts, the red lines represent the MIC of ketoconazole and the blue the MIC of bifonazole. (**a**) antifungal activity of the mushrooms from Presidente Prudente; (**b**) antibacterial activity of the mushrooms from Presidente Prudente; (**c**) antifungal activity of the mushrooms from Mogi-das-Cruzes; (**d**) antibacterial activity of the mushrooms from Mogi-das-Cruzes.

The red line represents ketoconazole for the antifungal activity and streptomycin for the antibacterial activity, while the blue line represents bifonazole for the antifungal activity and ampicillin for the antibacterial activity. The mushrooms from PP showed very uniform values and low variations from the first flush to the second (Figure 3a). The best result was sought against *B. cereus*, in which some percentages of supplementation displayed better activity than both positive controls. Furthermore, most samples of MC showed better results against *S. typhimurium* when compared to ampicillin. Interestingly, the antifungal activity for the mushrooms of PP (Figure 3b) showed different results, in which the higher supplementation showed better results than the positive controls in both flushes for the same fungi, *A. ochraceus* and *P. ochlorochloron* in the second flush. The antibacterial activity for mushrooms from MC, represented in Figure 3c, showed that the mushrooms in the second flush had lower antibacterial activity and that supplementation did not have much influence on the activity, although for *B. cereus*, *L. monocytogenes* and *E. coli*, the values of the mushrooms were very close to the activity of one of the positive controls. Regarding Figure 3d, the antifungal activity for the mushrooms from MC, the supplementation did not seem to have an influence on the antifungal activity, and while there seemed to be a decrease from the first to the second flush, very good results were sought against *P. ochrochloron*, in which the mushroom seemed be have higher activity in the first flush than both positive controls. Furthermore, the first flush was also between both positive controls for *A. ochraceus* and *P. funiculosum*.

Overall, *P. ostreatus* mushrooms seem to have a higher antibacterial than antifungal activity and, in some cases, supplementation with calcium silicate increase this activity, especially in PP against fungi and MC against bacteria.

### *3.4. Linear Discriminant Analysis*

A linear discriminant analysis (LDA) was used to further discriminate the differences induced by the supplementation with calcium silicate to the mushrooms from the two regions. The LDA discriminated the supplementation percentages (Figure 4) for both mushrooms.

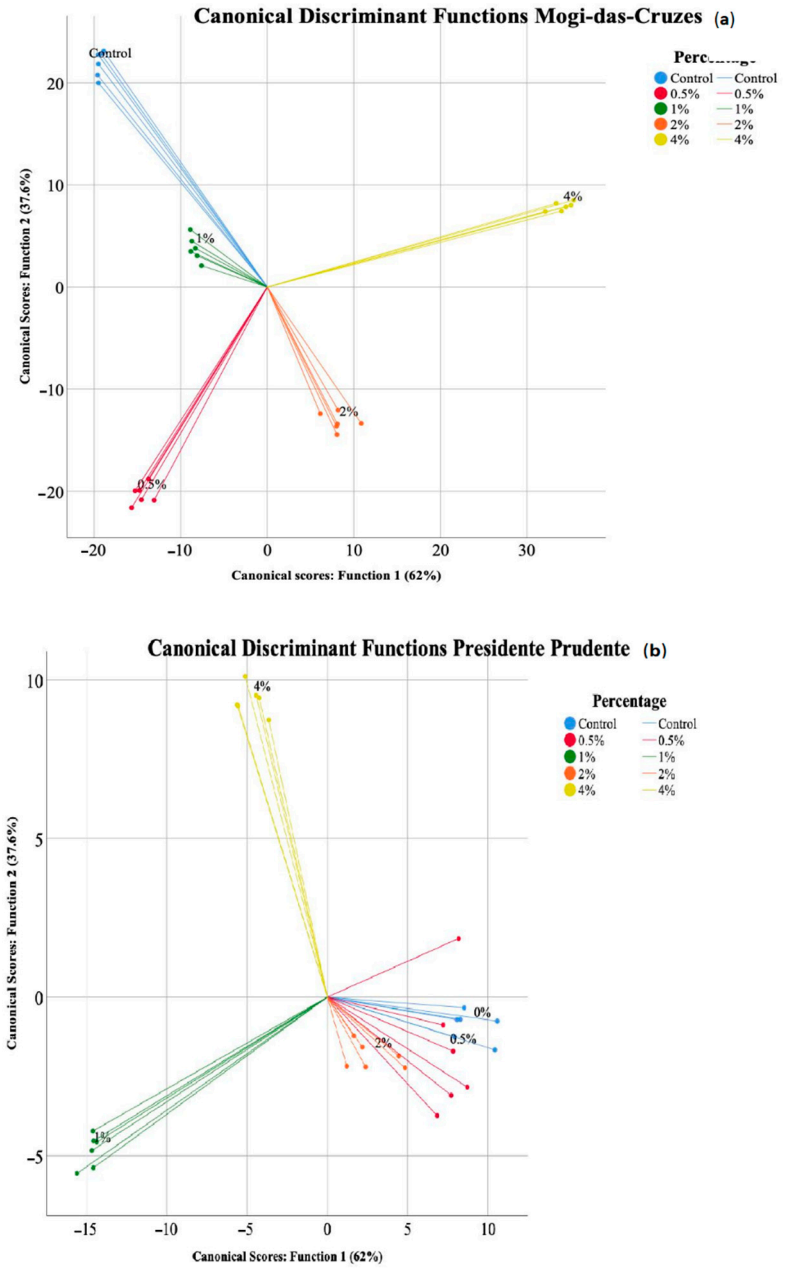



**Figure 4.** Linear discriminant analysis (LDA) of the different calcium silicate supplementation percentages for each mushroom, (**a**) Mogi-das-Cruzes and (**b**) Presidente Prudente.

Regarding the mushrooms from MC (Figure 4a), the model defined 4 functions that accounted for 100% of the variance, although the first two included 99.6% (function 1—62%, function 2—37.6%). Among the 10 parameters analysed, 8 had discriminating ability, namely *p*-coumaric acid, OxHLIA assay, malic acid, protocatechuic acid, TBARS assay,

oxalic acid, cinnamic acid and fumaric acid. The ones most correlated with function 1 were TBARS and OxHLIA assay as well as *p*-coumaric acid, while *p*-coumaric acid, protocatechuic acid and oxalic acid were highly correlated to function 2. By analysing Figure 4a a clear discrimination between the different supplemented mushrooms is visible, with the 4% calcium silicate supplemented mushrooms showing greater difference from the other samples, which was based on the antioxidant activity and *p*-coumaric acid values. Regarding the LDA for PP, shown in Figure 4b, the model also defined 4 functions that accounted for 100% of the variance, and the two first ones together accounted for 94.6% (function 1—72.5%, function 2—22.1%). Eight parameters showed discriminant ability, namely OxHLIA assay, cinnamic acid, protocatechuic acid, total organic acids, TBARS assay, *p*-coumaric acid, fumaric acid and oxalic acid. The parameters that best correlated to function 1 were *p*-coumaric acid, cinnamic acid and total organic acids, while *p*-coumaric acid, total organic acids and oxalic acid were best correlated with function 2. Figure 4b shows that contrary to Figure 4a, not all supplementation percentages were clustered, namely the control (0%) 0.5% and 2% supplementation were discriminated from 1 and 4% according to function 1, while the latter were separated according to function 2. Overall, the parameters were better at discriminating the supplementation from the mushroom from Mogi-das-Cruzes. Supplementation at 4% stands out as the most distinct from the other percentages and the control sample, revealing that an increase in supplementation probably over 4% might induce higher changes. Further studies with increasing supplementation percentages are needed to understand if they can improve on the chemical parameters of the mushrooms or have damaging effects.

## 4. Conclusions

Supplementation of mushroom substrate with minerals and other components has been carried out with relative success, aiming at controlling pests or increasing crop yields. Previous work has been carried out with calcium silicate studying the effects on biological yield and individual bioactive molecules [6]. In this work, the analysis of supplementation of calcium silicate was tested in terms of the potential bioactive properties that could arise or decrease from this supplementation. Overall, very slight changes were sought for the parameters analysed, implying that supplementation can have a beneficial effect on the antioxidant and antimicrobial activity of the mushrooms without drastically changing their chemical composition. For the mushrooms from MC, 1% of supplementation of calcium silicate seemed to promote an increase of cinnamic acid, although 0.5% of supplementation increased *p*-coumaric acid revealing that lower concentrations of calcium silicate are favoured in terms of secondary metabolites. The antimicrobial activity did not seem affected by the varying percentages of supplementation, although the mushrooms revealed some antimicrobial activity against *A. ocharaceus* and *P. ochrochloron*. The mushrooms from PP showed that higher supplementation favoured the increase of *p*-coumaric acid and malic acid and the antioxidant activity while also increasing the antimicrobial capacity against *A. ochraceus* and *P. ochrochloron*. The parameters used in the LDA had higher discriminating ability for the supplementation of the mushrooms from MC than from PP. Overall, supplementation of calcium silicate does not seem to negatively affect the mushrooms in terms of their bioactivities, organic, phenolic compounds or induce cytotoxicity, and might even have beneficial effects, namely their increase. From a sustainability point of view, the use of calcium silicate is moderately used in agriculture to help increase resistance to certain pests without the drawbacks of pesticides. Furthermore, it is a source of silicon for plants and mushrooms and using it as a stimulant for bioactive compounds in mushrooms stands as a valuable tool for their production. Thus, the use of calcium silicate can stand as an important supplement in agriculture, although further studies on vegetable crops and other mushrooms are needed to widen knowledge.

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Supervision; A.M.G.-P.—Supervision, I.C.F.R.F.—Supervision; L.B.—Supervision, Writing—Review and Editing. All authors have read and agreed to the published version of the manuscript.

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**Annex E**

Combined effects of irradiation and storage time on the nutritional and chemical parameters of dried *Agaricus bisporus* Portobello mushroom flour

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# Combined effects of irradiation and storage time on the nutritional and chemical parameters of dried *Agaricus bisporus* Portobello mushroom flour

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**Abstract:** Portobello variety of *Agaricus bisporus* mushrooms, appreciated for its taste, makes it desirable to be eaten fresh and also as flour in soups and gravies. Gamma and electron-beam radiation at four doses (1, 2, 5, and 10 kGy) were used to analyze its preservation effect on Portobello mushroom flour. A proximate analysis, as well as the impact on fatty acids, tocopherols, soluble sugars, organic acids, and ergosterol profiles, were performed every 3 months, during a storage period of 1 year. Gamma rays preserved mannitol (most abundant soluble sugar) over the 12 months, while electron beam radiation preserved organic acids. No significant changes were sought for any radiation type, and the slight changes extracted from the estimated marginal means reveal a tendency for irradiation as having preserving effects of nutrients and other important molecules. Thus, both irradiation types, up to 10 kGy are suitable for preservation of *A. bisporus* Portobello flour.

**KEYWORDS**
*Agaricus bisporus*, chemical profile, electron beam, food irradiation, gamma radiation

## 1 | INTRODUCTION

As the consumption of fresh mushrooms grows, an increase in their production is necessary, demanding additional research to clarify the role of mushrooms in human diets and their consumption benefits (PR Newswire, 2018). In the post-harvest stage, mushrooms quickly lose quality, as indicated by the loss of moisture, discoloration, degradation of nutrients, and hyphae development. This high perishability is a strong disadvantage that reduces their economic value. Accordingly, there is a growing need to extend their shelf life, both in fresh and processed forms, improving yields and economic competitiveness to producers and traders, and increasing their quality for consumers (Bernaś, 2018; Cardoso et al., 2019; Kic, 2018; Taofiq et al., 2017; Zhang et al., 2018).

  

Irradiation technology has proven to be scientifically feasible, practical, and worthwhile for several food products (fruits and vegetables, fresh or dried; Akram et al., 2013; Cardoso et al., 2019; Fernandes et al., 2017; Ferreira et al., 2018). Gamma rays and electron-beam radiation are techniques that have been validated previously and constitute an alternative to other preservation technologies (Ferreira et al., 2018) while maintaining the chemical profile, freshness, and overall security, although the beneficial and negative effects are always dose-dependent and case-specific; hence, the importance of testing doses within current legislation on different foods (Cardoso et al., 2019; Fernandes, Barreira, et al., 2014a, 2014b). Scientific reports have shown the benefits of this technology on the physical, chemical, nutritional and bioactive properties of several edible mushroom species mushrooms (Cardoso et al., 2019; Fernandes et al., 2013b, 2015, 2017; Fernandes, Barreira, et al., 2014a, 2014b, 2014c, Fernandes et al., 2016).

The *Agaricus bisporus* (J.E. Lange) Imbach, white mushrooms, are the ones within the Agaricaceae family with the least growth time in order to have the white and soft body, which when allowed to grow for longer periods, starts to develop a brown tone that tends to intensify. These brown mushrooms are called Portobello and have a more intense and textured taste than the white ones (Bernaś, 2018; Cardoso et al., 2019; Djekic et al., 2017; Wang et al., 2018). Additionally, this variety is rich in bioactive compounds (Cardoso et al., 2019; Djekic et al., 2017; Guan et al., 2016; Teichmann et al., 2007).

Interaction of ionizing radiation with natural matrices is multifactorial, where some molecules may protect others from radiation effects, requiring case-by-case studies (Antonio et al., 2018). In this way, validating both the processing technology and the processed product is a requisite to assure the feasibility of the preservation process under validation. Still, gamma and electron-beam irradiation does not induce deep changes in fresh or dried mushrooms (Cardoso et al., 2020). Accordingly, the main objective of this work was to understand the effects of different doses (1, 2, 5, and 10 kGy) of gamma and electron-beam irradiation on nutritional and chemical parameters of Portobello mushrooms flour stored for relatively long storage periods (0, 6, and 12 months).

## 2 | MATERIALS AND METHODS

### 2.1 | Gamma and electron-beam irradiation

*A. bisporus* Portobello fruiting bodies were acquired in a local market of Bragança, Northeast of Portugal in June 2017, and were divided into two groups of 15 mushrooms each and further submitted to a drying process. Samples were dried at 30°C in an oven for 4 days; each group was further subdivided into five subgroups (200 g per group): Control (non-irradiated, 0 kGy); sample 1, irradiated at 1 kGy, sample 2 (2 kGy), sample 3 (5 kGy) and sample 4 (10 kGy).

Gamma irradiation was performed at the ionizing radiation facility IRIS from Centro de Ciências e Tecnologias Nucleares (Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal) in a $^{60}$Co experimental chamber (model Precisa 22, Graviner Manufacturing Company Ltd., UK) with four sources and a total activity of 2.9 kCi (108 TBq, July 2017). The absorbed doses were measured by routine dosimeters (Batch X; Amber Perspex Harwell). Prior to irradiation, two dosimeters were added to each bag, and the samples were placed in line with the sources, rotated 180 degrees vertically and horizontally halfway through the irradiation to guarantee a uniform dose and to follow the recommended practices for food irradiation (dose uniformity ratio less than 3). The dosimeters were positioned according to a previous dose mapping of the irradiation chamber, and the absorbed doses were estimated using a calibration curve obtained for routine dosimeters.

The estimated absorbed doses, dose rate, and dose uniformity ratio ($D_{max}/D_{min}$) were, respectively, 1.2 ± 0.1, 1.9 ± 0.1, 5.0 ± 0.1, and 10.4 ± 0.3 kGy; 1.4 kGy/h and 1.3, respectively.

Electron-beam irradiation was carried out in the same facility using a LINAC equipment (Saturne 41, adapted for R&D, General Electric Co., Boston, MA, USA) with an electron beam of 10 MeV. Samples were placed at 60 cm from the beam exit and irradiated at dose rates ranging from 0.3 to 1.3 kGy/min (pulse duration 4 μs, pulse repetition frequency 20–100 Hz). The absorbed doses estimated by radiochromic film dosimeters FWT-60 (Far West Technology, Inc.) were the following: 1.0 ± 0.1, 2.0 ± 0.2, 4.7 ± 0.7, and 9.8 ± 0.1 kGy) with an uncertainty of 7% for the first dose and 10% for the other two doses. Previously to food irradiation experiments, a detailed dose mapping characterization of the radiation chamber was carried out, 60 cm of the beam, using routine radiochromic dosimeters that were calibrated against reference alanine dosimeters.

After irradiation and prior to the laboratorial analysis, the samples were ground to a fine dried powder (20 mesh) by means of an automated mill and mixed to obtain homogenized samples, and then kept in the dark at room temperature in airtight flasks until further analysis. One batch of the samples was analyzed immediately while the other two were stored for 6 and 12 months, respectively, at room temperature in the dark in airtight containers.

## 2.2 | Proximate composition

Carbohydrates, fat, protein, ash, and moisture were determined following AOAC (Association of Official Agricultural Chemists) procedures (AOAC, 2016). The crude protein content of the samples was determined by the macro-Kjeldahl method, the crude fat was extracted using a Soxhlet apparatus and subsequently weighed, the ash content was determined by incineration at 550 ± 15°C. Total carbohydrates were calculated by difference: Total carbohydrates = 100 (g of moisture + g of protein + g of fat + g of ashes); and energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

## 2.3 | Chemical composition

### 2.3.1 | Soluble sugars

For the analysis of soluble sugars, an extraction was performed with ethanol and water, followed by a filtration (Barros, Pereira, Calhelha, et al., 2013). The identification of soluble sugars was performed on a Knauer high-performance liquid chromatograph (HPLC; Smartline system 1000, Knauer, Berlin, Germany) coupled to a refractive index detector (RI; Knauer). The compounds were identified by comparing their retention times to the ones of commercial standards. The quantification was based on the RI signal response of each standard using the internal standard (IS) methodology and raffinose as the IS. The HPLC conditions were as following: The mobile phase was a mixture of acetonitrile/water (70:30, v/v) with a flow rate of 1 ml/min. The chromatographic separation was achieved using a Eurospher 100–5 $NH_2$ column (5 µm, 250 × 4.6 mm, Knauer) at 35°C. Data were analyzed using Clarity 2.4 software (DataApex, Podohradska), and the results were reported in g per 100 g of dry weight (dw).

### 2.3.2 | Organic acids

Organic acids were extracted following a procedure previously described by the authors Pereira et al. (2015) and quantified by ultrafast liquid chromatograph (Shimadzu 20A series, Shimadzu Corp., Kyoto, Japan) coupled to photodiode array detector, using 215 nm as the preferred wavelength. Results were expressed as g/100 g dw.

### 2.3.3 | Fatty acids

Fatty acids were determined by gas chromatography (GC) with flame ionization detection at 260°C after extraction and derivatization procedures previously described by Heleno et al. (2009). The analysis was carried out with a DANI GC 1000 instrument (DANI Instruments, Milan, Italy) equipped with a split/splitless injector and a Zebron-Kame column (30 m × 0.25 mm ID × 0.20 µm df, Phenomenex, Torrance, CA, USA) using the methodology previously reported by Cardoso et al. (2019). The results were expressed in the relative percentage of each fatty acid.

### 2.3.4 | Tocopherols

Tocopherols were quantified through HPLC after extraction with hexane, methanol, and water as previously described by Reis et al. (2012), using tocol as an IS. Chromatographic separation was performed with a polyamide II normal-phase column (250 × 4.6 mm; YMC model, Waters Corp., Milford, MA, USA) operating at 35°C, and the mobile phase employed was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min. This analysis was done using the equipment described for the soluble sugars, although coupled to a fluorescence detector (FP-2020; Jasco), designed for excitation at 290 nm and emission at 330 nm. Quantification was based on IS methodology, and the compounds were identified by chromatographic comparisons with commercial standards. Data were evaluated using Clarity 2.4 software, and the results were expressed in mg/100 g dw.

### 2.3.5 | Ergosterol

Ergosterol was identified and quantified according to the methodology described by Barreira et al. (2014), after an extraction procedure previously described by Guan et al. (2016), using the HPLC described above coupled to a UV detector (Knauer Smartline 2500) and using 280 nm as the preferred wavelength (Barros et al., 2013a). The results were expressed as mg/100 g dw.

## 2.4 | Statistical analysis

Throughout the manuscript, all assays were carried out with three replicates, and all data are expressed as mean ± standard deviation. Samples were analyzed through a two-way analysis of variance (ANOVA) with type III sums of squares, after verifying homoscedasticity through a Levene's test. The *post-hoc* test used was either a Tukey's Honest Significant Difference (HSD) test (homoscedastic samples) or Tamhane's T2 test (non-homoscedastic samples). By employing a two-way ANOVA, it is possible to verify the

influence of each factor: Storage time (ST) or radiation dose (RD), independently of the way the other factor varies. If a significant ($p < 0.05$) interaction (ST × RD) was detected, the potential tendencies had to be extracted from the plotted estimated marginal means (EMM). Inversely, if there is no significant interaction ($p > 0.05$), each factor was classified independently using the *post-hoc* tests described above. All statistical analysis was performed using a *p*-value of 0.05 and the IBM SPSS software, version 25 (IBM Corp.).

## 3 | RESULTS AND DISCUSSION

The tabled results include nutritional, soluble sugars, and ergosterol profiles (Table 1), tocopherols and organic acids (Table 2), and fatty acids (Table 3) and are divided into two sections, the top one belonging to gamma-irradiated samples and the bottom one to those treated with the electron beam. Each section is further divided to elucidate ST (upper part) and RD (lower part) effects with the values presented for each RD including all ST periods and *vice-versa*.

In dw basis, the most abundant nutrients were carbohydrates, followed by proteins (Table 1). The fat content was very low (<1.4 g/100 g (dw), which was similar to the values presented by other fresh mushroom species (Cardoso et al., 2019). For gamma-irradiated samples, there was a significant ($p < 0.05$) interaction (ST × RD) for moisture, crude fat, proteins, and carbohydrates, meaning that both RD and ST had a significant impact on the changes registered for these nutrients. Inversely, ash and energy did not show a significant interaction, being classified individually. Ash content decreased over ST, although the significantly higher values in irradiated samples, as previously observed in irradiated foods (Khan et al., 2018). Regarding the energy values of gamma-irradiated samples, this parameter tended to increase with ST but decreased for higher RD. Considering the EMM plots, it was possible to observe that the longer the ST, the lower the fat content (Figure 1(a)), but with RDs of 5 and 10 kGy, this nutrient seems to be preserved, which is a beneficial aspect, since the loss of fat content is related to rancidification of food. Similar results were reported by Fernandes et al. (2017). Storage had a role in the reduction of fat over time, but the treatment at 5 kGy seems to preserve the integrity of these molecules during storage.

In terms of the nutritional profile of electron-beam-irradiated samples, a significant interaction was also found for ash and energy, showing that electron-beam had similar effects to those of gamma radiation, with a significant decrease of ash and an increase of energy values over ST, while a reduction of these parameters was detected with the increase of RD. For nutrients with a significant interaction among factors, the EMM plots (Figure 1(b)), show a reduction of protein content for the stored samples, while a lower dose of irradiation (1 kGy) seems to slightly reduce this loss of protein content in stored samples. This behavior was also reported by (Fernandes et al., 2013a; Fernandes, Barreira, et al., 2014b).

In Table 1, the soluble sugars and ergosterol content are shown for both irradiation technologies. The detected sugars were fructose, mannitol, and trehalose, mannitol being the most abundant one, followed by trehalose. The same sugar profile had been identified previously in various instances for irradiated sugars Regarding gamma radiation, a non-significant interaction was found for mannitol and trehalose while mannitol was classified independently showing that while this sugar decreased over ST, the increase of RD preserved it. Trehalose did not show significant differences among ST or RD, but it can be observed that trehalose increased over the 12 months with no influence from RD. The EMM plot for total sugars, Figure 1(c), shows that the amount of these sugars reduces over time, although stored samples showed that gamma irradiation had increasing preserving capacity from 1 to 5 kGy, while the 10 kGy dose showed a better preserving potential in non-stored samples. Electron-beam-irradiated samples showed a significant interaction for all sugars with no observable tendency in the EMM.

Ergosterol was detected in samples irradiated by both technologies and showed a significant interaction with values around 239 mg/100 g for gamma irradiation and varying from 234 to 347 mg/100 g for electron beam. In a previous work by Cardoso et al. (2019), an increase in ergosterol was also recorded for irradiated samples. Overall, slight changes were detected in the nutritional and sugar profile of Portobello mushroom flour treated with gamma radiation or electron beam, clearly showing that ST had a higher impact than RD as can be observed in Figure 1.

Table 2 shows two classes of molecules detected in the mushrooms, namely, tocopherols and organic acids. Regarding tocopherols, all four isoforms were detected in gamma and electron-beam-irradiated mushrooms, with $\beta$-tocopherol showing the highest amounts and $\alpha$-tocopherol the lowest. A significative interaction was found for all isoforms, not allowing to present individual classifications. Considering the EMM plots, and starting by gamma radiation, a general tendency for preservation of tocopherols was found for doses of 1 kGy with a much deeper impact resulting from ST, which tended to reduce the amount of these bioactive molecules, (Figure 2(a)). This same tendency, a decrease in the bioactive molecules over time, had also been reported previously by Fernandes et al. (2017). In the case of electron-beam-irradiated mushroom flour, ST also

**TABLE 1** Centesimal composition, soluble sugars, and ergosterol quantities in gamma and electron-beam irradiated Portobello mushrooms

| | | Moisture (g/100 g fw) | Crude fat (g/100 g fw) | Proteins (g/100 g fw) | Ash (g/100 g fw) | Carbohydrates (g/100 g fw) | Energy (g/100 g fw) | Fructose (g/100 g dw) | Mannitol (g/100 g dw) | Trehalose (g/100 g dw) | Total sugars (g/100 g dw) | Ergosterol (mg/100 g dw) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gamma radiation** | | | | | | | | | | | | |
| Storage time (ST) | 0 months | 11 ± 2 | 1.4 ± 0.1 | 24 ± 1 | 9.6 ± 0.1$^{c}$ | 65 ± 1 | 369 ± 1$^{a}$ | 0.43 ± 0.05 | 34 ± 3$^{c}$ | 0.8 ± 0.1 | 36 ± 3 | 293 ± 39 |
| | 6 months | 11 ± 2 | 1.19 ± 0.1 | 21 ± 1 | 9.4 ± 0.1$^{b}$ | 68 ± 1 | 368 ± 2$^{a, b}$ | 0.34 ± 0.04 | 29 ± 3$^{b}$ | 0.6 ± 0.1 | 30 ± 3 | 275 ± 14 |
| | 12 Months | 12 ± 1 | 1.0 ± 0.1 | 21 ± 1 | 9.0 ± 0.3$^{a}$ | 96 ± 2 | 372 ± 2$^{b}$ | 0.28 ± 0.05 | 22 ± 2$^{a}$ | 2.0 ± 0.8 | 21 ± 4 | 238 ± 30 |
| *p*-value ($n = 25$) | Tukey's HSD test | 0.038 | <0.001 | <0.001 | <0.001 | <0.001 | 0.040 | <0.001 | <0.001 | 0.545 | <0.001 | <0.001 |
| Radiation dose (RD) | Control | 11 ± 3 | 1.2 ± 0.1 | 21 ± 2 | 8.5 ± 0.2$^{a}$ | 69 ± 2 | 372 ± 2$^{b}$ | 0.29 ± 0.07 | 24.8 ± 1.9$^{a}$ | 0.4 ± 0.1 | 24 ± 7 | 248 ± 51 |
| | 1 kGy | 12 ± 1 | 1.1 ± 0.2 | 21 ± 2 | 9.6 ± 0.3$^{b}$ | 68 ± 3 | 367 ± 1$^{a}$ | 0.33 ± 0.07 | 27.6 ± 1.7$^{a, b}$ | 0.6 ± 0.2 | 28 ± 6 | 283 ± 42 |
| | 2 kGy | 12 ± 2 | 1.1 ± 0.2 | 22 ± 2 | 9.5 ± 0.3$^{b}$ | 67 ± 2 | 367 ± 2$^{a}$ | 0.32 ± 0.08 | 29.5 ± 2.2$^{b}$ | 0.5 ± 0.3 | 30 ± 6 | 272 ± 26 |
| | 5 kGy | 10 ± 2 | 1.3 ± 0.2 | 22 ± 1 | 9.6 ± 0.2$^{b}$ | 67 ± 1 | 368 ± 1$^{a}$ | 0.39 ± 0.06 | 30.8 ± 1.7$^{b}$ | 0.6 ± 0.3 | 32 ± 4 | 282 ± 25 |
| | 10 kGy | 10 ± 1 | 1.3 ± 0.2 | 23 ± 1 | 9.6 ± 0.3$^{b}$ | 66 ± 1 | 368 ± 1$^{a}$ | 0.40 ± 0.06 | 30.3 ± 1.9$^{b}$ | 0.7 ± 0.3 | 31 ± 7 | 258 ± 27 |
| *p*-value ($n = 5$) | Tukey's HSD test | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.001 | 0.452 | <0.001 | <0.001 |
| ST × RD ($n = 50$) | *p*-value | <0.001 | <0.001 | <0.001 | 0.390 | <0.001 | 0.176 | <0.001 | 0.326 | 0.443 | <0.001 | <0.001 |
| **Electron-beam radiation** | | | | | | | | | | | | |
| ST | 0 months | 11 ± 2 | 1.29 ± 0.03 | 24.4 ± 0.7 | 9.3 ± 0.1$^{c}$ | 65.0 ± 0.9 | 369 ± 1$^{a}$ | 0.43 ± 0.06 | 23 ± 3 | 0.88 ± 0.05 | 34 ± 3 | 347 ± 27 |
| | 6 months | 10 ± 2 | 1.21 ± 0.03 | 19.7 ± 0.7 | 9.1 ± 0.1$^{b}$ | 69.9 ± 0.9 | 369 ± 1$^{a, b}$ | 0.38 ± 0.06 | 30 ± 4 | 0.5 ± 0.1 | 31 ± 4 | 300 ± 23 |
| | 12 months | 12 ± 1 | 1.14 ± 0.05 | 19.3 ± 0.5 | 8.9 ± 0.1$^{a}$ | 70.5 ± 0.7 | 370 ± 1$^{b}$ | 0.30 ± 0.05 | 20 ± 5 | 0.4 ± 0.1 | 21 ± 5 | 234 ± 28 |
| *p*-value ($n = 25$) | Tukey's HSD test | 0.007 | <0.001 | <0.001 | <0.001 | <0.001 | 0.045 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| RD | Control | 10 ± 2 | 1.17 ± 0.09 | 20 ± 2 | 8.5 ± 0.2$^{a}$ | 70 ± 2 | 372 ± 1$^{b}$ | 0.30 ± 0.06 | 21 ± 6 | 0.5 ± 0.2 | 22 ± 6 | 250 ± 48 |
| | 1 kGy | 12 ± 2 | 1.23 ± 0.07 | 22 ± 2 | 9.2 ± 0.3$^{b}$ | 67 ± 2 | 369 ± 1$^{a}$ | 0.40 ± 0.06 | 36 ± 7 | 0.5 ± 0.3 | 27 ± 8 | 304 ± 66 |
| | 2 kGy | 10 ± 1 | 1.21 ± 0.06 | 21 ± 2 | 9.2 ± 0.2$^{b}$ | 68 ± 3 | 369 ± 1$^{a}$ | 0.35 ± 0.07 | 27 ± 6 | 0.6 ± 0.2 | 28 ± 6 | 308 ± 54 |
| | 5 kGy | 12 ± 1 | 1.22 ± 0.06 | 21 ± 2 | 9.3 ± 0.2$^{b}$ | 68 ± 3 | 368 ± 2$^{a}$ | 0.37 ± 0.03 | 29 ± 5 | 0.6 ± 0.2 | 30 ± 6 | 298 ± 33 |
| | 10 kGy | 13 ± 1 | 1.24 ± 0.06 | 21 ± 3 | 9.2 ± 0.2$^{b}$ | 69 ± 3 | 369 ± 1$^{a}$ | 0.4 ± 0.1 | 33 ± 4 | 0.6 ± 0.2 | 34 ± 4 | 309 ± 46 |
| *p*-value ($n = 5$) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST × RD ($n = 50$) | *p*-value | 0.003 | 0.008 | <0.001 | 0.750 | <0.001 | 0.526 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

*Note*: In each row, different letters mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision rather as the range of the recorded values.

**TABLE 2** Tocopherol isoforms and organic acids quantities in gamma and electron-beam-irradiated Portobello mushrooms

| | | α-tocopherol (mg/100 g dw) | β-tocopherol (mg/100 g dw) | γ-tocopherol (mg/100 g dw) | δ-tocopherol (mg/100 g dw) | Total tocopherols (mg/100 g dw) | Oxalic acid (g/100 g dw) | Quinic acid (g/100 g dw) | Malic acid (g/100 g dw) | Total organic acids (g/100 g dw) |
|---|---|---|---|---|---|---|---|---|---|---|
| **Gamma radiation** | | | | | | | | | | |
| ST | 0 months | 7.1 ± 0.2 | 149 ± 12 | 130 ± 6 | 65 ± 3 | 352 ± 20 | 0.88 ± 0.07 | 4.4 ± 0.3 | 4.9 ± 0.1 | 10.2 ± 0.4 |
| | 6 months | 6.7 ± 0.3 | 67 ± 9 | 47 ± 2 | 51 ± 5 | 170 ± 14 | 0.82 ± 0.07 | 4.0 ± 0.4 | 4.5 ± 0.5 | 9.3 ± 0.9 |
| | 12 months | 3.2 ± 0.6 | 52 ± 15 | 25 ± 2 | 19 ± 4 | 99 ± 21 | 0.67 ± 0.06 | 3.2 ± 0.2 | 3.5 ± 0.1 | 7.3 ± 0.4 |
| *p*-value ($n = 25$) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| RD | Control | 4 ± 2 | 69 ± 46 | 63 ± 44 | 38 ± 21 | 174 ± 113 | 0.7 ± 0.1 | 3.5 ± 0.5 | 4.0 ± 0.6 | 8 ± 1 |
| | 1 kGy | 5 ± 2 | 95 ± 50 | 73 ± 52 | 46 ± 20 | 219 ± 120 | 0.78 ± 0.08 | 3.9 ± 0.4 | 4.5 ± 0.7 | 9 ± 1 |
| | 2 kGy | 5 ± 2 | 93 ± 44 | 66 ± 47 | 46 ± 20 | 211 ± 110 | 0.8 ± 0.1 | 4.2 ± 0.9 | 4.5 ± 0.7 | 9 ± 2 |
| | 5 kGy | 5 ± 1 | 93 ± 45 | 67 ± 47 | 47 ± 20 | 213 ± 110 | 0.8 ± 0.1 | 3.8 ± 0.5 | 4.5 ± 0.7 | 9 ± 1 |
| | 10 kGy | 5 ± 2 | 98 ± 43 | 67 ± 48 | 48 ± 20 | 218 ± 111 | 0.8 ± 0.1 | 3.7 ± 0.5 | 4.0 ± 0.7 | 9 ± 1 |
| *p*-value ($n = 5$) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST × RD ($n = 50$) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Electron-beam radiation** | | | | | | | | | | |
| ST | 0 months | 8.3 ± 0.9 | 140 ± 7 | 119 ± 4 | 61 ± 3 | 329 ± 14 | 0.91 ± 0.04 | 4.6 ± 0.2 | 4.9 ± 0.2 | 10.4 ± 0.5[c] |
| | 6 months | 5.1 ± 0.4 | 70 ± 7 | 58 ± 6 | 48 ± 3 | 181 ± 16 | 0.78 ± 0.06 | 3.4 ± 0.2 | 3.6 ± 0.4 | 7.8 ± 0.3[b] |
| | 12 months | 3.7 ± 0.9 | 49 ± 14 | 24 ± 1 | 17 ± 3 | 93 ± 18 | 0.71 ± 0.09 | 3.2 ± 0.2 | 3.5 ± 0.1 | 7.4 ± 0.2[a] |
| *p*-value ($n = 25$) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| RD | Control | 4 ± 2 | 69 ± 47 | 60 ± 39 | 37 ± 20 | 171 ± 106 | 0.7 ± 0.1 | 3.5 ± 0.6 | 3.6 ± 0.8 | 7.7 ± 0.3[a] |
| | 1 kGy | 6 ± 2 | 88 ± 40 | 68 ± 42 | 43 ± 21 | 205 ± 104 | 0.7 ± 0.1 | 3.9 ± 0.6 | 4.0 ± 0.8 | 8.6 ± 0.6[b] |
| | 2 kGy | 6 ± 2 | 91 ± 41 | 69 ± 43 | 43 ± 20 | 210 ± 104 | 0.84 ± 0.1 | 3.7 ± 0.7 | 4.0 ± 0.7 | 8.6 ± 0.5[b] |
| | 5 kGy | 6 ± 2 | 91 ± 39 | 68 ± 43 | 42 ± 18 | 207 ± 100 | 0.84 ± 0.1 | 3.7 ± 0.7 | 4.1 ± 0.6 | 8.7 ± 0.5[b] |
| | 10 kGy | 6 ± 2 | 92 ± 40 | 69 ± 42 | 44 ± 20 | 212 ± 102 | 0.86 ± 0.1 | 3.8 ± 0.7 | 4.1 ± 0.6 | 9.0 ± 0.2[b] |
| *p*-value ($n = 5$) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST × RD ($n = 50$) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.164 |

*Note*: In each row, different letters mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision rather as the range of the recorded values.

