Development of new functional bakery products with health benefits from yoghurt and curd cheese enrichment

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2021

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Lisbon, 2021

Carla Graça

General framework of the PhD thesis

The present PhD thesis include the research work carried out during the years 2017-2021 at the University of Lisbon, Department of Biosystems Sciences and Engineering of the Linking Landscape, Environment, Agriculture and Food (LEAF) research center unit from the Instituto Superior de Agronomia, Portugal, under the supervision of Professor´s Isabel Sousa and Anabela Raymundo. Part of the experimental laboratory work was performed at the Department of Food and Nutrition of the University of Helsinki, Faculty of Agriculture and Forestry, within an Internship Erasmus + Program for 7 months, supervised by Doctor Xin Huang and Professor Tuula Sontag-Strohm.

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This PhD thesis will present in detail the research carried out over 4 years, designed to meet the requirements of specific market niches, focused on nutritional-added needs and healthier lifestyle options, both inserted on a dietary personalized rationale towards a nutritional enrichment for a health-promoting and preventive diet.

All work performed was focused on the development of new bread formulations, as it is a prominent staple food with a worldwide relevance in human nutrition. The focus was not only to improve the technological properties but also, and foremost, the nutritional and functional components, in response to the increasing consumer´s demand for a healthier lifestyle.

The selection of the ingredients was based on their nutritional, functional, and bioactive contributions to functional foods, in which the potential of dairy products, were studied in detail for the development of new nutritional added-value baking products with bioactive benefits.

In this context, this PhD thesis aimed at the development of nutritional and functional added-value wheat bread by yoghurt and curd cheese enrichment, in which two types of fermentation processes were applied: baker´s yeast and sourdough fermentation. Gluten-free bread was also produced with those dairy products.

It is worth to highlight that the bread formulations developed are liable to be successfully extrapolated to develop other baking goods and easy to scale-up into baking industries.

A flowchart of the thesis is presented in Figure A, where the sequence of the different tasks performed and their connection is shown, to achieve the proposed goals. Selection and characterization the raw materials for the development of gluten-containing (wheat flour) and gluten-free bread (buckwheat, rice flour, and potato starch), using both the addition of yoghurt or curd cheese *per se*, the next step was the optimization of the formulation and processing conditions using the baker´s yeast as a leavening agent.

As for the sourdough fermentation, the raw material used (wheat flour and whole grain flour) were subjected to a long-fermentation time, using the presumptive lactic acid bacteria derived from

yoghurt as a starter. The breads developed, in terms of nutritional profile, impact on in *vitro* starch digestibility and glycemic response, bioactivities such as *in vitro* antioxidant and anti-inflammatory properties, and *in vitro* protein digestibility (to sourdough bread) were characterized.
Furthermore, the acceptance of the product, based on consumer´s preference, was analyzed using a sensory evaluation.

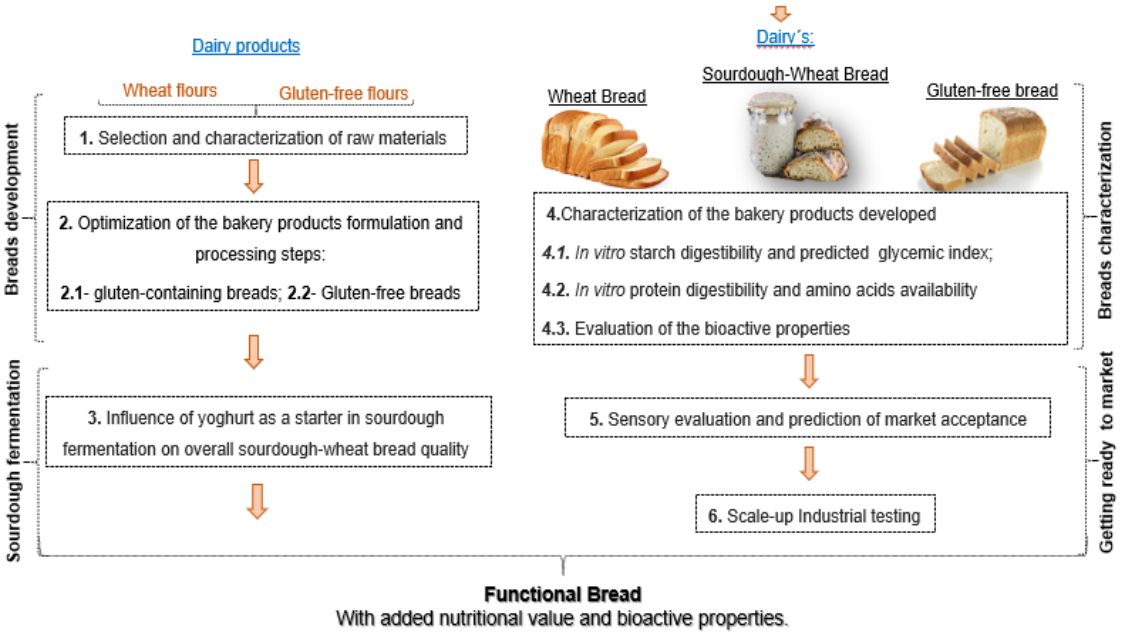


Figure A: Flowchart of activities developed throughout the PhD study

In terms of structure, the thesis' essay encompasses five main chapters (Figure C): chapter 1 of the general introduction and state of the art to harmonize the research work performed; chapter 2, 3 and 4 to present the compilation of scientific papers published (or submitted) obtained in each of the three different studies performed, and the overall conclusions and future perspectives in chapter 5.

Abstract

Together with the increasing consumer´s awareness about the relation between diet and health, the growing number of individuals with gluten and wheat-related illnesses, such as celiac and irritable bowel diseases, have been the main responsible driver´s for the continued innovation of food companies and the launching of healthy products, including gluten-free version, on the market. Additionally, the strategy of health-food development goes on not only by the usage of nutritional sources but also to the application of alternative processes to meet the consumer's expectations for natural, clean-label, nutritional and sustainable foods.

In those matters, following the current food market trends, the present dissertation was based on the development of new bread formulations, including gluten-free versions, with nutritional and functional added benefits, by the incorporation of nutritional sources, plain yoghurt and curd cheese. The study was extended to the field of sourdough fermentation, combining the yoghurt nutritional properties with the potential of its lactic acid bacteria to be used as a starter in sourdough fermentation.

At first approach, the influence of yoghurt and curd cheese (6%-50%) additions to improve the technological, nutritional, and sensory properties of the wheat bread, was studied. Subsequently, the influence of both dairy products (6%-25%) to reduce the wheat bread´s glycemic response, was detailed assessed. Ultimately, the impact of both dairies' additions (25%) to enhance the wheat bread´s bioactivity, was an important complementary study.

At second approach, the protein reinforcement by the incorporation of yoghurt and curd cheese enrichment (5%-20%) to overcome the technological challenge of gluten absence was evaluated, and so that resulted bread would improve nutritional/functional profile. Subsequently, the influence of both dairies (10%-20%) to reduce the glycemic index while improving the bread´s bioactivity, was as well appraised.

In the third approach, the usage of plain yoghurt (25%) as a starter in sourdough fermentation aiming for a more functional and easily digestible wheat bread, was studied. Two wheat dough matrices: 1) wheat flour and 2) wheat and whole-grain flour (50% ratio) were considered.

Overall, it can be stated that the development of new bread formulations, including gluten-free and sourdough-wheat bread, was successfully achieved, rendering higher nutritional added-value products with health-promoting benefits, that can be linked to a preventive consumer's diet and include health claims, responding well to the innovation needs of the bakery industry.

Keywords: market trends, bakery goods, dairy products, rheology properties, nutrition profile, bioactivity, health-promoting products

Resumo

A crescente consciencialização do consumidor face à relação entre alimentação e saúde, juntamente com o crescente número de indivíduos com doenças relacionadas com glúten e com trigo, como a doença celíaca e a síndrome do intestino irritável, têm sido as principais tendências responsáveis pela contínua inovação na indústria alimentar e lançamento no mercado de novos produtos alimentares com impacto na saúde, incluindo a versão sem glúten. A estratégia de desenvolvimento de alimentos saudáveis deve focar-se não só na utilização de fontes nutricionais, mas também na aplicação de processos alternativos, com potencial para gerar alimentos naturais, sustentáveis e com rótulo limpo (*clean label*).

Seguindo as tendências atuais de mercado, a presente dissertação baseou-se no desenvolvimento de novas formulações de pão, incluindo a versão sem glúten, com benefícios funcionais, através da incorporação de fontes nutricionais, o iogurte e o requeijão. O foco de estudo foi também direcionado para a aplicação de longos tempos de fermentação recorrendo à técnica de "massa-mãe", como um processo promissor de desenvolver um produto com benefícios nutricionais e rótulo limpo.

Numa primeira abordagem, estudou-se a influência da incorporação de iogurte e requeijão (6%-50%) nas propriedades tecnológicas, nutricionais e sensoriais do pão de trigo. Posteriormente, avaliou-se a influência de ambos os laticínios (6%-25%) na redução da resposta glicêmica, e na componente bioativa (25%) do pão de trigo.

Numa segunda fase, avaliou-se o impacto do reforço proteico pela incorporação de iogurte e requeijão (5%-20%) nas propriedades tecnológicas e no perfil nutricional do pão sem glúten. Posteriormente, estudou-se a influência de ambos os laticínios (10%-20%) na resposta glicémica e na bioatividade do pão.

Por último, o foco foi direcionado para o potencial das bactérias lácticas derivadas do iogurte (25%) como iniciador na fermentação da "massa-mãe" e a sua influência no desenvolvimento de pão de trigo mais funcional e facilmente digerível. Foram utilizadas duas matrizes de massa de trigo: 1) farinha de trigo e 2) farinhas de trigo integral (50%).

De uma forma geral, pode-se concluir que foi possível desenvolver novas formulações de pão, incluindo pão sem glúten, de valor nutricional agregado, funcional e com interessantes propriedades bioativas para o consumidor, que podem ser vinculados a uma dieta preventiva e incluir alegações de saúde, respondendo bem às necessidades de inovação da indústria de panificação.

Keywords: tendências de mercado, panificação, laticínios, propriedades reológicas, perfil nutricional, bioatividade, alimentos funcionais

Resumo alargado

O consumidor, como o principal impulsionador da valorização da cadeia alimentar, dita as tendências de mercado e consumo, direcionando as novas linhas de investigação e as empresas a adotar estratégias mais diversificadas, face às necessidades de mercado cada vez mais exigentes e seletivas.

Nos últimos anos, juntamente com a crescente consciencialização do consumidor face à interação alimentação - saúde, o crescente número de indivíduos com sensibilidade e/ou intolerância ao glúten, mas também os que consideram uma dieta isenta de glúten uma escolha mais saudável, têm sido tendências de mercado de destaque, responsáveis pela contínua inovação na indústria alimentar, gerando novos produtos, incluindo os alimentos sem glúten.

Além dos constrangimentos de mercado associados à limitada disponibilidade de produtos de qualidade, em comparação aos congéneres com glúten, os produtos sem glúten geralmente apresentam um baixo perfil nutricional, em termos de proteínas, vitaminas e minerais, elevado índice glicémico e um tempo de vida útil curto.

No entanto, o glúten não é o único responsável pelo desencadeamento de distúrbios gastrointestinais. Outros componentes que coexistem com o glúten, na mesma matriz alimentar, os designados hidratos de carbono de cadeia curta (*FODMAPs*, do inglês: *Fermentable Oligo, Di, Monossacarides And Polyois*), que são só parcialmente, ou não são de todo, absorvidos no intestino delgado, mas fermentáveis no colón pela microbiota endógena existente, originando vários sintomas gastrointestinais, de diferentes severidades, consoante a sensibilidade do individuo. A síndrome do intestino irritável é a condição inflamatória gastrointestinal mais comum associada a este grupo de pacientes. É de salientar que ambas as condições inflamatórias mencionadas podem degenerar em tumores. Assim, o desenvolvimento de novos produtos com compostos inibidores da resposta inflamatória, tem surgido como um relevante tópico nas áreas de alimentação e saúde. A atividade das enzimas metaloproteinases da matriz (MMPs), especificamente MMP-9, uma classe de gelatinases conhecidas por serem os principais modeladores dos processos de inflamação do intestino, está diretamente envolvida em doenças do intestino irritável e também são conhecidas por serem induzidas pelo glúten.

A estratégia de desenvolvimento de novos produtos promotores da saúde, deve também passar pela aplicação de processos alternativos para atender às expectativas do consumidor por alimentos naturais, com rótulo limpo, valor nutricional agregado e mais sustentáveis.

A aplicação de longos tempos de fermentação, utilizando a técnica de "massa-mãe" ("*sourdough fermentation"*), pode ser considerada uma alternativa promissora face às exigências do consumidor e do mercado de alimentos saudáveis, representando uma nova oportunidade para a indústria de alimentos.

Alinhando este trabalho com as tendências atuais de mercado, por forma a dar resposta à crescente procura do consumidor por alimentos mais saudáveis, a presente dissertação centra-se no desenvolvimento de novas formulações de pão de trigo, incluindo a versão sem glúten, de valor nutricional agregado e benefícios funcionais, pela incorporação de fontes nutricionais bem

reconhecidas: o iogurte natural e o requeijão. O foco de estudo foi direcionado para a aplicação de longos tempos de fermentação recorrendo à técnica de "massa-mãe", como um processo sustentável alternativo e promissor de gerar um rótulo limpo, vantagens tecnológicas e nutricionais, usando as bactérias lácteas, derivadas da incorporação de iogurte, como iniciador da fermentação.

Face ao exposto, este trabalho foi dividido em três grandes estudos, conforme detalhado na Figura B, que resume de forma esquemática os estudos desenvolvidos (I - III) e respetivas publicações científicas (ou em fase de publicação) resultantes.

A partir do **Estudo I**, que visou o desenvolvimento de novas formulações de pão com glúten, à base de farinha de trigo, foram realizados três diferentes estudos, articulados entre si, resultando em três artigos originais publicados em revistas científicas internacionais de revisão por pares:

No artigo 1, foi avaliada a influência da adição de iogurte ou de requeijão, nas propriedades tecnológicas, nutricionais e sensoriais do pão de trigo. Testaram-se diferentes níveis de incorporação de ambos os produtos lácteos, desde 6% a 30% (m / m) e níveis superiores de até 40% para iogurte e 50% para requeijão (m / m), com base na farinha de trigo.

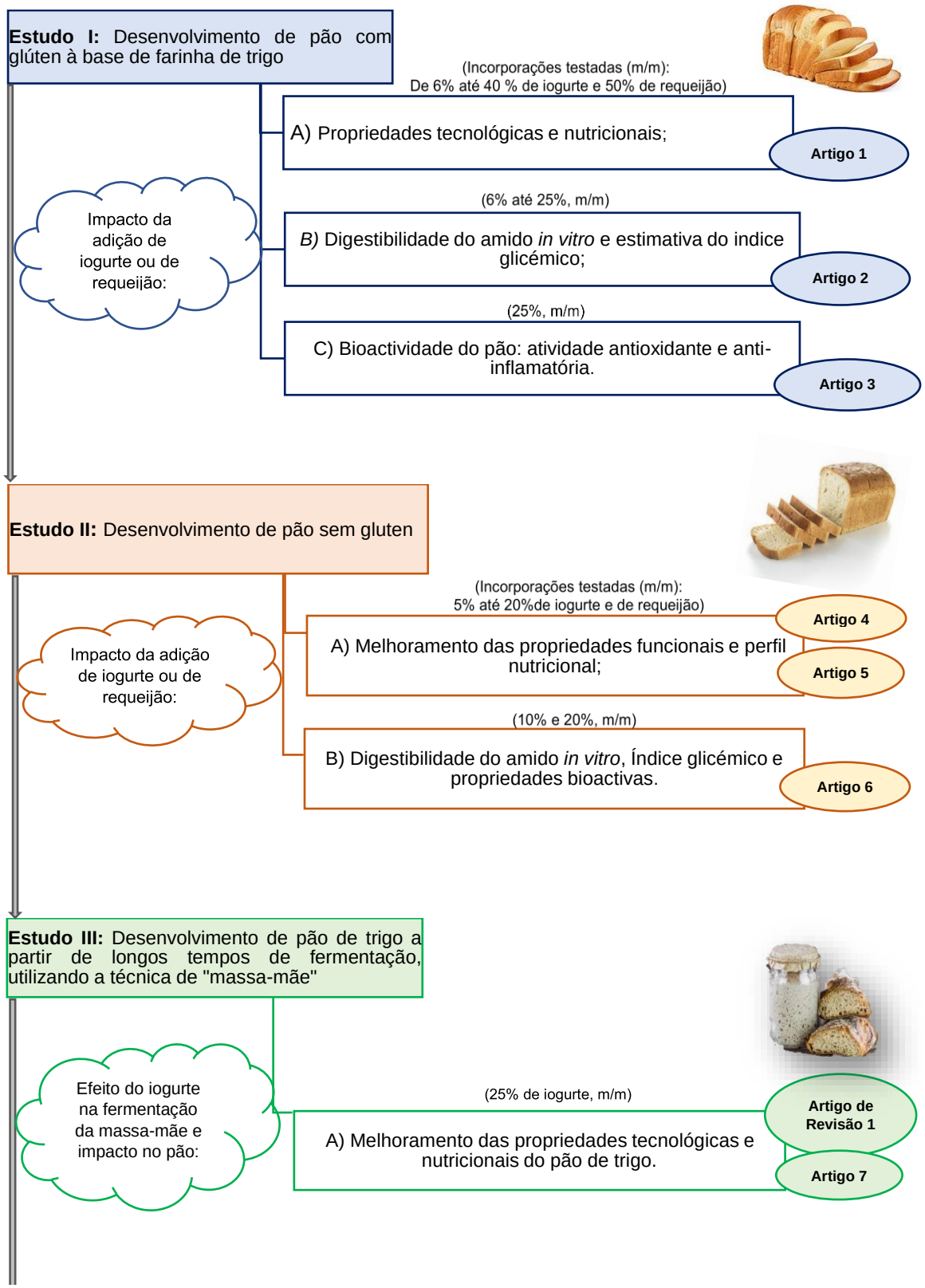
O iogurte revelou ser um potencial ingrediente de panificação tendo-se obtido um impacto positivo nas características reológicas da massa para todos os níveis de incorporação testados, enquanto a adição de requeijão apresentou um limite tecnológico de 18% de adição (m / m). O perfil nutricional do pão melhorou consideravelmente em termos de proteínas, minerais e oligoelementos, que são um fator importante para equilibrar a dieta diária do consumidor em geral. A boa aceitabilidade sensorial para os pães com 30% (m / m) de iogurte e 18% (m / m) de requeijão, revelou ser uma perspetiva interessante para futuras aplicações de mercado.

Posteriormente, **no artigo 2**, foi estudado em detalhe o impacto de ambos os produtos lácteos de 6% até 25% (m / m) nas propriedades tecnológicas do amido e o impacto na redução do índice glicémico do pão de trigo. O desempenho tecnológico do amido foi negativamente afetado pela incorporação de ambos os produtos lácteos (25%, m / m), promovendo uma redução considerável na digestibilidade do amido *in vitro*, que claramente se refletiu na redução da resposta glicémica do pão de iogurte (≈ 30%) e do pão de requeijão (≈ 38%), resultando num índice glicémico (IG) intermédio (IG: 55-69), e baixo (IG:<55), respetivamente.

Por último, **no artigo 3,** avaliou-se em detalhe o impacto da incorporação de iogurte e requeijão (25% m / m) na componente bioativa (compostos fenólicos, atividade antioxidante) do pão de trigo. Foram também avaliadas as propriedades anti-inflamatórias, com foco no potencial para inibir a atividade da gelatinase MMP-9, usando um ensaio de atividade gelatinolítica padrão.

O iogurte revelou ser um ingrediente com grande potencial para desenvolver um pão mais eficaz na capacidade sequestrante de radicais livres, enquanto o requeijão expressou melhores efeitos no desenvolvimento de um pão com potencial redutor. A inibição da atividade da MMP-9 foi significativamente melhorada pela incorporação de ambos os produtos lácteos, sugerindo um melhoramento das propriedades bioativas em termos de resposta anti-inflamatória.

Os artigos resultantes deste primeiro estudo são apresentados no capítulo 2 (C2).



Pão + funcional

Figura B: Esquema geral dos três estudos desenvolvidos e artigos científicos resultantes

A **partir do estudo II**, avaliou-se em detalhe o potencial dos produtos lácteos no desenvolvimento de novas formulações de pão sem glúten, e o seu impacto nas propriedades tecnológicas, nutricionais e bioativas. Igualmente, de forma articulada, este trabalho resultou em três estudos diferentes, que originaram três artigos originais publicados em revistas científicas internacionais de revisão por pares.

No artigo 4 e 5, estudou-se a influência do reforço proteico pela incorporação de iogurte e de requeijão no desenvolvimento de pães sem glúten, com intuito de ultrapassar o desafio tecnológico associado à remoção do glúten, a fim de melhorar a qualidade tecnológica e nutricional do produto final.

O enriquecimento pela incorporação de iogurte ou de requeijão, revelou ser uma alternativa promissora para melhorar o desempenho tecnológico no desenvolvimento de formulações de pão sem glúten, sugerindo o reforço das interações entre as macromoléculas na matriz proteica, mimetizando a malha do glúten. Relações lineares estudadas pela incorporação de iogurte (R^2> 0,9041) e de requeijão (R^2> 0,972) revelaram uma forte correlação entre os parâmetros de qualidade do pão e as propriedades reológicas da massa, indicando que os efeitos observados são proporcionais aos níveis de incorporação testados.

As melhorias significativas no perfil nutricional do pão sem glúten, que resultaram da adição de ambos os laticínios (20%, m / m) permitem alegações de saúde, com uma contribuição interessante para equilibrar a dieta do celíaco.

No artigo 6, avaliou-se a influência do iogurte e do requeijão (10% - 20%, m / m) na resposta glicémica e bioatividade do pão sem glúten (componente fenólica, propriedades antioxidantes e anti-inflamatórias). A resposta glicêmica foi consideravelmente reduzida, principalmente pela adição de requeijão, resultando num pão com índice glicémico intermédio (IG: 55-69). A bioatividade do pão, em termos de capacidade antioxidante e inibição da atividade de MMP-9, foi melhorada pela adição de ambos os produtos lácteos (20% m / m). Fortes correlações lineares (R^2> 0,922) entre a digestibilidade do amido *in vitro*, índice glicémico e capacidade antioxidante, suportam os resultados obtidos.

Os artigos resultantes do estudo II são apresentados no capítulo 3 (C3).

No estudo III, avaliou-se o potencial das bactérias lácticas derivadas da incorporação de iogurte (25%) como iniciador da fermentação da “massa-mãe” e a sua influência no desenvolvimento de pão de trigo mais nutritivo, funcional e facilmente digerível.

O artigo 7, fruto do trabalho realizado em colaboração com a Universidade de Helsínquia, teve como objetivo combinar as propriedades nutricionais do iogurte (25%, m / m) e a capacidade fermentativa da microbiota láctica de gerar condições ácidas *in situ*, com potencial de ativar as enzimas endógenas da farinha, e simultaneamente melhorar as propriedades tecnológicas e os benefícios nutricionais do pão de trigo. Foram estudadas duas matrizes de massa de trigo: 1) farinha de trigo e 2) uma misturada de farinhas de trigo e de trigo integral (50%, m / m).

Observou-se que a etapa prévia de preparação da “massa-mãe” com a incorporação de iogurte promoveu alterações consideráveis na estrutura proteica e composição química da massa, principalmente quando se utilizou farinha de trigo integral (*whole grain*). Este resultado pode estar

associado à maior atividade enzimática derivada do gérmen e do farelo. Foram obtidas vantagens nutricionais consideráveis, reduzindo o índice glicémico e aumentando a digestibilidade da proteína, promovendo uma maior biodisponibilidade de aminoácidos, em comparação com o pão de fermento.

Como suporte bibliográfico a este trabalho, foi escrito um artigo de revisão que compila o trabalho científico realizado na área do bioprocessamento e as suas vantagens ao nível tecnológico, benefícios nutricionais e impacto na saúde do consumidor.

Os artigos resultantes do estudo III são apresentados no capítulo 4 (C4).

De forma geral, pode-se concluir que os produtos desenvolvidos e estudados estão em linha com as novas tendências do mercado para o desenvolvimento de alimentos inovadores, com benefícios promotores da saúde, podendo ser vinculados a uma alimentação mais saudável, preventiva e incluir alegações de saúde, respondendo bem às necessidades de inovação da indústria de panificação, sendo também facilmente escaláveis para a produção industrial.

Keywords: tendências de mercado, panificação, laticínios, propriedades reológicas, perfil nutricional, bioatividade, alimentos funcionais.

Thesis outline

The present PhD thesis was divided into five main chapters, each one is described briefly below. Figure C represents a general diagram of the entire PhD study and the respective outputs.

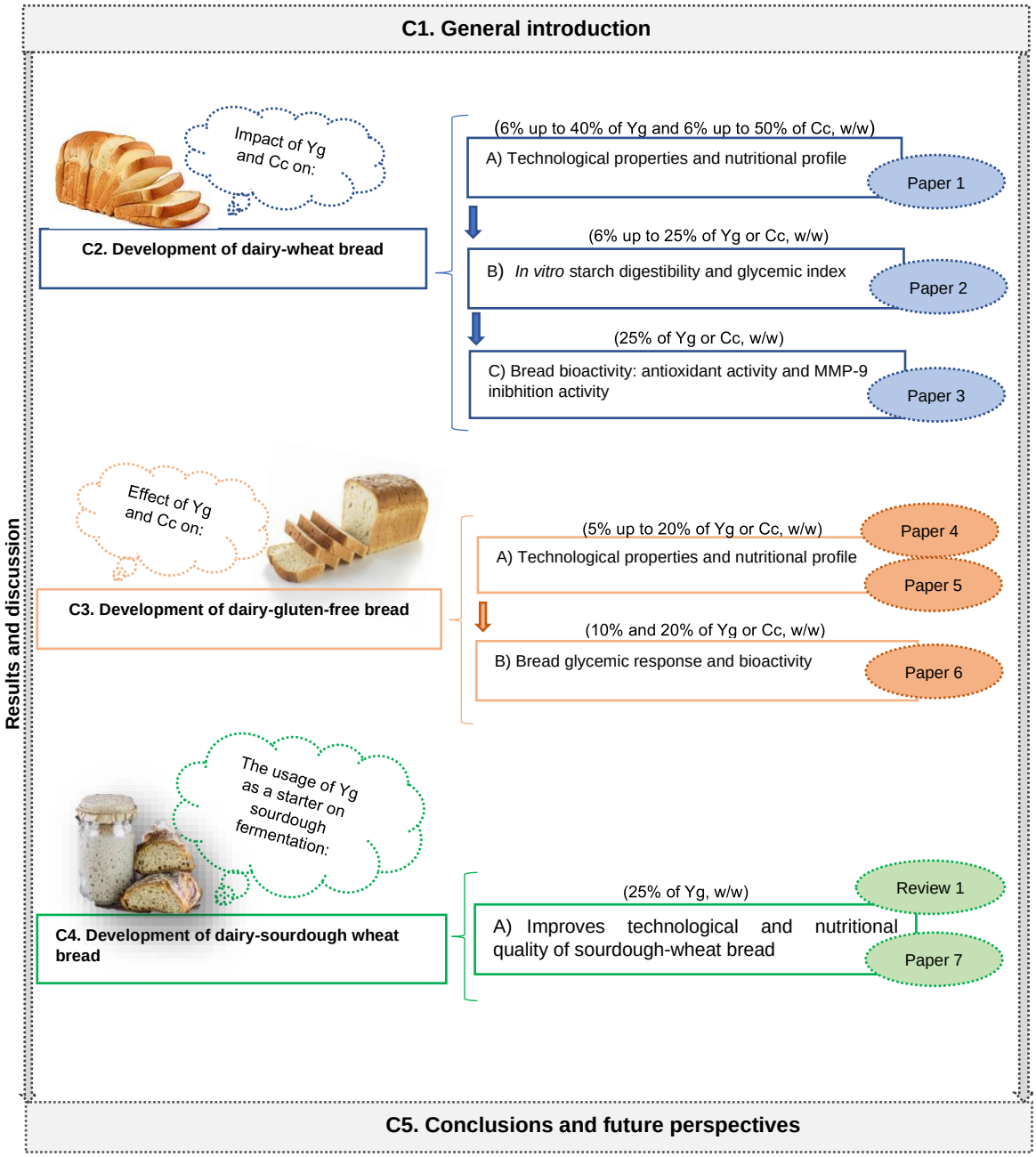


Figure *C*: General diagram of the PhD Thesis, with resulting papers delivered from each research study performed.

This PhD thesis is the compilation of the scientific works published in ISI Journals (or submitted to publication). Therefore, the introduction and state-of-the-art (C1) will briefly address some of the main aspects on which this thesis relies, since each publication has its own introduction section.

Chapter 1. Introduction and state of the art. This chapter (C1) presents the contextualization of the study as a general introduction and state of the art, in terms of food market trends as a mirror of consumer´s health demands. It is the highlighting of some of the specific market niches and its nutritional requirements and the alternative nutritional/functional ingredients sources to the development of added-value functional foods, healthy and sustainable foods, followed by the main and specific objectives of this work.

The **results and discussion** section are encompassed by three different chapters:

Chapter 2: Yoghurt and curd cheese as potential ingredients to improve technological properties and nutritional/functional profile of wheat bread.

From this chapter (C2), the capacity of yoghurt and curd cheese to improve the overall quality of wheat bread was studied. Small and large deformation dough rheology measurements, to assess in detail the impact of yoghurt and curd cheese addition on gluten-network reinforcement, were adopted. Dough network microstructure, by electronic scan microscope, was evaluated.

Technological properties based on bread texture behavior, during storage time, and post-baking quality attributes were characterized. From optimized bread samples, the nutritional composition, *in vitro* starch digestibility, mimicking the human digestive system, to estimate the glycemic response, and bioactive features, by *in vitro* antioxidant and anti-inflammatory properties, were studied in detail.

This first research study resulted in 2 papers (Paper 1 and 2) peer-reviewed and already published and 1 research paper accepted for publication (Paper 3), all in well-known scientific journals.

Chapter 3. Improving technological and nutritional/functional properties of gluten-free bread by yoghurt and curd cheese enrichment.

Chapter (C3) aimed to evaluate the functionality of the yoghurt and curd cheese dairy proteins to replace the gluten-like structure to improve the technological properties of gluten-free bread, with a concurrent influence on enhancing nutritional/functional and bioactive properties. Fundamental rheology measurements, based on viscoelastic behaviour and flow curves, to study the impact of dairy proteins on gluten-free dough structure, were performed. Pasting properties, to assess the influence of dairy proteins on starch performance behaviour, were evaluated. Resulted bread samples were characterized in terms of nutritional profile, texture rheological behaviour, bread quality attributes, *in vitro* starch digestibility and predicted glycemic index, *in vitro* antioxidant and anti-inflammatory potential.

Three research papers peer-reviewed (Paper´s 4, 5, and 6) were produced and are already published in recognized scientific journals.

Chapter 4. Yoghurt as a starter in sourdough fermentation to improve the technological and nutritional properties of sourdough-wheat bread.

Chapter (C4) was designed to explore the influence of yoghurt addition, as a starter on sourdough fermentation, to improve the technological and nutritional properties of sourdough-wheat bread, using the formulation optimized in subchapter 2.1. The idea was to evaluate the influence of presumptive lactic acid bacteria derived from yoghurt to generate *in situ* acid conditions capable to activate and stimulate endogenous flour enzymes to promote biochemical/composition dough changes, with a subsequent effect on technological and nutritional bread quality. The impact of *in situ* proteolytic activity generated during sourdough fermentation, on wheat protein pattern modification, small-sized peptides, and free amino nitrogen content, correlated with antioxidant activities, was studied in detail. Chemical/composition dough changes influence on technological properties (texture rheology behavior and baking parameters) and foremost on *in vitro* starch and protein digestibility measurements, were also evaluated.
This chapter comprises a detailed review paper (Review 1) compiling the main findings in the field of sourdough fermentation on the impact on gluten protein degradation, FODMAPs compounds reduction, as well as the nutritional and functional improvements, to guide and support the results obtained in the last research paper (Paper 7) obtained from the third study. Both submitted to highly quoted scientific journals to be published.

Chapter 5. Overall conclusions and future perspectives.

From chapter (C5), the main conclusions of the thesis are emphasized, and the perspectives for future work generated from this thesis are pointed out.

Table of contents

List of Tables

Chapter 2. Yoghurt and curd cheese as potential ingredients to improve technological properties and nutritional/functional profile of wheat bread

- **Research paper 1**

- **Research paper 2**

- **Research paper 3**

Chapter 3. Improving technological and nutritional/functional properties of gluten-free bread by yoghurt and curd cheese enrichment

- **Research paper 4:**

- **Research paper 5:**

- **Research paper 6:**

Chapter 4. Yoghurt as a starter in sourdough fermentation to improve the technological and nutritional properties of sourdough-wheat bread

- **Research paper 7:**

- **Review paper 1:**

List of Figures

- **Research paper 2:**

- **Research paper 3:**

- **Research paper 6:**

Chapter 4. Yoghurt as a starter in sourdough fermentation to improve the technological and nutritional properties of sourdough-wheat bread

- **Research paper 7:**

Figures as supplementary information (SI):

- **Review paper 1:**

List of publications

From this PhD thesis, seven research works, and one review paper have resulted, in which six of the research papers are published in International Scientific Journals Indexed in ISI-Web of Science, and the other two, including the review, are under review to be submitted for publication. The list of scientific works is presented as follows.

The transfer of acquired knowledge in an active way, was performed in National and International Scientific Conferences, by several oral (seven) and poster (two) communications, as follows listed.

A. Publications in international scientific journals with peer review

Graça, C., Raymundo, A., and Sousa, I. (2019). Wheat Bread with Dairy Products—Technology, Nutritional, and Sensory Properties*. Applied. Sciences, Eco Nov. Foods Spec. Issue*s, 9, 4101. https://doi.org/10.3390/app9194101 (Impact Factor: 2.474, Q2).

Graça, C., Raymundo, A. and Sousa, I. (2020). Yogurt as an Alternative Ingredient to Improve the Functional and Nutritional Properties of Gluten-Free Breads*. Foods Journal*, 9, 111. https://doi.org/10.3390/foods9020111 (Impact Factor: 4.092, Q1).

Graça, C., Raymundo, A., Sousa, I. 2020. Improving the Technological and Nutritive Properties of Gluten-Free Bread by Fresh Curd Cheese Enrichment. *Applied Sciences Journal, Special Issue in Food Sustainability: Using Byproducts from Food Industry and Unconventional Food Sources*, 10, 6868. https://doi.org/10.3390/app10196868 (Impact Factor: 2.474, Q2).

Graça, C., Mota, J., Lima, A., Ferreira, R.B., Raymundo, A., Sousa, I. (2020). Glycemic Response and Bioactive Properties of Gluten-Free Bread with Yoghurt or Curd-Cheese Addition. *Foods Journal, Food Nutrition Section*, 9, 1410. https://doi.org/10.3390/foods9101410 (Impact Factor: 4.092, Q1).

Graça, C., Raymundo, A., Sousa, I. (2021). Yoghurt and curd cheese addition to wheat bread dough: Impact on in vitro starch digestibility and estimated glycemic index*. Food Chemistry*, 339, 127887. https://10.1016/j.foodchem.2020.127887 (Impact Factor: 6.306, Q1).

Graça, C., Edelmann, M., Raymundo, R., Sousa, I., Coda, R., Sontag-Strohm, T., Huang, X. (2022). Yoghurt as a starter in sourdough fermentation to improve the technological and functional properties of sourdough-wheat bread. *Journal of Functional Foods*, 88, 104877. https://doi.org/10.1016/j.jff.2021.104877 (Impact factor:4.451; Q1).

Graça, C., Lima, A., Raymundo, A., Sousa, I. (2021). Sourdough Fermentation as a Tool to Improve the Nutritional and Health-Promoting Properties of Its Derived-Products. *Fermentation*, *7*, 246. https://doi.org/10.3390/ fermentation7040246 (Impact factor: 3.975; Q1).

- **To submit for publication**

Research paper:

Graça, C., Mota, J., Lima, A., Boavida-Ferreira, R., Raymundo, A., Sousa. (2020). Improving bioactive properties of wheat bread by yoghurt or curd cheese supplements: *Antioxidant, anti-inflammatory and antibacterial activities*. [S*ubmitted to Journal of Food Science and Technology, JFST-D-20-03129, 19 of November 2020 (*Impact Factor: 2.705, Q2*)*].

B. Presentations in scientific conferences

- **Oral communications**

Graça, C., Raymundo, A., Sousa, I. (2018). Yoghurt as a nutritional ingredient in bakery goods. Impact on rheology characteristics of the wheat bread dough. Annual European Rheology Conference (AERC 2018) – Sorrento, Italia; Abril 17-20, 2018. (https://rheology-esr.org/aercs/aerc-2018/).

Graça, C., Raymundo, A., Sousa, I. (2018). Modified milk proteins: potential ingredients in bakery foods. (2018). XIV Encontro de Química dos Alimentos (EQA) – Viana do Castelo, Portugal; November 6- 9, 2018. (http://xiveqa.eventos.chemistry.pt/).

Graça, C., Raymundo, A., Sousa, I. (2019). Improvement of the nutritional and functional profile of the bakery goods by adding dairy products. Impact on rheology characteristics of the wheat bread dough. Annual European Rheology Conference 2019 (AERC 2019) – Portoroz, Eslovenia; April 8 – 11, 2019. (https://rheology-esr.org/aercs/aerc-2019/).

Graça, C. Raymundo, A., Sousa, I. (2019). Impact of dairy products addition on rheology properties of wheat bread dough. Iberian Meeting on Rheology (IBEREO 2019) - Porto, Portugal, September 4-6, 2019. (https://paginas.fe.up.pt/~ceft/archives/3172).

Graça, C., Raymundo, A., Sousa, I. (2019). Wheat bread with dairy products; impact on dough, quality of bread and glycemic index. International Conference of Wheat Diversity and Human Health Istanbul, Turquía, October 22-24, 2019. (https://www.cimmyt.org/events/wheat-diversity-human-health/).

Graça, C., Mota, J., Lima, A., Boavida Ferreira, R., Raymundo, A., Sousa, I. (2020). *Bioactive properties and glycemic response of gluten-free bread enhanced by dairy products addition. 3rd International Conference on Food Bioactive and Health* – Parma, Italy, Junho 16-19, 2020 [Accepted for oral communication, but due to COVID-19 Pandemic it was cancelled and re-booked to 21st-24th June 2022- Parma, Italy, https://www.fbhc2020.com/].

- **Poster communications**

Graça, C., Raymundo, A., Sousa, I. (2019). *Dairy products as alternatives to reduce glycemic index of wheat bread. Book of Abstract of EuroFoodChem XX2019,* Poster FU_P24*,* Porto, Portugal, 17-19 junho, 2019 (http://xxeurofoodchem.eventos.chemistry.pt/).

Graça, C., Raymundo, A., Sousa, I. (2019). *Dairy products as ingredients in bakery foods to improve protein and mineral intake on daily diet*. Encontro com a Ciência e Tecnologia- Ciência´19, Lisboa, Portugal, 8-10 julho, 2019, Centro de Congressos de Lisboa (http://www.encontrociencia.pt/home/).

C. Publications performed in collaboration with other research projects, during the PhD

Marques, D.R., Kimberli, P. B., Graça, C., Monteiro, A. R. G. and Sousa, I. (2019). Fat Reduction in Extruded Snacks with the Use of Xanthan Gun. Springer Nature Switzerland AG 2020, F. J. Galindo-Rosales et al. (Eds.): IBEREO 2019, SPM, pp. 12–15, 2020. https://doi.org/10.1007/978-3-030-27701-7_3.

Graça, C., Marques, D., Sousa, I., Monteiro, A. R. G. (2020). Xanthan gum as an alternative to replace the fat for coating and flavoring the extruded snacks. Journal of Food Science and Technology. https://doi.org/10.1007/s13197-020-04542-y (Impact Factor: 2.702, Q2).

Nunes, C. M., Graça, C., Vlaisavljević, S., Tenreiro, A., Sousa, I., and Raymundo, A. (2019). Microalgae cell disruption: Effect on the bioactivity and rheology of wheat bread. *Algal Research*, 45, 101749. https://doi.org/10.1016/j.algal.2019.101749 (Impact Factor: 4.008, Q1).

Chapter 1. Introduction and state of the art

1.1. Consumer´s demands and food market trends

A rapid change in the dietary lifestyle has been observed along with industrialization, globalization, and economic development driving to an increased number of people suffering from poor health, in which the incidence of food related diseases such as allergies, intolerances, obesity, diabetes, and some types of cancer have been more recurrent (Goyal et al., 2015).

Consequently, consumer's awareness about what they eat, and the benefits derived from a healthy diet to enhance emotional, physical, mental well-being and health status, have been gaining considerable attention.

In a world where the increasing consumer demand and interest about the health-enhancing properties have been arising, scientific research and food companies have been moving towards the innovation and development of nutritional-added value products (Fardet, 2015), a trend that will promote a considerable influence on company strategies in incoming years and will continue to gain importance (Alexandrato and Bruinsma, 2012).

Food market trends are a mirror of consumer´s needs and choices and have been changing over the years. However, the relation between diet and health has been taking the market lead due to the consumer's nutritional demands and healthy-lifestyle choices.

Thus, aligning the research, innovation, and development of new products concept with the food market trends is a successful way of reaching consumers' expectations for healthy products, without failing the appealing and tasty properties.

1.1.1. Extending market niches to consumer trends

The consumers' behavior and food choices as well as the specific market niches are dictating the path towards the innovation and development of the new products in future markets.

Nowadays, as many consumers suffer from some type of intolerance, allergy, or other low-grade inflammatory conditions, the choice for "free-from" foods, has become more common (Savarese et al., 2021). In some individuals the symptoms of food allergies can range from mild reactions to severe responses, that even can include death. In other cases, the choice for "free-from" foods. particularly among people without a confirmed allergy or intolerance, may be driven by marketing- and media-fueled perception that these products are healthier, leading to a higher availability of these products on the market (Muir et al., 2019).

Consequently, this category of products has been slowly associated to conventional health-food products, in response to consumer´s appeals, which will lead to a continued market growth with an expected global rise of a 9.5% rate until 2025 (Mordor Intelligence, 2020).

The "free-from" food is a segment of the market characterized by wheat-free, gluten-free and other allergen-free products, with a special focus on bakery as the top category for gluten and wheat-free, with considerable increase expression on the distribution market channels (Mordor Intelligence, 2020; Innova Market Insights, 2021).

The development of gluten-free foods is on today's agenda and have been leading to an increasingly soaring demand for gluten-free products in the market, not only due to the growing number of individuals diagnosed with some type of gluten intolerance or sensitivity, but also by those non-celiac consumers based on food choices. All together, they represent of around 38% of avoidance and/or limiting of wheat or/and gluten-containing foods (Hendry, 2019).

Nevertheless, gluten-free foods are often less appealing, and the nutritional value and health promoting-properties are somehow left behind, being mainly starch-based foods with low protein content, higher in fat levels, with a high glycemic index, which is critical for celiac patients or individuals with some type of wheat / gluten sensitivity (Muir et al., 2019; Nyyssola et al., 2020).

Therefore, the development of nutritionally balanced gluten-free products, with sensory and technological improvements compared to their gluten-containing counterpart, is a clear opportunity for new research lines towards food industry's future innovation.

Apart from this, and despite the focus on children and young scholar's nutrition, the modern consumers are increasingly seeking solutions to living the fullest life, priming for health and well-being. Healthy aging claims are starting to be more common on food products and beverages, as elder generations are focusing on an healthy lifestyle, opting for nutritional and functional added-value food. A statistical data revealed that 76% of consumers aged between 26 and 55 years agreed that a fullest heathy lifestyle starts in what they eat and drink, and 56% of those already increased their consumption of functional foods to a better life-quality (Innova Market Insights, 2021).

Undoubtedly, functional food products with active health claims are increasing as a market niche to meet a huge variety of nutritional needs. Between 2018 to 2019, this healthy niche has represented a global growth of more than 11%, and it is clearly expected to continue to expand in next years (New Nutrition Business, 2020).

The increasing focus on the relationship between diet and health is clear, in a perspective not only of preventive care and health promoters, but also on heathy aging, which all together represents an opportunity for food companies increasing the commercial offerings of added-value functional products.

We are in an Era that nutrition is at the "heart of health", and the future of food is food with a purpose, a concept that is in line with the Innova Market Insights' third trend for 2021, "Tailored to fit", that arose as a spotlight to meet personalized nutrition, to fit the consumer's unique lifestyle choice, whether based on nutritional needs, lifestyle, or body composition (Innova Market Insights, 2021).

Closely connected with a healthy lifestyle is the "clean label foods", a concept that is going further a market trend, being a consumer´s expectation (Nutrition and Bioscience, 2021). Based on consumer´s expectation, food naturalness can be understood as traditional, natural and sustainably processed food, with familiar sound and natural ingredients (e.g., free-from or low-in additives, no chemical preservatives addition), health concepts (e.g., allergen friendly and health-friendly) and social responsible manufacturing (e.g, eco-friendliness as low carbon footprint) that are good both for themselves and for the planet (Amos et al., 2014; New Nutrition Business, 2020).

“Clean label foods” are linked with the first market trend for 2021 based on consumer´s evolving about the demand for more transparency, represented by 80% of the consumers actively seeking for healthiest food and 77% dodging chemically preserved foods (Renner et al., 2019). In light of transparency, ethical, environmental, and clean label foods, all together are the governing consumer demands in 2021 (Food Ingredients First, 2021).

Despite the importance of naturalness and the health issues around better life-quality, the increased sugar consumption over the last 20 years and the increasing number of people suffering from related-metabolic disease, deserve considerable attention. The world is facing an enormous problem of metabolic disease, such as obesity, cardiovascular disease, and diabetes, in which the main factor that is triggering these diseases is the excessive sugar consumption (World Health Organization, 2015, 2017). Therefore, in the next years, sugar reduction will become a norm, and no longer the exception.

Bakery products and similar are considered one of the main sugar sources on market processed foods. Thus, the search for alternative ingredients and/or processes that will not increase or can be useful to reduce the sugar content, without compromising the consumer´s eating experience in terms of taste, texture, and shelf-life, is a technological challenge (Sahin et al., 2019).

Beyond sugar-reduction, protein is another macronutrient attracting most attention in “better-for-you” treats, in which consumers are showing a growing interest to consume protein-fortified foods for improved health benefits, which is booming of around 20% per year the new products launched in the market (Innova Market Insights, 2021).

Bakery field, as source of staple foods with an important role in nutrition and worldwide-consumed, is a food sector that has been gaining traction in Europe’s markets, representing a promising strategy for protein-enrichment on consumer’s daily nutrition (Mitelut et al., 2021).

Therefore, innovation in taste, texture and shelf-life, as well as the research for low-sugar and novel protein-based ingredient sources, linked to a healthy and preventive diet, represent an unprecedented opportunity of giving everyday products with a health halo (Food Ingredients First, 2021).

Achieving nutritional balanced products with health-promoting benefits, is the focus of Nutrition hacking, the sixth Top Ten Trend, thought to go through consumer´s requirements, improving nutrition by recurring to sustainable and natural food technology applications, generating as much as possible clean labeling products (Innova Market Insights, 2021).

In the light of the above exposed, it is clearly understandable that safer, healthier, and nutritional added-value food, produced in a more natural, sustainable, and ethical manner, is today´s top priority of the consumer´s choice, and undoubtedly with great potential to take the lead of future market innovation.

Market trends are not all based on food and nutrition issues, but the above described were the main drivers of the present PhD thesis, to contribute to a portfolio of new nutritional and functional added-value bakery goods to future innovation lines of the bakery industry.

1.2. Avoidance of wheat and gluten-containing product

Cereal-based foods, like wheat and other gluten-based products, are staple foods widely consumed worldwide, as an important source of energy with an essential role in human nutrition. However, wheat and gluten-containing foods have been associated to a wide range of health issues, as allergy/sensitivity, gastrointestinal and autoimmune disorders, that have been led to a considerable avoidance of these products (Muir et al., 2019).

Celiac disease**,** as the most representative study of gluten intolerance, is a genetically related gastrointestinal inflammation via an autoimmune disease triggered by a life-long intolerance to gluten ingestion, a term that encompasses the cereal prolamins of wheat (gliadins), rye (secalin) and barley (hordein), affecting severely of around 1% of the population (Capriles and Arêas, 2014).

Cereal prolamins contain multiple proline and glutamine amino acid residues associated to the main triggers of these gastrointestinal symptoms in celiac patients, since those residues are proteolytic resistant on human digestion, thus, resulting in a wide variety of undigested peptides in the small intestine, that can be further deamidated by tissue transglutaminase. Subsequently, due to the expression of human leukocyte antigen (HLA), HLA-DQ2 and HLA-DQ8 molecules, in genetically susceptible individuals, with a high affinity to bind with deamidated gluten peptides, generate a sequence of events. These events can be: mucosal inflammation, small intestinal villous damage and atrophy (Marsh, 1992), increased intestinal permeability (Cobden et al., 1980) and malabsorption of macro and micronutrients (Green and Jabri, 2003; Briani et al., 2008). They can be as severe as small bowel cancer diseases, and/or other autoimmune disease (e.g., thyroid disease and diabetes) (Capriles and Arêas, 2014; Muir et al., 2019).

Figure 1 illustrates a schematic role of the gluten-toxic peptides in the small gut of individuals with celiac disease.

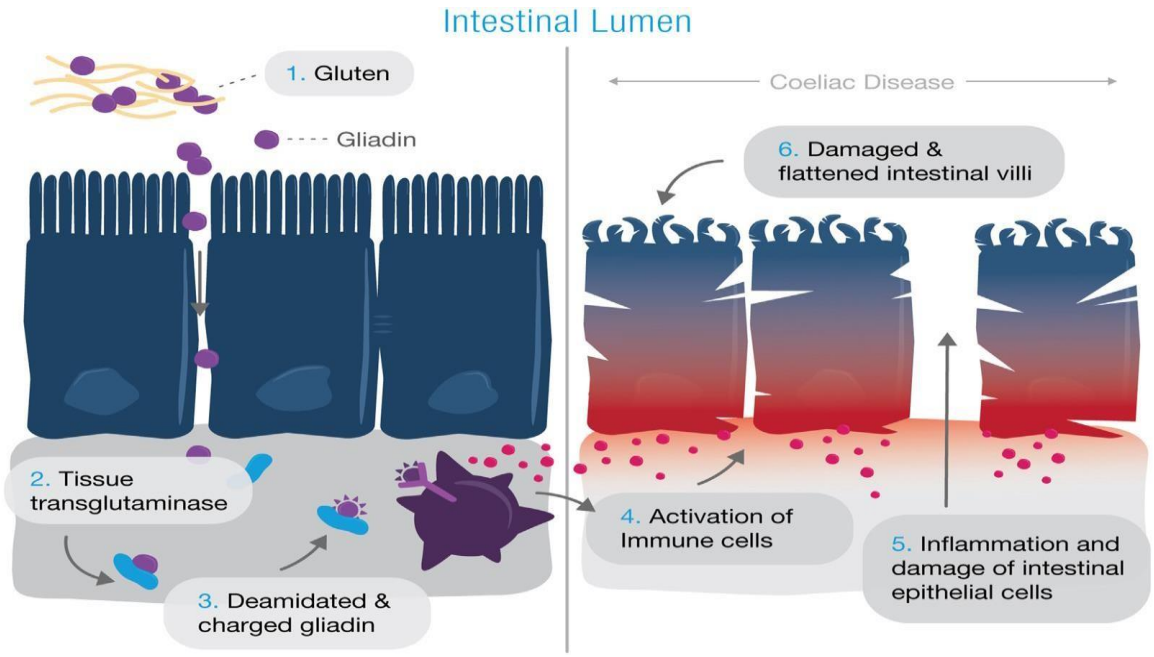


Figure 1: Role of gluten in celiac disease (Source: Muir et al. 2019)

Despite the huge advances made in understanding the clinical and nutritional aspects of celiac disease, at present the only available treatment is a life-long strict avoidance of gluten-containing foods (including wheat, rye and barley) which, in time, can result in clinical and mucosal recovery (Fasano and Catassi, 2012).

Nevertheless, recent evidence has been suggesting that gluten is not the only culprit of triggering gastrointestinal disorders. There are many other components that co-exist with gluten in wheat and gluten-containing foods, members of a short-chain carbohydrates group, named Fermentable Oligo-Di-Monosaccharides And Polyos (FODMAPs) (De Giorgio et al., 2015), that have been associated to triggering other clinical gastrointestinal conditions (Muir et al., 2019; Nyyssola et al., 2020). More details about this topic can be found in the Review paper at Chapter C4.

Other components on wheat-based products that can trigger gastrointestinal symptoms include amylase-trypsin inhibitors and wheat germ lectins, small glycoproteins that may activate the immune response system, inducing gastrointestinal/inflammation disorders (Junker et al., 2012).

The incidence of individuals who report gastrointestinal symptoms triggered by wheat and/or gluten-containing products ingestion, but who do not exhibit clinical symptoms of celiac disease (Muir et al., 2019), have been more recurrent. Gastrointestinal symptoms typically associated to this group known as non-celiac gluten sensitivity or wheat sensitivity, include bloating, abdominal pain/discomfort and altered bowel habit, and extra-intestinal symptoms as headache, anxiety, skin rash, dermatitis, and weakness (Volta et al., 2017; Muir et al., 2019).

Additionally, there is another group of individuals suffering from gastrointestinal disorders, known as irritable bowel diseases, representing around 10-15% of the population, characterized by flatulence, bloating, abdominal pain/discomfort and altered bowel habitat, as well as other common symptoms that includes excessive gas and urgency to defecate (Biesiekierski et al., 2013). While celiac disease has a considerable impact in life expectancy, irritable bowel clinical conditions can be associated to have a profound effect on life quality (Muir et al., 2019; Nyyssola et al., 2020).

The poor absorbed and rapid fermentation of FODMAPs in large intestine have been suggested to be a mechanism that triggers irritable bowel syndrome. There is a vast number of FODMAP compounds naturally present in many foods (Muir et al., 2019; Nyyssola et al., 2020). In wheat bread, the main compounds are fructans and galacto-oligosaccharides, that are not digested in the small intestine but are entirely delivered and rapidly fermented into the large intestine, promoting different gastrointestinal conditions, at different levels, depending on individual´s sensitivity (Nyyssola et al., 2020).

Muir et al., (2019), proposed a possible explanation of the role of FODMAPs in triggering symptoms in these patients, which is illustrated in Figure 2. Accordingly, FODMAPs as small molecules, tend to attract the water lumen of the small intestine, and since those compounds are not digested and absorbed in the small intestine, they pass undigested into the large intestine, being rapidly fermented by resident bacteria, with consequent gas production.

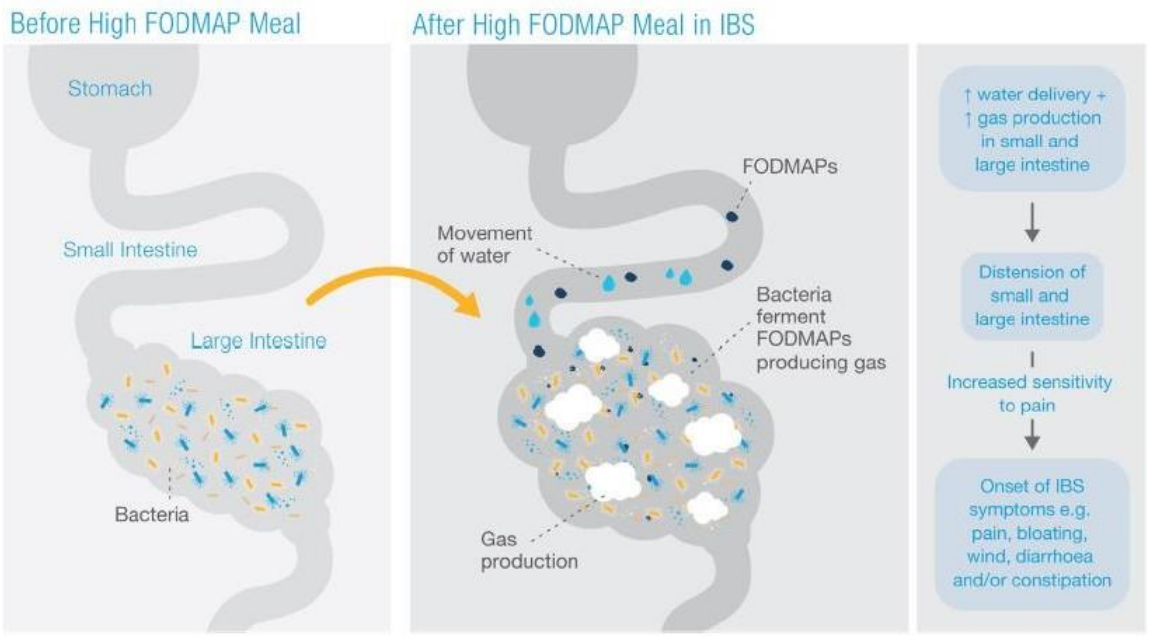


Figure 2: A possible representation of the FODMAPs role in triggering irritable bowel syndrome (IBS) (Source: Muir et al. 2019)

Subsequently, the increase on water and gas in the intestinal tract can cause the stretching of the intestine wall and its permeability, which can lead to defects in the intestinal epithelium causing the high hypersensitivity, pain, and other gastrointestinal symptoms, but foremost the development of gut inflammation in individuals susceptible to irritable bowel (Turner, 2009; Nighot et al., 2015).

Some studies have demonstrated that Matrix Metalloproteinases (MMPs), a group of zinc-dependent endopeptidases involved in the degradation/remodeling of extracellular tissue matrix and homeostasis (Ravi et al., 2007), play a central role in intestinal inflammation conditions in irritable bowel patients. Within the MMPs, the gelatinase MMP-9 demonstrated to be the main enzyme implicated in the early stages and development of inflammatory bowel disease, with its higher activities closely correlated with the disease severity and with the degree of inflammation (Lakatos et al., 2012; Annahazi et al., 2013). On this matter, studies on cell lines and animal models have shown that the inflammation development can be reduced with the administration of metalloproteinase inhibitors (Gan et al., 2001; Garc et al., 2006; Nighot et al., 2015). These findings have turned gelatinase inhibitors into very desirable pharmacological targets, however, previous efforts to target gelatinases MMP-2 and -9 were hampered by dose-limiting toxicity, insufficient clinical benefits, and severe side-effects, due to their lack of specificity and inhibition of MMP-dependent physiological processes (Bourguet et al., 2012; Ndinguri et al., 2012; Wang et al., 2012). Under this context, a substantial amount of research has turned towards the discovery of novel, non-toxic, food derived MMP inhibitors and their application, especially in food manufacturing, has emerged as an important branch of research.

It is worthy to note that these irritable bowel patients do not exhibit celiac clinical symptoms but respond well to a “gluten-free” diet, whose issue have been generating a considerable confusion among patients and health professionals.

The symptomatic improvements are wrongly attributed to gluten absence but rely on the lower amounts of FODMAPs on gluten-free (rice- and oat-based products) foods, in comparison with gluten-containing (specialty wheat and rye products) (Muir et al., 2019; Nyyssola et al., 2020).
Indeed, whether it is the increasing incidence of celiac disease, gluten allergy or sensitivity, inflammatory bowel symptoms, or the common belief that the avoidance of wheat and gluten-containing foods are an “healthier” option, all together have been driving to a considerable soaring demand for gluten-free products (Gallagher et al., 2003; Capriles and Arêas, 2014).
However, it is important to consider that despite the available gluten-free products they usually present nutritional deficiencies in micronutrients, protein, and dietary fiber. In addition, the absence of gluten translates into a major technological challenge, due to its structure-forming capacity, resulting in quality defects that negatively influence the consumer acceptance of final products (Kinsey et al., 2008; do Nascimento et al., 2013; Capriles and Arêas, 2014).

1.2.1. Gluten-free breadmaking as an emergent research topic

Gluten is a structure-building protein with a particular importance to the development of baked goods with high-quality and market acceptance. In wheat flour, it represents around 80% of proteins fraction, composed by glutenin and gliadin, both responsible for elasticity and extensibility of the dough, giving the viscoelastic properties needed for high quality baked products (Lazaridou et al., 2007; Capriles and Arêas, 2014).
Thinking about bread, it is a sponge-like structure, building up by a continuous matrix of gelatinized starch connected into a continuous coagulated gluten network, that support the foam-structure (Cauvain and Young 1999; Sluimer, 2005). Therefore, gluten is a crucial protein network, that plays an important role in breadmaking and responsible for bread’s appearance, texture, and quality.
The manufacture of gluten-free products, especially gluten-free bread, represents a huge technological challenge due to the importance of gluten proteins on dough structure, essential to form a viscoelastic network capable to retain the gases (CO_2), produced during the baker´s yeast fermentation stage, to support the dough-foaming structure expansion. The absence of gluten results in breads with a crumbly texture with fast staling rate, poor crust color, as well as other post-baking quality defects (Pico et al., 2019).
Furthermore, gluten-free products are more expensive than gluten-containing products, they have poor nutritional quality that can lead to nutritional insufficiencies, such as deficient values of protein, minerals and fiber, high contents of carbohydrates and fat, and with a high impact on glycemic human response and hence on metabolic diseases (Segura and Rosell, 2011).

Taking this into account, the research on possible alternatives to improve the technological and nutritional properties of the existing products, by the combination of gluten-free flours with structuring agents to produce a protein-structure like gluten network, is an important pre-requisite to gluten-free baking research and development.

Consequently, obtaining high technological and nutritional quality have been led the search for new ingredients, additives, and technologies to enhance the breadmaking performance and the nutritional profile of gluten-free products.

Figure 3 summarizes some approaches used to improve the structure, texture, acceptability, nutritive value, and shelf life of gluten-free goods. More detailed information can be found on review paper written by Capriles and Arêas (2014).

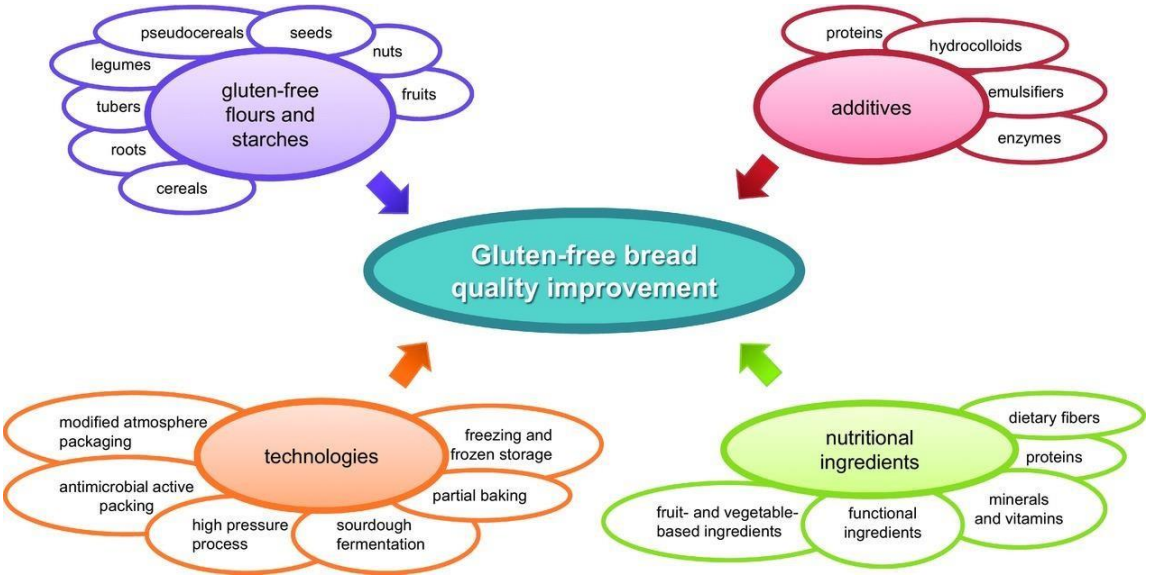


Figure 3: Technological and nutritional approaches to improve gluten-free bread making performance (Source: Capriles and Arêas, 2014)

Proteins are naturally good hydrocolloids and one of the main classes of molecules available to improve desirable textural attributes, based on crosslinking and aggregation mechanisms, with potential to replace synthetic hydrocolloids (Schoenlechner et at., 2010; Pico et al., 2019). Additionally, beyond the nutritional and health promoter´s benefits, the impact on food structure, due to their ability of water-binding and holding capacity, emulsifier, foaming, and gelling properties (Capriles and Arêas, 2014; Pico et al., 2019) make them interesting macromolecules to explore on new food´s design.

In the context of gluten-free breadmaking, protein-rich sources might be considered as promising structuring agents, since their ability to interact with water originating a gel network structure suitable to increase dough viscosity and strength, simulating a foaming-structure like gluten, capable to retain the gas during the critical steps of leavening and baking.

Moreover, as structuring agents, proteins represent a potential approach to reducing-sugar strategies, since it represents a technological challenge, especially in baked goods, due to structural interaction with all matrix components (Sahin et al., 2019).

Considering this, the search for protein ingredient sources with the potential for the development of nutritional/functional added-value products remains an important goal for research and development towards the improvement of technological, nutritional and sensory properties of bakery goods, including gluten-free goods.

1.3. Alternative nutritional/functional ingredient sources

Concerns over the ecologically responsible and ethical issues, as well as sustainable food sources, have led the search for new protein sources, which have been driving the academic research and companies interests towards the plant based-protein sources, as a promising approach to improve the sustainability of the food chain (Iriondo-De Hond et al., 2019).

Consequently, this trend has motivating the consumer to adopt vegetarian and vegan diets (Reipurth et al., 2019). Apart from this, it has also been promoting the growth in overall protein domain, with a clear potential for hybrid formulas made with both animal-based and plant-alternative proteins, based on consumer preferences for an “extra level of sophistication” (Food Ingredients First, 2020).

Despite the world challenges posed by the rising interest in plant-based diets, dairy products as a traditionally natural and nutritious food, remain key driver products in health-promoting benefits, and no competition between plant and dairy-derived proteins has been observed (Innova Market Insights, 2021). Evidence shows that the combination of both animal and plant proteins allows a more complete assimilation of amino acids, which address the requirements of the flexitarians, who are focused to reduce the consumption of animal protein sources. Supporting this, is the statistical market data revealing that 36% of global consumers prefer to include on diet both animal and vegetable products, while 25% would prefer a diet based on 100% of plant-based foods (Innova Market Insights, 2021).

Therefore, in a world in which the global population is projected to increase nearly 10 billion until the late 2050s (Alexandratos and Bruinsma, 2012), there is plenty market space for new tastier, healthier, and rich-protein foods opportunities by mixing both plant and animal proteins sources (Innova Market Insights, 2021).

Dairy products have long been associated with health benefits, largely allied with its nutritional composition based on high biological protein value and essential amino acids composition, with an interesting minerals and vitamins profile, beyond the antioxidant and anti-inflammatory bioactive peptides, with well-recognized influence as health-promoters on human body (Bordoni et al., 2017). In addition, the highly functionality and versatility of dairy proteins make them useful to a vast number of applications in many foods manufacturing (Gallagher et al., 2003).

The two major classes of proteins in milk and derived-dairy products are caseins and whey proteins. Depending on its emulsification and physicochemical properties, both can be readily used on food manufacturing in different purposes (Gallagher et al., 2003) whether for nutritional or functional benefits, including flavor and texture enhancement as well as shelf-life improvement (Kenny et al., 2001; Gallagher et al., 2003; Sharafi et al., 2017).

Some of the useful functional properties of dairy proteins include the emulsifying and stabilizing ability, the gelling properties, foaming capacity, water-absorption and binding capacity (Chandan, 1997; Karami and Akbari-adergani, 2019), able of forming a protein network with desirable swelling properties, a functional property that resemble to gluten network (Gallagher et al., 2003). Moreover, due to the water-holding capacity of casein and whey protein, its inclusion can result in more viscous matrixes, boosting flexiblising protein interaction effects. Batter viscosity is one of the key elements which is crucial to achieve high quality gluten-free products, especially bread (Capriles and Arêas, 2014).

As natural products obtained by cultures and enzyme activity, with no added sugar, dairy products can probably be considered as promising ingredients to face the sugar-reducing strategies, without compromise the consumer's food expectation, since as structuring and protein-reinforcement agents, can be suitable to improve both sensorial and texture properties.

Moreover, dairy proteins can be considered clean label ingredients with functionalities capable to meet the consumer requirements for healthier and natural-based products, responding well to the demanding for more transparency foods (Innova Market Insights, 2021).

During the last years, most of the chronic inflammatory diseases (e.g., obesity, diabetes, irritable bowel syndrome) have been strongly influenced by nutrition, in which the metabolism of food is intimately connected with inflammatory processes (Hotamisligil, 2006; Hernandez-Aguilera et al., 2013). It is not surprising that the search for bioactive nutrients able to modulate the inflammatory status on human's system has been emerging as an important research topic in food and nutrition sciences (Calder et al., 2011). Recent evidence has emphasized that dairy products can be a particularly interesting food type to study in the context of anti-inflammation activity (Labonte et al., 2013; Bordoni et al., 2017). Caseins and whey are protein chains composed of inactive bioactive peptides encrypted in the native structure that, once released by enzymatic, chemical, and/or microbial hydrolysis, can become biologically active promoting antioxidant, antihypertension, antimicrobial, antithrombotic, immunomodulatory effects (Irshad et al., 2015; Abd El-Salam and El-Shibiny, 2017), acting as health-promoters with disease prevention effects. Therefore, dairy protein sources can be a promising bakery ingredient for both nutritional and technological properties enhancement, including the gluten-free products, as a key role to circumvent the technological challenge of gluten absence and nutritional deficiencies of related products, contributing to the development of new nutritional-added-value "free-from" foods.

Yoghurt is one of the most popular fermented dairy products, characterized by a precipitated casein network obtained by the symbiotic acidification activity of *Streptococcus thermophilus and Lactobacillus bulgaricus subsp. delbrueckii* cultures (FAO, 2008). Widely consumed and appreciated on daily diet, with an important role in nutrition, it is a rich source of high-biological protein nutritionally good and easy digestible, with all nine essential amino acids, which is important to support metabolism by increasing energy expenditure and satiety (Halton and Hu, 2004; Leidy et al., 2015). Plain yoghurt is a source of natural sugars, mainly lactose (milk sugar) and galactose.

However, the lactose content is present in a much lower amount than in milk, due to the lactose fermentation, resulting in lactic acid and a few galactoses and glucose, sugars better tolerated by lactose intolerant individuals (He et al., 2008).

Additionally, it is a source of exopolysaccharides *in situ* produced by its dominant lactic acid bacteria fermentative activity, that have been associated to physiological benefits including antioxidant activities, anti-inflammatory properties and anticarcinogenic effects (Li et al., 2014; Adebayo-Tayo et al., 2018).

Apart from this, yoghurt is an excellent source of vitamins, such as vitamin B12, that is exclusively found in animal foods, and is also the main source of vitamin B2 (riboflavin) both connected to protect against heart disease (Powers, 2003; Ryan-Harshman and Aldoori, 2008). In terms of mineral profile, it is a good source of easily absorbable calcium, essential for healthy teeth and bones, as well as a source of phosphorus and magnesium, both playing an important role in human biological processes, such as regulating blood pressure, metabolism, and bone health (Weaver, 2013; de Baaji et al., 2015).

Curd cheese is a co-product obtained by the thermal denaturation and subsequent precipitation of the soluble whey proteins, a liquid by-product of cheese manufacturing, that is widely accepted for containing many valuable interesting components to improve human nutrition. Curd-cheese is a rich-source of proteins with an excellent nutritional and biological value (Madureira et al., 2007). This complex mixture of globular protein molecules presents a higher protein efficiency ratio and is an important source of essential amino acids (leucine, isoleucine, and valine), important for health-promoting benefits. It is also an interesting source of calcium, a mineral that plays an essential role in teeth and bone health, as well as on osteoporosis disease prevention (Önay-Uçar et al., 2014).

Biological properties and therapeutic potential of whey proteins derived from the encrypted peptides have been reviewed in detail (Madureira et al., 2007; Önay-Uçar et al., 2014). Health-promoting effects, as antimicrobial actions, immunomodulating on human system, anticarcinogenic activity and other metabolic active properties have been associated with such whey protein´s specific peptides and amino acids, predominantly derived by α-lactalbumin, lactoferrin, and bovine serum albumin, but particularly from β-lactoglobulin (Hernandez-Ledesma et al., 2008; Karami and Akbari-adergani, 2019).

In this context, the utilization of yoghurt and curd cheese as potential ingredients to the development of new nutritional-added value bakery goods, including gluten-free bread, as it can offers “advanced” or “complete” nutritional and bioactive advantages to fulfill the consumer´s nutritional needs but also for those who are prioritizing a healthy and preventive care lifestyle.

1.4. Healthy foods produced by sustainable processes

The increasing awareness about the relationship between diet and health have considerably moved the consumer´s choice to adopt an healthier diet, in which the functional foods have a prominent role on daily nutrition.

There is no specific definition for “functional or healthy foods” but generally, it is used for all the bioactive foods capable of providing health-benefits or preventing nutrition-related diseases beyond the basic nutrients. “Healthy food” is a term that has been used as an umbrella to encompass nutritional, functional and nutraceutical foods, responding well to the consumers need and continuous growth of food market niches (Vaughan and Judd 2003; Arias-Aranda and Romerosa-Martínez, 2010), expected to increase of 8% annually in Europe markets, between 2019 and 2023 (Statistica, 2019).

The strategy of health-food development goes on also to the application of alternative processes to meet the consumer expectations for natural, clean-label, added-nutritional, and easily digestible food, without losing the focus on sustainable production.

Sourdough fermentation is one of the oldest biotechnological application widely used as a leavening agent, dominated by lactic acid bacteria and yeast population. Sourdough-ingredients fermented as a natural, clean-label, sustainable and effective tool to ensure proper hygiene, rheology, sensory and shelf-life properties, with a simultaneous influence on functional/nutritional value of derived-end products. It can be considered a promising process to meet the consumer and healthy food market´s demand, representing a new opportunity for the food industry (Montemurro et al., 2019). Beyond the technological advantages of sourdough applications, it is known to enhance health benefits, such as decreasing the starch digestibility and reducing the glycemic index (Wang et al., 2019), increasing mineral bioavailability (Katina et al., 2005) and antioxidant activity (Gobbetti et al., 2014), improve the protein digestibility and nutritional indexes (Montemurro et al., 2019), and, beyond other positive benefits, it can have a considerable impact on the degradation/reduction of trypsin inhibitors and FODMAPs (Huang et al., 2020). For a more detailed revision of sourdough applications and their positive impact on technological and nutritional/functional aspects, a review paper is provided in Chapter 4.

Furthermore, sourdough fermentation is known to be an effective tool to generate clean label products, and it is associated with the antifungal compounds produced by lactic acid bacteria during fermentation (Axel et al., 2015), able to naturally prolong the shelf-life of end-products and to reduce the application of chemical preservative. Additionally, some lactic acid bacteria and yeast populations (Sahin et al., 2019) are characterized by *in situ* natural sugars alcohols (e.g., polyol) and/or bulking agent´s producers (e.g., exopolysaccharide, as dextran) (Wang et al., 2019), contributing to sweetness/flavor and structuring agents, enhancing dough stability and texture of the final product.

Therefore, a tailored sourdough fermentation with specific lactic acid bacteria could be a novel technological approach to produce more natural and clean label products, with low glycemic index and high protein digestibility and, in turn, an interesting approach to overcome quality losses in sugar-reduced baked products (Sahin et al., 2019).

The combination of lactic acid bacteria and/or nutritional ingredient sources, with the potential to further improvements on nutritional properties during sourdough fermentation, can be considered an interesting approach to the development of functional foods.

In this context, plain yoghurt can be an alternative ingredient for sourdough-bakery functional good manufacturing, both as an interesting source of lactic acid bacteria and of nutritional/functional properties.

1.5. Objectives

The present PhD thesis aimed at the development of new bakery good formulations, especially bread, including gluten-free bread, with health-promoting and preventive nutritional benefits, by the addition of high functional and nutritional added-value dairy proteins sources, in form of fresh plain yoghurt and curd cheese.

The study horizon was also expanded to the field of sourdough-ingredients fermentation, as a sustainable and natural food process, in which the goal was to explore the influence of the lactic acid bacteria derived from yoghurt incorporation as a starter on sourdough fermentation, to improve the technological and nutritional properties of sourdough-wheat bread.

The targeted products are aligned with new market trends for the development of innovative products, with an impact on the new health food market niches, responding well to the innovation needs of the bakery industry.

Based on the contextualization above detailed, this work was designed to respond to the following four main goals:

I. To meet the requirements of specific market niches with nutritional added needs, especially children, elderly, celiac patients and wheat/gluten sensitivity individuals;
II. Contribute to the valorization of curd cheese, a co-product obtained from whey, a cheese manufacturing by-product, and to the valorization of yoghurt, collaborating with the dairy industry to find new markets and add value to by-products such as whey protein;
III. Enhance the increase of dairy protein´s consumption with high added-value and bioactive properties on the human system, through their application in staple foods, as a potential vector to the enrichment of consumer´s nutrition;
IV. At last, but not least, to provide to the Bakery Industry a portfolio of innovative products with a positive impact on health issues, for the growing market of functional products with health-promoters benefits.

In terms of specific objectives, the present thesis aimed:

a) To understand how the protein-protein, protein-starch and hydrocolloid interactions can contribute to improve the rheological properties of the bread dough (see Paper 1), including gluten-free formulations (see Papers 4 and 5).
b) To evaluate the effect of yoghurt and curd cheese incorporation not only to improve the technological properties and nutritional profile but also on the influence on reducing the glycemic response of wheat bread (see Papers 2 and 6).
c) To study the influence of fermentation on the bread's dairy protein´s bioactive properties, particularly on the antioxidant and anti-inflammatory activities (see Papers 3 and 6);

d) To assess the influence of yogurt-derived lactic acid bacteria on generating acidulous conditions capable of activating/stimulate endogenous cereal enzymes proteolytic activity (see Paper 7);

e) To evaluate the influence of microbial and cereal enzymes in situ proteolytic activity on technological properties and on the nutritional profile, such as on antioxidant capacity, glycemic index, and protein digestibility (see Paper 7).

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Results and discussion

This section of results and discussion is encompassed by three different chapters (C2, C3 and C4), as a compilation of the scientific articles published and submitted for publication.

Chapter 2 (C2). Yoghurt and curd cheese as potential ingredients to improve technological properties and nutritional/functional profile of wheat bread

From this chapter, in which the addition of yoghurt or curd cheese to improve the technological, nutritional, functional, and bioactive properties of wheat bread was evaluated, three different studies were performed, resulted in three publications.

Therefore, the experimental work performed in this chapter will be presented in the following research papers:

- **Research paper 1**:

Graça, C., Raymundo, A., and Sousa, I. (2019). Wheat Bread with Dairy Products—Technology, Nutritional, and Sensory Properties. *Applied. Sciences, Eco Nov. Foods Spec. Issue*s, 9, 4101. https://doi.org/10.3390/app9194101. (Impact Factor: 2.474, Q2).

- **Research paper 2:**

Graça, C., Raymundo, A., Sousa, I. (2021). Yoghurt and curd cheese addition to wheat bread dough: Impact on in vitro starch digestibility and estimated glycemic index. *Food Chemistry*, 339, 127887. https://10.1016/j.foodchem.2020.127887 (Impact Factor: 6.306, Q1).

- **Research paper 3:**

Graça, C., Mota, J., Lima, A., Boavida-Ferreira, R., Raymundo, A., Sousa. (2020). Improving bioactive properties of wheat bread by yoghurt or curd cheese supplements: Antioxidant, anti-inflammatory, and antibacterial activities. [**Submitted to** Journal of Food Science and Technology, JFST-D-20-03129, 19 of November 2020 (Impact Factor: 2.705, Q2).

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Article

Wheat Bread with Dairy Products—Technology, Nutritional, and Sensory Properties

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Featured Application: Bakery industry, as nutritional and functional breads, with a considerable contribution to balance the daily diet for children and seniors in terms of proteins and minerals.

Abstract: As the relation between diet and health became a priority for the consumers, the development of healthy foods enriched with functional ingredients increased substantially. Dairy products represent an alternative for new products and can be used to enhance the functional and nutritional value of bakery products. The addition of yoghurt and curd cheese to wheat bread was studied, and the impact on the dough rheology, microstructure, bread quality, and sensory properties were evaluated. Dairy product additions from 10 to 50 g and higher levels up to 70 g of yoghurt and 83 g of curd cheese were tested. Replacements were performed on wheat flour basis and water absorption. It was observed that the yoghurt additions had a positive impact on the rheology characteristics of the dough. For curd cheese additions, the best dough evaluated on extension was the 30 g of wheat flour formulation. In both cases, the microstructure analysis supported the results obtained for doughs and breads. These breads showed a significant improvement on nutrition profile, which is important to balance the daily diet in terms of major and trace minerals and is important for health-enhancing and maintenance. Good sensorial acceptability for breads with 50 g of yoghurt and 30 g of curd cheese was obtained.

Keywords: dairy products; gluten network; rheology; nutrition profile; wheat bread

2.1. Introduction

Bakery products are staple foods, widely consumed in large quantities worldwide, with an important role in human nutrition [1]. Due to the increased awareness of health issues, the bakery industry is moving to provide functional and healthy foods, mainly via fortification with satiating and active ingredients, such as proteins, fibers, minerals, vitamins, and bioactive peptides [2] in response to an increasingly demanding consumer.

The incorporation of ingredients that exhibit functional properties, in addition to traditional nutrients, is an interesting alternative to the development of innovative bakery foods. Brazilian bread cheese is known worldwide, but the incorporation of dairy products (such as yogurt and curd cheese) in bread formulations is not common in the bakery markets.

Nutritional benefits of dairy products (DP) include increasing the amount of minerals with good assimilability (mainly Ca and P), vitamins (A and B12), protein, and essential amino acids (lysine, methionine, and tryptophan). Technology benefits may also be considered and can include improvement of dough handling properties and bread quality (flavor, crumb structure, and texture). These benefits result from the effect of protein and milk fat on the bread structure [3].

Good nutrition, especially adequate, easily digestible protein and mineral intake, is a determinant factor for the human health. Protein and minerals are considered key nutritional components in a well-balanced diet with an important contribution to maintain muscle mass and bone structure [4]. Some studies have reported the technological properties of dairy products as potential ingredients into a wide variety of products [5], such as infant formulas [6], that could aid in achieving dietary requirements for children and seniors.

Yoghurt (Yg) is a fermented milk product that consists of a casein network formed at the isoelectric point [7], being the acid obtained by the activity of the specific lactic acid bacteria (LAB), *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus* cultures. Yg is considered the most popular dairy product worldwide for its nutritional and health benefits, since it is a rich source of protein (casein), vitamins (B2, B6, and B12), and minerals (such as Ca, P, and K), and contributes to good microbiota. This product is a potential ingredient for bakeries, representing an interesting alternative for new bakery products [8], and can be incorporated into bread formulations as a fresh product.

Curd cheese (Cc) is a co-product obtained by the thermal denaturation and subsequent precipitation of the soluble whey proteins. These products are considered to have a high protein nutritional value, representing of 20–30% of the proteins present in bovine milk. This complex mixture of globular protein molecules (α-lactalbumin and β-lactoglobulin) presents a higher protein efficiency ratio than wheat proteins [9] and is considered to be an important source of essential amino acids (leucine, isoleucine, and valine). Previous work has shown significant increments in protein, ash, and mineral contents (Ca, K, Mg, and P) in bread with whey protein concentrate [10]. However, some authors reported that whey protein exerted some negative effects on bread quality by depressing the loaf volume and increasing the crumb firmness [11].

The aim of the present study was to evaluate the influence of yoghurt and curd cheese addition as potential ingredients for the design of new bread formulations and assess their effect on wheat dough (WD) rheology and bread quality, as well as the aging kinetics and nutritional profile.

Different contents of yoghurt and curd cheese were added to wheat flour dough, and the impact on dough rheology was assessed by small amplitude oscillatory measurements (SAOS) and extension properties. Dough microstructure was also evaluated. The breads produced were also characterized in terms of microstructure, texture profile, and shelf life. Sensory analysis was performed to evaluate the acceptability of the developed breads. The maximum addition of each dairy product was discussed in terms of the potential applications for the market.

2.1.2. Materials and Methods

2.1.2.1. Raw Material

Bread was prepared using commercial wheat flour—WF (EspigaT65) purchased from the Granel cereal milling industry (Alverca, Portugal) with (per 100 g) 13.5 g of moisture, 11.3 g of protein, and 0.6 g of ash (supplier Near Infra-Red results (Hägersten, Sweden)

The fresh yogurt (Yg) used was a commercial product from LongaVida, Portugal, with the label stating the nutritional composition per 100 g: 3.7 g of protein, 0.8 g of ash, 3.7 g of lipids, 5.5 g of carbohydrates, 0.06 g of salt, and 0.12 g of Ca. The dry extract of Yg was determined from the standard Portuguese method: NP.703-1982, corresponding to 11.5% of dry matter (88.5% of moisture).

Fresh curd cheese (Cc) from Lacticínios de Paiva (Lamego, Paiva, Portugal), was used with the following nutritional composition per 100 g: 10.7 g of protein, 3.5 g of ash, 0.2 g of fibber, 9.0 g of lipids, 3.0 g of carbohydrates, 0.2 g of salt, and 0.3 g of Ca. The dry extract of Cc was determined from the standard Portuguese method: NP.3544-1987, corresponding to 25.8% of dry matter (74.2% of moisture).

Commercial white crystalline saccharose (Sidul, Santa Iria de Azóia, Portugal), sea salt (Vatel, Alverca, Portugal), baker's dry yeast (Fermipan, Lallemand Iberia, SA, Setúbal, Portugal), and SSL-E481-sodium stearoyl-2 lactylate (Puratos, Portugal) were also used.

2.1.2.2. Bread Dough Preparation

Bread dough preparation was performed according to a previous study [12]. Water absorption (WA) was determined and optimized to 61.8 g/100 g of wheat flour, according to the farinograph tests (AACC 54–21.02). Control dough and doughs enriched individually with Yg and Cc were prepared considering additions of 10, 20, 30, and 50 g, and the highest levels were 70 g for Yg and 83 g for Cc. The maximum incorporation of the dairy products is determined by the level of water of the wheat dough, i.e., the water absorption with a value of 61.8%. Replacements of dairy products were based on a 100 g of wheat flour basis (baker´s formula), and the dry extract of each dairy product was considered to calculate the moisture coming from each dairy product addition, i.e., for 50 g of yoghurt addition the value for moisture is 44.2 g and dry matter 5.7 g, so we added 94.3 g of wheat flour and 17.6 g of water. The following other ingredients added were kept constant: salt: 1.0%; sugar: 0.6%; dry yeast: 2.4% and sodium stearoyl-2 lactylate: 0.3%. The different bread formulations tested are presented in Table 1.

Table 1. Bread formulations optimized to the control bread (CB) and different levels of yoghurt (Yg) and curd cheese (Cc) addition, considering the moisture content and dry extract of each dairy product (DP) *.

Ingredients	CB	Yg_{10g}	Cc_{10g}	Yg_{20g}	Cc_{20g}	Yg_{30g}	Cc_{30g}	Yg_{50g}	Cc_{50g}	Yg_{70g}	Cc_{83g}
Wheat flour	100.0	98.9	97.4	97.7	94.8	96.6	92.2	94.3	87.1	92.0	78.5
Deionized water	61.8	53.0	54.4	44.1	47.0	35.3	39.6	17.6	24.7	0.0	0.0
Yoghurt *	0.0	10.0	-	20.0	-	30.0	-	50.0	-	70.0	-
Curd cheese *	0.0	-	10.0	-	20.0	-	30.0	-	50.0	-	83.3

* 100 g of Yg: moisture—88.5 g; dry extract—11.5 g; * 100 g of Cc: moisture: 74.2 g; dry extract: 25.8 g.

2.1.3. Physical Characterization of the Wheat Dough

2.1.3.1. Extension Properties

Wheat dough extension properties (WDEP) were accessed by uniaxial extension tests using an SMS/Kieffer Dough and Gluten Extensibility Rig probe coupled to the texture analyzer (Texturometer TA-XTplus, Stable MicroSystems, Surrey, UK) with a load cell of 5 kg, according to the method described earlier [13] with some modifications. The pieces of dough were left to rest for 60 min at 30 °C. The measurement conditions were measure force in tension, test speed 1.0 $mm \cdot s^{-1}$, distance 70 mm, and triggers force 5 g. The force required to stretch the dough sample and the displacement of the hook were recorded as a function of time. The parameters of major importance were (i) R—resistance to deformation (N), (ii) E—extensibility (mm), and (iii) deformation energy ($N \cdot mm^{-1}$). The ratio number R/E ($N \cdot mm^{-1}$) was also calculated and reflects the balance between the levels of elastic and viscous components of the wheat dough [14].

2.1.3.2. Rheology Characterization

Fundamental viscoelastic behaviour of wheat dough (WD) after fermentation was studied by small amplitude oscillatory shear (SAOS) measurements using a controlled stress rheometer (Haake Mars III-Thermo Scientific, Karlsruhe, Germany) with an Universal Temperature Control-Peltier system to control temperature. The procedure was in agreement with the conditions previously optimized [12]. Stress sweeps were performed at 0.1 and 1.0 Hz to determine the linear viscoelastic region and select the stress to use. Frequency sweep tests, performed at 5 °C to inhibit the yeast fermentative activity, were applied to evaluate the effect of the Yg and Cc additions on dough structure. The evolution of the dough after fermentation time, expressed in terms of storage (G´) and loss (G") moduli, were obtained, ranging the frequency from 0.001 Hz to 100.0 Hz at a constant shear stress within the previously determined linear viscoelastic region of each sample. All determinations were repeated at least three times to ensure reproducibility of the results.

2.1.4. Technological Characteristics of the Bread

2.1.4.1. Bread Texture

Bread texture was evaluated using a texturometer TA-XTplus (Stable MicroSystems, Surrey, UK) in penetration mode. Each bread sample was cut into slices with a height of 20 mm and 120 × 100 mm rectangular shape, and slices rested for 15 min before testing. An acrylic cylindrical probe with 10 mm diameter pierced 5 mm of the sample at 1 mm/s of crosshead speed, with a load cell of 5 kg, according to the method earlier described in reference [12]. Comparison of the bread texture with different Yg and Cc contents was performed in terms of firmness.

Bread staling was evaluated measuring firmness during a storage time of 96 h, and the aging kinetics of the bread was described as a function of the dairy product incorporation according to a linear Equation (1)

$$\text{Firmness} = A * \text{time} + B, \quad (1)$$

where A can be considered the aging kinetics and B the initial firmness.

2.1.4.2. Quality Parameters of Bread

Bread moisture was determined according to the standard method AACC 44–15.02. Water activity (a_W) variations were determined at room temperature (Hygrolab, Rotronic).

Bake loss (BL) of breads, which is defined as the amount of water and organic material (sugars fermented and CO_2 released) lost during baking [15], was calculated, according to the Equation (2).

$$\textit{Bake loss (\%)} = [(W_{bb} - W_{ab})/W_{bb}] \times 100 \quad (2)$$

where
W_{ab}: weight of the loaf after baking;
W_{bb}: weight of the loaf before baking.

Bread volume was determined by rapeseed displacement according to the standard method AACC 10-05.01 after 2h of cooling down [8]. Specific bread volume was calculated using Equation (3).

$$\text{Specific volume (cm}^3\text{/g)} = \text{bread volume (cm}^3\text{)/bread weight (g)} \quad (3)$$

Knowing the weight and the volume of the bread, the specific weight (g/cm^3) can also be estimated using the Equation (4), i.e., the reciprocal of specific bread volume.

$$\text{Specific weight (g/cm}^3\text{)} = \text{bread weight (g)/bread volume (cm}^3\text{)} \quad (4)$$

All the experiments were done at least in triplicate.

2.1.5. Image Analysis of the Bread Slices

The gas cell number of the crumb breads was evaluated 120 min after production using image analysis technology. Images of three slices of breads (control, yoghurt, and curd cheese breads) were scanned full-scale using an Image Scanner (Xerox Corporation, Webster, NY, USA).

A threshold method was used for differentiating gas cells, and the segmentation was performed manually by binarization of grey-scale images into black-and-white images using the Otsu and default ImageJ algorithms, carried out in the ImageJ-based Fiji 1.46 software package [16].

2.1.6. Nutritional Composition of the Bread

Total nitrogen content was analyzed by the MicroKjeldahl method (ISO 20483:2006). The quantification was performed by molecular absorption spectrophotometric equipment (Skalar-San^{plus} System, Auto-Analyser, Giesen, Germany) [17,18].

Fat content was determined according to NP 4168. Ash content was determined by incineration at 550 °C in a muffle furnace (AACC Method 08-01.01).

Total mineral contents were determined by inductively coupled plasma atomic emission spectrometer (ICP-AES: Thermo System, ICAP-7000 series). All the experiments were done in triplicate.

2.1.7. Dough and Bread Microstructure

A scanning electron microscope (TM3030 ^{PLUS}—TabletUp Microspcope - Hitachi, Fukuoka Japan) was used to observe the microstructure of the control dough, doughs, and breads obtained with dairy product additions. Samples were placed on the specimen holder, dried automatically by the equipment, and the freezing model was applied (−14 °C). The observations were analyzed at 400 × of magnification, and 150 × of magnification, with scale bar of 200 μm for doughs and 500 μm for bread crumb, respectively.

2.1.8. Sensory Evaluation

Sensory evaluation of breads was carried out in a sensory evaluation room, in individual cabinets, under white light, and at room temperature by a panel of 25 untrained panelists, ages between 20–50, who were regular consumers of bread. Samples were analyzed 24 h after baking. Before analysis, the samples were sliced into equally sized slices (2 cm thick), coded, and then randomly served. Breads were evaluated based on their appearance and sensory acceptance, scoring with the descriptors: color, aroma, taste, texture, and overall acceptability on a five-point hedonic scale, where 1-"dislike extremely"; 3-"indifferent"; 5-"like extremely". Breads were considered acceptable if their mean scores for overall acceptance were above 3 (like or like extremely).

2.2. Statistical Analysis

The experimental results were statistically analyzed by determining the average value, standard deviation, and the significance level was set at 95% for each parameter evaluated. Statistical analysis (RStudio, Version 1.1.423) was performed by variance analysis, the one factor (ANOVA), and post-hoc comparisons (Tukey test).

2.3. Results and Discussion

2.3.1. Wheat Dough Extension Properties (WDEP)

The effect of yoghurt (Yg) and curd cheese (Cc) addition on wheat dough extension properties at 30 °C after 60 min of fermentation time was studied, and the results obtained are presented in Figure 1. It is clearly observed that the Yg and Cc have different impacts on dough extension properties, most probably due to the different nature and structure of the proteins added—acid precipitated caseins from Yg and soluble whey protein precipitated by thermal denaturation from Cc. The extension properties are positively affected by Yg addition and are adversely affected by Cc incorporations.

Considering Figure 1A, the dough resistance values were similar to control dough up to 50 g of Yg addition, whereas for 70 g a reduction of about 21% was observed compared to control.

For Cc additions, all the levels evaluated result in dough resistance values lower than the control, with a significant reduction of about 64% ($p < 0.05$) for the highest level of Cc added (83 g).

From Figure 1B, C at 20 g of Yg addition, a steep increase both in extensibility (B) and deformation energy (C) is observed and remained constant for the rest of the levels evaluated, with higher values compared to the control. Relative to Cc additions, lower values of extensibility and deformation energy than the control dough were observed, except at 30 g of addition, which showed values like the control.

In terms of R/E ratio values, the differences observed for Yg, and Cc additions are clearly reflected on Figure 1D. The resistance versus extensibility is an important ratio to evaluate the balance between

dough resistance (elasticity) and extensibility (viscosity), and a good combination of both parameters is required to obtain desirable dough properties and bread quality [13].

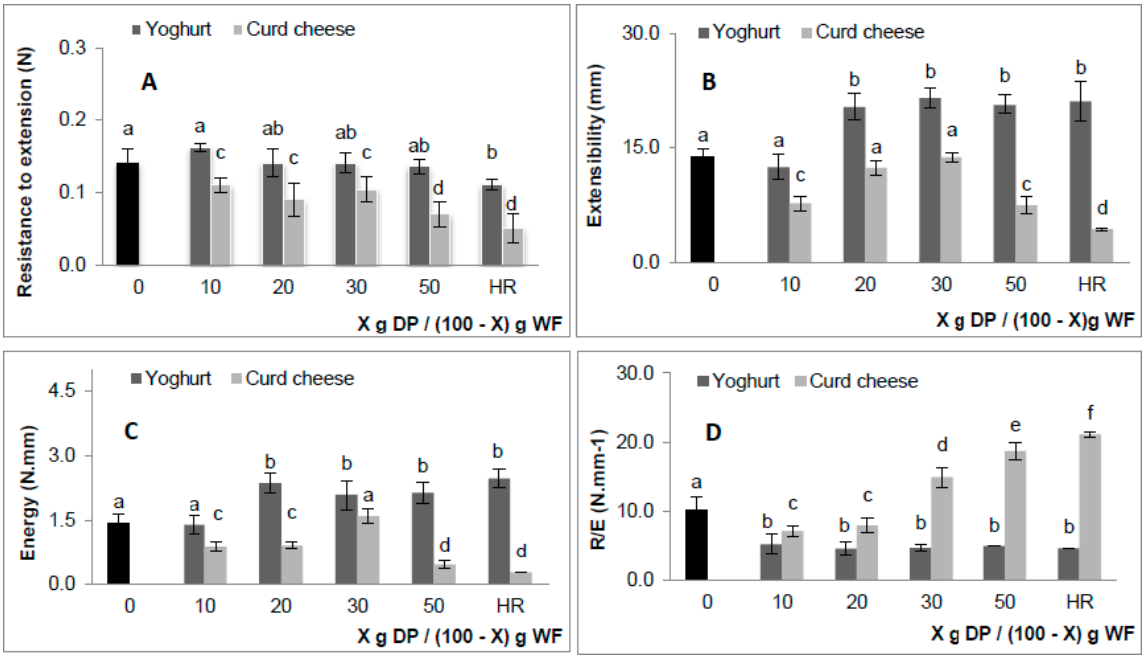

Figure 1. Effect of the Yg and Cc incorporation, after 60 min of fermentation time at 30 °C, on the extension properties of the dough, in terms of: (**A**)—resistance to extension (N), (**B**)—extensibility (mm), (**C**)—deformation energy (N.mm^{-1}) and (**D**)—ratio R/E (N.mm^{-1}), with different levels of dairy product (DP) additions: 10, 20, 30, 50, and higher replacements (HR) tested—70 g for Yg and 83 g for Cc—compared with control dough (0 g/100 g). Different letters indicate statistically significant differences at $p < 0.05$ (in Tukey test), compared with the control dough parameters (black bars).

Overall, the addition of Yg has a positive impact on the structure of the dough, which was seen by the increase of the extensibility and deformation energy. One can suggest a synergic interaction between the Yg proteins and wheat proteins on the gluten network development. In addition, the presence of the exopolysaccharides produced by the lactic acid bacteria present in yoghurt, can also act on the system as lubricants, together with gliadins, giving more stability and extensibility to the wheat dough. Similar results were observed by other authors [3,10] who also reported an increase in deformation energy due to the strengthening effect of hydrolyzed caseins and sodium caseinates from the added dairy product.

However, opposite results were obtained for Cc addition that affected the dough properties in extension, suggesting that denatured whey protein has an antagonistic effect with wheat proteins, strongly affecting the gluten matrix. These results can be attributed to the gluten proteins dilution effect [11,12] and protein competition for available water, impacting negatively on dough development [19]. Similar results were previously reported by other researchers [10], demonstrating that dairy by-products such as whey protein, in general, reduce the extensibility and the deformation energy. Therefore, it can be stated that the Yg addition had no adverse impact on the technology characteristics of the dough, which will have a positive impact on bread quality. The incorporation of the Cc implied a significant reduction on extensibility properties, which would promote a significant depression on bread volume.

2.3.2. *Dough Viscoelastic Behaviour*

The changes of dough viscoelastic properties after 60 min of fermentation time were monitored by a controlled stress rheometer. The variation of storage (G′) and dissipative (G″) moduli was recorded

by frequency sweeping from 0.001 to 100 Hz at a low constant stress value under linear conditions, and the G′ values were plotted at 0.1 Hz and 1.0 Hz to show the rheology changes observed by addition of the Yg and Cc to the wheat dough (Figure 2A1, B1).

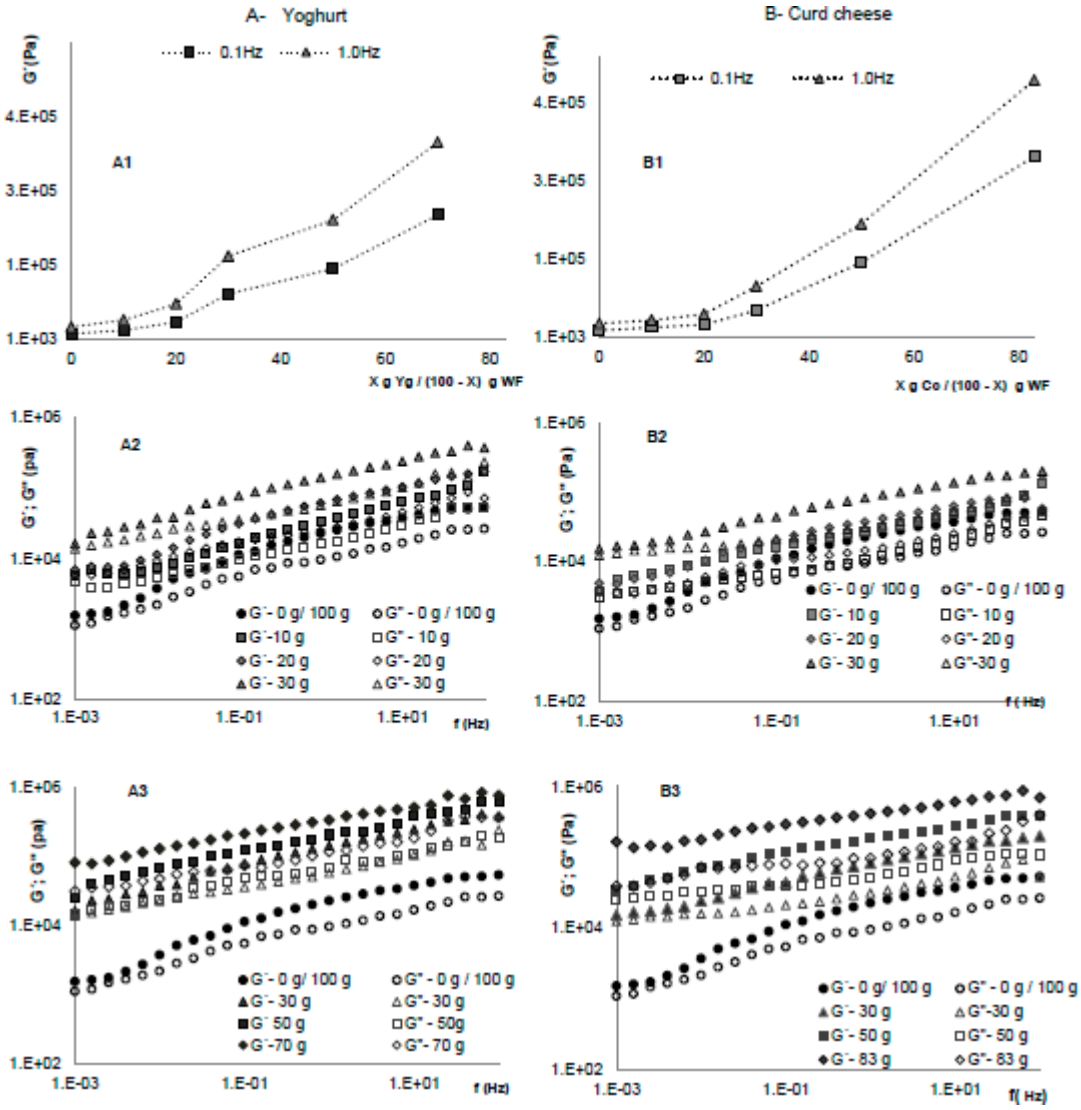

Figure 2. Variation of storage (G′) at 0.1 Hz and 1.0 Hz (**A1**–**B1**) and frequency sweep curves—change of the storage (G′—close symbols) and loss (dissipative (G″)—open symbols) moduli with frequency (**A2**, **A3**; **B2**, **B3**), for samples with differents levels of DP: A yoghurt doughs and B for curd cheese doughs.

As can be seen at Figure 2A1, B1, which are focused on G′ at 0.1 and 1.0 Hz, the behaviour of the doughs with Yg and with Cc were similar. In both, the steepest increase on slope arises from concentrations over 20 g of addition. The almost linear increase of G′ with Yg and Cc addition is characteristic of the dough structuring, indicating a reinforcement of higher density of the molecular links.

The presence of vegetable (cereals) and animal (milk) proteins adds complexity to the protein matrix with protein–protein interactions, which leads to an increasingly structured material [3,20].

Figure 2A2/A3, B2/B3 represent the mechanical spectra obtained with different Yg (A) and Cc (B) additions, where it can be observed that the G′ and G″ moduli increase with dairy product addition. In general, all the systems show a certain degree of dependence on the frequency range applied, and the addition of Yg and Cc promote the reinforcement of the dough structure, expressed in terms of viscoelastic functions (G′ and G″) increase.

The apparent disagreement between technology performances—empirical large amplitude extensional rheology (Kieffer Dough and Gluten Extensibility Rig) and small amplitude oscillatory fundamental rheology—can be explained, beyond the different nature and structure of the proteins in play, if the presence of a significant amount of exopolysaccharides in Yg is considered. In fact, the major differences in nature from Yg and Cc are the content of exopolysaccharides produced by lactic acid bacteria and protein structural characteristics of the Yg material. As was recently published [21], the impact of exopolysaccharides on the rheology of dough shows positive interactions with the gluten matrix development.

2.3.3. Evaluation of Bread Properties

2.3.3.1. *Bread Texture and Aging Kinetics*

The texture of the breads produced with different contents of yoghurt and curd cheese was evaluated in terms of firmness by a puncture test. The determination of firmness during a storage time of 96 h at room temperature was also performed to evaluate the effect of dairy product additions on the kinetics of bread aging (Figure 3A, B) [12]. From Figure 3A, B, it can be observed that both Yg and Cc additions had different impacts on the aging kinetics of the breads.

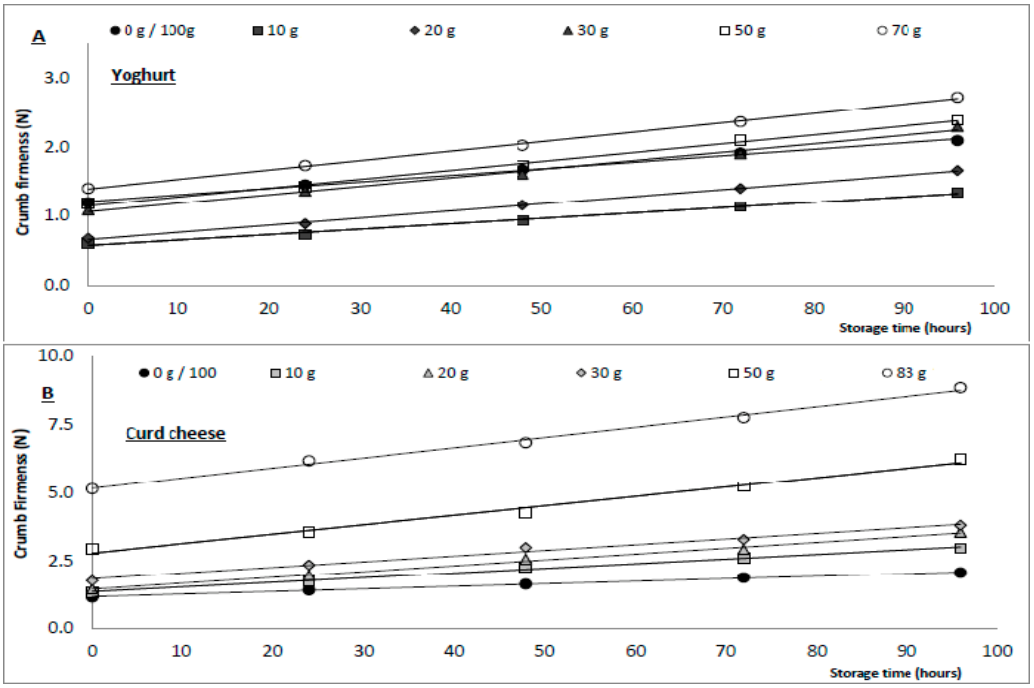

Figure 3. Variation of firmness (N) and aging kinetics for breads prepared with different contents of Yg 2.3.3.2. and Cc (**B**) addition, replacing the flour in bread formulation, at initial and 96 h of storage time, and respective linear equations: staling rate as crumb firmness with time for the different concentrations of Yg and Cc.