**TABLE 3** Individual fatty acids, saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) quantities in gamma and electron-beam irradiated Portobello mushrooms, expressed in relative percentage

| | | C15:0 | C16:0 | C18:0 | C18:1 | C18:2 | C20:0 | C22:0 | C24:0 | SFA | MUFA | PUFA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gamma radiation** | | | | | | | | | | | | |
| ST | 0 months | 1.4 ± 0.1 | 9 ± 1 | 4.0 ± 0.3 | 1.1 ± 0.1 | 72 ± 1 | 1.6 ± 0.3 | 1.6 ± 0.4 | 3.1 ± 0.2 | 25 ± 1 | 1.6 ± 0.1 | 72 ± 1 |
| | 6 months | 1.9 ± 0.2 | 10 ± 1 | 4.5 ± 0.5 | 1.1 ± 0.2 | 74 ± 3 | 1.6 ± 0.1 | 1.3 ± 0.1 | 1.1 ± 0.1 | 24 ± 2 | 1.5 ± 0.6 | 74 ± 3 |
| | 12 months | 1.7 ± 0.1 | 9 ± 1 | 4.0 ± 0.3 | 0.9 ± 0.1 | 75 ± 1 | 1.7 ± 0.1 | 1.33 ± 0.09 | 1.1 ± 0.1 | 24 ± 1 | 1.1 ± 0.1 | 75 ± 1 |
| *p*-value ($n = 25$) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| RD | Control | 1.6 ± 0.2 | 9.4 ± 0.4 | 4.0 ± 0.2 | 1.1 ± 0.1 | 74 ± 1 | 1.7 ± 0.1 | 1.6 ± 0.4 | 1 ± 1 | 24 ± 1 | 1.5 ± 0.3 | 74 ± 1 |
| | 1 kGy | 1.7 ± 0.4 | 10.2 ± 1.2 | 4.2 ± 0.6 | 1.1 ± 0.1 | 74 ± 1 | 1.5 ± 0.3 | 1.3 ± 0.1 | 2 ± 1 | 25 ± 1 | 1.4 ± 0.3 | 74 ± 1 |
| | 2 kGy | 1.6 ± 0.2 | 8.7 ± 0.5 | 4.0 ± 0.4 | 0.9 ± 0.1 | 75 ± 2 | 1.7 ± 0.1 | 1.5 ± 0.4 | 2 ± 1 | 23 ± 2 | 1.2 ± 0.3 | 76 ± 2 |
| | 5 kGy | 1.8 ± 0.2 | 10.1 ± 0.9 | 4.6 ± 0.6 | 1.1 ± 0.1 | 72 ± 2 | 1.6 ± 0.1 | 1.2 ± 0.2 | 2 ± 1 | 26 ± 1 | 1.7 ± 0.6 | 73 ± 2 |
| | 10 kGy | 1.6 ± 0.1 | 8.9 ± 0.5 | 4.1 ± 0.1 | 0.9 ± 0.1 | 75 ± 2 | 1.7 ± 0.1 | 1.4 ± 0.1 | 1 ± 1 | 24 ± 2 | 1.2 ± 0.2 | 75 ± 2 |
| *p*-value ($n = 5$) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ST × RD ($n = 50$) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Electron-beam radiation** | | | | | | | | | | | | |
| ST | 0 months | 1.2 ± 0.1 | 8.1 ± 0.3 | 4.2 ± 0.2 | 1.3 ± 0.3 | 75 ± 1 | 1.8 ± 0.1 | 1.5 ± 0.2 | 1.2 ± 0.2 | 12 ± 1 | 1.7 ± 0.3 | 75 ± 1 |
| | 6 months | 1.6 ± 0.1 | 9.8 ± 0.4 | 4.2 ± 0.1 | 0.4 ± 0.4 | 81 ± 1 | 1.0 ± 0.7 | 1.2 ± 0.1 | 1.0 ± 0.1 | 25 ± 19 | 0.5 ± 0.4 | 81 ± 1 |
| | 12 months | 1.7 ± 0.2 | 9.7 ± 0.3 | 4.0 ± 0.3 | 0.7 ± 0.1 | 76 ± 1 | 1.6 ± 0.2 | 1.3 ± 0.1 | 1.0 ± 0.1 | 23 ± 1 | 0.9 ± 0.1 | 76 ± 1 |
| *p*-value ($n = 25$) | Tukey's HSD test | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.810 | <0.001 | <0.001 |
| RD | Control | 1.5 ± 0.1 | 9 ± 1 | 3 ± 2 | 0.9 ± 0.2 | 78 ± 2 | 1.5 ± 0.1 | 1.2 ± 0.2 | 1.0 ± 0.1 | 21 ± 2 | 1.2 ± 0.4 | 78 ± 2 |
| | 1 kGy | 1.5 ± 0.2 | 9 ± 1 | 3 ± 2 | 0.9 ± 0.2 | 78 ± 3 | 1.6 ± 0.1 | 1.3 ± 0.1 | 0.9 ± 0.1 | 21 ± 2 | 1.2 ± 0.4 | 78 ± 2 |
| | 2 kGy | 1.5 ± 0.2 | 9 ± 1 | 3 ± 2 | 0.6 ± 0.4 | 77 ± 2 | 1.7 ± 0.1 | 1.3 ± 0.1 | 1.0 ± 0.1 | 22 ± 2 | 0.9 ± 0.6 | 77 ± 2 |
| | 5 kGy | 1.6 ± 0.3 | 9 ± 1 | 3 ± 2 | 0.6 ± 0.4 | 77 ± 4 | 1.2 ± 0.8 | 1.4 ± 0.2 | 1.3 ± 0.3 | 34 ± 22 | 0.9 ± 0.6 | 77 ± 4 |
| | 10 kGy | 1.4 ± 0.3 | 9 ± 1 | 3 ± 2 | 0.9 ± 0.8 | 77 ± 5 | 1.2 ± 0.8 | 1.5 ± 0.3 | 1.2 ± 0.1 | 22 ± 4 | 1.1 ± 0.9 | 77 ± 5 |
| *p*-value ($n = 5$) | Tukey's HSD test | <0.001 | 0.011 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.017 | <0.001 | <0.001 |
| ST × RD ($n = 50$) | *p*-value | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.016 | <0.001 | <0.001 |

*Note*: Presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision rather as the range of the recorded values.

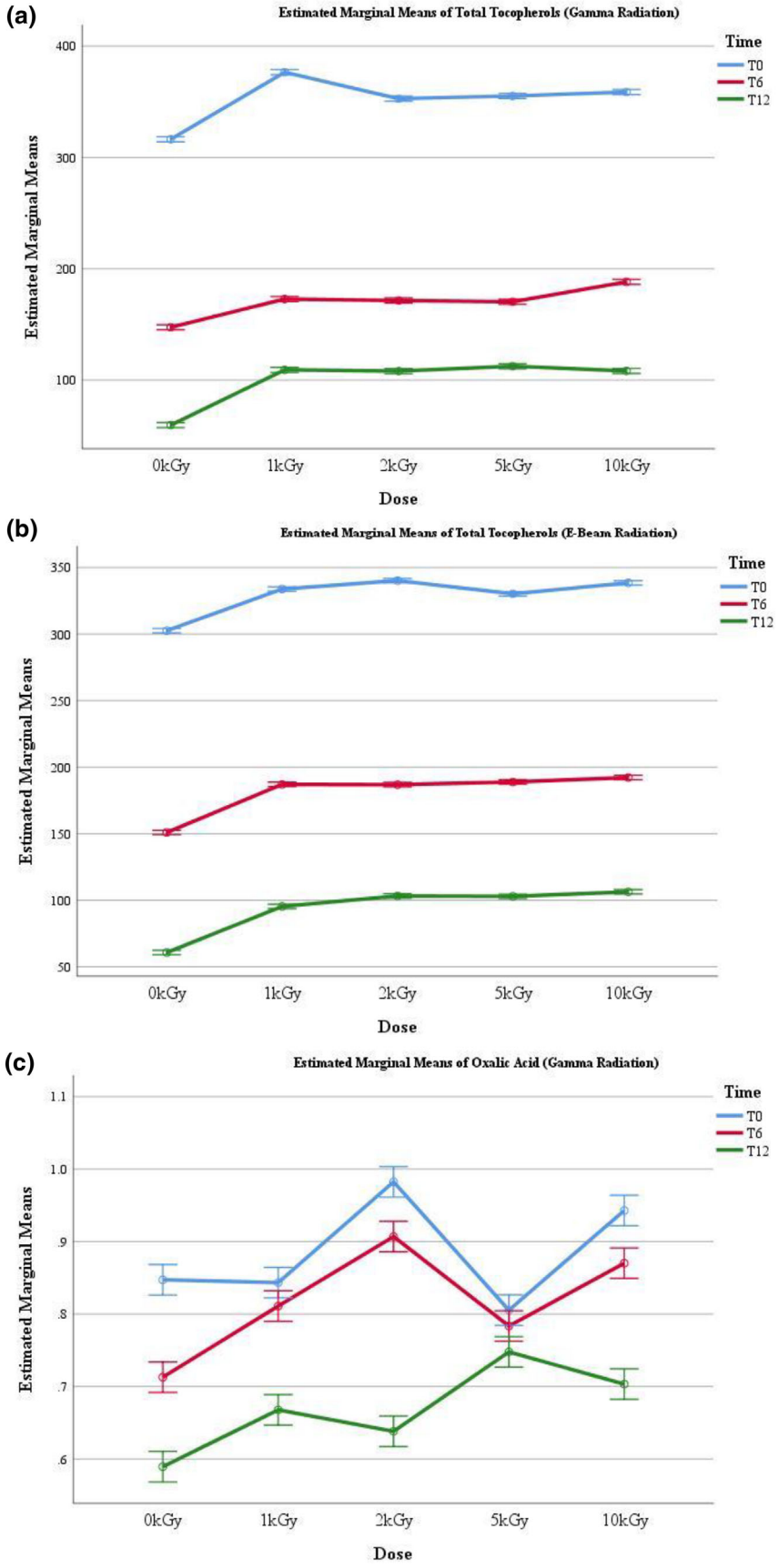


**FIGURE 1** Estimated marginal means (EMM) plots of (a) fat (gamma radiation), (b) proteins (electron-beam radiation), and (c) total sugars (gamma radiation)

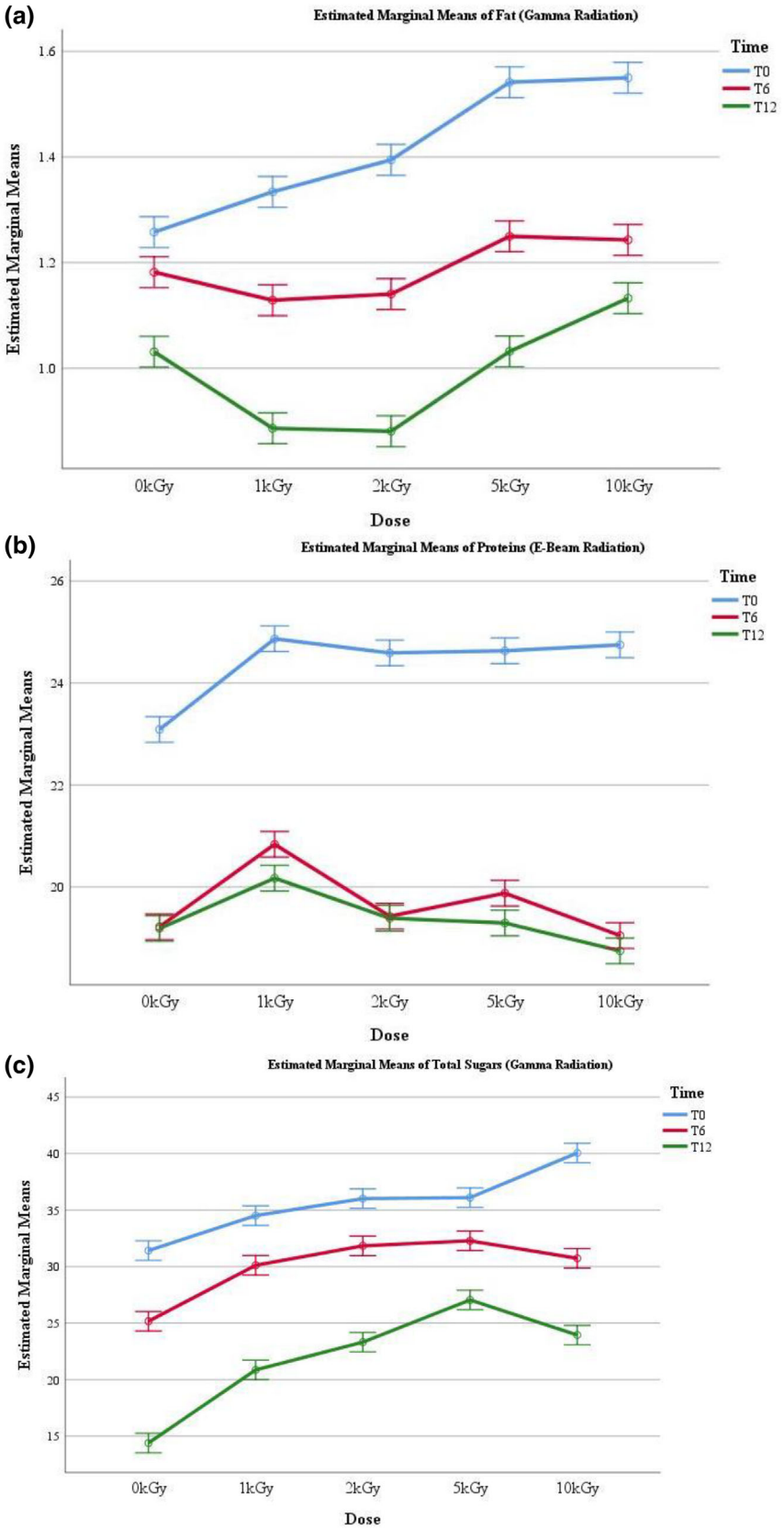


**FIGURE 2** EMM plots of (a) total tocopherols (gamma radiation), (b) total tocopherols (electron-beam radiation), and (c) oxalic acid (gamma radiation)

showed a higher impact than RD. All RDs showed preserving capabilities with an increase of about 50 mg/100 g (Figure 2(b)), while ST showed a high impact as seen in the gap between non-stored samples (blue line) and stored samples (red: 6 months, green: 12 months).

Organic acids are a group of simple organic molecules with acidic properties, present in all living organisms. In the irradiated mushrooms, three organic acids were detected: Oxalic, quinic, and malic acid. Quinic and malic acid were the most abundant, practically in *ex aequo*, while oxalic acid was not detected over 1 g/100 g. For both radiation processes, a significative interaction was found for all organic acids, and thus general conclusions were sought from the EMM plots. In Figure 2(c), (oxalic acid content in gamma-irradiated Portobello), the effect of ST had a higher influence than RD, although in this case, the dose of 2 kGy seem to have protecting capabilities (for the non-stored samples and 6 months stored ones, while samples stored for 12 months showed a better response at 5 kGy). This was also verified for quinic acid (results not shown). Regarding electron-beam-irradiated mushroom flours, and in line with the observed for the gamma-irradiated samples, organic acids showed a significative interaction in all cases. Irradiated samples (independently of RD) tend to present higher organic acid contents, while a significative decrease was observed over ST. Independently of RD, electron-beam radiation preserved organic acids in Portobello mushroom flour, while long STs reduce their amounts. These are important effects to take into account for these small molecules due to their relevance in taste, aroma, and overall appreciated properties of mushrooms. Barros et al. (2014) also mentioned that 1 kGy of gamma irradiation was effective to preserve organic acids in *Macrolepiota procera* mushrooms, pointing out that irradiation could be a complementary preserving technique.

Table 3 shows the individual fatty acids found in both mushrooms, as well as the groups of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA), expressed in relative percentage. Only the eight most abundant individual fatty acids are shown, from a total of 16 identified. In both cases, C18:2 was the most abundant fatty acid with a range of 72% to 81%, followed by C16:0. Overall, PUFA showed the highest amounts, also ranging from 81% to 82%, while SFA only registered a percentage of 12% to 34%, and MUFA being the lowest group, under 2%. This high incidence of PUFA shows the health beneficial unsaturated fat found in mushroom flour, which also helps their acceptance as healthy foods. Regarding the individual fatty acids, all samples showed a significant interaction between ST and RD, meaning that even though there were very low variations in the fatty acids, both ST and RD had an influence on the results. No tendencies could be extracted from the EMM plots, thus, concluding that due to the very low moisture, lipid peroxidation was very low, and the influence of the ST would not be drastic. Concomitantly, the influence of the different RDs did not have a great effect on the individual fatty acids.

## 4 | CONCLUSION

The main objective of this work was to assess the effect of different RDs on the chemical parameters of Portobello mushrooms flour (a sub-product of the fresh mushroom industry) stored at different intervals, as well as the difference between radiation sources, gamma rays, and electron beam. In this work it was possible to validate the use of both technologies with minimal effects on its main characteristics, allowing its preservation for longer times (up to 12 months) making it available to the food industry, allowing it to be incorporated into other foods, without compromising its main nutritional characteristics. Ionizing radiation using electron beam or gamma rays (up to a dose of 10 kGy) did not induce severe changes in the tested parameters, and the slight changes are all benefiting the preservation of specific nutrients like sugars and fat but also tocopherols or organic acids.

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### AUTHOR CONTRIBUTIONS

*Formal analysis, writing-original draft*: Rossana V. C. Cardoso. *Data curation, writing-review and editing*: Marcio Carocho. *Investigation*: Ângela Fernandes. *Data curation*: João C. M. Barreira. *Supervision*: Sandra Cabo Verde. *Investigation*: Pedro M. P. Santos. *Investigation, writing-review and editing*: Amilcar L. Antonio. *Supervision*: Ana M. Gonzaléz-Paramás. *Funding acquisition, project*

*administration, supervision*: Lillian Barros. *Funding acquisition*: Isabel C. F. R. Ferreira.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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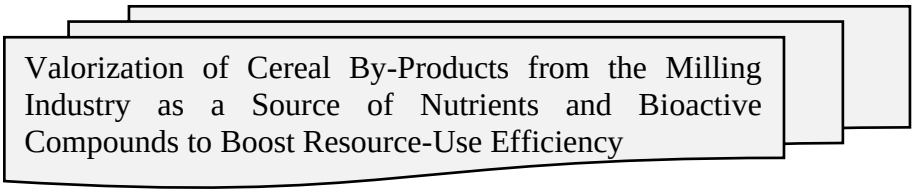
**Annex F**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*Article*

# Valorization of Cereal By-Products from the Milling Industry as a Source of Nutrients and Bioactive Compounds to Boost Resource-Use Efficiency

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**Abstract:** Cereal by-products (wheat germ, maize bran–germ mixture, rye bran, and wheat bran) from the flour milling industry were characterized for their nutritional value and chemical composition, as well as for antioxidant and antibacterial activities. Carbohydrates (including sucrose) were the major nutritional constituents (56.35–78.12 g/100 g dw), followed by proteins (11.2–30.0 g/100 g dw). The higher energy value (432.3 kcal/100 g dw) was presented by the wheat germ. This by-product also presented the highest citric acid content (0.857 g/100 g dw), the most abundant organic acid detected. Unsaturated fatty acids predominated in all samples given the high content of linoleic (53.9–57.1%) and oleic (13.4–29.0%) acids. Wheat germ had the highest levels of tocopherols (22.8 mg/100 g dw) and phenolic compounds (5.7 mg/g extract, with a high apigenin-*C*-pentoside-*C*-hexoside content). In turn, while the wheat bran extract was particularly effective in inhibiting the formation of thiobarbituric acid reactive substances (TBARS), the rye bran extract was the only sample capable of protecting erythrocytes from oxidative hemolysis. Regarding antibacterial properties, in general, the lowest minimum inhibitory concentrations were observed against methicillin-resistant *Staphylococcus aureus*. These results highlight the characterized by-products as sustainable ingredients for the development of novel bakery and functional food products and contribute to a better bioresource-use efficiency and circularity.

**Keywords:** cereal bran/germ; nutritional quality; chemical composition; antioxidant activity; antimicrobial activity; bioresource-use efficiency



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## 1. Introduction

Cereal grains are rich in phytochemicals and nutrients, such as phenolic acids, flavonoids, carbohydrates, dietary fibers, proteins, and tocopherols, among other constituents, which have a vital role in preventing cardiovascular and digestive system diseases, overweight and obesity, inflammation, type 2 diabetes, and some types of cancer [1]. Some studies have shown that consumers are increasingly aware of the relationship between diet and disease, and there is a trend towards a gradual decrease in the consumption of animal-derived protein and a demand for plant-based diets, which have well-known physical and environmental health benefits [2,3].

The food industry has focused on the production of functional foods based on different types of cereals, due to the growing consumer's demand for healthier foods [4,5]. For this purpose, both cereals and their constituents offer unlimited potential and are an excellent raw material for the production of functional foods and functional ingredients, in particular for the design of novel food products based on cereals or their by-products [6]. Cereals

include rice, wheat, rye, maize, barley, sorghum, millet, and oats, among others, and their global production is very extensive as they are the basis of many human diets worldwide. Therefore, the sector is taking into account the sustainability and the efficient use of the by-products generated by the crops or during cereal processing [7]. These result in valuable by-products during milling, such as bran, germ, coat, husk, or endosperm, which could be a good source of potentially marketable ingredients and bioactive compounds [6]. The milling industries commonly release these by-products in the field or direct them to animal feed, bioethanol production, cosmetics, meat substitutes, and nutraceutical/pharmaceutical products, among other applications [8].

During the milling operation, the endosperm is broken down into fine particles (flour), while the germ and bran are removed. The germ is a good source of vitamins (B and tocopherol), minerals, proteins, dietary fiber, carbohydrates, fatty acids, flavonoids, glutathione, and sterols [8–11]. The bran contains fibers and antioxidants, heteroxylans, cellulose, proteins, starch, phenolic acids, lipids, and minerals [12,13].

With the growing world population and given the limited resources our planet can provide, it is essential to produce enough food to meet the growing demands and needs of the human population and also to ensure food security. However, with restricted arable land, the agri-food industry by-products should become recycled within the food chain and, thus, be valorized as a sustainable source of food and food ingredients [6,14], also promoting the circular economy. Therefore, this study was performed to provide a detailed nutritional and chemical characterization of cereal by-products, namely wheat, maize, and rye bran and/or germ, currently produced in large quantities by food ingredients industrial groups, but which have low or no commercial value. Antioxidant and antibacterial activities were also evaluated after preparation of hydroethanolic extracts with these cereal by-products. Overall, it was intended to demonstrate that these milling by-products can have value-added potential in the food market as a low-cost material for the development of nutrient-rich ingredients and bioactive compounds.

## 2. Materials and Methods

### *2.1. Samples and Samples Preparation*

Cereal by-products (wheat germ, maize bran–germ mixture, rye bran, and wheat bran) were kindly supplied by the "Dacsa Group", a food ingredients industrial group from Almàssera-Valencia, Spain, in March 2018. The dry samples were reduced to a fine powder, packaged in sealed plastic bags, and stored at −20 °C until further analysis.

### *2.2. Standards and Reagents*

High-performance liquid chromatography (HPLC)-grade acetonitrile (99.9%), *n*-hexane (95%), and ethyl acetate (99.8%) were purchased from Fisher Scientific (Lisbon, Portugal). The fatty acid methyl ester standard mixture 47885-U, formic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), *L*-ascorbic acid, 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), and the tocopherol ($\alpha$, $\beta$, $\gamma$, and $\delta$ isoforms), free sugar, and organic acid standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phenolic compounds standards were acquired from Extrasynthèse (Genay, France). Thiamine, casamino acids, malt extract, and agar were obtained from Panreac AppliChem (Barcelona, Spain). PDA and PDB mediums were acquired from Oxoid microbiology products (Hampshire, UK). *p*-Iodonitrotetrazolium chloride (INT) was purchased from Panreac Applichem (Barcelona, Spain), Tryptic Soy Broth (TSB), and Mueller- Hinton (MH) from Biolab® (Budapest, Hungary). All other reagents and solvents were of analytical grade and obtained from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

### *2.3. Determination of Nutrients and Energy Value*

Fat, protein, and ash contents were determined by AOAC procedures [15]. The crude fat content was determined by Soxhlet extraction of the samples with petroleum ether

(AOAC 920.85). The ash (total mineral) content was determined by samples incineration at 550 ± 15 °C (AOAC 923.03). The crude protein content was analyzed using an automatic distillation and titration unit (Pro-Nitro-A model, JP Selecta, Barcelona) by the macro-Kjeldahl method (AOAC 978.04) (N × correction factor, namely 5.70 for wheat germ, 6.25 for maize bran/germ mixture, 5.83 for rye bran, and 6.31 for wheat bran) [16]. The results were expressed in g per 100 g of dry weight (dw).

Total carbohydrates were calculated by difference according to Equation (1).

$$\text{Carbohydrates (g/100 g dw)} = 100 - (\text{g protein} + \text{g fat} + \text{g ash}) \quad (1)$$

The energy was calculated according to the Regulation (EU) No 1169/2011 of the European Parliament and of the Council [17], as shown in Equation (2).

$$\text{Energy (kcal/100 g dw)} = 4 \times (\text{g protein} + \text{g carbohydrates}) + 9 \times (\text{g fat}) \quad (2)$$

### *2.4. Evaluation of Chemical Constituents*

#### 2.4.1. Organic Acids

The organic acids profile was analyzed by ultra-fast liquid chromatography (UFLC; Shimadzu 20A series, Kyoto, Japan) following a procedure previously described and optimized by Pereira et al. [18]. Briefly, the samples (1 g) were stirred with 25 mL of meta-phosphoric acid for 45 min and filtered, first through Whatman No. 4 filter paper and then through 0.2 µm nylon filters. Chromatographic separation was achieved in reverse phase on a C18 column (5 µm particle size, 250 × 4.6 mm; Phenomenex, Torrance, CA, USA). Detection was performed in a photo-diode array detector (PDA), at 215 and 245 nm (for ascorbic acid). The detected compounds were identified and quantified by chromatographic comparison of the peak area with calibration curves obtained from commercial standards of oxalic ($y = 1 \times 10^7x + 231{,}891$; $r^2 = 0.9999$; limit of detection (LOD) = 6.3 µg/mL; limit of quantification (LOQ) = 20.8 µg/mL), malic ($y = 950{,}041x + 6255.6$; $r^2 = 0.9999$; LOD = 15.9 µg/mL; LOQ = 52.9 µg/mL), ascorbic ($y = 4 \times 10^7x + 1 \times 10^6$; $r^2 = 0.9909$; LOD = 0.29 µg/mL; LOQ = 0.96 µg/mL), shikimic ($7 \times 10^7x + 175{,}156$; $r^2 = 0.9999$; LOD = 10.2 µg/mL; LOQ = 56.5 µg/mL), citric ($y = 1 \times 10^6x - 10{,}277$; $r^2 = 0.9997$; LOD = 4.4 µg/mL; LOQ = 14.5 µg/mL), and fumaric ($y = 185{,}062x + 117{,}588$; $r^2 = 1$; LOD = 42.5 µg/mL; LOQ = 141.7 µg/mL) acids. The results were expressed in g per 100 g dw.

#### 2.4.2. Free Sugars

Soluble sugars were analyzed in a HPLC system coupled to a refraction index (RI) detector as previously described by Spréa et al. [19]. Briefly, the samples (1 g) were spiked with melezitose (internal standard, 5 mg/mL) and extracted with 80% ethanol at 80 °C. The mixture was centrifuged and the supernatant was concentrated and defatted with ethyl ether. After concentration at 40 °C, the residues were dissolved in 5 mL of water and filtered through 0.2-µm nylon filters. Identification was achieved by comparing the sample retention times with those of the authentic standards, while quantification was based on the internal standard method, with calibration curves constructed with commercial standards of fructose ($y = 1.04x$; $r^2 = 0.999$; LOD = 0.05 mg/mL; LOQ = 0.18 mg/mL), glucose ($y = 0.935x$; $r^2 = 0.999$; LOD = 0.08 mg/mL; LOQ = 0.25 mg/mL), sucrose ($y = 0.977x$; $r^2 = 0.999$; LOD = 0.06 mg/mL; LOQ = 0.21 mg/mL), trehalose ($y = 0.991x$; $r^2 = 0.999$; LOD = 0.07 mg/mL; LOQ = 0.24 mg/mL), and raffinose ($y = 0.891x$, $r^2 = 0.9999$; LOD = 0.09 mg/mL; LOQ = 0.30 mg/mL). The results were expressed in g per 100 g dw.

#### 2.4.3. Fatty Acids

After transesterification of the lipid fraction obtained by Soxhlet extraction [19], the fatty acid methyl ester (FAME) mixture was analyzed by gas-liquid chromatography with flame ionization detection, using a YOUNG IN Chromass 6500 GC System apparatus equipped with a split/splitless injector, a flame ionization detector (FID), and a Zebron-

Fame column. Identification were made by chromatographic comparison of the retention times of the sample FAME peaks with those of the standard 47885-U (Sigma-Aldrich, St. Louis, MO, USA). The results were recorded and processed using Clarity DataApex 4.0 Software (Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

#### 2.4.4. Tocopherols

Tocopherols were analyzed accordingly to a procedure described by Spréa et al. [19], using the HPLC system coupled to a fluorescence detector (FP-2020; Jasco), programmed for excitation at 290 nm and emission at 330 nm. Briefly, the samples (500 mg) were spiked with a BHT solution (10 mg/mL) and tocol (internal standard, 50 µg/mL), and homogenized first with 4 mL of methanol and then with 4 mL of hexane. Then, 2 mL of saturated NaCl aqueous solution were added, the mixture was homogenized, centrifuged, and the upper layer was collected. The extraction was repeated twice with hexane. The extracts were dried under a nitrogen stream, redissolved in 2 mL of *n*-hexane, dehydrated, and filtered through 0.22-µm syringe filters. Chromatographic separation was performed in normal phase on a Polyamide II column (5 µm particle size, 250 × 4.6 mm; YMC, Kyoto, Japan). Identification was made by chromatographic comparison with authentic standards and quantification was based on the fluorescence signal response of each standard, using the internal standard (tocol) method and calibration curves constructed from commercial standards of α-tocopherol (y = 1.295x; $r^2$ = 0.991; LOD = 18.06 ng/mL; LOQ = 60.20 ng/mL), β-tocopherol (y = 0.396x; $r^2$ = 0.992; LOD = 25.82 ng/mL; LOQ = 86.07 ng/mL), γ-tocopherol (y = 0.567x; $r^2$ = 0.991; LOD = 14.79 ng/mL; LOQ = 49.32 ng/mL), and δ-tocopherol (y = 0.678x; $r^2$ = 0.992; LOD = 20.09 ng/mL; LOQ = 66.95 ng/mL). The results were expressed in mg per 100 g dw.

### *2.5. Evaluation of Phenolic Compounds and Bioactivities*

#### 2.5.1. Extracts Preparation

The dry material was used to prepare hydroethanolic extracts as described by Mariotti et al. [16]. The samples (2.5 g) were mixed with 30 mL of ethanol/water (80:20, *v/v*) and stirred for 1 h at 25 °C. The mixtures were then filtered through Whatman No. 4 filter paper and the residues were re-extracted with 30 mL of the same hydroalcoholic mixture. The extracts were first concentrated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) at 40 °C and then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

#### 2.5.2. Phenolic Compounds

Phenolic compounds were analyzed in the hydroethanolic extracts, which were re-dissolved in ethanol/water (80:20, *v/v*) to a final concentration of 10 mg/mL and filtered through 0.22-µm disposable filter disks. The analysis was performed in an HPLC system (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) coupled with a diode-array detector (DAD, using 280 and 370 nm as preferred wavelengths) and a Linear Ion Trap (LTQ XL) mass spectrometer (MS, Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Chromatographic separation was performed on a Waters Spherisorb S3 ODS-2 column (3 µm, 4.6 mm × 150 mm; Waters, Milford, MA, USA). The operating conditions were previously described by the Bessada et al. [20], as well as the identification and quantification procedures. The results were given as mg per g of extract.

#### 2.5.3. Antioxidant Activity

The thiobarbituric acid reactive substances (TBARS) formation inhibition and oxidative hemolysis inhibition (OxHLIA) assays were performed in vitro. Trolox was used as a positive control in both assays. For TBARS, the lyophilized extracts were re-dissolved in ethanol/water (80:20, *v/v*) and subjected to successive dilutions from 10 to 0.019 mg/mL. The lipid peroxidation inhibition was evaluated by the decrease in TBARS formation, using

porcine (*Sus scrofa*) brain cell homogenates. The color intensity of the malondialdehyde–thiobarbituric acid adduct in the mixtures was monitored spectrophotometrically at 532 nm. The inhibition ratio (%) was calculated using the following formula: $[(A-B)/A] \times 100$, where A and B correspond to the absorbance of control and sample solution, respectively [16]. The results were expressed in $IC_{50}$ values (mg/mL), which translate the extract concentration providing 50% of antioxidant activity. For OxHLIA, the anti-hemolytic activity was assessed as described by Silva de Sá et al. [21]. Briefly, an erythrocyte solution (2.8%, *v/v*; 200 µL) was mixed with 400 µL of either: (i) extract solution (0.106–3.4 mg/mL in PBS); (ii) PBS (control); (iii) water (for complete hemolysis); or (iv) trolox (7.81–250 µg/mL). After pre-incubation at 37 °C for 10 min with shaking, 200 µL of AAPH (160 mM in PBS) were added and the optical density was measured at 690 nm every ~10 min in a microplate reader (Bio-Tek Instruments, ELX800) until complete hemolysis. The results were expressed as $IC_{50}$ values (mg/mL), translating the extract concentration able to promote a $\Delta t$ hemolysis delay of 60 and 120 min.

#### 2.5.4. Antibacterial Activity

The antibacterial activity was evaluated by the broth microdilution method coupled to the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay [22]. The tested microorganisms were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal, and included three Gram-positive (*Enterococcus faecalis*, *Listeria monocytogenes*, and methicillin resistant *Staphylococcus aureus*) and five Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*) bacteria. The microorganisms' identification and susceptibility (Table S1) tests were performed as described in Supplementary Material. The minimum inhibitory concentration (MIC) was determined by the colorimetric microbial viability based on the reduction of the INT colorant (0.2 mg/mL). The minimum bactericidal concentration (MBC) was evaluated by plating the wells content without coloration in the MIC assay. Antibiotics were used as positive controls, namely ampicillin (20 mg/mL) and imipenem (1 mg/mL) for Gram-negative bacteria, and vancomycin (1 mg/mL) and ampicillin (20 mg/mL) for Gram-positive bacteria.

### *2.6. Statistical Analysis*

All analyses were performed in triplicate and the results were presented as mean ± standard deviation (SD) (except for antibacterial activity). Since the number of significant figures of the mean value is conditioned by the SD, the SD was rounded to one significant figure, which indicated in which decimal place the uncertain digit of the mean value was. The results were analyzed by a one-way analysis of variance (ANOVA) followed by a Tukey's HSD test, with $\alpha = 0.05$. A Student's *t*-test was applied when only two samples were compared. SPSS v. 23.0 was used for all statistical analysis.

## 3. Results and Discussion

Cereal by-products represent an unexploited source of nutrients and bioactive compounds and could serve as low-cost materials for the development of novel functional and fortifying ingredients for foods and functional foods, as well as for non-food products. Therefore, the macronutrient and micronutrient composition of wheat, rye, and maize bran and/or germ supplied by the milling industry are herein described, as well as their composition in phenolic compounds and in vitro antioxidant and antibacterial activities.

### *3.1. Compositional Features of the Cereal By-Products*

The results in Table 1 show the proximate composition of the different cereal by-products, including fat, protein, ash, and carbohydrates content and energy value. Carbohydrates were the most prevalent constituents, ranging from 56.35 g/100 g dw in wheat germ to 78.12 g/100 g dw in maize bran–germ mixture. The results are slightly higher

than the 46.07 g/100 g dw previously reported by Mahmoud et al. [23] for wheat germ sample from Egypt. Proteins were the second most abundant nutrients and the contents differed significantly between cereal by-products. Interestingly, the samples with the lowest carbohydrate content had the highest protein level. As shown in Table 1, 30.0 g/100 g dw were quantified in wheat germ, whose 100-g portions can provide 53.6 and 65.2% of the recommended dietary allowances of protein for adult male and female individual, respectively [24]. The two by-products with germ, namely wheat germ and the maize bran–germ mixture, presented higher quantities of crude fat (9.64 and 8.08 g/100 g dw, respectively) than the bran samples. Navarro et al. [25] and Mahmoud et al. [23] reported a higher fat content in maize bran–germ (10.74 g/100 g dw) and wheat germ (10.29 g/100 g dw) samples from Brazil and Egypt, respectively. Fats are important nutritional constituents of cereal by-products, being a source of essential fatty acids and energy, and play an important role in their quality [26]. Overall, in terms of energetic contribution, wheat germ presented the highest value (432.3 kcal/100 g dw) in accordance with the higher fat and protein contents, followed by the maize bran–germ mixture (429.8 kcal/100 g dw) and wheat bran (412.4 kcal/100 g dw). The rye bran samples showed the lowest energy value, reaching only 405.4 kcal/100 g dw. Mahmoud et al. [23] reported similar energy values for wheat germ (430.3 kcal/100 g dw), while the National Food Institute [27] presents lower values for wheat germ (379 kcal/100 g), wheat bran (291 kcal/100 g), and common rye flour (322 kcal/100 g). The INRAE-CIRAD-AFZ [28] composition tables present high energy values for maize germ (690 kcal/100 g) and bran (457 kcal/100 g). However, to the best of the authors' knowledge, this is the first study describing the energy value of the maize bran–germ mixture and rye bran.

**Table 1.** Proximate composition and energy value of the cereal by-products.

| | Wheat Germ | Maize Bran–Germ | Rye Bran | Wheat Bran |
|---|---|---|---|---|
| Fat (g/100 g dw) | $9.64 \pm 0.01^{a}$ | $8.08 \pm 0.01^{b}$ | $3.9 \pm 0.1^{d}$ | $5.05 \pm 0.02^{c}$ |
| Proteins (g/100 g dw) | $30.0 \pm 0.1^{a}$ | $11.2 \pm 0.2^{d}$ | $15.3 \pm 0.1^{c}$ | $16.4 \pm 0.1^{b}$ |
| Ash (g/100 g dw) | $3.97 \pm 0.05^{a}$ | $2.66 \pm 0.04^{d}$ | $3.54 \pm 0.04^{b}$ | $3.12 \pm 0.04^{c}$ |
| Carbohydrates (g/100 g dw) | $56.35 \pm 0.02^{d}$ | $78.12 \pm 0.04^{a}$ | $77.2 \pm 0.1^{b}$ | $75.42 \pm 0.01^{c}$ |
| Energy (kcal/100 g dw) | $432.3 \pm 0.1^{a}$ | $429 \pm 0.2^{b}$ | $405.4 \pm 011^{d}$ | $412.8 \pm 0.1^{c}$ |

In each line, different letters indicate significant differences ($p < 0.05$) between samples. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure.

Table 2 shows the free sugars and organic acids detected in the cereal by-products, namely five sugars and six organic acids characterized by HPLC-RI and UFLC-PDA, respectively. Regarding free sugars, sucrose was the most abundant in all cereal by-products, reaching 10.4 g/100 g dw in wheat germ, 3.84 g/100 g dw in the maize bran–germ mixture, and approximately 2.9 g/100 g dw in the bran samples. These results are in agreement with those of Rizzello et al. [29], who also reported sucrose as the main soluble sugar in wheat germ (7.2 g/100 g dw). Table 2 also shows that the free sugar profile of the different samples is different, as some of these water-soluble molecules were not detected in some of the studied cereal by-products. Trehalose and fructose were detected only in wheat germ (0.25 g/100 g dw) and maize bran–germ mixture (0.15 g/100 g dw), respectively, while rye bran did not contain glucose. Overall, the total amount of free sugars ranged from 3.51 g/100 g dw to 15.2 g/100 g dw in wheat germ, this last sample with a considerable difference towards the other cereal by-products. The National Food Institute [27] reported a similar value for a wheat germ sample from Denmark, namely 15.7 g/100 g dw of total free sugars. It is also interesting to note that free sugars correspond to 27% of the carbohydrate fraction of wheat germ and less than 6% in the remaining samples.