Bread aging kinetics was described as a function of the dairy products incorporation, and a positive linear relation ($R^2 > 0.98$) was observed, with parameters presented in Table 2, and clearly reflect the impact of the different levels of Yg and Cc addition on bread aging kinetics (A, the slope) and firmness (B, the interception).

As can be seen from Figure 3 A, B and the respective linearization's on Table 2, the Yg breads are clearly less firm than Cc breads: the initial firmness of the control bread (1.21 N) is higher than low incorporations of Yg up to 50 g (1.15 N), and for higher replacements (70 g), the firmness is slightly higher (1.39 N) than control.

Table 2. Parameters of linear relationship: bread aging kinetics (A) and firmness (B) obtained for control bread (CB—0 g/100 g) and for the different levels of yoghurt (Yg) and curd cheese (Cc) tested.

DP Levels	Aging Kinetics (A)	Firmness (B)	R^2
CB	0.94×10^{-2}	1.21	0.997
Yg_{10g}	0.78×10^{-2}	0.58	0.991
Yg_{20g}	1.03×10^{-2}	0.67	0.999
Yg_{30g}	1.23×10^{-2}	1.06	0.992
Yg_{50g}	1.28×10^{-2}	1.15	0.998
Yg_{70g}	1.36×10^{-2}	1.39	0.995
Cc_{10g}	1.70×10^{-2}	1.40	0.995
Cc_{20g}	2.14×10^{-2}	1.50	0.995
Cc_{30g}	2.08×10^{-2}	1.87	0.987
Cc_{50g}	3.45×10^{-2}	2.81	0.988
Cc_{83g}	3.72×10^{-2}	5.18	0.994

The Yg breads aging kinetics (A) ranging from 0.78×10^{-2} N/h for the 10 g up to 1.36×10^{-2} N/h for the 70 g of Yg addition and are like control bread (0.94×10^{-2} N/h). In the case of Cc breads, a completely different effect can be observed, i.e., all breads are firmer, and the rate of staling is higher than the control bread. The initial firmness up to 30 g of addition is like the control, but at 50 g of Cc addition, the increased is about 130%, and at 83 g, it goes up to 330%.

The rate of staling is also increasing substantially, ranging from 1.70×10^{-2} N/h for the 10 g, up to 3.72×10^{-2} N/h for the 83 g curd cheese addition, representing an increase of 120%.

Similar results were obtained in earlier studies [11], indicating that the whey protein significantly increase the bread crumb firmness. Our findings are also in agreement with those obtained by other authors [3,10] with the addition of dairy products in bread dough formulations.

This difference in bread texture with addition of Yg and Cc should reflect the complexity of the interactions of the major macromolecules at play and agree with the results observed in extension properties evaluation (Figure 1). Starting from the gluten matrix, when yoghurt is added, some reinforcement of the structure was seen by the increase of extensibility and deformation energy. Values of dough resistance were similar to the control, not interfering substantially on the R/E values. The opposite is observed for curd cheese breads, where R/E values increased steeply, revealing an imbalance of the elastic and viscous components of the wheat dough, clearly reflected in bread texture and aging kinetics.

2.3.3.3. *Quality Parameters of the Bread*

The quality features of breads, i.e., moisture content, water activity (aw), specific bread volume (SBV), and the percentage of bake loss (BL), were determined, as well as the crumb cells numbers. The results of quality parameters obtained for yoghurt and curd cheese breads are summarized in Table 3. No significant differences were observed in initial moisture content (43.5–42.6%) and water activity (0.95–0.92) for the different yoghurt breads and the control.

According to the results obtained for the Cc breads, significant differences were observed for the initial moisture content, varying between 43.5% and 38.9%, with a reduction of 11.0% of moisture, compared to the control bread and the higher Cc concentration (83 g). This effect can be explained by the water competition between wheat proteins and precipitated whey proteins during the dough development. Similar results were obtained by other authors [10] by testing the incorporation of dairy by-products on new bread formulations. Water activity had no significant variations ($p > 0.05$).

Table 3. Quality parameters, moisture, aw, bake loss, specific bread volume (SBV), and cell numbers of the control bread (CB—0 g/100 g) and breads produced with yoghurt (Yg) and curd cheese (Cc) addition *.

X g/(100 − X) g WF	Moisture (%)	aw	Bake Loss (%)	SBV (cm^3/g)	Cell Numbers
CB	43.50 ± 0.31 ^{a}	0.95 ± 0.01 ^{a}	16.16 ± 0.58 ^{a}	4.44 ± 0.09 ^{a}	2748.5 ± 52.3 ^{a}
Yg_{10g}	42.81 ± 0.11 ^{a}	0.93 ± 0.05 ^{a}	13.13 ± 1.86 ^{a}	4.40 ± 0.25 ^{a}	2875.0 ± 30.1 ^{ab}
Yg_{20g}	43.60 ± 1.34 ^{a}	0.92 ± 0.02 ^{a}	14.70 ± 1.06 ^{a}	4.10 ± 0.23 ^{ab}	2982.0 ± 65.4 ^{ab}
Yg_{30g}	43.65 ± 0.34 ^{a}	0.93 ± 0.03 ^{a}	13.15 ± 2.48 ^{a}	3.84 ± 0.26 ^{ab}	3015.3 ± 38.7 ^{b}
Yg_{50g}	43.30 ± 0.33 ^{a}	0.93 ± 0.05 ^{a}	12.07 ± 1.29 ^{b}	3.61 ± 0.36 ^{ab}	2799.7 ± 67.0 ^{a}
Yg_{70g}	42.60 ± 0.27 ^{a}	0.92 ± 0.04 ^{a}	11.55 ± 2.26 ^{b}	3.30 ± 0.23 ^{b}	2568.0 ± 85.0 ^{a}
Cc_{10g}	44.10 ± 0.24 ^{a}	0.95 ± 0.03 ^{a}	11.40 ± 0.26 ^{b}	3.90 ± 0.22 ^{b}	1131.0 ± 77.0 ^{b}
Cc_{20g}	43.10 ± 0.08 ^{ab}	0.94 ± 0.01 ^{a}	10.52 ± 1.15 ^{b}	3.47 ± 0.18 ^{bc}	1035.0 ± 88.2 ^{b}
Cc_{30g}	42.40 ± 0.50 ^{bc}	0.93 ± 0.05 ^{a}	10.70 ± 1.41 ^{b}	3.21 ± 0.04 ^{c}	1235.0 ± 83.1 ^{c}
Cc_{50g}	41.93 ± 0.20 ^{c}	0.94 ± 0.03 ^{a}	9.00 ± 0.60 ^{b}	2.84 ± 0.16 ^{d}	344.0 ± 15.5 ^{d}
Cc_{70g}	38.85 ± 1.48 ^{d}	0.95 ± 0.02 ^{a}	9.60 ± 1.27 ^{b}	1.28 ± 0.09 ^{e}	276.0 ± 25.0 ^{e}

* Different letters (a, b, c, d) within the same column, for each dairy product, indicate statistically significant differences at $p < 0.05$ (Tukey test), compared with the bread control parameters.

No significant differences ($p > 0.05$) in specific bread volume (SBV) values between control and Yg breads, up to 50 g, were observed (Figure 4A), as well as in the crumb cell numbers (Table 3). The highest replacement tested, 70 g Yg, presented slightly lower SBV (3.30 cm^3/g), compared to the control bread (4.44 cm^3/g), representing a decrease of 26% ($p < 0.05$). In terms of bake loss values, no significant differences, up to 50 g of Yg addition, were observed. However, for 70 g of Yg addition the bake loss was lower (11.6%), compared to control bread (16.0%), and this is in line with the slightly higher density observed, leading to a less weight loss during baking.

Figure 4. Breads obtained with different levels of yoghurt (**A**) and curd cheese (**B**) incorporation: 10 g, 20 g, 30 g, 50 g and 70 g Yg, and 83 g Cc/100 g wheat flour, compared to control bread (without dairy product, 0 g/100 g wheat flour).

These good bread volumes of the yoghurt bread are probably due to the synergic effect of the different proteins on the system. In addition, the contribution of exopolysaccharides, by building a structured polysaccharide network that interacts with the gluten matrix [21], can give more stability and extensibility to the wheat dough (Figure 1B), contributing to the gas retention and leading to a good appearance and desirable bread volume, as observed in Figure 4A.

The addition of Cc to the dough reduces significantly ($p < 0.05$) the specific bread volume (4.44–1.28 cm^3/g) during the baking process compared to control bread at the highest Cc concentration (83 g). As expected, the crumb gas cells for these breads decreased significantly ($p < 0.05$), varying from 2748.5 (control bread) to 276.0 (83 g of Cc), a reduction of 90% that is a consequence of the depression of 70% in bread volume, clearly observed in Figure 4B. These results, caused by the addition of precipitated whey protein, could be attributed to the antagonistic interaction between these proteins and the gluten complex, reducing the flexibility and the extensibility of the network (Figure 1B), which increases the density of the bread reducing the volume [1].

Our results agree with those obtained by other authors [3] who reported the effect of the whey protein on loaf volume decrease, and hence on the reduction of the gas cell numbers, but sodium caseinate and hydrolyzed casein additions had no significant impact on these values.

2.3.4. Microstructure of Doughs and Breads

Environment scanning electron microscopy was used to evaluate the microstructure of the doughs, obtained with different levels of yoghurt and curd cheese (30 g and 50 g), after 60 min of fermentation at 30 °C (Figure 5A1–A5). The microstructure of the breads crumb was also observed (Figure 5B1–B5). Figure 5A1–A5 show that dough is made up of the gluten network with small and large starch granules characteristic of the wheat dough.

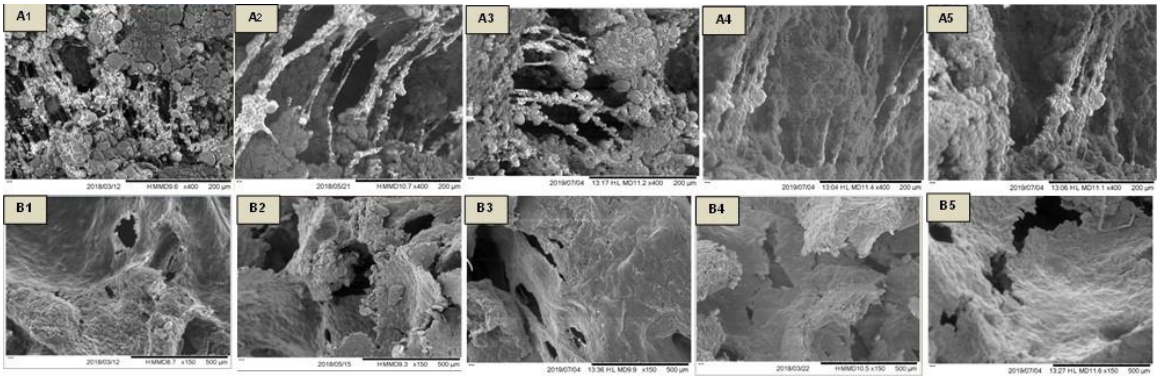

Figure 5. Scanning electron micrographs (400 ×, scale bar = 200μm for dough; 150 ×, scale bar = 500 μm for bread crumb): A—fermented dough (60 min/37 °C); **A1**—Control dough; **A2**—30 g Yg, A3—50 g Yg; A4—30 g Cc, A5—50 g Cc; B—breads: B1—control bread, B2—30 g Yg, B3—50 g Yg; B4—30 gCc, B5—50 g Cc, at first day of storage time.

Comparing the Yg doughs, the dough with 30 g and 50 g (Figure 5A2, A3) displayed a remarkable gluten film, with defined glutenins strands when compared to control dough, which is more evident at 30 g of Yg addition. These results agree with those obtained on the extension properties, supporting the improvement of the flexibility of the net responsible for the increase on the extensibility and deformation energy values (Figure 1B, C). With Cc additions, the gluten film became less notable, as shown in Figure 5 (A4—30 g and A5—50 g of Cc addition), revealing that denatured whey protein interfered with the development of the gluten network, reducing the extensibility of the net (Figure 1B) and thus affecting the gas retention. Therefore, with the structure morphology of these doughs, the reduction of the specific volume of the breads was expected, as observed (Figure 4B).

Figure 5 (B1–B5) show the changes of crumb bread structure of the Yg bread (B2–B3) and Cc bread (B4–B5) compared to the control bread (B1). A more complex cell structure with higher number of gas cells between starch granules and denatured gluten in bread crumb with 30 g of Yg compared to the control is also noticed. This microstructural feature is associated with a lower staling rate, a low degree of firmness, and better bread volume [8]. For breads with 50 g of Yg addition, a more sheet-like structure was observed, probably due to the higher casein's interactions and most probably by the presence of exopolysaccharides that interact with the gluten network, leading to a more

homogeneous structure [21]. For the Cc addition, Figure 5B4, B5, a heterogeneous and disaggregated bread crumb is observed, which is a consequence of the interference of the denatured whey protein in the gluten network.

The scanning electron microscopy images obtained for breads with Yg and Cc revealed that the interactions between dairy proteins with different nature and structures with gluten and starch matrix could partially explain and support the results obtained in dough extensibility and bread quality properties.

2.3.5. Nutritional Composition of the Breads

The nutritional composition, including the mineral contents, was determined for control and experimental breads obtained with 30 g and 50 g of Yg and Cc addition. A positive impact on protein and ash content was observed for both levels of dairy products tested. However, a remarkable effect was obtained for 50 g of addition in both cases, representing an increase of 7% and 30%, respectively, for Yg breads and 31% and 66% for Cc bread (Table 4). In terms of lipids, there was an increase of 28.0% for 50 g Yg addition and 163.0% of 30 g of Cc addition (the highest level to be considered in terms of bread quality). This increasing in fat is not high for the Yg breads but is considerable in the case of the Cc breads, as these are mainly saturated fatty acids from milk. This can be considered an additional restriction related with the curd cheese incorporation.

Table 4. Nutritional composition * and mineral content of breads produced with yoghurt and curd cheese: 30 g and 50 g of yoghurt (Yg) and curd cheese (Cc).

	Control Bread	**Yoghurt Breads**		**Curd Cheese Breads**	
(g/100 g)	**0 g**	**30 g**	**50 g**	**30 g**	**50 g**
Ash	1.75 ± 0.02 ^{a}	1.94 ± 0.14 ^{a}	2.28 ± 0.11 ^{b}	2.61 ± 0.02 ^{b}	2.90 ± 0.12 ^{c}
Lipids	1.92 ± 0.15 ^{a}	2.22 ± 0.21 ^{a}	2.46 ± 0.20 ^{ab}	5.06 ± 0.06 ^{c}	8.03 ± 0.16 ^{d}
Proteins	8.44 ± 0.15 ^{a}	8.50 ± 0.17 ^{a}	9.03 ± 0.57 ^{a}	9.85 ± 0.17 ^{b}	11.02 ± 0.32 ^{c}
Carbohydrates	44.30 ± 0.45 ^{a}	44.20 ± 0.45 ^{a}	43.4 ± 0.22 ^{a}	40.15 ± 0.80 ^{b}	32.38 ± 1.43 ^{c}
kcal	228.22 ± 1.39 ^{a}	230.75 ± 1.23 ^{a}	232.22 ± 1.83 ^{a}	244.73 ± 2.87 ^{c}	249.5 ± 2.13 ^{d}
Mineral Content (mg/100 g)					
Na (mg/g)	4.29 ± 0.07 ^{a}	4.06 ± 0.11 ^{b}	3.73 ± 0.28 ^{b}	4.74 ± 0.05 ^{c}	3.16 ± 3.39 ^{b}
K	215.32 ± 1.41 ^{a}	227.80 ± 4.85 ^{a}	257.25 ± 5.4 ^{b}	160.91 ± 3.39 ^{c}	117.58 ± 2.52 ^{c}
P	107.05 ± 1.99 ^{a}	115.80 ± 2.29 ^{a}	128.9 ± 1.05 ^{b}	144.06 ± 0.73 ^{c}	164.93 ± 5.84 ^{c}
S	96.8 ± 1.70 ^{a}	98.60 ± 2.33 ^{a}	102.01 ±1.16 ^{a}	117.11 ± 2.35 ^{b}	138.62 ± 3.07 ^{c}
Ca	79.76 ± 1.70 ^{a}	105.90 ± 2.68 ^{b}	120.5 ± 0.41 ^{b}	160.92 ± 2.44 ^{c}	195.33 ± 2.29 ^{d}
Mg	24.90 ± 0.77 ^{a}	25.72± 0.66 ^{a}	30.83 ± 0.05 ^{b}	26.54 ± 0.41 ^{a}	27.96 ± 0.40^{a}
Fe	2.09 ± 0.44 ^{a}	1.36 ± 0.23 ^{b}	1.50 ± 0.12 ^{b}	1.35 ± 0.14 ^{b}	1.17 ± 0.02 ^{b}
Zn	1.17 ± 0.03 ^{a}	1.16 ± 0.01 ^{a}	1.08 ± 0.01 ^{a}	1.24 ± 0.03 ^{a}	0.87 ± 0.01 ^{c}
Mn	0.48 ± 0.05 ^{a}	0.61 ± 0.02 ^{b}	0.68± 0.01 ^{b}	0.55 ± 0.03 ^{a}	0.63 ± 0.05^{b}
Cu	0.18 ± 0.02 ^{a}	0.64 ± 0.10 ^{b}	0.52 ± 0.08 ^{b}	0.19 ± 0.01 ^{a}	0.46 ± 0.05 ^{b}

* Different letters (a, b, c, d) within the same row indicate statistically significant differences at $p < 0.05$ (Tukey test), compared with the bread control parameters.

Milk and dairy products are valuable sources of minerals with a good assimilability [11] and exert several essential physiological functions in the human body. As they contain major minerals (Ca, K, Mg, and P) and trace elements (including Cu, Fe, Mn, Zn), incorporation of both dairy products promotes a significant improvement on mineral composition ($p < 0.05$) in general (Table 4).

In the breads with 50 g of addition, a significant increase was observed in Ca (Yg–51%, Cc–145%), K (Yg–20%), P (Yg–21%, Cc–54%), S (Yg–6%, Cc–43%), and Mg (Yg–24%, Cc–12%) compared to control bread. The fortification of the Yg and Cc breads with major and trace minerals is clearly noticeable, representing, in general, more than 15% of the recommended daily dosage for Ca (Yg–16%; Cc–24%), K (Yg–15%), and P (Yg–16%; Cc–18%).

For trace elements, interesting values were also noticed, especially for Cu (Yg–58%; Cc–46%) and Mn (Yg–32%; Cc–34%) (Reg. (CE), Nº 1924/2006; Dir. Nº 90/494 (CE)). These significant improvements will contribute to increase the minerals intake in not only daily diet of children's but also in adulthood and the elderly, where the aging process is associated with a gradual and progressive bone demineralization, along with lowered strength and physical endurance [4].

The results are supported by other researchers [11,22], which have demonstrated that the mineral and protein content of bread samples increased by the addition of different levels of whey protein, milk products, and dairy by-products.

2.3.6. Sensory Evaluation

The results of sensory analysis obtained for control bread and breads enriched with Yg (A) and Cc (B) are given in Figure 6. Appearance, flavor, crust color, aroma, texture, and overall acceptability of breads with 30 g and 50 g of both dairy products significantly differ in respect to control bread (0 g/100 g).

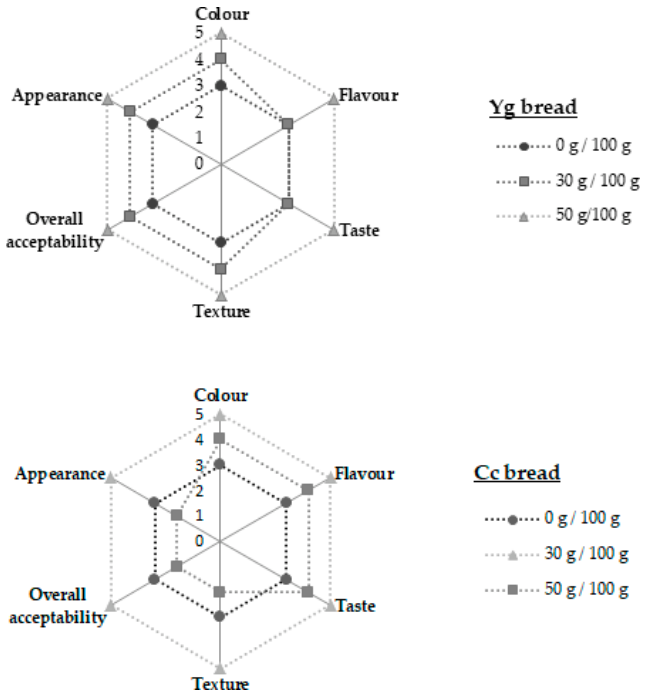

Figure 6. Sensory profile analysis of breads enriched with yoghurt (**A**) and curd cheese (**B**), with 30 g and 50 g of addition, compared with control bread (0 g/100 g).

Considering the Yg breads (Figure 6A), higher levels of yoghurt addition (50 g) caused a pleasant lactic aroma, taste, and crust color, with a significant positive influence on bread acceptability. This bread scored 5 for all attributes. In terms of texture, this Yg bread was classified as more crunchy, softer, and had a good alveoli distribution compared to the other breads (0 g and 30 g).

The best preference of Cc breads (Figure 6B) was obtained to 30 g of Cc addition, with a good acceptability in overall sensory attributes, and classified as having a better taste, crust color, and aroma. This bread also scored 5 for all attributes. For higher concentration tested (50 g), the excessive amounts of Cc proteins negatively affected the texture of this bread and the alveoli distribution, which influenced their sensorial acceptability. Results of sensory characteristics indicated that a partial replacement of wheat flour by 50 g of Yg and 30 g of Cc gave satisfactory overall consumer acceptability, and with respect to purchase intention, these breads were the best classified as "would buy for sure."

2.4. Conclusions

From the results of this work, it can be stated that all incorporations of the Yg resulted in good bread quality properties, and no significant adverse effect on texture and aging bread kinetics was observed. However, for Cc additions, from 30 g of Cc on, a significant increase of the crumb firmness and the aging kinetics was registered. The microstructure analysis supported the results obtained for the dough rheology and bread texture.

Considering nutritional composition, a significant increase in mineral contents was observed, indicating that the Yg and curd Cc can be used as nutritional and functional ingredients in bread formulations, which can have a significant impact on well-being. In terms of lipid content and based on higher levels to be considered as bread quality, the increase on Yg bread (50 g) was not significant, on the order of 28%, compared to control bread. However, for Cc breads (30 g), the increase in fat was much higher, around 168%, and these lipids coming from milk are mainly saturated fatty acids.

The incorporation of both dairy products studied were demonstrated to be an interesting alternative on the design of new bakery goods, with both technology properties and sensory acceptability. In terms of nutritional profile, Yg and Cc additions presented a considerable contribution to balance the daily diet, especially for children and seniors, in terms of protein, as well as in major and trace minerals, which are important to human health. However, the curd cheese addition promoted a significant increase in lipid content, which can constitute a limitation.

Author Contributions: C.G., conceived and planned the experiments; performed all samples preparation and analysis, data analysis, and interpretation of the results: and wrote the manuscript. A.R. and I.S. supervised the research work, contributed to the discussion of the data, and revised the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Yoghurt and curd cheese addition to wheat bread dough: Impact on *in vitro* starch digestibility and estimated glycemic index

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ABSTRACT

The effect of yoghurt and curd cheese additions on pasting properties, starch digestibility and estimated glycemic index of wheat bread were studied. Yoghurt and curd cheese incorporations (6% up to 25% w/w) promoted considerable changes on starch performance based on gelatinization and final dough consistency properties. These changes led to a significant impact on starch digestibility, reducing significantly the rapidly digestible starch while increasing the resistant starch. The estimated glycemic index reflected the changes promoted on starch performance from both dairy products additions, at higher level tested (25%): a significant reduction of around 30% for yoghurt bread and 38% for curd cheese bread, was obtained, resulting in medium to low (55–69) glycemic index breads. Correlations were found between pasting properties, starch digestibility and glycemic index, revealing that the effects observed are proportional to the levels of dairy products added. Microstructure images of the starch granules supported these findings.

2.5. Introduction

Bread has long been part of the human diet and nutrition for thousands of years (Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004), and is also rich in high-level of rapidly digestible starch which can impact on the glycemic response (Shumoy, Van Bockstaele, Devecioglu, & Raes, 2018). In this sense and considering that health issues have been a top priority for the consumers, it is important to search for new production strategies and/or new bakery's ingredients to reduce the glycemic response of starchy-rich foods.

Accordingly, dairies can be considered potential bakery's ingredients, since they are reported as low glycemic index products (GI < 55) (Wolter, Hager, Zannini & Arendt, 2014), in addition to rich protein sources with essential amino acids profile, that can be alternative strategies to reduce the glycemic response of the bakery goods. Yoghurt (Yg), is considered the most popular dairy product (DP) worldwide for its nutritional and health benefits, since it is a source of protein (casein), exopolysaccharides (EPS), vitamins (B2, B6, and B12), and minerals (such as Ca, P, and K), representing an alternative for healthier bakery products (Sharafi et al., 2017; Graça et al., 2019; 2020).

Curd cheese (Cc) is a cheese co-product, obtained by the thermal denaturation and subsequent precipitation of the soluble whey protein (WP), essentially composed by β-lactoglobulin, α-lactalbumin and bovine serum albumin (20–25% of the milk proteins). A considerable impact on protein and mineral profile enhancement on wheat bread, by Cc additions, was earlier reported (Graça et al., 2019).

Recent evidence has shown that the enrichment of the wheat bread formulation by protein-rich ingredients, can reinforce the interaction between starch and proteins, further enhanced by baking process, limiting the accessibility of α-amylase to starch granules and probably reducing the glycemic response (Fardet et al., 2006; Chung, Lin, Hoover, Warkentin, & Vandenberg, 2008). Furthermore, the presence of fiber and/or other microbial exopolysaccharides, in addition to starch–protein interactions, may also contribute to reducing the GI (Fardet et al., 2006; Lynch, Coffey, & Arendt, 2018).

Therefore, the inclusion of yoghurt and curd cheese as bakery ingredients can be an approach to control the enzymatic attack on starch via encapsulation mechanisms by protein-starch interaction, impacting on starch gelatinization performance during the baking process.

This work aimed to study the influence of plain yoghurt or fresh curd cheese additions (6% up to 25%), to reduce the glycemic response of the wheat bread. The impact of Yg or Cc on starch performance, by heating–cooling cycles, was firstly assessed. The physical integrity of starch granule's structure, after heating–cooling cycles, was evaluated by scanning electron microscopy. Subsequently, the starch digestibility of the obtained wheat bread was evaluated by an *in vitro* digestion model, and the glycemic index was calculated. Correlations between pasting properties, starch digestibility and glycemic index were tested to acquire additional information about the processes involved.

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2.5.1. Materials and methods

2.5.1.1 Raw materials

Bread was prepared using commercial wheat flour Type 65 from Granel Cereal Milling Industry, Alverca, Portugal (13.5% moisture, 11.5% protein and 25% of carbohydrates, w/w).

The plain yoghurt (Yg) used is a product from Nestlé LongaVida, Portugal (88.5% moisture, 4% protein, 5.5% carbohydrates, w/w). The dry extract of Yg was determined from the Standard Portuguese Method: NP.703–1982 (Standard Portuguese Norm), corresponding to 11.5% of dry matter.

The fresh curd cheese (Cc) used was from Lacticínios do Paiva, Lamego, Paiva, Portugal (75% moisture, 11% protein, 3% carbohydrates, w/w). The dry extract of Cc was determined from the Standard Portuguese Method: NP.3544–1987 (Standard Portuguese Norm), corresponding to 25% of dry matter.

Commercial white crystalline saccharose (Sidul, Santa Iria de Azóia, Portugal), sea salt (Vatel, Alverca, Portugal), baker's dry yeast (Fermipan, Lallemand Iberia, SA, Setubal, Portugal), and SSL-E481-sodium stearoyl-2 lactylate (Puratos, Portugal) were also used.

2.5.2. Bread dough preparation

The bread dough was prepared according to Graça et al. (2019) 2.4% yeast and 0.6% sugar were added to warm (distilled) water and dissolved well; 1.0% salt, 0.3% SSL and 6%, 18% or 25% of dairy products were incorporated into 58.5–53.0% wheat flour and mixed with 31.3–19.0% (distilled) water, according to each bread formulation, to complete 36.6% of wheat flour water absorption, previously optimized (Table 1. Supplementary Materials).

The bread dough preparation was performed in triplicate by randomized sampling to cover the variability of the raw materials used.

2.5.3. Pasting properties

The effect of Yg and Cc addition on starch rheology behaviour of the wheat dough was evaluated using the microdoughLab equipment applying the mixing and heating–cooling cycles, according to following setting conditions were: sample homogenization for 30 s, mixing curve at 30 °C for 360 s, heating from 30 to 95 °C for 390 s, holding at 95 °C for 60 s, cooling down to 30 °C for 390 s, at similar temperature rate (0.17 °C/s). Paddle speed was 63 rpm for the first 30 s, and then set steady at 120 rpm for running the analysis. The dough consistency values (expressed in torque units, mNm) produced by kneading the wheat dough is measured, in real time to study its physic behavior, delivering the follow parameters (AACC, 54-60.01): water absorption (%) - the percentage of water required to reach the optimal dough consistency, dough development time (C1) or maximum dough consistency, which is driven by the gluten matrix; protein weakening (C2), at this stage the temperature starts to rise, and the lower consistency is attained, due to the heat denaturation of proteins; starch gelatinization (C3), under constant shear and increase of temperature the starch granules starts to swell, and will break, loose amylose and gelatinize, increasing the torque values; cooking stability (C4) or the minimum torque reached at this phase of cooking, in which the amylase activity is dominating as well as the stabilization of the amylose network; starch gelling (C5) or final consistency peak torque produced by further cooling, in which gelation taking the lead with possible retrogradation/crystallization of amylose and continuous increase of torque and final consistency (Huang et al., 2010).

Triplicates were performed to ensure reproducible results.

2.5.4. In vitro starch hydrolysis

2.5.4.1 Total starch (TS)

The total starch in bread crumb samples: control, Yg and Cc breads, was determined enzymatically following the method described by Goni et al. (1997).

All the incubation steps were performed in a controlled shaking water - bath equipment (Thermo-Scientific- Model: 2871, Waltham, MA, U.S.A).

Ground bread (50 mg) was dispersed in 6 ml of 2 M KOH and shaken (30 min at room temperature); 3 ml of 0.4 M sodium acetate buffer (pH 4.75) and 60 µl of amyloglucosidase (3300U/mL) (EC-3.2.1.3, Sigma-Aldrich, Chemical Company, St Louis, MO, USA) were added; the suspension was incubated (45 min at 60 °C), under controlled shaking water-bath. Triplicates were performed.

2.5.4.2 Resistant starch (RS)

Resistant starch was estimated according to the methodology described by Goni et al. (1997).

Grounded bread sample (100 g) was incubated (60 min at 40 °C) with a pepsin solution, from porcine gastric mucosa (EC-3.4.23.1, Sigma-Aldrich, Chemical Company, St Louis, MO, USA) (1 g/10 ml buffer KCL-HCL: 40 000U/mL), to protein interference removal. Starch was hydrolyzed by adding pancreatic α-amylase (EC-3.2.1.1, Sigma-Aldrich, Chemical Company, St Louis, MO, USA) (40 mg α-amylase per ml Tris–maleate buffer: 200U/mL) and incubated (16 h at 37 °C); ob tained samples were washed three times with deionized water, and the pellet was separated by centrifugation to further digestion with KOH 4 M; this solution (at pH 4.75) was incubated (45 min at 60 °C) with amyloglucosidase (3300U/ml). Total and resistant starches were measured as glucose release, using a glucose-oxidase-peroxidase (GODPOD) reagent ki t (K-Glox Megazyme Bray, Co, Wicklow, Ireland). Th e absorbance (510 nm) was spectrophotometric measured.

Table 1
Variation of the rheology parameters of wheat starch by the addition of dairy products, evaluated during mixing and heating–cooling circles on microdoughLab analysis.

		C1	C2	C3		C4	C5
Samples	WA (%)	DD (mNm)	PW (mNm)	SG (mNm)	GT (°C)	CS (mNm)	FV (mNm)
CD	52.4 ± 0.5^{a}	128.0 ± 2.9^{a}	77.7 ± 1.2^{a}	250.0 ± 6.0^{a}	83.9 ± 0.8^{a}	221.7 ± 11.0^{a}	594.0 ± 4.4^{a}
$Yg_{6\%}$	51.6 ± 2.1^{a}	135.3 ± 0.1^{a}	76.3 ± 2.1^{a}	244.0 ± 1.7^{a}	81.8 ± 1.5^{a}	237.7 ± 2.5^{a}	593.3 ± 2.1^{a}
$Yg_{18\%}$	45.4 ± 0.4^{b}	131.3 ± 4.2^{a}	70.1 ± 3.2^{a}	220.5 ± 2.2^{a}	81.8 ± 1.5^{a}	197.6 ± 6.1^{a}	589.0 ± 3.1^{a}
$Yg_{25\%}$	39.3 ± 1.2^{d}	132.7 ± 2.3^{a}	27.3 ± 3.2^{b}	185.3 ± 4.6^{b}	79.7 ± 1.8^{a}	65.5 ± 2.1^{b}	600.0 ± 7.6^{a}
$Cc_{6\%}$	51.8 ± 2.3^{a}	130.7 ± 0.2^{a}	73.1 ± 1.2^{a}	217.5 ± 5.1^{a}	78.2 ± 1.7^{a}	106.3 ± 9.6^{d}	551.5 ± 3.6^{a}
$Cc_{18\%}$	49.6 ± 0.1^{ab}	127.0 ± 2.0^{a}	71.7 ± 0.6^{a}	179.7 ± 7.1^{b}	73.5 ± 0.7^{b}	97.1 ± 5.1^{d}	495.0 ± 6.9^{b}
$Cc_{25\%}$	50.7 ± 0.6^{ab}	127.3 ± 5.8^{a}	69.7 ± 3.5^{a}	112.7 ± 1.2^{c}	69.4 ± 1.5^{c}	54.0 ± 1.5^{e}	86.7 ± 6.7^{c}

WA- water absorption (%); DD- dough development (C1); PW – protein weakening (C2); SG- starch gelatinization (C3); GT- gelatinization temperature; CS- cooking stability (C4); FV- final viscosity (C5). Different letters within the same column are statistically different ($p < 0.05$).

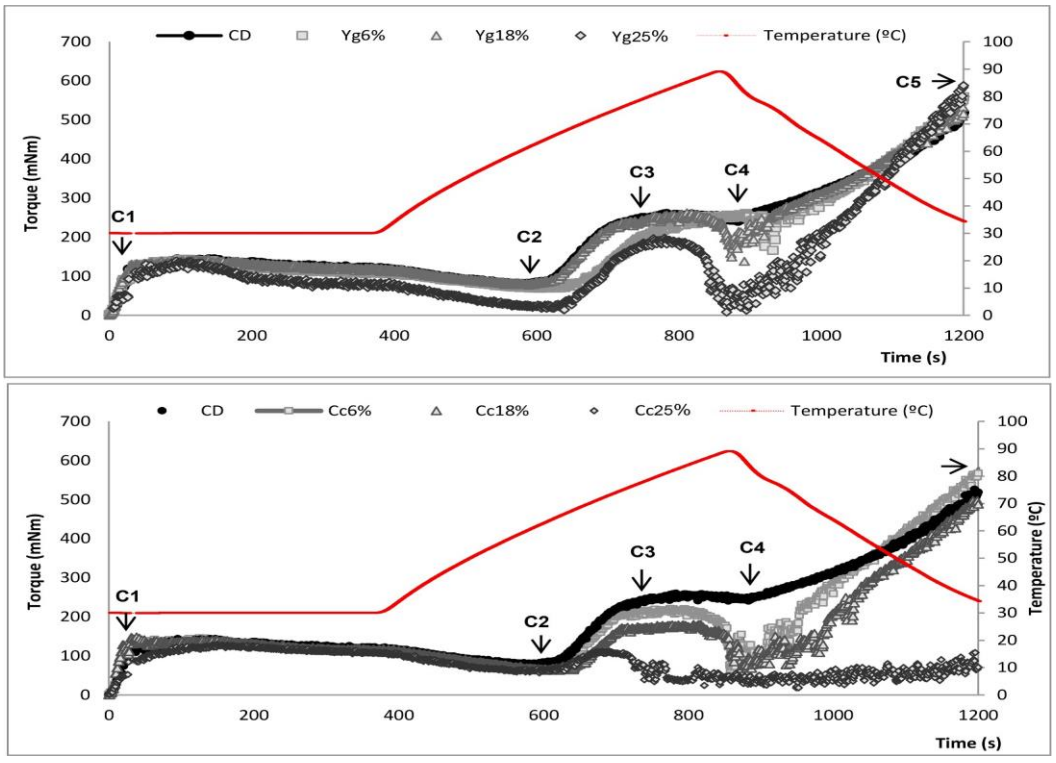

Fig. 1. Effect of Yg (A) and Cc (B) addition on pasting properties of wheat starch, determined by MicroDoughLab analysis: CD- control wheat dough; C1- dough development time; C2- protein weakening; C3- starch gelatinization; C4- cooking stability; C5- final viscosity.

measured using a microplate reader (Spectramax, Bio-TEK, Multi-Detection Synergy HT, UK). Starch was calculated as glucose (mg) × 0.9 (conversion factor). Triplicates were performed.

2.5.4.3 In vitro starch digestibility and estimated glycemic index

To evaluate the effect of the DP addition on the starch digestibility and

to predicted glycemic index of the bread, an *in vitro* starch hydrolysis based in the procedure described by Goni et al. (1997), was applied. Three phases were simulated: 1) to chewing phase, bread sample (100 mg) was milled; 2) to gastric phase, the grounded bread was dispersed in 10 ml of 0.1 M KCL-HCL buffer (pH 1.5) and 200 µl of

pepsin solution (1 g/10 ml KCL-HCL buffer), followed by incubation (60 min at 40 °C); 3) to pancreatic phase, 25 ml of 0.1 M of tris-maleate

buffer (pH 6.9) and 5 ml of a pancreatic α-amylase solution (3U/ml tris-maleate buffer) was added; followed by incubation (37 °C). Triplicates of 1 ml were taken at every thirty minutes (30–180 min) and placed into boiling water (5 min) to inactivate the enzyme reaction; followed by refrigeration conditions (4 °C) until the end of incubation time (180 min). For each aliquot taken, 3 ml of 0.4 M sodium acetate buffer (pH 4.75) and 60 µl of amyloglucosidase (3300 U/ ml) were added, followed by incubation (45 min at 60 °C); the volume was adjusted to 10 ml with distilled water, mixed and centrifuged (3000 × g/10 min); the supernatant was taken for glucose determination.

The glucose content was measured using a glucose oxidase–peroxidase (GODPOD) kit as described for total and resistant starch procedure. Triplicates were performed.

The *in vitro* digestion kinetics was calculated in accordance with the procedure established by Goni et al (1997).

A nonlinear model, as expressed by Eq. (1), was employed to describe the kinetics of starch hydrolysis, where C was the concentration at *t* time, *C* was the equilibrium concentration, *k* was the kinetic constant and t was the time.

$$C = C\infty\,(1 - e^{-kt}) \quad (1)$$

The hydrolysis index (HI), was obtained from Eq. (2), dividing the area under hydrolysis curve of the breads (AUC 0–180 min) by the area under curve of the reference food (fresh white wheat bread) over the same period time.

$$HI = \frac{\text{AUC of product}}{\text{AUC Reference food}} \times 100 \quad (2)$$

The estimation of glycemic indices (eGI) was calculated according to Eq. (3), proposed earlier by Goni et al. (1997).

$$eGI = (0.549 \times HI) + 39.71 \quad (3)$$

2.5.5 Microstructure of the dough – Starch granules size

A scanning electron microscope (SEM) (TM3030 ^{PLUS}- TabletUp Microspcope- Hitachi, Japan) was used to observe the starch granules physical integrity of control dough, Yg and Cc doughs, after the heating–cooling cycles applied. Samples were placed on the specimen holder, dried automatically by the equipment, and the freezing model was applied (−14 °C). The observations were analyzed at 800× of magnification, with scale bar of 100 µm. Triplicates were performed.

2.5.6 Statistical analysis

The experimental results were statistically analyzed by determining the average value, standard deviation, and the significance level was set at 95%, for each parameter evaluated. Statistical analysis (RStudio, Version 1.1.423) was performed by applying variance analysis, the one factor (ANOVA), and post-hoc comparisons (Tukey test). The experimental data was fitted to nonlinear model and the relation between continuous variables was carried out by correlation matrix using the Pearson product moment correlation distribution to significant $p < 0.05$.

2.6 Results and discussion

2.6.4 Pasting properties of wheat starch

The impact of Yg and Cc incorporation to wheat dough on gluten and starch performance, was studied based on pasting properties microdoughLab procedure.

From Fig. 1 the heating–cooling cycles obtained show the different impacts of the Yg (A) and Cc (B) on gluten proteins and on starch behavior, compared with control dough.

The impact observed by Yg addition is much lower than that observed for the Cc addition, most probably due to the different structure of the proteins involved on the starch-gluten matrix as well as the differences on protein content (Bertolini et al., 2005; Noisuwan et al., 2008). Some peak values reflect the influence of the Yg and Cc on pasting properties of doughs (Fig. 1), and these parameters are summarized in Table 1. The incorporation of Yg on wheat dough, up to 18% (w/w) of addition, has no significant effect ($p > 0.05$) on protein weakening (C2), starch gelatinization (C3), cooking stability (C4) and final consistency (C5), with values similar to the control ones. For higher levels of Yg tested (25%, w/w) a significant decrease ($p < 0.05$) on C2, meaning further protein weakening (27.3 ± 3.2 mNm), com- pared to control dough (77.7 ± 1.2 mNm), was observed, most probably due to the dilution effect of the gluten proteins (Graça et al., 2018; 2019). The lubrication effect of gluten by the exopolysaccharides (EPS) produced by lactic acid bacteria in Yg can be also considered (Lynch et al., 2018) to explain this observed torque reduction. A significant decrease ($p < 0.05$) on peak consistency (C3), by starch gelatinization (185.3 ± 4.6 mNm), was also registered, compared to control dough (250.0 ± 6.0 mNm), representing a reduction of 26%. During cooking stability (C4), a phase separation process was observed, promoting a significant decrease in torque units of Yg dough (65.5 ± 2.1 mNm) compared to control dough (221.7 ± 11.0 mNm), that represents 70.5% of torque variation. However, during cooling stage, the Yg dough structure reinforces with values of final torque (C5) slightly higher (600.0 ± 7.6 mNm) than the control dough (594.0 ± 4.4 mNm). This result suggests that the caseins and the EPS may have strong interactions with starch molecules, which upon cooling increase the consistency by becoming entrapped in the gelled matrix, by EPS-amylose-casein-gluten interactions. Probably, this be- haviour was mediated by the depletion flocculation mechanism (phase separation phenomena), as earlier described in similar complex systems (Nunes, Raymundo, & Sousa, 2006; Considine, Noisuwan, Hemar, Wilkinson, Bronlund, & Kasapis, 2011).

According to the results obtained for Cc addition, at all levels tested, on stages of dough development (C1) and protein weakening (C2), no significant effects were observed, presenting values like those obtained to control. However, as the temperature increases, the addition of different Cc levels promoted significant changes on starch performance, in terms of starch gelatinization, cooking stability and final consistency. Considering higher levels of Cc tested (25%, w/w), where the impact was more evident, a strong effect on starch performance was noticed: starting from starch gelatinization, a significant decrease on peak consistency (112.7 ± 1.2 mNm) was registered, compared to control dough (250.0 ± 6.0 mNm), representing a reduction of 55%. The cooking stability (C4) and the final consistency (C5) were also negatively affected, representing a decrease in torque units of 49.2% and 84.3%, respectively. These results suggested that denatured Cc proteins have a thermodynamic incompatibility with hydrolyzed starch molecules, hindering a matrix structuration during cooling, which could be attributed not only to the dilution effect of the starch but also to different causes like: i) the starch started to swell but due to the high competition for available water, from denatured Cc proteins, swelling was hindered (Noisuwan et al., 2008) by insufficient hydration; ii) starch-protein interactions, reducing the volume portion occupied by swelled granules in dough and the concentration of the amylose molecules (Chung et al., 2008).

In Cc doughs, the starch should be less damaged than in the Yg dough, as it can be seen by the lower values of torque for starch gelatinized (C3) and final peak consistency (C5), therefore less available.

According to the results obtained, it can be stated that the different nature and structure of the dairy proteins added in the form of the Yg and Cc, promoted different effects on starch rheology behaviour. These changes were probably a result of complex dough structure modification, which can be attributed to the macromolecules interactions, and/ or phase separation mechanisms, due to the incompatibility phenomena between the molecular chains involved on dough matrix.

2.6.5 In vitro starch digestibility of wheat bread

The impact of yoghurt and curd cheese addition, at different levels, on *in vitro* starch digestibility was evaluated based on the procedure well described earlier by Goni et al. (1997).

The levels (g/100 g) of total starch (TS), resistant starch (RS) and digestible starch (DS) of the developed breads were determined andare summarize in Table 2.

The TS content in the dairy breads ranged from 60.0 to 50.0 g/100 g for Yg breads and from 50.0 to 40.0 g/100 g for Cc breads, compared to control bread (65.0 g/100 g). Resistant starch (RS) content varied among the dairy breads with a range of 5.0–7.0 g/100 g for Yg breads and 6.1–7.6 g/100 g for Cc breads, compared to the control one (4.4 g/ 100 g). Comparing the control bread and maximum levels of both dairy products tested (25%, w/w) the increase on RS content was higher in Cc breads (73.0%) than in Yg breads (60%).

This significant increase in RS content, could be attributed to the effect on the starch performance, probably by entrapping the granules and hindering the complete gelatinization, possibly due to the establishment of starch-proteins interaction further enhanced during the baking stage (Fardet et al., 2006; Chung, Lin, Hoover, Warkentin, & Vandenberg, 2008). In addition, the competition to available water should also be considered to justify this starch behaviour, since a strong depletion on starch gelatinization was observed, which can be associated to the insufficient starch water uptake. RS acts as a soluble fiber, since it is not absorbed in small intestine but is fermented in the large intestine by bacteria microbiome, which is considered as beneficial to human health (Lehmann & Robin, 2007). These results agree with those

Obtained in pasting properties where a strong negative effect on starch gelatinization was observed for higher levels of Cc addition tested.

Table 2
Total Starch (TS), Resistant Starch (RS) and Digestible Starch (DS) of control (CB), yoghurt (Yg) and curd cheese (Cc) breads.

Samples	TS (g/ 100 g)	RS (g/100 g)	DS (TS-RS) (g/100 g)
CB	65.0 ± 1.1^{a}	4.4 ± 0.4^{a}	60.3 ± 0.3^{a}
$Yg_{6\%}$	60.0 ± 4.1^{a}	5.0 ± 0.5^{a}	55.0 ± 2.2^{a}
$Yg_{18\%}$	59.0 ± 1.3^{a}	5.2 ± 1.0^{a}	54.0 ± 1.5^{a}
$Yg_{25\%}$	49.4 ± 0.9^{b}	7.0 ± 1.1^{b}	43.0 ± 2.0^{b}
$Cc_{6\%}$	50.0 ± 2.7^{b}	6.1 ± 0.3^{b}	47.0 ± 1.8^{b}
$Cc_{18\%}$	47.3 ± 1.3^{b}	6.4 ± 0.5^{b}	41.0 ± 2.6^{b}
$Cc_{25\%}$	40.0 ± 3.5^{c}	7.6 ± 0.6^{c}	31.3 ± 2.1^{c}

Different letters within the same column are significantly different ($p < 0.05$).

In terms of digestible starch (DS), both Yg and Cc addition at the higher levels tested (25%, w/w) promoted a significant reduction ($p < 0.05$) of 29.0% and 48.0%, respectively. From the results presented, the dilution effect of the starch (TS and DS) is evident, and these results are also in agreement with those obtained on pasting properties. The starch fractions variation observed can be attributed to the inter- action between dairy proteins (caseins from Yg and denatured whey proteins from Cc, additions) and starch molecules, restricting the granules swelling and extension of the starch gelatinization. This results in a lower susceptibility to enzymatic attack, which may be partially responsible for the low starch digestibility observed (Chandrashekar & Kirleis, 1988; Reshmi et al., 2017).

As it is well-known, the rate of starch digestion is the main factor that influences the glucose release and absorption during digestion (Jenkins et al., 1982). Based on *in vitro* hydrolysis the digestible starch can be distinguished into two different fractions: rapidly digested starch (RDS) - hydrolyzed within the first 30 min of digestion and slowly digested starch (SDS) - digested within the following 100 min. RDS has a significant impact on glycemic response, whereas the SDS presents low impact, due to the slow glucose release over the hydrolysis time (Englyst et al., 1996).

The influence of both DP on digestible starch fractions (RDS and SDS) was evaluated, and the results are illustrated at Fig. 2. The Yg (A) and Cc (B) additions had a positive influence on digestible starch fractions: from DP additions of 18% (w/w), a significant reduction ($p < 0.05$) on RDS was observed, and a remarkable effect was achieved for higher level of both DP tested (25%, w/w), representing a reduction of 62% for Yg and 73% for Cc breads, compared to the control bread. Since the starch gelatinization, during baking process, is the main mechanism responsible for the RDS fraction increase (Shumoy et al., 2018), these results should reflect the dilution effect on starch granules and/or the reinforcement of starch- protein interaction pro- moted by the Yg and Cc addition, driving to an inhibition of the gelatinization process, that may lead to a significant reduction on RDS. Based on SDS fraction and considering the higher level of the Yg and Cc additions tested (25%, w/w), an increase of 54.2% on Yg bread was attained, whereas for Cc bread only 23.3% of increase was observed, compared to the control bread. This lower increase of SDS to Cc bread could be attributed to the negative effect of Cc on starch gelatinization, driving to starch granules becoming more resistance to enzymatic attack, increasing the resistant starch content (Table 2).

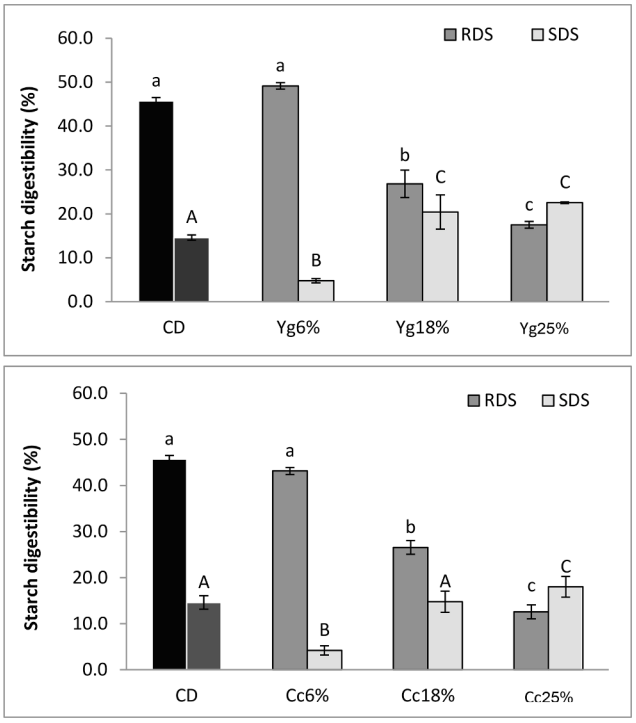

Fig. 2. Effect of the Yg (A) and Cc (B) addition on digestible starch fractions: rapidly digestible starch (RDS) and slowly digestible starch (SDS). Different letters in each bar indicate significant differences ($p < 0.05$).

In general, higher values of SDS are desirable over the RDS, due to nutritional aspects related to the slowly digestible of the starch in the small intestine, inducing a gradual insulin production after ingestion (Jenkins et al., 1982). Similar results were obtained by other authors (Giuberti, Gallo, Cerioli, Fortunati, & Masoero, 2015) using bean flour to produce gluten-free pasta.

Based on the results presented, it was noticeable that the impact of DP on starch performance, mainly on gelatinization and final consistency values, is reflected on digestible starch fractions. Therefore, a possible correlation between the addition of DP on pasting properties and *in vitro* starch hydrolysis was investigated by Pearson´s correlation analysis ($p < 0.05$). A negative correlation was found between pasting properties with both DP additions: starch gelatinization ($r_{Yg} = -0.971$; $r_{Cc} = -0.979$), cooking stability ($r_{Yg} = -0.928$) and final consistency ($r_{Yg} = -0.688$; $r_{Cc} = -0.989$). A positive correlation was observed for Cc addition on cooking stability ($r_{Cc} = 0.574$). In terms of starch digestibility, a negative correlation was also obtained between RDS and both DP ($r_{Yg} = -0.846$; $r_{Cc} = -0.992$), whereas a positive correlation was observed for SDS ($r_{Yg} = 0.671$; $r_{Cc} = 0.887$) and RS ($r_{Yg} = 0.855$; $r_{Cc} = 0.915$), supporting the relations aforementioned.

These correlations telling that the effect observed on pasting properties is proportional to the quantity of DP added.

Considering the possible relation between pasting properties and digestible starch fraction, for both DP tested, RDS is positively correlated with starch gelatinization ($r_{Yg} = 0.947$; $r_{Cc} = 0.988$) and final consistency ($r_{Yg} = 0.985$; $r_{Cc} = 0.896$), whereas SDS showed a negative correlation (starch gelatinization: $r_{Yg} = -0.826$; $r_{Cc} = -0.825$; final consistency: $r_{Yg} = -0.891$; $r_{Cc} = -0.911$).