**Table 2.** Composition in sugars and organic acids of the cereal by-products.

| | Wheat Germ | Maize Bran–Germ | Rye Bran | Wheat Bran |
|---|---|---|---|---|
| Free sugars (g/100 g dw) | | | | |
| Fructose | nd | 0.15 ± 0.02 | nd | nd |
| Glucose | 0.18 ± 0.01 $^{c}$ | 0.16 ± 0.01 $^{b}$ | nd | 0.14 ± 0.01 $^{a}$ |
| Sucrose | 10.4 ± 0.1 $^{a}$ | 3.84 ± 0.01 $^{b}$ | 2.92 ± 0.03 $^{c}$ | 2.9 ± 0.1 $^{c}$ |
| Trehalose | 0.25 ± 0.01 | nd | nd | nd |
| Raffinose | 4.65 ± 0.03 $^{a}$ | 0.4 ± 0.1 $^{d}$ | 0.59 ± 0.01 $^{c}$ | 1.69 ± 0.01 $^{b}$ |
| Total of free sugars | 15.2 ± 0.1 $^{a}$ | 4.4 ± 0.1 $^{c}$ | 3.51 ± 0.02 $^{d}$ | 4.7 ± 0.1 $^{b}$ |
| Organic acid (g/100 g dw) | | | | |
| Oxalic acid | 0.090 ± 0.001 $^{b}$ | 0.105 ± 0.001 $^{a}$ | 0.0471 ± 0.0001 $^{d}$ | 0.0593 ± 0.0001 $^{c}$ |
| Malic acid | nd | nd | tr | tr |
| Ascorbic acid | tr | tr | nd | nd |
| Shikimic acid | nd | nd | nd | tr |
| Citric acid | 0.857 ± 0.002 $^{a}$ | 0.204 ± 0.001 $^{d}$ | 0.424 ± 0.001 $^{c}$ | 0.539 ± 0.001 $^{b}$ |
| Fumaric acid | tr | tr | tr | tr |
| Total of organic acids | 0.946 ± 0.002 $^{a}$ | 0.309 ± 0.001 $^{d}$ | 0.471 ± 0.001 $^{c}$ | 0.598 ± 0.001 $^{b}$ |

In each line, different letters indicate significant differences ($p < 0.05$) between samples. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure. nd—not detected; tr—traces (below LOQ).

Regarding organic acids, malic, ascorbic, shikimic, and fumaric acids were detected in trace amounts and just in some samples (Table 2). Citric acid was the main compound identified in the cereal by-products, and its levels contribute greatly to the total amounts of organic acids recorded in wheat germ (0.946 g/100 g dw), followed by wheat bran (0.598 g/100 g dw), rye bran (0.471 g/100 g dw), and maize bran–germ mixture with the lowest levels (0.309 g/g 100 g dw). Oxalic acid was also detected in all samples, especially in the maize bran–germ mixture (0.105 g/100 g dw). To the best of the authors knowledge, this is the first report describing the organic acids composition of these by-products. However, wheat is recognized as a complete cereal because it has much higher soluble sugars and organic acids than other cereals, regardless of the cereal part under analysis (bran or germ). Sugars and organic acids are important quality indicators that can be related to preservation and storage conditions [30,31].

Table 3 shows the lipophilic compounds detected in the cereal by-products, namely fatty acids and tocopherols. The GC-FID analysis allowed the detection of 13 fatty acids, whose contents are presented in relative percentage. Linoleic acid (C18:2n6c) was the most abundant fatty acid, with levels up to 53% in all samples. Similar C18:2n6c contents (55.1 and 54.4%) were reported by Mahmoud et al. [23] for a wheat germ oil sample from Egypt and by Abdelghany et al. [32] for soybean seed accessions collected in China, respectively. This essential fatty acid is precursor of eicosanoids, which take part in many biological processes, and required for normal human health. Oleic acid (C18:1n9c) ranked second and prevailed in the maize bran–germ mixture (29%). This omega-9 fatty acid was also previously reported as the second most abundant in soybean in a slightly lower relative percentage (24.6%) [32]. Regarding saturated fatty acids (SFA), palmitic acid (C16:0) was the major contributor and prevailed in wheat bran and germ samples (with approximately 18%), while stearic acid (C18:0) predominated in the maize bran–germ mixture (2.96%). In turn, the higher amounts of monounsaturated fatty acids (MUFA) were quantified in the maize bran–germ mixture (29.2%). In general, polyunsaturated fatty acids (PUFA) predominated in all samples, with levels ranging from 55.9 to 65.4%. The fatty acid profiles observed in the characterized cereal by-products are in agreement with previous reports [33–36]. Fatty acids are good food quality indicators, since lipid peroxidation affects the overall quality and odor of cereals when stored for a long period [30,37].

**Table 3.** Composition in fatty acids and tocopherols of the cereal by-products.

| | Wheat Germ | Maize Bran–Germ | Rye Bran | Wheat Bran |
|---|---|---|---|---|
| Fatty acids (relative %) | | | | |
| C14:0 | 0.12 ± 0.01 $^{b}$ | nd | 0.14 ± 0.01 $^{a}$ | 0.126 ± 0.001 $^{b}$ |
| C15:0 | 0.073 ± 0.003 $^{c}$ | nd | 0.15 ± 0.01 $^{a}$ | 0.104 ± 0.001 $^{b}$ |
| C16:0 | 18.02 ± 0.02 $^{a}$ | 10.6 ± 0.3 $^{c}$ | 15.59 ± 0.04 $^{b}$ | 18.3 ± 0.2 $^{a}$ |
| C16:1 | 0.17 ± 0.02 $^{c}$ | 0.101 ± 0.004 $^{d}$ | 0.27 ± 0.02 $^{a}$ | 0.20 ± 0.01 $^{b}$ |
| C18:0 | 0.86 ± 0.01 $^{c}$ | 2.955 ± 0.001 $^{a}$ | 1.3 ± 0.1 $^{b}$ | 1.32 ± 0.01 $^{b}$ |
| C18:1n9c | 13.4 ± 0.1 $^{d}$ | 29.0 ± 0.1 $^{a}$ | 17.31 ± 0.04 $^{b}$ | 16.07 ± 0.04 $^{c}$ |
| C18:2n6c | 57.1 ± 0.1 $^{a}$ | 54.8 ± 0.2 $^{c}$ | 53.9 ± 0.1 $^{d}$ | 56.3 ± 0.2 $^{b}$ |
| C18:3n3 | 8.0 ± 0.1 $^{b}$ | 1.04 ± 0.01 $^{d}$ | 8.6 ± 0.1 $^{a}$ | 5.17 ± 0.05 $^{c}$ |
| C20:0 | 0.205 ± 0.001 $^{c}$ | 0.48 ± 0.01 $^{a}$ | nd | 0.24 ± 0.01 $^{b}$ |
| C20:1 | 1.35 ± 0.03 $^{b}$ | 0.168 ± 0.005 $^{d}$ | 1.456 ± 0.003 $^{a}$ | 1.0 ± 0.1 $^{c}$ |
| C22:0 | 0.25 ± 0.02 $^{c}$ | 0.36 ± 0.03 $^{b}$ | 0.42 ± 0.01 $^{a}$ | 0.41 ± 0.01 $^{a}$ |
| C20:5n3 | 0.30 ± 0.02 $^{c}$ | nd | 0.47 ± 0.01 $^{a}$ | 0.40 ± 0.02 $^{b}$ |
| C24:0 | 0.20 ± 0.01 $^{d}$ | 0.46 ± 0.01 $^{b}$ | 0.49 ± 0.03 $^{a}$ | 0.29 ± 0.01 $^{c}$ |
| SFA | 19.73 ± 0.02 $^{b}$ | 14.9 ± 0.3 $^{d}$ | 18.1 ± 0.1 $^{c}$ | 20.8 ± 0.2 $^{a}$ |
| MUFA | 14.9 ± 0.1 $^{d}$ | 29.2 ± 0.1 $^{a}$ | 19.03 ± 0.02 $^{b}$ | 17.3 ± 0.1 $^{c}$ |
| PUFA | 65.4 ± 0.1 $^{a}$ | 55.9 ± 0.2 $^{d}$ | 62.9 ± 0.1 $^{b}$ | 61.9 ± 0.3 $^{c}$ |
| Tocopherols (mg/100 g dw) | | | | |
| α-Tocopherol | 13.46 ± 0.01 $^{a}$ | 3.38 ± 0.02 $^{d}$ | 4.1 ± 0.1 $^{c}$ | 5.23 ± 0.04 $^{b}$ |
| β-Tocopherol | 9.27 ± 0.04 $^{a}$ | 0.12 ± 0.03 $^{d}$ | 1.27 ± 0.01 $^{c}$ | 2.99 ± 0.01 $^{b}$ |
| γ-Tocopherol | nd | 1.61 ± 0.03 $^{a}$ | nd | 0.140 ± 0.001 $^{b}$ |
| δ-Tocopherol | 0.046 ± 0.001 | nd | nd | nd |
| Total of tocopherols | 22.8 ± 0.1 $^{a}$ | 5.10 ± 0.02 $^{d}$ | 5.4 ± 0.1 $^{c}$ | 8.35 ± 0.04 $^{b}$ |

In each line, different letters indicate significant differences ($p < 0.05$) between samples. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure. SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; nd—not detected.

The results of the tocopherols composition are also shown in Table 3. *α*-Tocopherol was found in all cereal by-products and as the predominant isoform, especially in wheat germ (13.46 mg/100 g dw), where it was possible to detected *δ*-tocopherol. The study of Górnaś et al. [38] reports lower *α*-tocopherol values for wheat and rye bran (1.9 and 1.4 mg/100 g dw, respectively) than those achieved in the present study. In turn, higher levels were obtained by Ansolin et al. [39] in wheat germ oil (28.5 mg/100 g) and by Navarro et al. [25] in maize bran–germ oil (13 mg/100 g dw). Wheat bran and germ samples stand out with the highest amounts of *β*-tocopherol (2.99 and 9.27 mg/100 g dw, respectively). Only two samples contained *γ*-tocopherol, namely the maize bran–germ mixture and wheat bran (1.61 and 0.14 mg/100 g dw, respectively). The tocopherol profiles herein reported are in agreement with those previously reported [9,25,38]. Overall, wheat by-products showed the highest concentration of tocopherols, with 22.8 mg/100 g dw quantified in the germ and 8.35 ± 0.04 mg/100 g dw in the bran. Based on the α-tocopherol values in Table 3 and the recommended dietary allowances for vitamin E of 15 mg/day for healthy adults [24], it could be concluded that a 100-g portion of wheat germ contributes 61.8% to the intake of this liposoluble vitamin.

### *3.2. Phenolic Composition of the Cereal By-Products*

Cereal by-products are important sources of phytochemicals such as phenolic compounds, which have gained major attention since they exhibit a wide range of biological activities, including antioxidant and antimicrobial effects [40,41]. The chromatographic information (retention time, wavelengths of maximum absorption in the visible region ($\lambda_{max}$), and mass spectral data) used in the tentative identification of the phenolic compounds detected in the hydroethanolic extracts prepared with the cereals by-products are shown in Table 4. Eleven phenolic compounds were tentatively identified in the extracts, including 4

phenolic acids (*p*-coumaroyl, caffeic, and chlorogenic acid derivatives), 6 flavonoids (luteolin and apigenin *C*-glycosylated derivatives) and one unknown compound that was found in maize bran–germ and rye bran samples. Regarding phenolic acids, peak 1 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 487 and a unique $MS^2$ fragment at *m/z* 163 (loss of two hexosyl moieties), coupled to a characteristic UV spectrum of p-coumaric acid at 301 nm, the peak was tentatively identified as *p*-coumaric acid dihexoside. Peaks 2 and 3 were tentatively identified as caffeic acid hexoside, presenting a pseudomolecular ion $[M-H]^-$ at *m/z* 341 and $MS^2$ fragments at *m/z* 179, 161, and 135, that correspond to the break of the caffeic acid unit and the loss of one hexosyl moiety (341−179 = 162 u). Finally, peak 9, with $[M-H]^-$ at *m/z* 515 and $\lambda_{max}$ 311 nm (characteristic of chlorogenic acid derivatives), was tentatively identified as 3,5-*O*-dicaffeoylquinic acid using the hierarchical fragmentation pattern previously reported by Clifford et al. [42,43].

**Table 4.** Phenolic compounds tentatively identified in the hydroethanolic extracts of the cereal by-products. It is presented the retention time (Rt), wavelengths of maximum absorption in the visible region ($\lambda_{max}$), and mass spectral data.

| Peak | Rt (min) | $\lambda_{max}$ (nm) | $[M-H]^-$ (*m/z*) | $MS^2$ (*m/z*) | Tentative Identification |
|---|---|---|---|---|---|
| 1 | 4.7 | 301 | 487 | 162 (100) | *p*-Coumaric acid dihexoside |
| 2 | 4.8 | 311 | 341 | 179(100), 161(15), 132(5) | Caffeic acid hexoside |
| 3 | 5.43 | 311 | 341 | 179(100), 161(15), 132(5) | Caffeic acid hexoside |
| 4 | 11.45 | 345 | 579 | 459(35), 429(10), 357(5), 327(10), 309(5) | Luteolin-*O*-pentoside-*C*-hexoside |
| 5 | 12.11 | 287 | 385 | 267 (100), 249(20) | Unknown compound |
| 6 | 12.86 | 336 | 563 | 545(43), 473(100), 443(7), 383(31), 353(28), 311(5) | Apigenin-*C*-pentoside-*C*-hexoside |
| 7 | 13.21 | 326 | 563 | 545(20), 473(92), 443(100), 383(28), 353(25), 311(5) | Apigenin-*C*-hexoside-*C*-pentoside |
| 8 | 14.21 | 336 | 563 | 545(32), 473(100), 443(98), 383(38), 353(31), 311(5) | Apigenin-*C*-pentoside-*C*-hexoside |
| 9 | 17.11 | 324 | 515 | 353(60), 191(100), 179(30), 173(5), 161(5), 135(5) | 3,5-*O*-Dicaffeoylquinic acid |
| 10 | 19.65 | 331 | 769 | 563(11), 545(81), 425(100), 335(31) | Sinapic acid ester of apigenin-*C*-diglycoside |
| 11 | 20.53 | 331 | 769 | 563(10), 545(89), 425(100), 335(12) | Sinapic acid ester of apigenin-*C*-diglycoside |

The flavonoids group represented the majority of the compounds identified in the cereal by-products, being apigenin derivatives the ones with higher expression in all samples (expect in maize bran). Peaks 6, 7, and 8 all presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 563, and characteristics $MS^2$ fragment at *m/z* 473 (90 u), 383 (90 u), 353 (30 u), that correspond to the loss of units in multiples of 30, leading to the identification of *C*-glycosylated derivatives. The differentiation between the three peaks, take into account the abundance of 100% in $MS^2$ fragment at *m/z* 473 in peaks 6 and 8, and the $MS^2$ fragment at *m/z* 443 in peak 7, which lead to the tentative identification of apigenin-*C*-pentoside-*C*-hexoside (peaks 6 and 8) and apigenin-*C*-hexoside-*C*-pentoside (peak 7) [44]. Peaks 10 and 11 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 769 and an $MS^2$ fragmentation pattern coherent with that previously described by Hirawan and Beta [45] in whole-wheat Spaghetti, being both tentatively identified as sinapic acid esters of apigenin-*C*-diglycoside. Finally, the last flavonoid found was peak 4 tentatively identified as luteolin-*O*-pentoside-*C*-hexoside ($[M-H]^-$ at *m/z* 579), using the chromatographic profile previously described by Roriz et al. [46].

As presented in Table 5, the flavonoids group represents the majority (in content) of the compounds found in all samples (except for maize bran), more specifically, 81% (4.7 mg/g extract) in wheat germ and 77% (3.4 mg/g extract) in wheat bran of the total phenolic compounds. These concentrations are mainly due to the presence of apigenin-*C*-pentoside-*C*-hexoside, with 2.95 mg/g of extract in the wheat germ and 2.09 mg/g of extract in wheat bran. In maize bran and rye bran, phenolic acids were detected in higher concentrations, 0.046 mg/g extract in maize bran (peak 2 corresponded to the only phenolic compound found in this sample) and 0.93 mg/g extract in rye bran (mainly due to the

presence of *p*-coumaric acid dihexoside, in agreement with previous reports found in the literature [47–49].

**Table 5.** Phenolic compounds content in the hydroethanolic extracts of the cereal by-products.

| Peak | Tentative Identification | Content (mg/g Extract) | | | |
|---|---|---|---|---|---|
| | | Wheat Germ | Maize Bran–Germ | Rye Bran | Wheat Bran |
| 1 | *p*-Coumaric acid dihexoside | nd | nd | 0.57 ± 0.02 | nd |
| 2 | Caffeic acid hexoside | 0.088 ± 0.001 $^{b}$ | 0.046 ± 0.001 $^{c}$ | nd | 0.115 ± 0.006 $^{a}$ |
| 3 | Caffeic acid hexoside | nd | nd | nd | nd |
| 4 | Luteolin-*O*-pentoside-*C*-hexoside | 0.121 ± 0.004 * | nd | nd | 0.084 ± 0.001 |
| 5 | Unknown compound | nd | nq | nq | nd |
| 6 | Apigenin-*C*-pentoside-*C*-hexoside | 0.602 ± 0.01 * | nd | nd | 0.39 ± 0.01 |
| 7 | Apigenin-*C*-hexoside-*C*-pentoside | 1.004 ± 0.058 * | nd | nd | 0.80 ± 0.05 |
| 8 | Apigenin-*C*-pentoside-*C*-hexoside | 2.9 ± 0.1 $^{a}$ | nd | 0.067 ± 0.002 $^{c}$ | 2.1 ± 0.1 $^{b}$ |
| 9 | 3,5-*O*-Dicaffeoylquinic acid | 0.34 ± 0.01 $^{a}$ | nd | 0.293 ± 0.001 $^{b}$ | 0.35 ± 0.01 $^{a}$ |
| 10 | Sinapic acid ester of apigenin-*C*-diglycoside | 0.16 ± 0.01 * | nd | nd | 0.140 ± 0.001 |
| 11 | Sinapic acid ester of apigenin-*C*-diglycoside | 0.470 ± 0.001 $^{a}$ | nd | 0.057 ± 0.001 $^{c}$ | 0.413 ± 0.007 $^{b}$ |
| | Σ Phenolic acids | 1.066 ± 0.002 $^{a}$ | 0.046 ± 0.001 $^{d}$ | 0.93 ± 0.02 $^{c}$ | 1.017 ± 0.001 $^{b}$ |
| | Σ Flavonoids | 4.7 ± 0.1 $^{a}$ | nd | 0.067 ± 0.002 $^{c}$ | 3.4 ± 0.1 $^{b}$ |
| | Σ Phenolic compounds | 5.7 ± 0.1 $^{a}$ | 0.046 ± 0.001 $^{d}$ | 0.418 ± 0.003 $^{c}$ | 4.3 ± 0.1 $^{b}$ |

Calibration curves used in quantification: apigenin-6-C-glucoside (y = 107025x + 61531, $r^2$ = 0.9989, LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL; peak 6, 7, and 8); caffeic acid (y = 388345x + 406369, $r^2$ = 0.9939, LOD = 0.78 µg/mL, and LOQ = 1.97 µg/mL; peaks 2 and 3); chlorogenic acid (y = 168823x − 161172, $r^2$ = 0.9999, LOD = 0.20 µg/mL, and LOQ = 0.68 µg/mL; peak 9); luteolin-6-C-glucoside (y = 4087.1x + 72589, $r^2$ = 0.9988, LOD = 0.20 µg/mL, and LOQ = 0.45 µg/mL; peak 4); *p*-coumaric acid (y = 301950x + 6966.7, $r^2$ = 0.9999, LOD = 0.68 µg/mL, and LOQ = 1.61 µg/mL; peak 1); sinapic acid (y = 197337x + 30036, $r^2$ = 0.9997, LOD = 0.17 µg/mL, and LOQ = 1.22 µg/mL; peaks 10 and 11). Statistically significant differences ($p < 0.05$) between more than two samples were assessed by a one-way ANOVA, using Tukey's significant difference (HSD), and are indicated by different letters; significant differences ($p < 0.001$) between two samples * were assessed by a Student's *t*-test. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure. nd—not detected; nq—not quantified.

Wheat germ and bran were the samples presenting the highest concentration of phenolic compounds (5.7 and 4.4 mg/g extract, respectively), and also a higher concentration of total phenolic acids (1.066 and 1.017 mg/g extract, respectively). The phenolic compounds of wheat were previously studied by different authors, with different phenolic acids and results obtained. Zou et al. [50] reported ferulic acid as the major compound found in wheat germ (250 µg/g of extract) and 554 µg/g extract of total phenolic acids. Vaher [51], in bran spring wheat, also reported ferulic acid was the main one (268.9 µg/g dw) and the amount of total phenolic acids was 569 µg/g dw. López-Perea et al. [52] reported caffeic acid as the major compound (0.86 µg/g dw) and the total of phenolic compound was 100 µg/g dw in wheat bran. Skrajda-Brdak et al. [49] reported ferulic acid (222.1 µg/g dw) as the main compound and the total phenolic acids content was 273.2 µg/g dw for common wheat.

### *3.3. Bioactivity of the Cereal By-Products*

Whole cereal grains have been described as having great bioactive properties due to their fractions, bran and germ, that comprise health-promoting bioactive compounds [41]. Therefore, the antioxidant activity of the cereal by-product hydroethanolic extracts was evaluated in vitro by the thiobarbituric acid reactive substances (TBARS) formation inhibition, using porcine brain tissue homogenates, and the oxidative hemolysis inhibition assay (OxHLIA), using sheep erythrocytes. The results are presented in Table 6 and were expressed in $IC_{50}$ values, translating the extract concentration providing 50% of antioxidant activity in the TBARS assay or the concentration able to protect 50% of the erythrocyte population in OxHLIA. The cereal by-product with the best TBARS formation inhibition

capacity was the wheat bran (with the lowest $IC_{50}$ value of 0.26 mg/mL), followed by the maize bran–germ mixture and rye bran. The lowest activity was shown by the wheat germ extract, not agreeing, therefore, with the highest content of phenolic compounds quantified in this sample (Table 5), but coinciding with the highest levels of citric acids (Table 2) and tocopherols (Table 3). For OxHLIA, only rye bran presented capacity to protect the erythrocyte membrane form the free radicals generated in the system by the thermal decomposition of AAPH. The other extracts did not delay hemolysis compared to the used negative control (PBS). It should be noted that the lower $IC_{50}$ values of trolox are due to the fact that this positive control is a pure antioxidant compound, while the natural extracts tested are complex mixtures of different constituents, some of which have no activity. The antioxidant activity of the studied cereals has been demonstrated by previous studies, but must of them used chemical methods, such as reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and Folin–Ciocalteu assays, among others [53–55]. However, cell-based assays have been described as more suitable to measure the antioxidant activity of natural products than chemical methods [56]. It is also worth noting that, according to some studies, cereal phenolic acids display antioxidant properties in vitro due to the presence of an aromatic phenolic ring [41].

**Table 6.** Antioxidant activity ($IC_{50}$ values, mg/mL) of the cereal by-product hydroethanolic extracts.

| | Wheat Germ | Maize Bran–Germ | Rye Bran | Wheat Bran | Trolox |
|---|---|---|---|---|---|
| TBARS | $4.8 \pm 0.1^{a}$ | $0.62 \pm 0.01^{c}$ | $0.98 \pm 0.01^{b}$ | $0.26 \pm 0.01^{d}$ | $0.023 \pm 0.001^{e}$ |
| OxHLIA, $\Delta t$ 60 min | na | na | $0.58 \pm 0.02$ | na | $0.020 \pm 0.001$ |
| OxHLIA, $\Delta t$ 120 min | na | na | $1.02 \pm 0.04$ * | na | $0.041 \pm 0.001$ |

For TBARS, statistically significant differences ($p < 0.05$) between samples were assessed by a one-way ANOVA, using Tukey's significant difference (HSD), and are indicated by different letters; for OxHLIA, significant differences ($p < 0.001$) between the two samples * were assessed by a Student's *t*-test. The number of significant figures of each mean value was conditioned by the standard deviation, which was rounded to one significant figure. na—no activity.

The antibacterial activity of the cereal by-products was also tested against Gram-positive and Gram-negative bacteria and the results are presented in Table 7 as minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively). In general, the extracts were not very effective against the selected foodborne microorganisms. The best results were achieved against methicillin-resistant *Staphylococcus aureus* with the maize bran–germ moisture extract (MIC of 2.5 mg/mL) and rye bran and wheat bran extracts (MIC of 5 mg/mL). MBC values above 20 mg/mL were obtained in all cases. Among the tested microorganisms, the Gram-positive bacteria appear to be more susceptible to the extracts than the Gram-negative bacteria. The positive controls ampicillin, imipenem, and vancomycin yielded much lower MIC and MBC values, as expected for commercial antibiotics. Despite the low efficacy of the tested extracts, isolated compounds such as phenolic acids can display remarkable antimicrobial effects [41], which highlights their potential for application as preservatives in food and food-packing materials, among others. Regarding previous studies, Călinoiu and Vodnar [57] reported better results for heat processed wheat bran, with an MIC of 1.875 mg/mL against *Enterococcus faecalis*. In turn, a moderate antibacterial effect toward *Pseudomonas aeruginosa* was achieved with heat processed wheat bran and fresh wheat bran extracts, with MIC values of 3.75 mg/mL and 7.5 mg/mL, respectively. The same authors attributed moderate antibacterial activity to heat processed wheat bran against *Escherichia coli* (MIC of 3.75 mg/mL) and *Salmonella typhimurium* (MIC of 7.5 mg/mL).

**Table 7.** Antibacterial activity (mg/mL) of the cereal by-product hydroethanolic extracts.

| | Wheat Germ | | Maize Bran–Germ | | Rye Bran | | Wheat Bran | | Ampicillin | | Imipenem | | Vancomycin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Gram-negative bacteria | | | | | | | | | | | | | | |
| *Escherichia coli* | 10 | >20 | 10 | >20 | 20 | >20 | 20 | >20 | <0.15 | <0.15 | <0.0078 | <0.0078 | nt | nt |
| *Klebsiella pneumoniae* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | 10 | 20 | <0.0078 | <0.0078 | nt | nt |
| *Morganella morganii* | 20 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | <0.0078 | <0.0078 | nt | nt |
| *Proteus mirabilis* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | <015 | <0.15 | <0.0078 | <0.0078 | nt | nt |
| *Pseudomonas aeruginosa* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | 0.5 | 1 | nt | nt |
| Gram-positive bacteria | | | | | | | | | | | | | | |
| *Enterococcus faecalis* | 10 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | <0.15 | <0.15 | nt | nt | <0.0078 | <0.0078 |
| *Listeria monocytogenes* | 10 | >20 | 20 | >20 | 20 | >20 | 10 | >20 | <0.15 | <0.15 | <0.0078 | <0.0078 | nt | nt |
| MRSA | 10 | >20 | 2.5 | >20 | 5 | >20 | 5 | >20 | <0.15 | <0.15 | nt | nt | 0.25 | 0.5 |

MRSA—Methicillin-resistant *Staphylococcus aureus*; MIC—minimum inhibitory concentration; MBC—minimal bactericidal concentration; nt—not tested.

## 4. Conclusions

This study contributes to the valorization of cereal by-products generated worldwide by the flour milling industry. The findings herein described are valid arguments to support the use of the industry's cereal germ and bran by-products as underexploited alternative sources of nutrients and bioactive compounds with potential health benefits for consumers. Among the studied cereal by-products, wheat germ has emerged as an interesting source of protein and vitamin E, given the high contribution to the recommended dietary allowances. Wheat germ, maize bran–germ mixture, rye bran, and wheat bran could be directed to human nutrition as a sustainable way to promote the development of novel and functional foods, the bioresource-use efficiency, and the circular bioeconomy in this so important food sector. The results can also be useful to complete food and/or feed composition tables.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050972/s1, Table S1: Resistance profile of the tested Gram-positive and Gram-negative bacteria to different antibiotics. Minimum inhibitory concentration (MIC) in μg/mL.

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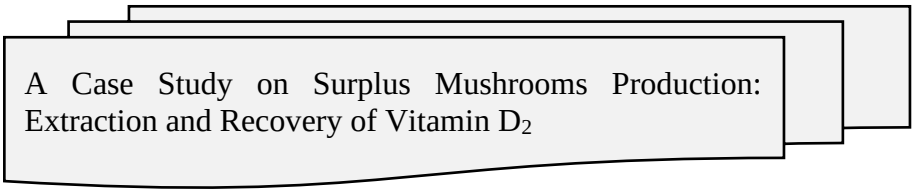
**Annex G**


A Case Study on Surplus Mushrooms Production: Extraction and Recovery of Vitamin $D_2$

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# A Case Study on Surplus Mushrooms Production: Extraction and Recovery of Vitamin $D_2$

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**Abstract:** The presented case study illustrates the possibility of adding value to the biological surplus remaining from the mushroom cultivation industry. In essence, the unused mushroom parts were submitted to UV-C irradiation, with the purpose of increasing the vitamin $D_2$ content and validating its extraction. Vitamin $D_2$ concentration in three different mushroom species (*Agaricus bisporus*, *A. bisporus* Portobello, and *Pleurotus ostreatus*) was obtained by high-performance liquid chromatography (HPLC), by means of an ultraviolet (UV) detector. The method was validated using an *A. bisporus* Portobello sample, and its reproducibility and accuracy were confirmed. Independently of the UV-C irradiation dose, the effect on vitamin $D_2$ concentration was significant, allowing it to increase from less than 4 μg/g dry weight (dw) to more than 100 μg/g dw in all mushroom species. These results are good indicators of the feasibility of industrial surplus mushrooms as sustainable vitamin $D_2$ food sources, besides contributing to strengthen the circularity principals associated to the mushroom production chain.

**Keywords:** surplus mushroom; natural resources; UV-C irradiation; vitamin $D_2$; natural-based ingredients; circular economy



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## 1. Introduction

The global mushroom market share is dominated by *Agaricus bisporus* (J.E.Lange) Imbach (button mushroom), *Lentinula edodes* (Berk.) Pegler (shiitake mushroom) and *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. (oyster mushroom) [1]. The production of mushrooms and truffles is dominated by Asia (78.2%), followed by Europe (14.7%), and then the Americas (6.2%) [2]. During mushroom production, a percentage as high as 20% of surplus might be generated [3]. These mushrooms have low industrial application, because they are in an advanced stage of maturation, or they have deformed lids and/or stems that do not meet the specifications established by retailers, so they are considered mushrooms of low economic value. These unused mushrooms are often discarded, even though their high nutritional compounds (e.g., proteins, carbohydrates) and valuable chemical compounds (e.g., amino acids, polysaccharides, sterols) are not compromised [3–6].

Currently, the disposal procedures (such as incineration, burying, and landfilling) employed to eliminate these surplus mushrooms generate some cost and may have an environmental impact; these techniques can cause water source contamination, acidification, eutrophication, air pollution, depletion of natural resources, eco-toxicity, among others [3,6].

In this sense, innovative alternatives to add value to this surplus mushroom production need to be explored. The irradiation of surplus mushrooms to obtain vitamin $D_2$ is a sustainable strategy to increase vitamin D availability. In Europe, for example, assessments

of vitamin D intake showed that for 77–100% of adults (19–64 years old) and 55–100% of elderly adults (>64 years old), vitamin D intake is inadequate [7,8]. In recent years, with advances in the food industry, in parallel with consumer demand for natural-based options, fortified or enriched foods with natural vitamin $D_2$ are an innovative alternative, particularly for specific groups such as vegans [9,10]. In addition, surplus mushroom production can be used to prepare vitamin $D_2$-enriched extracts that could be applied by the pharmaceutical industry as nutritive supplements [3].

In their natural state, mushrooms present very low concentrations of vitamin $D_2$ [11]. Nonetheless, several researchers have found them to be a rich source of ergosterol (a precursor of vitamin $D_2$), which can be converted into vitamin $D_2$ by artificial UV irradiation [11,12]. Studies assessing the effects of radiation on ergosterol conversion into vitamin $D_2$ using UV light are mostly available for cultivated species namely the ones with high production value [11,13]. There are several examples of mushroom species where some amounts of vitamin $D_2$ have been developed after irradiation [11,13–16]. To the best of the authors' knowledge, the present study provides the first report of the use of surplus mushrooms as a sustainable source of vitamin $D_2$.

For natural vitamin $D_2$ to be a real and promising alternative, it is necessary to find suitable methodologies for its extraction, and to develop effective recovery processes. Several technologies are available to extract and quantify vitamin $D_2$ [17]. However, the effectiveness of the employed methodologies is affected by factors such as time, temperature, power, and solvent type. It is therefore important to combine the best operational conditions to achieve the best vitamin $D_2$ recovery indices [3,18].

In view of the growing consumer demand for natural-based ingredients, the objective of this case study was to set the UV-C irradiation and extraction conditions that maximize vitamin $D_2$ contents in the surplus mushrooms production and, meeting the concept reduce-reuse-recycle to minimize the surplus in the mushrooms production sector. The bioactive effects and potential toxicity of vitamin $D_2$-enriched extracts were also evaluated.

## 2. Materials and Methods

### *2.1. Samples Information, UV-C Irradiation and Reagents*

The surplus production from *P. ostreatus* and *A. bisporus* (Portobello and white mushroom) were supplied by Ponto Agrícola, Baião, north of Portugal. Subsequently, the fresh samples were sliced (2 to 3 mm) and divided into the following four groups with twenty specimens in each group: control (non-irradiated, 0.0 mJ/cm$^2$), sample 1 (200 mJ/cm$^2$), sample 2 (800 mJ/cm$^2$) and sample 3 (3200 mJ/cm$^2$) [12,19].

The irradiation was performed at the Centro de Investigação de Montanha of Instituto Politécnico de Bragança, Portugal and took place in an ultraviolet (UV-C) radiation chamber (JP Selecta, Barcelona, Spain) with the following different exposure times: 0, 2, 6 and 10 min. Before analysis, the samples were lyophilized and reduced to a fine, dried powder, and mixed to obtain homogenized samples.

The standard of pure vitamin $D_2$ was purchased from Acrōs Organics (Fair Lawn, NJ, USA). HPLC-grade acetonitrile (99.9%) and n-hexane (95%) were purchased from Fisher Scientific (Lisbon, Portugal). Dimethyl sulfoxide was purchased from Fisher Scientific (Loughborough, UK), sulforhodamine B and ellipticine were acquired from Sigma-Aldrich (St. Louis, MO, USA), four human tumor cell lines were acquired from Leibniz-Institut DSMZ and one non-tumoral cell line was obtained from ATCC, LGC Standards (Middlesex, UK).

Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). Methanol and acetonitrile were of HPLC grade, from Lab-Scan (Dublin, Ireland).

### *2.2. Method Proof Assays*

The sensitivity and linearity of the HPLC analysis were determined and the method was validated by the instrumental precision, repeatability, and accuracy, using the best

extract obtained. Precision was accessed after six extractions of the same sample; each one being analyzed twice in the same day. The repeatability was accomplished by analyzing the mushroom sample, six times in the same day. The accuracy of the method was evaluated by the standard addition procedure (percentage of recovery), with three additional levels (25%, 50%, and 100% of the peak/area concentration), each one in triplicate. The standard mixture (vitamin $D_2$) was added to the sample and the extraction procedure was carried out [20].

### *2.3. Extraction Methodology and Chromatographic Analysis*

Vitamin $D_2$ was extracted and analyzed according to the method described by Huang et al. [21], with some modifications. Mushroom powder (2.5 g) was mixed with 10 mL of dimethyl sulfoxide and ultrasound-oscillated at 45 °C for 30 min. Then 10 mL of methanol and water (1:1, *v*/*v*) and 20 mL of hexane were added, and the mixture was ultrasound-oscillated at 45 °C for 30 min and centrifuged at 5200× *g* for 5 min (Centurion K24OR, West Sussex, UK). The residue was extracted twice with 20 mL of hexane and centrifuged. The combined filtrate was rotary evaporated (Hei-VAP Advantage, Heidolph, Germany) at 40 °C to dryness, redissolved in 1 mL of methanol (Fisher Scientific, Loughborough, UK), and filtered using a 0.1 µm Whatman nylon filter (Millipore, Billerica, MA, USA) before HPLC injection.

The HPLC system (Knauer system, Smartline 1000, Berlin, Germany) coupled to a UV detector (Knauer Smartline 2500) was used under the same conditions described and optimized by Barreira et al. [22]. Chromatographic separation was performed with an Inertsil 100A ODS-3 reverse phase column (5 µm, 250 × 4.6 mm, BGB Analytik AG, Boeckten, Switzerland) at 35 °C. The mobile phase used was acetonitrile/methanol (70:30, *v*/*v*) at a flow rate of 1 mL/min, with an injection volume of 20 µL and the wavelength was 280 nm. Subsequently, the results were analyzed using the Clarity 2.4 software (DataApex, Pod Ohradska, Czech Republic). Vitamin $D_2$ was quantified based on a calibration curve obtained from a commercial standard vitamin $D_2$, and the results were expressed in µg per g of dry weight (dw).

### *2.4. Bioactivity of the Vitamin $D_2$-Enriched Extract*

According to the extraction results, the most potent extract (*A. bisporus* Portobello irradiated with UV-C, 6 min, 3200 mJ/cm$^2$) was chosen to test the bioactivity in cell lines. In this assay, four human tumor cell lines (MCF-7—breast adenocarcinoma, NCI-H460—non-small cell lung cancer, AGS—gastric cancer, and CaCo-2—colorectal adenocarcinoma) and one non-tumoral cell line of bone origin (h-FOB 1.19—human osteoblasts) were used.

Cell proliferation in the presence and absence of functional extract and pure vitamin $D_2$ was assessed using the sulforhodamine B (SRB) assay. For this assay, the extract prepared above was dissolved in water at a concentration of 8 mg/mL, the procedures were performed as described previously by the authors [23,24], and the final concentrations were 400, 100, 25 and 6.25 mg/mL. Ellipticin was used as a positive control. Absorbance was measured at 495 nm and the results were expressed as $GI_{50}$ values (sample concentration that inhibited 50% of cell growth) in µg per mL.

### *2.5. Statistical Analysis*

All analyses (extractions) were performed in triplicate and each replicate was also quantified three times. Data were expressed as mean ± standard deviation, presenting the significant numbers in agreement with the magnitude of the corresponding standard deviation.

All statistical tests were applied considering a 5% significance level (IBM SPSS Statistics for Windows, Version 22.0. Armonk; IBM Corp., Armonk, NY, USA). The results were compared through two-way analysis of variance (ANOVA) with type III sums of squares, performed using the general linear model (GLM) procedure. The analyzed statistical factors were "exposure time" (ET) and "ultraviolet radiation" (UV-C) and their effects were

classified through the HSD Tukey's test. The statistical interaction among these two factors was verified in all cases.

## 3. Results and Discussion

### *3.1. Method Validation*

For this case study, before the surplus mushroom extract analysis, the correlation coefficient ($R^2$), linearity range, and limits of detection and quantification (LOD and LOQ, respectively) of the methodology employed to determine vitamin $D_2$, were fully validated (Table 1). After the linearity check (linearity range: 0.78–50 µg/mL), a seven-level calibration curve (y = 11.909x + 6.9688) was made, using the peak/area ratio versus concentration of the standard concentration (µg/mL), reaching a correlation coefficient of 0.9992. The average of the double determinations for each level was used.

**Table 1.** Calibration parameters of the method for vitamin $D_2$ detection and quantification, and method validation parameters using *Agaricus bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm$^2$).

| Calibration Curve | Correlation Coefficient ($R^2$) | Linearity Range (µg/mL) | Limit | |
|---|---|---|---|---|
| | | | LOD [1] (µg/mL) | LOQ [2] (µg/mL) |
| y = 11.909x + 6.9688 | 0.9992 | 0.78–50 | 1.67 | 5.07 |
| Precision CV, % ($n$ = 6) | Accuracy (recovery, %) | | Precision CV, % ($n$ = 6) | |
| 0.82 | 1.35 | | 94 | |

[1] LOD: limit of detection of the chromatographic method; [2] LOQ: limit of quantification of the chromatographic method; CV: coefficient of variation.

The LOD, calculated as the concentration corresponding to three times the standard error of the calibration curve, divided by the inclination, was 1.67 µg/mL, while the LOQ, i.d., the concentration corresponding to ten times the calibration error, divided by the inclination, was 5.07 µg/mL.

In order to evaluate the instrumental precision, the mushroom sample (*A. bisporus* Portobello, irradiated for 6 min at 3200 mJ/cm$^2$) was injected six times, and the chromatographic method proved to be precise, according to the coefficient of variation (CV) of 0.82%. Repeatability was evaluated by applying the whole extraction procedure six times to the same sample, and the CV value obtained was low (1.35%). The method accuracy was evaluated by the standard addition procedure (% of recovery). The standard mixture was added to the samples in three concentration levels (25%, 50% and 100% of the peak/area concentration, each one in duplicate) before the extraction. The method showed good recovery results, with an average of 94% (Table 1).

### *3.2. Conversion Conditions*

The starting point is the use of surplus mushrooms as a sustainable material to obtain vitamin $D_2$, avoiding the use of mushroom suitable for commercialization.

In this sense, Table 2 presents the vitamin $D_2$-enriched extracts content in different mushroom species, exposed to different UV-C radiation doses and exposure times.

As it is mandatory in any two-way ANOVA, the possible interaction among the assayed factors was verified (ET × UV-C). Since the interaction proved to be significant ($p < 0.050$) in all the cases, it became obvious that the effect of one factor depends on the level of the second.

Therefore, the variation induced by every single factor could not be classified. Nonetheless, it was possible to observe some evident trends, as confirmed by the individual $p$-values of each factor. A significant increase (from less than 4 µg/g dw to more than 100 µg/g dw in all the cases) in vitamin $D_2$ concentration was observed with the application of this irradiation type, most likely due to the conversion of ergosterol naturally present in the assayed mushroom. Furthermore, there were no significant differences in the result of using 200, 800 or 3200 mJ/cm$^2$, which indicates that the vitamin $D_2$ increase may be

achieved with the least energetic consumption, making this processing approach more competitive and with minimal environmental impact.

**Table 2.** Vitamin $D_2$ content in different mushroom species exposed to different UV-C radiation doses and exposure times (ET). The results are presented as mean ± SD [1].

| | | Vitamin $D_2$-Enriched Extracts (μg/g dw) | | |
|---|---|---|---|---|
| | | ***A. bisporus*** | ***A. bisporus* Portobello** | ***P. ostreatus*** |
| ET/min | 0 | 3.77 ± 0.02 | 3.7 ± 0.2 | 2.38 ± 0.04 |
| | 2 | 84 ± 7 | 109 ± 6 | 97 ± 11 |
| | 6 | 125 ± 8 | 124 ± 11 | 125 ± 11 |
| | 10 | 122 ± 1 | 127 ± 5 | 119 ± 7 |
| ANOVA *p*-value [2] | | <0.001 | <0.001 | <0.001 |
| UV-C ($mJ/cm^2$) | 0 | 3.77 ± 0.02 | 3.7 ± 0.2 | 2.38 ± 0.04 |
| | 200 | 104 ± 22 | 113 ± 9 | 107 ± 20 |
| | 800 | 111 ± 21 | 119 ± 9 | 114 ± 12 |
| | 3200 | 116 ± 19 | 128 ± 10 | 119 ± 14 |
| ANOVA *p*-value [2] | | <0.001 | <0.001 | <0.001 |
| ET × UV-C *p*-value [3] | | <0.001 | 0.035 | <0.001 |

[1] Results are reported as mean values of each parameter (ET or UV-C), combining all exposure times and irradiation doses (from ET or UV-C). [2] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one ET or UV-C. [3] The interaction among factors was significant in all cases; thereby the statistical classification could not be indicated.

With regard to exposure time, there were no significant differences upon irradiating mushrooms during 6 or 10 min, but the intermediate assayed time was better than the 2 min. Hence, the optimal exposure time, considering the results obtained with the surplus of assayed mushroom species, turned out to be 6 min. The origin of the mushroom, applied dose, time after harvest, positioning of the mushrooms to the light source, fresh or dried samples, whole or sliced samples, the method by which vitamin $D_2$ has been extracted, among others, influence the results obtained [12].

### *3.3. Vitamin $D_2$-Enriched Extracts*

As for the mushrooms evaluated in this work (e.g., for 6 min at 3200 $mJ/cm^2$), vitamin $D_2$-enriched extract levels in the Portobello *A. bisporus* samples reached a maximum concentration of 124 μg/g dw, and in the white *A. bisporus* and *P. ostreatus* samples they reached values of 125 μg/g dw (Table 2, Figure 1).

In UV-C-irradiated *P. ostreatus* samples, Hu et al. [13] reported a maximum concentration of approximately 24 μg/g dw of vitamin $D_2$ content. Teichmann et al. [25] reported 10.14 μg/g dw in white *A. bisporus* samples, Guan et al. [26] reported 13.4 and 9.5 μg/g dw in white and Portobello *A. bisporus* samples, respectively, and Jasinghe and Perera [14] reported 34.4 μg/g dw in white button mushrooms. Similarly, for UV-C-irradiated shiitake (*Lentinula edodes*) mushroom, Xu et al. [27] obtained an increase in vitamin $D_2$ content, until 20.11 μg/g dw.

Concerning UV-B irradiation, Urbain et al. [28] and Urbain et al. [29] obtained 56.8 and 67.1 μg/g dw of vitamin $D_2$, respectively, in button mushrooms; Nölle et al. [30] reported that fresh whole *A. bisporus*, followed by freeze-drying, obtained 45 μg/g dw of vitamin $D_2$, and slicing before UV-B irradiation led to a ten-fold increase.

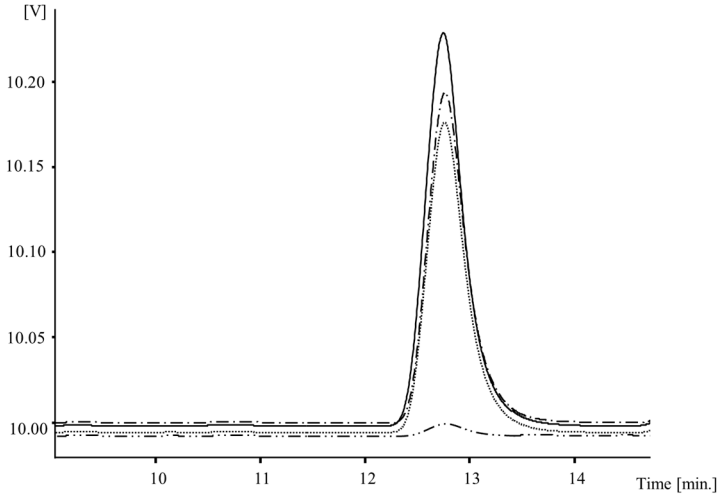


**Figure 1.** Vitamin $D_2$-enriched extracts chromatogram profile of *Agaricus bisporus* Portobello (-), white *A. bisporus* (.-.-) and *Pleurotus ostreatus* (...) irradiated with UV-C (6 min at 3200 mJ/cm$^2$), and *A. bisporus* Portobello control samples (..-..-).

There are dissimilarities in the irradiation process and conditions to maximize the photoconversion of ergosterol into vitamin $D_2$ in mushrooms, and most of these cited studies were performed with the whole intact mushroom, with a longer irradiation time (20 min) and higher irradiation dose.

In this sense, based on the case study considered in this work, we make the first attempt to establish the irradiation conditions and extraction procedure needed to maximize ergosterol conversion to vitamin $D_2$ from surplus mushroom production, avoiding the need to use mushroom samples that are suitable to be commercialized.

### *3.4. Bioactivity of the Vitamin $D_2$-Enriched Extract*

The in vitro cytotoxicity of the vitamin $D_2$-enriched extract and pure vitamin $D_2$ was analyzed. The effect of the vitamin $D_2$-enriched extract and pure vitamin $D_2$ in human tumoral cell lines (MCF-7, NCI-H460, AGS and CaCo) and non-tumoral bone cell line (h-FOB 1.19) growth are presented in Table 3. The $GI_{50}$ values represent the extract concentrations that cause a 50% inhibition of cell growth.

**Table 3.** Antiproliferative and cytotoxicity activities of vitamin $D_2$-enriched extracts using *Agaricus bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm$^2$) and pure vitamin $D_2$ (mean ± SD, $n$ = 9).

| | MCF-7 | NCI-H460 | AGS | CaCo | h-FOB 1.19 |
|---|---|---|---|---|---|
| Vitamin $D_2$-enriched extracts ($GI_{50}$ μg/mL) | >400 | 293 ± 17 $^b$ | 82 ± 9 $^c$ | 377 ± 24 $^a$ | >400 |
| Vitamin $D_2$ pure ($GI_{50}$ μg/mL) | >400 | >400 | >400 | >400 | >400 |

The cytotoxicity results were expressed as $GI_{50}$ values, corresponding to the sample concentration that inhibited 50% of the net cell growth. In row, different letters mean significant differences ($p < 0.05$).

The sample of pure vitamin $D_2$ tested did not reveal cytotoxicity at the evaluated concentrations ($GI_{50}$ values > 400 μg/mL) for all the cell lines tested (tumoral and non-tumoral). However, the vitamin $D_2$-enriched extract presented effective activity in the AGS (82 μg/mL) tumoral cell line, and moderate activity in the NCI-H460 (293 μg/mL) and CaCo (377 μg/mL) tumoral cell lines.

The results obtained indicate that these effects may be related to the compounds (including ergosterol, phenolic compounds, organic acids, etc.) present in the mushroom extract, since the mushrooms are a rich source of bioactive compounds [31]. It is noteworthy

that neither vitamin $D_2$-enriched extracts or pure vitamin $D_2$ presented cytotoxicity against the non-tumoral bone cell, h-FOB 1.19 ($GI_{50}$ > 400 µg/mL).

## 4. Conclusions

Based on the case study considered, vitamin $D_2$-enriched extracts were obtained by HPLC-UV, using a methodology that proved to be reproducible and accurate. Vitamin $D_2$ was identified and quantified, and *A. bisporus* Portobello was the species with the highest total content. The recovery of vitamin $D_2$ from surplus mushrooms presents an interesting valorization and sustainable approach.

The use of vitamin $D_2$-enriched extracts from surplus mushroom production could benefit several bio-based industries, since the applications of vitamin $D_2$ from this sustainable material are nonexistent. Accordingly, the development of food applications of mushroom vitamin $D_2$-enriched extract, from surplus mushroom production, can be considered and valued. It could be of added value to promote the agricultural sector or the pharmaceutical industries.

**Author Contributions:** R.V.C.C.: formal analysis, writing—original draft. Â.F.: methodology, software, validation, investigation, data curation, writing—review and editing. J.C.M.B.: data curation, software, writing—review and editing. R.M.V.A.: data curation, software, writing—review and editing. F.M.: methodology. A.M.G.-P.: visualization, supervision. I.C.F.R.F.: supervision, project administration. L.B.: conceptualization, validation, investigation, writing—review and editing, visualization, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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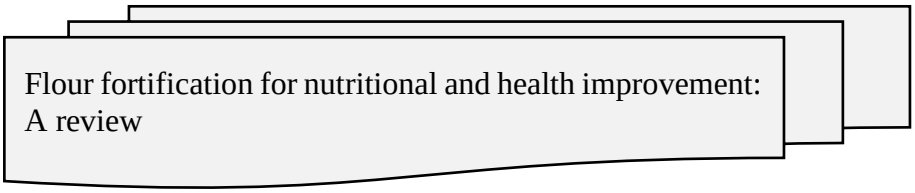
**Annex H**


Flour fortification for nutritional and health improvement:
A review

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# Flour fortification for nutritional and health improvement: A review

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ABSTRACT

Deficiencies of micronutrients, essentially vitamins and minerals, have mainly cause several illnesses, especially in children and women worldwide. Governments and world organizations have focused great efforts to address these shortcomings and improve the health of the populations. This malnutrition can be combated by fortifying basic foods that are consumed daily. Thus, flours, especially wheat, maize and rice, are a popular and common food in the world's populations diet and are gaining considerable attention as a suitable vehicle for micronutrient fortification. There are some studies that show the effectiveness of flour fortification in combating micronutrient deficiencies and several diseases and the flour fortification strategies are directed to meet nutritional and health needs of the populations. The main points discussed in this review are food fortification, with great focus in flour fortification, health benefits, and legislative issues. This review also highlights multifaceted issues related to flour fortification to meet nutritional needs and to improve the health of vulnerable populations.

## 1. Introduction

Vitamins and minerals are essential elements for the growth, metabolism and maintenance of a healthy body and deficiencies of these nutrients have resulted in irreversible physical and cognitive consequences, since these compounds play an important role in the normal functioning of almost all organs (Looman et al., 2019; Wahengbam, Das, Green, Shooter, & Hazarika, 2019). The FAO, IFAD, UNICEF, WFP, and WHO (2018) have estimated that billions of people are deficient in different types of vitamins and minerals. Revealing that in several countries, women and children suffer from severe deficiencies caused by inadequate amounts of vitamins, amino acids and minerals, more precisely vitamin A, vitamin D, iron, zinc and probably other micronutrients. It has also been reported that several micronutrient deficiencies cohabit and are more prevalent in developing countries (Black et al., 2013; Blumfield, Hure, Macdonald-wicks, Smith, & Collins, 2013; Farhat, Lees, Macdonald-Clarke, & Amirabdollahian, 2019; Radziejewska & Chmurzynska, 2019; Ramakrishnan, 2002). A study in pregnant Nepalese women showed vitamin B6 deficiency and in villages in India showed zinc, iron and folate deficiency (Jiang, Christian, Khatry, Wu, & West, 2005; Pathak, Kapoor, Saxena, Kumar, & Gupta, 2004). Moreover, according to WHO, millions of pre-school children worldwide and pregnant women suffer from vitamin A deficiency (WHO, 2009); otherwise, it is also estimated that, globally, about million people are affected by iodine deficiency (Li & Eastman, 2012).

Micronutrient deficiencies can lead to unfavourable health consequences, such as growth problems, immune competence, mental and physical development, and poor reproductive outcomes. It has also been reported to be related to an increased occurrence, severity and mortality of infectious diseases such as malaria, diarrhoea, pneumonia, among others. These deficiencies have numerous unfavourable results across all populations and age groups, with children and women of reproductive age being more vulnerable (Black et al., 2013; Gibson & Hotz, 2002; Looman et al., 2019).

Many strategies have been used to combat micronutrient deficiencies, such as exclusive breastfeeding during babies first 6 months, control of parasitic infections, food fortification, food diversification, and nutritional supplementation (Black et al., 2013; Hemery et al., 2018; WHO, 2000). Particularly, fortification is a method of incorporating nutrients or non-nutritive bioactive components into food products (Dwyer et al., 2015). Food fortification is one of the methods that has been applied increasingly and addressed to all age groups, being widely used to minimize micronutrient deficiency. By placing micronutrients in food products consumed daily, it reaches target populations, from which daily dietary requirements of micronutrients are scarcely satisfied (WHO & FAO, 2006). It is an impressive public health

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strategy with interesting cost-effectiveness ratios and has the advantage of being installed in the usual dietary patterns, without a major change in eating or health practices and is generally well accepted by the populations (Berner, Keast, Bailey, & Dwyer, 2014; WHO & FAO, 2006).

The development of new products has a strategic role in the food industry, because consumers are increasingly demanding food products with high nutritional value that provide health benefits (Páramo-Calderón et al., 2019). As a staple food common in many countries, flour is often considered one of the most suitable vehicles for multi-micronutrient fortification (Hemery et al., 2018). In addition, fortified flours can be a major source of bioactive compounds, since flour can be fortified with many micronutrients, reducing the risk of multiple deficiencies where they exist and improve health benefits (Akhtar, Anjum, & Anjum, 2011; Oghbaei & Prakash, 2012; Serdula, 2010a, 2010b).

Wheat and maize flours can be fortified with several micronutrients and reduce vitamin, mineral and other micronutrients deficiencies when identified as health public problem. It is estimated that the amount of industrial flour fortified is 97% in the Americas, 21% in Southeast Asia, 6% in Europe, 4% in the Western Pacific, 31% in Africa, and 44% in the Mediterranean area in 2007–2008 (WHO, 2009). Efforts made by some countries to adopt mandatory fortification of flours with some micronutrients are useful in combating these deficiencies. These initiatives are an excellent example for other countries, considering their own programs, however, when it comes to food fortification, appropriate legislation is needed to ensure impact and safety, and the intended benefits to health (Luthringer, Rowe, Vossenaar, & Garretta, 2015; Serdula, 2010a). Therefore, it is necessary to explore how nutrient fortification contributes to the context of the current nutrient intakes. The aim of this review is to evaluate the impact of flour fortification for nutritional and health improvement, detailing the prevalence of micronutrient deficiencies, health consequences, and global trends and experiences regarding flour fortifications.

## 2. Food fortification

Food fortification has a long history of use in some countries and has successfully controlled micronutrient deficiencies (WHO & FAO, 2006). World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), defines fortification as the practice of deliberately increasing the amount of a vital micronutrient, i.e. vitamins, minerals, amino acids among others, in a food regardless of whether the nutrients are initially in the food before processing or not, with the intention of improving the nutritional quality of the food and providing a public health benefit with little health risk (Whiting, Kohrt, Warren, Kraenzlin, & Bonjour, 2016; WHO & FAO, 2006). This strategy can lead to relatively rapid improvements in the number of people with food deficiencies within a population, since the benefits are potentially large and consists of very cost-effective public health interventions (Dwyer et al., 2015; WHO, 2009).

The advantages of fortification to public health has been demonstrated over time by the scientific community, helping in the micronutrients deficiency correction and prevention or reduction of these deficiencies in a population, having the capacity to improve nutritional status and dietary intake and consequently improving eating habits and lifestyles (FAO et al., 2018; WHO & FAO, 2006).

To have consolidated results in food fortification, the micronutrient used must have a good availability of absorption by the organism, ideal organoleptic characteristics (not changing the colour and the flavour of the fortified food), be easily accessible, should belong to the usual diet of the population and have a good acceptance.

The most common fortified foods are cereals and cereal products, milk and dairy products, fats and oils, tea and other beverages and various condiments such as salt, soy sauce, sugar and infant formulas (Pacho, Spohrer, Mei, & Serdula, 2015; Whiting et al., 2016; WHO & FAO, 2006). In the early 1920s salt iodization was adopted in Switzerland and the United States of America and today it is used in most countries. From the 1940s, fortification of cereal products with thiamine, riboflavin, and niacin became a common practice. In Denmark, margarine was fortified with vitamin A and in the United States milk with vitamin D. Also, one procedure that significantly reduced the risk of iron deficiency anaemia in young children, was the fortification of foods with iron (Vlaic et al., 2019; WHO & FAO, 2006). Nowadays, fortification of foods with different types of micronutrients has spread throughout the world, for example, folic acid fortification has spread in the Americas, a tactic adopted by the United States and Canada and approximately 20 Latin American countries (FAO et al., 2018; Vlaic et al., 2019).

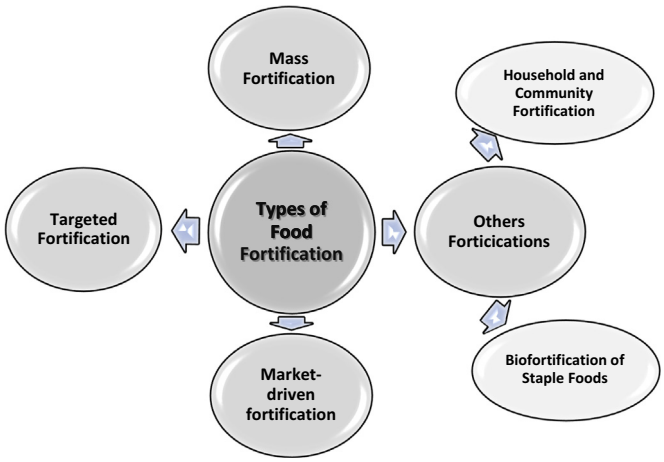


**Fig. 1.** Different types of food fortification.

### 2.1. Types of fortification

According WHO and FAO, food fortification includes different forms (Fig. 1): i) mass fortification (fortify foods that are quite consumed by the general population), ii) targeted fortification (fortify food targeted to specific population groups, e.g. small children), and iii) market-driven fortification (ensure that food is available in the market). There are other types of fortification such as household fortification (addition of micronutrients to homemade foods, namely a fusion of supplements and fortification) and biofortification of staple foods (breeding and genetic modification of plants to improve the content and the absorption of nutrients) (WHO & FAO, 2006).

Generalizing, mass fortification is often mandatory, targeted fortification is mandatory or voluntary and depends on the importance for public health, and market-driven fortification is always voluntary, but controlled by regulatory limits (Liyanage & Hettiarachchi, 2011; Marques, Marques, Xavier, & Gregório, 2012; WHO & FAO, 2006).

According to Liyanage and Hettiarachchi (2011), commercial and industrial fortification includes available products, such as flour, rice, cooking oils, sauces, butter, etc. and the procedure takes place throughout the manufacturing process. On the contrary, the biofortification encompasses the creation of micronutrient cultures using traditional breeding and/or biotechnology techniques (for example, transgenic “Golden Rice” containing higher amounts of iron and significant levels of beta-carotene (Lonnerdal, 2003). There is also microbial biofortification, which includes the use of probiotic bacteria, which ferment to produce β-carotene in foods or directly in the intestine. For example, the use of animal feed enriched with these bacteria (more specific lactic acid bacteria), so that meat, milk and bioproducts are enriched with vitamin A (Liyanage & Hettiarachchi, 2011; Sasson, 2005). Moreover, there is home fortification, where micronutrients obtained from packages or tablets can be incorporated when cooking and or consumed in a homemade meal to fill micronutrient deficiencies in the populations (Liyanage & Hettiarachchi, 2011; Marques et al.,

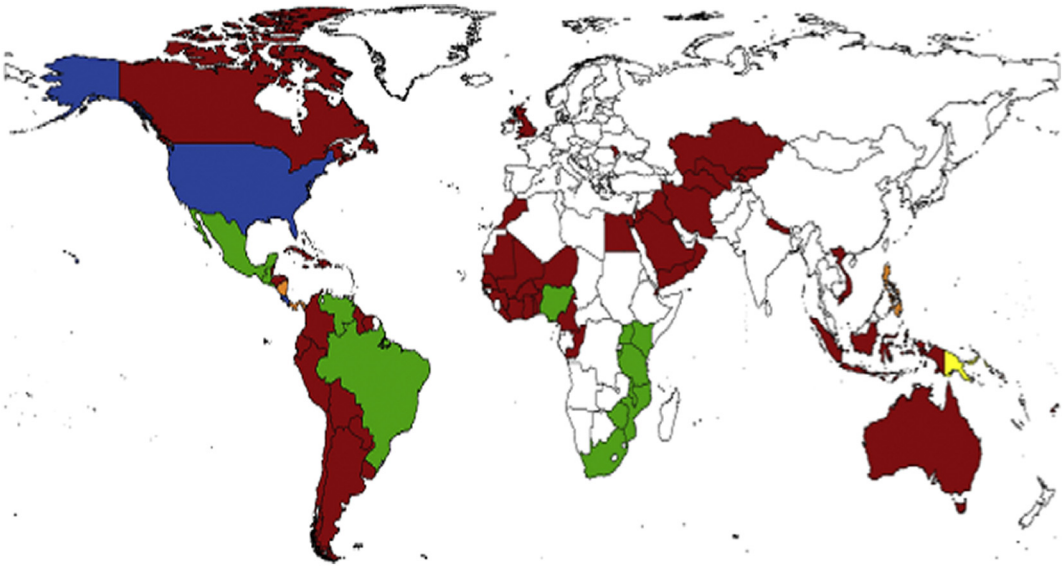

**Fig. 2.** Mandatory fortification of different types of flour around the world: 65 countries – wheat flour, 14 countries – wheat flour and maize flour, 4 countries – wheat flour and rice (Nicaragua, Panama, Philippines, Solomon Islands), 2 countries – wheat flour, maize flour and rice (Costa Rica and the United States), 1 country – rice (Papua New Guinea) (FFI, 2019a, 2019b, 2019c, 2019d, 2019e).

2012; WHO & FAO, 2006).

### 2.2. Issues and challenges of nutrient deficiencies

The greatest modifiable threat to global health and survival is represented by malnutrition, especially among children in the world's poorest countries (Prentice et al., 2008). Given the increasing demands of pregnant and lactating women and young children, this group is among the most vulnerable to micronutrient deficiencies (Black et al., 2013).

WHO reports that worldwide approximately 19.1 million pregnant women are deficient in vitamin A and it is estimated that 82% of these women have zinc intake inappropriate to meet the normal needs of pregnancy; 100 million women of reproductive age have iodine deficiency; 190 million pre-school children are also deficient in vitamin A; iron deficiency is globally found worldwide and approximately 1.62 billion people are anaemic (mostly among pre-school children and pregnant women). Deficiencies of vitamin B6 and B12 have also been observed in many countries (McLean, de Benoist, & Allen, 2008; WHO, 2000, 2009).

Wide spreading of infectious diseases and mortality from pneumonia, malaria, diarrhoea and measles are linked to micronutrient deficiency. Proper nutrition influences health status and helps prevent many diseases, being responsible for providing nutrients (including vitamins, minerals and others nutrients), and contributing to the proper functioning of the body (Jakubowska & Staniewska, 2015). The implications of these shortcomings are not only limited to health standards, but also have effects on the economy, through secondary physical and mental disabilities and modified work productivity (Das, Salam, Kumar, & Bhutta, 2013). Currently the approach to nutritional issues has undergone considerable changes.

Nowadays solutions are already known (such as fortified foods), but require some challenges like political dedication, economic advancement, and scientific research, in order to range a plausible solution. In this sense, several agencies worldwide are focusing their attention on the fight against micronutrient deficiencies, since they are a short-term resolution (FAO, 2017; Prentice et al., 2008). Some challenges, such as choosing appropriate fortification vehicles, having a good policy, targeting populations, avoiding excessive consumption in non-target groups, and monitoring nutritional status, are among relevant factors in improving health and will have the possibility to save billions of lives (Dwyer et al., 2015; Osendarp et al., 2018; Prentice et al., 2008; WHO & FAO, 2006).

## 3. Fortification of flour around the world

Worldwide, millions of tons of flours are used for human consumption each year, they are consumed as noodles, breads, pasta and other flour products (Pacho et al., 2015; Serdula, 2010b). In 2016, according to the Food Fortification Initiative, out of the 250 metric tons of industrially milled wheat flour, 26 metric tons of industrially milled maize flour, and 171 metric tons of industrially milled rice, 34%, 57%, and 1%, respectively for each one of the flours was fortified. Between 2016 and 2017, 87 countries have decided to fortify at least one of these cereals (FFI, 2016, 2018; Marks et al., 2018).

The first cereal product to be largely fortified was wheat flour, and the first recommendations about cereals fortification from the World Health Organization (WHO) referred to maize and wheat flour. At the beginning of 2015, 83 countries demanded the fortification of wheat flour, of which 14, required simultaneously the fortification of maize flour. Most of these countries require fortification of wheat flour and maize with at least iron and folic acid, excluding Australia that does not require fortification of flour with iron, and Congo, Nigeria, Philippines, United Kingdom, and Venezuela do not require flour fortification with folic acid (Pacho et al., 2015; WHO, 2009).

Today, just 86 countries worldwide have legislation requiring the fortification of at least one grain of industrially milled cereal, depending on the country the flour is fortified with vitamins, minerals, amino acids and other micronutrients. Within these, 85 countries fortify wheat flour alone or in combination with other grains, taking away the country Papua New Guinea which is mandated only for rice flour fortification (Fig. 2) (FFI, 2019c).

Each country adopts its fortification standard depending on the geographic region, income status, food vehicle(s) and nutrient(s). The total number of nutrients fortified in flours are determined according to the fortification pattern (Table 1) (GFDx, 2019).

Nutrition International directs and supports flour fortification efforts in developing countries through various programs and partners such as: Global Alliance for Improved Nutrition (GAIN), UNICEF, World Food Program (WFP), Food Fortification Initiative (FFI), Unite State Centres for Disease Control and other organizations, working within different countries (South Africa, Yemen, Iran, India, Pakistan, Nepal,

**Table 1**
Nutrients in food fortification standards of some country (GFDx, 2019; WHO, 2013).

| Country | Food vehicle | Nutrients |
|---|---|---|
| Afghanistan | Wheat flour | Vitamin B12, Folate (B9), Iron, Zinc |
| Bolivia | Wheat flour | Niacin (B3), Riboflavin (B2), Thiamine (B1) |
| Costa Rica | Wheat flour | Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1) |
| | Rice | Folate (B9), Vitamin B12, Niacin (B3), Selenium, Thiamine (B1), Vitamin E, Zinc |
| | Maize flour | Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1) |
| Tanzania | Wheat flour | Vitamin B6, Vitamin B12, Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1), Vitamin A, Zinc |
| | Maize flour | Vitamin B12, Folate (B9), Iron, Zinc |
| Colombia | Wheat flour | Calcium, Folate (B9), Niacin (B3), Riboflavin (B2), Thiamine (B1) |
| Philippines | Wheat flour | Iron, Vitamin A |
| | Rice | Iron |
| Nigeria | Wheat flour | Zinc, Vitamin A, B6, B12, Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1) |
| | Maize flour | Zinc, Vitamin A, Thiamine (B1), Riboflavin (B2), Niacin (B3), Iron, Folate (B9), Vitamin B12 And B6 |
| Nepal | Wheat flour | Folate (B9), Iron, Vitamin A |
| United States of America | Maize flour | Calcium, Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1), Vitamin D |
| | Rice | Thiamine (B1), Vitamin D |

Bolivia, Central and South America and the Middle East, Indonesia, Nigeria, among others countries). They support and expand fortification programs of flours with different micronutrients to combat the deficiency of these nutrients and improve health disorders (Nutrition International, 2019).

### 3.1. Types of fortified flours

Since the early days, cereals and cereal products have been the main components of the human diet throughout the world. Major cereal crops include wheat, rice, maize and barley. Maize (or corn) is the most produced, but it is less important than wheat and rice, which are the most important cereals for human nutrition (Preedy, Watson, & Patel, 2011).

Cereals are common staple foods since they are versatile, tasty, always available on the market, accessible and culturally acceptable, are consumed every day by all age groups, being a great fortification vehicle.

Cereals are commonly consumed after being processed by milling industries. When the flours are fortified at the industrial level (flour production), the different produced food products in the food preparation industries (e.g. bakeries) are easier to prepared. This makes the cereal industrial unit much more powerful in this type of fortification, than the a bakery, or even supplements (Johnson, Mannar, & Ranum, 2004; Pacho et al., 2015).

Wheat flour was the first cereal grain product to be extensively fortified and the recommendations of the World Health Organization (WHO) is that fortification of wheat, maize and rice flour are an opportunity to improve health (WHO, 2009). Table 2 presents some types of flours or derived products that have already been fortified and it is found that the work on fortifications has been made mostly in wheat flour.

In the market there are many flours that can be fortified or used to fortify other flours, the following topics highlight the main produced and/or consumed cereals flours worldwide.

#### 3.1.1. Wheat flour

The third largest cereal production in the world is wheat, after maize and rice, and is the second most consumed by the populations after rice. Wheat flour is considered one of the most appropriate vehicles for multi-micronutrient fortification because of its worldwide consumption and given the high consumption of bread and pasta worldwide (Awika, 2011; Peña-Rosas, Field, Burford, & De-Regil, 2014).

The industrial processed fortification of wheat flour, when properly implemented, is an effective, simple and inexpensive strategy to provide vitamins and minerals to the world population, thereby improving the nutritional quality of food supply and providing a public health benefit (Cardoso et al., 2019; FFI, 2019b; Nuria Mateo Ansón, 2010; Peña-Rosas et al., 2014).

Globally the effort to begin fortifying wheat flour was launched during the 1940s as a way to improve the health of populations (Bishai & Nalubola, 2002). Wheat flour has been fortified with different micronutrients such as iron, folic acid, B-complex vitamins, vitamin A, D and C, zinc, calcium among others, in different parts of the world (Akhtar et al., 2011; FFI, 2019b, 2019c; Johnson et al., 2004; Jungjohann et al., 2015; Marks et al., 2018; Pacho et al., 2015; Peña-Rosas et al., 2014; Rebellato et al., 2017; Serdula, 2010b; WHO, 2009). For example, pasta made with white wheat flour, enriched with *Oreochromis niloticus* L. (high levels of essential amino acids and polyunsaturated fatty acids) flour, improves the nutritional composition of the pasta without compromising its sensorial quality (Monteiro et al., 2019).

#### 3.1.2. Rye flour

Rye is essentially a European cereal and globally the largest production is also centred in Europe. In the baking industry, it is the second most used cereal worldwide, after wheat. In the Middle East, rye is developed as a secondary crop, it has a great wintering capacity and high tolerance to drought, cold, and develops well on low fertility soils. With these characteristics, it becomes a low-risk and economical crop (Cardoso et al., 2019; Carena, 2009; Kaminski, da Silva, do Nascimento Júnior, & Ferrão, 2011; Redant, Buggenhout, Brijs, & Delcour, 2017).

The interest in rye has been increasing because of its nutritional profile, due to its high levels in dietary fibre. Beside its excellent nutritional quality, it also has many health benefits, decreasing the absorption of triglycerides and blood cholesterol levels; reduction in blood glucose; prevention of constipation, and prebiotic effects, among others. These characteristics, allow the classification of rye products as functional foods, proving a relationship between diet and health (Carena, 2009; EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), 2011; Grossmann & Koehler, 2016; Kaminski et al., 2011; Moniz et al., 2018; Preedy et al., 2011; Redant et al., 2017). There are some studies that have presented the fortification of rye flour with cellulose fibre (Fuckerer, Hensel, & Schmitt, 2015, 2016) and with folic acid (Gujska & Majewska, 2005) to increase its health benefits.

#### 3.1.3. Rice flours

Rice is the staple food for more than half the world's population and is the main cereal in many developing countries. In most Asian countries, rice provides between 50% and 80% of the caloric intake. In South and Southeast Asia, most women and children are anaemic and the nutritional value of rice has a significant impact in their health (FFI, 2019e; Preedy et al., 2011; Sasson, 2005; WHO, 2018a).

**Table 2**
Flour fortification with micronutrients and their health benefits.

| Flours | Micronutrients[a] | Health benefits[b] | References |
|---|---|---|---|
| Wheat | Minerals<br>Vitamins<br>Proteins<br>Fibre<br>Fatty acids | Increases the content or bioavailability of a specific micronutrients<br>Prevention/reduction/improvement of the incidence of anaemia or specific mineral deficiency<br>Functional food with therapeutic protective effects against diabetes and cardiovascular diseases<br>Effect on the prevention of colon cancer<br>Antioxidants activity<br>Prevention of civilization diseases (e.g.: hypertension, obesity) | Chiplonkar et al. (1999), Gujska and Majewska (2005), Towo, Mgoba, Ndossi, and Kimboka (2006), Butt, Arshad, Alam, and Nadeem (2007), Gigante and Victora (2007), Naves et al. (2007), Akhtar, Anjum, Rehman, Sheikh, and Farzana (2008), Pachón, Stoltzfus, and Glahn (2008), Sadighi et al. (2008), Anton, Lukow, Fulcher, and Arntfield (2009), Škrbić, Milovac, Dodig, and Filipčev (2009), Berry et al. (2010), Kaminski et al. (2011), Barbosa, Taddei, Palma, Ancona-Lopez, and Braga (2012), Buzzo et al. (2012), Fares and Menga (2012), Erukainure, Ebuehi, Adeboyejo, Aliyu, and Elemo (2013), Danza et al. (2014), Peña-Rosas et al. (2014), Gawlik-Dziki et al. (2015), Pacho et al. (2015), Prodhan et al. (2015), Muhammad et al. (2016), Sui, Zhang, and Zhou (2016), Bolarinwa, Aruna, and Raji (2017), Kurek, Wyrwisz, Karp, and Wierzbicka (2017), Rebellato et al. (2017), Reshmi, Sudha, and Shashirekha (2017), Aranibar et al. (2018), Armellini et al. (2018), Benjakul and Karnjanapratum (2018), Emaleku, Omueti, and Emaleku (2018), Garrod et al. (2019), and Hemery et al. (2018)<br>Jakubczyk, Świeca, Gawlik-Dziki, and Dziki (2018), Moniz et al. (2018), Rebellato et al. (2018), Sheikholeslami, Karimi, Komeili, and Mahfouzi (2018), and Bryszewska et al. (2019) |
| Rye | Fibre<br>Vitamins | Increases the content of a specific micronutrients | Gujska and Majewska (2005), Fuckerer et al. (2015), and Fuckerer et al. (2016) |
| Maize | Minerals<br>Vitamins | Increases the content or bioavailability of a specific micronutrients<br>Prevention/reduction/improvement of the incidence of anaemia or specific mineral deficiency<br>Antioxidants activity | Chiplonkar et al. (1999), Hansen, Bæch, Thomsen, Tetens, and Sandström (2005), Towo et al. (2006), Andang'o et al. (2007), Gigante and Victora (2007), Naves et al. (2007), Bilgi Boyaci et al. (2012), Buzzo et al. (2012), Pacho et al. (2015), Giménez, Drago, Bassett, Lobo, and Sammán (2016), and Kumar, Xavier, Lekshmi, Balange, and Gudipati (2018) |
| Rice | Minerals<br>Vitamins<br>Fibre | Increases the content or bioavailability of a specific micronutrients<br>Antioxidants activity | Hettiarachchi et al. (2004), Sharif, Rizvi, and Paraman (2014), Igoumenidis, Lekka, and Karathanos (2016), Kumar et al. (2018), and Mounjouenpou et al. (2018) |
| Millet | Minerals<br>Vitamins | Increases the content or bioavailability of a specific micronutrients | Chiplonkar et al. (1999), Tripathi and Platel (2010), Tripathi, Chetana, and Platel (2010), and Tripathi and Platel (2011)<br>Tripathi, Platel, and Srinivasan (2012), Tripathi and Platel (2013), and Oluyimika, Kruger, White, and Taylor (2019) |
| Sorghum | Minerals<br>Proteins<br>Fatty acid<br>Amino acids | Increases the content or bioavailability of a specific micronutrients<br>Increases the content of protein and fatty acid<br>Antioxidant activity | Chiplonkar et al. (1999), Tripathi and Platel (2010), Tripathi and Platel (2011), Tripathi et al. (2012), Tripathi and Platel (2013), and Abdualrahman et al. (2019) |

[a] In this section, the micronutrients have been used directly or they are derived from other foods that are used to fortify the flours.
[b] Some of the health benefits that can be found in referenced works.