The impact of Yg and Cc addition on starch gelatinization and on final consistency should be considered to explain the results obtained, since the starch swelling and subsequent structure breakdown increase the accessibility of enzyme attack into the granules, consequently, increasing the RDS content. The gelatinized starch is more susceptible to hydrolysis generating higher RDS content and lower SDS and RS contents (Englyst et al., 1996; Chung et al., 2008).

The results presented show that the Yg and Cc additions, at higher level, promoted significant changes on starch physical behaviour which were reflected on *in vitro* starch digestibility, contributing to reduce significantly the RDS fraction, increasing the SDS and RS fractions, and this must have an impact in reducing the glycemic response of bread.

2.6.6 Hydrolysis kinetics and estimated glycemic index of the breads

The influence of the Yg and Cc additions on starch hydrolysis kinetics was studied and the glycemic index of the obtained wheat breads was calculated.

Nonlinear parameters of the starch hydrolysis kinetics were obtained by fitting the experimental data (Eq. (1)) that describes well the impact of dairy products on starch digestion dynamic (Goni et al., 1997). These fitted parameters included equilibrium concentration ($C\infty$), kinetics constant (k), hydrolysis index (HI) and estimated glycemic index (eGI), that are summarize at Table 3.

From Table 3, it can be observed that the incorporation of Yg and Cc had a significant ($p < 0.05$) impact on equilibrium concentration reduction ($C\infty$), varying from 52.7 to 32.2% for Yg breads and from 47.6 to 20.0% for Cc additions, compared to control bread (54.3%). These results are in line with those obtained for the starch fractions discussed above, where a considerable reduction on total starch was registered.

Table 3
Nonlinear parameters: Equilibrium concentration C∞, Kinetics constant (K), Hydrolysis index (HI) and estimated glycemic index (eGI) of control, yoghurt (Yg) and curd cheese (Cc) breads.

Samples	C∞	*k*	R^2	HI	eGI*
CB	54.3 ± 1.4^{a}	0.065 ± 0.004^{a}	0.960	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
$Yg_{6\%}$	52.7 ± 0.6^{b}	0.104 ± 0.018^{b}	0.996	100.3 ± 0.9^{a}	94.7 ± 0.5^{a}
$Yg_{18\%}$	47.5 ± 0.9^{c}	0.034 ± 0.002^{c}	0.986	81.0 ± 0.5^{b}	84.1 ± 0.3^{b}
$Yg_{25\%}$	32.2 ± 0.9^{d}	0.019 ± 0.002^{c}	0.957	47.5 ± 0.4^{c}	65.7 ± 0.2^{c}
$Cc_{6\%}$	47.6 ± 1.4^{c}	0.077 ± 0.001^{a}	0.998	88.1 ± 2.1^{d}	88.0 ± 1.1^{d}
$Cc_{18\%}$	39.5 ± 1.0^{e}	0.035 ± 0.003^{c}	0.986	67.4 ± 0.5^{e}	76.6 ± 0.3^{e}
$Cc_{25\%}$	20.0 ± 0.3^{f}	0.033 ± 0.006^{c}	0.975	33.8 ± 0.8^{f}	58.2 ± 0.4^{f}

*eGI = (0.549 × HI) + 39.71; Different letters within the same column are statistically different ($p < 0.05$).

The kinetics constant (*k*), indicative of the enzymatic hydrolysis rate in the early stage, in general showed decreasing values with increasing levels of Yg and Cc: Yg breads ranging from 0.104 to 0.019, and for Cc breads varying between 0.077 and 0.033 compared to control bread (0.065). Lower values of *k* suggest that the increase of dairy product additions to dough are probably promoting higher resistance to enzymatic starch hydrolysis, and the decrease in digestibility, may be explained by the starch–protein interaction, are limiting the starch digestion, during enzymatic hydrolysis (Chung et al., 2008).

These results are in line with those obtained on digestible starch fractions, where a significant reduction on RDS and an increase on RS were registered. Similar results were obtained by Barine & Yorte (2016), by incorporation of "Amala" and Plantain on bread formulations.

Hydrolysis index (HI) calculated from the rate of starch hydrolysis over time and the respective estimated glycemic index (eGI) are also presented in Table 3. For Yg and Cc breads, up to 18% (w/w) of addition, higher values of HI resulted in higher eGI, ranging from 94.7 to 84.1 to Yg breads and 88.0–76.6 to Cc breads, compared to control bread (100). The lowest HI and eGI was recorded at higher levels of Yg and Cc (25%, w/w) tested, with eGI of 65.7 and 58.2, representing a reduction of 31.0% (Yg) and 38.4% (Cc), respectively. GI of foods can vary between ≤ 55, and 56–69 and ≥ 70, that are classified as low, medium, and high GI, respectively (Atkinson et al., 2008; Jenkins et al., 2008). According to this classification, the dairy breads developed with maximum incorporations (25%, w/w) can be classified in medium GI ($Yg_{25\%}$) and almost low GI ($Cc_{25\%}$). These results of GI could be attributed to the low RDS and high SDS and RS fractions values obtained, by *in vitro* starch digestibility.

The findings agree with those registered on pasting properties by the addition of DP which are reflected on digestible starch fractions and support the results obtained on glycemic index of the dairy breads. Similar relations were reported by other authors (Reshmi et al., 2017) showing that the fortification of bread with pomelo fruit segments reduced the glycemic index of the breads. Low GI foods is desirable not only for consumers with diabetes, but also for all consumers in general, since it prevents the incidence of chronic metabolic diseases (Brand-Miller et al., 2009).

2.6.7 Microstructure of the wheat dough- starch granules size and structure integrity

Environment scanning electron microscopy (ESEM) was used to investigate the impact of the higher levels of Yg and Cc addition (25%, w/w) on starch granules size and structure integrity to explain and support the results, discussed above. The control, Yg and Cc doughs were collected after applying the heating/cooling cycles on microdoughLab analysis. From Fig. 3 A-C, the impact of the Yg (B) and Cc (C) addition on granules structure, compared to control dough (A), can be observed. Considering the control dough (A), the starch granules structures seem to be less intact on dough surface, as well as covered by a gummy structure acting such as a filling effect, aggregating the starch granular structure. This gummy structure resulted, most probably, from the amylose molecules released by the swelled starch breakdown and gelatinization during heating stage.

In the case of Yg dough (B), the ESEM image seems to show some swollen and unbraked starch granules and, although they also look aggregated by this gummy structure, it seems to be less noticeable than in control dough. However, comparing the images of control and Yg doughs, slight differences can be observed.

With Cc addition (C), a different picture can be seen, there is a higher number of intact starch granules preserved, and less aggregated, compared to control and Yg doughs. In addition, the gummy structure around swollen starch granules are much less perceptible, as in control and Yg dough, suggesting that the gelatinization process was negatively affected by Cc addition.

These results agree with those obtained on pasting properties evaluation, where a significant negative influence on starch gelatinization, either for Yg and Cc addition, was observed.

The ESEM images obtained could partially explain and support the results obtained by the addition of the Yg and Cc and their impact on pasting properties, on *in vitro* starch digestibility as well as on the GI values of the dairy breads.

2.7 Conclusion

Considering the importance of reducing the glycemic index of the bread, this study was conceived to evaluate the incorporation of yoghurt and curd cheese on wheat bread, as an alternative approach to reduce the glycemic response. The findings showed that higher additions (25%, w/w) of yoghurt and curd cheese negatively affected the starch gelatinization performance, and this effect was reflected on *in vitro* starch digestibility. A significant reduction on rapidly digestible starch was observed, while resistant starch increased, for both Yg and Cc breads, being stronger on the latter. In line with these results, are the estimated values of the glycemic index from the dairy breads, resulting in Yg breads with medium GI (55–69) and Cc breads with almost low GI (≤55).

Improvements observed in the glycemic response of the wheat dairy breads, can probably be associated not only to the dilution of starch, promoted by the dairy products additions, but also to further starch-protein interaction enhanced by baking, limiting the gelatinization process and the enzymatic attack to starch granules. The microstructure images obtained for starch granules surface, where higher integrity of the starch granules was observed for Cc dough, give some additional support to these findings.

Correlations were found between pasting properties, starch digestibility and glycemic index, meaning that the effects observed are proportional to the quantity of the dairy products added.

In summary, the incorporation of Yg and Cc showed to be an interesting strategy to reduce the glycemic response of the wheat bread.

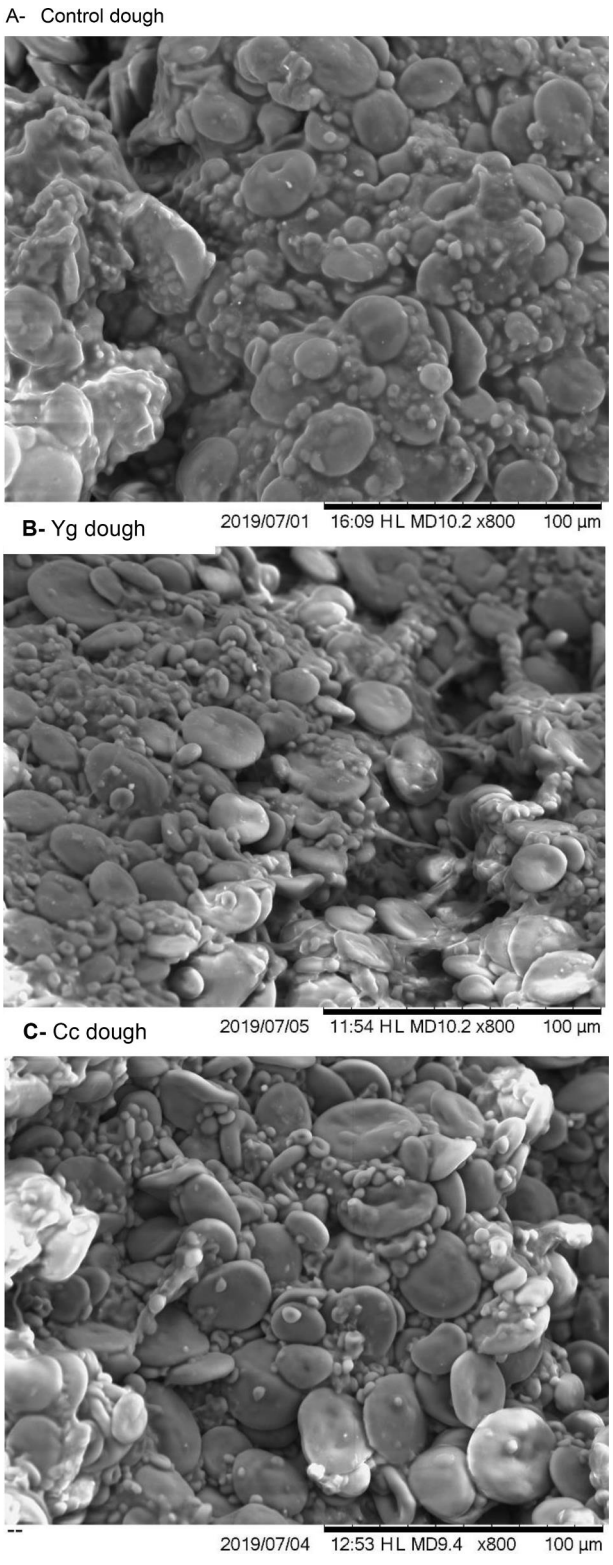

Fig. 3. Scanning electron micrographs (800x of magnification, scale bar = 100 µm): A- control dough; B- yoghurt dough and C- curd cheese dough (25%, w/w), evaluated after the heating–cooling cycles by microdoughLab analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127887.

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Improving bioactive properties of wheat bread by yoghurt or curd cheese supplements

Antioxidant, anti-inflammatory, and antibacterial properties

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Abstract

This research work aimed to evaluate the impact of yoghurt or curd cheese addition (at 25% addition) to enhance the wheat bread bioactivity: antioxidant, anti-inflammatory and antibacterial properties. Bread antioxidant capacity was evaluated by DPPH and FRAP methods. Total phenolic compounds were also assessed. Anti-inflammatory properties were investigated by enzymatic inhibitory activity, using a specific MMP-9 as a matrix metalloproteinase. The antibacterial effect against two pathogenic microorganisms, *E. coli,* and *L. monocytogenes*, was also determined.

In terms of antioxidant capacity, yoghurt addition showed higher potential to produce bread more effective in radical scavenging capacity, whereas curd cheese bread expressed better reducing power potential. Phenolic compounds content increased in both cases, being higher by curd cheese incorporation. MMP-9 inhibition activity was higher in dairy bread than in control bread, suggesting an improvement in anti-inflammatory properties. Antibacterial effects were not found.

The incorporation of yoghurt or curd cheese showed to be an interesting bakery´s ingredient to improve the bioactive properties of the wheat bread, with the potential to be part of the consumer´s nutritional and preventive diet.

Additionally, this research work provides relevant information which helps in providing direction towards novel research lines in human well-being.

Keywords: Wheat bread, Dairy products, Bioactive properties, Functional bread, Preventive diets

2.8 Introduction

Consumers are increasingly aware of what they eat, making the selection of healthier foods for disease prevention as a top priority.

Bread is a staple food with widespread consumption, representing a good vehicle to enrich with bioactive compounds sources, not only to improve the daily diet but also to prevent nutrient deficiencies.

Low-grade chronic inflammatory diseases (L-GCIs) such as obesity, cardiovascular diseases, and type-2 diabetes are linked to metabolic pathologies, directly influenced by nutrition, since the metabolism of food is associated with inflammatory processes (Hotamisligil, 2006; Schwander et al., 2014). L-GCIs can be modulated by some nutrients, such as polyunsaturated fatty acids (German and Dillard, 2006), proteins (Chatterton et al., 2013b), glycans (Newburg, 2013), bioactive compounds, vitamins and minerals (Hirai et al., 2010), which can either reduce or alleviate the inflammatory symptoms.

Matrix metalloproteinase (MMPs) are a family of zinc-dependent endopeptidases involved in connective tissue remodelling (Markle et al., 2010; Vandooren et al., 2013). Specifically (MMP)-9, a subgroup of MMPs gelatinases, has been recognized as key mediators in inflammation processes, especially involved in bowel inflammatory diseases (BIDs) (Malla et al., 2008; Garg et al., 2009; Moore et al., 2013).

Consequently, in the last decade, food and nutrition sciences have emerged as a relevant research topic, and the search for bioactive nutrients and MMPs inhibitors in food has become an important branch of research in academic as well as in industrial settings.

In the case of BIDs, an effective and convenient approach could be achieved, at least partly, via the long-term ingestion of natural food sources of specific MMP-9 inhibitors, that probably can be available on colon after the digestion process.

Dairy products (DP) represent a particularly interesting food in the context of inflammation diseases, especially those deriving from fermentation (e.g., yoghurt or cheese) whose metabolites produced by lactic acid bacteria (LAB) fermentation may contribute to an anti-inflammatory activity on humans immune processes (Augustin and Udabage, 2007; Ceapa et al., 2013; Bordoni et al., 2017). Regarding other physiological properties, DP anticarcinogenic, antimicrobial and antioxidant activities have also been reported (Poppi et al., 2010). Nutritionally, they have an excellent composition and bioavailability of amino acids, vitamins and minerals, with high values of digestibility and absorption (Zinsly et al., 2001).

Yoghurt (Yg) has recognized nutritional properties and functional benefits since it is a source of protein (e.g., caseins), exopolysaccharides (EPS), vitamins (B2, B6, and B12), and minerals (such as Ca, P, and K), representing an alternative for bakery products fortification (Graça et al., 2019; 2020a).

Curd cheese (Cc) is a dairy co-product obtained by the thermal denaturation and precipitation of the soluble whey proteins (WP), considered a rich source of protein and minerals. In addition, WP presents bioactive properties associated to their amino acid residues, which can contribute to enhance the bioactivity features of the bread (Mann et al., 2015).

In a previous study (Graça et al., 2021) it was demonstrated that incorporation of Yg or Cc (at 25% of addition, w/w) significantly reduced starch digestibility and hence the glycemic index of wheat bread. However, the impact of these DP to improve the bioactive properties of the wheat bread, such as antioxidant capacity, anti-inflammatory and antibacterial properties, was not reported. The present work is complemental, as the influence of Yg or Cc at 25% (w/w) of addition, to improve the bioactive properties of the wheat bread is analyzed.

The effect of DP enrichment on both antioxidant capacity of the bread by scavenging effects, using the DPPH (2,2-diphenyl-1-picryl-hydroxyl-hydrate) methodology, and the reducing power by ferric ion reducing power (FRAP) assay were evaluated. Total phenolic compounds (TPC) were also determined. The gelatinolytic activity of MMP-9 inhibition was measured by activity fluorometric quantification (DQ-gelatin assay), to assess the anti-inflammatory potential of the wheat bread obtained. Antibacterial assays, by serial dilutions in a microplate, were performed, to assess the influence of DP in the pathogenic microorganism's growth, such as *Escherichia coli (E. coli)* and *Listeria monocytogenes (L. monocytogenes).*

2.8.4 Materials and methods

2.8.4.2 Raw materials

Bread was prepared using commercial wheat flour (WF) T65 (Granel Cereal Milling Industry, Alverca, Portugal), plain yoghurt (Yg) (Nestlé, LongaVida, Portugal) and fresh curd cheese (Lacticínios do Paiva, Lamego, Paiva, Portugal), based on the raw materials and respective chemical compositions earlier described by Graça et al. (2019). Other dry ingredients used were: commercial white crystalline sucrose (Sidul, Santa Iria de Azóia, Portugal), sea salt (Vatel, Alverca, Portugal), baker's yeast (Fermipan, Lallemand Iberia, SA, Setubal, Portugal), and SSL- E481-sodium stearoyl-2 lactylate (Puratos, Portugal).

2.8.4.3 Bread dough preparation

The bread dough was prepared according to the method earlier described by Graça et al. 2019; 2021: 2.4 % yeast and 0.6 % sugar were added to warm (distilled) water and dissolved well; 1.0 % salt, 0.3 % SSL and 25 % of Yg or Cc were incorporated into 56.5 - 53.0 % wheat flour and mixed with 15.6 % – 19.0 % (distilled) water, according to each bread formulation, to complete 36.6 % of wheat flour water absorption, previously optimized (Table 1).

Water absorption (WA) was determined in the control dough (61.8 g water / 100 g wheat flour, corresponding to 36.6% w/w in the final formulation), applying the mixing curves results according to microdoughLab assays performed in Perten instruments (Hägersten, Sweden). Control dough and doughs enriched with Yg or Cc were prepared considering the addition of 40 g of DP (wet basis), corresponding to 25% w/w of bread formulation. Replacements of Yg or Cc were based on 100 g of wheat flour basis (baker´s formula), and on WA previously determined, according to the procedure previously optimised by Graça et al. (2019).

The dry extract and moisture deriving from each DP added were considered to optimize the bread formulations, i.e., for 25% Yg addition the value of moisture is 21.0% (w/w) and dry matter is 3.0% (w/w). Therefore, 15.6% (w/w) water was added to 56.5% (w/w) wheat flour, to achieve the 36.6% (w/w) WA. The amounts of other ingredients added were kept constant (Table 1).

2.8.5 Bioactivity assays

2.8.5.2 Antioxidant activity

To evaluate the antioxidant activity of wheat bread, methanolic extracts of ground bread samples (2 g) were first prepared by mixing the sample with 20 mL methanol, followed by centrifugation (8,000 *g*) at 4 ºC during 20 min. After filtration (0.2 µm filter), the extracts were stored at −4 °C until the experiments were conducted.

The scavenging effect of bread extracts was determined using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) methodology (Sánchez-Moreno et al., 1998).

According to this method, gluten-free bread extracts or an L-ascorbic acid solution (100 µL) were added to 1 mL of DPPH solution in methanol (90 µL/L), and the mixture was diluted to 1.9 mL with methanol. After 1h in the dark, the absorbance was measured at 515 nm. Mean values of the antioxidant capacity were reported as mg ascorbic acid equivalents (AAE) per g of bread extract.

The reducing power of bread extracts was determined using the ferric ion reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996). In this case, the bread extract or an L-ascorbic acid solution (90 µL) and 2700 µL of methanol were added to 2700 µL of FRAP reagent and the absorbance was measured at 595 nm after 30min in a shaking water-bath at 37 ºC. The mean values of reducing power were reported as mg of ascorbic acid equivalents (AAE) per g of bread extract.

The total phenolic content (TPC) of bread extracts was also evaluated, using the method reported by Oktay et al. (2003). The bread extract or gallic acid solution (150 µL) was added to 150 µL of 0.1 M Folin–Ciocalteu reagent and mixed with 300 µL of sodium carbonate (7.5% w/v) after 10 min. The mixtures were incubated in the dark at room temperature for 2 h, and then the absorbance was measured at 760 nm. TPC was expressed in mg of gallic acid equivalents (AGE) per g of bread extract.

Triplicates for each antioxidant activity assay were performed to ensure reproducibility of the results.

2.8.5.3 MMP-9 inhibition

To evaluate the MMP-9 inhibitory activity of different wheat breads, a buffer-solution extraction was performed. Bread samples (5 g) were added to 30 mL of Tris-HCl buffer solution, 100 mM, and pH 7.5, in a ratio of 1/6 (w/v) This solution was kept stirring for 4 h at 4 °C. Samples were then centrifuged in a Beckman J2-21M/E centrifuge at 12,000 *g* for 30 min at 4 ºC, and the supernatant was collected and stored at −20 °C. The MMP-9 inhibitory capacity of the yoghurt and curd cheese individually was also evaluated, applying the same procedure described above.

MMP-inhibition activity was tested using the dye-quenched (DQ)-gelatin assay as described before by Lima et al. (2016). The fluorogenic DQ-gelatin substrate (Invitrogen, CA, USA) was dissolved in milli-Q water at 1 mg/mL. All solutions and dilutions were prepared in assay-buffer (50 mM Tris-HCl buffer, pH7.6, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% v/v Tween 20).

A black micro-assay plate (Grainer bio-one, 96-well) was used, and each well was loaded with 0.1 mM (for a final volume of 200 µL) MMP-9 (Sigma) and 80 µL of each bread extract, and the plate was incubated for 30 min at 37 °C to allow inhibition. The dairy products and bread extract volume was kept constant (80 µL), equivalent to the following protein content (mg/5g of dairy product or bread): 8.64 ± 0.40 mg to Yg and 83.0 ± 3.15 mg to Cc, 6.8 ± 1.1 mg to CB, 12.20 ± 0.73 mg to YgB and 10.05 ± 0.07 mg to CcB.

Subsequently, to quantify the remaining MMP-9 catalytic activity, DQ-gelatin (at a final concentration of 2.5 µg/mL) was added to each well and the plate was incubated again, for 1 h. In the presence of gelatinolytic activity, the DQ-gelatin substrate is hydrolyzed and releases fluorescence. Fluorescence levels were measured (excitation 485 nm/emission 530 nm). In each experiment, both positive (without bread extracts) and negative (without enzyme) controls were included for all samples to correct for possible gelatinolytic activities present in the bread extracts. Assays were performed in triplicate and all data were corrected by subtraction of their corresponding negative controls.

2.8.5.4 Antibacterial activities

Two microorganisms were used in the assay, *Escherichia coli* (NCTC 9001) and *Listeria monocytogenes* (NCTC 11994). The microorganisms were grown for 24-48 h, a 37 °C in Brain Heart Infusion medium (BHI) (Biokar, França). The inoculum preparation was carried out by measuring absorbance (Boeco S-20, Hamburg, Germany), at 546 nm, obtaining a final absorbance of 0.12 ($2x10^8$ UFC.mL^{-1}) (Gottlieb et al., 2008). The bacterial suspensions containing 10^5 UFC.mL^{-1} were obtained by serial dilutions in Müller-Hinton's medium (Biokar, France).

The determination of minimal inhibitory concentrations (MICs) was performed as described by Lima et al. (2016), with some modifications. This assay was performed using flat-bottom 96-well clear microtiter plates, and 50 µL of Müller-Hinton medium was distributed, from the 2nd to the 12th well. In the 1st well of each line, 100 µL of each sample was placed. The sample dilutions were performed from the 1st to the 10th well, removing 50 µL from one well and diluting it 1:2 in the next well. Then, 50 µL of a bacterial suspension containing a concentration of about $2x10^5$ UFC.mL^{-1} were inoculated, from the 2nd to the 11th well. The 11th well was used as a positive control (50 µL of Müller-Hinton medium + 50 µL of bacterial suspension) and the 12th well was used as a negative control (100 µL of Müller-Hinton medium). The plates were incubated 24 h at 37 °C, and then the absorbance was read at 546 nm, in a microplate reader (Synergy HT, Biotek, USA).

2.8.6 Statistical analysis

All experimental data were shown by mean value ± standard error, and statistical analysis (RStudio, Version 1.1.423) using variance test in one factor (ANOVA), by Tukey test (post-hoc comparisons, were assessed. The significance level was assumed to be 5% ($p < 0.05$).

2.9 Results and discussion

2.9.4 Effect of yoghurt or curd cheese enrichment on wheat bread bioactivity

2.9.4.2 Antioxidant activity

The influence of yoghurt or curd cheese enrichment of wheat bread on antioxidant activity (AC), based on DPPH and FRAP methods, is presented in Figure 1 A and B.

Compared to CB (DPPH: 9.4 $mg \cdot mg^{-1}$ AAE, FRAP: 19.8 $mg \cdot mg^{-1}$ AAE), the incorporation of Yg (DPPH: 17.7 $mg \cdot mg^{-1}$ AAE, FRAP: 23.0 $mg \cdot mg^{-1}$ AAE) and Cc (DPPH: 12.8 $mg \cdot mg^{-1}$ AAE, FRAP: 28.9 $mg \cdot mg^{-1}$ AAE), led to an increase in the AC of the bread, corresponding to an enhancement in bread bioactivity of around 90% and 16% for Yg addition, and about 36% and 45% for Cc incorporation, respectively. The AC increment in Yg breads could result from the α-tocopherol (vitamin E) present in cow's milk, since this is a strong antioxidant (Tijerina-Sáenz et al., 2009). Additionally, it is known that the hydrophobic and aromatic amino acid residues of the WP exhibit bioactive properties (Mann et al., 2015) that probably improved the AC of the bread with Cc. Other research group demonstrated well the potential of fermented dairy products as rich antioxidant sources with recognized impact on human health (Qian et al., 2011).

The addition, Yg or Cc promoted an increase in total phenolic compounds (TPC) (Fig. 1C), which amounted to 11.2 $mg \cdot mg^{-1}$ AGE for YgB25% and to 14.0 $mg \cdot mg^{-1}$ AGE for CcB25%, when compared to 5.30 $mg \cdot mg^{-1}$ for CB, representing an increase of 115% and 170% respectively.

From the antioxidant capacity results obtained by yoghurt or curd cheese addition into bread dough, it can be stated that both improved the bread bioactivity properties, and probably can give an additional support to the consumer´s nutritional and preventive daily diet.

Similar results were found in previous works (Graça et al., 2020b) studying the effects of dairy product addition to gluten-free bread bioactivity fortification.

2.9.4.3 MMP-9 Inhibition activity

The impact of Yg or Cc enrichment of wheat bread based on the inhibition of MMP-9 activity, using the DQ-gelatin assay was evaluated, to assess the anti-inflammatory properties of the bread. The MMP-9 inhibitory capacity of yoghurt and curd cheese was assessed individually (Figure 2), in which of around 13.3 % and 21.6 % of inhibition effect were obtained, respectively, compared to control (C+: 100%).

Figure 2 shows that both dairy bread extracts had significant higher impact ($p < 0.05$) on MMP-9 inhibition activity, compared to the control (C+). The highest inhibition on MMP-9 activity was obtained in the CcB25% (27.5%), followed by the YgB20% (19.7%), compared to control bread (7.4%).

The improvement in the bread anti-inflammatory activity can probably derive for the dairy products incorporation into bread dough, and the presented data suggests that it can be further enhanced by fermentation activity. Considering the MMP-9 inhibition activity results obtained for yoghurt and curd cheese *de per si* (Figure 2), and those registered for dairy´s bread (after fermentation and baking), it shows that the fermentation process can probably boost the releasing of bioactive peptides with effect on MMP-9 inhibition activity.

In cereal systems fermentation the oligopeptides are released mainly by the activity of endogenous flour endoproteases, whereas secondary proteolysis releases free amino acids and small-sized (bioactive) peptides and occurs through microbial peptidase activity (Arte et al., 2015).

One can hints that the enrichment with both DP can be an approach to improve the bioactivity of wheat bread in terms of anti-inflammatory properties and probably, contributing partly to the long-term ingestion of natural food sources of specific MMP-9 inhibitors.

These results agree with those previously obtained by Lima et al. (2017), based on the inhibition of MMP-9 by phenolic compounds and proteins extracted from cooked soybeans.

Additionally, several studies have been shown the anti-inflammatory properties of dairy products, especially those obtained by fermentation processes, associating them as anti-inflammatory agents on humans' immune processes (Ceapa et al., 2013; Bordoni et al., 2017).

Recent studies revealed anti-inflammatory properties of the dairy products in subjects with metabolic disorders associated to low-grade chronic inflammation, such as cardiovascular diseases (Panagiotakos et al., 2010) type-2 diabetes (Panagiotakos et al., 2010) and obesity-related inflammatory conditions (Labonte et al., 2014).

It can be stated that the MMP-9 inhibition activity by wheat bread enriched with Yg or Cc can probably have a contribution to inflammatory preventive diets, such as low-grade chronic inflammatory diseases and specially to those suffering from irritable bowel disorders.

2.9.4.4 Antibacterial activities

Some food poisoning reports are related to bacterial contamination, especially Gram-bacteria such as *Escherichia coli* and Gram + bacteria such as *Listeria monocytogenes* (Pandey and Singh, 2011).

The influence of Yg or Cc addition on the growth inhibition of these foodborne pathogens on wheat bread, was tested. Wheat bread enrichment by each DP did not exhibit any growth inhibition against the microorganisms tested (data not shown).

2.9.5 Conclusion

From the present work, the influence of Yg or Cc enrichment into wheat dough to improve the antioxidant capacity, anti-inflammatory properties, and antibacterial activity of the wheat bread, was studied.

Bioactivity of the wheat bread with each DP addition was improved in terms of both antioxidant capacity and phenolic compounds, revealing higher values in comparison with control bread.

Higher MMP-9 inibhition activity by Yg or Cc bread enrichment was achieved, in comparison with control bread, suggesting that these DP can be interesting baker´s ingredients to improve anti-inflammatory properties of wheat bread.

Nevertheless, further *in vitro,* and *in vivo* assays to consolidate these anti-inflammatory properties achieved would be tested in future surveys.

In addition, the optimization of the breadmaking steps, such as fermentation time and baking conditions are going to be considered in future studies, to boost the bioactivity of the bread.

In summary, the positive results obtained revealed that these dairy baking goods can probably be linked to healthier foods to consumers in general, to fulfill the nutritional deficiencies, and to be part of a prevention diet.

Author Contributions: C.G. conceived and planned the experiments; performed all samples preparation and analysis, data analysis, interpretation of the results, and wrote the manuscript; J.M. contributed with bioactivity analysis and discussion, A.L. and R.B.F revised the manuscript, A.R. and I.S. supervised and validated the work, data discussion and writing—review and editing.

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Table 1: Bread formulation for control bread (CB) and bread obtained with yoghurt (YgB) or curd cheese (CcB) addition at 25% w/w (40 g / x g WF) considering the dry extract (DE) and moisture (M) deriving from both DP additions (Yg: 11.5% w/w DE and 88.5% w/w M; Cc: 25.8% w/w DE and 74.2% w/w M)

Ingredients (%, w/w)	CB	YgB25%	CcB25%
Wheat flour added	59.2	56.5	53.0
Deionized water added*	36.6	15.6	19.0
Yg or Cc dry extract	0.0	3.0	6.1
Yg or Cc moisture**	0.0	21.0	17.6
Water absorption***	36.6	36.6	36.6
Other ingredients ******	4.3	4.3	4.3

* Water added to achieve the 36.6% w/w of water absorption (61.8 g water/100 g wheat flour); ** Water deriving from Yg or Cc additions; *** Sum of the water added and the water deriving from Yg or Cc additions; **** salt –1.0% w/w (1.7 g); sugar – 0.6% w/w (1.0 g); dry yeast – 2.4% w/w (4.0 g) and sodium stearoyl-2 lactylate (SSL) – 0.3% w/w (0.5 g), corresponding to 4.3% w/w (7.2 g), of the bread formulation

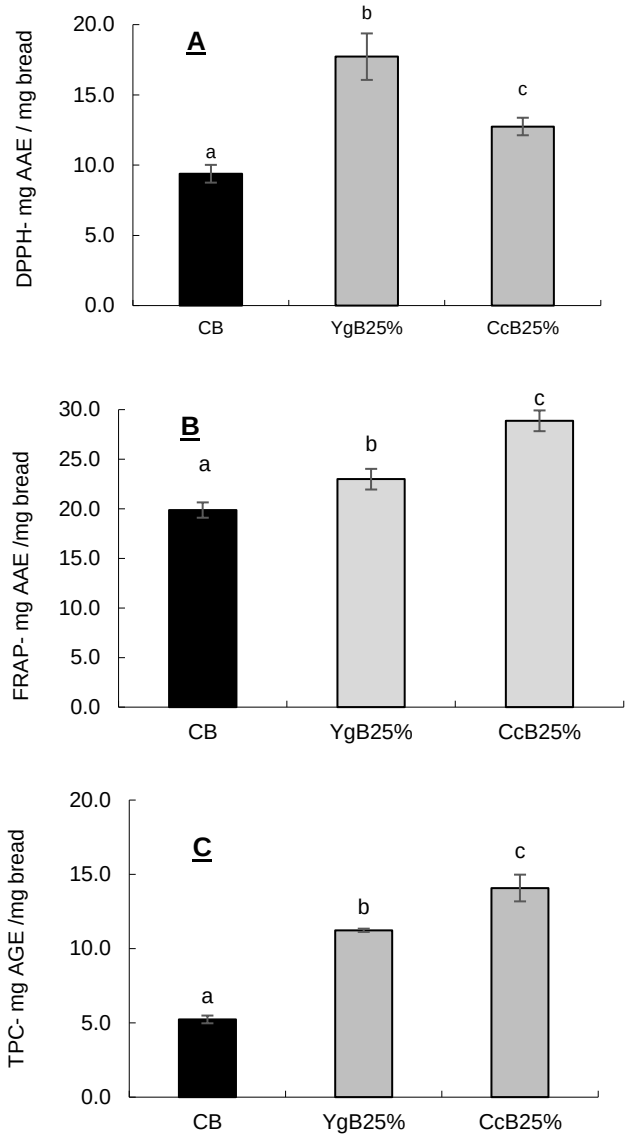

Figure 1: The antioxidant capacity of wheat bread was measured using the follow methodologies: A - DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate; $mg \cdot mg^{-1}$ ascorbic acid equivalents- AAE), B - FRAP (ferric ion reducing antioxidant power; $mg \cdot mg^{-1}$ ascorbic acid equivalents), and C - TPC (total phenolic content; $mg \cdot mg^{-1}$ gallic acid equivalents- GAE) of fresh breads enriched with 25% (w/w) yoghurt (YgB25%) or curd cheese (CcB25%) were compared to control bread (CB). Different letters in the same graph correspond to significant differences ($p<0.05$)

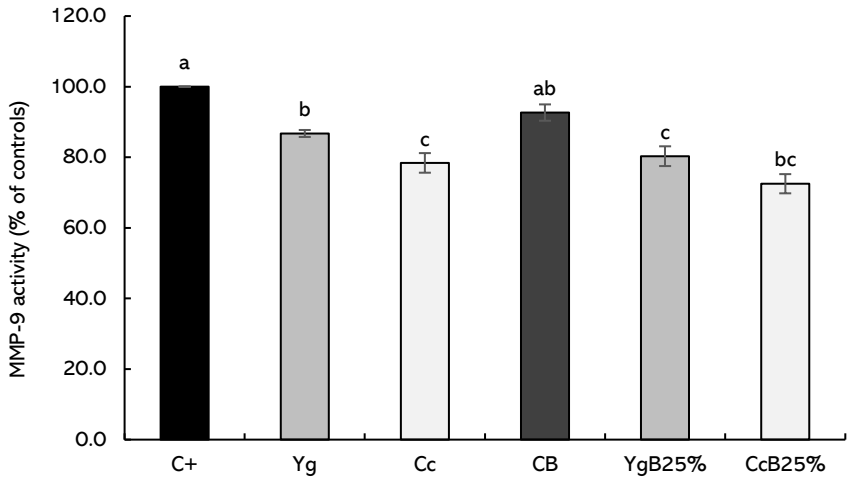

Figure 2: Effect of bread extracts on the proteolytic activity of MMP-9 after 1 h of incubation as determined by the DG-gelatin assay: yoghurt (Yg), curd cheese (Cc), control bread (CB), and bread enriched with 25% (w/w) yoghurt (YgB25%) or curd cheese (CcB25%). The control (C+) does not inhibit MMP-9, resulting in 100% proteolytic activity for this protease. MMP activities are expressed by relative fluorescence as a percent of controls and represent the averages of at least three replicate experiments (n = 3) ± standard deviation. Different letters correspond to significant differences ($p < 0.05$)

Chapter 3 (C3): Improving technological and nutritional/functional properties of gluten-free bread by yoghurt and curd cheese enrichment

In this chapter, the functionality of modified dairy proteins of yoghurt or curd cheese was evaluated to replace the gluten-like structure as a potential strategy to overcome the technological challenge of gluten-free bread manufacturing, while it can give an interesting contribution to fulfil the nutritional and functional deficiencies of gluten-free bread.

The work performed was divided into three different studies, resulted in three scientific works, that are presented in the following published research papers:

- **Research paper 4:**

Graça, C., Raymundo, A. and Sousa, I. (2020). Yogurt as an Alternative Ingredient to Improve the Functional and Nutritional Properties of Gluten-Free Breads*. Foods Journal*, 9, 111. https://doi.org/10.3390/foods9020111 (Impact Factor: 4.092, Q1).

- **Research paper 5:**

Graça, C., Raymundo, A., Sousa, I. 2020. Improving the Technological and Nutritive Properties of Gluten-Free Bread by Fresh Curd Cheese Enrichment. *Applied Sciences Journal, Special Issue in Food Sustainability: Using Byproducts from Food Industry and Unconventional Food Sources*, 10, 6868. https://doi.org/10.3390/app10196868 (Impact Factor: 2.474, Q2).

- **Research paper 6:**

Graça, C., Mota, J., Lima, A., Ferreira, R.B., Raymundo, A., Sousa, I. (2020). Glycemic Response and Bioactive Properties of Gluten-Free Bread with Yoghurt or Curd-Cheese Addition. *Foods Journal, Food Nutrition Section*, 9, 1410. https://doi.org/10.3390/foods9101410 (Impact Factor: 4.092, Q1).

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Yogurt as an Alternative Ingredient to Improve the Functional and Nutritional Properties of Gluten-Free Breads

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Abstract: Absence of gluten in bakery goods is a technological challenge, generating gluten-free breads with low functional and nutritional properties. However, these issues can be minimized using new protein sources, by the addition of nutritional added-value products. Fresh yogurt represents an interesting approach since it is a source of protein, polysaccharides, and minerals, with potential to mimic the gluten network, while improving the nutritional value of gluten-free products. In the present work, different levels of yogurt addition (5% up to 20% *weight/weight*) were incorporated into gluten-free bread formulations, and the impact on dough rheology properties and bread quality parameters were assessed. Linear correlations ($R^2 > 0.9041$) between steady shear (viscosity) and oscillatory (elastic modulus, at 1 Hz) values of the dough rheology with bread quality parameters (volume and firmness) were obtained. Results confirmed that the yogurt addition led to a significant improvement on bread quality properties, increasing the volume and crumb softness and lowering the staling rate, with a good nutritional contribution in terms of proteins and minerals, to improve the daily diet of celiac people.

Keywords: gluten-free bread; yogurt; rheology

3.1. Introduction

Celiac disease is an immune enteropathy caused by the ingestion of gluten in genetically susceptible individuals, and it is estimated to affect about 1–3% of the population worldwide [1].

Currently, the gluten-free (GF) diet is the only option for people suffering from gluten-related disorders. For other reasons, a high number of nonceliac individuals are also adopting this diet. Subsequently, there has been a significant increase of research work to improve the functional and nutritional properties of GF products [2,3]. However, several studies stated that the consumers remain unsatisfied with the quality of the GF products in the market [4], highlighted their insufficiency on overall appearance and nutritional values, compared to the gluten-containing counterparts [5,6].

Gluten-free products, especially breads being mainly based on refined flours and starches, are generally characterized by poor technological quality attributes, including dry, crumbling texture, color, and mouth feel [7], and undergo fast staling [8].

In terms of nutritional profile, normally they present deficient values of protein and minerals and higher carbohydrates and fat content than recommended [9].

Rice flour has been widely proposed as an alternative for making gluten-free breads (GFB) due to its hypoallergenic protein, soft taste, and white color [10]. However, rice-based bread presented low quality attributes, in terms of volume and hard crumb [11]. Alternative flours from pseudocereal

sources, such as amaranth, buckwheat, quinoa, sorghum, and teff, have been applied to improve the nutritional profile of the GFB [12].

In fact, gluten possesses unique viscoelastic properties which are crucial for the water-holding capacity of the dough and gas retention during fermentation [13]. To mimic this structure-building potential, hydrocolloids such as hydroxy-propril-methylcellulose, carboxy- or methylcellulose, locust bean, guar gum, and xanthan gum, as well as enzymes [8,14], are currently used to improve the viscoelastic properties and technological quality of the end-products [15]. Although these functional additives contribute to the gas retention during the fermentation process, improving the bread volume, they can be insufficient in terms of desirable bread texture and nutritional value.

Addition of new protein sources, e.g., dairy dry powders incorporation (skim milk powder, dry milk, whey protein concentrate) was shown to improve the volume, appearance, and sensory aspects of the loaves [16,17]. A previous work [18] showed a positive impact of dairy products addition, such as fresh yogurt, on gluten-containing bread where a significant improvement on functional properties and nutritional value was noticeable.

Yogurt (Yg), is considered the most popular dairy product worldwide for its nutritional and health benefits, since it is a source of protein (casein), exopolysaccharides (EPS), vitamins (B2, B6, and B12), and minerals (such as Ca, P, and K), representing an interesting alternative for new bakery products [18,19]. In this context, incorporation of fresh Yg in gluten-free bread formulations can be an interesting approach to mimic the gluten network, while improving the nutritional value of gluten-free breads.

The aim of the present work was to explore the potential of fresh yogurt addition on GF bread making and to overcome the technological challenge involved in gluten removal, improving the GFB quality. Different levels of Yg addition to a GF dough formulation, previously optimized, was tested, and the impact on dough, based on steady shear behavior and oscillatory measurements, was assessed. The effect on GFB quality, by the evolution of the crumb firmness and staling rate, during the storage time, as well as from bread quality parameters and nutritional profile, was also evaluated.

3.2. Materials and Methods

3.2.1. Raw Materials

Gluten-free bread was prepared using rice flour (composition per 100 g: Moisture content 10.9 g, protein 7.0 g, lipid content 1.3 g, carbohydrates 80.0 g, fiber 0.5 g), buckwheat flour (composition per 100 g: Moisture 13.1 g, protein 13.3 g, lipid content 3.4 g, carbohydrates 61.5 g, fiber 10.0 g, salt 0.08 g, phosphorus 0.35 g, and magnesium 0.23 g), and potato starch (composition per 100 g: Moisture content 17.5 g, protein 0.2 g, lipid content 0.1 g, carbohydrates 80.0 g).

The fresh yoghurt (Yg) used was a commercial product from LongaVida, Portugal (composition per 100 g: Moisture content 88.5 g, protein 3.7 g, lipid content 3.7 g, carbohydrates 5.5 g, fiber 0.8 g, 0.06 g of salt, and 0.12 g of calcium). The dry extract of Yg was determined from the standard Portuguese method: NP.703-1982 (Standard Portuguese Norm), corresponding to 11.5% of dry matter.

Commercial white crystalline saccharose (Sidul, Santa Iria de Azóia, Portugal), sea salt (Vatel, Alverca, Portugal), baker's dry yeast (Fermipan, Lallemand Iberia, SA, Setúbal, Portugal), vegetable oil (Vegê, Sovena Group, Algês, Portugal), and xanthan gum (Naturefoods, Lisboa, Portugal) were also used.

3.2.2. Bread Dough's Preparation and Bread making

Gluten-free bread dough was prepared in a thermo-processor (Bimby-Vorwerk, Cloyes-sur-le-Loir, France), according to the procedure earlier described [20], with some modifications. First, the yeast was activated in warm water on the processor cup, during 2 min at position 3. The rest of the ingredients were added and homogenized during 1 min at position 6, and the kneading was carried out during 10 min. Hydroxy-propril-methylcellulose (HPMC) was replaced by xanthan gum and the fermentation time was reduced to 20 min. Preliminary assays showed that 20 min of fermentation

was enough to obtain expanded doughs ready to bake. Further fermentation time led to dough structure breakdown, losing the gas (CO_2) produced and retained during this bread making step. Bread baking conditions: Oven with convection at 180 °C during 30 min.

Different GFB formulations tested are summarized in Table 1. Other ingredients added were kept constant: Salt, 1.5%; sugar, 2.8%; dry yeast, 2.8%; xanthan gum, 0.5%; and vegetable oil, 5.5%.

Table 1. Gluten-free bread formulations of control bread (CB) and breads obtained with different levels of yogurt (Yg) addition (YgB), considering the dry extract coming from Yg (11.5%) to replace on flour basis**.**

Ingredients	CB	$YgB_{5\%}$	$YgB_{10\%}$	$YgB_{15\%}$	$YgB_{20\%}$
Buckwheat	16.6	16.4	15.3	14.4	13.3
Rice	24.8	24.6	23.0	21.5	19.9
Potato starch	13.8	13.7	12.8	12.0	11.1
Yoghurt	0.0	5.0	10.0	15.0	20.0
Added water *	37.5	32.6	31.7	30.7	31.1
Yoghurt water **	0.0	4.7	8.5	11.6	14.1
Total water absorption ***	37.5	37.3	40.3	42.3	45.2

* Determined by mixing curves performed in the MicrodoughLab equipment; ** water coming from Yg addition; *** sum of water added and water coming from Yg addition.

Fixing the viscosity torque values of the gluten-free control dough (16.0 ± 2.0 milliNewton meter - mNm), previously optimized, the water flour absorption was determined for each Yg bread formulation tested (based on 14% of moisture basis), considering the water coming from Yg additions, applying the mixing curves procedure by MicrodoughLab assays (Perten Instruments, Hägersten, Sweden). Control dough and doughs obtained with Yg incorporations, were prepared considering the levels 10, 20, 30, and 40 g of Yg into dough, corresponding to 5% up to 20% *w*/*w* (weight/weight) in overall percentage. Replacements were based on gluten-free flours basis, substituting the dry extract of each Yg percentage on 100 g of flour [18].

3.2.3. Dough Rheology Measurements

All rheological measurements were carried out in a controlled stress rheometer (Haake Mars III—Thermo Scientific, Karlsruhe, Germany), with a universal temperature control Peltier system to control temperature, using a serrated parallel plate sensor system (PP35 and 1-mm gap), to overcome the slip effect [21,22].

The rheology features of the dough were evaluated after 20 min of fermentation time, and all the assays were conducted at 5 °C of temperature to inactivate the yeast fermentative activity.

The impact of the yogurt addition on dough viscosity behavior was assessed by flow curves, under shear steady conditions ranging the shear rate from 1.0×10^{-6} to 1.0×10^{3} s^{-1}.

The Carreau model was used to model the flow curves obtained, applying the Equation (1):

$$\eta = \eta_0/[1 + (\dot{\gamma}/\dot{\gamma}_c)^2]^{s} \quad (1)$$

where η is the apparent viscosity (Pa s), γ is the shear rate (s−1), η_0 is the zero-shear rate viscosity (Pa s), $\dot{\gamma}_c$ is a critical shear rate for the onset of the shear-thinning behavior (s−1), i.e., the value corresponding to the transition from Newtonian to shear-thinning behavior, and s is a dimensionless parameter related to the slope of this region.

Frequency sweep was applied to evaluate the impact of the Yg addition on dough structure, and the evolution of the viscoelastic functions, storage (G') and loss (G'') moduli, were obtained ranging the frequency from 0.001 Hz to 100.0 Hz, at a constant shear stress (10 Pa), within the linear viscoelastic region of each sample, previously determined (at 1 Hz).

All rheology determinations were repeated at least three times to ensure the reproducibility of the results.

3.2.4. Quality Assessment of the Gluten-Free Breads

3.2.4.1. Bread Firmness and Staling Rate

Bread texture was evaluated using a texturometer TA-XTplus (Stable MicroSystems, Surrey, UK) in penetration mode, according to the method earlier described [18,23].

Comparison of the bread texture, with different contents of Yg, was performed in terms of firmness, and the staling rate of the breads was evaluated, measuring the firmness during a storage time, during 96 h (4 days).

Staling bread rate was described as a function of the Yg incorporation by a linear Equation (2)

$$\text{Firmness} = A \times \text{time} + B, \quad (2)$$

where A can be considered the staling rate and B the initial firmness of the bread.

3.2.4.2. Quality Parameters of the Gluten-Free Bread

Resulting gluten-free breads were evaluated based on after baking quality parameters, such as moisture, water activity (aw), bake loss (BL), and specific bread volume (SBV) (cm^3/g), as earlier described [18].

Bread moisture was determined according to the standard method (American Association of Cereal Chemists (AACC 44–15.02)). Water activity (aw) variations were determined at room temperature (Hygrolab, Rotronic, Bassersdorf, Switzerland). Bread volume was measured by the rapeseed displacement standard method AACC 10-05.01, after two hours of bread cooling down. Specific volume (cm^3/g) was calculated as the ratio between the volume of the bread and its weight. Weight loss during baking (baking loss) was assessed by weighing the bread forms before and after baking. These measurements were carried out in triplicates.

Bread crumb color was recorded using a Minolta colorimeter (Chromameter CR-300, Minolta—Osaka, Japan) after calibration with a white calibration plate (L* = 97.21, a* = −0.14, b* = 1.99). The data collected from three slices of each bread measured at three different locations of the slices were averaged and expressed using illuminative D65 by L* a* b* scale, where: L* indicates lightness, a* indicates hue on a green (−) to red (+) axis, and b* indicates hue on a blue (−) to yellow (+) axis.

3.2.4.3. Nutritional Composition of the Gluten-Free Breads

Nutritional profile characterization of the gluten-free breads was based on protein (International Organization for Standardization (ISO-20483:2006)), lipids (NP 4168), ash (AACC Method 08-01.01), carbohydrates (calculated by difference), and total minerals contents (ICP-AES-Inductively Coupled Plasma-Atomic Emission Spectrometry: Thermo System, ICAP-7000 series) as described in a previous study [18]. All the experiments were performed in triplicate.

3.2.5. Statistical Analysis

The experimental data were statistically analyzed by determining the average values and standard deviation, and the significance level was set at 95% for each parameter evaluated. Statistical analysis (RStudio, Version 1.1.423, Northern Ave, Boston) was performed by applying variance analysis, the one factor (ANOVA), and post hoc comparisons (Tukey test). Experimental rheology data was fitted to nonlinear Carreau model, using the TA Instruments' TRIOS software.

3.3. Results and Discussion

3.3.1. Dough Rheology Measurements

3.3.1.1. Steady Shear Flow Curves

The effect of the yogurt addition, at different levels, on steady shear behavior of the gluten-free doughs was evaluated, and the flow curves obtained are presented in Figure 1. It can be observed

that all systems showed a typical shear-thinning behavior: An initial Newtonian region with constant viscosity at low shear rate, and as the shear rate values increase the dough viscosity began to decrease, following a straight-line decay. Similar results were obtained from the study of the different hydrocolloids' (e.g., xanthan gum) and dairy proteins' interaction on rheology properties of GFB formulations [17].

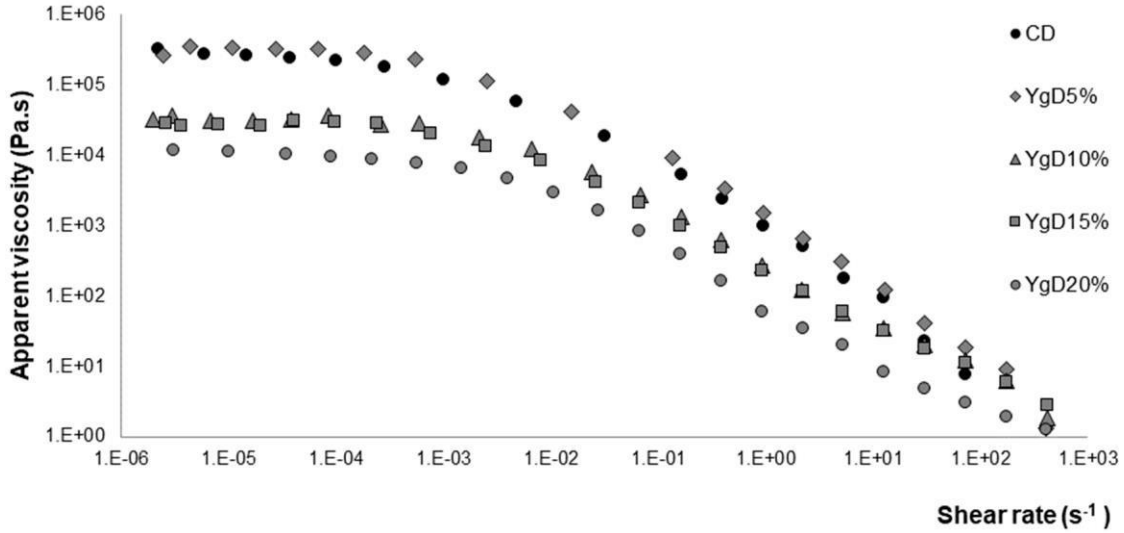

Figure 1. Flow curves, under steady shear conditions, obtained for control dough (CD) and doughs obtained with different levels of Yg addition (YgD).