Such as wheat and maize flour fortification, rice fortification with vitamins, minerals and other micronutrients is a public health opportunity, in order to prevent deficiencies in these compounds and severe diseases (de Pee, 2014; FFI, 2019e; Forsman, Milani, Schondebare, Matthias, & Guyondet, 2014).

According to GFDx (2019) some countries like Costa Rica, Nicaragua, Panama, Papua New Guinea, Philippines, United States of America, Bangladesh, India and Venezuela fortify rice with zinc, vitamin A, vitamin B1, B2, B3, B6, B9, and B12, iron, calcium, and vitamin D.

However, rice is very difficult to fortify because most of the grains are not processed. Therefore, the alternative is to fortify rice flour instead of rice grain, which can be fortified with the same methods used for the other flours. In Sri Lanka, rice flour is processed, and its cost is approximately equal to wheat flour, leading to a growing interest by the population. Mounjouenpou et al. (2018) fortified rice flour biscuits with 20% baobab (*Adansonia digitata* L.) pulp flour, which has been shown to be very rich in minerals, such as potassium, magnesium, iron and calcium, as also vitamin C and total fibre. According to the authors the biscuits with baobab appears to be a very low cost alternative and an accessible way to combat micronutrient deficiencies in Africa. Fortification of rice flour has been proposed in countries such as the Philippines and Guyana, which have a very significant consumption of this product (Hettiarachchi, Hilmers, Liyanage, & Abrams, 2004; Johnson et al., 2004; Marks et al., 2018).

#### 3.1.4. Maize flours

Maize is grown all over the world and millions of tonnes are produced, actually, different types of maize, varying colours, are cultivated globally. The United States, China, India, France, Brazil, Argentina, and Indonesia are the main maize producing countries. It is also the main food preferred by billions of consumers in sub-Saharan Africa and Latin America. Tons of maize flours are milled annually, and its consumption is performed in many forms. After wheat and rice, maize is the third most frequently consumed cereal in the world (Sasson, 2005; WHO, 2009).

Around 65% of industrially processed maize in the world is fortified (FFI, 2018, 2019d) and this fortification has been practiced for several years in many countries, where this ingredient is used for the preparation of many common dishes. Between the African and American continent about 16 countries have mandatory legislation to fortify maize flour (Enzama, Afidra, Johnson, & Verster, 2017; FFI, 2019d). Maize flour can be fortified with several micronutrients, such as iron, folic acid, vitamin A and B, zinc, among others; some of them are used to replace nutritional contents and others are used to prevent deficiencies of certain micronutrients relevant to health (GFDx, 2019; WHO, 2016). For example tortilla made from maize flour incorporated with *Moringa oleifera* Lam. flour showed a significantly increased in the protein and lipid content of this food product and an increase in total polyphenolic content as well as its antioxidant activity (Páramo-Calderón et al., 2019).

### 3.2. Flour fortification as a supplement

Supplementation is a set of substances (vitamins, minerals or other nutrients) that serve to complement your diet when the natural intake of these components is insufficient; this term is used to describe large doses of micronutrients, usually in the form of capsules, tablets or

syrups. It has the advantage of offering the ideal amount of specific micronutrients in an absorbable form and is often the fastest way to combat nutrient deficiency (Bailey, Fulgoni, Keast, Lentino, & Dwyer, 2012; Dwyer et al., 2015; WHO & FAO, 2006).

Worldwide, supplementation programs have been widely used to provide iron, folic acid, vitamin A among other nutrients to pregnant women, infants, children under 5 years, among other groups of individuals (Datta & Vitolins, 2016). However, as for the more water-soluble vitamins and minerals, the populations or target populations need to consume the supplements more often for the control and combat for this type of micronutrient deficiencies. Flour fortification with micronutrients (Table 2) has been widely implemented worldwide, due to the extensive consumption of these foods in different forms and for being a cost-effective and sustainable strategy (Datta & Vitolins, 2016; Marks et al., 2018; Pacho et al., 2015).

As it has been described earlier, there are different types of micronutrients that can be used to supplement food, the following topics highlight the major micronutrients used to fortify flours worldwide.

#### 3.2.1. Vitamins

Vitamins are essential nutrients for growth and maintenance of life, since our organism does not have the capacity to synthesize them, we must guarantee their ingestion through food or supplements. These compounds are present in very small quantities and are linked to various processes related to the transfer and storage of energy, protection and strengthening of the body's defences, protection against various diseases, bone and tissue formation, formation and maintenance of cellular structure and functions, visual system, activity of other nutrients, etc. (Das et al., 2013; Johnson et al., 2004; Verma, 2015).

It has been reported the use of fortified flours with different vitamins to reduce nutritional deficiencies and to prevent and control various diseases. For example, rice has been fortified with vitamin A, to improve the iron status and vitamin A nutrition of populations, while maize flour has been fortified with B vitamins, contributing in the elimination of beriberi and pellagra in many countries, also vitamin B12 added as a fortifier for flour retains high bioavailability when baked in bread, which when consumed contributes in the diminution in this vitamin deficiency (Akhtar et al., 2011; Das et al., 2013; Garrod et al., 2019; Johnson et al., 2004; Peña-Rosas et al., 2014; Serdula, 2010a; WHO, 2018a), vitamin D has been used to fortified chapattis consumed by residents of private sunbeds in Romania, showing higher serum vitamin D levels and significantly increased bone density (Allen, Dangour, Chalabi, & Tedstone, 2015; Ritu & Gupta, 2015; WHO, 2016).

#### 3.2.2. Minerals

Minerals (sodium, potassium, calcium, iron etc.) are nutrients important to the body and are responsible for the proper functioning of metabolism. They favour the balance and maintenance of basic bodily functions such as conduction of nerve impulses, cellular activity and maintenance, and structural functions in the body, nail, tooth, and bone formation (Akhtar et al., 2011; Peña-Rosas et al., 2014; WHO, 2009; WHO & FAO, 2006). The different essential minerals are classified according to their concentration in the body and the requirement in the diet. The differences in the required mineral quantity has a great influence on the cost and on other aspects of mineral fortification of the flours. The main minerals for our organism are calcium, phosphorus, sodium, potassium, chloride and magnesium, while those that are needed in smaller quantities are iron and zinc, and the trace elements needed are iodine, copper and selenium (BNF, 2019; Peña-Rosas et al., 2014; Serdula, 2010a; WHO, 2016).

There are several studies demonstrating the use of minerals to strengthen different types of flours in order to reduce their deficiencies and prevent/control some diseases. Iron deficiency leads to anaemia and is one of the most prevalent public health problems in the world, and for example, in Iran, the fortification of flour with iron is considered a strategy to combat this deficiency (Blanco-rojo & Vaquero, 2019; Sadighi et al., 2008; WHO & FAO, 2006). Currently, every wheat flour sold in many countries are enriched with iron in order to reduce iron deficiency and anaemia caused by low intakes of bioavailable iron (Rebellato, Klein, Wagner, & Lima Pallone, 2018). Zinc is being added to maize flour in South Africa and Mexico and in wheat flour in Mexico, South Africa, Central Asia and Indonesia to address the problem of their deficiency (delayed growth and increased risk of disease), especially in children (Johnson et al., 2004; WHO, 2017; WHO & FAO, 2006). The fortification of calcium in whole wheat flour in Asia has been performed in order to increase the amount of calcium in the body and to combat some diseases, such as osteoporosis (Muhammad et al., 2016; WHO, 2017).

#### 3.2.3. Other nutrients

There are many other micronutrients with health benefits that can be used to fortify flours. Fibre deficiency has a negative impact on quality of life and can lead to serious health problems. Food fibres can be classified as soluble and insoluble fibres. Both types of fibre have several health benefits, including maintaining intestinal integrity and overall health, lowering blood cholesterol levels, controlling blood sugar levels, and providing non-caloric volume that may help in the loss of weight ratio by replacing caloric components. In this sense, fortification of flours with fibre can bring many health benefits (Anderson et al., 2009; Salmean, Zello, & Dahl, 2013).

Proteins belong to a category of compounds that are essential to life through its specific action. They are important for cell walls, muscles, blood, hair, internal organs, such as the heart and brain, among others, also for hormones, enzymes and antibodies, and replacement of waste cells. Essential amino acids can only be acquired through food, because the body cannot produce them by itself. One advantage may be the fortification of products with plant proteins, for example, the fortification of wheat flour with chickpeas, amaranth, quinoa, lentils and mushroom powder, etc. (Preedy et al., 2011; Prodhan, Linkon, Al-Amin, & Alam, 2015).

Essential fatty acids are also an important nutrient, especially for the cardiovascular system. Essential fatty acid deficiency is uncommon, but include scaly dermatitis, alopecia, thrombocytopenia and, in children, intellectual disability. Diets in the West are deficient in omega-3 fatty acids. Although there is not much mentioned about fortified flours with essential fatty acids, there are products derived from flour that have been incorporated with these compounds (e.g.: breads fortified with oils containing omega-3). Nevertheless, there are other products that have been fortified with essential fatty acids that include meat, oil, butter, jelly, different types of sauces, etc. (Preedy et al., 2011; Vlaic et al., 2019).

### 3.3. Flour fortification with bioactive compounds

The usefulness of food fortification is mostly given to bioactive compounds such as vitamins, minerals, phenolic compounds, essential amino acids, dietary fibre, among other group of compounds. By fortifying day-to-day foods we can guarantee the necessary micronutrients ingestion in order to stay healthy (Del Pino-García, Rico, & Martín-Diana, 2018).

There are many natural sources with bioactive compounds that can be used to fortify flours, a good example could be mushrooms, which can provide relevant amounts of B vitamins, ergosterol (precursor of vitamin D2) and minerals such as selenium, potassium, copper and zinc. Mushrooms are nutritious foods and an excellent source of bioactive compounds, and they have already been used to fortify wheat flour in order to increase the availability of different micronutrients (Cardwell, Bornman, James, & Black, 2018; Prodhan et al., 2015). Moreover, wheat and maize flours have been fortified with egg shell powder as a source of calcium, with finger millet (excellent source of bioactive compounds), due to its high concentration in fibres, minerals, protein, and calcium (Naves, Fernandes, Prado, & Telxeira, 2007; Oghbaei &

**Table 3**
Some natural sources used as flour fortifiers[a].

| Natural sources (origin) | | Class of bioactive compounds[b] | References |
|---|---|---|---|
| Mushrooms | *Agaricus bisporus*<br>*Pleurotus ostreatus*<br>*Boletus edulis*<br>*Cantharellus tubaeformis*<br>*Pleurotus sajor caju* | Carotenoids<br>Phytosterols<br>Tocopherols<br>Flavonoids<br>Phenolic acid | Archana and Neetu (2016), Cardwell et al. (2018), Chaudhari, Wandhekar, Shaikh, and Devkatte (2018), Chauhan, Kumar, Kumar, and Kumar (2018), Condé, Oliveira, and Oliveira (2017), Ishara, Sila, Kenji, and Buzera (2018), Mahamud, Shirshir, and Hasan (2012), Prodhan et al. (2015), Sonkar and Singh (2015), and Sulieman et al. (2019) |
| Plants | *Aloe vera* L.<br>*Artocarpus integer (Thunb.)* Merr.<br>*Avena sativa* L.<br>*Chenopodium quinoa* W.<br>*Hordeum vulgare* L.<br>*Ipomoea batatas* Lam.<br>*Moringa oleífera* Lam.<br>*Pennisetrum glaucum* (L.) R. Br.<br>*Abelmoschus esculentus* (L.) Moench. (Flour)<br>*Glycine* max (L.) Merr. (Proteins)<br>*Hordeum vulgare* L. (Brewer's Spent Grain)<br>*Lupinus spp* (Lupine Kernel Fibre)<br>*Malus domestica* Borkh. (Pomace)<br>*Mangifera indica* L. (Flours)<br>*Musa spp* (Flours) | Fatty acid | Bolarinwa, Aruna, and Raji (2019), Izydorczyk and McMillan (2019), Montemurro, Pontonio, and Rizzello (2019), Oyeyinka and Oyeyinka (2018), Preedy and Watson (2019), Preedy et al. (2011), Sonkar and Singh (2015), and Urade (2019) |

[a] The natural sources mentioned are already used to fortify flours or can be used for the same purpose.
[b] Classes of bioactive compounds that may be related to plants and/or mushrooms.

Prakash, 2012; Preedy et al., 2011).

Several non-wheat cereal flours are also used for substitution of a portion of wheat flour to improve the micronutrients content in breads, e.g.: rice, maize, amaranth, barley, oat, rye, emmer, buckwheat, spelt and sorghum. There are also other natural source that can be used to fortify flour and develop different types of products, as they contain a large number of bioactive compounds, for example chicken bones, used as a sources of calcium (Muhammad et al., 2016), these natural products, help to decrease micronutrient deficiency, and control/prevent several diseases (Berner et al., 2014). Table 3 shows some of the natural sources, namely plants and mushrooms, used as fortifiers and the class of bioactive compounds that can be targeted.

### 3.4. Health benefits of fortified flour

Flour fortification in the field of public health is a very attractive tactic and has the advantage of reaching massively a risk populations through existing food distribution systems, without widely changing existent patterns of consumption (Das et al., 2013; Preedy et al., 2011).

The benefits of fortification of flour or derivative flour products (Table 2), act in a positive way over the entire life cycle of the population, especially in children and pregnant women, preventing the birth of children with intellectual disabilities or malformations or deficiencies (Vlaic et al., 2019). It is one of the most efficient ways of combating malnutrition and controlling various diseases linked to vitamin or mineral deficiency (Santos & Pereira, 2007; Vlaic et al., 2019). For example vitamin A fortified cereal flours can be effective to reduce this vitamin deficiency (Ranum, 2001); as previously described, flour fortification with iron is a strategy to combat iron deficiency in Iran (Sadighi et al., 2008); folic acid fortification programs in Chile have resulted in significant declines in the occurrence of pregnancies affected by neural tube defect (Berry, Bailey, Mulinare, Bower, & Dary, 2010).

If consumed regularly, many are the health benefits that can be gained from fortified flour consumption, especially because it can help to maintain body reserves of nutrients in a more efficient way than supplements. Since fortified flours provide nutrients that are similar to those provided by an effective and balanced diet, fortified foods will contain “natural” amounts of nutrients and this does not happen with supplements by its own (Dwyer et al., 2015; Preedy et al., 2011; Whiting et al., 2016).

Flour fortification has the potential to improve the nutritional status of a large portion of the population, regardless of the social class, as they are a staple food widely distributed and consumed worldwide, and, this fortification does not require changes in existing food patterns of populations, being a very cost-effective method. It is also more efficient in reducing the risk of multiple deficiencies that can result from deficits in food supply or a poor diet. It is a major benefit primarily for women of childbearing age, during periods of pregnancy and lactation (increasing the rate of vitamins and minerals in breast milk and reducing the use of supplements) that need adequate amounts of micronutrients, as well as growing children, which need nutrients daily for growth and development (Berner et al., 2014; Das et al., 2013; Datta & Vitolins, 2016; Dwyer et al., 2015; FFI, 2018; Johnson et al., 2004; Preedy et al., 2011). Moreover, it is also usually possible to add one or more micronutrient fortification (multi-nutrient fortification) without adding significantly to the total cost of the flour at the point of manufacture (Das, Salam, Kumar, Lassi, & Bhutta, 2014; Dwyer et al., 2015; Whiting et al., 2016; WHO, 2009).

## 4. Legislation and major issues regarding quality control

The main objectives of food law are to protect consumer's health, facilitate trade, and protect consumer's fraud. In the case of fortified meals, the population should be protected from consuming toxic levels of micronutrients or nutritionally ineffective. Food fraud is committed to deceive consumers, it often arises from the need for competition between manufacturers, businesses, food establishments and large food retailers. Legislations are applied to require appropriate control in the fortification process to ensure that micronutrient levels are adequately within acceptable limits. The legislation also serves to prevent fortification with nutrients from unsafe or nutritionally unnecessary products, and the constant vigilance of the flours fortification industry, food fraud vulnerability assessments, brings benefits to the industry and ensures the safety and integrity of the supply chain of fortified flours

(Esteki, Regueiro, & Simal-Gándara, 2019; Marks et al., 2018; Spink, 2019).

The wide variety in each country's particularities and the public health goals worldwide have resulted in the development of many different approaches in the regulation of fortified foods. In most countries, fortification standards (in this case flour) are established by law or through cooperative arrangements. In some countries the fortification of food is achieved without any form of management guidance or quality control. With the increase distance between marketing food, whether fortified or not, from its place of production to the final consumer by Global Trade, has made it difficult to track the source of unintentional contamination and quality control concerns, and food safety (Esteki et al., 2019; Johnson et al., 2004). Quality control is performed to evaluate if the fortified product is following the established technical standards, using objective and measurable indicators. It typically consists of collecting samples of fortified food, depending on the production system, and determining its micronutrient content, since it is important to routinely collect and analyse the samples in order to verify and control whether the technical standards are being met. Quality control focuses on purely public health optics and, in this case, concentrates mainly on indicators and criteria that are relevant to the food fortification process (Johnson et al., 2004; Nestel & Nalubola, 2002; Verma, 2015; WHO & FAO, 2006).

The management tools available in order to establish an appropriate level of control over food fortification are food laws and related measures, as well as a broader food control system. This management has the function of protecting public health, being generally recommended that all forms of fortified foods be adequately regulated, to ensure that food fortification is safe and effective for certain population groups, mostly those with micronutrient deficiency risks (Marques et al., 2012; WHO & FAO, 2006). Food fortification techniques follow the principles established by the Codex Alimentarius to ensure food security (FAO & WHO, 2015). Any legislation on food fortification should also include the World Trade Organization (WTO) Agreement on the Application of Sanitary Measures (SPS) and the WTO Agreement on Technical Barriers to Trade (TBT), which have added new values to standards, guidelines, Codex codes and recommendations (FAO & WTO, 2017; Orriss, 1998; WHO & FAO, 2006).

In the legal context, fortification can be classified as mandatory or voluntary, which refers to the level of obligation imposed on producers of fortified foods to comply with the government's purposes evidenced by law (Datta & Vitolins, 2016; WHO & FAO, 2006). Mandatory fortification is where a manufacturer is legally obliged to add one or more micronutrients to a specific food or food product. Compulsory fortification can reach the general population or a specific group, depending on the consumption criterion of that food. For example, fortification of a staple food, such as flours, would increase consumption of a micronutrient in the general population, while fortification of complementary infant foods would only increase the intake of micronutrients from a target group. In controversy voluntary fortification occurs when a producer freely decides to fortify food, however, it varies depending on the micronutrient and the socio-political and legal environment present. Governments are advised to impose an appropriate degree of control over voluntary fortification, it should not only be consistent with general regulatory objectives but should also consider the General Principles of Codex for the addition of essential nutrients to food. Voluntary fortification is moved by a desire of the industry to promote trade and the consumer to increase consumption of micronutrients as a means to gain health benefits (FAO & WTO, 2017; Hennessy, Walton, & Flynn, 2013; Marks et al., 2018; WHO & FAO, 2006).

In general, and according to the FFI, for wheat flour 82 countries have mandatory fortification and 10 countries have voluntary fortification, for maize flour 16 countries have mandatory fortification and 4 countries have voluntary fortification, while for rice 7 countries have mandatory fortification and 11 countries have voluntary fortification. The list of countries that have mandatory or voluntary fortification can be consulted in FFI (2019a) and WHO (2018b).

## 5. Concluding remarks and future perspectives

Micronutrient deficiency is a serious problem that has adverse consequences and leads to economic and health tragedies for populations that are in risk around the world. Food fortification programs are extremely important to overcome and ensure the correct intake of micronutrients by these groups of population, so fortification of food is seen as an excellent methodology in the correction of nutritional deficiencies.

In general, flours are a potential vehicle for fortification, because of their high consumption worldwide. Nevertheless, the success of flour fortification is based on the correct evaluation of the prevalence of micronutrient deficiency, political opinions and their implementation, selection of fortifiers, levels of fortification, usual level of flour consumption and products derived from that staple food, fortification of other food vehicles, feasibility, cost and acceptability studies. Systems of monitoring and inspection of fortified products are also necessary.

Despite being fortification a mandatory priority for the international corporations to eliminate micronutrient deficiency, it remains a problematic issue in several countries. Thus, food fortification should be included into the national health and nutrition plans of each country, as a strategy to overcome micronutrients deficiency. These fortifications should include the incorporation of different micronutrients in the staple foods, in order to meet different purposes of millions of people around the world, being considered a low-cost, effective strategy with a low toxicity risk.

Moreover, the studies that have been performed until now, clearly show the high potential of fortifying flours as an alternative to combat micronutrient deficiencies, and consequently to control and/or eliminate various diseases, thus bringing nutritional benefits and improving health in general.

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## Author contributions

I.C.F.R. Ferreira and A.M.G.P. structured the manuscript; R.V.C·C wrote the manuscript with collaboration of A.F. and L. Barros; I.C.F.R. Ferreira, L.B. and A.M.G.P. reviewed the manuscript.

## Conflict of interest

The authors declare that they do not have any conflict of interest.

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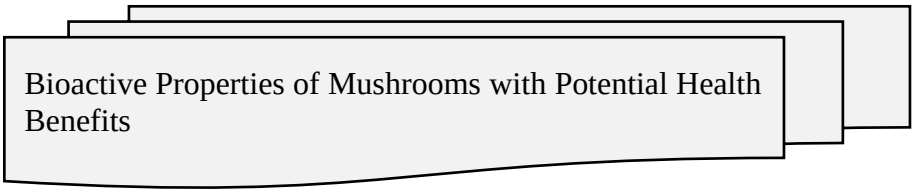
**Annex I**


Bioactive Properties of Mushrooms with Potential Health Benefits

CHAPTER 5

# *Bioactive Properties of Mushrooms with Potential Health Benefits*

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## 5.1 Introduction

Macrofungi are a group of organisms from the Fungi kingdom generally grouped in the families Ascomycota and Basidiomycota, which under special conditions develop a fruiting body of sufficient size to be seen with the naked eye and grow under the soil.[1] In 2019, global production of mushrooms and truffles was around 11.9 million tons worldwide. China is by far the largest producer of mushrooms globally, producing around 8.93 million metric tons of mushrooms and truffles annually. For producing regions, Asia is the largest producer, and is responsible for 82.8% of total global production, followed by Europe, the Americas, Oceania, and Africa with 12%, 4.5%, 0.4%, and 0.3%, respectively. Poland is the largest European producer

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of mushrooms, with a total production of 362 million metric tons, representing 3.05% of the total global volume.[2]

For a long time, mushrooms have played an important role in several aspects of human activity. Particularly, edible mushrooms are used extensively in cooking and make up part of a low-calorie diet. In different world regions, the demand and consumption of mushrooms are mostly related to different characteristics such as organoleptic properties, attractive taste and flavour, and a very accessible and marketable product.[1] Wild and cultivated mushrooms contain a huge diversity of biomolecules with nutritional (since they are rich in minerals, proteins, fibres, and carbohydrates, and are low-caloric foods due to a low content in fat) and/or medicinal properties. Due to these properties, they have been recognized as functional foods and as a source for developing medicines and nutraceuticals.[3–5] In order to survive, mushrooms possess the capacity to generate and accumulate a variety of functional compounds, such as carbohydrates (polysaccharides, glucose, fructose, maltose, rhamnose, arabinose, sucrose, mannitol, trehalose, and xylose), proteins (lectins), fatty acids (linoleic, oleic and palmitic acids), phenolic compounds (caffeic, gallic, cinnamic, melatonin, *p*-hydroxybenzoic, *p*-coumaric, and protocatechuic acids), indole compounds (L-tryptophan and 5-hydroxy-L-tryptophan), vitamins (vitamins B complex, vitamin C, and tocopherols), terpenoids (carotenoids such as β-carotene and lycopene), enzymes (laccases, cellulases, lignins, peroxidases, ribonucleases), and unique molecules (such as ergothioneine and glutathione). These bioactive molecules have immunoregulatory effects, and cardio- and liver-protective, antifibrotic, anti-inflammatory, antidiabetic, antiviral, anti-diabetes, anti-angiogenic, antioxidant, antitumour, and antimicrobial properties.[1,3,6–8] The molecular structure, molecular weight, size, branching pattern, conformation, solubility, and intra- and intermolecular association of these compounds are important for showing biological responses.[3] Their pharmacological action and therapeutic interest in promoting human health have been known for thousands of years. In the recent past, mushrooms have become increasingly important as potential natural agents to prevent and treat numerous diseases, such as cancer, cardiovascular diseases, diabetes mellitus and neurodegenerative diseases.[1,5]

In this respect, this chapter aims to analyse and summarize current knowledge and trends regarding the bioactive properties of mushrooms. In addition, important biological properties are discussed with their potential health benefits, which could be useful for the full use of mushrooms. A compilation of the published information from 2015 onwards has been explored.

## 5.2 Mushroom Consumption and Potential Health Benefits

The shift towards natural-based diets has been accelerating at varying rates due to the increased awareness of the health and environmental impacts of

excessive and ultra-processed foods.[9] Since ancient times, mushrooms have traditionally been collected in various geographical locations for human consumption; and this practice has increased in recent years, mainly due to the growing awareness of their therapeutic and beneficial effects.[10,11] Edible, medicinal, and wild mushrooms are the major components of the global mushroom industry. Medicinal and edible mushrooms are mostly found in higher basidiomycetes with a saprophytic growth habit, which allows them to grow on various agricultural waste-based substrates.[12] Edible mushrooms are the leading component, accounting for approximately 54% of global mushroom production, while medicinal and wild mushrooms make up 38% and 8% of the total, respectively.[13,14] *Agaricus*, *Lentinula*, *Pleurotus*, *Auricularia*, and *Flammulina* represent the five main genera, constituting over 85% of the world's commercial mushroom production. Other mushroom genera with well-documented nutritional and medicinal properties include *Ganoderma*, *Grifola*, *Trametes*, *Clitocybe*, *Antrodia*, *Cordyceps*, *Xerocomus*, *Calvatia*, *Hericium*, *Volvariella*, *Schizophyllum*, *Inonotus*, *Inocybe*, *Lactarius*, *Albatrellus*, *Russula*, *Boletus*, *Cantharellus*, *Lactarius*, *Morchella*, *Macrolepiota*, and *Fomes*.[15,16] *Agaricus bisporus* (white and brown button mushroom) represents about 40% of worldwide production, followed by *Lentinula edodes*, *Pleurotus ostreatus*, and *Flammulina velutipes*.[17] The global mushroom market share is expected to grow significantly in the coming years due to these nutritional advantages and health-promoting benefits. In addition, mushroom production and processing generate a large volume of by-products in the form of caps, stipes, mushrooms that do not fit with commercial standards, and spent mushroom substrate (SMS), being predominantly discarded with a high environmental impact and treatment costs for the industry.[18,19] These mushroom by-products constitute a real economic loss due to the abundance of bioactive compounds, which can be extracted and utilized as value-added ingredients. Hence, some integral approaches for the sustainable valorization of these by-products in developing innovative food and pharmaceutical formulations have been adopted further to improve the economic performance of the mushroom processing industry and promote a circular economy in line with current consumer preferences towards sustainability. The widespread utilizations of mushroom extracts and their associated metabolites in the development of nutraceutical and functional food formulations are due to the extensive range of nutritional, preventive, therapeutic, and structurally diverse bioactive compounds.[10] These biomolecules include polysaccharides (chitosans, β-glucans, fucogalactan, and lentinan), proteins (lectin and cordymin), fatty acids (linoleic, oleic, and palmitic), terpenoids and steroids (ergosterol, betulin, zhankuic acid, and ganoderic acid), phenolic compounds (caffeic acid, gallic acid, cinnamic acid, inotilone, hispolon, *p*-hydroxybenzoic acid, *p*-coumaric acid, and protocatechuic acid), vitamins (thiamine, riboflavin, biotin, and tocopherols), and other unique molecules (ergothioneine, cordycepin, and hispidin).[16,20] Mushrooms have

also been well characterized in their mineral content and have been shown to be rich in elements such as potassium, phosphorus, calcium, magnesium, sodium, iron, zinc, and copper.[21]

Apart from their desirable status in the culinary realm, their health-promoting properties are also significant, namely antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, cytotoxic, antimutagenic, cardioprotective, hepatoprotective, antidiabetic, and anti-ageing properties, validated via *in vitro* and *in vivo* studies (see Figure 5.1).[16,22–24] Studies have indicated that mushrooms exposed to ultraviolet (UV), gamma, and electron-beam irradiation under certain conditions convert ergosterol to vitamin D2.[25,26] These irradiated mushrooms effectively maintain serum 25(OH)D levels and are currently being utilized as food fortification ingredients to reduce vitamin D deficiency.[27] *In vivo* studies in a mouse model of osteoporosis have shown that the consumption of vitamin D2-enhanced mushrooms have also been associated with improved bone health.[28]

The use of mushroom extracts in the development of food formulations is widespread in baked goods (pasta, bread, and cookies), sausages, hamburgers, dairy products (cheese), beverages, and soups.[29,30] Additionally, considering the need to reduce foods from animal origins to enable better global sustainability, there is a growing search for plant-based protein

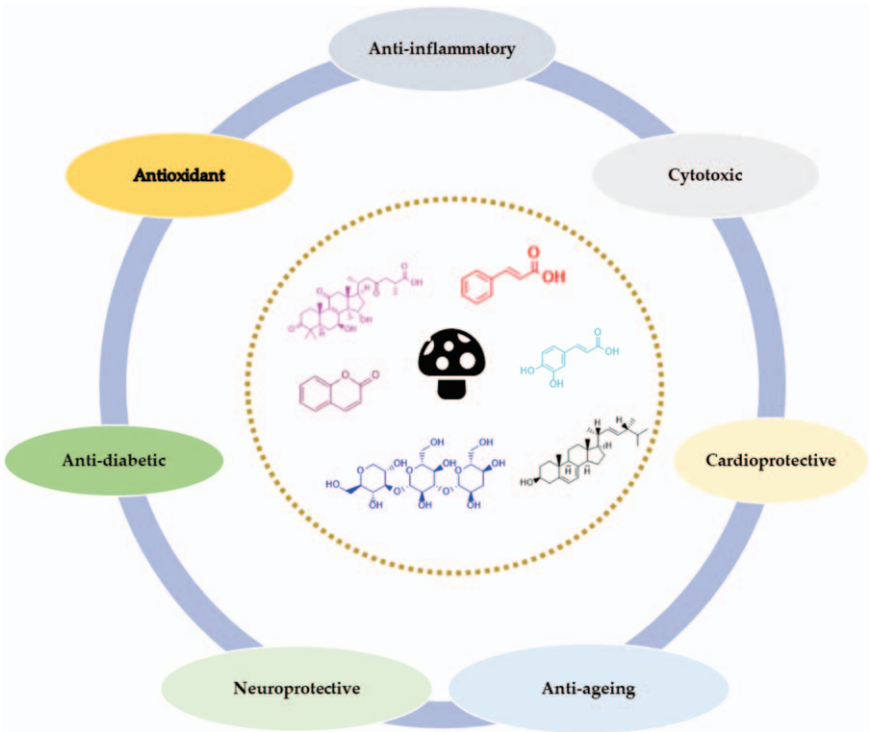


**Figure 5.1** Bioactive properties of mushroom extracts.

sources as an alternative to animal protein, and mushrooms represent a viable option for obtaining high-quality protein with a complete profile of essential amino acids, often covering the recommended dietary requirement.[31] Several commercially available products containing blends of mushroom extracts or their metabolites have been developed into patents and sold in the market as daily health supplements.

## 5.3 Extracts and Individual Compounds from Mushrooms with Bioactive Properties

### 5.3.1 Antioxidant Activity

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) accompany several cellular metabolic processes. Oxygen is an essential component for the oxidation of organic compounds and energy production,[32] and when a small amount of oxygen is reduced, it produces highly reactive chemical substances called ROS. Thus, ROS represents the most important class of all free radicals generated in living organisms.[33–35] They are related to several physiological processes and are beneficial to cells when produced in small quantities. However, in situations in which the balance between the production of these reactive species and the antioxidant defences is destroyed, oxidative stress begins.[36,37] ROS are highly reactive and when in excess they can cause irreversible damage to the structure of cells. The production of free radicals can be of endogenous or exogenous origin, referring to metabolic interactions or environmental factors, respectively (see Figure 5.2). Free radicals are related to several human diseases and participate as essential elements in many of them, which shows how extensive is the oxidative damage caused by them.[38,39] Natural or synthetic antioxidants are chemical compounds that significantly delay or prevent oxidation even at low concentrations compared to an oxidizable substrate. Organisms have antioxidant defence and repair systems that eliminate free radicals and protect the body from oxidative damage. The harmful effect of free radicals occurs when they are in excessive amounts in the body, exceeding its capacity to neutralize them with its natural defence systems (see Figure 5.2). Antioxidants have the power to intercept free radicals formed by cell metabolism or by exogenous sources, preventing oxidative stress and consequent damage to cells. Thus, antioxidant supplements, or natural products with antioxidant capacity, can help reduce oxidative damage in the body, preventing hundreds of diseases, including many types of cancer, diabetes, obesity, and cardiovascular diseases.[40–42] The appreciation of the relationship between food and health is growing every day, and healthy nutrition focused on natural products, mainly of vegetal origin, can help combat free radicals. Many natural products have bioactive molecules with various beneficial health claims.[38,43]

Mushrooms have been present in the human diet for many years and have long been valued as tasty, nutritious, healthy, and valuable food. They are rich

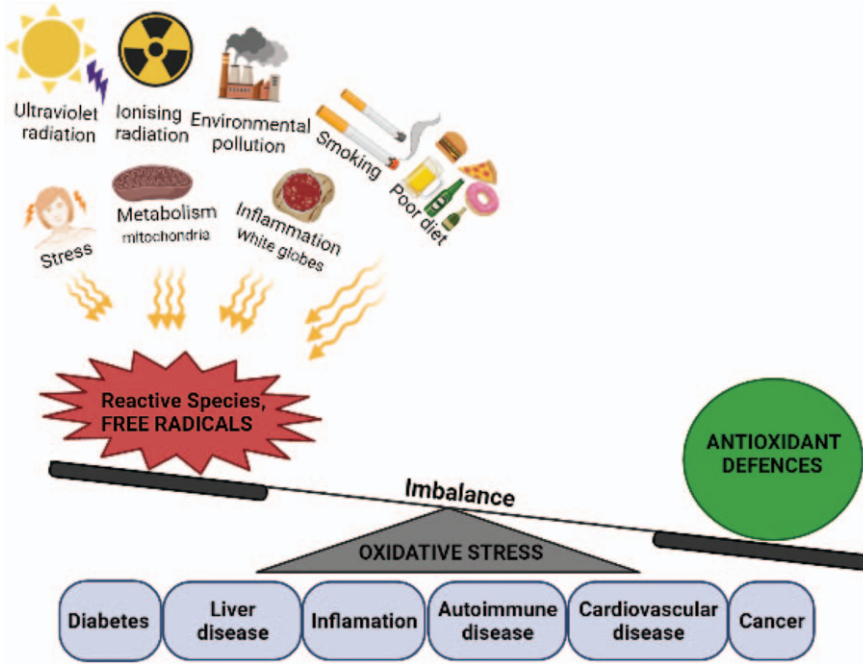


**Figure 5.2** The results of the imbalance between reactive species and antioxidant defences.

in bioactive molecules with health benefits, and in recent years the amounts consumed have increased significantly, involving many species.[43,44] Mushrooms are rich in antioxidants, including polysaccharides, phenols, proteins, peptides, carotenoids, ergosterol (pre-vitamin $D_2$), and vitamins C and E.[32,36,37,45,46] Several studies are available on the antioxidant properties of mushrooms, such as *Agaricus bisporus* samples,[47,48] *Agaricus campestris* L.,[49] *Pleurotus ostreatus*,[47,50–52] *Lentinula edodes*,[53,54] *Macrolepiota procera*,[49] *Pleurotus pulmonarius* (Fr.),[55] among others (see Table 5.1). Therefore, with the growing interest in the beneficial health effects of antioxidants, it is essential to study the antioxidant potential of the most consumed mushroom species worldwide and the varieties of existing methods to determine the antioxidant activity of extracts or substances obtained in *in vitro* and *ex vivo* conditions.[36,44,56] Several methods have been developed to evaluate the antioxidant capacity of edible and medicinal mushroom species (see Table 5.1).

Given the *in vitro* condition, the most commonly applied are the DPPH radical scavenging (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{\bullet+}$ radical scavenging capacity (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid), reducing power (RP), and β-carotene bleaching 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid. Polysaccharides such as heteroglycan, β-glucan, and lentinan obtained from different edible and medicinal mushrooms have presented high ferrous ion chelating, radical scavenging, and reducing power properties that have

AQ:1 **Table 5.1** Antioxidant potential in mushroom extracts.[a]

| Mushroom species | Extracts | Methods | Ref. |
|---|---|---|---|
| *A. bisporus*, *F. velutipes*, *L. edodes*, and *A. brasiliensis* | Ethanolic | DPPH, ABTS and FRAP | 44 |
| *A. bisporus* and *P. ostreatus* | Water, methanolic and ethanolic | ABTS, and FRAP | 218 |
| *A. campestris* and *B. edulis* | Methanolic extract | DPPH and RP | 219 |
| *Agaricus* sp., *A. cylindracea*, *B. loyo*, *C. lebre*, *C. espinosae*, *F. velutipes*, *G. gargal*, *L. deliciosus*, *L. edodes*, *M. procera*, *M. cónica*, *Pleurotus* sp., *Ramaria* sp., *Suillus* sp., *T. terreum,* and *X. chrysenteron* | Dichloromethane and methanolic | DPPH | 220 |
| *A. silvaticus*, *H. rufescens*, and *M. giganteus* | Methanol and ethyl acetate extract | DPPH, ABTS, FRAP, and catalase | 221 |
| *Amanita* sp., *L. volemus*, *Russula* sp., *Termitomyces* sp., *T. crissum*, *V. volvacea, A. hygrometricus*, *A. trappei*, *A. auricula*, *C. cibarius*, *C. Craterellus*, and *Lentinus* sp. | Methanolic extract | DPPH and FRAP | 222 |
| *A. lanipes* | Methanolic extract | ABTS and TOS | 223 |
| *B. edulis* | Ethanolic and water extract | DPPH, FRAP, hydroxyl oxide, nitric oxide, and superoxide anion | 224 |
| *B. edulis, B. pinophilus*, *B. aureus*, *A. mellea*, *T. aestivum, L. piperatus*, *L. deliciosus*, *P. eryngii*, *R. botrytis*, and *R. virescens* | Ethanolic extract | DPPH, chelating, RP, and inhibition of lipid peroxidation | 225 |
| *B. griseipurpureus* | Dichloromethane and methanolic extract | DPPH, ABTS, and ORAC | 226 |
| *C. cinereus*, *C. pistillaris*, *C. nebularis*, and *H. punicea* | Methanol, ethanol, and water extract | DPPH, ABTS, and RP | 227 |
| *C. cylindracea*, *F. velutipes*, *L. duriusculum*, *Pleurotus* sp., *Agaricus* sp., and *L. edodes* | Methanolic | DPPH | 48 |
| *C. cornucopioides* | Acetone | DPPH, superoxide anion, and RP | 228 |
| *G. lucidum* and *A. bisporus* | Water extract | DPPH | 229 |
| *H. marmoreus*, *A. vaginata*, *A. auricular*, *G. lucidum*, *L. edodes*, *L. polychrous*, *L. squarrosulus*, *P. ostreatus*, *P. ostreatus*, *P. pulmonarius*, *P. sajor-caju*, *A. cytindracea*, *T. fusiformis*, *F. velutipes*, and *V. volvacea* | Hexane, ethyl acetate, ethanol, methanol, and water extract | ABTS | 230 |
| AQ:2 *I. obliquus*, *G. frondosa*, *Ganoderma* sp., *L. edodes*, *T. versicolor*, and *H. erinaceus* | Reishi, hydroalcoholic, and water | ORAC, FRAP, DPPH, and NanoCerac | 53 |

**Table 5.1** (*Continued*)

| Mushroom species | Extracts | Methods | Ref. |
|---|---|---|---|
| *L. leucothites* | Ethanolic extract | DPPH | 231 |
| *M. lobayensis* | Ethanolic extract | DPPH, ABTS superoxide radical, hydroxyl radical quenching, chelating ability of metal ion, RP, and TAC | 232 |
| | Methanolic extract | DPPH, ABTS, RP, and chelating ability of ferrous ion | 233 |
| *M. giganteus* | Methanolic extract | DPPH, RP, β-carotene bleaching, and TBARS | 109 |
| *M. cognata* and *M. stridula* | Ethyl acetate, methanolic, and water | DPPH, ABTS, and RP, phospho-molybdenum, and metal chelating | 234 |
| *P. eryngii* and *S. belinii* | Methanolic extract | DPPH, RP, TBARS, and β-carotene bleaching | 235 |
| *P. florida* | Methanolic, ethanolic, water, ethyl acetate, and hexane | DPPH and RP | 52 |
| *P. ostreatus* | Water | DPPH, ABTS, RP, metal chelating, and hydroxyl radical | 38 |
| | Ethanolic | DPPH and hydroxyl radical scavenging | 236 |
| | Water | DPPH | 50 |
| | Ethyl acetate and *n*-butanol extract | DPPH and ABTS | 237 |
| *P. ostreatus* var. *Florida* | Ethanolic | TBARS and OxHLIA | 51 |
| *P. ostreatus* and *A. bisporus* | Methanolic extract | DPPH, RP, β-carotene, TBARS | 47 |
| *Pleurotus* sp. | Hydroalcoholic extract | DPPH, ORAC, ABTS, and β-carotene bleaching | 238 |
| *P. pulmonarius* | Ethanolic extract | TBARS, Conjugated dienes formation, DPPH, and RP | 55 |
| *R. subalpina* | Methanolic extract | DPPH, ferrous ion chelating, and RP | 239 |
| *Russula* sp. | Methanolic and ethanolic | OxHLIA and TBARS | 240 |
| *T. indicum* | Methanol and ethanol extract | DPPH and ABTS | 241 |
| *T. equestre* | Water and methanol extract | DPPH | 242 |

[a]TBARS, thiobarbituric acid reactive substances; OxHLIA, oxidative haemolysis inhibition assay; DPPH, 2,2′-diphenyl-1-picrylhydrazyl; RP, reducing power; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; ABTS, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid; TAC, total antioxidant capacity; TOS, total oxidant status.

contributed to their multifunctional health-promoting function. Some of these polysaccharide-rich mushrooms include *Termitomyces clypeatus*,[57] *Hohenbuehelia serotina*,[58] and *Meripilus giganteus*.[59] An optimized extract of *Pleurotus ostreatus* rich in heteropolysaccharides, β-glucans, α-glucans, and oligosaccharides, besides presenting a solid radical scavenging activity, also showed a promising capacity to protect cells from oxidative damage induced by hydrogen peroxide ($H_2O_2$).[50] In addition to the well-reported *in vitro* antioxidant effects of these mushrooms and their related metabolites, some *ex vivo* and *in vivo* studies involving animal models have also shown promising results. Among these, Popović *et al.*[60] evaluated antioxidative activity using an aqueous suspension of *Coprinus comatus*, with oxidative stress being induced in rats using alloxane and carbon tetrachloride. Jayakumar *et al.*[61] conducted a review of the *in vivo* antioxidant activity of *Pleurotus ostreatus*. The antioxidant potential was evaluated using an acute state of oxidative stress in animal models induced by carbon tetrachloride ($CCl_4$) and a chronic state of stress (ageing). In addition to the significant reduction in lipid peroxidation, the levels of enzyme antioxidant defences such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), which are involved in the destruction of ROS, were significantly elevated. Recently, the *in vivo* antioxidant effect of *Coprinus comatus* was developed using an animal model of $CCl_4$-induced hepatotoxicity.[62] The authors reported that oral ingestion of its methanolic extract suppressed $CCL_4$-induced liver damage, reduced lipid peroxidation, and increased levels of GSH-Px. The beneficial antioxidant and renoprotective effects of an acidic hydrolytic polysaccharide extract from *Lentinula edodes* extract were also conducted using an *in vivo* model. The extract significantly enhanced the levels of SOD, GSH-Px, and CAT enzymes, while also suppressing the level of malondialdehyde (MDA), a vital indicator of lipid peroxidation, responsible for cell membrane disruption and oxidative induced cell damage.[63] These findings imply that mushrooms extract contains various molecular lead compounds with potent antioxidant effects and they are currently used as additives in nutraceutical formulations to manage diseases associated with oxidative stress.

### 5.3.2 Anti-inflammatory, Neuroinflammatory, and Neuroprotective Activities

Inflammation is a complex biological process triggered in response to external stimuli such as infections, injuries, irritants, ultraviolet radiations, and toxins.[64] However, persistent inflammation has been associated with diabetes, arthritis, obesity, metabolic syndrome, cancer, and several cardiovascular diseases.[65] Pro-inflammatory cells (mainly macrophages and monocytes) play a key role in natural immunity by releasing specialized inflammatory mediators, which include vasoactive amines, eicosanoids, pro-inflammatory cytokines, and acute-phase proteins, which mediate the inflammatory process by preventing further tissue damage and ultimately

resulting in healing and restoration of tissue function. However, oversecretion of these inflammatory mediators causes accumulation of oxidative products and increased cytotoxicity, leading to tumour development, DNA damage, and programmed cell death.[65] External stimuli such as bacterial lipopolysaccharides, viral proteins, toxins, or endogenous proteins activate cascades of various transcription factors, namely activator protein 1 (AP-1), signal transducer and activator of transcription 3 (STAT3), and nuclear factor kappa β (NF-κB). NF-κB comprises five structurally related proteins, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel, responsible for the expression of a vast array of inflammatory mediators.[64] In the absence of stimuli, NF-κB complexes (usually in the forms of p60 and p65 subunits), are confined in the cytoplasm and attached to inhibitory proteins, including from the IκB family. Once the toll-like receptor (TLR) ligands are stimulated, IκB proteins are rapidly phosphorylated, which leads to the ubiquitination and degradation of IκBα (see Figure 5.3). This frees the NF-κβ complex (mainly p65 and p50) to translocate from the cytosol into the nucleus to instantaneously provoke the release of pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and cytokines like interferon-gamma (IFN-γ), interleukins (IL-1β and IL-6), and tumour necrosis factor alpha (TNF-α).[66]

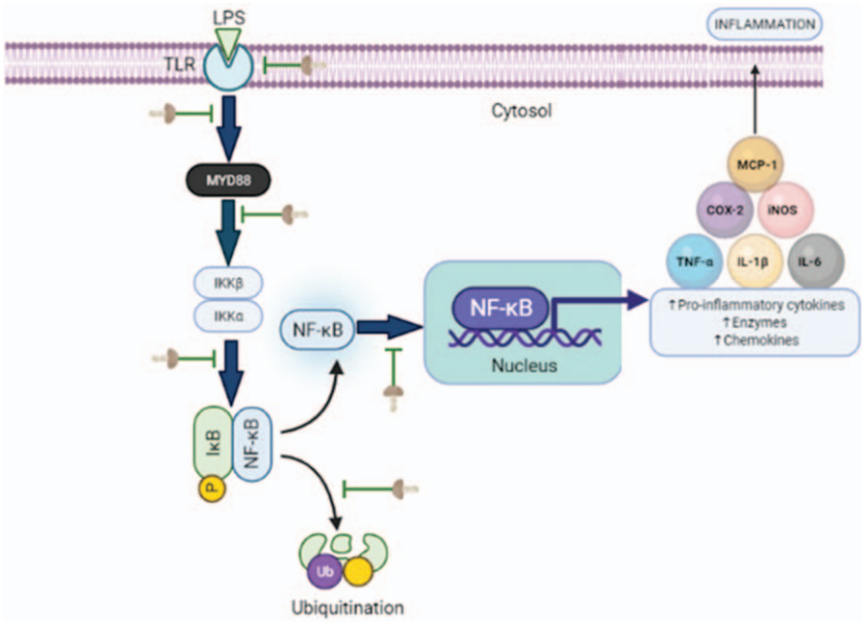


**Figure 5.3** The nuclear factor-kappa beta (NF-κβ) pathway. Abbreviations: toll-like receptors (TLR), lipopolysaccharides (LPS), myeloid differentiation protein 88 (MyD88), IL (interleukins), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumour necrosis factor (TNF-α), monocyte chemoattractant protein-1 (MCP-1), IkappaB kinase (IKK).

The mechanism behind the inflammatory process is mainly controlled by the NF-κβ pathway and trying to inhibit some of these specific steps in the pathway leading to NF-kβ release is crucial to suppressing the inflammation process (see Figure 5.3).

*In vitro* models such as lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells, mouse splenocytes, NIH/3T3 cell, HaCaT, RBL-2H3, and IFN-γ activated murine macrophages have been utilized as effective models to evaluate the anti-inflammatory activities of mushroom extracts and their related metabolites (see Table 5.2).[67–70] Additionally, numerous animal models such as a dextran sulfate sodium (DSS)-induced colitis model, carrageenan-induced mice models, formalin-induced mice models, and an acetic acid-induced mice model have been utilized to determine the anti-inflammatory properties of bioactive ingredients derived from mushroom (see Table 5.2).[71] Polysaccharide and sulfated polysaccharide-rich extracts obtained from *Antrodia cinnamomea* significantly reduced levels of TNF-α, IL-1β, and IL-6 in LPS-induced RAW 264.7 macrophage cells.[72,73] At the same time, a more distinct anti-inflammatory effect via downregulation of NF-κβ expression was reported for its oligosaccharide and ergostatrien-3β-ol rich extracts.[74,75] Polysaccharide rich fractions from *Grifola frondosa*,[76] *Hericium erinaceus*,[77] *Lentinula edodes* (acidic polysaccharide and β-D-glucan),[78,79] *Phellinus baumii* (heteropolysaccharide),[80] and *Schizophyllum commune* (exopolysaccharide)[71,81] presented interesting anti-inflammatory effects in RAW 264.7 macrophage cells, human monocyte THP-1 cell line, carrageenan-induced paw oedema, and DSS-induced colitis models. Sterol-rich extract from *G. lucidum* shows anti-inflammatory effects via attenuation of the NF-κβ pathway by restraining degradation of IκB-α and phosphorylation of NF-κβ p65.[82] Its phenolic-rich extract significantly suppressed gene expression of COX-2, TNF-α, iNOS, IL-1β, IL-6, IL-10, and NF-κB in RAW 264.7 macrophage cells and a DSS-induced colitis model.[83] The mechanism behind the anti-inflammatory effect of mushroom extracts and their bioactive metabolites has been associated with the downregulation and dephosphorylation of NF-κB, inhibition of mRNA expression of TLR4, inhibition of NF-κB translocation into the nucleus, and degradation of IκB-α.[82] Due to the current pharmaceutical practices involving highly purified compounds, the utilization of complex mixtures of bioactive compounds with unknown concentrations is difficult to reconcile. Moreover, the recovery of complex mushroom-derived compounds such as polysaccharides requires energy-consuming and costly extraction processes. Hence, low-molecular-weight compounds, such as *N*6-(2-hydroxyethyl) adenosine (HEA), cordycepin, methyl 9-oxo-(10*E*,12*E*)-octadecadienoate, polyozellin, and poricoic acid C, also have been studied (see Table 5.2) and known to inhibit inflammatory mediator production, block steps leading to NF-κβ activation, and suppress *in vivo* induced inflammation. As shown in Table 5.2, different research studies have highlighted mushrooms' very promising anti-inflammatory effects, mainly based on their extracts and bioactive compounds. Nevertheless, intensive preclinical and clinical research into these bioactive molecules is still lacking.

**Table 5.2** Anti-inflammatory and neuroinflammatory effects of mushroom extracts and their individual compounds.

| Species | Bioactive form | Bioactive property | Model | Key findings and suggested mechanism of action | Ref. |
|---|---|---|---|---|---|
| *A. blazei* | Polypeptide | Anti-inflammatory effect | NIH/3T3 cell | ↓ downregulate the expression of NF-κBp65 and TLR4<br>↓ levels of TNF-α, IL-1β and IL-6 | 68 |
| *A. rugosum* | Ethanolic extract | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ production of TNF-α and NO | 69 |
| *A. cinnamomea* | Triterpenoid-enriched fractions | Neuroprotection | LPS induced BV2 microglia cells | ↓ production of NO,<br>↓ mRNA expression of iNOS and COX-2 | 86 |
| | Sulfated polysaccharide | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ levels of TNF-α, IL-1β and IL-6 | 73 |
| | Polysaccharides and sulfated polysaccharides | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ levels of TNF-α and IL-6 | 72 |
| | Oligosaccharides | Anti-inflammatory effect | RAW 264.7 macrophage cells and LPS-induced mouse model | ↓ mRNA expression of IL-6, IL-8, IL-1, TNF-α, and MCP-1<br>↓ activation of MAPK and Akt signalling pathways | 74 |
| | Ergostatrien-3β-ol | Anti-inflammatory effect | Mouse skin ischaemia model induced by skin flap surgery | ↓ gene expression of iNOS, IL-6, TNF-α, IκB, and NF-κB | 75 |
| *A. mellea* | Xylosyl 1,3-galactofucan | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ levels of TNF-α and cytokine monocyte chemotactic protein-1 (MCP-1) | 243 |
| | Ethyl acetate extract | Neuroprotection | LPS induced BV2 microglia cells | ↓ production of NO, IL-6, TNF-α, and IL-1β<br>↓ phosphorylation of IκB-α and NF-κB p65 | 84 |
| *C. cicadae* | *N*6-(2-Hydroxyethyl) adenosine (HEA) | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ gene expression of TLR4, IκB, p-IκB, and COX-2<br>↓ production of TNF-α and PGE2 | 244 |

| | | | | | |
|---|---|---|---|---|---|
| *C. militaris* | Cordycepin | Neuroprotection | LPS induced BV2 microglia cells and hippocampal neurons from the brain of prenatal mouse | ↓ levels of TNF-α and IL-1β<br>↓ mRNA expression of iNOS and COX-2<br>↓ mRNA expression of p65, IκBα, and phosphorylated IκBα (p-IκBα) | 66 |
| *C. africanus* | Polyoxygenated cyathane diterpenoids | Neuroprotection | LPS induced BV2 microglia cells | ↓ production of NO | 87 |
| *D. indusiata* | Polysaccharide | Anti-inflammatory effect | DSS-induced colitis model | ↓ levels of TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-17, IL-4, and IL-10<br>↓ dephosphorylation of NF-κB and MAPK,<br>↓ mRNA expression of iNOS, COX-2, TNF-α, and IL-6 | 245 |
| *F. fomentarius* | Methyl 9-oxo-(10*E*,12*E*)-octadecadienoate | Anti-inflammatory effect | Murine macrophage cells | ↓ secretion of NO and PGE2,<br>↓ downregulation of iNOS and COX-2,<br>↓ levels of TNF-α and IL-6<br>↓ activation of STAT3 | 70 |
| *G. lucidum* | Polysaccharide | Neuroprotection | LPS- and Aβ-induced BV2 microglia and primary mouse microglia cells | ↓ mRNA expressions of IL-1β, IL-6, iNOS and MCP-1<br>↓ mRNA expressions of TGFβ | 85 |
| | Ganodermanontriol | Anti-inflammatory effect | Ana-1 macrophage cell line | ↓ levels of IL-1β, IL-6, TNF-α, and PEG2<br>↓ translocation of NF-κB<br>↓ mRNA expression of TLR4 | 246 |
| | Phenolic rich extract | Anti-inflammatory effect | RAW 264.7 macrophage cells and dextran sulfate sodium (DSS)-induced colitis model | ↓ gene expression of COX-2, TNF-α, iNOS, IL-1β, IL-6, IL-10, NF-κB | 83 |

**Table 5.2** (*Continued*)

| Species | Bioactive form | Bioactive property | Model | Key findings and suggested mechanism of action | Ref. |
|---|---|---|---|---|---|
| *G. frondosa* | Polysaccharide-rich extract | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ production of NO, PGE2, IL-6, TNF-α and IL-1β<br>↓ activation of NF-κB | 76 |
| *H. erinaceus* | Polysaccharide | Anti-inflammatory effect | DSS-induced colitis model | ↓ Serum levels of NO, IL-6, IL-1β, and TNF-α<br>↓ mRNA expression of COX-2 and iNOS<br>↓ phosphorylation of NF-κB p65, IκB-α, MAPK and Akt | 77 |
| | Erinacine C | Neuroprotection | BV-2 microglial cells | ↓ production of NO, IL-6, and TNF-α<br>↓ mRNA expression of iNOS<br>↓ mRNA expression of NF-κB and IκB-α | 88 |
| *I. obliquus* | Lanostane-type triterpenoids | Neuroprotection | BV-2 microglial cells | ↓ production of NO<br>↓ mRNA expression of iNOS | 89 |
| *L. edodes* | Acidic polysaccharides | Anti-inflammatory effect | LPS-induced mouse model | ↓ serum levels of TNF-α, IL-6, and IL-1β | 78 |
| | Polysaccharides | Anti-inflammatory effect | LPS-induced mouse model | ↓ serum levels of TNF-α, IL-6, and IL-1β | 247 |
| | α- and β-D-Glucans | Anti-inflammatory effect | Human monocyte THP-1 cell line | ↓ secretion of TNF-α, IL-6, and IL-1β | 79 |
| *L. rhinocerus* | Extract | Neuroprotection | HT22 Hippocampal neuronal cell model | ↓ number of apoptotic cells | 248 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *P. baumii* | Heteropolysaccharide | Anti-inflammatory effect | RAW 264.7 macrophage cells and DSS-induced colitis model | ↓<br>↓ | phosphorylation of STAT-1<br>mRNA expression of iNOS and TNF-α | 80 |
| *P. linteus* | Hispidin | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ | translocation of NF-κB and IκB degradation | 249 |
| *P. eryngii* | Polysaccharide | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ | production of IL-1β, IL-6, IL-10, and TNF-α | 250 |
| *P. multiplex* | Polyozellin | Anti-inflammatory effect | *In vitro* cells (HaCaT and RBL-2H3) and DFE/DNCB-induced AD animal model | ↓ | gene expression levels of TNF-α, IFN-γ, IL-4, IL-13, IL-31, IL-17A, STAT1, and NF-κB | 67 |
| *P. cocos* | Poricoic acid C | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓<br>↓<br>↓ | production of NO<br>gene expression of iNOS and COX-2<br>downregulation of NF-κB | 251 |
| | Lanostane triterpenoids | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓<br>↓ | production of NO and PGE2<br>mRNA expression of iNOS and COX-2 | 252 |
| *P. cubensis* | Psilocybin-rich extract | Anti-inflammatory effect | Human U937 macrophage cells | ↓ | level of TNF-α, IL-1β, IL-6, IL-10, and COX-2 | 253 |
| *S. commune* | Exopolysaccharide | Anti-inflammatory effect | DSS-induced colitis model | ↓ | serum levels of IFN-γ, IL-4, IL-10, and IL-17 | 254 |
| *X. nigripes* | Aqueous and hydroethanolic mycelial extract | Anti-inflammatory effect | RAW 264.7 macrophage cells | ↓ | production of NO, IL-6, TNF-α and COX-2 | 255 |

Neuroinflammation is a critical defence mechanism against infectious agents and neuronal injuries in the central nervous system (CNS). When microglia cells are activated in response to brain injury or immunological stimuli, such as LPS, amyloid beta (Aβ), and lipoteichoic acid, pro-inflammatory cytokines and neurotoxic mediators are released.[44] However, prolonged release of these inflammatory mediators may lead to aberrant phagocytosis, causing neuronal damage, thereby contributing to the pathogenesis of neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease (AD), Huntington's disease, motor neuron disease, prion disease, and multiple sclerosis.[84,85] Presently, the use of non-steroidal anti-inflammatory drugs against neurodegenerative disorders only slows down the disease progression transiently, and as such, there is increasing attention to finding potent and safe neuroprotective agents from natural sources that are able to prevent or treat these disorders. Mushroom extracts have been reported to display neuroprotective effects mainly via *in vitro* studies conducted in LPS and Aβ induced BV2 microglial cells, whose mechanisms of action are largely associated with downregulation of the gene expression of different inflammatory mediators.[84] Bioactive compounds such as cordycepin, polysaccharides, and phenolic acids have been reported to present a neuroprotective effect in different *in vitro* and *in vivo* models.[66,85] Mushroom-derived triterpenes (see Table 5.2) such as triterpenoid-enriched fractions from *Antrodia cinnamomea*,[86] polyoxygenated cyathane diterpenoids from *Cyathus africanus*,[87] erinacine C from *Hericium erinaceus*,[88] and lanostane-type triterpenoids from *Inonotus obliquus*[89] have been reported to display neuroprotective effects in BV2 microglial cells via downregulation of mRNA expression of IκB-α, NF-κβ, p65, iNOS, and COX-2. Studies have also evidenced the potential of medicinal and edible mushrooms as ''brain food'' being utilized to manage neurodegenerative disorders. The reported neuroprotective effects of these mushrooms and their bioactive secondary metabolites have been associated with their potential to reduce beta amyloid-induced neurotoxicity, and to promote nerve growth factor (NGF) synthesis, anti-acetylcholinesterase, and anti-neuroinflammatory activity.[90] Inhibitors of acetylcholinesterase improve cholinergic function by preventing the breakdown of acetylcholine into choline and acetate. Extracts or compounds with strong inhibitory capacity against acetylcholinesterase have been widely utilized in the management of AD.[91] The most common inhibitors of acetylcholinesterase utilized as treatment options for the management of AD include galantamine, rivastigmine, and donepezil that are ineffective in the long term with several associated side effects, including hepatotoxicity, nausea, diarrhoea, lack of appetite, gastrointestinal toxicity, and other side effects.[92,93] Hence, several mushroom extracts have been utilized as complementary and alternative medicines in the form of nutraceuticals and functional foods loaded with bioactive molecules that can potentially suppress the severity of AD. Submerged polysaccharide-rich extracts from two different *Schizophyllum commune* strains were examined for their acetylcholinesterase inhibitory capacity.[94] The extracts presented promising inhibitory capacity with $IC_{90}$ values of $79.73 \pm 26.34$ $\mu g\,mL^{-1}$ in

comparison to donepezil (87.92%), the positive control. Water and hydroethanolic extract from *Pholiota adiposa*, among other studied mushrooms, also presented up to 30.9 and 35% acetylcholinesterase (AChE) inhibitory activity, respectively.[95] Butyrylcholinesterase (BChE) inhibitors have also been reported to be viable therapeutic options in managing AD by promoting normal cholinergic function and preventing the breakdown of acetylcholine. Six macrofungi, namely *Coprinus comatus*, *Macrolepiota mastoidea*, *Agaricus campestris*, *Lycoperdon utriforme*, *Macrolepiota procera*, and *Leucoagaricus leucothites* were reported to prevent both AChE and BChE inhibition, and the promising results were expressed as galantamine equivalent.[96] A cholinesterase inhibitory effect of methanol extract rich in phenolic compounds from fruiting bodies of *Pleurotus pulmonarias* was reported to present a strong inhibitory effect against AChE and BChE, with the latter being more effective than galantamine.[97] Bio-guided fractionation performed on the fruiting bodies of mushroom has presented very promising AChE inhibitory activity. *Pleurotus florida* yielded a resveratrol-rich extract that inhibits acetylcholinesterase activity and combat oxidative stress in an *in vivo* animal model,[98] while a gallic acid-rich fraction obtained from *Ganoderma mediosinense* hydromethanolic extract inhibited AChE ($IC_{50}$, $0.10 \pm 0.02$ mg mL$^{-1}$).[99] Regarding the contribution of terpenoids and related compounds from mushrooms on AChE inhibitory activity, five novel meroterpenoids, possessing a γ-lactone motif obtained from *Ganoderma lucidum* presented AChE inhibitory activities with $IC_{50}$ values of 7.37–59.86 μM. Several lanostanoids obtained from the ethyl acetate extract of *Haddowia longipes* showed very low inhibitory capacity against AChE (<40%) at 100 μM.[100] Based on the beta-amyloid cascade hypothesis, several bioactive compounds obtained from mushrooms have shown promising inhibitory effects against Aβ aggregation, thereby reducing Aβ-induced neurotoxicity.[101] Ten monoterpenoids were isolated from the chloroform extracts of *Albatrellus yasudae*, and all the tested compounds presented potential Aβ40-aggregation inhibitory capacity with $IC_{50}$ between 12.3–221 μM, with the carboxyl and farnesyl groups exhibiting a more significant contribution to the bioactivity.[101] A growing body of scientific evidence has shown that dietary interventions involving mushroom extracts and their associated metabolites have shown promising potential to suppress the severity of neurodegenerative disorders due to their neuroprotective, antioxidant, and anti-inflammatory effects. However, studies involving clinical trials are significantly lacking and should be conducted to strengthen their utilization further in combatting these diseases.

### 5.3.3 Antimicrobial Properties and Impact of Bioactives on Gut Microbiota

Multiple-drug resistance in human pathogenic microorganisms has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used to treat infectious diseases. This situation has forced

scientists to search for new antimicrobial substances from various sources to be used as novel antimicrobial chemotherapeutic agents. The scientific community, while searching for new therapeutic alternatives, has studied many kinds of mushrooms and has found several therapeutic activities.[5,102,103]

Although many natural/synthetic antimicrobial agents have been isolated/developed to kill pathogenic microorganisms effectively, global antimicrobial resistance is an increasing public health problem.[104] Mushrooms need antibacterial and antifungal compounds to survive in their natural environment. Therefore, antimicrobial compounds could be isolated from many mushroom species and could be a benefit on humans.[3,8]

Despite the huge diversity of antibacterial compounds, bacterial resistance to first-choice substances has been drastically increasing. Moreover, the association between multiresistant microorganisms and infections highlights the problem and the urgent need for solutions. Natural resources have been exploited in recent years, and among them mushrooms could be an alternative source of new antimicrobials.[5,103,104] The research into mushrooms is extensive, and hundreds of species have demonstrated a broad spectrum of pharmacological activities, including antimicrobial activity. The two most used methodologies to assess the antimicrobial activity of mushroom extracts include the microdilution method and the disk diffusion method. The microdilution method comprises microdilutions of the extract in a liquid medium using microplates to determine the minimum inhibitory concentration (MIC) values. In the disk diffusion method, the extract is incorporated in disks at different concentrations, and the halo of growth inhibition is determined and represented by internal zone diameter (IZD) values.[5] Therefore, the results for antimicrobial activity may be expressed in different units.

Several mushroom extracts have been reported to have antimicrobial activity against Gram-positive and Gram-negative bacteria (see Table 5.3). It is presented as a mean value and, in parentheses, the range of variability of the literature data. *A. blazei* commercial sample from Brazil should be highlighted, as its ethanolic extract revealed MIC mean $= 0.144$ mg mL$^{-1}$ and minimum bactericidal concentration (MBC) mean $= 0.225$ mg mL$^{-1}$ against Gram-positive bacteria, like *S. aureus*, *B. cereus*, *M. flavus*, and *L. monocytogenes*, even lower than the standard ampicillin, and MIC mean $= 0.138$ mg mL$^{-1}$ and MBC mean $= 0.225$ mg mL$^{-1}$ against Gram-negative bacteria, such as *E. coli*, *S. enteritides*, *S. typhimurium*, and *E. cloacae*.[105] *A. bisporus* ethanolic extracts from a commercial sample from Portugal showed an inhibitory effect upon all the tested Gram-positive bacteria (MIC $= 10$ mg mL$^{-1}$).[106] *A. mellea* methanolic extract also revealed antimicrobial properties against Gram-positive and Gram-negative bacteria strains (MIC mean $= 7.19$ and $9.49$ mg mL$^{-1}$, respectively).[107] The methanolic extract of *H. tessulatus* showed activity against two Gram-positive bacteria (*S. aureus* and *B. subtilis*, MIC mean $= 7.5$ mg mL$^{-1}$) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *S. typhi*, and *K. pneumonia*, MIC

AQ:3 **Table 5.3** Mushroom extracts with reported antibacterial activity against Gram-positive and Gram-negative bacteria.[a]

| Mushroom | Origin | Samples | Extracts | Activity against Gram (+) and (−) bacteria | Method | Ref. |
|---|---|---|---|---|---|---|
| *A. bisporus* | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus*<br>Gram (−) bacteria: *E. coli* and *S. enteritidis* | MIC = 100 (−)<br>MIC = 150 (100–200) | 44 |
| | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*<br>Gram (−) bacteria: *S. typhimurium*, *P. aeruginosa*, *E. coli* | MIC = 59.52 (−)<br>MBC = 59.52 (−)<br>MIC = 59.52 (−)<br>MBC = 59.52 (−) | 256 |
| | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus*<br>Gram (−) bacteria: *E. coli* and *S. enteritidis* | MIC = 125 (50–200)<br>MIC = 200 (−) | 44 |
| | Portugal | Commercial | Ethanol | Gram (+) bacteria: Enterococcus faecalis, MSSA and MRSA<br>Gram (−) bacteria: *E. coli* and *P. aeruginosa* | MIC = 10 (−)<br>MIC ≥ 20 | 106 |
| *A. brasiliensis* | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus*<br>Gram (−) bacteria: *E. coli* and *S. enteritidis* | MIC = 75 (50–100)<br>MIC = 200 (−) | 44 |
| *A. blazei* | Brazil | Commercial | Ethanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus* and *L. monocytogenes*<br>Gram (−) bacteria: *E. coli*, *S. enteritides*, *S. typhimurium*, *E. cloacae* | MIC = 0.575 (0.075–0.20)<br>MBC = 0.225 (0.10–0.40)<br>MIC = 0.138 (0.025–0.40)<br>MBC = 0.225 (0.05–0.60) | 105 |
| *A. mellea* | Serbia | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *L. monocytogenes*, and *M. flavus*<br>Gram (−) bacteria: *P. aeruginosa*, *E. coli*, *S. typhimurium*, and *E. cloacae* | MIC = 7.19 (3.75–12.50)<br>MBC = 13.61 (6.25–21.7)<br>MIC = 9.49 (3.12–15)<br>MBC = 16.95 (6.25–25) | 107 |
| *B. edulis* | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*<br>Gram (−) bacteria: *S. typhimurium*, *P. aeruginosa*, *E. coli* | MIC = 20.92 (13.49–28.34)<br>MBC = 28.34<br>MIC = 20.92 (13.49–28.34)<br>MBC = 28.34 (−) | 256 |
| *C. cibarius* | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus* | MIC = 59.52 (−)<br>MBC = 59.52 (−) | 256 |

**Table 5.3** (*Continued*)

| Mushroom | Origin | Samples | Extracts | Activity against Gram (+) and (−) bacteria | Method | Ref. |
|---|---|---|---|---|---|---|
| | | | | Gram (−) bacteria: *S. typhimurium*, *P. aeruginosa*, *E. coli* | MIC = 59.52 (−)<br>MBC = 59.52 (−) | |
| *F. velutipes* | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus* | MIC = 37.5 (25–50) | 44 |
| | | | | Gram (−) bacteria: *E. coli* and *S. enteritidis* | MIC = (−) | |
| *G. lucidum* | Portugal | Commercial | Ethanol | Gram (+) bacteria: *E. faecalis*, *L. monocytogenes*, MSSA, and MRSA | MIC = 10 (5–20) | 257 |
| | | | | Gram (−) bacteria: *E. coli*, *E. coli* ESBL, *K. pneumoniae*, *K. pneumoniae* ESBL, *M. morganii*, *P. aeruginosa*, and *P. mirabilis* | MIC = 16 (1–20) | |
| *H. tessulatus* | Bangladesh | Commercial | Methanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | MIC = 7.5 (7–8) | 108 |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa*, *S. typhi*, and *K. pneumonia* | MIC = 7.25 (6–9) | |
| *L. piperatus* | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus* | MIC = 41.83 (26.99–56.68)<br>MBC = 56.68 (−) | 256 |
| | | | | Gram (−) bacteria: *S. typhimurium*, *P. aeruginosa*, *E. coli* | MIC = 41.83 (26.99–56.68)<br>MBC = 56.68 (−) | |
| *L. edodes* | Bangladesh | Commercial | Methanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | MIC = 14.06 (1.56–12.50) | 108 |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa*, *S. typhi* and *K. pneumonia* | MIC = 3 (−) | |
| | Portugal | Commercial | Ethanol | Gram (+) bacteria: Enterococcus faecalis, MSSA and MRSA | MIC = 3.33 (2.5–5) | 106 |
| | | | | Gram (−) bacteria: *E. coli* and *P. aeruginosa* | MIC ≥ 20 | |
| | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus* and *B. cereus* | MIC = 100 (−) | 44 |
| | | | | Gram (−) bacteria: *E. coli* and *S. enteritidis* | MIC = 3 (2–4) | |
| *M. giganteus* | Serbia | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus*, and *L. monocytogenes* | MIC = 0.23 (0.0125–0.60)<br>MBC = 0.48 (0.035–0.60) | 109 |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium*, and *E. cloacae* | MIC = 0.819 (0.025–2.50)<br>MBC = 1.72 (0.035–5.00) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *M. conica* | Portugal | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus*, and *L. monocytogenes* | MIC = 2.05 (0.70-3.75)<br>MBC = 3.99 (0.95-7.5) | 110 |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium*, and *E. cloacae* | MIC = 3.16 (0.70–5.62)<br>MBC = 4.23 (0.95–7.50) | |
| | Serbia | Wild | Methanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, *M. flavus*, and *L. monocytogenes* | MIC = 3.99 (0.95–3.75)<br>MBC = 7.97 (1.87–15.00) | |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium*, and *E. cloacae* | MIC = 6.56 (3.75–7.5)<br>MBC = 13.13 (7.50–15.00) | |
| *M. esculenta* | India | Wild | Butanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | IZD = 11.5 (8–15) | 111 |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa*, and *P. vulgaris* | IZD = 14 (11–19) | |
| | | | Ethyl acetate | Gram (+) bacteria: *S. aureus* and *B. subtilis* | IZD = 11 (8–14) | |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa*, and *P. vulgaris* | IZD = 8 (−) | |
| | | | Butanol | Gram (+) bacteria: *S. aureus* | MIC = 0.25 (−)<br>MBC = 0.75 (−) | |
| | | | | Gram (−) bacteria: *E. coli* and *P. aeruginosa* | MIC = 0.5 (0.25–0.75)<br>MBC = 0.75 (0.5–1) | |
| | | | Ethyl acetate | Gram (+) bacteria: *S. aureus* | — | |
| | | | | Gram (−) bacteria: *E. coli* and *P. aeruginosa* | MIC = 0.75 (−)<br>MBC = 1.25 (−) | |
| *P. ostreatus* | Bangladesh | Commercial | Methanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | MIC = 6.5 (6–7) | 108 |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa*, *S. typhi*, and *K. pneumonia* | MIC = 6.5 (5–8) | |
| | Portugal | Commercial | Ethanol | Gram (+) bacteria: *Enterococcus faecalis*, MSSA, and MRSA | MIC = 5 (2.5–10) | 106 |
| | | | | Gram (−) bacteria: *E. coli* and *P. aeruginosa* | MIC ≥ 20 | |
| | Portugal | Commercial | Hydroethanol | Gram (+) bacteria: *S. aureus*, *B. cereus*, and *L. monocytogenes* | MIC = 0.32 (0.25–0.35)<br>MBC = 0.58 (0.35–0.70) | 51 |
| | | | | Gram (−) bacteria: *E. coli*, *E. cloacae*, and *S. typhimurium* | MIC = 0.28 (0.25–0.35)<br>MBC = 0.47 (0.35–0.70) | |