The experimental data presented in Figure 1 were fitted well by the Carreau model ($R^2 > 0.967$), and the values of the main parameters that characterize the flow behavior are summarized in Table 2.

Table 2. Carreau model parameters obtained for control dough (CD) and doughs obtained with different levels of Yg addition (YgD)*.

Yg levels (%)	η_0 (k Pa s)	$\gamma \cdot_C$ (s^{-1})	s (slope)	R^2
CD	290.00 ± 8.4 ^{a}	1.70E^{-3} ± 2.50E^{-4} ^{a}	0.27 ± 0.03 ^{a}	0.988
$YgD_{5\%}$	295.04 ± 13.4 ^{a}	2.20E^{-3} ± 1.41E^{-4} ^{a}	0.23 ± 0.03 ^{a}	0.980
$YgD_{10\%}$	41.90 ± 1.8 ^{b}	2.52E^{-3} ± 1.34E^{-4} ^{a}	0.19 ± 0.03 ^{ab}	0.977
$YgD_{15\%}$	28.70 ± 0.8 ^{c}	2.40E^{-3} ± 1.70E^{-4} ^{a}	0.19 ± 0.04 ^{ab}	0.976
$YgD_{20\%}$	9.50 ± 0.3 ^{d}	1.43E^{-3} ± 1.80E^{-4} ^{a}	0.17 ± 0.06 ^{b}	0.967

* Different letters (a, b, c, d) within the same column indicate significant statistical differences at $p \leq 0.05$ (Tukey test) compared with the control bread values.

As it can be observed from the zero-shear rate viscosity values (η_0), higher dough viscosities were obtained for the control dough (CD) and yoghurt dough at 5% ($YgD_{5\%}$, lower level of Yg tested). According to previous works [17,24], GF doughs obtained with xanthan gum (XG) addition showed high viscosities and flow behavior indexes due to the complex aggregates formed by strong molecular linkages. However, a balance must be reached since high viscosity may retain bubbles in the batter, but it may also restrict expansion during baking [25].

Nevertheless, increasing the amounts of Yg resulted in a significant decrease of dough viscosity (η_0), varying from 290.00 k Pa s (CD) to 9.50 k Pa s ($YgD_{20\%}$), representing a reduction of around 96.60%. One can suggest that the Yg incorporation promoted a dilution effect of starch–cereal protein–xanthan gum interaction density, decreasing the dough viscosity under shear conditions. In addition, the presence of the casein and of the exopolysaccharides (EPS) coming from Yg (produced

by lactic acid bacteria) impacted the system, improving the lubrication and flexibility of the dough network. These effects can also be explained by the ability of EPS to bind water and retain moisture, contributing to the increase in the water-holding capacity [26] and, possibly, reducing the rigidity of the linkages between the different molecules of the GF dough matrix. Similar findings were obtained by other authors [27], investigating the addition of caseins and albumins on GFB formulations, where a considerable reduction on dough viscosity was obtained.

In terms of critical shear rate, no significant differences were observed. All the dough systems' viscosity began to decrease around 2.0×10^{-3} s^{-1}. This behavior suggests that, although the addition of Yg can promote the dilution effect on molecular links density as well as reducing the stiffer complex aggregates, it seems to have no effect on the breakdown of the dough matrix.

3.3.1.2. Dough Viscoelastic Behavior

The changes on viscoelastic behavior, expressed in terms of elastic (G′) and viscous (G′′) moduli, of the gluten-free doughs, with different levels of Yg addition, were evaluated on fermented doughs, by oscillatory frequency sweep measurements.

The comparison of the mechanical spectra of control dough (CD) and doughs obtained with Yg additions (YgD) are represented in Figure 2.

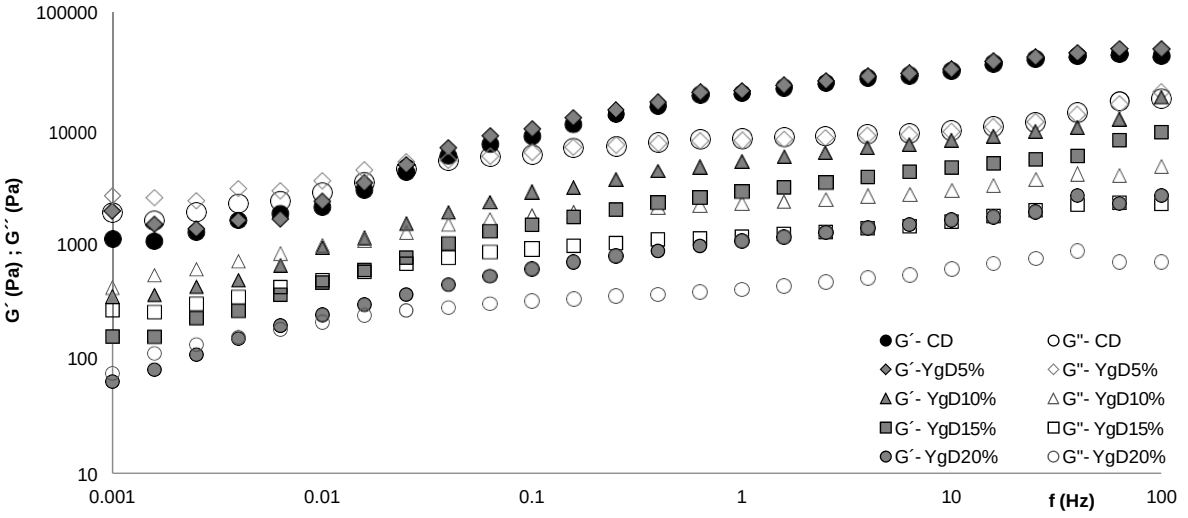

Figure 2. Changes in viscoelastic functions, elastic (G′) and viscous (G′′) moduli, promoted by different levels of Yg addition, $YgD_{5\%}$ up to $YgD_{20\%}$, compared to control dough (CD).

Figure 2 shows that the elastic (G′) and viscous (G′′) moduli values obtained for Yg doughs, from 10% of Yg addition, were lower than control dough (CD), indicating a formation of weaker structures more like batter. No significant differences in viscoelastic profile for lower values of Yg tested ($YgD_{5\%}$) were observed, comparing with CD.

These results are aligned with those obtained by steady shear flow curves, discussed above, where a significant reduction of dough viscosity was registered from 10% on of Yg addition. Similar results were obtained by other researchers [10,28], using different gluten-free flours, proteins sources, and hydrocolloids in GFB formulations.

Analyzing in detail the results (Figure 2) at low frequencies, all the dough systems displayed a viscoelastic fluid behavior with values of G" higher than G′, characteristic of an entangled network [29–31], probably formed by the proteins', exopolysaccharides', and starch molecules' interaction. However, with the frequency increase, the crossover of both moduli occurred and the dominance of the G′ over the G′′ was observed, expressing a typical pseudo-gel behavior [28–30], with high frequency dependence [32]. These results agree with previous findings obtained by the study of the rheology evolution of gluten-free flours and starches during bread fermentation and baking [33].

It can be observed that the frequency values of the G′ crossing over the G′′ were reduced by the Yg addition to dough, varying from 0.025 Hz for CD to 0.004 Hz for $YgD_{20\%}$ (higher level tested). Based on these results, and although the Yg addition promoted significant changes on dough structure, some reinforcement of the molecular linkages on the dough matrix should be considered. This may be explained by the presence of casein and exopolysaccharides coming from Yg that are acting on the system through three probable mechanisms:

- Improving the orientation and disentanglement of the molecular linkages on the dough matrix, under oscillatory conditions [34], by lubrification and flexiblising effects,
- Reducing the stiffer network structure formed by xanthan gum, giving more flexibility to the dough network [17], and
- Reinforcing the dough molecular bonds, by additional casein and exopolysaccharides interaction, giving more stability to the dough [13,17].

These results showed that the Yg additions promoted considerable changings in viscoelastic properties that resulted in the improvement of the dough network capacity to incorporate and retain gas bubbles produced by yeast′s fermentative activity, consequently, reducing significantly the viscoelastic properties of the dough under oscillatory conditions. This observed behavior resulted in better texture properties and higher specific volumes of the bread [13].

From previous work, it was already stated that interactions between protein and polysaccharides led to similar changes in the viscoelastic functions (G′ and G′′) profile of gluten-free bread doughs [10]. These findings are also in line with those obtained by other researchers [17] evaluating the effect of different hydrocolloids and proteins on rheology properties of GFB formulation.

3.3.2. Evaluation of the Gluten-Free Bread Properties

3.3.2.1. Bread Texture and Staling Rate

The texture of the GFB produced with different contents of Yg addition was evaluated based on bread crumb firmness by a puncture test, and subsequent bread staling rate obtained from the evolution of the bread firmness, during the storage time of 96 h at room temperature [18,23].

From Figure 3, it can be observed that the Yg addition had a significant ($p \leq 0.05$) positive impact to increase the bread crumb softness: Initial and final values of control bread firmness were higher than all levels of Yg tested, varying from 2.25 Newton (initial) to 6.50 Newton (final) for CB and 0.82 Newton (initial) to 1.35 Newton (final) for higher levels of Yg tested on breads ($YgB_{20\%}$), representing a decrease of 64% and 80% of crumb firmness values, respectively.

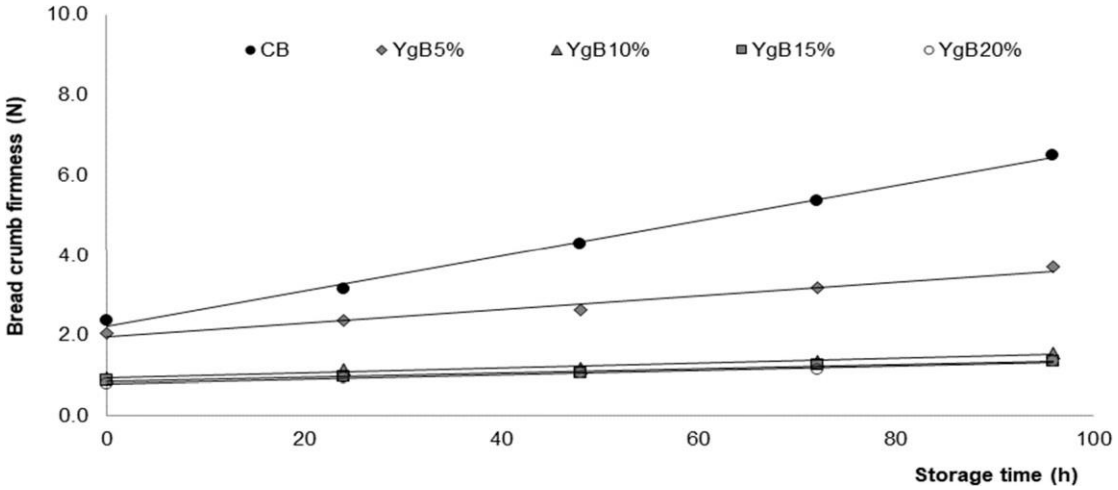

Figure 3. Variation of bread crumb firmness during 96 h of storage time, at room temperature, obtained for breads with different levels of Yg addition ($YgB_{5\%}$ up to $YgB_{20\%}$) compared to control bread (CB).

Bread staling rate was described as a function of time ($R^2 > 0.974$). The linear parameters presented in Table 3 clearly reflect the impact of Yg additions on bread staling rate (A, the slope) and initial firmness (B, the interception).

Table 3. Bread stalling parameters: A, bread staling rate (Newton/h), and B, initial bread firmness (N), obtained for control bread (CB) and breads with different levels of Yg tested (YgB)*.

Yg levels	B—Initial Firmness (N)	A—Staling Rate (N/h)	R2
CB	2.25 [a]	0.044 [a]	0.997
$YgB_{5\%}$	1.90 [b]	0.020 [b]	0.974
$YgB_{10\%}$	0.95 [c]	0.006 [c]	0.975
$YgB_{15\%}$	0.85 [c]	0.005 [c]	0.979
$YgB_{20\%}$	0.82 [c]	0.005 [c]	0.993

*Different letters (a, b, c) within the same column indicate significant statistical differences at $p \leq 0.05$, (Tukey test), compared with the control bread parameters.

The staling rate of the Yg breads were significantly lower than for the (CB), ranging from 0.044 N/h (CB) to 0.005 N/h for higher level of yoghurt tested yoghurt bread ($YgB_{20\%}$), representing a reduction of 90%. Similar results were obtained by other authors [35] evaluating the impact of the dairy powders on loaf and crumb characteristics and on shelf life of GFB. These findings are also in line with those obtained by a previous work [18] evaluating the impact of fresh dairy products' addition on technological, nutritional, and sensory properties of wheat bread. Good results of bread texture and staling rate can be explained by the presence of exopolysaccharides, coming from the Yg addition, since it has been well demonstrated that EPS improve bread texture properties, and such effects are related to its ability to bind water and retain moisture, retarding the starch crystallization and, hence, the increase of bread firmness [26,36–38].

It can be stated that the Yg addition increased the bread crumb softness and delayed the staling rate of the GFB, leading to an increase of shelf life, which is an important industrial advantage.

3.3.2.2. Quality Parameters of Gluten-Free Bread

The impact of the Yg addition on gluten-free quality parameters, such as crumb color, moisture, water activity (aw), bake loss (%), and specific bread volume (SBV), was evaluated. Results obtained are summarized in Table 4.

Table 4. Gluten-free bread quality parameters: Crumb color (L*, a*, b*), moisture, water activity (aw), bake loss (BL), and specific bread volume (SBV) of the control bread (CB) and breads produced with different levels of Yg (YgB) addition*.

Samples	L*	a*	b*	Moisture (%)	aw	BL (%)	SBV (cm^3/g)
CB	59.52 ± 2.85 ^{a}	7.81 ± 0.23 ^{a}	11.40 ± 0.20 ^{a}	42.00 ± 0.33 ^{a}	0.959 ± 0.006 ^{a}	10.00 ± 0.41 ^{a}	1.80 ± 0.08 ^{a}
$YgB_{5\%}$	59.93 ± 2.83 ^{a}	6.21 ± 0.08 ^{ab}	13.61 ± 1.20 ^{a}	43.20 ± 0.11 ^{a}	0.979 ± 0.001 ^{a}	9.35 ± 0.93 ^{a}	2.10 ± 0.03 ^{b}
$YgB_{10\%}$	62.52 ± 2.75 ^{ab}	5.26 ± 0.17 ^{b}	19.60 ± 0.75 ^{b}	46.40 ± 0.20 ^{b}	0.978 ± 0.002 ^{a}	8.44 ±1.24 ^{ab}	2.40 ± 0.05 ^{c}
$YgB_{15\%}$	68.02 ± 1.60 ^{b}	4.16 ± 0.11 ^{c}	22.90 ± 0.50 ^{bc}	48.00 ± 0.04 ^{c}	0.981 ± 0.005 ^{a}	7.50 ± 0.30 ^{b}	2.43 ± 0.08 ^{c}
$YgB_{20\%}$	68.60 ± 2.65 ^{b}	4.05 ± 0.15 ^{c}	25.32 ± 0.73 ^{c}	50.00 ± 0.83 ^{d}	0.985 ± 0.003 ^{a}	7.30 ± 0.94 ^{b}	2.50 ± 0.06 ^{d}

*Different letters (a, b, c, d) within the same column, for each level of Yg, indicate statistically significant differences at $p \leq 0.05$ (Tukey test), compared with the control bread parameters.

Bread crumb color was significantly changed by Yg addition, giving a lighter color (higher L* values) with more pronounced yellow tone (higher b* values) while the red tone became less dominant (lower a* values).

In terms of bread moisture, an increase in moisture content was observed (from 10% on, of Yg addition), varying from 42.0 for CB to 50.0 for $YgB_{20\%}$ (higher level tested), corresponding to an increase of 20%. Related to water activity, no significant ($p \geq 0.05$) differences were registered for all levels tested, compared to CB.

The bake loss (BL) represented the amount of water and organic material (CO_2 and other volatiles) lost during baking [12]. One can see (Table 4) that increasing the amounts of Yg led to a significant decrease of BL ($p \leq 0.05$), corresponding to an increase of 27% on bread yield, comparing CB with $YgB_{20\%}$. Presumably, as discussed above, the dough network formed by the Yg addition, probably via starch molecules, vegetable protein, casein, and EPS (coming from Yg), had a higher ability to trap water and it may cause an increase in water-holding capacity, consequently, a decrease on the BL percentage [39–41]. These results agree with those published by other authors [26] on the combination of dairy proteins with transglutaminases on GFB formulation.

Based on specific bread volume (SBV), a significant improvement was observed by Yg addition from 10% on, varying between 1.80 cm^3/g for CB to 2.50 cm^3/g for $YgB_{20\%}$, representing an increase of around 40%. These improvements on the SBV were probably due to the increase in water-holding capacity, contributing to the starch molecules being more prone to form a uniform continuous starch–protein matrix, which was further enhanced during baking [12,41]. In addition, the contribution of the caseins and exopolysaccharides, coming from Yg addition, cannot be excluded, probably by building a structured starch–protein–EPS matrix [38,39], improving the flexibility of the dough network and the capacity to retain the gases produced during the fermentation process, contributing to keeping the bread structure with uniform gas cell distribution, resulting in better bread volumes than CB, as can be observed in Figure 4.

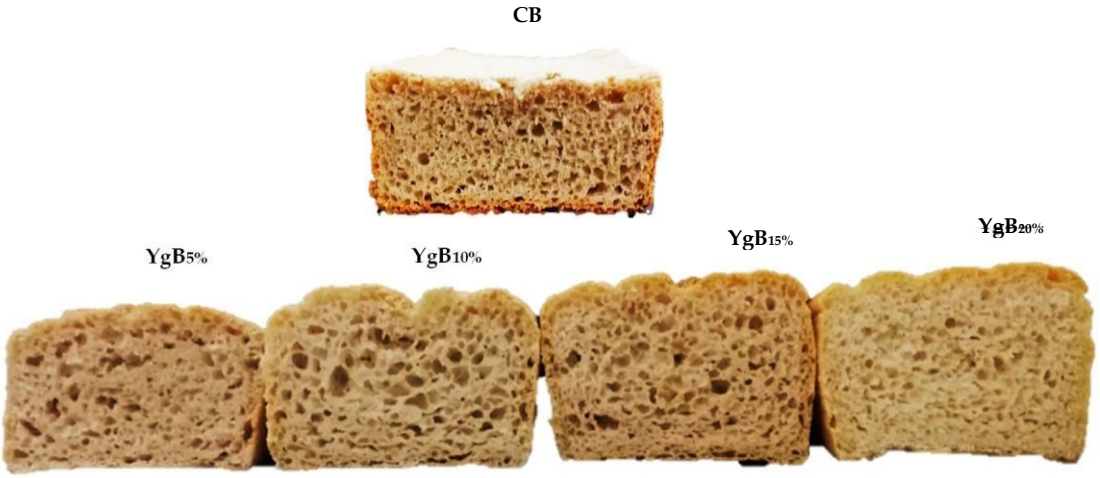

Figure 4. Control bread (CB) and breads obtained with different levels of Yg addition (YgB): 5, 10, 15, and 20% (*w/w- weight/weight*).

Our results agree with those published by other authors [26] reporting the effect of dairy proteins (caseins and albumins) with transglutaminase additions on the improvement of crumb texture and better SBV.

Resuming, it can be stated that the Yg addition improved the bread quality of the gluten-free bread in all baking quality parameters evaluated.

3.3.3. Relationship between Bread Quality Parameters and Dough Rheology Properties

Bread crumb texture and volume are considered the most important baking properties by the consumers [12] and the incorporation of fresh Yg showed to be a potential ingredient to improve these bread quality parameters.

The results presented along this work suggested a relationship between bread quality parameters and dough rheology properties. Linear correlations ($R^2 > 0.9041$) between the bread firmness (BF) and specific bread volume (SBV) with steady shear (dough viscosity (DV)) and oscillatory (G, elastic modulus at 1Hz) values of dough rheology was obtained. The linear correlations are illustrated in Figures 5.

As can be observed from Figures 5, while the control dough (only with xanthan gum) exhibited higher dough viscosity (DV) and elasticity (G′ at 1Hz) values, the specific volume of this bread was not high, and the crumb firmness was harder than the Yg doughs obtained. Previous works [13,17] reported that, although xanthan gum had the most pronounced effect on viscoelastic properties of the dough, the bread texture and volume were negatively affected.

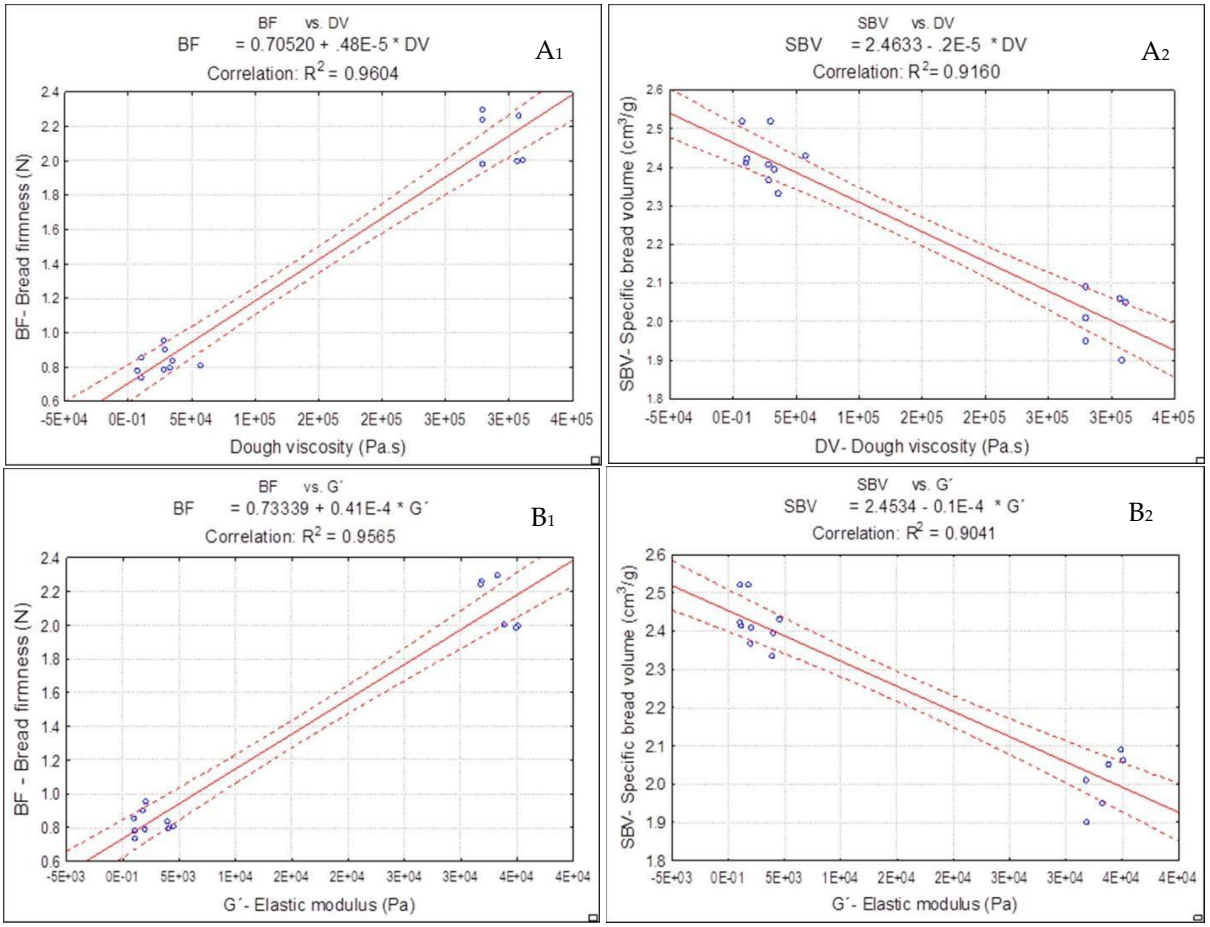

Figure 5. Linear correlation between bread firmness (BF) and specific bread volume (SBV) with dough viscosity (DV) and elastic modulus at 1 Hz (G′): A1) BF vs. DV, A2) SBV vs. DV, B1) BF vs. G′, B2) SBV vs. G′.

In opposite, lower values of dough viscosity (DV) and elastic modulus (G′ at 1 Hz) by the Yg addition to dough resulted in breads with lower values of crumb firmness (B1) and staling rate and with higher specific volumes (B2) than control bread.

These linear correlations support the results presented along this work, showing a strong correlation between bread quality parameters and dough rheology properties. It can be stated that the dough system was improved by Yg addition, which resulted in softer breads with better volumes, as aforementioned.

These findings disagree with those obtained by other researchers [11,17] evaluating different gums and emulsifiers on gluten-free bread formulations, where higher dough viscosity and elastic values resulted in lower firmness values of the bread. It suggests that the quality of the GFB depends strongly on the type of the ingredients used and the interactions between the macromolecules into play, to mimic the structure building-like gluten matrix.

These relations between the dough rheology and bread quality can be useful to predict the behavior of the dough and provide important information for the GF bread making industry.

3.3.4. Nutritional Composition of the Gluten-Free Breads

The nutritional composition of the gluten-free breads, including the mineral profile, were determined for control bread (CB) and breads obtained with 10% and 20% of Yg addition (YgB). A significant ($p \leq 0.05$) reinforcement on protein and ash content was observed for both levels of Yg

tested. However, a remarkable effect was obtained for 20% *(w/w)* of Yg addition in both cases, representing an increase of 52% and 55%, respectively. Nutritional composition and minerals profile of the gluten-free breads are summarized in Table 5.

Table 5. Proximate nutritional composition and mineral profile for control bread (CB) and breads enriched with 10% ($YgB_{10\%}$) and 20% ($YgB_{20\%}$) of yogurt*****.

g/100 g	CB	YgB10%	YgB20%	
Proteins	5.34 ± 0.21 ^{a}	6.90 ± 0.02 ^{b}	8.10 ± 0.20 ^{c}	
Lipids	4.83 ± 0.29 ^{a}	5.20 ± 0.84 ^{a}	5.60 ± 0.22 ^{a}	
Ash	1.40 ± 0.03 ^{a}	1.74 ± 0.17 ^{ab}	2.12 ± 0.27 ^{b}	
Carbohydrates	45.61 ± 1.12 ^{a}	39.00 ± 2.25 ^{b}	34.20 ± 1.58 ^{c}	
Kcal	250.30 ± 1.40 ^{a}	237.07 ± 2.13 ^{b}	226.52 ± 2.89 ^{c}	
	Minerals content mg/100 g			15% RDV (mg/100g) **
Na (g/100 g)	0.41 ± 0.03 ^{a}	0.42 ± 0.01 ^{a}	0.53 ± 0.02 ^{b}	
K	412.0 ± 8.02 ^{a}	497.30 ± 5.92 ^{b}	515.40 ± 16.40 ^{b}	300.0
P	131.81 ± 5.40 ^{a}	133.90 ± 0.90 ^{a}	141.24 ± 2.00 ^{b}	120.0
Mg	48.70 ± 1.02 ^{a}	50.24 ± 0.56 ^{a}	52.32 ± 0.82 ^{b}	45.0
Ca	35.93 ± 2.06 ^{a}	52.20 ± 0.81 ^{b}	92.60 ± 2.60 ^{c}	120.0
Fe	6.94 ± 0.12 ^{a}	4.74 ± 0.67 ^{b}	3.10 ± 0.05 ^{c}	2.1
Cu	0.23 ± 0.01 ^{a}	0.25 ± 0.02 ^{a}	0.30 ± 0.03 ^{a}	0.2
Mn	0.51 ± 0.01 ^{a}	0.60 ± 0.02 ^{a}	0.70 ± 0.03 ^{b}	0.3
Zn	1.12 ± 0.01 ^{a}	1.05 ± 0.08 ^{a}	0.98 ± 0.01 ^{b}	2.3

*Different letters used (a, b, c, d) indicate statistically significant differences, within the same row, at $p \leq 0.05$ (Tukey test), compared with the bread control; **according to the recommended daily values (RDV) established by Regulation (European Community), N° 1924/2006; Directive N° 90/494 (CE).

In terms of lipids, no significant ($p \geq 0.05$) differences were obtained. Based on carbohydrates content, a significant decrease for higher levels of Yg tested (20% *w/w*) was observed, representing a decrease of 25%, due to the dilution effect of the starch content.

Regarding the minerals profile, a significant improvement ($p \leq 0.05$) of major (Ca, K, P, Mg) and trace minerals (Fe, Cu, Mn) was obtained, representing, in general, more than 15% of the recommended daily values (Regulation (European community)), No. 1924/2006; Directive No. 90/494 (CE). Considering the higher level of Yg tested ($YgB_{20\%}$), a significant increase in Ca (158.0%), K (25.1%), P (7.0%), and Mg (4.1%) can be noticed as well as in Fe (55.3%), Cu (30.4), and Mn (37.3%), compared to control bread (Table 5). Similar results were obtained by other authors [18] testing the addition of dairy products on wheat bread.

These results showed that the Yg addition can be used to enhance the nutritional value of the GFB, increasing the amount of protein and minerals profile with a good contribution to reducing the carbohydrates intake.

3.3.5. Conclusions

Gluten-free bread formulations, with different levels of yogurt addition, were evaluated using dough rheology measurements and baking quality parameters.

The results of the present work showed that the functionality of gluten-free breads, in terms of bread making performances, quality parameters, and nutritional profile can be successfully improved by the addition of fresh yogurt.

Although, the yogurt incorporation significantly reduced the rheology properties of doughs, such effects resulted in significant improvements in the overall quality of the corresponding breads.

Good linear correlations between bread firmness, specific volume with flow behavior, and viscoelastic functions were found, supporting the results obtained.

Resuming, the Yg showed to be a potential ingredient to improve the quality of gluten-free breads, resulting in softer breads with higher volume and lower staling rate, compared to control bread.

Related to the nutritional composition, the addition of Yg revealed to be an attractive ingredient to enhance the nutritional value of GF breads, increasing the protein and mineral contents and reducing the carbohydrates intake, with a good contribution to improve the daily diet of the celiac people.

Author Contributions: C.G. conceived and planned the experiments; performed all samples preparation, analysis, data analysis, and interpretation of the results; and wrote the manuscript. A.R. and I.S. supervised the research work, contributed to the discussion of the data, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Improving the Technological and Nutritive Properties of Gluten-Free Bread by Fresh Curd Cheese Enrichment

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Abstract: Replacing wheat flour in the breadmaking process is a technology challenge since the elimination of gluten has a strong influence on bread quality. Protein's addition is often used to form a protein network capable of mimicking gluten-like structure, giving to dough a foaming support. This study aimed to evaluate the potential of denatured whey proteins coming from fresh curd cheese addition, to strengthening gluten-free dough structure, enhancing the breadmaking performance. Curd cheese additions were tested (5% up to 20%, weight/weight) and the effect on dough rheology behavior and bread quality was evaluated. Findings obtained revealed that the technology and nutritional properties of the bread can be enhanced by curd cheese addition, and such effects should be related to the composition and functionality of denatured whey proteins. Considering higher levels of curd cheese (20%) tested, improvements on bread quality was observed, leading to a considerable increase in bread volume (73%), softness (65%), with a significant reduction on staling kinetics (70%), comparing with control bread. Additionally, an improvement in nutritional value in terms of proteins (80%) and minerals content (P—50.0%, Mg—6.0%, and Ca—360.3%) was obtained, which can give an additional contribution to the nutritional daily requirements of celiac patients. Linear correlations between dough rheology properties and bread quality attributes were found, supporting the good breadmaking performance obtained.

Keywords: curd cheese enrichment; whey protein; mimic gluten structure; dough rheology; bread texture; gluten-free bread

3.4.Introduction

The demand for gluten-free products, especially bread, has been growing since the number of celiac disease diagnoses, as well as other gluten intolerance cases, have been increasing, requiring the absence of gluten from the diet [1].

Celiac disease is characterized by a small gut inflammation via autoimmune response to specific peptides of gliadin, one of the gluten proteins [2]. This gluten intolerance can lead to several health-associated disorders, as the damage and atrophy of the gut villi results in malabsorption of nutrients [2], with a great impact on health.

Gluten is the main structure-forming protein in flour, responsible for the dough viscous-elasticity features that contribute to a good appearance and crumb structure of the baked goods [3]. The protein fractions in the gluten network are glutenin and gliadin: the former gives elasticity to dough, while gliadin produces a viscous, fluid mass, under hydration conditions. The gluten matrix is a protein structure determinant of wheat dough properties such as dough resistance to stretch, extensibility,

gas retention capacity, bread volume, and softness, responsible for the quality of final products. Therefore, gluten removal results in major technological problems leading to low quality and short shelf life of the gluten-free products [1]. The technological challenge of gluten removal has led to the search for alternatives to mimic gluten structure in the manufacture of gluten-free bakery goods [1–7].

In addition, the nutritive value of the gluten-free bread (GFB) presents, generally, a deficient nutritional profile of protein and minerals, but high contents of carbohydrates and fat [4].

To overcome this challenge, several approaches were taken in the last few years, as the use of gluten-free cereal flours (e.g., rice and corn flours) [5], pseudocereals (e.g., quinoa, amaranth, buckwheat) [6], and starches (corn, potato, cassava) [5–8]. To mimic the gluten-like structure, the usage of certain hydrocolloids such as hydroxypropyl-methylcellulose and guar and/or xanthan gum [5,7], have been widely tested to enhance the dough viscoelastic features and GFB technological properties [8]. Proteins are naturally good hydrocolloids and one of the main molecule classes available to improve desirable textural attributes, promoting crosslinking and aggregation mechanisms, with a great impact on food structure. They are extensively used in different gluten free formulations, such as egg albumin and whey protein [1,9], due to their functionality, based on water binding and holding capacity, emulsifier, foaming, and gelling properties. In addition, proteins present a key role in Maillard reactions, which are responsible for color development, needed to improve the appearance of GFB crust [10].

Dairy proteins are interesting ingredients to enhance the nutritional properties of GFB due to their protein content and balanced amino acids profile, minerals content as calcium and phosphorus, as well as functional properties on baking performance [11].

A recent work showed that the incorporation of fresh dairy products on wheat bread formulations increased the nutritional value and improved the texture and other bread quality attributes [12]. Other researchers showed relevant enhancements in breadmaking, by the addition of whey protein (WP) on GFB formulations, reporting the capacity to increase water absorption, improving the handling properties [13]. Good thermal gelation capacity of WP inducing viscoelastic gels by protein network, capable of supporting dough foaming, was also reported [14].

Curd cheese (Cc) is a coproduct obtained by the thermal denaturation and subsequent precipitation of the soluble whey proteins. These products are considered to have a high protein nutritional value, representing of 20–30% of the proteins present in bovine milk (beta-lactoglobulin, alfa-lactalbumin) and are considered to be an important source of essential amino acids (leucine, isoleucine, and valine) and minerals (e.g., Ca and P) [15].

Therefore, the enrichment of GFB with fresh Cc can be an interesting approach to enhance their nutritional value, and the combination between vegetable (cereal) and animal proteins (denatured whey protein from Cc) could represent an alternative to mimic a foam structure support like the gluten network, improving bread quality.

This research work aimed to evaluate the influence of the fresh Cc addition on gluten-free breadmaking performance, to improve the GFB quality. Various levels of Cc addition into a gluten-free dough were tested, and the effect on dough rheology properties, by mixing curves, steady shear flow behavior, and small amplitude oscillatory assays, was assessed. The impact on GFB quality, by evaluating the loaf firmness and staling kinetics during the storage time, as well as by the after-baking quality attributes and the nutritive value, were also evaluated.

3.4.1. Materials and Methods

3.4.1.1. Raw Materials

GFB were prepared using rice flour (Próvida, Pêro Pinheiro, Portugal), buckwheat flour (Próvida, Pêro Pinheiro, Portugal), and potato starch (Colmeia do Minho, Paio Pires, Portugal according to the GFB recipes earlier described by Graça et al. [16]. Detailed nutritional composition of the gluten-free flours used were described earlier by other authors [16].

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The fresh Cc lactose-free used was a commercial product from Lacticínios do Paiva (Lamego, Paiva, Portugal), obtained by the soluble protein thermal denaturation/precipitation of the whey resulting from the production of the lactose free cheeses; the dry extract of Cc (31.2%, dry matter) and respective nutritional composition were determined and described in previous works [12,16].

Other ingredients used were commercial saccharose (Sidul, Santa Iria de Azóia, Portugal), salt (Vatel, Alverca, Portugal), dry yeast (Fermipan, Setúbal, Portugal), vegetable fat (Vegê, Sovena Group, Algés, Portugal), and xantham gum (XG) (Naturefoods, Lisboa, Portugal) [12,16].

3.4.1.2. Gluten-Free Bread Dough Preparation

GFB dough formulations were prepared according to the procedure earlier described by Graça et al. [16]: the yeast was activated in warm water; dry ingredients were incorporated, well mixed, and kneaded for 10 min; dough was fermented/leavened for 20 min at 30 °C, followed by baking at 180 °C during 30 min under convection, according to the breadmaking conditions earlier described [16]. Baking trials were performed in triplicates.

The GFB formulations tested are presented in Table 1.

Table 1. Bread formulations tested: control bread (CB) and curd cheese breads (CcB).

Ingredients (% *w*/*w*)	CB	CcB5%	CcB10%	CcB15%	CcB20%
Buckwheat	16.6	14.6	12.6	10.6	9.6
Rice	25.0	22.0	20.0	16.6	13.0
Potato starch	14.0	12.0	11.0	10.4	8.0
Curd cheese	0.0	5.0	10.0	15.0	20.0
Water added **	37.0	35	32.0	31.0	30.0
Curd cheese water ***	0.0	4.0	7.0	9.0	12.0
Total water absorption ****	37.0	39.0	39.0	40.0	42.0

Other ingredients kept constant: 7.4%; ** Determined by MicrodoughLab mixing curves; *** Water deriving for curd cheese incorporation; **** Total water absorption = water added + water deriving from Cc incorporation.

Considering the water coming from the different Cc additions tested, the water added (WAD) was determined by MicrodoughLab mixing curves, and the water absorption was calculated according to the procedure earlier described by Graça et al. [16]. Cc doughs were prepared by successive incorporations of 10, 20, 30, and 40 g of Cc into dough (5% up to 20% *w*/*w*). Replacements were based on gluten-free flours basis, substituting the dry extract of each Cc percentage on 100 g of flour [16]. Ingredients kept constant: salt—0.8%; saccharose—1.6%; yeast—1.6%, XG—0.3%, and vegetable fat— 3.1% (sum of ingredients = 7.4%) (Table 1).

3.4.2. MicrodoughLab Measurements

3.4.2.1. Mixing Curves

Mixing dough behavior of control dough and doughs produced with Cc additions, was studied using the MicrodoughLab equipment (Perten instruments, Hägersten, Sweden), according to the specific protocol: 4 g of gluten-free flours mixed for 20 min at 30 °C under constant speed, 63 rpm. The parameters recorded from the mixing curves that characterize the dough rheology behavior are (AACC, 54–60.01): (WA) water absorption, or the amount of water required to reach the optimal dough consistency (mL of water/100 g of flour, at 14.0% of moisture basis), (DDT) dough development time or required time to reach the maximum dough consistency, (DST) dough stability during mixing, corresponding the time at which dough kept the maximum consistency; (DSF) dough softening degree or mixing tolerance index. Mixing curves were triplicated.

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3.4.3. Dough Rheology Characterization

The dough rheology measurements were assessed according to the method earlier described by Graça et al. [16]. Briefly, rheology measurements (Haake Mars III—Thermo Scientific, Karlsruhe, Germany, Universal Temperature Control-Peltier system), on the fermented dough (20 min), using a serrated parallel plate as sensor system (PP35 and 1 mm gap), to avoid the split effect [17], and under cold conditions (at 5 °C) to inactivate the yeast, were performed in triplicates.

3.4.3.1. Steady-Shear Dough Behavior

The influence of the Cc addition on dough viscosity behavior was evaluated according to the method earlier described by Graça et al. [16]: shear steady conditions, varying the shear rate varied from 1.00×10^{-6} to 1.00×10^{3} s^{-1}.

Experimental rheology data were compared and model by Carreau Model, applying Equation (1):

$$\eta = \eta_0/[1 + (\dot{\gamma}/\dot{\gamma}_c)^2]^s \quad (1)$$

where the fitted parameters obtained corresponds to: η is the apparent viscosity (Pa. s), $\dot{\gamma}$ is the shear rate (s^{-1}), η_0 is the zero-shear rate-viscosity (Pa. s), $\dot{\gamma}_c$ is the critical shear rate at the onset of the shear-thinning behavior (s^{-1}), i.e., the value corresponding to the transition from Newtonian to shear-thinning behavior and s is a dimensionless parameter related to the slope of this region.

3.4.3.2. Dynamic-Shear Dough Behavior

The impact of the Cc enrichment on dough linear viscoelastic properties, by the elastic (G') and viscous (G") moduli changes, by small-amplitude oscillatory measurements was applied, according to the method earlier described by other authors [16]: 0.001 to 100.0 Hz of frequency ranging, at 10 Pa of shear stress within the linear viscoelastic region (determined at 1 Hz).

In all rheology methods described above, triplicates were performed.

3.4.4. After-Baking Quality Parameters of the GFB

3.4.4.1. Bread Texture and Staling Kinetics

Bread crumb texture (BCT) was evaluated following the methods earlier described Graça et al. [12,16,18]: penetration mode, by puncture test, using a Texturometer Stable MicroSystems (Surrey, UK). Triplicates of each 10-bread crumb firmness measurement repetitions were performed.

BCT of the different Cc breads was compared in terms of firmness, and bread staling kinetics (during a storage time of 96 h), in accordance with a procedure previously described [12,16].

Applying the linear Equation (2) the bread staling kinetics was described as a function of time,

$$\text{Firmness} = A \times \text{time} + B \quad (2)$$

where A (N/h) corresponds to the staling kinetics and B (N) the initial firmness of the GFB [16].

3.4.4.2. Breadmaking Properties

Post-baking quality parameters of the GFB: moisture (%), water activity (aw), bake loss (BL), and specific bread volume (SBV) (cm^3/g), were evaluated, according to the procedures previously reported [12,16]. Triplicates were performed.

Bread crumb and crust color were recorded by Minolta colorimeter using illuminative D65 (Chromameter CR-400, Minolta-Japan), as earlier described [16].

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Differences in bread color (ΔE) between control bread (CB) and curd cheese breads (CcB) was calculated by Equation (3):

$$\Delta E = [(L^* CcB - L^* CB)^2 + (a^* CcB - a^* CB)^2 + (b^* CcB - b^* CB)^2]^{1/2} \quad (3)$$

3.4.4.3. Nutritional Value of the GFB

Nutritionally, the obtained GFBs were characterized based on the proteins (ISO-20483:2006), lipids (NP 4168), ash (AACC Method 08–01.01), carbohydrates (estimated by difference), and total minerals (ICP-AES-Inductively Coupled Plasma-Atomic Emission Spectrometry) contents, following the Standard Methods described previously by Graça et al. [12,16]. Triplicates were performed.

3.4.5. Statistical Analysis

Statistical analysis of the experimental data (average values and standard deviation) (RStudio, Version 1.1.423) using variance test in one factor (ANOVA), by Tukey test (post-hoc comparisons, at 95% of significant level), were assessed. A Nonlinear Rheology Model, to fit the experimental rheology data was applied (Carreau model using TA Instruments/TRIOS software).

3.5. Results and Discussion

3.5.1. MicrodoughLab Mixing Curves

The impact of Cc additions on dough mixing properties was evaluated and the mixing parameters, summarized in Table 2, were used to characterize the effect of Cc additions on mixing behavior.

Table 2. Comparison of Microdoughlab mixing parameters: WA—water absorption (%), DDT—dough development time (min), DST—dough stability (min), DSF—dough softening degree (mNm), for control dough (CD) and curd cheese doughs (CcD5% up to CcD20%).

Samples	WA (%)	DDT (min)	DST (min)	DSF (mNm)
CD	66.30 ± 0.60 ^{a}	0.80 ± 0.10 ^{a}	0.80 ± 0.10 ^{a}	3.30 ± 0.61 ^{a}
CcD5%	66.70 ±0.60 ^{a}	0.70 ±0.10 ^{a}	0.60 ±0.20 ^{a}	3.00 ±0.12 ^{a}
CcD10%	67.70 ± 0.50 ^{a}	0.70 ± 0.20 ^{a}	0.60 ± 0.10 ^{a}	3.00 ± 0.63 ^{a}
CcD15%	69.30 ± 0.50 ^{ab}	0.90 ± 0.50 ^{ab}	0.60 ± 0.15 ^{a}	5.30 ± 0.60 ^{b}
CcD20%	70.30 ± 0.60 ^{b}	1.00 ± 0.10 ^{b}	0.60 ± 0.10 ^{a}	6.00 ± 0.30 ^{b}

Different superscripts (^{a}, ^{b}) indicate significantly statistical differences between CB and CcB. (at $p \leq 0.05$, Tukey test).

From Table 2, it can be observed that up to 10% of incorporation, no significant ($p > 0.05$) impact on water absorption (WA), dough development time (DDT), dough stability (DST), and dough softening (DSF), was registered. However, for higher levels of Cc tested, 15% and 20%, the WA increased, probably due to the increased amounts of proteins in dough matrix, requiring more water to improve the connectivity of the network developed by starch-protein-xanthan gum, reinforced by hydrogen bonds [19]. The DDT also increased with 15% of Cc addition, resulting from the increase in solids content associated with difficulties in dough homogenization. In terms of dough stability, no significant differences were observed. However, Cc additions led to a significant ($p < 0.05$) increase of dough softening after 15% of Cc addition, reflecting the weakening of dough structure. Although the Cc addition promoted a slight impact on dough mixing properties, in terms of DDT and DSF, these effects can be considered as an additional support of denatured WP (coming from Cc) to dough plasticity or extensibility, which can be crucial during the fermentation process to dough expansion and gas retention.

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3.5.2. Dough Rheology Characterization

3.5.2.1. Gluten-Free Dough Flow Behavior

Figure 1 shows the impact of Cc additions, on steady-shear flow behavior of gluten-free doughs, in comparison to control dough (CD).

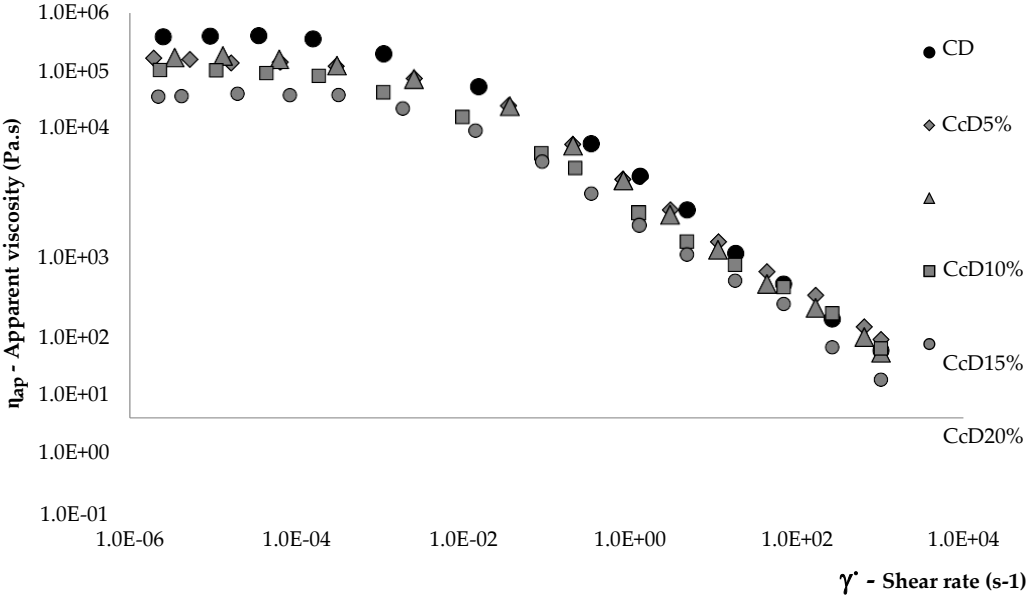

Figure 1. Flow rheology behavior of the control dough (CD) and Cc doughs (CcD): 5% up to 20%, *w*/*w*.

The flow curves indicated that all these systems behaved as typical structured materials, showing a Newtonian plateau region at low shear rates and dough viscosity began to decrease as the shear rate values increase, reflecting a typical shear-thinning behavior. These results agree with those obtained by other authors [19,20], using different gluten-free flours and hydrocolloids on GFB formulations.

Carreau equation was adjusted to the experimental data from Figure 1 ($R^2 > 0.975$) and the obtained rheology fitted parameters, summarized in Table 3, were used to characterize the dough flow behavior: Cc incorporations to dough promoted a significant reduction on apparent viscosity values (η_0) ranging from 390.2 k Pa s for CD to 37.3 k Pa s for CcD20% (higher Cc level tested), representing a decrease in viscosity of around 90.0%. Considering the significant reduction in dough viscosity observed, promoted by the Cc addition to dough, the dilution effect of starch-proteins-xanthan gum linkage density, of the GFB reference, should be invoked to support these findings.

Table 3. Fitted rheology parameters estimated for control dough (CD) and curd cheese doughs (CcD5% up to CcD20%), by applying Carreau Model**.**

	η_0 (k Pa s)	$\gamma_{.c}$ (s^{-1})	s (Slope)	R^2
CD	390.2 ±8.7 ^{a}	$3.3 \times 10^{-3} \pm 1.3 \times 10^{-4}$ ^{a}	0.27 ± 0.06 ^{a}	0.987
CcD5%	166.4 ± 5.0 ^{b}	$3.1 \times 10^{-3} \pm 4.0 \times 10^{-4}$ ^{a}	0.26 ± 0.03 ^{a}	0.987
CcD10%	178.0 ± 6.6 ^{b}	$3.2 \times 10^{-3} \pm 2.3 \times 10^{-4}$ ^{a}	0.23 ± 0.03 ^{ab}	0.980
CcD15%	101.5 ± 1.7 ^{c}	$3.6 \times 10^{-3} \pm 4.8 \times 10^{-4}$ ^{a}	0.21 ± 0.04 ^{b}	0.996
CcD20%	37.3 ± 4.0 ^{d}	$3.6 \times 10^{-3} \pm 2.3 \times 10^{-4}$ ^{a}	0.18 ± 0.09 ^{b}	0.975

Different superscripts (^{a}, ^{b}, ^{c}, ^{d}) indicate significant statistical differences between CD and CcD (at $p \leq 0.05$, Tukey test).

Based on the values of critical shear rate ($\gamma_{.c}$), the transition of the Newtonian to shear-thinning behavior of all systems occurred around 3.4×10^{-3} s^{-1}.

Concerning the dough flow index (slope), the addition of Cc up to 10% did not significantly ($p > 0.05$) change the dough's flow behavior, presenting values like CD (0.27–0.23). Nevertheless, as the amount of Cc increases, the flow index was significantly reduced, varying from 0.27 (CD) to 0.18

for high Cc level tested (CcD20%). This increase in shear thinning behavior is probably due to the formation of longer and more flexible polymer-chains [21].

Similar findings were reported by other researchers [22] to evaluate the effect of other hydrocolloids addition on the rheology features of the GF cakes. According to a previous study [23], lower flow indexes led to a quick decrease in dough viscosity, which can improve the capacity of dough to expand and retain more gas during baking.

3.5.2.2. Gluten-Free Dough Viscoelastic Behavior

Rheology behavior of the GF doughs focused on storage (G') and loss (G") moduli changes with oscillatory frequency, was assessed on fermented doughs. Results are illustrated in Figure 2A–D, where different viscoelastic functions can be observed between CD and Cc doughs obtained.

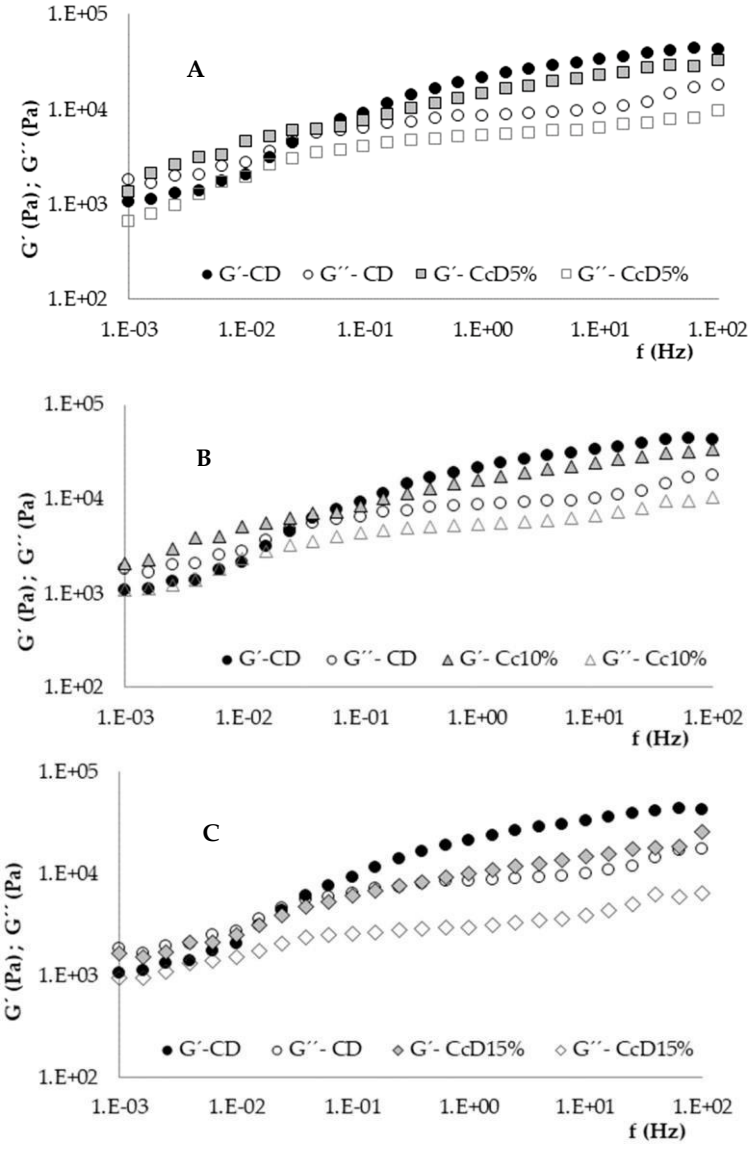

Figure 2. *Cont.*

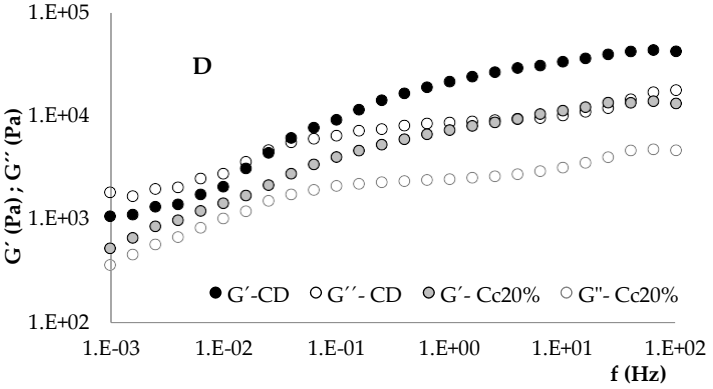

Figure 2. Rheology changes in viscoelastic functions, storage (G', full symbols) and loss (G", empty symbols) moduli, by the additions of curd cheese to dough (CcD5% up to CcD20% *w*/*w*), compared with viscoelastic profile of the control dough (CD) (black round symbols): (**A**) CD vs. Cc5%; (**B**) CB vs. Cc10%; (**C**) CB vs. Cc15%; (**D**) CB vs. Cc20%.