**Table 5.3** (*Continued*)

| Mushroom | Origin | Samples | Extracts | Activity against Gram (+) and (−) bacteria | Method | Ref. |
|---|---|---|---|---|---|---|
| | Romania | Wild | Methanol | Gram (+) bacteria: *S. aureus* and *B. cereus*<br>Gram (−) bacteria: *S. typhimurium*, *P. aeruginosa*, and *E. coli* | MIC = 59.52 (−)<br>MBC = 59.52 (−)<br>MIC = 59.52 (−)<br>MBC = 59.52 (−) | 256 |
| *P. sajor-caju* | Brazil | Commercial | Hydroethanol | Gram (+) bacteria: *E. faecalis*, *L. monocytogenes*, MSSA, and MRSA<br>Gram (−) bacteria: *P. aeruginosa*, *E. coli*, ESBL, *K. pneumoniae*, *K. pneumoniae* ESBL, and *M. morganii* | MIC = 10 (−)<br>MIC = (−) | 258 |
| *P. squamosus* | Portugal | Wild | Methanol | Gram (+) bacteria: *S. aureus, B. cereus, M. flavus*, and *L. monocytogenes*<br>Gram (−) bacteria: *E. coli, P. aeruginosa, S. typhimurium*, and *E. cloacae* | MIC = 2.00 (0.20–3.13)<br>MBC = 4.00 (0.39–6.25)<br>MIC = 2.05 (0.39–3.13)<br>MBC = 4.10 (0.78–6.25) | 259 |
| | Serbia | Wild | Methanol | Gram (+) bacteria: *S. aureus, B. cereus, M. flavus*, and *L. monocytogenes*<br>Gram (−) bacteria: *E. coli, P. aeruginosa, S. typhimurium*, and *E. cloacae* | MIC = 1.23 (0.40–1.50)<br>MBC = 2.44 (0.75–3.00)<br>MIC = 0.80 (0.40–1.50)<br>MBC = 1.79 (1.14–3.00) | |
| | Romania | Wild | Methanol | Gram (+) bacteria: *B. cereus*, *M. flavus*, and *S. aureus*<br>Gram (−) bacteria: *E. coli*, *P. aeruginosa*, *S. typhimurium*, *L. monocytogenes*, and *E. cloacae* | MIC = 10.6 (1.2–20.4)<br>MBC = 21.2 (2.4–40.8)<br>MIC = 16.44 (0.61–20.4)<br>MBC = 32.8 (1.2–40.8) | 260 |
| *R. integra* | Serbia | Wild | Ethanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis*, and *S. warneri*<br>Gram (−) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 4.61 (0.78–12.50)<br>MBC = 7.42 (1.56–12.50)<br>MIC = 7.81 (3.12–12.50)<br>MBC = 9.38 (6.25–12.50) | 240 |

| | | | | | |
|---|---|---|---|---|---|
| | | | Methanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis*, and *S. warneri* | MIC = 3.74 (0.39–12.50)<br>MBC = 5.40 (0.78–12.50) |
| | | | | Gram (−) bacteria: *E. cloacae* and *S. maltophilia* | MIC = (−)<br>MBC = (−) |
| *R. nigricans* | Serbia | Wild | Ethanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis*, and *S. warneri* | MIC = 3.66 (1.56–7.50)<br>MBC = 6.73 (3.12–12.50) |
| | | | | Gram (−) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 3.91 (1.56–6.25)<br>MBC = 7.81 (3.12–12.50) |
| | | | Methanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis*, and *S. warneri* | MIC = 3.27 (0.39–12.50)<br>MBC = 5.01 (0.78–12.50) |
| | | | | Gram (−) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 3.91 (3.12–12.50)<br>MBC = 6.25 (−) |
| *R. rosea* | Serbia | Wild | Ethanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis*, and *S. warneri* | MIC = 2.93 (0.20–12.50)<br>MBC = 4.26 (0.39–12.50) |
| | | | | Gram (−) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 9.38 (6.25–12.50)<br>MBC = 12.50 (−) |

**Table 5.3** (*Continued*)

| Mushroom | Origin | Samples | Extracts | Activity against Gram (+) and (−) bacteria | Method | Ref. |
|---|---|---|---|---|---|---|
| | | | Methanol | Gram (+) bacteria: *M. luteus*, *R. mucilaginosa*, *S. agalactiae*, *S. angiosus*, *S. conselatus*, *S. dysgalactiae*, *S. oralis*, *S. parasanquinis*, *S. pseudopneumoniae*, *S. pyogenes*, *S. salivarius*, *S. aureus*, *S. hominis*, and *S. warneri* | MIC = 3.41 (0.20–12.50)<br>MBC = 5.30 (0.39–12.50) | |
| | | | | Gram (−) bacteria: *E. cloacae* and *S. maltophilia* | MIC = 6.25 (−)<br>MBC = 12.50 (−) | |
| *T. aestivum* | Spain | | Ethyl acetate | Gram (+) bacteria: *L. monocytogenes* | IZD = 8.5 | |
| *T. gennadii* | Spain | Wild | Methanol | Gram (−) bacteria: *S. flexneri* | IZD = 8.1 | 261 |
| *T. magnusii* | Italy | | Water | Gram (+) bacteria: *S. aureus* and *M. luteus* | IZD = 13.9 (8.1–19.7) | |
| *T. melanosporum* | Spain | | Water | Gram (+) bacteria: *S. aureus* | IZD = 8.7 | |
| *V. bohemica* | India | Wild | Butanol | Gram (+) bacteria: *S. aureus* and *B. subtilis* | IZD = 10.5 (9–12) | 111 |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa* and *P. vulgaris* | IZD = 11.7 (9–15) | |
| | | | Ethyl acetate | Gram (+) bacteria: *S. aureus* and *B. subtilis* | IZD = 13.5 (12–15) | |
| | | | | Gram (−) bacteria: *E. coli*, *P. aeruginosa* and *P. vulgaris* | IZD = 11 (9–13) | |
| | | | Butanol | Gram (+) bacteria: *S. aureus* | MIC = 0.25 (−)<br>MBC = 0.5 (−) | |
| | | | | Gram (−) bacteria: *E. coli* and *P. aeruginosa* | MIC = 0.25 (−)<br>MBC = 0.75 (−) | |
| | | | Ethyl acetate | Gram (+) bacteria: *S. aureus* | — | |
| | | | | Gram (−) bacteria: *E. coli* and *P. aeruginosa* | MIC = 0.75 (−)<br>MBC = 1 (−) | |

[a](−) MIC and MBC not found for the concentrations tested; MSSA, meticillin-sensitive *Staphylococcus aureus*; MRSA, meticillin-resistant *Staphylococcus aureus*; ESBL, extended spectrum β-lactamases; MIC/MBC, mg mL$^{-1}$; IZD, inhibition zone diameters (mm).

mean = 7.25 mg mL$^{-1}$).[108] *L. edodes* hydroethanolic extract demonstrated good activity against two Gram-negative bacteria, *E. coli* and *S. enteritidis* (MIC mean = 3 mg mL$^{-1}$).[44] The methanolic extracts of *M. giganteus* wild samples from Serbia showed an antibacterial effect against Gram-positive and Gram-negative bacteria with MIC means of 0.23 and 0.819 mg mL$^{-1}$, respectively.[109] *M. conica* methanolic extracts from wild samples from Serbia and Portugal also presented an inhibitory effect upon all the tested Gram-positive and Gram-negative bacteria.[110] *P. ostreatus* hydroethanolic extracts from commercial samples from Portugal showed an antibacterial effect against Gram-positive and Gram-negative bacteria with MIC means of 0.32 and 0.28 mg mL$^{-1}$, respectively.[51]

Shameem *et al.*[111] tested the antimicrobial activity of butanol extract from *M. esculenta* and revealed a significant antibacterial effect against *S. aureus* with MIC and MBC means of 0.25 and 0.75 mg mL$^{-1}$, respectively and, *E. coli* and *P. aeruginosa* presented MIC and MBC means of 0.75 and 1.25 mg mL$^{-1}$, respectively.

Data available from the literature indicate that mushroom species showed antimicrobial activity against Gram-positive and Gram-negative bacteria, particularly, against pathogenic microorganisms, including bacteria associated with nosocomial infections (*P. aeruginosa*, *L. monocytogenes*, *S. aureus*, *K. pneumoniae*, *M. morganii*, *P. mirabilis*, *etc.*) and multiresistance (MRSA).

As Table 5.4 shows, different extracts obtained from several mushroom species have been described in the literature as possessing antifungal activity. Stojković *et al.*[109] reported antifungal activity of *M. giganteus* methanolic extracts against *A. fumigatus*, *A. versicolor*, *A. ochraceus*, *A. niger*, *T. viride*, *P. funiculosum*, *P. ochrochloron*, and *P. aurantiogriseum* with MIC and MFC means of 0.138 and 0.31 mg mL$^{-1}$, respectively. Ethanolic extract of *A. blazei* showed activity against *A. fumigatus*, *A. versicolor*, *A. ochraceus*, *A. niger*, *C. crusei*, *P. funiculosum*, and *P. verrucosum* var. *cyclopium* with MIC and MFC means of 0.75 and 0.94 mg mL$^{-1}$.[105] *P. ostreatus* hydroethanolic extract seems promising against some fungal mentioned before with MIC and MFC means of 0.30 and 0.49 mg mL$^{-1}$, respectively.[51]

AQ:4

Most studies on mushrooms with antibacterial and antifungal activities describe the action of the extracts, focused on screening without identifying the compounds responsible for this activity.[5] Prebiotics are substances that induce the growth or action of microorganisms that contribute to the well-being of their host. Particularly, mushrooms are considered a potential source of prebiotics as they contain phenolic compounds and several active polysaccharides, such as chitin, hemicellulose, β- and α-glucans, mannans, xylans, and galactans, which make them the right choice for prebiotics. Different bioactive compounds from mushrooms have been shown to alter gut microbiota and improve health status. The gut microbiota comprises trillions of bacteria that contribute to nutrient acquisition and energy regulation. Current research indicates that diet is the most important factor as it significantly affects the composition, diversity, and richness of gut microbiota. The microorganisms present in the gut play an important role in

AQ:5 **Table 5.4** Mushroom extracts with reported antifungal activity.[a]

| Mushroom | Origin | Samples | Extracts | Activity against | Method | Ref. |
|---|---|---|---|---|---|---|
| *A. mellea* | Serbia | Wild | Methanol | *A. niger, A. ochraceus, A. versicolor, A. fumigatus, T. viride, P. funiculosum, P. ochrochloron, P. verrucosum* var. *cyclopium*, and *C. albicans* | MIC = 7.28 (6.25–15)<br>MFC = 12.81 (6.25–25) | 107 |
| *A. blazei* | Brazil | Commercial | Ethanol | *A. fumigatus, A. versicolor, A. ochraceus, A. niger, C. crusei, P. funiculosum*, and *P. verrucosum* var. *cyclopium* | MIC = 0.57 (0.20–0.80)<br>MFC = 0.94 (0.40–1.60) | 105 |
| *G. lucidum* | Portugal | Commercial | Ethanol | *C. albicans* | MIC ≥20 | 257 |
| *H. tessulatus* | Bangladesh | Commercial | Methanol | *C. albicans* and *S. cerevisiae* | MIC = 5.5. (5–6) | 108 |
| *L. edodes* | Bangladesh | Commercial | Methanol | *C. albicans* and *S. cerevisiae* | MIC = 1 (–) | |
| *M. giganteus* | Serbia | Wild | Methanol | *A. fumigatus, A. versicolor, A. ochraceus, A. niger, T. viride, P. funiculosum, P. ochrochloron*, and *P. aurantiogriseum* | MIC = 0.138 (0.025–0.30)<br>MFC = 0.31 (0.050–0.40) | 109 |
| *M. conica* | Portugal | Wild | Methanol | *P. aurantiogriseum, A. versicolor, A. niger*, and *T. viride* | MIC = 3.59 (0.78–6.25)<br>MFC = 5.94 (1.56–12.5) | 110 |
| | Serbia | | | | MIC = 9.06 (1.56–12.5)<br>MFC = 13.12 (3.12–25.00) | |
| *M. esculenta* | India | Wild | Butanol | *A. niger, C. albicans, C. kruesie, C. paraloposis*, and *A. fumigates* | IZD = 17.25 (11–18) | 111 |
| | | | Ethyl acetate | *A. niger, C. albicans, C. kruesie, C. paraloposis* and *A. fumigates* | IZD = 18 (–) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *P. squamosus* | Portugal | Wild | Methanol | *A. fumigatus, A. ochraceus, A. versicolor, A. niger, P. funiculosum, P. ochrochloron, P. aurantiogriseum*, and *T. viride* | MIC = 1.46 (0.78–3.13)<br>MFC = 2.93 (1.56–6.25) | 259 |
| | Serbia | Wild | Methanol | *A. fumigatus, A. ochraceus, A. versicolor, A. niger, P. funiculosum, P. ochrochloron, P. aurantiogriseum*, and *T. viride* | MIC = 0.71 (0.40–1.50)<br>MFC = 1.41 (0.75–3.00) | |
| *P. ostreatus* | Bangladesh | Commercial | Methanol | *C. albicans* and *S. cerevisiae* | MIC = 4 (–) | 108 |
| | Portugal | Commercial | Hydroethanol | *A. fumigatus*, *A. ochraceus*, *A. niger*, *P. funiculosum*, *P. ochrochloron*, and *P.v. cyclopium* | MIC = 0.30 (0.08–0.75)<br>MFC = 0.49 (0.35–0.70) | 51 |
| *V. bohemica* | India | Wild | Butanol | *A. niger, C. albicans, C. kruesie, C. paraloposis*, and *A. fumigates* | IZD = 14 (9–22) | 111 |
| | | | Ethyl acetate | *A. niger, C. albicans, C. kruesie, C. paraloposis*, and *A. fumigates* | IZD = 10 (7–14) | |

[a]MIC/MFC, mg mL$^{-1}$; IZD, inhibition zone diameters (mm).

the health of the digestive system and influence the immune system. The medicinal metabolites from mushrooms can act as immunomodulatory agents to activate gut microbiota.[112–114] These bioactive ingredients can modulate the gut microbiota and improve body health through various regulatory mechanisms, including enriching microbiota diversity, promoting beneficial bacteria, increasing the cacteroides/firmicutes ratio, decreasing harmful bacteria, and increasing short-chain fatty acid-producing bacteria.[114]

### 5.3.4 Immunomodulatory and Cytotoxic Properties

The immune system is an essential building pillar of the body and is responsible for recognizing, drowning, and fighting against diseases and toxic microorganisms. Through what is called immunity of two types, acquired or innate immune systems, the immune system acts in multifaceted immune responses to control and eliminate harmful organisms or substances (see Figure 5.4).[115,116] Immunomodulators are substances that work efficiently with the immunologic system to increase or decrease the host response against certain microorganisms.[117] In the immune system, macrophages are the first cells to recognize pathogens. When macrophages are activated, they can fight infectious agents directly by phagocytosis or indirectly by synthesizing and secreting nitric oxide (NO), reactive oxygen species (ROS), interleukins (IL), and tumour necrosis factor-α (TNF-α), which act as regulators of the immune system.

Lately, edible mushrooms have significantly been investigated, as there has been a considerable increase in their production and consumption due to their taste, aroma, and nutritional values, and they possess several bioactive molecules, with stimulant, therapeutic, and medicinal properties for health, which make them potential sources of pharmaceutical drugs and functional foods.[118,119] Edible mushrooms are more aptly considered nutraceutical foods, and nowadays, there is great interest in the medicinal use

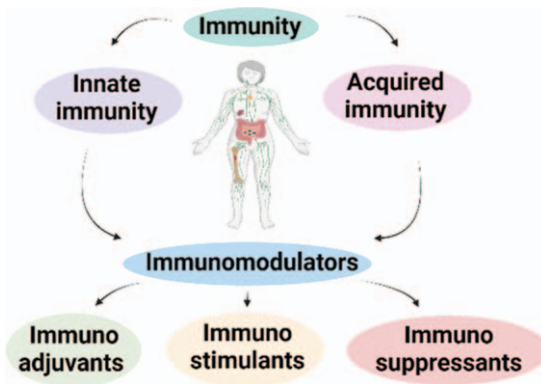


**Figure 5.4** Immunity and immunomodulator types.

of those mushrooms which have bioactive compounds with anti-inflammatory, antidiabetic, anticancer, immunomodulatory properties, among others.[120–122] Mushrooms belonging to the Basidiomycota and Ascomycota phylum stand out for their anticancer and immunomodulatory properties. These include *Pleurotus ostreatus*, *Agaricus blazei* Murrill, *Polyporus umbellatus*, *Dictyophora indusiata*, *Lentinus edodes*, *Ganoderma lucidum*, *Inonotus obliquus*, *Boletus edulis*, *Grifola frondosa*, *Calocybe indica*, *Hericium erinaceus*, *Phellinus linteus*, *Ganoderma formosanum*, *Sparassis crispa*, and *Cordyceps militaris*, among others (see Table 5.5).[118,123] The anticancer activities of mushroom polysaccharides (see Table 5.5) have been extensively studied in recent years. The activation mechanisms of these polysaccharides against cancer cells involve a complex sequence of responses, inducing both innate and adaptive immune systems.[124] Polysaccharides obtained from natural matrices possess immense beneficial properties, such as antitumour and immunomodulatory activities. They help the body resist diverse biological tensions and increase immunity against the progression of cancer cells, thus boosting immunity by stimulating the central biological systems.[125] Immunomodulatory polysaccharides have been reported to present beneficial effects on the regulation of macrophage immune function.[126,127] When activated by mushroom polysaccharides, the innate immune system stimulates the body's defence mechanisms by activating macrophages, T-lymphocytes, B-lymphocytes, cytotoxic T-lymphocytes (CTL), and natural killer cells to produce cytokines such as tumour necrosis factor-alpha (TNF-$\alpha$), interferon-gamma (IFN-$\gamma$), and interleukin 1 beta (IL-1$\beta$), and they have invariable antiproliferative activity.[124,128] Edible mushrooms have been much highlighted because of their immense benefits and bioactive compounds, and principally their immunomodulatory activity. Additionally, some species have been studied recently for their immunomodulatory and cytotoxic properties. Polysaccharides of mushrooms are found principally in the cell walls, in the fruiting bodies where they concentrate the most significant quantity,[129,130] and glycogen, cellulose, and starch are the most commonly occurring polysaccharides.[131] $\beta$-Glucans are glucose polymers linked up together through 1,3 linear $\beta$-glycosidic chains and are essential active constituents derived from mushrooms. Furthermore, depending on the source of $\beta$-glucans, variations in chain size occur, and many of these variations will determine their bioactive properties. Therefore, because of their health benefits, there has been growing interest in their use in treating disease.[127,132] Lately, edible mushrooms have significantly been investigated, as there has been a considerable increase in their production and consumption due to their taste, aroma, and nutritional values, and they possess several bioactive molecules, with stimulant, therapeutic, and medicinal properties for health, which make them potential sources of pharmaceutical drugs and functional foods.[118,119] Edible mushrooms are more aptly considered nutraceutical foods, and nowadays, there is great interest in the medicinal use of these mushrooms that have bioactive compounds with anti-inflammatory, antidiabetic, anticancer,

**Table 5.5** Immunomodulatory and cytotoxic effect of mushroom extracts and their compounds.

| Species | Compounds | Experimental models | Immunomodulatory/cytotoxic effects | Ref. |
|---|---|---|---|---|
| *A. auricula-judae* | Polysaccharide | RAW 264.7 cells | Promote the release of NO and secretion of TNF-α, IL-6, and IL-10 | 262 |
| *A. bitorquis* | Polysaccharide | RAW 264.7 cells | Promoted viability and phagocytic ability, increasing levels of NO, ROS, TNF-α | 122 |
| *A. bisporus* | Polysaccharide | SW 620 cells | Cell growth inhibition | 263 |
| *A. bisporus* and *L. edodes* | — | PC3 cells | Decreased nuclear and total NF-κB activity/ decreased the proliferation of cells | 264 |
| *A. blazei* | — | MIAPaCa-2, PCI-35, and PK-8 cells | Inhibited cell proliferation and upregulated of pro-apoptotic | 265 |
| *A. blazei, H. erinaceus*, and *G. frondosa* | — | KG1a, HL 60, Meg 01, INA-6, RPMI-8226, and U226 cells | Caused a cytotoxic and immunomodulatory effect in cells | 266 |
| *A. camphorata* | — | Mice, PLC/PRF/5, HepG2, SMMC-7721, Huh7, MHCC 97, MHCC 97L, HCCLM3, and MIHA cells | Downregulated protein levels of phosphorylated and total STAT3 and JAK2 in cells/reduced cell viability, induced apoptosis, and retarded migration and invasion in cultured cells | 267 |
| | — | SW620 and SW480 cells | Inhibitory effect on emigration and invasion of cancer cell | 268 |
| | — | B16-F0 cells | Exhibited cytotoxic effect and inhibited the migration ability of cells. | 269 |
| *A. cinnamomea* | — | Mice, LLC, and CL1-5 cells | Inhibited tumour growth and metastasis | 270 |
| *A. cinnamomea* | — | Mice and T47D cells | Inhibited cells proliferation, induced autophagy, decreased tumour volume, and inhibited tumour growth | 271 |
| *A. mellea* | Polysaccharide | RAW264.7 cells | Increases phagocytosis of macrophages and induces secretion of NO, ROS, TNF-α, IL-1β, and IL-6 | 129 |
| *B. edulis* | Phenolic and flavonoid | MCF-7 cells | Showed antiproliferative effects | 224 |
| | — | MCF-7 cells | Antiproliferative effects against the cell line | 224 |
| *C. cibarius, C. comatus, L. perlatum*, and *L. deliciousus* | — | SVGp12, U87MG, and LN-18 cells | Inhibition of proliferation and induction of apoptosis | 121 |
| *C. militaris* | Polysaccharide | RAW 264.7 cells | Release of NO, TNF-α, IL-6, and IL-10, and mainly induced M1 polarization of macrophages | 272 |

| | | | | |
|---|---|---|---|---|
| | Polysaccharide | Mice, splenic lymphocytes, RAW264.7 cells, YAC-1 lymphoma cell, and natural killer (NK) cells | Promoted the proliferation of lymphocytes and promoted lymphocyte secretion of the NO, TNF-α, and IL-2, strengthened the phagocytosis of macrophages and induced M1 polarization/ enhanced cytotoxicity of NK cells | 273 |
| *C. versicolor* | Polysaccharide | RAW 264.7 cells | Induction of NO production and induced nitric oxide synthase (iNOS) and TNF-αmRNA expression level | 274 |
| *C. radicata* | Polysaccharide | RAW 264.7 cells | Improved the proliferation and phagocytosis and induced the secretion of NO, iNOS, TNF-α, IFN-γ, IL-6, IL-1β and IL-10 | 275 |
| *C. versicolor* and *L. edodes* | Polysaccharide | Peripheral blood mononuclear cells | Reduction of Th2 cytokines and IL-10 in cells cultures | 128 |
| *F. velutipes* | Polysaccharide | RAW 264.7 cells | Regulate the expression of NO, TNF-α, IL-6 | 276 |
| *G. frondosa* | Polysaccharide | Mice and H22 hepatoma cells | Increasing the percentage of $CD4^+CD8^+$ T cells, B cells, and Treg cells, and the secretion of IL-2, IL-12p70, TNF-α, and IFN-γ/inhibit liver tumours growth | 134 |
| *G. lucidum* | Polysaccharide | Mice, MDA-MB-231, and 4T1 cells | Significantly suppressed the growth of tumours | 277 |
| | Polysaccharide | Mice | Produced better protection of the spleen and thymus, promoting haematopoiesis, and improving IgA levels in serum | 278 |
| | Polysaccharide | GBM8901, U87, MDA-MB-231, and 4T1 cells | Inhibited the growth of cancer cells, inducing apoptosis, inhibited cell migration and cell cycle arrest | 277 and 279 |
| | Polysaccharide | Mice | Protect spleen and thymus and promoting haematopoiesis, and improve IgA levels | 278 |
| *G. neo-japonicum* | Terpenoid and sterol | CCD-18Co, HCT 116, and HT 29 cells | Exerted a potent cytotoxic effect on colonic carcinoma cells | 280 |
| *G. applanatum* | — | SEC and Caco-2 cell | Decrease in the volume of the tumour mass and tumour growth | 281 |
| *G. atrum* | Polysaccharide | T lymphocytes | Increased the secretion of IL-2, IFN-γ, and IL-12 | 282 |
| *G. leucocontextum* | Polysaccharide | RAW 264.7 cells | Activating mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K)/ Akt, and nuclear factor-kappa B (NF-κB) signalling pathways | 283 |

**Table 5.5** (*Continued*)

| Species | Compounds | Experimental models | Immunomodulatory/cytotoxic effects | Ref. |
|---|---|---|---|---|
| *G. tsugae* | — | AN3 CA, HEC-1-A and KLE cells | Inhibited cell proliferation, induced cell cycle arrest, and inhibited the Akt signalling pathway | 284 |
| *G. frondosa* | Polysaccharide | Mice | Improve or reverse the CTX-induced immunosuppression, enhance the spleen and thymus indices, spleen lymphocyte proliferation and cytokine production | 285 |
| | — | SMMC-7721 cells | Reduction of cell viability and induction of cell apoptosis | 286 |
| | Polysaccharide | RAW 264.7 cells | Increased TNF-α, IL-6, IFN-γ, MIP-1β, MIP-1α, and MIP-2 levels | 287 |
| *G. lucidum* | Polysaccharides and triterpenes | Mice, SNU719, and MKN1-EBV cells | Induced lytic reactivation of the Epstein–Barr virus – risk factor of gastric cancer/induced cancer cell apoptosis | 288 |
| | Polysaccharide | Rats/ RG2 glioma cells and human myelogenous leukaemia cells K562 | Increase the concentration of serum IL-2, TNF-α, and INF-γ/enhance the cytotoxic activity of NK cells and T cells in glioma-bearing rats. | 289 |
| | — | Mice, HEK293T, and HEK293FT Cells, MDA-MB-231 cell, and 4T1 cell | Inhibited Wnt-induced hyperproliferation of cancer cells | 290 |
| | Triterpenoids | Mice and HCT16 cells | Inhibited cell migration, proliferation, and xenograft tumour growth | 291 |
| *H. erinaceus* | Polysaccharide | Human monocytic cell lines THP-1 | Enhance the levels of TNF-α, IL-1β, and IL-6 and induce the proliferation of lymphocytes | 292 |
| *I. baumii* | — | SMMC-7721 cells | Induce apoptosis and autophagy in tumours cells, and inhibit the growth of tumours | 293 |
| *I. obliquus* and *P. linteus* | — | Mice, yeast, RAW264.7, A2780, HeLa, AML1, S18, and CL-81 cells | Enhanced the phagocytosis of macrophages/ inhibitory effect on tumour development and progression | 294 |
| *L. edodes* | Polysaccharide | Mice and MCF-7 cells | Induction of apoptosis | 295 |
| | — | Hep-2 and MRC-5 cells | Inhibition of cell proliferation and induced depolarization of mitochondria | 296 |
| *L. volemus* | Polysaccharide | RAW 264.7, H1299, and MCF-7 cells | Induced the secretion of NO, IL-6, and TNF-α/inhibit the proliferation of cells | 136 |

| | | | | |
|---|---|---|---|---|
| *L. tigris* | — | Mice and MCF-7, A549, PC3, and 184B5 cells | Show antiproliferative effect and inhibited the growth of cells | 297 |
| *M. importuna* | Polysaccharide | RAW 264.7 and 4T1 cells | Promote the secretion of TNF-α, IL-6, and NO and enhance the phagocytosis of macrophages/ enhance the antitumour activity of doxorubicin (DOX) and inhibit the growth of tumours | 298 and 299 |
| *M. oreades* | — | MCF-7, MDA-MB-231, and HT-29 cells | Inhibited all human cancer cell lines | 300 |
| *M. giganteus* | — | Jurkat and HL-60 cells | Induced apoptosis in Jurkat and HL-60 cell and induced cell-cycle arrest | 301 |
| *M. esculenta* | Fatty acids and sterols | A549, H1264, H1299, and Calu-6 cells | Induction of apoptosis | 302 |
| *P. highking* | — | MCF-7 cells | Reduced the proliferation and viability of cells and reduced size and number of the tumour spheres | 303 |
| *P. nameko* | Polysaccharide | Mice and MCF-7 cells | Activated the death receptor pathway and mitochondrial apoptosis pathway/inhibited malignant proliferation of tumours | 304 |
| *P. eryngii* | Polysaccharide | THP-1 cells | Increased the secretion of IL-1β, IL-10, and NO | 305 |
| | Protein | Mice, CCD-18Co, HCT116, and MC38 cells | Suppressed tumour development and proliferation-induced cell cycle arrest and led to cellular apoptosis | 306 |
| | Polysaccharide | Mice | Increased concentrations of TNF-α, IFN-γ, IL-1, IL-2, and IL-6 | 307 |
| *P. highking* | — | MDA-MB-231 and HCC-1937 cells | Reduced the number and size of the tumour-spheres, suppressed the migratory ability of the cells, and reduced the mRNA expression of Ki-67, MMP-9, and vimentin in the treated tumour-sphere cells | 308 |
| *P. ostreatus* | — | KG-1 and Jurkat cells | Inhibited cell proliferation, migration, expression, and promoted apoptosis | 309 |
| *P. ostreatus* | — | KG-1 cells | Induced apoptosis and increased anticancer effects | 310 |
| *P. ferulae* | — | Mice, H22 and HepG2 cells | Inhibited the growth of cells, increased ROS generation, and suppressed migration | 311 |
| *P. sajor-caju* | — | HCT116 and MRC-5 cells | Induced apoptosis and cell cycle arrest | 312 |

**Table 5.5** (*Continued*)

| Species | Compounds | Experimental models | Immunomodulatory/cytotoxic effects | Ref. |
|---|---|---|---|---|
| *P. grammocephalus* | Polysaccharide | Mice and RAW 264.7 cells | Activation of macrophage and splenocyte and thymocyte stimulatory properties | 120 |
| *P. rhinocerus* | Polysaccharide | Murine immature BMDCs | Induced BMDC maturation, upregulated the expression of membrane phenotypic marker, stimulated the release of MIP-1α, MIP-2, and IL-2, and upregulation of the expression of IL-2, IL-6, MIP-1α, MIP-2, RANTES, IL-12p40p70, IL-12p70, TIMP-1, IFN-γ, KC, MCP-1, and GCSF | 313 |
| *S. imbricatus* | — | Mice and MDA-MB-231, MDA-MB-468, MCF-7, MT-1, and 4T1 cells | Inhibited the proliferation and invasive properties of breast cancer cells and decreased the tumour volume and weight in mice | 314 |
| *S. commune* | Polysaccharide | RAW264.7 cells | Produced NO and cytokines by upregulating mRNA expression levels | 315 |
| *T. lobayense* | Polysaccharide | RAW264.7 cells | Enhanced the phagocytic uptake capacity, increased NO production, increased secretion of IL-6 and TNF-α | 316 |
| *T. versicolor* | Polysaccharide | Hela cells | Induction of cell death by apoptosis | 317 |
| *T. heimii* | Polysaccharide | Mice, Vero, and HCT cells | Diminished hyperplasia in 1,2-dimethylhydrazine (DMH)-induced colon cancer | 318 |

immunomodulatory properties, among others.[120–122] The polysaccharide from *Pleurotus citrinopileatus* revealed antitumor activity on H22 tumour-bearing mice.[133] *Coprinus comatus* and *Lactarius deliciosus* showed an inhibitory effect on U87MG and LN-18 glioblastoma cells viability.[121] *Grifola frondosa* contains promising immunomodulatory agents and effective antitumour polysaccharides.[134] The polysaccharide of *Agaricus bitorquis* shows an immunomodulatory effect on RAW264.7.[122] *Pholiota nameko* induces apoptosis of human breast adenocarcinoma.[135] Polysaccharides from *Lactarius volemus* were investigated for antiproliferative and immunomodulatory activities on RAW264.7 murine macrophages, human lung cancer cells H1299, and human breast cancer cells MCF-7.[136] More studies involving mushrooms and their immunomodulatory and cytotoxic properties are presented in Table 5.5.

### 5.3.5 Cardioprotective Properties

Cardiovascular diseases (CVD) are among the leading cause of death in the world, afflicting the heart and blood vessels. They include high blood pressure, coronary heart disease, cerebrovascular disease, atherosclerosis, heart failure, and peripheral vascular disease.[137] Diets rich in functional ingredients such as antioxidants, dietary fibre, omega-3 polyunsaturated fatty acids (PUFAs), vitamins, minerals, and phenolic compounds have been associated with a lower risk of cardiovascular disease in numerous studies.[138,139] Cholesterol has long been considered a significant risk factor for heart diseases. Plant sterols, also known as phytosterols, are found in several plant species and have been shown to compete with dietary cholesterol by inhibiting absorption and enabling its elimination from the body.[137] Mycosterols including ergosterol (ergosta-5,7,22-trien-3*b*-ol), ergosta-5,8,22-trien-3-ol, ergosta-7,22-dien-3-ol, ergosta-5,7-dien-3-olm and fungisterol (ergosta-7-en-3-ol) either occurring in their free or esterified forms have gained prominence in recent years due to their well-reported beneficial effects on human health.[140,141] Because of their structural similarities to the dietary mixed micelles (DMM) during *in vitro* digestion, these biomolecules have a cholesterol-lowering impact by competing with cholesterol. In simulated gastrointestinal digestion utilizing Caco-2 cells, ergosterol-rich extracts produced by supercritical fluid extraction (SFE) from *Agaricus bisporus* were found to successfully displace cholesterol (up to 67%) from the DMM.[142] The extract mentioned above's precise mechanism of action was linked to a significant reduction in mRNA expression of sterol regulatory element-binding protein 2 (SREBF2), a protein-coding gene that regulates the expression of numerous genes involved in cholesterol homeostasis, absorption, and biosynthesis.[139] A diet supplemented with an antioxidant-rich extract from *A. brasiliensis* was reported to significantly reduce the total cholesterol level *in vivo* and downregulate mRNA expression of cholesterol metabolism genes, including SREBP-2, HMG-CoA reductase, LDLR, and PPAR-$\alpha$.[143] The effect of ergosterol peroxide derived from *Ganoderma lucidum*

on SREBP-1 mRNA expression in 3T3-L1 preadipocyte cells has also been described.[144] The cholesterol-lowering effect of lard functionalized with extracts from *P. ostreatus* and *L. edodes* rich in β-glucans, water-soluble polysaccharides, and ergosterol was analysed.[138] A significant reduction (up to 22 – 42%) in plasma levels of total cholesterol was observed in the studied *in vivo* model. However, mRNA expressions of cholesterol-related genes (NPC1L1 and ABCG5) in the jejunum, cecum, and liver were not suppressed. Although the precise molecular mechanism is still not completely defined, the cardioprotective effect was associated with hindering the bioavailability of cholesterol and inhibiting the expression of several genes responsible for cholesterol absorption and metabolism in intestinal models.[143,144]

Atherosclerosis is a multifaceted disease that contributes to the pathogenesis of CVDs, characterized by endothelial dysfunction, lipid deposition, inflammatory cell infiltration, smooth muscle cell proliferation, intravascular wall neoangiogenesis, and plaque development.[145] Although the exact cause of atherosclerosis is unknown, persistent inflammation of the artery walls triggered by procoagulant proteins, proinflammatory cytokines, metalloproteinases, and growth factors have all been identified as major contributions to the disease's aetiology.[145,146] Commonly consumed mushrooms, such as *A. bisporus*, *L. edodes*, and *P. ostreatus*, abundant in nutrients, soluble dietary fibres, and several health-promoting bioactive compounds, are beneficial in suppressing inflammation, improving lipid profiles, and ameliorating heart disease and atherosclerosis.[146] The potential of *A. bisporus* (portobello) and *L. edodes* extracts rich in ergothioneine in ameliorating atherosclerosis was conducted *in vivo*. The results showed a significant decrease in plasma lipid concentrations (cholesterol and triglycerides), mRNA expression of vascular cell adhesion molecule 1 (VCAM-1), and mean aortic lesion area.[146] *In vivo* studies involving the infusion of angiotensin II in a mouse model and subsequent supplementation with a diet rich in *Grifola gargal* extract presented a significant decrease in the weight of the heart, vessels, and atheromatous lesions. In addition, the extract also induces the secretion of transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF), both vital signalling molecules responsible for promoting angiogenesis.[147] Several studies have shown that mushroom extracts and their associated bioactive molecules possess antioxidant, anti-inflammatory, and lipid-lowering effects that can potentially slow down the progression of atherosclerosis, thereby potentiating a cardioprotective effect.[148] The mechanism behind these cardioprotective properties of mushroom extracts remains obscure but it has been attributed to suppression of mRNA expression of several genes related to cholesterol absorption and metabolism, decreased oxidative damage due to their potent radical scavenging and metal chelating effect, and downregulation of the expression of inflammatory mediators.[143,146] To the best of our knowledge, no studies have been conducted in clinical settings to evidence the high therapeutic potential of these mushroom metabolites against any cardiovascular diseases. Hence, more work still needs to be performed to

determine these bioactive ingredients' clinical effectiveness and potential toxicity effect.