Starting for CD, at low frequency, a poorly structured system can be observed, since the values of G" are higher than G', characteristic of a typical viscoelastic fluid behavior [22]. Nevertheless, the crossover of both moduli occurred with the frequency increase, and the dominance of the G' over the G" was observed, expressing a weak gel behavior [24]. For Cc doughs, a typical weak-gel structure can be observed [25], where the G' values were greater than the G" over the whole range of frequency applied, indicating the predominance of the solid-elastic behavior [24], with high frequency dependence [26]. The values of both G' and G" were decreasing with Cc incorporations. The viscoelastic behaviors observed suggested that the presence of denatured WP, coming from Cc addition, are promoting a new network or/and protein agglomerates, giving more complexity to dough matrix. On the other hand, the increasing amount of lipids coming from the Cc additions enhance extensibility by the lubrication effect on the macromolecular chains resulting in the reduction of the viscoelastic parameters, increasing the plasticity of the doughs. The viscoelasticity of the gluten-free dough (GFD) depends strongly on intra and inter molecular interactions, i.e., a network with a desirable viscoelastic behavior, required for breadmaking. The chemical composition and functional properties of Cc proteins and their interactions are responsible for the changes in dough viscoelastic behavior.

From the results obtained, it can be stated that the Cc addition into dough promoted significant changes in dough viscoelastic profile, that probably can result in higher dough protein network ability to inflate and retain more CO_2, due to a flexible protein network, reducing considerably the dough viscoelastic parameters, under oscillatory measurements. Similar reports were obtained by other authors [27], where the addition of different protein sources to dough produced smaller G' and G" values, causing the reduction of consistency. This behavior can result in better quality properties based on the bread texture and volume [28].

3.5.3. Quality Characterization of the Gluten-Free Bread

3.5.3.1. Bread Crumb Texture and Hardening Kinetics

Bread crumb firmness is an important parameter used to evaluate the bread quality, that influences the consumers acceptability. To measure the effect of Cc addition on bread firmness, during 4 days of storage time (96 h), a puncture test was applied [12,16,18], to mimic the teeth chewing of the human sensory perception.

The mean values of bread crumb firmness (BCF) of CB and CcB are illustrated in Figure 3, showing that for all Cc amounts tested, bread crumb was softer than for CB, as the Cc levels increase in GFB formulations.

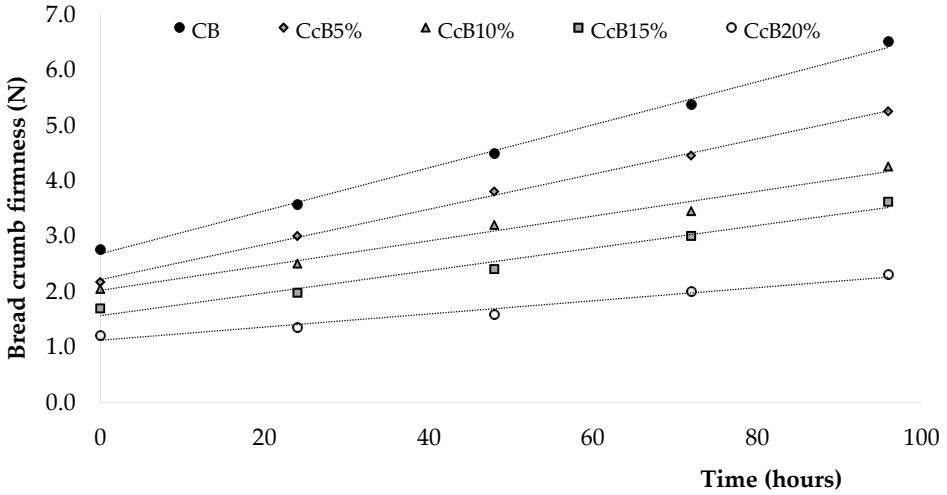

Figure 3. Evaluation of the gluten-free bread (GFB) crumb firmness progression, (96 h at room temperature), between control bread (CB) and curd cheese bread (CcB5% up to CcB20%).

Initial and final values of BCF varied from 2.70 N to 6.50 N for CB and 1.12 N to 2.30 N for CcB20%, representing a reduction on the firmness of around 55% (initial) and 65% (final). This behavior suggests a high functionality of the denatured WP in breadmaking performance. Higher firmness values of CB seem to be consistent with high viscosity and viscoelastic moduli values exhibited for CD, probably due to stiffer structure effect promoted by xanthan gum, reducing the dough expansion, consequently leading to firmer breads. Similarly, Demirkesen et al. [21] reported an increase of BCF by xanthan gum addition in GFB.

Staling of bread during the storage time is a consequence of humidity loss, and subsequent starch crystallization/retrogradation phenomena, leading to an increase of crumb firmness values [28].

Linear correlations were determined to describe the staling kinetics of the GFB, under controlled storage conditions (96 h at room temperature). The obtained linear parameters, summarized in Table 4, reflects the influence of Cc incorporations on the bread staling rate (Equation (2).

Table 4. Linear parameters derived by linear correlation ($R^2 > 0.972$): A—bread staling rate (N/h) and B—initial bread firmness (N), for control bread (CB) and curd cheese bread obtained (CcB).

Cc Levels	Staling Rate (N/h)	Firmness (N)	R^2
CB	0.040 ± 0.001^{a}	2.70 ± 0.23^{a}	0.996
CcB5%	0.032 ± 0.002^{ab}	2.21 ± 0.40^{ab}	0.998
CcB10%	0.022 ± 0.001^{c}	1.90 ± 0.30^{b}	0.981
CcB15%	0.020 ± 0.003^{c}	1.57 ± 0.09^{c}	0.979
CcB20%	0.012 ± 0.002^{d}	1.12 ± 0.15^{d}	0.972

Different superscripts (^{a}, ^{b}, ^{c}, ^{d}) indicate significant statistical differences between CB and CcB: CcB5% up to CcB20% (at $p \leq 0.05$, Tukey test); A and B linear parameters were obtained by Equation (2): Firmness = A × time + B.

The Cc bread staling rate values were substantially lower than CB value, ranging from 0.032 N/h for lower incorporation (CcB5%) to 0.012 N/h for higher additions (CcB20%), compared to 0.040 N/h obtained for CD, are duction about70%ofstaling. Results reported by Gallagher et al. [13], by evaluating the effect of dairy powder addition on GFB shelf-life, are similar with those obtained. These findings are also in line with those obtained by other researchers [12], by testing the fresh dairy products addition to improve the wheat bread quality.

The functionality of the Cc protein should be considered to explain these results of bread texture and bread aging rate, whose positive effects can be associated to the capability of these proteins to bind with water and retain moisture, retarding the starch retrogradation and hence the increase of bread hardness [14]. On the other hand, these results suggest that the denatured WP relaxes the

protein-starch-XG network, giving to dough a foaming support to retain the gases within, improving the bread texture by increasing volume (Figure 4) and softening effects [29]. Figure 4 shows the bread obtained with different levels of Cc addition, in comparison with CB.

Figure 4. Comparation of the resulted breads obtained for control bread (CB) and those breads obtained by different additions of curd cheese (CcB): CcB5% up to CcB20%.

3.5.3.2. After-Baking Parameters of the Gluten-Free Bread

Breadmaking performance of GFB produced with Cc additions was assessed by bread quality parameters: moistness (%) water activity (aw), baking loss (BL, %), specific bread volume (SBV, cm^3/g), crust and crumb color. Results are summarized in Table 5.

Table 5. Bread quality parameters: moisture, water activity (aw), baking loss (BL), specific bread volume (SBV), and bread crust and crumb color, for control bread (CB) and curd cheese breads (CcB5% up to CcB20%).

	CB	**CcB5%**	**CcB10%**	**CcB15%**	**CcB20%**
Moisture (%)	42.85 ± 0.33 ^{a}	43.02 ± 0.60 ^{a}	45.50 ± 0.43 ^{b}	47.80 ± 0.93 ^{c}	51.00 ± 0.13 ^{d}
aw	0.960 ± 0.006 ^{a}	0.951 ± 0.004 ^{ab}	0.943 ± 0.003 ^{b}	0.913 ± 0.005 ^{c}	0.892 ± 0.002 ^{c}
BL (%)	12.00 ± 0.40 ^{a}	11.00 ± 0.04 ^{ab}	10.22 ± 1.10 ^{b}	9.60 ± 0.80 ^{b}	9.00 ± 0.30 ^{b}
SBV (cm^3/g)	1.50 ± 0.11 ^{a}	1.90 ± 0.08 ^{a}	2.10 ± 0.11 ^{b}	2.30 ± 0.15 ^{bc}	2.50 ± 0.06 ^{c}
Crust color					
L*	59.60 ±2.90 ^{a}	58.60 ±1.64 ^{a}	51.80 ±2.30 ^{b}	46.44 ±3.13 ^{c}	36.80 ±2.50 ^{d}
a*	8.40 ± 1.00 ^{a}	9.40 ± 0.51 ^{a}	10.10 ± 0.94 ^{b}	11.80 ± 0.80 ^{bc}	12.80 ± 0.30^{c}
b*	29.10 ± 1.43 ^{a}	29.10 ± 1.20 ^{a}	29.30 ± 1.10 ^{a}	21.20 ± 2.53 ^{b}	21.10 ± 2.40^{b}
ΔE		1.41 ± 0.90 ^{a}	8.00 ± 1.02 ^{b}	15.70 ± 1.80 ^{c}	24.60 ± 1.30 ^{d}
Crumb color					
L*	60.52 ± 2.90 ^{a}	63.81 ± 2.32 ^{a}	67.36 ± 1.90 ^{b}	69.59 ± 0.62 ^{b}	70.90 ± 1.91^{b}
a*	3.49 ± 1.05 ^{a}	5.49 ± 0.75 ^{a}	6.63 ± 0.89 ^{b}	7.83 ± 0.20 ^{c}	1.81 ± 0.23 ^{d}
b*	11.40 ± 0.20 ^{a}	11.10 ± 0.64 ^{a}	12.15 ± 1.30 ^{a}	18.53 ± 1.50 ^{b}	23.40 ± 1.10^{c}
ΔE		3.90 ± 0.95 ^{a}	7.60 ± 1.10 ^{b}	12.30 ± 1.20 ^{c}	16.00 ± 1.23^{c}

Different superscripts (^{a}, ^{b}, ^{c}, ^{d}) indicate significant statistical differences between CB and CcB: CcB5% up to CcB20% (at $p \leq 0.05$, Tukey test).

Moisture percentage of the breads ($p < 0.05$) increased by successively Cc additions, varying from 43.0% (CcB5%) to 51.0% (CcB20%), compared to 42.5% of CB, an increase of 21% in moisture. Water is the most important plasticizer in food structure, acting as chain polymer lubricant, reducing stiffer linkage forces by increasing flexibility of structure effects [30]. Water activity (aw) was slightly reduced as the Cc levels increase in bread formulations, from 0.960 for CB to 0.890 for CcB20% (upper Cc amount), a decrease of 7.3%, that will contribute to improving the preservation of bread during storage.

Additionally, a significant increase on yield for Cc bread was obtained, around 25% compared to CB, as the consequence of less weight loss during baking (BL). Improvement in water holding capacity has been previously reported in GFB systems, by protein sources addition, enhancing the bread yield after baking [27].

The enrichment of the bread formulation by Cc proteins addition enhanced the appearance of the breads, in terms of specific bread volume (SBV), from 1.50 cm^3/g for CB to 2.60 cm^3/g for CcB20%, a considerable increase about of 73%. These improvements in SBV could be justified by the increase in water absorption, improving the handling properties and subsequently led to a flexible starch-protein matrix further enhanced by starch gelatinization during baking [13].

The findings also agree with those reported by Sahagún and Gómez [27] evaluating the effect of other protein sources on physicochemical and quality properties of GFB.

Other important features of the GFB are the crust and crumb color, also highly associated with bread consumers acceptance. Crust and crumb color results (Table 5) reveal that the addition of Cc had a considerable impact on both quality bread attributes. The inclusion of Cc substantially ($p < 0.05$) increased the darker crust color of the GFBs (lower L* values). In fact, it could be related to the increments in protein levels by Cc additions, since the Maillard reaction occurs between amino acids and reducing sugars [31]. Based on bread crust tone, Cc addition promoted an increase of redness (higher a* values) reducing slightly the yellowness of the crust (lower b*). The differences in color (ΔE) between Cc breads and CB, were calculated by Equation (3), resulting in ΔE values higher than 5, indicating significant bread color differences by the increments in Cc. In agreement, are previous reports about GFB with egg white and whey proteins addition [1]. In contrast, on crumb color, lighter breads (L* values) with a remarkable yellowness tone (b* values) whilst the reddish tone decreased (a* values), was obtained. Differences in color were also confirmed by the values ΔE higher than 5. Similar results were previous reported Graça et al. [16] by adding yoghurt to improve the GFB quality properties.

3.5.4. The Relationship between Bread Quality and Dough Rheology Features

This study showed that the bread volume and crumb texture can be improved by denatured WP coming from Cc incorporation to dough, acting as lubricant agents in dough matrix. In fact, results discussed above suggest some linear correlation between bread quality attributes and dough rheology results, that can be taken into consideration to support the enhancements of the GFB.

Linear relationships ($R^2 > 0.900$) were found between bread firmness (BF) with dough viscosity (DV) and elastic modulus (G', at 1 Hz), and bread volume (SBV) with both DV and G', at 1 Hz, as can be observed from Figure 5A, B.

Improvements of BF and SBV were obtained for Cc doughs with lower viscosity and elasticity values, resulting in softer breads with better volumes.

It can be stated that the Cc incorporation to dough had a significant impact on GFB quality attributes, and such effects are probably due to additional support of the denatured WP to flexible effects on macromolecule interactions, improving the dough matrix performance to produce higher quality breads than CB.

These finding are aligned with those published by Graça et al. [16], reporting positive effects of the casein protein, present in yoghurt, on GFB quality properties.

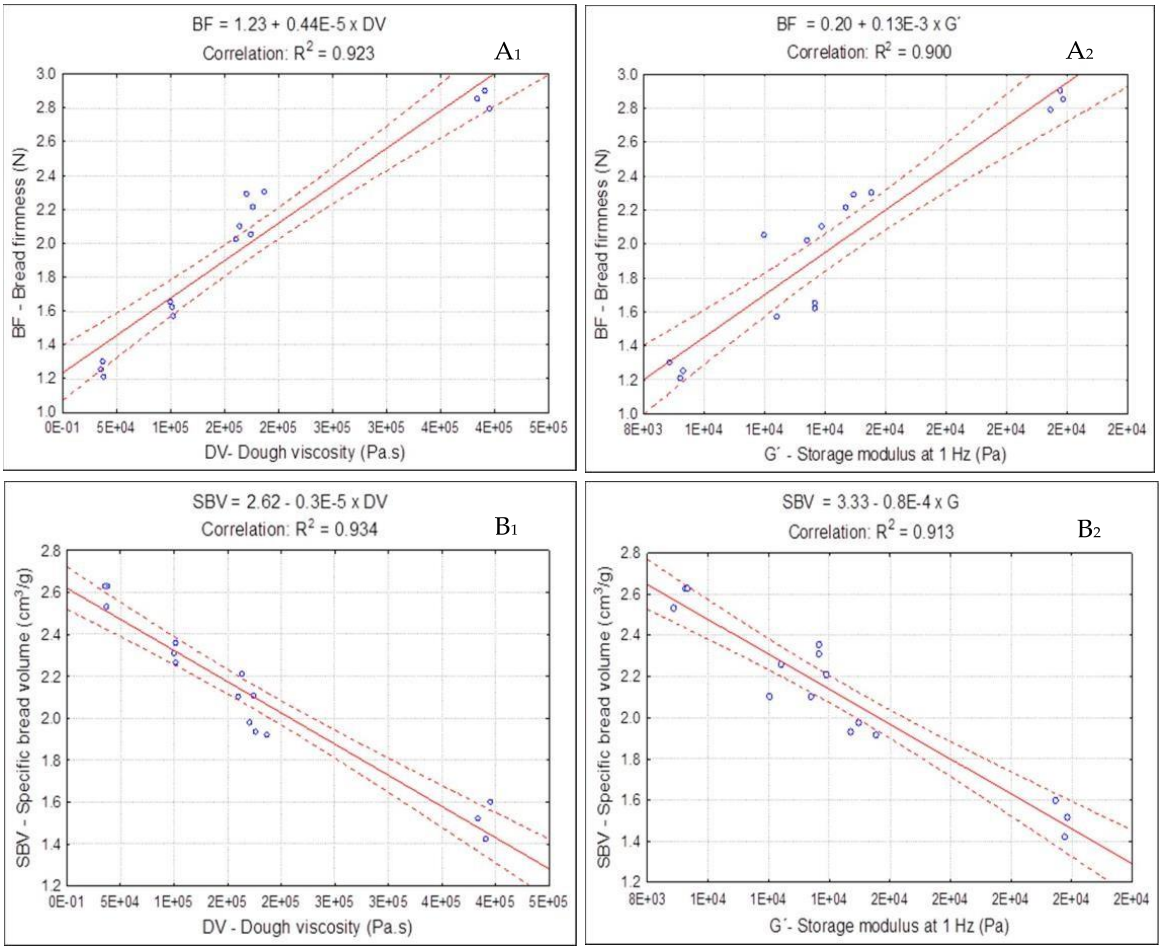

Figure5. Linear relationship between bread quality attributes (BF—bread firmness and SBV—specific bread volume) with dough rheology properties (DV—dough viscosity and G'—elastic modulus, at 1 Hz): (**A_1**)—BF vs. DV; (**A_2**)—BF vs. G'; (**B_1**)—SBV vs. DV; (**B_2**)—SBV vs. G'.

3.5.5. Nutritional Value of the Gluten-Free Bread

The impact of Cc addition to improve the nutritional and minerals profile of GFB was evaluated, and the results obtained are summarized in Table 6.

The increments of protein by Cc incorporation to GFB was noticeable: comparing CB to CcB20% (higher level tested), the protein content varied from 5.34 to 9.40 g/100 g, respectively, representing an increase around 80%. Carbohydrates levels varied from 45.9 to 31.5 g/100 g, representing a considerable reduction of 31.4% for CcB20%, compared to CB. According to a recent work [32], in which the supplementation of wheat bread with curd cheese to reduce the glycemic response was performed, these results can suggest that the incorporation of Cc as baking ingredients in GFB formulations can be an alternative to obtain breads with reduced glycemic index.

In terms of lipids content, a significant increase of 35% for upper levels of Cc tested, was obtained. A considerable increase in ash content was also observed, and this result is clearly reflected in terms of major minerals profile. As it can be observed from Table 6, a significant increase in phosphorus (50.0%), magnesium (6.0%), and calcium (360.3%) were registered for Cc bread, that can cover more than 15% of recommended daily value as stated in European community (CE) Regulation N° 1924/2006 based on nutrition and health claims made on foods [33], and in the Regulation (UE) N° 432/2012 that specifies a list of permitted health claims made on foods [34].

In terms of trace elements (Fe, Cu, Mn, and Zn), since their prevalence in buckwheat flour is higher than in curd cheese, their proportions were diluted by successive curd cheese additions.

A balanced gluten-free diet should provide adequate levels of macro- and micronutrients, since the diagnosis of celiac patients is often associated to deficiencies in protein levels and mineral components, caused by the atrophy and subsequent damage of small gut villus [35–37].

These nutritional improvements obtained by the addition of Cc on GFB formulation can give an additional contribution to fulfill the nutritional daily diet requirements in terms of protein and major mineral profile of celiac patients and also gluten-sensitive individuals.

Table 6. Nutritional value (g/100 g), including mineral profile (mg/100 g), for control bread (CB) and Cc breads (CcB5% up to CcB20%).

Nutrients (g/100 g)	CB	CcB10%	CcB20%	
Proteins	5.34 ± 0.21 ^{a}	7.60 ± 0.12 ^{b}	9.40 ± 0.10 ^{c}	
Lipids	4.30 ± 0.29 ^{a}	5.30 ± 0.61 ^{b}	5.80 ± 0.20 ^{c}	
Ash	1.40 ± 0.03 ^{a}	2.03 ± 0.11 ^{b}	2.90 ± 0.07 ^{c}	
Carbohydrates	45.90 ±0.95 ^{a}	39.60 ±1.50 ^{b}	31.50±1.20 ^{c}	
Kcal	245.80 ±1.80 ^{a}	236.20 ±1.10 ^{a}	213.40 ±2.10 ^{b}	
	Minerals (mg/100 g)			15% RDV * (mg/100 g)
Na (g/100 g)	0.41 ± 0.03 ^{a}	0.35 ± 0.01 ^{a}	0.36 ± 0.02 ^{b}	-
K	412.0 ± 8.02 ^{a}	315.35 ± 5.92 ^{b}	290.35 ± 10.40 ^{b}	300.0
P	131.80 ± 5.40 ^{a}	177.00 ± 0.90 ^{a}	197.40 ± 2.00 ^{b}	120.0
Mg	50.24 ± 1.02 ^{a}	50.60 ± 0.56 ^{a}	53.20 ± 0.82 ^{b}	45.0
Ca	35.93 ± 2.06 ^{a}	151.00 ± 0.81 ^{b}	165.38 ± 2.60 ^{c}	120.0
Fe	6.94 ± 0.12 ^{a}	3.68 ± 0.67 ^{b}	3.50 ± 0.05 ^{c}	2.1
Cu	0.23 ± 0.01 ^{a}	0.10 ± 0.02 ^{b}	0.07 ± 0.03 ^{c}	0.2
Mn	0.51 ± 0.01 ^{a}	0.40 ± 0.02 ^{a}	0.32 ± 0.03 ^{b}	0.3
Zn	1.12 ± 0.01 ^{a}	0.70 ± 0.08 ^{b}	0.65 ± 0.01 ^{b}	2.3

Different superscripts (^{a}, ^{b}, ^{c}) indicate significant statistical differences between CB and CcB: CcB5% up to CcB20%, (at $p \leq 0.05$, Tukey test). * Recommended daily values (RDV) established by Reg. (CE), No. 1924/2006 and Reg. (EU) No. 432/2012.

3.6. Conclusions

The findings from this work showed that the quality of the GFB can be substantially improved by Cc supplementation, and such effects are probably associated to the functionality of denatured WP and lipids from the Cc, enhancing the viscoelastic properties of the GFD, giving additional support to dough foaming structure.

The weakening effects observed on dough rheology features, promoted by Cc lipids incorporation led to a significant improvement in bread softness with higher specific volumes than CB. Linear correlation between dough viscosity and storage modulus, with bread firmness and specific volume, supported these findings, showing that the positive effects observed are proportional to the increased levels of the curd cheese into the gluten-free dough.

Nutritionally, a considerable protein enrichment as well as major minerals profile, especially calcium, phosphorus, and magnesium contents, were obtained, whereas the carbohydrates amount was significantly reduced.

Considerable improvements in terms of bread quality of the gluten-free bread were achieved by successive increments of curd cheese, especially on the higher levels tested (15% and 20% *w*/*w*).

In summary, it can be stated that the breadmaking performance, technology properties, quality attributes, as well as the nutritive value of the gluten-free bread, can be substantially improved by fresh Cc enrichment.

Author Contributions: Conceptualization, Methodology, Investigation, Data Curation, Writing—Original Draft Preparation, C.G.; Project Administration, Investigation, Supervision, Validation, Writing—Review and Editing, A.R. and I.S. All authors have read and agree to the published version of the manuscript.

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Glycemic Response and Bioactive Properties of Gluten-Free Bread with Yoghurt or Curd-Cheese Addition

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Abstract: The influence of flour replacement by yogurt or curd-cheese additions (from 10% to 20%, *w/w*) on the glycemic response and bioactivity improvements of gluten-free bread was evaluated. Starch digestibility, measured by an in vitro digestion model, was applied to determine the effect on starch fractions. The bread glycemic index was calculated. Bread antioxidant capacity (2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and ferric-ion-reducing antioxidant power (FRAP) methods) and total phenolic compounds were assessed. Anti-inflammatory properties according to enzymatic matrix metalloproteinase (MMP)-9 inhibitory activity were also studied. Considering the higher level of both dairy products tested (20%, *w/w*) and comparing with control bread results, a reduction of around 35% in the glycemic response of curd cheese bread was achieved, resulting in intermediate index level (glycemic index (GI) 55–69), with yogurt bread still showing a high glycemic index (GI > 70). In terms of bread bioactivity, curd cheese bread expressed better reducing power effects, whereas yogurt bread showed more effective radical-scavenging capacity. An increase in bread phenolic compounds by yogurt (55.3%) and curd cheese (73.0%) additions (at 20%) were also registered. MMP-9 inhibition activity was higher in the dairy bread than in control bread, suggesting an improvement in terms of anti-inflammatory properties. The supplementation of the gluten-free bread by yogurt or curd cheese was shown to be a promising strategy to reduce the glycemic response and to improve the bioactive properties of the bread, that which can contribute to preventive diets of celiac patients and irritable bowel syndrome individuals.

Keywords: gluten-free bread; dairy products; starch digestibility; bioactivity; celiac disease; irritable bowel disease; preventive diets

3.7. Introduction

Wheat and gluten-containing products have been associated with a wide range of gastrointestinal disorders. Celiac disease (CD) is the most studied form of gluten intolerance, characterized by a small gut inflammation via an immune response to specific peptides of gliadin, one of the gluten proteins [1]. This inflammation–immune process results in several health problems such as intestinal mucosal damage and villous atrophy [2], leading to malabsorption of macro- and micronutrients [3], which can also lead to small bowel cancer and other associated autoimmune diseases (e.g., diabetes, osteoporosis, and skin disorders) [4].

Furthermore, gluten is not the only triggering component in gastrointestinal disorders. Many other components coexist with gluten in wheat and gluten-related food, such as members of the

short-chain carbohydrate group (e.g., fructans), collectively termed FODMAPs (fermentable oligo-, di-, monosaccharides, and polyols), which are associated with several gastrointestinal symptoms in non-celiac gluten sensitivity (NCGS). Irritable bowel syndrome is the common gastrointestinal inflammatory condition associated with non-celiac gluten sensitivity symptoms, characterized by abdominal pain, bloating symptoms, diarrhea, and irregular bowel microbiota [1], strongly impacting the quality of life of these patients.

Accordingly, there has been an increase in demand for gluten-free products, not only due to the greater prevalence of celiac disease but also due to the prevalence of irritable bowel syndrome diseases, since gluten-free grains and derived products tend to be lower in FODPAMs [1].

The recent development of gluten-free foods has focused on research aimed at overcoming the technical challenge of gluten removal from bakery products [5,6], whose nutritional value and health-promotion properties were somehow left behind, being mainly starch-based foods with low protein content, higher in fat levels, with a high glycemic index (GI) [7,8].

The glycemic response depends on several intrinsic properties of flour, such as starch grain structure and molecular size, protein and/or lipid content, and the amylose–amylopectin ratio [9]. Previous studies reported that the physical interaction between proteins and starch can decrease the glycemic response by forming a physical barrier that reduces the accessibility of enzymatic attack to starch granules, limiting the degree of starch hydrolysis [8,10], which can be an approach to reduce the glycemic response of gluten-free bread [11].

Additionally, recent works [12,13] showed that some polyphenol compounds (e.g., phenolic compounds, anthocyanins) can reduce digestive enzymes, which can be a strategy to reduce the glycemic index in starchy foods while improving bioactive properties.

In the last decade, the search for anti-inflammatory inhibitors in food has been an important branch of research since it can be a promising strategy for preventive diets.

Matrix metalloproteinases (MMPs) are a zinc-dependent family of endopeptidases, highly involved in the biological human process of connective-tissue remodeling [14]. Specifically, MMP-9 is known to be a key moderator in bowel inflammation processes [15] and carcinogenic processes [16], directly involved in irritable bowel diseases. It is worth noting that chronic inflammatory conditions can degenerate into tumors [17].

Accordingly, the search for new protein-rich sources that contribute to reducing the glycemic index and for bioactive ingredient sources capable of an inhibitory effect against MMP-9 is an important topic of research, since it can contribute to a preventive diet, not only for celiac patients but also for those suffering from irritable bowel disorders.

Dairy products (DP) are promising protein sources, with recognized nutritional properties and functional benefits. Additionally, they present bioactive compounds associated with human physiological properties, such as antioxidant [18,19], anticarcinogenic, and antimicrobial activities [20]. Furthermore, a few studies showed particular interest in these products, especially those deriving from lactic acid bacteria (LAB) fermentation (e.g., yogurt or cheese), in which secondary metabolites may contribute to an anti-inflammatory activity toward human immune processes, due to the bioactive peptides generated by LAB fermentative activity [21–24].

Yogurt (Yg) is one of the most nutritious dairy products, widely consumed around the world, due to its functional benefits to the human diet, highlighting it as an alternative ingredient for bakery product supplementation [25,26].

Curd cheese (Cc) is a derived dairy product obtained via the manufacturing of soluble whey proteins, characterized by a rich protein source, essential amino acids (e.g., leucine and lysine), and minerals [27], and aromatic amino acids with bioactive properties [28].

This work aimed to study the influence of flour replacement with yogurt or curd-cheese additions (from 10% to 20%, *w/w*) in terms of a reduction in glycemic index and an improvement in the bioactivity of gluten-free bread. The impact on starch performance (pasting properties), determined using heating–cooling microdoughLab assays, was first assessed. To mimic starch digestibility in the

human body, an in vitro digestion model was applied. The glycemic index of the gluten-free bread was calculated. The antioxidant capacity of gluten-free bread was evaluated based on scavenging effects (2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH)) and ferric-ion-reducing antioxidant power (FRAP). Total phenolic content (TPC) was also determined. The gelatinolytic activity of MMP-9 inhibition was assessed using fluorometric quantification (dye-quenched (DQ) gelatin assay) to assess the anti-inflammatory potential of the gluten-free bread obtained. Linear correlations were tested among pasting properties, starch digestibility, glycemic index, and antioxidant capacity, to acquire additional information about the processes involved.

3.7.1. Materials and Methods

3.7.1.1. Raw Materials

Gluten-free bread was prepared according to the bread formulations earlier described [29], using rice flour (Próvida, Pêro Pinheiro, Portugal), buckwheat flour (Próvida, Pêro Pinheiro, Portugal), and potato starch (Colmeia do Minho, Paio Pires, Portugal).

The fresh plain yogurt (from cow milk) used is a product from LongaVida, Portugal; The yogurt dry extract (11.5%, dry matter) was determined as described earlier [29].

The fresh curd cheese (from whey cow milk) used was a commercial product from Lacticínios do Paiva (Lamego, Paiva, Portugal); The dry extract of curd cheese (31.2%, dry matter) was determined as described earlier [29].

The dry matter of both dairy products was determined to be considered in the optimization of the gluten-free bread formulations since the replacement was based gluten-free flour basis.

Other ingredients used in bread formulations [29] were commercial saccharose (Sidul, Santa Iria de Azóia, Portugal), salt (Vatel, Alverca, Portugal), dry yeast (Fermipan, Setúbal, Portugal), vegetable fat (Vegê, Sovena Group, Algés, Portugal), and xantham gum (Naturefoods, Lisboa, Portugal) [28,29].

3.7.1.2. Bread Dough Preparation

Gluten-free bread dough formulations were prepared in according to the procedure earlier described by Gretel.[29]:the yeast is activatedin warm water; dry ingredients were incorporated, well mixed, and kneaded during 10 min; fermentation/leavened (34 °C) of the dough during 20 min at 30 °C was performed, followed by baking at 180 °C during 30 min under convection, according to the breadmaking conditions earlier described [29].

Considering the water coming from each level of the dairy products tested, the water added (WAD) was determined by microdoughLab mixing curves, and the water absorption was calculated according to the procedure earlier described by Graça et al. [29]. Doughs enriched with yogurt or curd cheese were prepared considering incorporations of 20 g and 40 g of each dairy product, which corresponds to 10% to 20%, *w/w*. Replacements were based on gluten-free flour basis, i.e., substituting the dry matter coming from each percentage of yogurt or curd cheese added, on 100 g of flour, as earlier described [26,29]: Ingredients kept constant were salt—1.5 g (0.8%, *w/w*), sugar—2.8 g (1.6%, *w/w*), dry yeast—2.8 g (1.6%, *w/w*), xanthan gum—0.5 g (0.3%, *w/w*), and vegetable oil—5.5 g (3.1%, *w/w*).

The different gluten-free bread formulations tested are presented in Table 1.

Table 1. Gluten-free bread formulations of control bread (CB), yogurt (YgB), and curd cheese bread (CcB) (the dry extract derived from yogurt (11.5%) and curd cheese (31.2%) additions was considered to replace on flour basis).

* Ingredients (% *w*/*w*)	CB	$YgB_{10\%}$	$YgB_{20\%}$	$CcB_{10\%}$	$CcB_{20\%}$
Buckwheat	16.6	14.0	11.0	12.6	10.0
Rice	25.0	21.0	17.0	20.0	13.0
Potato starch	14.0	11.0	9.0	11.0	8.0
Yogurt/Curd cheese	0.0	10.0	20.0	10.0	20.0
Total water absorption **	37.0	37.5	40.0	39.0	42.0

* Other ingredients kept constant: 7.4%; ** Total water absorption: water added + water originated from Yg or Cc addition, according to the procedure earlier described [29].

The nutritional composition of the different bread formulations, presented in Table 1, was determined in previous work [29], recently published in *Foods*.

3.7.2. Pasting Properties of Gluten-Free Dough

The effect of yogurt and Cc additions (from 10 to 20% *w*/*w*) on the starch physical behavior of the gluten-free bread dough, in comparison to control dough (CD), was studied by MicrodoughLab measurements (Perten, instruments, Hägersten, Sweden), according to the method earlier described [30], with some modifications. Mixing and heating-cooling curves were applied, according to the following set of conditions: sample homogenization for 30 s, mixing curve at 30 °C for 360 s, heating up from 30 to 95 °C for 390 s, standing at 95 °C for 60 s, cooling down to 50 °C for 390 s, at similar temperature rate (0.17 °C/s). Paddle speed was 63 rpm for running the analysis. MicrodoughLab parameters, that characterize the consistency of the dough during mixing, heating (cooking), and cooling phases were recorded in torque units (mNm) (AACC, 54–60.01): dough development or maximum torque (C1) was reached during mixing at 30 °C, the minimum torque of dough when subjected to mechanical and thermal conditions by heat denaturation of proteins (C2), peak torque of starch gelatinization (C3), cooking stability or minimum torque during the heating period (C4), and final consistency peak torque produced after cooling stage at 50 °C (C5). Tests were performed in triplicates.

3.7.3. In Vitro Starch Hydrolysis

3.7.3.1. Digestible Starch Fraction

The effect of both dairy products on digestible and resistant starch of the gluten-free bread obtained, in comparison to gluten-free control bread, was evaluated by in vitro starch digestion according to the procedure earlier described [31] and subsequently applied by other researchers [30,32]. Briefly, ground bread crumb samples (100 mg) were dispersed in HCL-KCL buffer (0.1 M; pH 1.5) and incubated (37 °C for 1 h) with pepsin (1 g/10 mL HCL-KCL, 220 U/mL) to prevent protein interactions with starch, simulating the gastric phase. Then, 25 mL of tris-maleate buffer solution (0.1 M, pH 6.9) was added to this mixture to stop the enzyme reaction and to create ideal conditions to initiate the small intestine digestion or pancreatic phase, simulated by adding 5 mL of α-amylase solution (3 U/mL), followed by incubation at 37 °C. Aliquots were collected every 30 min (1 mL, 0–180 min), and the enzymatic reaction was stopped immediately by water-bath boiling (5 min), and kept under cold conditions (until the 180 min of incubation).

Aliquots were treated with 3 mL of sodium acetate buffer (0.4 M; pH 4.75), and 60 µL of amyloglucosidase (3300 U/mL) were added, followed by incubation for 45 min at 60 °C, under constant stirring; volumes were adjusted to 10 mL with distilled water and centrifuged (3.000 *g*/10 min at room temperature, 21 °C ± 2 °C). The supernatant (digestible starch fraction) was used for glucose determination.

3.7.3.2. Resistant Starch

Resistant starch was determined according to the methodology earlier described [31] and subsequently applied by other authors [30,32]: The ground bread crumb sample (100 g) was incubated (60 min at 40 °C) with a pepsin solution from porcine gastric mucosa (40,000 U/mL; 1 g/10 mL KCL- HCL buffer), to reduce the protein interference. Subsequently, pancreatic α-amylase (40 mg α- amylase: 200 U/mL) was added and incubated during 16 h at 37 °C, for starch hydrolysis. After hydrolysis, the pellet was isolated by centrifugation and further subjected to digestion with 4 M KOH as described by Goni et al. [31]. This solution was incubated for 45 min at 60 °C, in the presence of amyloglucosidase (3300 U/mL) to hydrolyze the remaining resistant starch to glucose. The pH conditions (pH 4.75) were adjusted according to the enzymatic activity requirements of amyloglucosidase.

Hydrolyzed starch was measured as the amount of glucose released, using the Megazyme GODPOD reagent kit, according to described earlier [30]. Starch was calculated as glucose (mg) × 0.9 (the conversion factor). Tests were performed in triplicates and applied in three breads.

3.7.3.3. In Vitro Starch Digestion and Estimation of Gluten-Free Bread Glycemic Index

The in vitro digestion kinetics was calculated according to the procedure established earlier [31]. A nonlinear model as expressed by Equation (1) was employed to describe the starch hydrolysis kinetics: C is the concentration at t time, C∞ the equilibrium concentration, k the kinetic constant, and t the time:

$$C = C\infty \ (1 - e^{-kt}) \quad (1)$$

The hydrolysis index (HI) was obtained from Equation (2), dividing the estimated area under the hydrolysis curve (AUC 0–180 min) obtained for gluten-free bread and reference food (white wheat bread) [30–32]:

$$HI = \frac{\text{AUC of product}}{\text{AUC Reference food}} \ 100 \quad (2)$$

The estimation of glycemic indices (eGI) was calculated according to Equation (3) [31]:

$$eGI = (0.549 \times HI) + 39.71 \quad (3)$$

3.7.4. Antioxidant and Anti-Inflammatory Activities of the Gluten-Free Bread

3.7.4.1. Antioxidant Activity

The antioxidant activity of the gluten-free bread was evaluated on ground breadcrumb samples (2 g) by preparing methanolic extracts (20 mL of methanol), followed by centrifugation (8000 g/4C/20 min) and filtration (0.2 μm filter). The extracts were stored at −4 °C. The scavenging effect of bread extracts was determined using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) methodology [33]: Bread extracts or an acid ascorbic solution (100 μL) were added to DPPH solution (1000 μL) in methanol (90 μmol/L), and the mixture was diluted with methanol (1900 μL). The absorbance (515 nm) was measured after 60 min in a dark room.

Bread extract reducing power was evaluated applying the ferric ion reducing antioxidant power (FRAP) method [34]: The bread extract or acid ascorbic (90 μL) and methanol (2700 μL) were added to FRAP reagent (2700 μL), and the absorbance (at 595 nm) was measured after 30 min in a shaking water-bath (37 °C). Results of scavenging activity and reducing power were expressed as mg ascorbic acid equivalents (AAE) per gram of bread extract.

The total phenolic content (TPC) of bread extracts was assessed following the method earlier described [35]: The bread extract or gallic acid (150 μL) was added to Folin–Ciocalteu reagent (150 μL, at 0.1 mol/L) and, after 10 min, was well mixed with sodium carbonate (300 μL at 7.5% *w/v*, 300 μL), followed by room temperature dark room incubation (2 h).

Absorbance (760 nm) was measured, and TPC results were reported in mg of gallic acid equivalents (AGE) per gram of bread extract.

Tests were performed in triplicates for each antioxidant activity assay, in three breads, to ensure reproducibility of the results.

3.7.4.2. MMP-9 Inhibition Activity

To evaluate the MMP-9 inhibitory activity of different crumb bread, a buffer-solution extraction was performed: Bread samples (5 g) were added to Tris-HCl buffer solution (30 mL at 100 mM, pH 7.5, ratio 1/6 (*w/v*)), and it was kept stirring for 4 h at 4 °C. After centrifugation (12,000 g for 30 min at 4 °C) (Beckman J2-21M/E), the supernatant was collected and stored (−20 °C). The MMP-9 inhibitory capacity of the yogurt and that of the curd cheese were also evaluated, applying the same procedure described above.

MMP-inhibition activity was tested using the dye-quenched (DQ)-gelatin assay as described before [36]. The fluorogenic DQ-gelatin substrate (Invitrogen, CA, USA) was dissolved in Milli-Q water at 1 mg/mL. All solutions and dilutions were prepared in assay-buffer (50 mM Tris-HCl buffer, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% *v/v* Tween 20). A black micro-assay plate (Grainer bio-one, 96-well) was used, and each well was loaded with 0.1 mM (for a final volume of 200 µL) MMP-9 (Reference: M8945, Sigma-Aldrich, Chemical Company, St Louis, MO, USA), and 80 µL of each bread extract was incubated (30 min at 37 °C). The dairy products and bread extract volume was kept constant (80 µL), equivalent to the following protein content (mg/5 g of dairy product or bread): 8.64 ± 0.40 mg to Yg and 83.0 ± 3.15 mg to Cc, 8.6 ± 1.0 mg to CB, 13.0 ± 0.05 mg to yogurt bread (YgB) and 17.0 ± 0.51 mg to curd cheese bread (CcB).

Subsequently, remaining MMP-9 catalytic activity quantification, the DQ-gelatin (final concentration of 2.5 µg/mL), was added to each plate well followed by incubation, for 1 h. Since the gelatinolytic activity is present, the DQ-gelatin substrate is hydrolyzed and releases fluorescence (fluorescence measurement conditions: excitation 485 nm/emission 530 nm). To correct possible gelatinolytic activity in the bread extracts, positive (without bread extracts) and negative (Without enzyme) controls were also included, and all experimental data were corrected by subtraction the respective negative controls. Tests were performed in triplicates and applied in three breads.

3.7.5. Statistical Analysis

Statistical analysis in the experimental data (average values and standard deviation) (RStudio, Version 1.1.423, Northern Ave, Boston, MA, USA) using variance test in one factor (ANOVA), by Tukey test (post hoc comparisons, at 95% of a significant level), were assessed. A Nonlinear Rheology Model, to fit the experimental rheology data, was applied (Carreau model using TA Instruments\TRIOS software, Waters, Lukens Drive, New Castle, PA, USA).

3.8. Results and Discussion

3.8.1. Pasting Properties

Starch behavior changes, induced by the addition of yogurt or Cc, were assessed by heating-cooling cycles, to simulate the mixing and baking steps of the breadmaking process.

Figure 1 clearly shows that the impact of Cc additions on starch performance is much greater than the effect promoted by yogurt incorporations, which can be attributed to the different structure of the proteins involved on the starch-protein matrix, as well as to differences in protein and lipid content [8] between these two dairy products [26]. Protein and lipids content in yogurt and curd cheese bread, at 10 and 20% of addition, varied from 6.9 ± 0.12 g to 8.10 ± 0.20 g and 7.60 ± 0.15 to 9.50 ±0.10 g for protein content and 5.20 ± 0.84 g to 5.60 ± 0.22 g and 8.90 ± 0.80 g to 10.20 ± 0.30 g for lipids content, in comparison with control bread, 5.34 ± 0.21 g of protein and 4.83 ± 0.29 g of lipids.

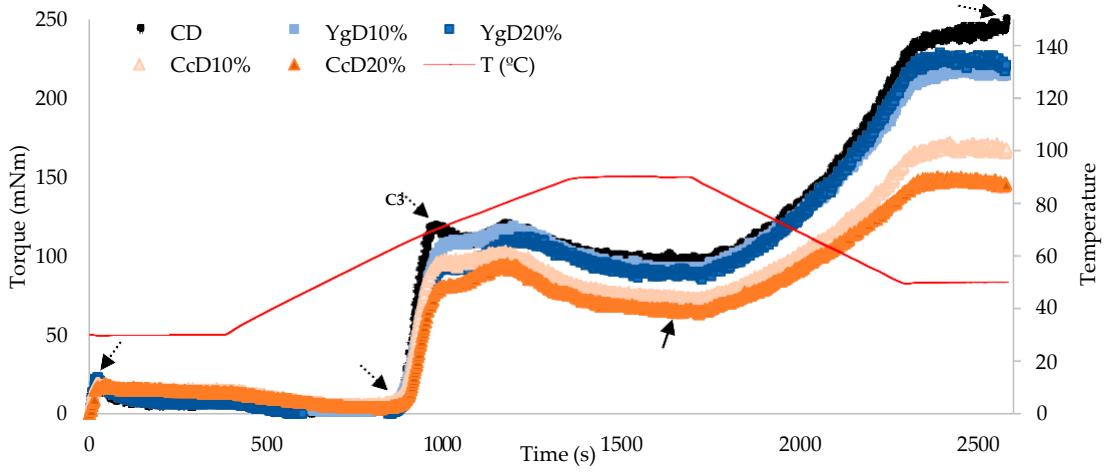

Figure 1. MicrodoughLab heating-cooling curves, expressing the impact of dairy products addition (Yg or Cc) in different amounts (10 and 20%, *w/w*) on starch behavior of gluten-free doughs, compared to control dough: CD—control dough, YgD—yogurt dough, and CcD—curd cheese dough.

During the first seconds of mixing, at constant temperature (30 °C), the torque was very low for all the tested doughs. This result was expected since these doughs comprise mixtures of flours without gluten. Starting the heating stage, at about 60 °C, the systems tended to phase separation, resulting in a further decrease of dough consistency (measured as torque—C2). The values of C2 were too low, namely, for yogurt dough (YgD), expressing a difficulty for the equipment to measure such low torque values, compared to control dough (control dough = 4.3 mNm ± 0.6 mNm). These results suggest that the caseins and exopolysaccharides (EPS) coming from yogurt addition are acting as lubricants with destabilizing effects on the dough matrix [37], reducing considerably the torque under mechanical shear stress. Cc doughs are superimposing the ones from control dough, with mNm values ranging from 6.3 ± 0.6 and 5.7 ± 1.2, showing no significant ($p > 0.05$) differences from control dough.

As the heating phase proceeds (up to 95 °C), starch granules start gelatinizing and take the dominance on torque values, whereas dough consistency increases as a result of starch swelling [38]. Significant impact on starch gelatinization performance was obtained for higher levels of dairy products tested: YgD20%, 101.0 mNm ± 8.5 mNm, and CcD20%, 92.0 mNm ± 1.7 mNm, in which a reduction of around 13% and 21%, respectively, compared to control dough (CD: 116.0 mNm ± 5.0 mNm), was registered. The diminished starch gelatinization observed can probably be attributed to the interaction between starch and protein coming from dairy product additions, reducing the starch availability to swell and break, affecting considerably the dough consistency (in torque values).

Because of continuous starch granule physical breakdown, due to the mechanical shear stress, further reduction in torque units occurred (C4 value), and at this phase of cooking, the amylase activity takes the dominance, followed by the amylose network stabilization [39]. No significant differences ($p < 0.05$) were obtained on C4 values by yogurt additions, presenting values close to control dough (98.0 mNm ± 2.1 mNm). However, for curd cheese incorporations, significant differences in C4 torque values were noticed, varying from 77.0 mNm ± 3.2 mNm for CcD10% to 65.0 mNm ± 0.6 mNm for Cc20%, compared to control dough, representing a reduction around of 22% and 34%, respectively. Subsequently, on cooling (from 95 °C to 50 °C), starch retrogrades, and the torque values increase (C5). In terms of final consistency values (C5), slight differences were obtained for yogurt additions, varying from 230 mNm ± 15.1 mNm for Yg10% to 220 mNm ± 16.1 mNm for Yg20%, compared to control dough (250 mNm ± 5.6 mNm). Nevertheless, curd cheese additions promoted a significant impact on C5 values, ranging from 165.3 mNm ± 5.5 mNm for Cc10% to 150.0 mNm ± 1.5 mNm for Cc20%, representing a reduction of around 34% and 40%, compared to control dough values.

This considerable reduction in final torque can be attributed to a sort of protective effect of curd cheese proteins on the starch granules hindering gelatinization, reducing the damage of starch granules, and lowering amylose concentrations on continuous phase. Besides, it suggests that the starch granules in the curd cheese dough should be less available to enzymatic hydrolysis than in yogurt doughs. Besides, curd cheese lipids may induce some lubricant effect, further reducing the final consistency.

Linear correlations ($R^2 = 0.922$) were found between the final consistency and dairy products additions, meaning that the effect on reducing final consistency is proportional to the level of dairy products added (Linear correlations presented in Section 3.8.4).

The impact of curd cheese additions on starch behavior was remarkable compared to yogurt incorporations, and it could be attributed not only to the dilution effect on starch but also to the following scenarios:

(i) Insufficient hydration of starch granules resulting from the competition for free water by denatured whey proteins (derived from curd cheese additions [40]);
(ii) Starch-protein interactions, reducing the accessibility of enzymatic attack to starch granules, thus limiting the degree of starch hydrolysis [8];
(iii) Amylose-lipid complexes formed during cooling, lowering the final peak consistency [41].

Results suggested that the different dairy proteins added by yogurt and curd cheese incorporations, promoted different effects on starch performance, mainly on starch gelatinization (C3) and on final dough consistency (C5), being higher for curd cheese incorporations, that probably can have an impact in reducing the glycemic response of bread.

3.8.2. In Vitro Starch Digestion of the Gluten-Free Bread

The influence of yogurt and curd cheese additions to gluten-free bread dough on digestible and resistant starch fractions was evaluated, and the results obtained are presented in Figure 2.

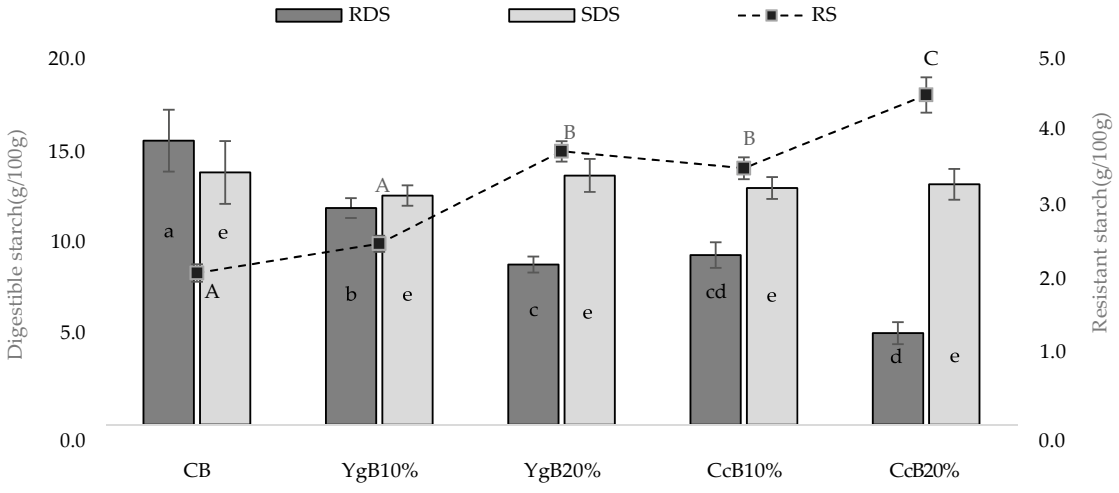

Figure 2. Effect of dairy product additions (10% or 20% *w/w* of yogurt or curd cheese) on starch fractions variation of GFB: rapidly (RDS) and slowly (SDS) digestible starch and resistant starch (RS). Different letters (a–e; A–D) indicate significant statistical differences at $p < 0.05$ (Tukey test).

Starch digestibility can be characterized into rapid digestion (RDS)—hydrolyzed within the first 30 min of human digestion; slowly digestion (SDS)—digested within 60–180 min; and resistant starch (RS)—not enzymatically hydrolyzed during human digestion (0–180 min) [42].

From Figure 2, significant ($p < 0.05$) differences on the RDS and RS fractions were achieved, whereas no significant impact was registered in SDS fraction, compared to CB.

Starting from control bread, the dominant starch fraction was RDS followed by SDS and RS.

However, upon the addition of yogurt and curd cheese to the dough, this pattern changed, essentially for higher levels (20% *w/w*) tested, where the SDS took the dominance, followed by the RDS and RS. The SDS fraction, being slowly digested, is more desirable than RDS since it promotes a gradual increase in plasma glucose and insulin levels [43].

Figure 2 also shows that a remarkable effect in the RDS reduction was achieved for yogurt and curd cheese additions (10% and 20%, *w/w*), where a significant reduction of about 24% and 44% for yogurt bread and 40% and 67% for curd cheese bread were registered, compared to control bread. Moreover, considerably increases in RS values of around 20% and 80%, and 70% and 110%, respectively, were also obtained.

Yoghurt and curd cheese are known to be rich-protein sources (even richer in the latter's case). Therefore, protein-starch interactions within bread structure, further boosted by the baking process, might affect the digestive enzymes' accessibility to starch granules, hindering their hydrolytic performance.

These significant decreases of RDS fraction and subsequent increase in RS levels can most probably be linked to the negative impact promoted on starch gelatinization performance (C3). Since the starch was less available to swell and break, probably due to the starch-protein interaction established, the accessibility of enzymatic attack to starch granules was diminished, thus limiting the degree of starch hydrolysis.

Linear correlations were studied between the RDS (R^2 = 0.993) and RS (R^2 = 0.933) results with the protein content of yogurt and curd cheese bread, at 10% and 20% addition (Linear correlations presented in Section 3.8.4), showing that the variations in both starch fractions are proportional to the increments in protein content by dairy products additions.

Similar results were obtained by other authors [10,11,30], on gluten-free bread enrichment with rich-protein sources.

Additionally, the competition for available water by denatured whey protein should also be invoked to explain these results, lowering the amount of accessible water in the dough system, reducing starch gelatinization due to limited moisture, and impacting negatively on RDS content.

Therefore, the increase in RS levels can be associated with the diminished catalytic activity of digestive enzymes due to the less accessibility to starch granules since the starch gelatinization performance was reduced [44].

These results are in line with those obtained in pasting property studies, where a significant effect on starch gelatinization, as well as on final dough consistency values, was obtained, with a major impact by curd cheese addition. Linear correlations (R^2 > 0.900) were found among dairy product additions to the dough, and RDS and RS fractions, as well as the relation between starch fractions and final consistency results (Linear correlations presented in Section 3.8.4). These findings agree with those recently obtained [30] on the evaluation of the impact of dairy products to improve the glycemic response to wheat bread.

3.8.2.1. Hydrolysis Kinetics and Estimated Glycemic Index of Gluten-Free Bread

The gluten-free bread, prepared with the addition of yogurt or curd cheese, and control bread were subjected to in vitro hydrolysis to simulate starch digestibility, and after specific digestion times (30 to 180 min), starch hydrolysis was estimated by the amount of glucose released. Hydrolysis curves and starch hydrolysis vs. time are plotted in Figure 3.

All digested bread samples exhibited similar behavior, in which the extent of glucose release displayed an almost linear rise between 30 and 60 min of hydrolysis, followed by a gradually diminished as hydrolysis time proceeded (90–180 min).

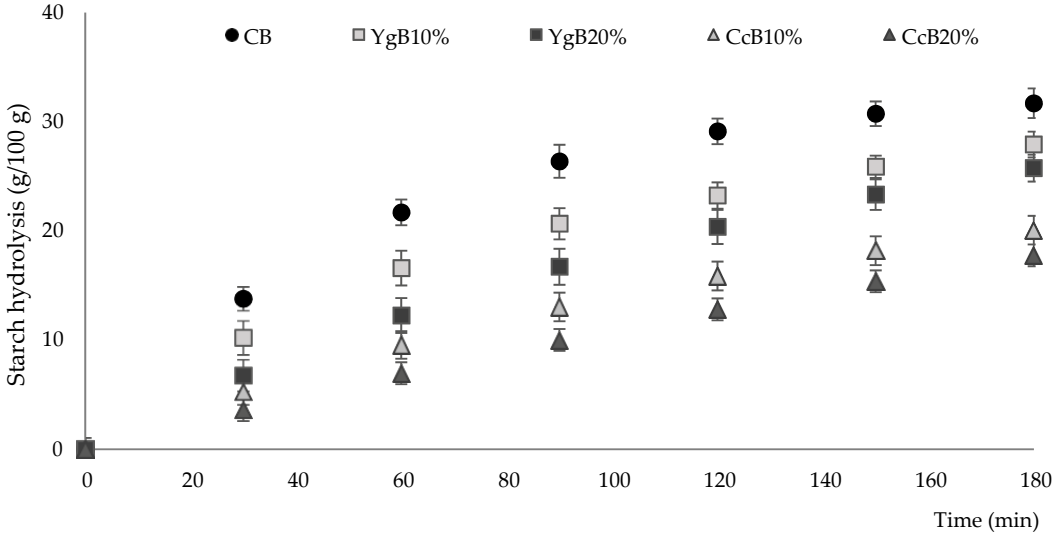

Figure 3. Effect of dairy product additions (10% or 20% *w/w* of yogurt or curd cheese) on starch hydrolysis pattern during in vitro starch digestibility (YgB10% and YgB20%, square symbols) and curd cheese (CcB10% and CcB20%, triangular symbols), compared to control bread (CB, round symbols). Different letters (a–e; A–D) indicate significant statistical differences at $p < 0.05$ (Tukey test).

The influence of yogurt and curd cheese supplementation, in vitro kinetics starch digestion of the bread, was also evaluated, based on primary and secondary parameters derived by fitting the experimental data ($R^2 > 0.915$) to a nonlinear model (Equation (1)) [31]. These fitted parameters included equilibrium concentration (C∞), kinetics constant (k), hydrolysis index (HI), and estimated glycemic index (eGI) and are summarized in Table 2.

Table 2. Nonlinear parameters that characterize the in vitro digestibility of gluten-free bread: equilibrium concentration (C∞), kinetic constant (k), hydrolysis index (HI), area under the hydrolysis curve after 180 min (AUC 0–180) and estimated glycemic index (eGI) of control (CB); yogurt (YgB10% and YgB20%) and curd cheese (CcB10% and CcB20%) bread.

Samples	C∞ *	K *	R^2	AUC 0–180	HI (%)	IG **
CB	33.1 ± 0.9 ^{a}	0.0179 ± 0.002 ^{a}	0.915	4122.9 ± 61.2 ^{a}	100.0 ± 0.0 ^{a}	100.0 ± 0.0 ^{a}
YgB10%	28.0 ± 0.8 ^{b}	0.0153 ± 0.002 ^{a}	0.972	3239.6 ± 36.0 ^{b}	78.6 ± 1.3 ^{b}	82.8 ± 0.7 ^{b}
YgB20%	27.0 ± 3.0 ^{b}	0.0068 ± 0.001 ^{b}	0.984	2766.2 ± 83.0 ^{c}	67.1 ± 1.7 ^{c}	76.5 ± 0.9 ^{c}
CcB10%	20.0 ± 1.2 ^{c}	0.0068 ± 0.003 ^{b}	0.984	2185.1 ± 28.0 ^{d}	53.0 ± 1.0 ^{d}	68.0 ± 0.5 ^{d}
CcB20%	18.0 ± 0.4 ^{c}	0.0029 ± 0.001 ^{c}	0.985	1728.3 ± 21.2 ^{e}	42.0 ± 0.1 ^{e}	62.7 ± 0.1 ^{e}

Different superscripts (a, b, c, d, e) indicate significantly statistical differences at $p < 0.05$, (Tukey test), compared to control bread (CB). * C∞ and k were obtained by nonlinear Equation (1): $C = C\infty\ (1 - e^{-kt})$ [31]. ** eGI was estimated by linear Equation (3): $eGI = (0.549 \times HI) + 39.71$ [31].

Incorporation of yogurt and curd cheese had a significant ($p < 0.05$) influence in C∞ reduction, varying from 28.0 g/100 g to 27.0 g/100 g for yogurt additions, and 20.0 g/100 g to 18.0 g/100 g for curd cheese incorporations, compared to 33.1 g/100 g obtained for CB. Considering the highest levels of yogurt and curd cheese tested (20%, *w/w*), these variations represented a decrease of around 18% and 46% of the equilibrium concentration, respectively, compared to control bread.

The k values that express the enzymatic hydrolysis rate in the early stages decrease substantially by the additions of yogurt or curd cheese: yogurt bread ranging from 0.015 to 0.007 and curd cheese bread varying between 0.007 and 0.003, compared to CB (0.018). Lower values of k suggest that the increase of dairy product in the dough is promoting a higher resistance to enzymatic starch hydrolysis, and this may be explained by a starch–protein interactions, limiting starch digestibility.

These results are in line with those obtained on digestible starch fractions, where a significant reduction in RDS and an increase in RS were noticed. Comparable findings were obtained recently by other researchers [45] by "Amala" and plantain enrichment on bread formulations.

Table 2 further shows that the additions of yogurt or curd cheese substantially influenced the starch hydrolysis index (HI), resulting in values considerably lower than control bread. However, a remarkable impact was achieved by curd cheese additions, varying from 53% to 42%, compared to control bread (100%).

These results were reflected in estimated values for the glycemic index, resulting in an intermediate glycemic index for curd cheese bread (GI 55–69), a considerable reduction of about 35% for both dairy products levels tested (10% and 20%, *w/w*). Despite the variations obtained for yogurt levels tested (10% and 20%, *w/w*), the estimated values of the glycemic index are still showing that they maintain a high glycemic response (GI > 70).

3.8.3. Effect of Yoghurt and Curd Cheese Enrichment on Gluten-Free Bread Bioactive Properties

3.8.3.1. Antioxidant Activity

The antioxidant capacity (AC) of gluten-free bread enriched with yogurt or curd cheese additions was tested by DPPH and FRAP methods. From Figure 4A, B, it can be observed that both dairy products tested promoted different effects on AC: Yogurt additions showed higher potential to produce bread more effective in radical scavenging capacity (RSC), whereas curd cheese bread expressed better reducing power effects. Compared to CB (DPPH: 24.0 mg· mg^{-1} AAE, FRAP: 10.0 mg· mg^{-1} AAE), the incorporation of the higher level (20%, *w/w*) of yoghurt (DPPH: 57.0 mg· mg^{-1} AAE, FRAP: 15.0 mg· mg^{-1} AAE) and of curd cheese (DPPH: 41.0 mg· mg^{-1} AAE, FRAP: 17.0 mg· mg^{-1} AAE) led to an increase in bread AC, representing an improvement of around 140% and 50% for yoghurt addition and about 71% and 70% for curd cheese incorporation, respectively. The RSC increment on yogurt bread can probably derive from α-tocopherol (vitamin E) since the bioactive properties of this compound contribute to oxidative stability properties [18]. Additionally, it is known that the hydrophobic and aromatic amino acids of the whey protein present bioactive properties [28] that probably can improve the AC of the bread with curd cheese.

Phenolic compounds (PC) are regarded as an important class of secondary metabolites, some of which exhibits health-promoting benefits, including antioxidant activity [19]. The addition of yogurt and curd cheese increased total phenolic compounds (TPC) (Figure 4C): For the highest dairy product level tested (20%, *w/w*) ranged from 13.2 mg· mg^{-1} AGE for YgB, and 15.0 mg· mg^{-1} AGE for CcB, compared to 8.50 mg· mg^{-1} in control bread, representing an increase of 55.3% and 73.0%, respectively. The increase of PC in bread, deriving from dairy products additions, probably contributed to enhancing the antioxidant activity of the bread, expressed as FRAP activity. The linear correlation ($R^2 = 0.855$) calculated between TPC and FRAP activity supports this relation (Section 3.8.4, Table 3). Similar results were found by other researchers [10] studying the effect of *Lycium ruthenicum* addition to bread fortification and in vitro digestibility impact.

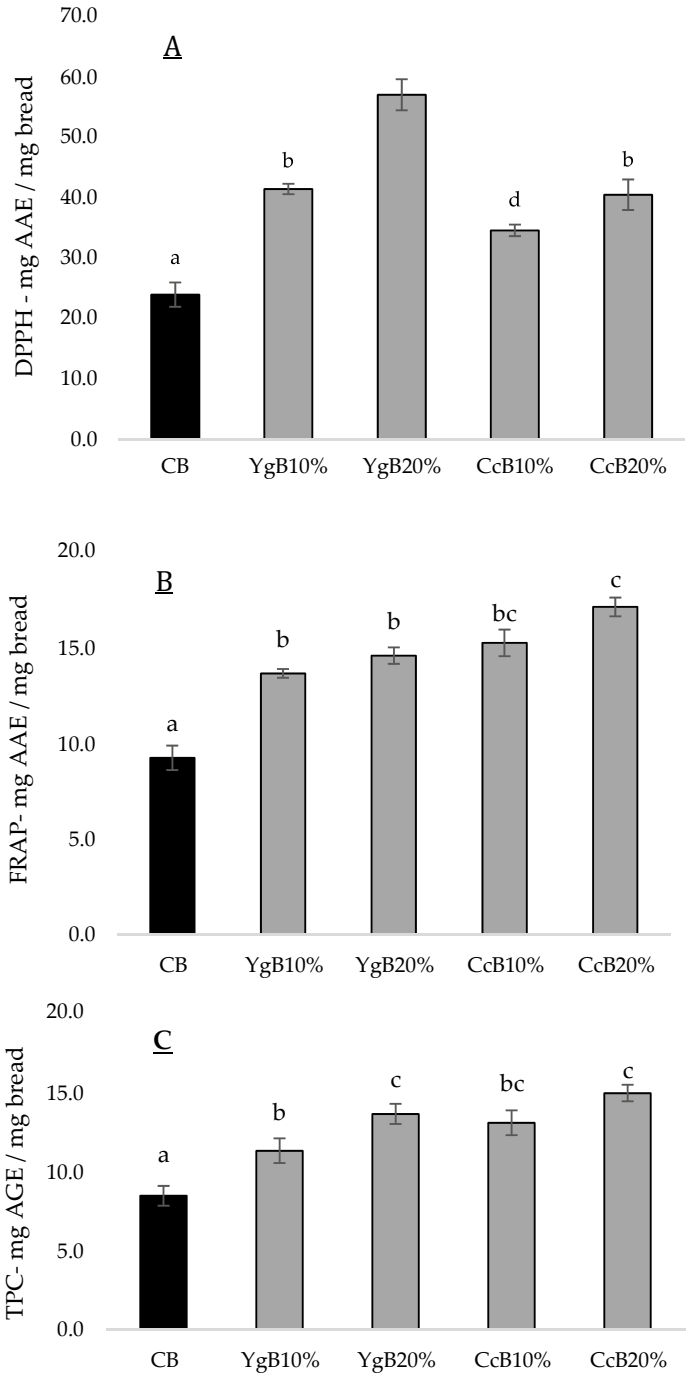

Figure 4. Antioxidant capacity measured with the following methodologies: (**A**)—DPPH, (**B**)—FRAP (both expressed as mg· mg^{-1} ascorbic acid equivalents—AAE) and (**C**)—TPC (total phenolic content, expressed as mg· mg^{-1} gallic acid equivalents—GAE) of fresh breads with 10% (*w/w*) or 20% (*w/w*) yoghurt (YgB10%, YgB20%) or curd cheese (CcB10%, CcB20%), compared to control bread (CB). Different letters indicate significant statistically differences ($p < 0.05$).

Table 3. Linear correlations were found between pasting properties, starch digestibility, antioxidant capacity, and glycemic index results and dairy product additions ($R^2 > 0.850$; $p = 0.05$).

Parameters Correlated	Linear Equation	R^2
FC vs. DP	FC = 218.48 − 3.49 × DP	0.922
RDS vs. DP	RDS = 13.52 − 0.27 × DP	0.910
RDS vs. PBC	RDS = 29.3 − 2.58 × PBC	0.993
RS vs. DP	RS = 2.30 + 0.05 × DP	0.940
RS vs. PBC	RS = - 1.25 + 0.60× PBC	0.933
RS vs. FC	RS = 7.52 − 0.02× FC	0.900
GI vs. FC	GI = 23.14 + 0.27× FC	0.900
GI vs. RDS	GI = 55.32 + 2.68× RDS	0.944
GI vs. RS	GI = 121.60 − 12.91 × RS	0.952
GI vs. DP	GI = 89.88 − 0.99× DP	0.922
FRAP vs. TPC	FRAP = 0.60 + 1.26 × TPC	0.850
GI vs. TPC	GI = 134.76 − 5.43 × FRAP	0.900
GI vs. FRAP	GI = 132.80 − 3.97× FRAP	0.920

Table legend: FC—final consistency; RDS—rapidly digestible starch; RS—Resistant starch; DP—Dairy products; PC—protein bread content GI—Glycemic index; FRAP—Ferric ion reducing antioxidant power; TPC—Total phenolic compounds.

3.8.3.2. MMP-9 Inhibition Activity

The impact of yogurt or curd cheese supplementation on gluten-free bread was evaluated by its capacity to inhibit MMP-9 enzyme activity. The DQ-gelatin assay was used to evaluate the anti-inflammatory capacity of bread. The results obtained are illustrated in Figure 5.

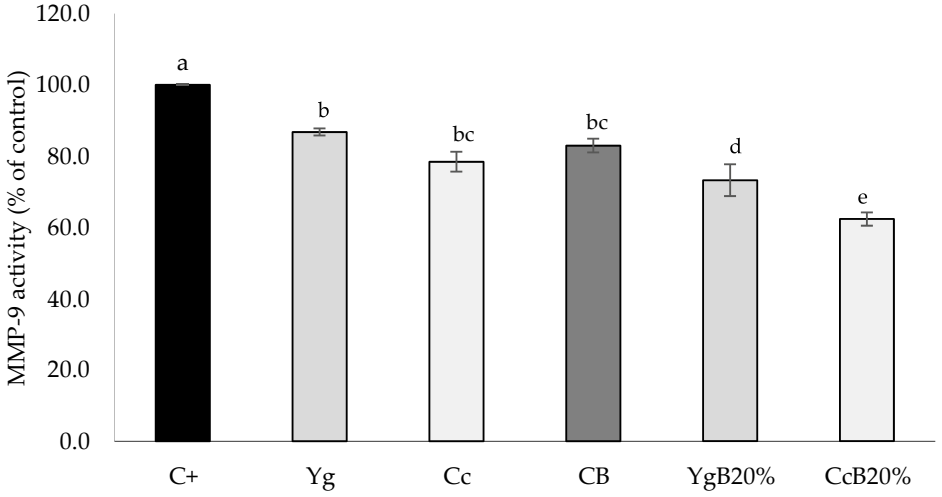

Figure 5. Effect of gluten-free bread extracts on the proteolytic activity of MMP-9 after 60 min of incubation time: Yoghurt (Yg), curd cheese (Cc), control Bread (CB), and bread enriched with 20% (*w/w*) yogurt (YgB20%) or curd cheese (CcB20%). The control (C+) does not inhibit MMP-9. MMP-9 activities were expressed by relative fluorescence as a control percentage (averages of at least. Different letters indicate significant differences ($p < 0.05$), between three replicate experiments ($n = 3$) ± standard deviation of fresh bread.

The MMP-9 inhibitory capacity of yogurt and curd cheese was assessed individually (Figure 5), in which around 13.3% and 21.6% of inhibition effect were obtained, respectively, compared to control (C+).

Figure 5 also shows that all bread extracts had a significant effect ($p < 0.05$) on MMP-9 activity inhibition, compared to the control (C+). The highest inhibition of MMP-9 activity was obtained in CcB20% (40%), followed by YgB20% (30%), and finally by the control bread (17%). One can suggest

that the supplementation of the control bread with each one of the two dairy products tested can be an interesting approach to improve its bioactivity in terms of anti-inflammatory properties, and these properties can probably be further enhanced by the breadmaking fermentation process.

Several studies have been shown the anti-inflammatory properties of dairy products, especially those obtained by fermentation processes, associating them as anti-inflammatory agents on humans' immune processes [22,24].

These results agree with those obtained in a recent study [46], which showed inhibition of this enzyme by *Limonium tetragonum* extract and are also in line with those reported by other authors [47] based on the inhibition of MMP-9 with phenolic compounds and proteins from cooked soybean.

It can be stated that the MMP-9 inhibition activity exhibited by gluten-free bread with yogurt or curd cheese may constitute an interesting contribution to inflammatory bowel disease preventive diets. Besides, the incorporation of these nutritional dairy products creates an advantage since it can be consumed by the entire population, even by celiac patients, improving their daily diet.

3.8.4. Correlations between Starch Behavior, Glycemic Index, and Bread Bioactivity

According to the results presented along with this research work, a considerable impact on glycemic index reduction by yogurt or curd cheese additions to gluten-free bread was obtained.

The relationships among pasting properties, starch digestibility, antioxidant capacity, and glycemic index should be considered, to give additional support and consolidate these findings.

Linear correlations found between pasting properties, starch digestibility, antioxidant capacity, and glycemic index results (R^2 > 0.850) are presented in Table 3.

Strong correlations between the dairy products addition and final consistency (FC) (R^2 = 0.922), RDS content (R^2 = 0.910), RS fraction (R^2 = 0.940), and the glycemic index (R^2 = 0.922) of the breads were obtained. In addition, glycemic index results were also strongly correlated with all these parameters, FC (R^2 = 0.900), RDS (R^2 = 0.940) and RS (R^2 = 0.950). These linear relations reflect a strong correlation between dairy product addition and starch performance changes that, in turn, led to a considerable impact on in vitro starch digestibility and, consequently, on the glycemic index of the gluten-free bread. According to previous studies [11,12] digestive enzyme inhibition caused by some phenolic compounds was found to be an interesting alternative to maintain a low glycemic index diet, especially for starch-based foods, via inhibition of α-amylase. The possible relationships between the TPC and FRAP activity with glycemic index values were also studied. Linear correlations (Table 3) obtained between glycemic index and TPC (R^2 = 0.900) and glycemic index and FRAP (R^2 = 0.920), suggest that the enrichment in PC and the increase of FRAP activity by the addition of dairy product probably promoted an additional effect on the reduction of the glycemic index of the gluten-free bread.
These results agree with those published by other authors [11,12] reinforcing their findings.

3.9. Conclusions

From this study, in which the influence of yogurt or curd cheese supplemented to gluten-free bread was evaluated, to reduce the glycemic response and to improve bioactivity, encouraging results were obtained.

Considering the glycemic index results, the impact of curd cheese addition to dough was greater than those obtained by yogurt additions, significantly reducing the glycemic response to intermediate values of the glycemic index (glycemic index: 55–69).

Improvements in the glycemic response can probably be associated not only to the dilution effect of starch granules and physical interactions between starch and proteins but also to the presence of some bioactive metabolites (e.g., phenolic compounds), derived from the dairy products additions, that we're able to slow the enzyme's hydrolysis activity performance while improving the bioactivity of gluten-free bread.

It was possible to improve the bioactivity of bread with both dairy products additions, in terms of antioxidant capacity and phenolic compounds.

The enrichment of gluten-free bread with yogurt or curd cheese resulted in effective inhibition of MMP-9 activity, suggesting that both can be interesting baker's ingredients to improve the bioactivity of bread in terms of anti-inflammatory properties and possibly anticarcinogenic effects. Nevertheless, further in vitro and in vivo assays to consolidate these anti-inflammatory properties achieved would be tested in future surveys.

The gluten-free bread obtained can give an important nutritional contribution to the celiac and irritable bowel syndrome patients' daily diet as well as to an inflammation preventive diet strategy.

These findings can be a new window of research to prove the positive impact of dairy product addition to reduce the glycemic index and increase the antioxidant and anti-inflammatory potential of foods.

In summary, the incorporation of yogurt or curd cheese in gluten-free bread showed to be an interesting strategy to improve the glycemic response and anti-oxidative and anti-inflammatory activities, thus contributing to fulfill the needs of the celiac individual's daily diet.

Author Contributions: Conceptualization, methodology, investigation, data curation, writing—original draft preparation, C.G.; methodology and data curation on anti-inflammatory properties, J.M.; manuscript revision, A.L. and R.B.F.; project administration, investigation, supervision, validation, writing—review and editing, A.R. and I.S. All authors have read and agreed to the published version of the manuscript.

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Chapter 4. Yoghurt as a starter in sourdough fermentation to improve the technological and nutritional properties of sourdough-wheat bread

This chapter contains the experimental data obtained from the study of the influence of the presumptive lactic acid bacteria derived from yoghurt addition as a starter on sourdough fermentation, and its concurrent effect to improve the technological and nutritional properties, in terms of antioxidant capacity, *in vitro* starch and protein of the sourdough-wheat bread.

The work presented in this chapter was included in the following research study:

- **Research paper 7:**

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Yoghurt as a starter in sourdough fermentation to improve the technological and functional properties of sourdough-wheat bread

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ABSTRACT

The incorporation of yoghurt as a starter in sourdough wheat bread, to improve technological and nutritional properties, was investigated. Two bread dough matrices were considered: endosperm wheat flour and blended with whole-grain flour. Two fermentation's types were performed, two-stage sourdough bread and yeast bread fermentation.

Compared with yeast dough, yoghurt-sourdough fermentation promoted considerable changes in chemical composition, particularly when whole-grain flour was conjoined with wheat flour: higher protein proteolysis degree, increase of peptide and free amino nitrogen content, solubilization of phenolic compounds (46–53%), increase of DPPH radical scavenging (54–65%) and ferric-reducing power (85–88%), were observed. As a baking ingredient, yoghurt-sourdough improved the bread crumb softness (15–12%) and delayed the staling (40–35%). Nutritionally, the glycemic index was reduced (18–32%) while protein digestibility (6–12%) and free amino acids bioavailability (50–100%) were enhanced. The addition of yoghurt and sourdough fermentation techniques offered a promising tool to improve wheat bread's technological and functional properties.

1. Introduction

The increasing consumer demand for well-balanced nutritional foods, has been driving both scientific researchers and the food companies towards the development of health-promoting goods, recurring for more natural ingredients and sustainable processes. Bread, as a staple food and an important component on daily diet, represents a good vector to nutritional enrichment applications (AIBI, 2013). Sourdough is one of the oldest bioprocessing techniques characterized by a mutual relationship between a complex microbiota dominated by lactic acid bacteria (LAB) and yeast population, widely used in baking applications as acidifying and leavening bread dough agent, until the late 19th century (Ganzle, Loponen, & Gobbetti, 2008). However, the introduction of baker's yeast in 1871 resulted in a gradual replacement of sourdough fermentation, by a short-term dough process resultant in a less tasty, nutritional, and digestible bread (Sapone, Bai, Ciacci, Dolinsek, & Green, 2012), with health implications for wheat-sensitive consumers (Huang et al., 2020).

The current renaissance of sourdough as bioprocessing is encouraged by its beneficial effect on flavor, texture, shelf-life, and health-promoting properties of derived-foods, since it is considered a promising process to meet the consumer's requirement and represents a new opportunity for food industry (Montemurro, Coda, & Rizzello, 2019). Additionally, it is known to generate peculiar characteristics in the derived-foods, mainly associated to *in situ* organic acids synthesis, activation of the endogenous flour enzymes as well as the LAB secondary metabolic activity (Ganzle et al., 2008), which advantages have been well conferred by several studies: (i) delay of starch digestion and

Abbreviations: W, wheat flour; WW, whole grain flour; WD, wheat dough; WWD, wheat, whole grain dough; WSD, wheat sourdough; WWSD, wheat, whole grain sourdough; WSYD, wheat yoghurt sourdough; WWSYD, wheat whole grain yoghurt sourdough; WB, Wheat yeast bread; WWB, wheat, whole grain yeast bread; WSB, wheat sourdough bread; WWSB, wheatwhole grain sourdough bread; WWSYD, wheat whole yoghurt sourdough; LAB, lactic acid bacteria.

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decrease of the end-products glycemic response (Coda, Varis, Verni, Rizzello, & Katina, 2017; Wang et al., 2019), (ii) increase of the *in vitro* protein digestibility and free amino acids bioavailability (Coda, Rizzello, Curiel, Poutanen, & Katina, 2014; Wang et al., 2019; Gobbetti et al., 2019), and (iii) increments on soluble phenolic compounds correlated with antioxidant capacity enhancement (Gobbetti et al., 2019; Coda et al., 2017) and (iv) decrease of others anti-nutritional factors as the Fermentable, Oligo-, Di-, Mono-saccharides and Polyols (FODMAPs) and α-amylase/trypsin inhibitors, as the well-documented trigger pro-inflammatory compound (Laatikainen et al., 2017; Huang et al., 2020).

In cereals, the nutritional compounds, such as proteins, vitamins and polyphenol compounds, derives from the germ and bran of the grain. Although these fractions are usually removed during milling, to prevent sensory and technological adverse implications, it is a source of protein and fiber than can be useful for other food applications (Arte, Huang, Nordlund, & Katina, 2019; Arte et al., 2015).

Sourdough LAB fermentation has been extensively studied as a promising bioprocess to overcome these adverse effects, in which great impact on both nutritional and technological properties of derived products, have been reported (Coda et al., 2017; Wang et al., 2019). Although the refined wheat flour promotes great technological properties and sensorial stability, it is easy to realize that the absence of these fractions leads to the loss of most of nutritional and beneficial healthy compounds (Fardet, 2010).

Yoghurt is a natural and nutritional fermented product (FAO Anonymous, 2010), widely consumed and appreciated on daily diet, since it is a source of high-biological protein easy digestible, essential amino acids profile and health-promoting benefits, which is important to support metabolism by increasing energy expenditure and satiety (Kristensen et al., 2016). Additionally, it is a source of exopolysaccharides *in situ* produced by its dominant lactic acid bacteria fermentative activity, that have been associated to physiological benefits including antioxidant activities and anti-inflammatory properties (Li et al., 2014). The fortification of refined-derived wheat products with dairy products, such as plain yoghurt, was successfully reported on both technological and nutritional properties of gluten-containing (Graça, Raymundo, & Sousa, 2019) and gluten-free (Graça, Raymundo et al., 2020) bread, rendering a significant improvement of the glycemic response and bioactive properties (Graça, Mota et al., 2020; Graça, Raymundo, & Sousa, 2021).

In that matters, plain yoghurt addition as a starter process on sourdough fermentation might be an alternative ingredient for new sourdough-bakery applications.

The aim of this study was to incorporate plain yoghurt as a starter in sourdough fermentation so that the resulted bread would have improved nutritional and technological properties. Two wheat bread dough formulations made of wheat flour (W, 100%, flour weight (fw)) and blended with whole grain flour (WW, 50:50%, fw), both with 40% (fw) of yoghurt addition, were performed. As control, baker's yeast fermented bread was used. We performed microbial and chemical changes after dough fermentation, and technological and nutritional quality measurement on breads. Including: (i) the changes on wheat proteins pattern promoted *in situ* by yoghurt-sourdough fermentation, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), (ii) the degree of proteolysis via the free amino nitrogen and peptides content, (iii) the changes on the soluble, bound, total phenolic content and antioxidant activity. Bread characterization included: (i) technological properties based on post-baking quality parameters and, (ii) nutritional features, focused on (a) *in vitro* starch and predicted glycemic index and (b) *in vitro* protein digestibility and free amino acids content, comparing with yeast breads.

2. Materials and methods

2.1. Materials

Endosperm wheat flour was from Sunnuntai Erikoisvehnäjauho, (Raisio, Finland; nutritional composition: total carbohydrate 70 g/100 g, protein 12 g/100 g, lipids 3 g/100 g, dietary fiber 4 g/100 g) and whole grain flour was from Graham Täysjyvävehnäjauho (Myllyn Paras, Hyvinkää, Finland; nutritional composition: total carbohydrates 56 g/100 g, protein 14 g/100 g, lipids 3 g/100 g, dietary fiber 12 g/100 g).

Plain yoghurt was from Bulgarian Jogurtti (Valio, Finland; nutritional composition: total carbohydrates 4.5 g/100 g; protein 3.4 g/100 g; lipids 3.5 g/100 g) produced from the symbiotic cultures of S*treptococcus thermophilus and Lactobacillus delbrueckii* subsp. *bulgaricus*.

Dry yeast was from Sunnuntai Kuivahiiva (Raisio Finland), sugar from Dansukker (Kantvik, Finland) and salt from AkzoNobel (Jozo, Finland).

2.2. Bread-making

To study the incorporation of yoghurt (40% based on flour weight, Table SI1) in sourdough fermentation and yeast fermentation, four types of bread formulations were performed, including two sourdough breads (WSB, wheat sourdough bread; WWSB, wheat whole grain sourdough bread) and yeast breads (WB, wheat yeast bread; WWB, wheat whole grain yeast bread). The yeast breads were directly fermented with all ingredients for one hour, while the sourdough breads consisted of two-stage fermentation, the first stage was yoghurt incorporated sourdough fermentation for 24 h and the second stage with additional yeast fermentation for one hour. The bread formulations and fermentation conditions are described in supplementary information 1 (SI1). A chemically acidified dough was performed, in which instead of deionized water, 1 M of sodium acetate buffer at pH 4.0 and antibiotics, as cycloheximide (at 0.01% w/w) and chloramphenicol (at 0.01% w/w), were added (based on flour water absorption), to prevent microbial growth, were performed (Arte et al., 2015).

The water absorption for wheat-based (65%, fw) and a wheat-whole grain based (66.5%, fw) breads formulations, by applying farinograph's mixing curves (Brabender GmbH and Co.KG, Germany), was determined according to AACC method 54-21 (AACC 2000). The yoghurt-derived water was taken in consideration (35.4%) to optimize the bread formulations (SI1).

Bread-making, according to the procedure earlier described by Wang et al. (2018) with some modifications, were performed: all ingredients were mixed in a DIOSNA mixer bowl (Dierks & Sohne GmbH, Germany) for 3 min at low speed and 4 min at fast speed. Afterwards, 150 g of each dough was molded mechanically and rested for 60 min at 35 °C with 75% of relative humidity (RH), in a proofing cabinet (Lillnord, Order, Denmark). The breads were baked in a rotating rack oven (Sveba Dahlen, Fristad, Sweden) at 180 °C for 15 min with 15 s of steaming at the beginning. After baking the breads were allowed to cool down for 2 h at room temperature, before further technical analysis. Triplicates from each of three irrespective biological fermentation were performed.

3. Dough characterization

In supplementary information SI2 summarised a general scheme of the work.

3.1. Microbiological analysis and pH determination

To assess the cell density of endogenous flour and yoghurt-derived presumptive LAB, the sourdough (10 g), before (0 h) and after (24 h) fermentation process, were homogenized with 90 mL of sterile saline (NaCl at 0.9% w/v) in a Stomacher 400 lab blender (Seward Medical, London). In detail, dough's serial dilutions were made, and the enumeration of the main microbial groups was carried out: mesophilic lactic acid bacteria (LAB) were obtained by plating on MRS agar (NEOGEN, Heywood, United Kingdom) at 30 °C for 48 h. Thermophilic LAB were counted on MRS agar at pH 5.4 (NEOGEN, Heywood, United Kingdom) after incubation at 40 °C for 48 h, and on M17 agar (NEOGEN,

Heywood, United Kingdom) at 37 °C for 48 h. The dough pH values were measured by a pH meter 92 (MeterLab™, Radiometer, Copenhagen, Denmark), suspending an aliquot of 5 g dough in 10 mL of distilled water. Triplicates were performed.

3.2. Chemical analysis

The yeast-bread, sourdough, sourdough-yeast, and chemically acidified dough samples, after frozen storage (at —20 °C and subsequently at — 70 °C), were subjected to freeze-drying (Christ, Gamma 2–20, Osterode, Germany), grounded and homogenised before further analysis, as follows described.

3.2.1. Determination of sugars and organic acids

Free sugars and organic acids were analyzed by high performance liquid chromatography (HPLC), according to the method earlier described by Rizzello, Cassone, Di Cagno, and Gobbetti (2008). A preliminary sample preparation was performed as follows: first to remove the protein fraction, a water/salt soluble extraction were performed (Weiss, Vogelmeier, & Go¨rg, 1993): 1 g of each sample in 4 mL of 50 mM Tris–HCl (pH 8.8) under a constant shaking at 4 °C for 1 h and then centrifuged (20,000*g*, 15 min);1 mL of clear supernatant was collected and 1 mL of 5% perchloric acid was added to precipitate the proteins. The mixture was kept in 4 °C overnight and then centrifuged (13,000*g*, 15 min); 1 mL of clear supernatant was then syringe-filtered (filter 0.45 µm, Merck). The separation was performed on a Hi-Plex H column (Agilent, CA, USA; 300 × 6.5 mm) at 65 °C in an HPLC (Waters e2695, Milford, MA, USA) equipped with a dual detector system, an ultraviolet (UV) detector plus a refractive index detector (2414 RI detector, Waters). The elution was isocratic phase for 25 min with 10 mM H_2SO_4 at a flow rate of 0.6 mL/min. The volume injection was 20 µl. Glucose (Sigma), galactose (Sigma) and lactose (AnalaR) were used as standards for sugars quantification, and lactic acid (Fluka BioChemika5) and acetic acid (VWR Prolabor Chemicals) were used for organic acids quantification.

3.2.2. Free amino nitrogen and peptides content

Water-soluble extracts, to follow the degree of proteolysis during the yeast-bread and sourdough-yeast fermented dough's, through the free amino nitrogen (FAN) by ninhydrin method (ASBC, 1992) and the peptides content following the o-phtaldialdehyde (OPA) procedure (Church, Swaisgood, Porter, & Catignani, 1983) was applied. The concentration of solubilized protein, by a DC protein assays kit, using bovine serum albumin as a standard, was quantified (Lowry, Brock, & Lopez, 1951).

3.2.3. Electrophoresis

Sequential water/salt extracts based on the original method of Osborne and modified by Weiss et al. (1993), was used to albumins/globulins, gliadins and glutenins extraction: 0.1 g of each lyophilized dough samples was mixed with 0.5 mL of 0.4 M NaCl with 0.067 M of Na_2HPO_4/KH_2PO_4 (pH 7.6) for 30 min at room temperature followed by centrifugation (20,000*g*, 5 min). This supernatant contains the A/G fraction. The pellet was washed twice with 1 mL of deionized water, centrifuged as described above and the supernatant was discarded. Afterwards, 1 mL of ethanol/water solution (60:40, v/v) was added to the obtained pellet, mixed for 30 min at 50 °C, and centrifuged as described. This supernatant contains the gliadin fraction. Subsequently, to extract the glutenin fraction, 1 mL of 0.1 M Tris-HCl (pH 7.5) was added, sonicated during 30 min at 60 °C and vigorously vortex every 5 min, followed by centrifugation as above.

The protein concentration of the extract was determined according to the method of Lowry et al. (1951) (Albumins/Globulins and Gliadins: DC™ Protein Assay; Glutenins: RC-DC™ Protein Assay, BIO-RAD Kit), using bovine serum albumin as the standard. The different protein fraction extracts were diluted with SDS sample buffer, and 10× sample reducing agent (Novex, Bolt™, CA, USA) and boiled for 3 min. Subsequently, the protein extract (12.5 µg protein/well) was separated by SDS-PAGE, using a gradient gel NuPage Bis-Tris 4–12% (for glutenins separation) or a Bis-Tris 10% gel (for albumins/globulins and gliadins separation) with MES (2-(N-morpholino) ethane sulfonic acid) running buffer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) (Laemmli, 1970). Running condition was 200 V for 35 min. The gel was stained by Commaasie Brilliant Blue R-250, and the image captured by Alpha Imager HP (Proteinsimple, Minneapolis, USA).Mark 12™ unstained protein standard (Thermo Fisher Scientific, MA, USA) served as molecular markers (myosin 200 kDa, β-galactosidase 116.3 KDa, phosphorylase b 97.4 kDa, bovine serum albumin 66.3 kDa, glutamic dehydrogenase 55.4 kDa, lactate dehydrogenase 36.5 kDa, carbonic anhydrase 31 kDa, trypsin inhibitor 31.5 KDa, lysozyme 14.4 kDa, aprotinin 6 kDa, insulin B chain 3.5 KDa and insulin A chain 2.5 KDa.

3.2.4. Phenolic compounds and antioxidant activity

Soluble free, insoluble ester bound, and total phenolic compounds (PC) were evaluated, according to the method earlier described by Svensson, Sekwati-Monang, Lutz, Schieber, and Gänzle (2010) with some modifications described by Wang et al. (2019). From the soluble phenolic compounds fraction, the antioxidant capacity was assessed.

(a) Extraction of soluble free and insoluble ester bound phenolic compounds

The soluble free PC fraction was extracted by mixing 50 mg of the lyophilized samples with 1.5 mL 80% (v/v) aqueous methanol, sonicated (5 min at 60 Hz at room temperature) in an ultrasonic bath (Branson, Merck), followed by 1 h shaking and centrifugation (20,000*g*, 15 min) (Sorval rotor SS-34). Three sequential extractions of the pellet were combined following the same condition. The supernatants obtained were dried under nitrogen in a Reacti-Therm heater/stirrer (Thermo Scientific, USA) at 25 °C and the solids were dissolved in 5 mL of methanol (99.9%) for 1 h prior to analysis. From the remaining pellets, an alkaline hydrolysis to extract the insoluble ester bound fraction was performed, by mixing with 0.9 mL of 2 M NaOH, sonicated for 30 min in water bath and 150 min of constant shaking, at room temperature. The pH was adjusted to pH 2 (6 M HCl), followed by the addition of 0.4 mL of ethyl acetate to the hydrolysates and centrifuged for 15 min (20,000*g*, 15 min). The procedure was performed twice, and the collected supernatants were treated as described for free soluble fraction.

(b) Determination of phenolic compounds

Free, ester bound, and total (soluble + esterified) PC content of the different extracts obtained were determined by Folin–Ciocalteu (FC, Merck) method (Singleton, Orthofer, & Lamuela-Raventós, 1999): 200 µl of extracts were mixed with 1 mL of FC reagent (1:10 with Milli-Q water) in a glass tube; 0.75 mL of aqueous Na_2CO_3 (7.5%, w/v) was added, vortexed and incubated for 1 h in dark conditions. The absorbance was measured at 765 nm (UV spectrophotometer-UV1800, Shimadzu, Japan) at 24 °C, against a methanol blank solution. The phenolic content was expressed as mg gallic acid equivalents (GAE)/100 g freeze-dried dough sample.

(c) Determination of antioxidant capacity

The antioxidant capacity was evaluated on methanol soluble PC extracts, based on free-radical scavenging effect against 1,1-diphenyl-2-picrylhydrazyl (DPPH), as described by Cuendet, Hostettmann, Potterat, and Dyatmiko (1997) and based on Fe^{3+} reducing power, following the method of Oyaizu (1986) with slight modifications described by Coda et al. (2014).

The methanolic DPPH fresh prepared (at 100 µM) was added (2.5

mL) to each extract sample (500 µl), vortexed and incubated without light (30 min at room temperature). The absorbance was measured against methanol blank solution at 517 nm, and the scavenging capacity was expressed as follows: DPPH scavenging activity (%) [(blank absorbance-sample absorbance)/blank absorbance]×100. The value of absorbance was compared with 75 ppm butylated hydroxytoluene (BHT), used as the antioxidant reference.

The ferric-reducing power consists of the reduction of Fe^{3+} to Fe^{2+} by electron transfer rather than hydrogen donor. The Fe^{3+} reducing power was determined by mixing the extract sample with 0.75 mL of 0.2 M potassium phosphate buffer (pH 6.6) and 0.75 mL of 1% (w/v) of potassium hexacyanoferrate, followed by water bath incubation at 50 °C for 20 min; the reaction was stopped by adding trichloroacetic acid (0.75 mL,10% w/v) followed by centrifugation (20,000 g, 10 min). The supernatant obtained (1.5 mL) was mixed with distilled water (1.5 mL) and ferric chloride solution (0.1 mL at 0.1%, w/v) for 10 min. The reducing power was measured at 700 nm, and the higher absorbance of the reaction mixture indicated greater reducing power. The value of absorbance was compared with 75 ppm ascorbic acid (AC), used as the antioxidant reference (Graça, Raymundo et al., 2020; Graça, Mota et al., 2020). Triplicates of chemical measurements from three biological replicates, were performed.

4. Bread quality characterization

4.1. Technological properties evaluation

The influence of yeast-bread and sourdough-yeast fermentation on wheat bread technological properties was assessed, by texture profile analysis (TPA) and post-baking quality attributes. The bread firmness (25 mm of crumb bread cubes, from the bread center part) was evaluated by a Texture analyzer (TA, TA-XT2i, Stable Micro Systems Ltd., UK), on first and fourth days of storage, using a 5 kg load cell and 36 mm diameter probe, according to the TPA method as previous described (Wang et al., 2019).

Bread post-baking quality attributes: staling kinetic (N/day), considering the increase of firmness during storage time (staling rate = [firmness (day 4 — day 1)/days of storage]), the baking loss (% bake loss = (dough weight — bread weight) × 100/dough weight), the specific bread volume (mL/g) and density (kg/cm^3) obtained with a laser-based scanner equipment (Volscan Profiler 300, Stable Micro Systems, UK), were assessed. The moisture content of bread crumb during the storage days was measured by following the two-stage moisture air oven procedure described on AACC method 44-15.02 (AACC International, 2000), and the bread moisture loss was then calculated (moisture loss (%) = [moisture content (day 1 — day 4)/days of storage]). Each type of bread was prepared in three individual replicates, and each replicate was measured in triplicates.

4.2. Nutritional analysis

4.2.1. Total starch, in vitro starch digestibility and predicted glycemic index

The total starch of the yeast-bread and sourdough-yeast bread crumb samples was determined by enzymatic method described by Goñi, Garcia-Alonso, and Saura-Calixto (1997), with modifications as follows: ground crumb bread (100 mg from the crumb part) was washed with aqueous ethanol (80% v/v) followed by the addition of 2 mL of 2 M KOH, under shaking (at 4 °C for 30 min). Subsequently, 3 mL of 0.1 M sodium acetate buffer (pH 6.9) was added and incubated under controlled shaking water-bath (at 37 °C for 45 min) with 1 mL of pancreatic α-amylase (5 U/mL in distilled water). Then, 3 mL of 0.1 M sodium acetate buffer (pH 4.75) and 60 µl of amyloglucosidase (3300 U/mL in distilled water) were added and incubated under controlled shaking water-bath (at 60 °C for 45 min). After centrifugation (10,000 rpm/15 min), the reducing sugar content by DNS color reagent was spectrophotometric (540 nm) determined, using maltose as standard.

The *in vitro* starch digestibility was determined according to the method described by Germaine et al. (2008): bread portion corre- sponding to 1 g of starch (1 g/[(1-moisture content (%)) starch con- tent (%)]), were chewed (15 times in 15 s) and the mouth was rinsed (5 mL of deionized water for 30 s); then, 94 mL of sodium phosphate buffer (0.05 M, pH 6.9) was added to the suspension followed by constant shaking (120 rpm) and incubation (at 37 °C) with pancreatic α-amylase (110 U/mL in water). The pH was adjusted with 1 M of HCl or NaOH. The starch digestibility was measured as sugars release during the hydrolysis time, by taken aliquots of the digested solution (2 mL) at times 0, 30, 60, 90, 120 and 180 min. After centrifugation (20,000 g, 10 min) the supernatant was mixed with DNS color reagent to determine the reducing sugars as described for total starch.

The area under the curve (AUC) was calculated for each of the starch hydrolysis curves obtained, and the hydrolysis indexes (HI) was calculated (HI = (AUC of bread sample/AUC of wheat control bread) × 100), considering the wheat bread as a food reference (AUC was given a HI = 100).

The predicted glycemic index (pGI) was calculated according to Eq. (1), proposed earlier by Goñi et al. (1997):

$$eGI = (0.549 \times HI) + 39.71 \quad (1)$$

4.2.2. Total protein, in vitro protein digestibility and free amino acid profile

Total bread protein content was analyzed with Dumas's combustion method using a LECO CN828 element analyzer (Elementar Analysensystem GmbH, Germany). The conversion factor to convert nitrogen to protein was 5.7. *In vitro* protein digestibility (IVPD) was analyzed according to the protocol described by Akenson and Stahmann (1964), by subjecting the bread sample (3 g) to a two-step proteolysis: (i) pepsin hydrolysis with 15 mL of 0.1 M HCl at pH 2.0, and 1.5 mg pepsin (pepsin from porcine pancreas, P7012-25G, Sigma Aldrich), incubated for 3 h at 37 °C; 2 M NaOH to adjust the pH to 8.0 was used; (ii) Pancreatin digestion with 4 mg of pancreatin (from porcine pancreas, P7545-100G, Sigma-Aldrich) dissolved with 7.5 mL sodium phosphate buffer (pH 8.0), incubated for 24 h at 37 °C with constant shaking. The enzymatic reaction was stopped by the addition of trichloroacetic acid (10 mL at 20% w/v). The undigested protein was precipitated and centrifuged (13,000 g, 20 min).

The pellet which contained the undigested protein fraction, was kept in oven overnight (70 °C) and the protein content was analyzed by Dumas's combustion method. The IVPD (%) was expressed as the percentage of total protein, which was solubilized after *in vitro* enzymatic digestion. The supernatant, which contains the digested protein fraction, was used to evaluate the free amino acids (FAAs) profile released during digestion 2 mL of supernatant was taken, mixed with 250 µl of 6 M sulfosalicylic acid to precipitate the remain soluble proteins, sonicated (5 min) and centrifuged (19,000*g*/10 min. The pH of supernatant was adjusted to between 7 and 10 with NaOH.

FAAs were determined using pre-column derivatization method (Waters AccQ-Tag™ Ultra, Waters Corporation, Millford, MA). An aliquot (10 µl) of sample was mixed with AccQ-Tag™ Ultra borate buffer (60 µl) and internal standard (D-Norvaline; Sigma-Aldrich, Germany). 20 µl of reconstituted AccQ-Tag™ Ultra derivatization reagent was added, and sample was incubated at 55 C for 10 min.

The chromatographic determination of FAAs was performed on an Acquity UPLC system (Waters Corporation, Milford, MA, USA) according to the manufacturer's instructions for amino acid analysis (Waters, 2007, Revision B). The UPLC equipped with photodiode array (PDA) detector, and an ACCQ-TAG™ ULTRA C18 column (100 mm × 2.1 mm, 1.7 µm, Waters) at a flow rate of 0.7 mL/min within 10 min of run time, at 55 °C of column temperature, was used. The injection volume was 1 µl and the detection wavelength was set at 260 nm.

The FAAs were separated using a gradient mobile phase consisting in

5% AccQ-Tag™ Ultra Eluent A (A) and AccQ-Ta™g Ultra Eluent B (B), according to the following gradient conditions: 0–0.54 min, 99.9% A–0.1% B; 5.74 min, 90.9% A–9.1% B; 7.74 min, 78.8% A–21.2% B; 8.04 min, 40.4% A–59.6% B; 8.50–8.64 min, 10% A-90% B; 8.70–10 min, 99.9% A–0.1% B.

Amino acids were identified by their retention times. Quantification of amino acids was based on the internal standard method where norvaline was used as an internal standard. An amino acid standard hydrolysate provided by Waters, containing 2.5 mM of each amino acid (except cysteine 1.25 μM) was diluted to appropriate concentrations. Six-point linear calibration curves for each amino acid were prepared by calculating ratio of the peak area to the area of the internal standard. The results were expressed in mg per Kg of fresh digested bread (Motta et al., 2019).

In vitro starch and protein digestibility triplicate measurements, from each of the three breads obtained in two irrespective biological fermentation assays, were carried out.

5. Statistical analysis

The data obtained for dough microbiological and chemical characterization, bread technological and nutritional analyses are reported as an average of parallel and irrespective measurements (each performed in triplicate). The statistical differences were assessed with one-way analysis of variance (ANOVA) by Tukey's significant test differences at $p < 0.05$ using statistical software Statistica for Windows (Statistica 8.0, Windows).

6. Results

6.1. Microbiological analysis and pH determination

The cell density of presumptive *L. bulgaricus and S. thermophilus*, deriving from yoghurt addition, and endogenous flour *LAB spp.* before (0 h) and after (24 h) sourdough fermentation was determined in wheat and wheat-whole grain sourdough formulations. After sourdough fermentation with endosperm wheat flour, the thermophilic LAB showed an increase of roughly 2 log cycles. The LAB cell density on MRS ranged from 6.7 ± 0.8 (0 h) to 8.8 ±1.1 (24 h) log cfu g^{-1}, and on M17 the LAB increased from 6.9± 0.8 (0 h) to 9.0 ±1.0 (24 h) log cfu g^{-1}. The wheat-whole sourdough showed higher increase of thermophilic LAB cell density, roughly 3 log cycles. The LAB cell density ranged from 6.7 ±1.1 (0 h) to and 9.5 ±1.0 (24 h) log cfu g^{-1} on MRS and 6.9 ± 0.8 (0 h) to 9.6 ±1.0 (24 h) on M17.

The endogenous *LAB* spp. in both wheat and wheat-whole sourdough formulations ranged roughly from 6.0 ± 0.9 log cfu g^{-1} to 8.1 ± 1.0 log cfu g^{-1}, an increase of around 2 log cycles.

6.2. Chemical analysis

6.2.1. Sugars and organic acids

The utilization of sugars, glucose, lactose and galactose, and the organic acid production during yeast -dough and sourdough fermentation, was confirmed by HPLC analysis (Table 1). The higher sugar's utilization during sourdough fermentation, in both wheat and wheat-whole grain dough formulations (WSD, WWSD, 24 h) was attained, and further utilized during the yeast-fermented last stage (WSYD, WWSYD, 24 h + 1 h): glucose was practically depleted (0.30 ± 0.07 and 0.20 ± 0.06 mg/g) and small amounts of lactose (0.60 ± 0.08 and 0.70 ± 0.08 mg/g), and galactose (1.90±0.06 mg/g) were detected. Dough pH underwent a dynamic decrease, varying from 5.31±0.05 to 3.82 ± 0.06 for wheat dough and 5.10 ± 0.03 to 3.70 ± 0.01 for wheat-whole grain dough, being the pH-drop faster on the later, during the first 6 h of fermentation (data not shown). Sugar's consumption by LAB with a consequent acid production promoted a notable ($p < 0.05$) dough acidification and pH drop, in which the lactic acid was the dominant organic. For both yeast-fermented dough (WD, WWD, 1 h) and particulary, for sourdough-yeast fermentation (WSYD, WWSYD, 24 h + 1 h), the lactic acid content increased around 4–5-fold higher from 5.80 ± 0.21 and 5.61 ± 0.20 to 21.40 ± 1.50 and 26.52 ± 0.30 g/mg of lyophilized dough, respectively. Chemically acidified doughs results, for both free sugars and organic acids, were comparable with unfermented control dough, with no significant ($p > 0.05$) differences.

Table 1
Sugar's content: lactose, glucose, galactose (mg/g of freeze-dried dough) and organic acids amount: lactic and acetic acid (mg/g of lyophilized dough) obtained for yoghurt wheat (W) and wheat-whole grain (WW) dough formulations in study: baker's yeast (WD, WWD), sourdough (WSD, WWSD), sourdough-yeast fermented (WSYD, WWSYD), and for the chemically acidified doughs (WCD, WWCD), at different fermentation times: 0 h, 1 h, 24 h, 24 h + 1 h.