### 5.3.6 Antidiabetic Properties

Diabetes mellitus (DM) is a chronic metabolic disease, and millions of people worldwide suffer from this disease and its complications, with its predominance constantly increasing. DM is one of the most common life-threatening endocrine diseases caused by defects in insulin secretion, insulin resistance, or both.[149,150] There are different types of DM: type 1, earlier recognized as insulin-dependent DM, where hormonal insulin is not produced due to damage to pancreatic β-cells, and people with this type of DM need daily insulin administration;[151,152] type 2 diabetes, previously known as non-insulin-dependent DM, is more predominant and is recognized by a progressive reduction in insulin secretion by pancreatic β-cells and a relative diminution in the sensitivity of target tissues to the action of this hormone. Type 2 DM can be prevented or delayed by lifestyle intervention.[153] Other forms of DM include gestational DM, which occurs due to any degree of glucose intolerance with it beginning or first being recognized during pregnancy;[154] other types of DM can also be induced by genetic defects, endocrine disorders, other types of diseases, therapy, and malnutrition.[155]

DM is an irreversible disease with no cure, however glycaemic control is fundamental for both the prevention of diabetic complications and the reduction of mortality. In uncontrolled DM, hyperglycaemia is a common occurrence and eventually leads to infections, serious injuries, dysfunction, and multiple organ failure, particularly in the heart, kidneys, eye, blood vessels, and nerves.[156,157] Depending on the nature of DM, insulin and other synthetic medicines are widely used in its treatment. However, with prolonged medicine administration, some insulin resistance and side effects cases may develop, which leads to the search for more natural, safe, and efficient alternatives.[158,159]

The treatment of DM and its chronic and vascular complications have a significant economic impact on people's lives and the wider economy. However, the best weapon is the prevention of DM, focusing on avoiding or delaying its onset and complications with dietary control and exercise.[151] Thus, with concern to treating this disease, the key is in identifying the individually targeted factors that can lead to better glycaemic control. In addition to regular oral and injectable medications, natural product-based therapy is highly indicated because of its efficiency, few adverse effects, and low cost. Thus, due to the benefits of current antidiabetic medicines, the acceptance and use of these natural products are increasing dramatically.[149,160]

Natural products, including mushrooms, are of great help in managing DM[152,161] (see Figure 5.5). They have been reported to potentially control DM by reducing oxidative stress, mimicking insulin action, increasing insulin secretion and activity, regulating glucose transport type 4, glucose uptake,

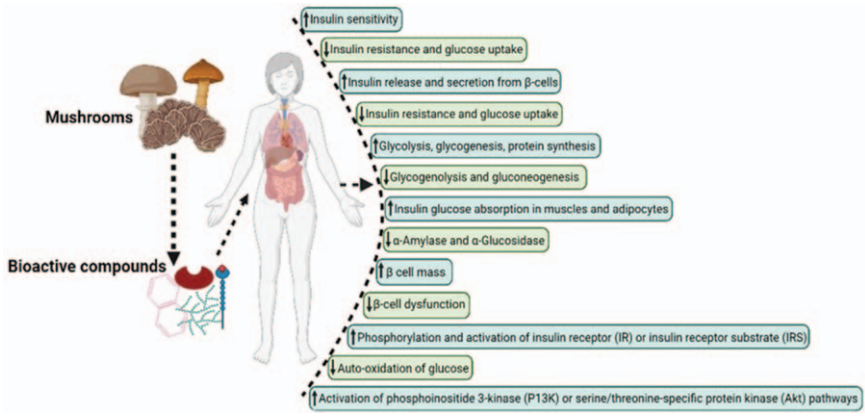


**Figure 5.5** Favourable effects of mushroom bioactive compounds on DM.

inhibiting α-amylase and α-glucosidase, and protecting pancreatic β-cells.[153,162] Over the years, several bioactive compounds have been isolated from mushrooms (see Table 5.6), such as polysaccharides, dietary fibre, and other compounds of therapeutic interest such as lectins, lactones, terpenoids, alkaloids, metal chelating agents, and also several enzymes such as laccase, superoxide dismutase, glucose oxidase, and peroxidase, with favourable effects on glucose homeostasis[153,159,163] (see Figure 5.5). In addition, mushrooms have been prestigious as a source of these compounds and have been highly valued as potent sources of antidiabetic and hypoglycaemic compounds, promising in treating diabetes as biological antihyperglycaemic agents (see Table 5.6).[162]

Several species of mushrooms are known for their hypoglycaemic or antidiabetic activities (see Table 5.6), including *Agaricus bisporus*,[164] *Agaricus subrufescens*,[165] *Auricularia auricular*,[166,167] *Antrodia cinnanomea*,[168] *Coprinus comatus*,[169] *Cordyceps militaris*,[170] *Ganoderma lucidum*,[171] *Grifola frondosa*,[172] *Inonotus obliquus*,[173] *Lentinus edodes*,[174] *Pleurotus ostreatus*,[175] *Pleurotus eryngii*,[176] *Pleurotus citrinopileatus*,[177] *Pleurotus pulmonarius*,[178] and *Wolfiporia extensa*.[179] The bioactive compounds in mushrooms have significantly contributed to regulating and managing various diseases, including DM. Thus, the various mushrooms presenting health benefits provide an opportunity to plan and develop new formulations and therapies for DM.[153] However, extensive studies are needed to investigate the untapped mushrooms and extract and purify antidiabetic biomolecules that can be used to treat and manage DM.

### 5.3.7 Anti-ageing Properties

Skin ageing is a complex biological process that is induced through two independent intrinsic and extrinsic routes, responsible for the progressive loss of structural integrity, elasticity, and physiological functions of the

**Table 5.6** Antidiabetic effect of mushroom extracts on rodents.

| Samples | Parts/components | Antidiabetic effect | Ref. |
|---|---|---|---|
| *A. auricula* and *A. polytricha* | Polysaccharides | Reduction of fasting blood glucose, promotion of insulin synthesis, and reduction of the level of plasma glucose | 167 |
| *A. auricular* | Polysaccharides | Reduced HbA1 serum levels | 319 |
| | Dried fruiting body/ polysaccharides | Reduction of blood glucose level | 166 |
| *A. bisponus* and *P. ostreatus* | Powder | Decrease in serum glucose level | 150 |
| *A. bisporus* | Ethanol and methanol extract | Decrease in the levels of glucose | 164 |
| *A. cinnamomea* | Mycelium powder | Decreased plasma glucose levels and improved insulin resistance | 168 |
| *C. militaris* | Acidic-extractable polysaccharides | Decreased blood glucose, improved glucose and insulin resistance, enhanced antioxidant enzyme activities, and attenuated injuries of the liver, kidney, and pancreas | 320 |
| *C. comatus* | Ethanol extract | Reduced the blood glucose and glycosylated haemoglobin (HbA1c) levels and increased the plasma insulin level | 169 |
| *C. gigantea* | Methanol extract | Reduced the blood glucose level | 321 |
| *C. ventricosum* | Mycelial polysaccharides | Decrease blood glucose levels | 322 |
| *C. rutilus* | Ethanol extract | Decrease blood glucose concentration | 323 |
| *G. frondosa* | Polysaccharide-chromium(III) | Regulated the mRNA expression related to glucose | 172 |
| | Polysaccharides | Regulated mRNA expression levels of the genes responsible for hepatic glucose metabolism | 324 |
| | Polysaccharides | Decrease in fasting blood glucose levels and reduction of the inflammatory factor content, and prevented renal fibrosis | 325 |
| | Polysaccharides | Decreased fasting blood glucose levels and glucose tolerance | 326 |
| | Mycelium/polysaccharides | Reduced fasting blood glucose levels | 327 |

**Table 5.6** (*Continued*)

| Samples | Parts/components | Antidiabetic effect | Ref. |
|---|---|---|---|
| | Extracts | Improved glucose intolerance | 328 |
| | Heteropolysaccharide | Improved blood glucose levels and glucose intolerance | 329 |
| | Polysaccharides | Decreased the fasting blood glucose level, improved oral glucose tolerance, alleviated insulin resistance, and protected against liver and kidney injury | 330 |
| *G. lucidum* | Polysaccharides | Decreased the levels of fasting blood glucose and insulin | 331 |
| | Spores | Reduced the blood glucose and oxidative stress | 171 |
| | Beta-glucans | Decreased blood glucose levels | 332 |
| | Ethanol extract | Decreased blood glucose level, increased insulin level, and decreased HbA1c level | 333 |
| *G. pfeifferi* and *G. resinaceum* | Aqueous and ethanol extracts/ polysaccharides | Prevented significant increase in glycaemia values | 334 |
| *I. obliquus* | Polysaccharides | Decreased fasting glucose level, an improvement in the liver, kidney, pancreas, glucose tolerance capacity, and increased hepatic and muscle glycogen | 173 |
| *I. obliquus* | Polysaccharides | Reduced fasting blood glucose levels, improved glucose tolerance ability, increased hepatic glycogen level, and ameliorated insulin resistance | 335 |
| *L. edodes* | Powder | Increased maternal insulin levels | 336 |
| *L. edodes* | β-Glucans extract | Reduced blood glucose levels | 174 |
| *L. edodes* | Freeze-dried powder | Improved the glucose tolerance, potentiating insulin secretion, and reduced insulin resistance | 337 |
| *L. rhinocerotis* | Freeze-dried powder | Reduced elevated blood glucose concentrations | 338 |

| | | | |
|---|---|---|---|
| *M. conica* | Methanol extract | Reduced elevated blood glucose and improved liver and kidney damages | 339 |
| *P. ostreatus* | Ergosterol | Improved insulin resistance, reduced fasting blood glucose levels, and protected pancreas and liver | 340 |
| | Powder | Reduction of the serum glucose level | 341 |
| *P. ostreatus* and *L. subnudus* | Dried mushrooms | Reduction of fasting blood glucose, the activity of α-amylase, α-glucosidase, and angiotensin-1 converting enzyme | 175 |
| *P. cocos* | Gold nanoparticles | Reduced blood glucose status | 179 |
| *P. linteus* | Extract/polysaccharides | Improves insulin resistance | 342 |
| *P. pulmonarius* | Mycelia | Reduced blood glucose levels, decreased injuries in the pancreas, liver, and kidney | 178 |
| *P. eryngii* | Polysaccharides | Showed renoprotective effects, decreased glucose levels, and improved antioxidant status | 343 |
| *P. citrinopileatus* | Aqueous extract | Inhibitory activities on α-amylase, α-glucosidase | 344 |
| *P. tuberregium* | Aqueous extract | Reduced plasma sodium and raised plasma potassium levels and had no deleterious effects on the liver and kidney | 345 |
| *S. luridus* | Polysaccharides | Decreased blood glucose levels and attenuated kidney and liver injures | 346 |
| | Ethanol extract | Decreased blood glucose levels | 347 |
| *T. melanosporum* | Aqueous extract | Reduced glucose levels and attenuated oxidative stress | 348 |

skin.[180] Ultraviolet radiation is the main environmental mutagen responsible for the oxidative damage to cellular components such as cell walls, lipid membranes, mitochondria, and skin DNA by generating free radicals. These free radicals (reactive oxygen species) activate many skin disease-related enzymes, such as collagenase, elastase, hyaluronidase, tyrosinase, and xanthine oxidase, and increase the expression of matrix metalloproteinases (MMPs) responsible for degrading the different components of the extracellular matrix (collagen, elastin, fibronectin, and hyaluronic acid), thus resulting in skin damage and premature skin ageing.[181] These ECM components are responsible for accelerating wound healing, contributing to mechanical resilience and maintaining the structural integrity of the skin.[182] Biomolecules with strong free radical scavenging and key enzyme inhibitory effects are vital ingredients in developing nutraceutical or cosmeceutical formulations to prevent skin ageing.[183] Numerous extracts and compounds obtained from edible and medicinal mushrooms, such as *Antrodia camphorata*, *Auricularia auricula-judae*, *Ganoderma lucidum*, *Inonotus obliquus*, *Lentinula edodes*, *Phellinus vaninii*, *Pholiota nameko*, *Piptoporus betulinus*, *Pleurotus citrinopileatus*, *Russula capensis*, *Schizophyllum commune*, *Tricholoma magnivelare*, and *Tricholoma matsutake*, have been widely investigated and found to possess strong antioxidant, anticollagenase, antihyaluronidase, antielastase, wound healing, anti-inflammatory, antimicrobial, antityrosinase, and photoprotective effects against UV-B and UV-A radiation as shown in Table 5.7. These biological activities are associated with major cell signal transduction pathways related to ageing, inflammation, hyperpigmentation, and extracellular matrix degradation (see Table 5.7).[184,185]

Inhibition of enzymes such as collagenase, hyaluronidase, and elastase has been associated with maintaining the skin's structural integrity. Fractions prepared from *P. citrinopileatus* (*n*-butanol, aqueous, and methanol extract) showed up to 9.7%, 10.8%, and 25.4% hyaluronidase inhibition activity, respectively.[186] In comparison, an aqueous and acetonic extract prepared from the mycelial suspension of *Trametes lactinea* presented 88% antihyaluronidase activity at 100 $\mu g\,mL^{-1}$.[187] Aqueous extracts obtained from *P. ostreatus*, *G. lucidum*, and *Auricularia polytricha* presented over 50% inhibition of hyaluronidase enzyme.[188] To the authors' best knowledge, only these findings have been reported so far on the hyaluronidase inhibition activity of mushroom extracts or their individual compounds. Polysaccharide-rich extracts obtained from *Auricularia auricula-judae*, *G. lucidum*, and *Pholiota nameko* have been reported to significantly inhibit collagenase enzyme activity and promote collagen synthesis in a fibroblast cell model.[189–191] Extracts obtained from different mushrooms have also been reported to present a potent elastase inhibitory effect, as shown in Table 5.7. MMPs are specialized enzymes produced by epithelial cells, fibroblasts, and inflammatory cells and are associated with degrading the ECM, collagen proteolysis, and contribute to other destructive processes such as tumour invasion and inflammation.[192] The studies presented in Table 5.7 showed

**Table 5.7** Anti-ageing effect of mushroom extracts and their individual compounds.

| Species | Bioactive form | Bioactive property | Key findings and suggested mechanism of action | Ref. |
|---|---|---|---|---|
| *A. camphorata* | Extract | Wound healing | – Cell viability of fibroblast cells was maintained at 100 $\mu g\,mL^{-1}$<br>– Closure of 60% was observed in the wound healing scratch assay | 349 |
| *A. auricula-judae* | Water-soluble polysaccharide-rich extract | Wound healing and collagen synthesis | – Fibroblast and keratinocyte cell migration and proliferation were promoted at 20 $\mu g\,mL^{-1}$<br>– Collagen synthesis increased up to 40% at 25 $\mu g\,mL^{-1}$<br>– Wound closure was accelerated in mice wound-healing model | 189 |
| *G. lucidum* | Crude polysaccharide | Antityrosinase, antielastase, and anticollagenase | – Extract effectively inhibited tyrosinase, elastase, and collagenase up to 50% at 0.37, 1.01, and 0.49 $mg\,mL^{-1}$, respectively | 190 |
| | Ethanolic extract | Wound healing | – Cell viability of keratinocyte cells was maintained at 640 $\mu g\,mL^{-1}$<br>– At 10 $\mu g\,mL^{-1}$, significant improvement in the cell migration capacity was observed in the scratch assay | 205 |
| | Cold water and hot water extract | Antityrosinase and antihyaluronidase | – Tyrosinase inhibition activity reached 45%<br>– Up to 72% inhibition of hyaluronidase was observed | 188 |
| *I. obliquus* | Triterpenoid-rich fractions | Antityrosinase and melanin inhibition | – Petroleum ether fraction (3.81 $\mu g\,mL^{-1}$) showed a stronger tyrosinase inhibitory effect than kojic acid (5.23 $\mu g\,mL^{-1}$)<br>– Fractions presented little or no cytotoxicity on B16 melanoma cells<br>– Cellular tyrosinase activity and melanin content were inhibited in α-MSH-stimulated B16 melanoma cells | 195 |

**Table 5.7** (*Continued*)

| Species | Bioactive form | Bioactive property | Key findings and suggested mechanism of action | Ref. |
|---|---|---|---|---|
| *L. edodes* | Hydroethanolic extract | MMP inhibition assay | – Cell viability was maintained in HaCaT keratinocyte cells at 50 $\mu g\,mL^{-1}$<br>– The expressions of MMP-1 and MMP-9 were significantly inhibited<br>– The expression of type I procollagen in UVA and UVB-irradiated HaCaT keratinocytes was increased | 193 |
| *P. vaninii* | Methanolic extract | Antityrosinase, antielastase, and anticollagenase | – The cell viability of B16-F10 murine melanoma cells was maintained up to 85% at 750 $\mu g\,mL^{-1}$<br>– The tyrosinase inhibitory activity ranged from 55.83% to 96.16% at 0.125–2.0 $mg\,mL^{-1}$<br>– Melanin synthesis was suppressed (71.18% to 27.61%) in B16-F10 melanoma cells at 25–500 $\mu g\,mL^{-1}$<br>– Elastase and collagenase were inhibited up to 71.24 and 63.02% at 2.0 $mg\,mL^{-1}$, respectively | 200 |
| *P. nameko* | Polysaccharides | Anticollagenase and wound healing | – Collagenase was inhibited up to 61% at 500 $\mu g\,mL^{-1}$<br>– Cell migration and proliferation in L929 mouse fibroblast cells were maintained<br>– A significant increase in closure rate (54.75%) was observed in the wound-healing scratch assay | 191 |
| *P. betulinus* | Water-soluble β-D-glucan | Wound healing | – Cell viability was maintained at 1000 $\mu g\,mL^{-1}$<br>– Cell migration was enhanced presenting a closure of 55% in the wound-healing scratch assay | 207 |
| *P. citrinopileatus* | Methanol extract | Antityrosinase, antihyaluronidase, | – Cell viability of B16 melanoma cells was maintained at 100 $\mu g\,mL^{-1}$ | 186 |

| | | | | |
|---|---|---|---|---|
| | | and melanin inhibition | – The tyrosinase inhibitory activity ranged from 2.5% to 10.5% at 0.125–2.0 mg $mL^{-1}$<br>– Melanin content was suppressed in B16 melanoma cells<br>– Hyaluronidase enzyme was inhibited up to 25% at 100 μg $mL^{-1}$ | |
| *R. capensis* | Aqueous and methanolic extract | Anticollagenase and wound healing | – Extract (100 μg $mL^{-1}$) mildly inhibits dermal fibroblast proliferation and significantly improved cell migration<br>– Extract inhibited collagenase up to 20% at 200 μg $mL^{-1}$<br>– Fibroblast migration in MRHF cells was enhanced and up to 40% wound closure rate was observed | 204 |
| *S. commune* | Cold water extract | Antityrosinase and antihyaluronidase | – Tyrosinase inhibition activity reached 98% comparable to kojic acid (94.4%)<br>– Up to 40% inhibition of hyaluronidase was observed | 188 |
| *T. fuciformis* | Polysaccharides | Collagen synthesis | Collagen levels were elevated in UVA- and UVB-exposed animal models | 350 |
| *T. magnivelare* | Methyl cinnamate | Antityrosinase and melanin inhibition | – Cell viability of B16-F10 melanoma cells was maintained<br>– Total melanin production was significantly suppressed in a concentration-dependent manner | 201 |
| *T. matsutake* | Mycelial extract | Antielastase | – Extract presented potent elastase inhibitory effect (81.4 ± 3.92%) similar to phophoramidon (89.6 ± 7.74%) used as positive control<br>– Cell viability of human fibroblasts was maintained at 100 μg $mL^{-1}$<br>– mRNA expressions of MMP-1 and MMP-3 in fibroblast treated cells were decreased in a dose-independent manner | 192 |

that mushroom extracts and their associated metabolites present potential anti-ageing effects by inhibiting collagenase, elastase, and hyaluronidase. The anti-elastase activity has been attributed to downregulation of mRNA expression of MMPs (MMP-1, MMP-3, and MMP-9) and increased expression of type 1 procollagen in UVA- and UVB-irradiated keratinocyte cells.[192,193] Hence, more work is still needed to determine the mechanism behind the anticollagenase potential of these mushroom extracts or their individual compounds.

Tyrosinase is the key enzyme involved in the synthesis of melanin. Melanin production is crucial for preventing UV-induced skin damage, and the overproduction of this pigment has resulted in hyperpigmentation.[194] Bioactive ingredients with the capacity to bind to the active site of the tyrosinase enzyme and cause enzyme inactivation have become the most prominent and successful targets for melanogenesis inhibitors. Besides the competitive and non-competitive inhibition potential against tyrosinase, mushroom extracts have also been reported to decrease melanocyte metabolism, resulting in reduced melanin synthesis. Fractions from *Inonotus obliquus* containing betulin, trametenolic acid, inotodiol, and lanoserol are reported to present very little or no cytotoxic effect in B16 melanoma cells.[195] The extract reduced cellular tyrosinase activity and significantly lowered melanin content in α-MSH-stimulated B16 melanoma cells. Several extracts obtained from medicinal and edible mushrooms, as shown in Table 5.7, are potent inhibitors of tyrosinase enzyme, being also effective in suppressing melanin production in cultured melanocytes and other *in vitro* models. These findings make these extracts potential ingredients to develop formulations against diseases resulting from aberrant melanin production. Most tyrosinase inhibition activities currently reported have also presented incomparable/irreproducible $IC_{50}$ values due to varied assay conditions, including using different substrates (L-dihydroxyphenylalanine and tyrosine) and substrate concentrations, various incubation times, and the use of other assay ingredients. Cosmetics or skin-lightening formulations commercially available contain hydroquinone, kojic acid, arbutin, azelaic acid, ellagic acid, and resveratrol.[196] Despite being widely used, these compounds have been reported to alter melanosome formation, promote their destruction, and present unsatisfactory clinical efficacy.[197] The lack of efficacy of the available tyrosinase-targeting compounds might be associated with most studies being conducted exclusively using tyrosinase isolated from the mushroom *Agaricus bisporus* (mushTYR).[198] In the past, mushTYR was the only commercially available enzyme, but recent studies have shown that its catalytic activities and substrate specificities are significantly different from human tyrosinase (humTYR). Very few studies have been published on humTYR inhibitors, with thiamidol being one of the most promising candidates.[199] To the best of our knowledge, most tyrosinase inhibition properties of mushrooms or their associated metabolites heavily relied on enzymatic assays performed using mushroom tyrosinase (mushTYR),[190,200,201] mainly because there were no available humTYR-based assays

and mushTYR assays are relatively inexpensive. Hence, with the recent advances in humTYR structural information,[199,202] more work needs to be carried out to find bioactive ingredients from mushrooms with the best humTYR and melanin inhibition potential to develop effective and safe cosmeceutical and nutricosmetic formulations. All signalling pathways associated with melanogenesis are controlled by the master regulator, microphthalmia-associated transcription factor (MITF), a melanocyte-specific transcription factor that is responsible for differentiation of melanocyte cells that modulates the expression of tyrosinase and tyrosinase-related proteins (TRP-1 and TRP-2).[203] Hence, most tyrosinase-inhibiting bioactives tend to target the downregulation of MITF expression to inhibit the whole process of melanogenesis, and as such more targeting of the expression of these melanogenesis-related proteins, including MITF, TRP-1, and TRP-2 should be conducted.

The wound-healing effect of mushrooms has been one of the most studied beneficial effects, and is well described in different scientific findings.[204,205] The wound-healing process is characterized by a complex sequence of cellular and molecular events involving inflammation, fibroplasia, neovascularization, collagen deposition, epithelialization, and wound contraction.[189] Most mushroom extracts and their compounds have been investigated (see Table 5.7), targeting relevant wound repair mechanisms such as cells (fibroblasts and keratinocyte) proliferation and migration, inhibition of reactive oxygen species (ROS) production, modulation of inflammatory mediators secretion, and deposition of ECM components (collagen and hyaluronic acid).[189,204–207] The wound-healing potential of most mushroom species has been associated with their rich content in polysaccharides such as water-soluble β-D-glucan from *P. betulinus*, a low-molecular-weight polysaccharide from *P. nameko*, and water-soluble polysaccharide from *A. auricula-judae*.[189,191,207] However, their related mechanism of action has not been fully elucidated. Nonetheless, mushrooms' anti-ageing and wound-healing potential have increasingly been reported, linked to their richness in triterpenes, polysaccharides, and phenolic compounds. These multifunctional bioactive properties have intensified the use of mushrooms and their related metabolites as components in cosmetic formulation development, a niche that has received increased attention in recent years.

### 5.3.8 Hepatoprotective Properties

The liver is a vital and complex organ of the human body, maintaining homeostasis by regulating various physiological functions such as carbohydrate, protein, and fat metabolism, detoxification, secretion of bile, and storage of vitamins. The liver is also involved in most biochemical pathways related to growth, combatting diseases, nutrient supply, energy provision, and reproduction.[208]

Liver damage is a widespread disease that, in most cases, involves oxidative stress. Normally, host cells are protected from oxygen-derived radical

injury by naturally occurring free radical scavengers and antioxidants. When these natural defences are overwhelmed by excessive generation of pro-oxidants, a situation of oxidative stress evolves, and cellular macromolecules suffer oxidative damage.[208,209]

Both reactive oxygen and nitrogen species are the major initiators of oxidative stress, and they appear due to various pathogenic diseases, exposure to radiation, tissue injury, *etc.* Most of the hepatotoxins induce tissue injury after having been metabolized to free radicals and cause subsequent cell damage through a mechanism of covalent binding and lipid peroxidation. Oxidative stress has been implicated in the pathogenesis of acute and chronic liver injury in a variety of pathophysiological conditions such as hepatotoxin exposure, intrahepatic cholestasis, alcoholic liver injury, hepatocellular carcinoma, liver ischaemia and viral hepatitis, fibrosis, cirrhosis, steatohepatitis and biliary disease, inflammation, and necrosis of the liver, among others. Thus, maintaining a healthy liver is crucial for overall health and human well-being.[208–210]

Clinically, serum enzymes [aspartate aminotransferase (AST) and alanine aminotransferase (ALT), hepatic lipids (total cholesterol and triglycerides (TG)], and enzymes involved in alcohol metabolism [alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)] are commonly used as biochemical markers for early diagnosis of hepatic injury. Serum ALT and AST activities are elevated when a hepatic injury occurs since ALT and AST could leach out of hepatocytes into the blood circulation.[211]

Carbon tetrachloride ($CCl_4$), a selective hepatotoxic chemical agent, is one of the most widely used toxins for the experimental induction of liver fibrosis in laboratory animals. The principal causes of $CCl_4$-induced hepatic damage are lipid peroxidation, decreased activities of antioxidant enzymes, and the generation of free radicals leading to liver fibrosis and cirrhosis.[209,212]

As mentioned earlier, the particular characteristics of growth and development of mushrooms in nature result in the accumulation of a great variety of secondary metabolites. All these related activities give mushrooms great potential as generators of bioactive compounds helpful in promoting human health. This includes their consumption as foods and their role as producers of biomolecules with specific pharmacological properties in laboratory cultures under strictly controlled conditions.[208]

The hepatoprotective properties and the greatest contributions described by different authors in several mushroom species are summarized in Table 5.8.

The protective effects of *L. deliciosus* and *A. cylindracea* supplementation against $CCl_4$-induced oxidative stress were investigated by measuring the adenosine deaminase (ADA) and myeloperoxidase (MPO) levels and histopathological changes in liver and kidney tissues in an *in vivo* model.[213] The $CCl_4$-induced histopathology was increased significantly in the *A. cylindracea* and $CCl_4 + A.$ *cylindracea* groups due to the toxic effects of *A. cylindracea*. On the other hand, *L. deliciosus*-treated rats exhibited significantly fewer

**Table 5.8** Main mushrooms with reported hepatoprotective properties and the main contribution.

| Mushroom species | Liver injury | Main role | Ref. |
|---|---|---|---|
| *A. cylindracea* | $CCl_4$ | *A. cylindracea* supplementation causes severe pathology in liver and kidney tissues in rats | 213 |
| *C. comatus* | Alcohol | A modified polysaccharide from *C. comatus* significantly attenuated the hepatic and serum lipid levels, enhanced antioxidant enzyme activities, markedly improved alcohol metabolism system and inflammatory response, and mitigated alcohol-induced liver injury histopathologically | 217 |
| *G. lucidum* | $CCl_4$ | *G. lucidum* extract can significantly prevent the $CCl_4$-induced liver and kidney damage, restored MDA, $H_2O_2$ contents, SOD, CAT, and GSH levels | 214 |
| *H. marmoreus* | $CCl_4$ | All extracts prepared from garlic and buna-shimeji with low and high contents of S-allyl-L-cysteine sulfoxide and arginine or ornithine significantly suppressed $CCl_4$-induced hepatic injury in rats | 215 |
| *L. deliciosus* | | Supplementation with *L. deliciosus* exhibits beneficial effects in liver and kidney tissues of $CCl_4$-treated rats | 213 |
| *L. sulphureus* | Acute alcohol | Both hot-water-extractable polysaccharides and enzymatic-extractable polysaccharides confer effective hepatoprotection against acute alcohol-induced alcoholic liver disease | 211 |
| *P. ostreatus* | $CCl_4$ | The crude phenolic-rich extract treatments showed significant increases in SOD and GPx hepatic activities | 212 |
| | | Daily supplementation of powdered oyster mushroom reduced dyslipidaemia, hepatotoxicity, and haematotoxicity | 216 |
| *P. tuber-regium* | $CCl_4$ | The activity of aspartate aminotransferase, alanine transaminase, and alkaline phosphatase, and the concentration of bilirubin significantly increased in *P. tuber-regium* extract-treated hepatotoxic rats | 210 |

degenerative and necrotic changes in liver and kidney in the $CCl_4 + L.$ *deliciosus* group compared to rats in the $CCl_4$-treated group. Purified polysaccharide isolated from *L. deliciosus* is a potential source of antioxidants and natural immunostimulants, which could explain the protective effect of this mushroom.[213]

In another study, $CCl_4$-induced oxidative stress was tested in an adult male Sprague–Dawley rat. The authors found that $CCl_4$ toxicity increased blood urea, uric acid, and creatinine levels compared to the control samples. Pretreatment of *G. lucidum* extracts significantly decreased urea, uric acid, and creatinine levels, proposing that it would counteract nephrotoxicity

caused by $CCl_4$. Otherwise, treatment with *G. lucidum* extract restored malondialdehyde (MDA), hydrogen peroxide ($H_2O_2$) contents, superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) levels.[214] The restoration activities of oxidative stress parameters by *G. lucidum* can protect the enzymes; this species contains several antioxidant compounds and exhibits significant strong scavenging free radical activity.[214]

The combination of the garlic-derived amino acid, *S*-allyl-L-cysteine sulfoxide (ACSO), and ornithine or arginine from *H. marmoreus* on $CCl_4$-induced hepatic injury was examined by Yamaguchi *et al.*[215] Some mushrooms have highly active arginase that catalyses the hydrolysis of arginine to ornithine and urea, accumulating a considerable amount of urea in the fruit bodies to regulate osmotic pressure. All extracts prepared from garlic and *H. marmoreus* with low and high contents of ACSO and arginine or ornithine significantly suppressed $CCl_4$-induced hepatic injury in rats. The co-administration of ornithine/arginine with ACSO suppressed $CCl_4$-induced hepatic injury in rats more potently than a single administration of ACSO, probably owing to synergistic effects.

In another study, the hepatoprotective activity of the crude phenolic-rich extract (CPRE) isolated from *P. ostreatus* on $CCl_4$-induced oxidative stress was investigated in albino rats and several biochemical parameters were evaluated. Administration of $CCl_4$ for 28 days exhibited a significant increase in serum markers of liver damage [*i.e.*, ALT, AST, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), urea, creatinine, total lipids and TG]. $CCl_4$ exposure significantly decreased the hepatic antioxidant enzyme activities such as SOD and glutathione peroxidase (GSH-Px). Contrarily, the CPRE treatments showed a significant increase in these hepatic activities.[212] The potential antioxidant activity of CPRE isolated from *P. ostreatus* against several oxidation systems *in vivo* contributed to its hepatoprotective effects in $CCl_4$-induced liver injury in male albino rats.[212]

Ubhenin *et al.*[216] evaluate the hepatoprotective, haematoprotective, and hypolipidaemic effects of *P. ostreatus* in $CCl_4$-induced liver injury in Wistar rats. The results revealed that $CCl_4$ caused a significant increase in lipid peroxidation due to the significantly elevated level of MDA in the hepatic tissues, whereas the levels or activities of reduced GSH, CAT, SOD, GPx, and glutathione *S*-transferase (GST) in the liver tissues were significantly reduced. Dyslipidaemia and haematotoxicity were manifested by a significant increase in the serum levels of TG, total cholesterol, very low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein cholesterol (LDL-C), and white blood cells count. The ability of the powdered *P. ostreatus* to mitigate against $CCl_4$-induced hepatotoxicity is probably due to its antioxidant and enzyme modulatory effects.

The hepatoprotective effects of modified polysaccharide from *C. comatus* on alcohol-induced liver injury were investigated. The mushroom extract significantly increased the SOD, GSHPx, and CAT activities, which may be because the extract activated nuclear factor erythroid 2-related factor-2 in mouse livers. These results indicated that the activities of antioxidant

enzymes might be one of the main mechanisms for contributing to the protective effects of modified polysaccharides from *C. comatus* against alcohol-induced liver injury.[217]

Chemical screening and analysis of the hepatoprotective efficacy of *P. tuber-regium* extracts in $CCl_4$ hepatotoxic rat was evaluated by the authors.[210] When *P. tuber-regium* extract was administered to hepatotoxic rats, AST, ALT, and ALP activity significantly decreased, and the concentration of serum albumin and protein significantly increased compared to the hepatotoxic group. *P. tuber-regium* extract possessed antioxidant activity in a dose-dependent manner, possibly due to a relatively high concentration of bioactive compounds like flavonoids, tannins, alkaloids, and phenolics.[210]

The hepatoprotective effects and antioxidant activities of hot-water-extractable polysaccharides and enzymatic-extractable polysaccharides from *L. sulphureus* in acute alcohol-induced alcoholic liver disease mice were investigated by the authors.[211] The elevation of AST and ALT activities could be attenuated by supplementation of both hot-water-extractable polysaccharides and enzymatic-extractable polysaccharides. Polysaccharides confer effective hepatoprotection against acute alcohol-induced alcoholic liver disease, possibly by reducing oxidative stress. Enzymatic-extractable polysaccharides showed superior effects, indicating that enzymatic hydrolysis has a potential effect on enhancing bioactivities.[211]

## 5.4 Conclusion and Future Trends

This chapter focuses on the beneficial biological effects of mushroom extracts and their related metabolites. Mushrooms have received significant interest in developing functional foods due to their diverse nutritional profile and a variety of bioactive molecules with effective therapeutic and medicinal properties. Some of these functional ingredients containing mushroom extracts or their metabolites have been utilized to prevent and treat a variety of diseases, and they continue to be patented and sold as a daily health supplement. A considerable body of literature has demonstrated the potential health beneficial effect of consuming mushroom extracts and their associated metabolites. Mushroom consumption has demonstrable antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, antidiabetic, anti-ageing, neuroprotective, hepatoprotective, and cardioprotective effects through a variety of signalling pathways that have the potential to contribute to the suppression or delay of disease severity. Despite this, there is a significant gap in validating some of these beneficial effects in preclinical or clinical settings to evidence their therapeutic effectiveness. In addition, the safety and toxicity profile of mushroom extracts used for different applications, although widely conducted, still needs more convincing research involving improved models. Also, there remains the need to standardize extract preparation and consumption method to avoid inconsistencies in the desired therapeutic effect.

## Abbreviations

| | |
|---|---|
| AChE | Acetylcholinesterase |
| AP-1 | Activator protein 1 |
| ADA | Adenosine deaminase |
| ALT | Alanine aminotransferase |
| ALP | Alkaline phosphatase |
| ADH | Alcohol dehydrogenase |
| ALDH | Aldehyde dehydrogenase |
| α-MSH | Alpha-melanocyte-stimulating hormone |
| AD | Alzheimer's disease |
| Aβ | Amyloid beta |
| AST | Aspartate aminotransferase |
| ABTS | 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid |
| BMDCs | Bone-marrow-derived dendritic cells |
| BChE | Butyrylcholinesterase |
| $CCl_4$ | Carbon tetrachloride |
| CVD | Cardiovascular diseases |
| CAT | Catalase |
| CNS | Central nervous system |
| COX-2 | Cyclooxygenase-2 |
| CTL | Cytotoxic T-lymphocytes |
| DM | Diabetes mellitus |
| DMM | Dietary mixed micelles |
| DPPH | 2,2′-Diphenyl-1-picrylhydrazyl |
| DMH | 1,2-Dimethylhydrazine |
| DSS | Dextran sulfate sodium |
| DOX | Doxorubicin |
| ESBL | Extended-spectrum β-lactamases |
| ECM | Extracellular matrix |
| FSG | Fasting serum glucose |
| FSI | Fasting serum insulin |
| FRAP | Ferric reducing antioxidant power |
| GSH | Glutathione |
| GSH-Px | Glutathione peroxidase |
| GST | Glutathione *S*-transferase |
| HbA1c | Glycosylated haemoglobin |
| GCSF | Granulocyte colony stimulating factor |
| HbA1 | Haemoglobin subunit alpha 1 |
| H22 | Hepatoma cells |
| HOMA-IR | Homeostasis model assessment of insulin resistance |
| HEA | Hydroxyethyl adenosine |
| $H_2O_2$ | Hydrogen peroxide |
| Humtyr | Human tyrosinase |
| iNOS | Inducible nitric oxide synthase |
| IFN-γ | Interferon gamma |

| | |
|---|---|
| IL | Interleukins |
| IL-1β | Interleukin 1 beta |
| IL-10 | Interleukin 10 |
| IZD | Internal zone diameter |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharides |
| LDL-C | Low-density lipoprotein cholesterol |
| KC | Keratinocyte chemoattractant |
| MIP-1α | Macrophage inflammatory protein-1 alpha |
| MIP-1β | Macrophage inflammatory protein-1 beta |
| MIP-2 | Macrophage inflammatory protein-2 |
| MDA | Malondialdehyde |
| MMP | Matrix metalloproteinases |
| MMP-9 | Matrix metallopeptidase 9 |
| MITF | Microphthalmia-associated transcription factor |
| MSSA | Meticillin-sensitive *Staphylococcus aureus* |
| MRSA | Meticillin-resistant *Staphylococcus aureus* |
| mRNA | Messenger ribonucleic acid |
| MBC | Minimum bactericidal concentration |
| MIC | Minimum inhibitory concentration |
| MAPKs | Mitogen-activated protein kinases |
| MCP-1 | Monocyte chemoattractant protein 1 |
| Mushtyr | Mushroom tyrosinase |
| MYD88 | Myeloid differentiation 88 |
| MPO | Myeloperoxidase |
| NGF | Nerve growth factor |
| NO | Nitric oxide |
| NF-κβ | Nuclear factor kappa B |
| OxHLIA | Oxidative haemolysis inhibition assay |
| ORAC | Oxygen radical absorbance capacity |
| PI3K | Phosphatidylinositol-3-kinase |
| PIH | Post-inflammatory hyperpigmentation |
| PUFA | Polyunsaturated fatty acids |
| PGE2 | Prostaglandin E2 |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RP | Reducing power |
| RANTES | Regulated on activation, normal T-cell expressed and secreted |
| ACSO | *S*-Allyl-L-cysteine sulfoxide |
| SFE | Supercritical fluid extraction |
| SOD | Superoxide dismutase |
| SREBF2 | Sterol regulatory element-binding protein 2 |
| STAT3 | Signal transducer and activator of transcription 3 |
| TBARS | Thiobarbituric acid reactive substances |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |

| | |
|---|---|
| TIMP-1 | Tissue inhibitor of metalloproteinase 1 |
| TGF-β | Transforming growth factor beta |
| TG | Triglycerides |
| TLR | Toll-like receptor |
| TLR4 | Toll-like receptor 4 |
| TAC | Total antioxidant capacity |
| TOS | Total oxidant status |
| TNF-α | Tumour necrosis factor alpha |
| TRP | Tyrosinase-related proteins |
| UV | Ultraviolet |
| VCAM-1 | Vascular cell adhesion molecule 1 |
| VEGF | Vascular endothelial growth factor |
| VLDL-C | Very low-density lipoprotein cholesterol |

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