Dough samples	Glucose	Lactose	Galactose	Lactic acid	Acetic acid	Dough pH
WD 0 h	20.0 ± 0.4^{a}	6.8 ± 0.1^{a}	10.2 ± 0.3^{a}	4.3 ± 0.2^{a}	ND	5.3 ± 0.1^{a}
WD 1 h	4.0 ± 0.2^{b}	3.4 ± 0.3^{b}	4.0 ± 0.2^{b}	5.8 ± 0.2^{b}	2.9 ± 0.1^{a}	5.1 ± 0.0^{a}
WS 24 h	0.5 ± 0.0^{c}	0.9 ± 0.1^{c}	2.4 ± 0.2^{c}	21.8 ± 1.6^{c}	1.5 ± 0.1^{b}	3.8 ± 016^{b}
WSYD 24 h + 1 h	0.3 ± 0.1^{c}	0.6 ± 0.1^{c}	1.9 ± 0.1^{c}	21.4 ± 1.5^{c}	2.8 ± 0.1^{a}	4.3 ± 0.1^{b}
WCD 24 h	19.5 ± 0.2^{a}	6.8 ± 0.2^{a}	9.2 ± 0.1^{a}	4.5 ± 0.1^{a}	ND	4.0 ± 0.1^{b}
WWD 0 h	20.0 ± 0.30^{a}	6.5 ± 0.1^{a}	10.0 ± 0.1^{a}	4.1 ± 0.1^{a}	ND	5.1 ± 0.0^{a}
WWD 1 h	4.1 ± 0.2^{b}	2.1 ± 0.1^{b}	4.2 ± 0.1^{b}	5.6 ± 0.2^{b}	2.9 ± 0.3^{a} 0.0	5.0 ± ^{a}
WWS 24 h	0.4 ± 0.1^{c}	1.0 ± 0.2^{c}	2.3 ± 0.4^{c}	27.7 ± 1.0d	2.0 ± 0.2^{b}	3.7 ± 0.0^{b}
WWSYD 24 h + 1 h	0.2 ± 0.1^{c}	0.7 ± 0.1^{c}	2.0 ± 0.1^{c}	27.5 ± 0.3d	3.0 ± 0.1^{a}	4.1 ± 0.0^{b}
WWCD 24 h	19.1 ± 0.5^{a}	6.8 ± 0.3^{a}	9.5 ± 0.2^{a}	4.5 ± 0.2^{a}	ND	4.0 ± 0.1^{b}

Data values with different subscript letters (a–c) in the same column differ significantly (Tukey test at $p < 0.05$).
ND – Not detected.

6.2.2. Peptides and free amino nitrogen content

To follow the degree of proteolysis during yeast-bread and sourdough fermentation, the peptides content and free amino nitrogen (FAN) concentration was determined, in water-soluble extracts (Table 2). Comparing with the yeast-bread (1 h) and sourdough-yeast fermented (24 h + 1 h) doughs, both wheat (WD 1 h, WSYD 24 h + 1 h) and wheat-whole grain (WWD 1 h, WWSYD 24 h + 1 h) formulation, were characterized by a peptide content ranging from 1.2 ± 0.1 to 6.2 ± 0.1 g/100 g and 1.5 ± 0.2 to 10.0 ± 0.3 g/100 an increase of around 5- and 8-fold, respectively. The FAN concentration can be clearly distinguished, from 92.0 ± 1.2 to 319.0 ± 13.3 g/100 g, and 98.7 ± 7.7 to 671.5 ± 17.5 g/100 g, an increase of around 3- and 6-fold higher, correspondingly. Moreover, the peptide and FAN content showed a significant ($p < 0.05$) increase in chemically acidified doughs, but considerably ($p < 0.05$) lower when compared with sourdough (24 h). No significant ($p > 0.05$) differences can be noticed between unfermented control (0 h) with yeast-fermented doughs (1 h).

6.2.3. Change of the wheat protein pattern after yeast-dough and sourdough fermentation

The variation of wheat polypeptide pattern, before (0 h), after yeast-dough (1 h), sourdough (24 h) and sourdough-yeast (24 h + 1 h) fermentation, was characterized by electrophoresis analysis (Fig. 1). A chemically acidified and incubated dough, at the same fermentation conditions, were performed. In general, for both dough matrix in study, the sourdough 3 at pH 3.8 (A1-C1) and 3.7 (A2-C2) showed the more intensive hydrolysis, being higher for wheat-whole grain sourdough

Table 2
Peptides content and free amino nitrogen, free soluble, insoluble ester bound and total phenolic compounds (PC), DPPH radical scavenging activity (%) and ferric-reducing power (ABS values) results obtained for yoghurt wheat (W) and wheat-whole grain (WW) dough formulations in study: yeast bread dough (WD, WWD), sourdough (WSD, WWSD), sourdough-yeast fermented (WSYD, WWSYD), and for the chemically acidified doughs (WCD, WWCD), at different fermentation times: 0 h, 1 h, 24 h, 24 h + 1 h.

Chemical parameters	Peptide content	Free Amino Nitrogen	Free Soluble PC	Insoluble ester Bound PC	Total PC	DPPH	Reducing power
Samples	(mg/g)	(mg/g)	(mg EAG/100 g)			(% RSC)*	(mg EAC/100 g)
WD 0 h	1.0 ± 0.0^{a}	87.3 ± 2.0^{a}	83.6 ± 0.5^{a}	95.5 ± 0.8^{a}	174.3 ± 0.8^{a}	39.8 ± 0.7^{a}	30.7 ± 0.2^{a}
WD 1 h	1.2 ± 0.1^{a}	920 ± 1.21^{a}	89.8 ± 0.5^{a}	95.0 ± 0.6^{a}	179.1 ± 1.0^{a}	40.6 ± 0.7^{a}	32.3 ± 0.3^{a}
WSD 24 h	5.6 ± 0.1^{b}	278.9 ± 12.1^{b}	121.7 ± 0.7^{b}	66.0 ± 1.2^{b}	189.3 ± 1.9^{a}	52.1 ± 0.4^{b}	60.2 ± 0.8^{b}
WSYD 24 h + 1 h	6.2 ± 0.0^{b}	319.0 ± 13.3^{b}	130.7 ± 1.7^{b}	58.0 ± 2.2^{b}	188.7 ± 1.5^{a}	54.1 ± 1.1^{b}	61.5 ± 0.2^{b}
WCD 24 h	40 ± 0.1^{c}	187.5 ± 16.3^{c}	101.3 ± 0.9^{ab}	80.50 ± 1.1^{c}	183.4 ± 2.7^{a}	44.4 ± 1.2^{a}	32.3 ± 0.4^{a}
WWD 0 h	1.2 ± 0.0^{a}	91.7 ± 7.0^{a}	111.7 ± 0.9^{b}	121.8 ± 1.8d	227.1 ± 1.6^{b}	45.7 ± 0.7^{a}	51.3 ± 1.6^{c}
WWD 1 h	1.5 ± 0.0^{a}	98.7 ± 7.7^{a}	122.4 ± 2.1^{b}	121.4 ± 1.7d	232.2 ± 1.3^{b}	46.5 ± 0.9^{a}	54.6 ± 0.7^{c}
WWSD 24 h	9.1 ± 0.1^{d}	606.7 ± 13.2^{d}	176.9 ± 1.0^{c}	69.6 ± 2.5^{b}	240.4 ± 1.5^{b}	62.8 ± 0.8^{c}	87.2 ± 1.2^{d}
WWSYD 24 h + 1 h	10.0 ± 0.3^{d}	671.5 ± 17.5^{d}	186.8 ± 1.5^{c}	58.9 ± 0.9b	247.9 ± 1.9^{b}	64.8 ± 1.4^{c}	90.7 ± 0.6^{d}
WWCD 24 h	7.2 ± 0.3^{b}	332.9 ± 7.3^{b}	118.7 ± 2.3^{b}	108.3 ± 1.5^{d}	232.92 ± 8.5^{b}	48.6 ± 0.7^{a}	53.0 ± 1.0^{c}
AR's: BHT/AC**						54.5 ± 0.7^{b}	105.5 ± 1.2^{e}

Data values in the same column with different superscript letters (a–e) differ significantly (Tukey test at $p < 0.05$).
* Percentage of DPPH radical scavenging capacity.
*** AR's: BHT/AC: Antioxidant references: butylated hydroxytoluene/ascorbic acid.

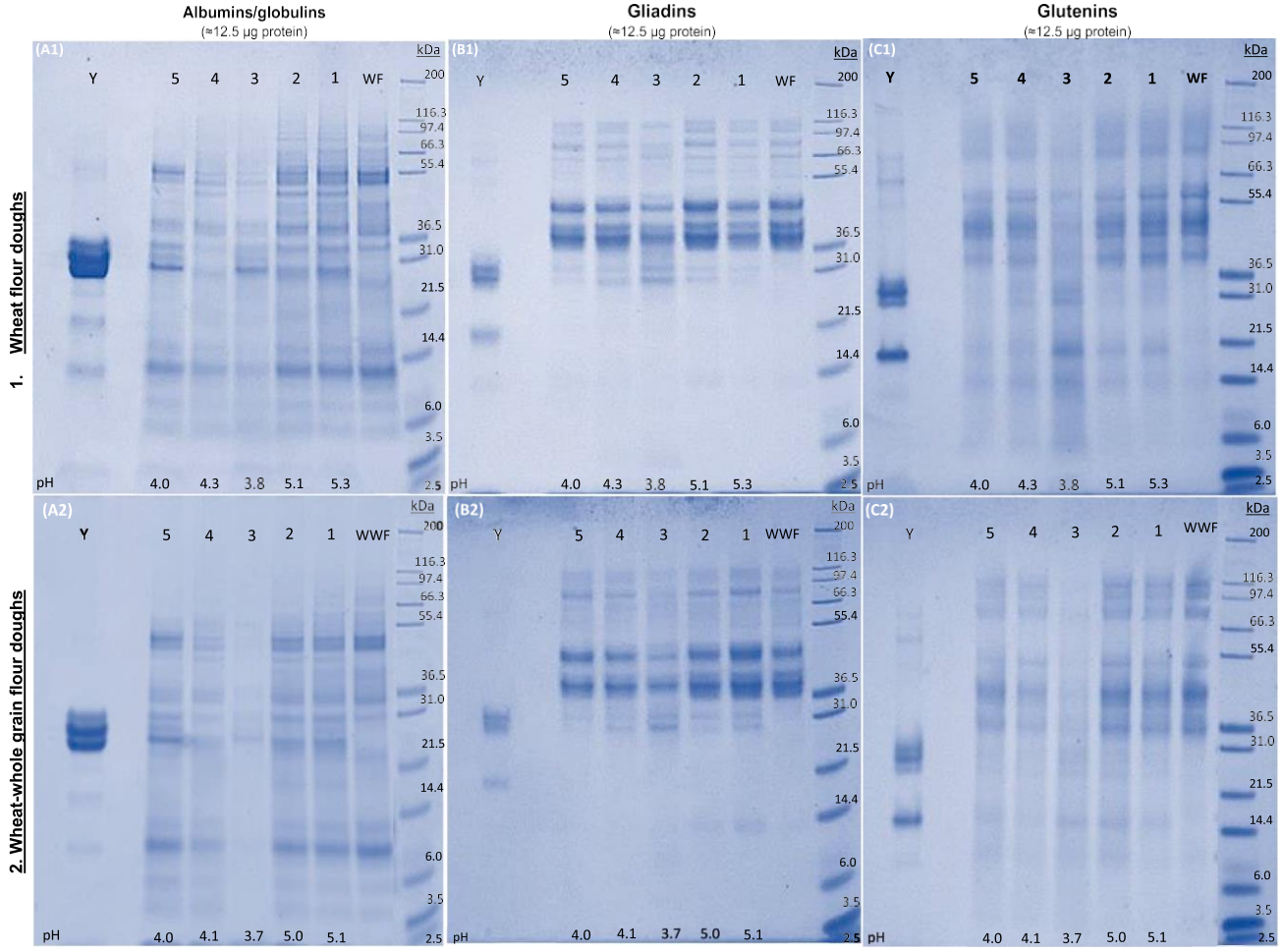

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) showing the changes of (A) albumins/globulins, (B) gliadins and (C) glutenins protein extracts of baker's yeast and sourdough-yeast fermented doughs, made of (gels A1-C1) wheat flour (100%, f.w.) and (gels A2-C2) a blending of wheat and whole grain flour (50:50, f.w.); The same amount of protein (≈12.5 µg/well) was pipetted. in each well Gels legend: WF-wheat flour, WWF-wheat-whole grain flour, doughs: 1-baker's yeast control dough before fermentation (0 h), 2-baker's yeast fermented dough at 35 °C for 1 h, 3-sourdough at 40 °C for 24 h, 4-sourdough-yeast fermented dough at 40 °C for 24 h + 35 °C for 1 h, 5-chemically acidified dough at pH 4.0 fermented at 40 °C for 24 h, and Y-yoghurt protein extract. The pH values obtained for each dough at different fermentation time are indicated at below of the gels.

(A2-C2). Considering albumins/globulins (A2) an almost complete degradation after 24 h of yoghurt-sourdough fermentation can be observed. Yoghurt-proteins bands (31.0–14.4 kDa) remained intact after wheat sourdough (A1) fermentation, while after wheat-whole sourdough (A2) a less intensity can be notice. As for gliadins fraction (B2), even though a mild hydrolysis occurred during fermentation, new hydrolysates protein bands can be observed between 36.5 and 31.0 kDa. Based on glutenins fractions (C2), high molecular protein bands between 116.3 and 97.4 kDa were less observed, but new protein bands from hydrolysates can be observed between 31.0 and 3.5 KDa. These results agree with those obtained in FAN and small-sized peptides content, in which the higher amounts were obtained for wheat-whole sourdough matrix.

After yeast-fermentation (dough 2) and chemically acidified doughs (dough 5), the wheat protein pattern remained present, showing the same band gel profile as unfermented dough (dough 1, at 0 h) and native flours (WF-wheat flour and WWF-wheat-whole grain flour). Sourdough-yeast fermented before baking (doughs 4) showed a less protein bands intensity comparing with yeast-bread fermented (doughs 2), indicating that the proteases presumably activated during sourdough fermentation remained active at pH 4.1, changing the wheat protein profile of the added wheat flour during the yeast fermentation step. Only minor differences between chemically acidified (doughs 5) and yeast-bread fermented doughs (doughs 2), can be observed, whereas major variations can be noticed comparing with sourdough fermentation (doughs 3).

6.2.4. Phenolic compounds and antioxidant activity

After yeast-dough fermentation (1 h), for both wheat (WD) and wheat-whole grain (WWD) doughs, no significant ($p > 0.05$) variations can be distinguished among the different PC fractions PC, comparing with control unfermented dough (WD 0 h) (Table 2). However, after yoghurt-sourdough fermentation a significant ($p < 0.05$) decrease of bound compounds content was registered, and further reduced during yeast leavening step (24 h + 1 h), of 39.0% for WSYD and 51.3% for WWSYD, with a markedly increase of soluble PC fractions of 45.5% and 52.6% respectively, comparing with yeast-dough fermented (1 h). The total phenolic content increased slightly, but not significantly ($p > 0.05$).

Likewise, considerable PC variations can be noticed between chemically acidified and sourdough (24 h) fermented ones, being higher in the latter.

Increments on soluble PC fractions was accompanied by a significant ($p < 0.05$) enhancement on scavenging activity towards DPPH free radical and ferric-reducing power (Table 2). Sourdough-containing samples exhibited a marked increase of the radical-scavenging activity compared to yeast-dough fermented and that of the chemically acidified doughs: the highest DPPH inhibition effect for sourdough-yeast fermented (24 h + 1 h), was registered, reaching values comparable to BHT activity (54.5 ± 0.7%) for WSYD (54.1 ± 1.1%) and higher values (64.8 ± 1.4) for WWSYD, exhibiting higher capacity to quench DPPH radical.

Although the ferric-reducing power activity of sourdough samples were lower comparing with ascorbic acid reference (Table 2), it was significantly ($p < 0.05$) improved after sourdough-yeast fermentation (24 h + 1 h), of around 84.6% for WSYD and 87.6% for WWSYD, while for yeast-bread (1 h) and chemically acidified fermented doughs no significant ($p > 0.05$) differences were registered. The reducing power activities of soluble PC extracted from sourdough samples was notably higher, comparing to chemically acidified doughs.

6.3. Bread quality characterization

6.3.1. Technological properties: post-baking quality parameters

Experimental breads replacing wheat flour by yoghurt-sourdough addition (50% fw, SI1), were prepared, and the impact on technological properties based on post-baking quality parameters for both wheat (WSB) and wheat-whole grain bread (WWSB), was assessed. Control baker's yeast fermented breads, were used (WB, WWB). Results obtained are summarized in Table 3.

Table 3
Comparison of the technological and nutritional properties obtained for baker's yeast and sourdough-yeast fermented breads, prepared from (100%, fw) wheat flour (WB, WSB) and a mixture of (50:50%, fw) of wheat-whole grain flour (WWB, WWSB).

		Post-baking parameters			
Bread quality parameters	**CWB***	**WB**	**WSB**	**WWB**	**WWSB**
Baking loss (%)		13.4 ± 1.1^{a}	12.3 ± 0.7^{a}	11.9 ± 0.4^{a}	10.8 ± 0.6^{a}
Density (Kg/cm^3)		342.2 ± 3.3^{a}	373.2 ± 5.5^{ab}	331.8 ± 7.7^{a}	398.0 ± 8.4^{b}
Specific volume (mL/g)		3.7 ± 0.1^{a}	2.7 ± 0.3^{b}	3.8 ± 0.1^{a}	2.5 ± 0.1^{b}
Firmness (N/day 1)		1.6 ± 0.3^{a}	2.5 ± 0.3^{b}	2.8 ± 0.2^{b}	3.4 ± 0.2^{c}
Firmness (N/day 4)		6.5 ± 0.3^{a}	5.5 ± 0.4^{b}	6.1 ± 0.3^{a}	5.4 ± 0.3^{b}
Staling rate (N/day)		1.2 ± 0.1 ^{a}	0.7 ± 0.1^{b}	0.8 ± 0.1^{b}	0.5 ± 0.1^{b}
Moisture content (%/day1)		40.0 ± 0.04^{a}	40.0 ± 0.0^{a}	41.0 ± 0.1 ^{a}	41.0 ± 0.3 ^{a}
Moisture content (%/day4)		36.6 ± 0.2 ^{a}	37.7 ± 0.3^{b}	38.6 ± 0.2 ^{b}	39.5 ± 0.1^{c}
Moisture loss (%/day)		0.7 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.4 ± 0.1^{b}
pH		5.20 ± 0.2	4.30 ± 0.1	5.10 ± 0.3	4.10 ± 0.3
		Nutritional parameters			
Total starch (% DW)	66.4 ± 1.0^{a}	65.7 ± 0.7^{a}	65.2 ± 1.3^{a}	56.3 ± 0.8^{b}	55.4 ± 0.8^{b}
HI (%)	100.0 ± 0.0^{a}	81.8 ± 0.4^{b}	68.5 ± 1.3^{c}	57.1 ± 0.7^{d}	45.6 ± 1.0^{e}
pGI (%)	94.5 ± 0.00^{a}	84.5 ± 0.2^{b}	77.2 ± 0.8^{c}	71.0 ± 0.4 ^{c}	64.7 ± 0.5 ^{d}
Total protein (% DW)		12.6 ± 0.1 ^{a}	12.8 ± 0.2 ^{a}	12.6 ± 0.4^{a}	12.9 ± 0.1^{a}
IVPD (%)		78.9 ± 0.8^{a}	84.0 ± 0.6^{ab}	78.9 ± 0.19^{a}	88.2 ± 0.3^{b}
TFFAs (mg/Kg)		935.5 ± 44.3^{a}	1403.0 ± 59.3^{b}	944.4 ± 47.0^{a}	1921.0 ± 75.0^{c}

Data values with different subscript letters (a-e; A-B) in the same row differ significantly (Tukey test at $p < 0.05$).
CWB*-control wheat bread (food reference); HI: Hydrolysis index (%); pGI: predicted glycemic index (%); IVPD: *In vitro* protein digestibility (%); TFFAs: Total free amino acids (mg/kg of digested bread).

In general, yoghurt-sourdough addition as ingredient on WSB and WWSB formulations, resulted in denser bread with heterogenous gas-cells distribution (SI3), accompanied by a significant ($p < 0.05$) reduction of 8.0% and 16.3% on loaf-specific volume, as well as harder crumb firmness, respectively, comparing with WB and WWB. However, after storage, sourdough-containing bread presented a softer crumb texture, by 15% (WSB) and 12% (WWSB) of less firm than control's yeast-crumb breads. Accordingly, sourdough bread staled significantly ($p < 0.05$) slower than baker's yeast-ones, representing a reduction of 40% (WSB) and % (WWSB). In line with those results, the sourdough bread showed the least moisture loss, of around 22% and 36% related to yeast-fermented bread, respectively.

6.3.2. Nutritional properties

The assessment of bread's nutritional properties was mostly focused on mimicking the *in vitro* starch and protein digestibility, further characterized based on the predicted glycemic index and free amino acids profile, respectively (Table 3). Starting from the *in vitro* starch digestibility results, control wheat bread (CWB) as a food reference was used, corresponding to hydrolysis index (HI) of 100%.

Sourdough fermentation induced a significant reduction on starch digestibility: comparing baker's yeast (WB, WWB) and sourdough-yeast

fermented breads (WSB, WWSB) with control wheat bread (CWB), a significant ($p < 0.05$) lower starch hydrolysis over the digestion time (0–180 min, SI4) was obtained, ranging from 81.8 0.4% ± to 68.5 ± 1.3%, and 57.1 ±0.7% to 45.6 ± 0.7%, respectively, related to CWB (100%). Notably, yoghurt-sourdough bread underwent a considerably lower starch hydrolysis of 32% for WSB and of 54% for WWSB, compared with CWB. Correspondingly, the predicted glycemic index (pGI) of sourdough breads was reduced, particularly for WWSB, resulting in intermediate glycemic index (GI: 64.7) a significant reduction ($p < 0.05$) of 32%, comparing with CWB (GI: 94.5) (D'Alessandro & De Pergola, 2014).

As for *in vitro* protein digestibility (IVPD), sourdough fermentation led to a slight ($p < 0.05$) improving on protein digestibility, in the range of 84.0 ± 0.6% for WSB and 88.2 ± 0.3% for WWSB, comparing with baker's yeast breads (WB, WWB: 78.9%), an increase of 6.4 and 11.4%, respectively.

The digestible protein fraction obtained from IVPD analysis was further characterized focused on the free amino acids (FAAs) profile and total free amino acids (TFFAs, sum of FAAs), comparing the yeast-bread and sourdough-yeast fermentation processes impact.

Fig. 2 showed that the sourdough fermentation in both wheat bread formulations led to a significant ($p < 0.05$) increase of all FAAs concentration, particulary for WWSB, with particular emphasis on doubling the amounts of all essential amino acids: His, The, Lys, Met, Val, Iso, Leu, Phe, and Trp. Moreover, it can be also noticing the increase of Pro, Glu and Cys concentration of around 3–2.5–5-fold, respectively.

Accordingly, digested sourdough's breads were characterized by a significant ($p < 0.05$) increase of TFAAs concentration varying from 1403 ± 60 mg/kg for WSB and 1921 ± 75 mg/kg for WWSB, related to 935.5 ±44.3 and 944.4 ±47.0 mg/kg for yeast's bread formulations, a notable increase of around 50% and 100%, respectively.

7. Discussion

In this study, we evaluated the utilization of yoghurt as a starter on sourdough fermentation focused on technological and nutritional properties of wheat-sourdough bread, considering two different bread dough matrices: refined-wheat and blended with whole grain flour. To the best of our knowledge, the utilization of yogurt was only used as a bakery ingredient in the yeast-bread dough system (Graça et al., 2019; Graça, Raymundo et al., 2020; Graça, Mota et al., 2020). Therefore, this study reports for the first time the effect of yoghurt as a starter in bio-processing and its concurrent effect on overall sourdough-bread quality.

The presumptive yoghurt-derived LAB strains showed an efficient cell density growth in both dough matrix in study, particularly when the whole grain flour was added. The differences can probably be traced on flour composition, since whole grain flour typically harbors more microbes and may have a higher buffering capacity due to its higher mineral content comparing to refined-wheat flour (Wang et al., 2019) which provided better conditions for microbial growth. Consequently, the faster microbial) acidification promoted a faster pH-dynamic reduction over the fermentation time, generating ideal conditions to the activation of several endogenous flour proteases, resulting in more intense proteolysis on this dough system, notably to albumins/globulins and glutenins fractions degradation. Whole grain flour contains endogenous proteases derived from germen and aleurone layer (Fardet, 2010) which can be a possible explanation to support the higher wheat protein pattern changes observed (Fig. 1, A2-C2). In wheat flour, proteolysis has been associated with serine carboxypeptidases and mainly with aspartic proteinases (Huang et al., 2020; Jiang et al., 2021) mostly located in the endosperm layer. Additionally, wheat bran aspartic proteinases (Jiang et al., 2021; Yilmaz-Turan et al., 2020; Ganzle & Gobbetti, 2013), serine carboxylases (Galleschi & Felicioli, 1994; Breddam, 1988) and carboxypeptidase (Mikola, 1983) might have had contributed to the proteolytic events occurred (Sapone et al., 2012).

The variations on wheat protein profile can probably be linked with the increase of peptides concentration, as hydrolysates products of the breakdown of the high-molecular-weight protein into low-molecular-weight peptides, possibly attributed to proteolytic events occurring during yoghurt-sourdough fermentation. Subsequently, the free amino nitrogen concentration also increased, mostly due to enhanced peptides degradation to amino acids, as consequence of microbial activity to meet their demand of complex nitrogen (Ganzle et al., 2008; Ganzle & Gobbetti, 2013). Our findings are aligned with those reported by Zhao, Guo, and Zhu (2017) showing that the sourdough LAB fermentation was relevant to increase the peptides and amino acids concentration.

Phenolic compounds and antioxidant capacity results were notable improved in both dough systems, but, then again, particularly higher when whole-grain flour was included.

Microbial acidification and subsequent proteolysis during yoghurt-sourdough fermentation had a markedly impact to release the bound phenolic compounds, and consequently to increase the soluble fraction content. The *in vitro* antioxidant capacities, DPPH scavenging activity and ferric reducing power of both dough matrices, can probably be correlated to the soluble compound's increments, which agrees with previous reports (Jiang et al., 2021; Shumoy, Gabaza, Vandevelde, &

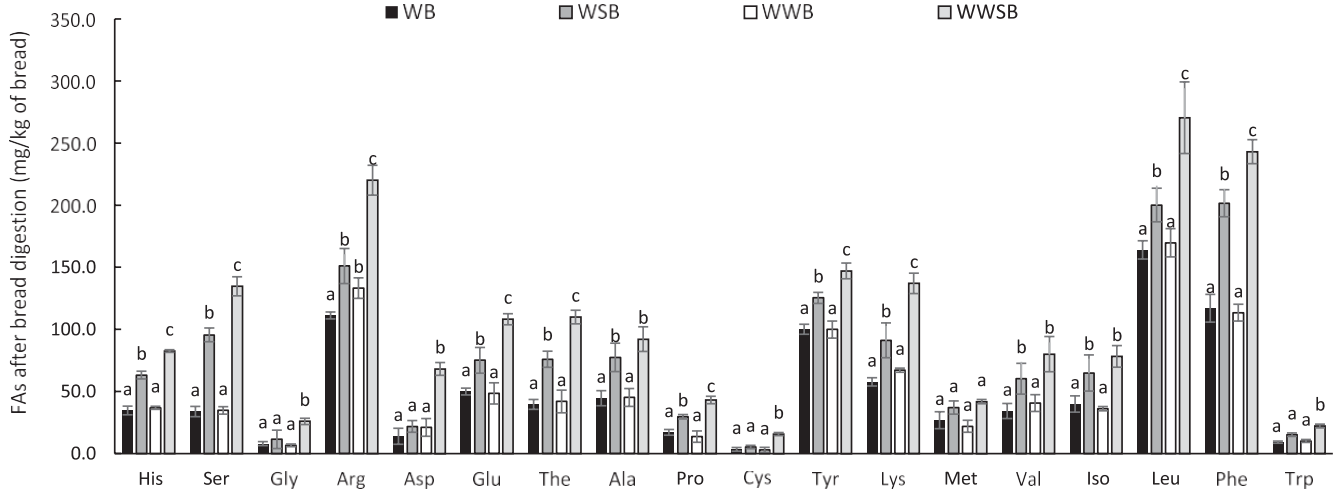

Fig. 2. Free amino acids (FAs) concentration of digested protein fraction obtained after IVPD analysis of the baker's yeast and sourdough-yeast fermented breads, for wheat (WB, WSB) and a blending of wheat-whole grain (WWB, WWSB) bread formulations. Data values with different subscript letters (a-c) in the same amino acid (e.g., His) differ significantly (ANOVA Tukey test at $p < 0.05$).

Raes, 2017; Laatikainen et al., 2017).

Findings obtained suggests that the higher proteolytic activity, probably derived from whole grain flour, contributed to the solubilization of higher phenolic compounds amount by the breakdown of aleurone bran layer and germen cell wall structure (Shumoy et al., 2017; Rizzello, Coda, Mazzacane, Minervini, & Gobbetti, 2012).

The contribution of LAB peptidades activity to release the antioxidant peptides encrypted in native proteins, with DPPH chelating and ferric-reducing power properties, was also previously reported (Sanjukta, Rai, Muhammed, Jeyaram, & Talukdar, 2015; Arte et al., 2015; Yilmaz-Turan et al., 2020).

Proteolysis of wheat proteins during sourdough fermentation can influence the overall quality of the baked goods. The addition of yoghurt-sourdough as bread-making ingredient, resulted in lower specific bread volume, markedly for WWSB, in which the proteolytic events were more intense (Fig. 1), affecting mostly the glutenins fraction. However, yoghurt-sourdough addition considerable improved the texture attributes of the both bread formulation in terms of crumb softness and delayed staling rate, which can constitute an advantage in terms of shelf-life extension, comparing to yeast-fermented breads. Sourdough fermentation have been reported as a promising bioprocessing to generate natural hydrocolloids *in situ* produced by LAB (Wang et al., 2019), not only to efficiently improve the bread quality properties but also to circumvent the labelling-packaging requirements (Arendt, Ryan, & Dal Bello, 2007; Patel & Prajapati, 2013; Wang et al., 2019). Additionally, the addition of sourdough led to more acidic bread with pH values of around pH 4, which was previously reported as an advantage to reduce the formation of amylose microcrystal reducing the starch retrogradation and hence the ageing process, while retard the microbial spoilage (Arendt et al., 2007).

In terms of nutritional bread quality, the incorporation of yoghurt-sourdough into wheat bread formulations led to a notably decrease of starch digestibility and further increased the protein digestibility, compared to yeast-fermented breads. The lower starch digestibility has been attributed mostly to the organic acids produced during sourdough fermentation (Bjorck & Elmstahl, 2003) in which lactic acid, has been linked to inactivate the amylase performance, reducing its effect on starch granules breakdown, hence limiting the gelatinization process and digestion rate (Bjorck & Elmstahl, 2003; Fekri, Torbati, Khosrowshahi, Shamloo, & Azadmard-Damirchi, 2020). The increase of soluble fibre and insoluble fiber solubilization has been reported as a result of sourdough fermentation, generating a more viscous environment that hampers enzymes activity performance, delaying the starch digestion and absorption rates, consequently reducing the glycemic response (Lu, Walker, & Muir, 2004). Even though abundant literature (Bjorck & Elmstahl, 2003; Lu et al., 2004; Fekri et al., 2020) have been reporting the effect of sourdough fermentation to reduce the glycemic response of final products, the inclusion of fiber rich sources like whole grains, as an additional factor can further boost this effect (Polese et al., 2018).

The improved protein digestibility obtained was probably a result of the enhanced proteolytic activity during yoghurt-sourdough fermentation, which contributed to increase the protein solubilization, make the polypeptides more susceptible to partially degradation into small peptides and easily digestible (Annor, Tyl, Marcone, Ragaee, & Marti, 2017; Rizzello et al., 2019). In this study, the FAAs concentration, analyzed from the digested protein fraction obtained, was notable higher comparing with yeast-fermented bread, particularly when whole grain flour was blended with wheat flour. These results suggest that yoghurt-sourdough fermentation promoted a pre-digestion process, probably driven by the mutual effect of bacterial and/or cereal enzymes, improving the protein digestibility and amino acids bioavailability (Rizzello et al., 2014) particularly when enzymes sources, as whole grain flour, were included. Our results are consistent with those reported by Rizzello et al. (2019) showing that sourdough-containing bread is more easily digestible compared to yeast-fermented bread.

8. Conclusion

In conclusion, this study showed that the yoghurt-sourdough as a bread-making ingredient was a promising approach to improve the technological and nutritional properties of wheat bread. Sourdough fermentation promoted drastic chemical modifications in proteins and phenolics compared with yeast fermentation. The yoghurt-sourdough wheat bread resulted in improved crumb softness and delayed staling. Nutritionally, it gains considerable advantages, such as the higher *in vitro* starch and protein digestibility, intermediate-low glycemic index and higher bioavailability of amino acids.

CRediT authorship contribution statement

Carla Graça and Xin Huang, conceived and planned the experiments: Carla Graça performed all samples preparation and analysis, data analysis, and interpretation of the results assisted by Xin Huang, Minnamari Edelmann, Rossana Coda and Tuula- Sontag-Strohm. Carla Graça wrote the manuscript. Minnamari Edelmann contributed to the free aminoacids analysis. Rossana Coda revised the manuscript, Anabela Raymundo, Isabel de Sousa revised the manuscript. Xin Huang supervised and validated the work, data discussion and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Ethical statement

Our research work entitled “Yoghurt as a starter in sourdough fermentation to improve the technological and functional properties of sourdough-wheat bread”, did not involve the use of human subjects neither animal experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104877.

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fermentation

MDPI

Review

Sourdough Fermentation as a Tool to Improve the Nutritional and Health-Promoting Properties of Its Derived-Products

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Abstract: Cereal products are staple foods highly appreciated and consumed worldwide. Nonetheless, due to the presence of gluten proteins, and other co-existing compounds such as amylase-trypsin inhibitors and fermentable short-chain carbohydrates in those products, their preference by consumers has substantially decreased. Gluten affects the small gut of people with celiac disease, triggering a gut inflammation condition via auto-immune response, causing a cascade of health disorders. Amylase-trypsin inhibitors and fermentable short-chain carbohydrate compounds that co-exists with gluten in the cereal-based foods matrix have been associated with several gastrointestinal symptoms in non-celiac gluten sensitivity. Since the symptoms are somewhat overlapped, the relation between celiac disease and irritable bowel syndrome has recently received marked interest by researchers. Sourdough fermentation is one of the oldest ways of bread leavening, by lactic acid bacteria and yeasts population, converting cereal flour into attractive, tastier, and more digestible end-products. Lactic acid bacteria acidification in situ is a key factor to activate several cereal enzymes as well as the synthesis of microbial active metabolites, to positively influence the nutritional/functional and health-promoting benefits of the derived products. This review aims to explore and highlight the potential of sourdough fermentation in the Food Science and Technology field.

Keywords: sourdough fermentation; lactic acid bacteria; acidification; nutritional advantages; functional properties

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1. Introduction

Wheat and gluten-containing products have been associated with a wide range of gastrointestinal disorders, reducing their consumption worldwide and leading to considerable soaring demand for gluten-free products [1]. Indeed, wheat and other gluten-containing foods have been recognized for triggering a wide range of health problems, from which gluten intolerance observed in celiac disease (CD) is the most important [1]. CD is characterized by the small gut inflammation condition via an autoimmune response, triggered by specific gliadin peptide fractions of the gluten network proteins, which affects around 1 to 3% of the population [2] inducing mucosal inflammation, small gut villous atrophy and malabsorption of macro and micronutrients [3]. The only medical treatment available is a strict and life-long restriction of gluten-containing foods, including not only wheat but also rye and barley [4].

Nonetheless, recent evidence suggests that gluten is not the only culprit in triggering gastrointestinal disorders [1]. Many other components co-exist with gluten in wheat and gluten-containing foods, members of a short-chain carbohydrates group, named

FODMAPs, an acronym that stands for Fermentable Oligo-Di-Monosaccharides And Polyols [5], that have also have been associated with several gastrointestinal symptoms in non-celiac gluten sensitivity (NCGS), commonly known as wheat sensitive (WS) individuals, even though they do not exhibit clinical markers of CD or wheat protein sensitivity [1]. Irritable bowel syndrome (IBS) is the most common gastrointestinal disorder in NCGS individuals, affecting of around 10–15% of the population, characterized by flatulence, bloating, abdominal pain/discomfort and altered bowel habitat, that can profoundly affect the life quality of these patients [6].

The rapid fermentation of FODMAPs in the large intestine is suggested as a mechanism that triggers IBS symptoms. Amylase-trypsin inhibitors (ATI's) and other non-gluten proteins have been associated as pro-inflammatory effect compounds capable of triggering gastrointestinal symptoms in humans. However, it is still not clear whether the wheat-related symptoms are due to wheat proteins (ATI´s and gluten) or FODMAPs or even a synergic combination of both [7,8].

One method to degrade gluten proteins fractions and to decrease the amount of FODMAPs and possibly the bioactivity of ATIs in bread, are the prolonged fermentation processes in breadmaking. Sourdough fermentation is a long-term fermentation and represents one of the oldest biotechnology processes, dating back to ancient Egypt, characterized by a synergic activity between lactic acid bacteria (LAB) and yeast populations [9]. During the last century, sourdough fermentation was widely replaced by industrial fast-tracked processes, using large quantities of chemical and/or baker's yeast leavening agents [10]. Under these leavening agents, the main polymeric cereal components (e.g., proteins, starch) and short-chain carbohydrates compounds (e.g., fructans) undergo very mildly or absent hydrolysis/degradation resulting in less easily digestible foods with possible consequences to human health and life quality [11]. Compared to the other leavening agents, the sourdough can positively influence the bread sensory quality, generating more natural bread with a clean label and increasing the nutritional and functional properties [12,13].

The impact of sourdough fermentation has been associated with organic acids synthesis, the activation of the flour endogenous enzymes and the microbial secondary metabolic activity [14,15]. Along with the advantages related to the sourdough process, the increase of the in vitro protein digestibility, nutritional indexes, and amount of soluble fibre [16,17], the decrease of the glycemic index [15,18], phytate content [16,19] trypsin inhibitors, and other anti-nutritional factors reduction [8,20–22], and increments on soluble phenolic compounds correlated with antioxidant capacity enhancement [23–25] have been described (For review: Montemurro et al. [26]).

In cereal sourdough fermentation, oligopeptides are released mainly by the activity of cereal endoproteases, during primary proteolysis, whereas the release of small-sized peptides and free amino acids occurs through microbial peptidase secondary metabolic activity, especially that of LAB (lactic acid bacteria) [12,14,27]. LAB possess different enzymatic activities that can be an interesting tool to increase the free amino acids profile and generate several bioactive peptides with antimicrobial and antioxidant properties and can also modulate inflammatory processes [28–30]. This effect is strain-specific and thereby very dependent on the microorganisms used for sourdough fermentation [31–33].

In this review, the role of sourdough LAB fermentation to improve the digestibility of bakery goods, based on their impact on cereals prolamins degradation and anti-nutritional factors by synergic proteolytic activity between LAB and endogenous cereal proteases, will be described. Evidence in nutritional, functional, and health-promoting properties by LAB proteolytic activity, will also be reported.

2. Cereal Prolamins: Celiac Disease and Wheat Sensitivity

Wheat, barley, and rye are closely related cereals belonging to the *Triticale genus* [34]. Cereal prolamin of wheat (gliadins), barley (hordeins) and rye (secalins) are the frequent causes of food allergies and autoimmune disorders known as gluten sensitivity or

intolerance. Wheat proteins induce classical inflammation conditions via immune responses that affect the skin, gut, or respiratory tract and could also induce anaphylaxis or asthma [11]. The ingestion of prolamin-containing foods is the causal factor in CD or WS individuals, leading to the atrophy of the small intestine villi [35]. The 33-mer peptide from α-gliadin has frequently been described as the most important CD-immunogenic sequence within gluten [36].

Wheat proteins, glutenins, and gliadins, are the major storage proteins of the wheat grain: gliadins are alcohol-soluble proteins and glutenins are soluble in dilute acids [37]. The gliadins are monomeric as they contain only intramolecular disulphide bonds, and are grouped into α-, γ- and ω-type gliadins, based on their amino acid composition. Glutenins are highly polymeric proteins, divided into high molecular weight (HMW) and low molecular weight (LMW) fractions [38]. The term prolamins refer to the proline (Pro) and glutamine (Gln) rich alcohol-soluble proteins, typically found in cereals. Prolamins are further divided into three subgroups, based on their molecular weights and sulphur contents: the HMW prolamins, s-rich prolamins, and s-poor prolamins (Table 1). Within the wheat gluten proteins, the HMW prolamins include the HMW glutenins, whereas the LMW glutenins and α- and γ -gliadins belong to the s-rich prolamin subgroup. The s-poor prolamins include the ω-gliadins. Cysteine residues are only present in α-gliadins and γ-gliadins monomers [14,39].

Table 1. The prolamins of the cereal's grains (wheat, rye, barley) (Adapted from Loponen, [40]).

Prolamins of the cereals grains			
	Wheat	Rye	Barley
HMW prolamins	HMW glutenins	HMW secalins	D-hordeins
S-rich prolamins	LMW glutenins	-	B-hordeins
S-rich prolamins	α- and γ-gliadins	γ-secalins	γ-hordeins
S-poor prolamins	ω-gliadins	ω-secalins	C-hordeins
	Gluten proteins	Secalins	Hordeins

3. FODMAPs: Non-Celiac Gluten Sensitivity and Irritable Bowel Symptoms

Short-chain dietary carbohydrates are additional components that co-exist with gluten in wheat and gluten-containing foods, considered as undigested compounds in the human small intestine but colon-fermented by microbiota bacteria, to short-chain fatty acids and gases. These components known as FODMAPs can promote beneficial effects, as dietary fibres or prebiotic effects [41], but also adverse impacts on human health [42], mainly for individuals with functional gastrointestinal disorders, as irritable bowel symptoms (IBS) [43].

Accordingly, IBS is a gastrointestinal disorder characterized by both abdominal pain and abnormal bowel habit, in which the ingestion of the FODMAPs leads to a cascade of symptoms related to bloating, distension, excessive gas production, and urgency to defecate, implying severe effects on patient's life quality [44].

FODMAPs are natural compounds present not only in wheat and gluten-containing foods but also in many other food groups and raw materials (some examples are given in Table 2). They often comprise the dietary non-digestible, osmotically active, and readily fermentable carbohydrates of galactooligosaccharides (α-GOS), fructans and fructooligosaccharides (FOS), fructose in excess of glucose, lactose, and polyols (sugar-alcohols) (for review: Ispiryan et al. [43]).

Alfa-galactooligosaccharides (α-GOS), are a large group of oligosaccharides and the most common polymeric FODMAP compound found in foods, being ubiquitous in pulse seeds and legumes as raffinose, stachyose and verbascose. Gastrointestinal discomfort in IBS patients as well as in healthy individuals is due to the absence of α-galactosidase [43].

Similarity, non-digestible fructans, made up of fructose units with a single D-glucosyl unit at the end, generally is referred to as fruto-oligosaccaharides (2-9 fructose

units) or oligofructose (>10 fructose units), both present mainly in grain and cereals foods [20]. Wheat and rye, especially whole grains, are the major sources of the dietary intake of fructans [20]. Since humans lack the enzymes hydrolyzing fructans to fructose (exo- and endo- inulinase and invertase) these polymers cannot be digested and absorbed in the intestine [45]; (for review: Nyyssola et al. [46]).

The disaccharide lactose consisting of galactose and glucose molecules linked by a β (1–4) glycosidic bond, is the main FODMAPs in dairy products. A high fraction of human adults are lactose intolerant, due to the decreased intestinal lactase activity, leading to gastrointestinal disorders in many individuals [47]. Lactose can also be found in cereal-based products depending on their formulation ingredients [48]. Enzymes belonging to β-galactosidases catalyze the hydrolysis of lactose to its monosaccharide components [46].

Another group of FODMAPs that can trigger gastrointestinal symptoms are the sugar polyols (known as sugar-alcohols) generally present in stone fruits, some vegetables (e.g., sorbitol) and can also be produced during the fermentation of cereal-based products (e.g., mannitol) [49]. Mannitol is found in fruits, such as watermelon and peach as a minor component [1], and in sourdough fermentation products (as bread), due to the conversion of the fructose to mannitol through mannitol dehydrogenases by heterofermentative lactobacilli fermentation [50].

Finally, fructose is a ubiquitous monosaccharide found in a wide variety of fruits and vegetables either in free form or as a part of sucrose, linked with glucose. Fructose is regarded as FODMAPs, when it is present in excess of glucose since the intake of glucose together with fructose considerably boost their absorption [46].

All these components belong to the FODMAPs group, characterized as gastrointestinal disorders agents since they are slowly digested, or not digested at all, in the small intestine, due to certain limitations of the human digestion system, passing undigested to the colon, causing gastrointestinal discomfort in IBS patients and probably in many others called non-celiac gluten sensitivity [1].

Omitting these products from the diet should be the simplest solution to avoid gastrointestinal symptoms. However, evidence has shown that although the levels of FODMAPs in food products need to be reduced to tolerated levels, they should not be eliminated, since they act as a dietary fibre and prebiotic on the human body (especially fructans and α-GOS in particular), with beneficial effects to the gut microbiota, vital for good immunological responses and producers of several metabolites, like essential fatty acids [1,46].

Table 2. Examples of FODMAP containing foods [g/100 g DM].

		FODMAPs Contents [g/100 g DM]						
	Products	**Fructans**	**GOS**	**Fructose (FEG)**	**Lactose**	**Polyols Sorbitol**	**Polyols Mannitol**	**Reference**
1.	Gluten-containing cereal							
	Whole wheat	1.88	0.14	-	na	0.04	0.01	
	Whole barley	1.38	0.56	-	na	nd	nd	
	Rye	3.61	0.13	-	na	0.01	nd	Ispiryan et al. [43]
	Spelt	0.85	0.13	-	na	nd	nd	
2.	Gluten-free cereals and pseudocereals							
	Corn starch	nd	nd	-	na	nd	nd	
	Potato starch	nd	nd	-	na	nd	nd	
	Quinoa	nd	0.09	-	na	0.28	nd	Ispiryan et a. [43]
	Buckwheat	nd	0.01	-	na	0.17	nd	
3.	Seeds from pulses							
	Lentil	3.98	1.44	-	na	0.95	nd	
	Chickpea	nd	2.11	-	na	nd	nd	
	Soy	nd	3.55	-	na	0.06	nd	Ispiryan et al. [43]
	Faba bean	nd	3.45	-	na	0.03	nd	

4.	Fruits						
	Pear	nd	nd	2.3-5.0	na	2.3-60	nd	
	Apple	nd	nd	0.14-0.76	na	0.70-0.83	nd	
	Peach	nd	nd	0.0-4.2	na	0.68-0.99	nd	Muir et al. [1]
	Blackberries	nd	nd	nd	na	4.6	nd	
5.	Dairy products							
	Yoghurt	na	na	na	2.9–4.2	na	na	
	Curd cheese	na	na	na	1.8	na	na	Gille et al. [51]
	Bovine milk	na	na	na	4.1–5.0	na	na	
6.	Cereal products and gluten-free alternatives [g / 100 g FW]							
	White wheat bread	0.44	0.01	0.19	nd	0.01 *		
	Wheat sourdough bread Gluten-free white bread	0.11	nd	nd	nd	0.21*		Ispiryan et al. [43]
		nd	nd	nd	nd	0.03*		

FODMAPs determination via High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD); Food Groups 1–5, results referred to dry matter (DM); Food group 6- results referred to fresh weight (FW); FEG–fructose in excess of glucose; nd—Not detected or values below 0.005 g/100 g DM; na—Not analyzed; *sum of polyols: xylitol; sorbitol and mannitol.

4. Effect of Sourdough Fermentation in Alleviating Symptoms of Celiac Disease and Wheat Sensitivity

4.1. Proteolytic Enzymes from Dormant and Germinated Wheat Grains

Generally, proteolytic enzymes (proteases) are grouped into proteinases and peptidases [14]. The proteases are divided into exoproteases and endoproteases: exoproteases hydrolyze only peptide bonds near the terminal ends of polypeptides, or hydrolyze small peptides, whereas endoproteases are those that cleave peptide bonds located in the central part of proteins. According to their chemical structures and active sites, the four main classes of proteinases are aspartic, cysteine, serine, and metalloproteinases [40]. Peptidases (e.g., serine carboxypeptidase), hydrolyze specific peptide bonds or completely break down peptides to amino acids [14].

Dormant wheat grains contain proteolytic activities that are derived mainly from aspartic proteinases and serine carboxypeptidases [40,52], which are activated by moisture, within a medium temperature range, under acidic and mild pH conditions, pH 3.0–3.5 and pH 4.0–4.5, respectively [53]. Both proteases are present in the endosperm and are partially associated with gluten proteins. Their activation cause changes in the gluten structures, mainly on glutenins subunits [40].

The hydrolysis of prolamins requires specific enzyme activities, and cereal grains naturally contain those specific proteases to hydrolyze gluten proteins, synthesized during the cereal grain germination. Grain's germination process induces the production of endogenous cereal enzymes, and, in general, the cysteine proteinases have been considered the most important group of proteases to hydrolyze the prolamins (especially gliadins) in germinated wheat grains. Nevertheless, the presence of aspartic protease intensifies the overall proteolysis. The cysteine and aspartic proteinase activities are strongly dependent at a pH range between 3.8–5.0 (from pH 3.8 the total proteolytic activities are higher) [53].

Previous studies have shown that cysteine proteinases in wheat grain are capable to hydrolyze both gliadins and glutenins [54], whereas the wheat aspartic proteinases predominantly degrade just glutenins [53,55].

In addition to the cysteine proteinase, serine and metalloproteinase activities also occur in germinated wheat grains [40]. Both these proteases operate efficiently at pH values close to neutral (6.0–8.0). Of all the peptidases, carboxypeptidases are active under mildly acidic conditions and probably can be suitable to hydrolyze proline-containing

toxic peptides prolamins as well as derived peptides [14], although low specific activities were achieved [56].

4.2. Prolamin Proteolysis in Wheat Sourdough Fermentation

In the second half of the last century, fast leavening processes by chemicals and/or baker's yeast almost replaced the use of sourdough. Using these leavening agents, the main polymeric cereal components (e.g., proteins) undergo very mildly or no hydrolysis during processing.

Sourdough fermentations offer nearly ideal conditions for the degradation of cereal prolamins since it is a pH-dynamic semi-fluid system that allows the activation and stimulation of cereal proteases, according to its optimal-pH range. Additionally, microbial acidification during fermentation increases the solubility of the prolamins, which makes them more susceptible to proteolytic breakdown [15].

Evidence from sourdough applications has shown the efficiency of this system to degrade the gluten network since this proteolytic system efficiently degrades glutenins as well as the gliadin fraction [57].

The degradation of the toxic 33-mer peptide (considered the most immunogenic peptide responsible for triggering celiac disease) [58] have been reported to be possible by strain-specific and thereby very dependent on the sourdough LAB strains used [32].

Additionally, surveys based on extensive prolamin hydrolysis showed that when wheat germinated grains were used as raw materials in sourdough fermentation, considerable proteolysis occurred, in which, around 95% of the prolamins were hydrolyzed [14,53]. Previous studies have shown that cysteines proteinases of germinated wheat grain, activated by sourdough fermentation, were capable to hydrolyze both gliadins and glutenins [53–55,59], whereas the wheat aspartic proteinases predominantly degrade only glutenins [40].

The hydrolysis of glutenins, during sourdough fermentation, results in depolymerization and subsequent solubilization [14], being mainly dependent on the acidification by pH changes (14–15). LMW glutenins are partially hydrolyzed during sourdough fermentation and the degradation of HMW glutenins is virtually quantitative [40,57,60].

Loponen et al. [53] reported that when germinated wheat grains, with their high and diverse proteolytic activities, were used as a raw material in sourdough fermentation, extensive prolamins proteolysis occurred after six hours of fermentation with LAB, resulting in a virtual disappearance of the protein bands in the alcohol-soluble fraction.

These studies evidence the concept that a combination of germinated grain and sourdough fermentation can probably be used to hydrolyze prolamins to levels that might be better tolerated by wheat sensitivity patients.

The quantification of the prolamin contents in sourdoughs samples using the R5-ELISA method confirmed the observations from the SDS-PAGE analyses), where the prolamin concentration of the germinated wheat sourdough fermentation decreased significantly, during the first 6h of fermentation (27,000 ppm to 3700 ppm). After 12 h of germinated wheat sourdough dough fermentation, the prolamin content was only 1200 ppm, whereas in the control sourdough the prolamin concentration remained at 24,000 ppm [53].

However, it is worth noting, according to the upper limit of gluten content in food, that it would not be safe for patients with CD, as the daily gluten intake should be between 10–20 parts per million of gluten [1]. Even though the sourdough application can contribute to an extensive gluten network degradation, the levels reached are not enough to ensure them as safe products for celiac individuals' diets.

In this work, evidence that the toxicity of wheat prolamins, or their hydrolysis products, was reduced or eliminated by using germinated raw material in sourdoughs, was not investigated. Nevertheless, Hartmann et al. [61] showed that the pool of proteases presents in germinated grains, including wheat, hydrolyzed typical gliadins peptides into

fragments that were not harmful to celiac patients. Mandile et al. [62], also performed a clinical study in young people with CD utilizing sourdough products produced from the combination of selected LAB to degrade wheat gluten, in which no immune responses or clinical symptoms from celiac patients over 60 days, was registered.

These studies suggested that sourdough by selected LAB can be used as an important pre-digested prolamins system, making the IgE-binding proteins more degradable by digestion enzymes [12,62–64], improving the digestibility of the gluten proteins.

4.3. Combining Cereal Endogenous Enzymes and LAB Sourdough Fermentation

The synergic proteolytic activity between cereal endogenous proteases (primary proteolysis) and strain-specific intracellular peptidases from LAB, which provides several proline-specific peptidases (secondary proteolysis), have been associated with the complete degradation of gluten during sourdough fermentation [27,60,63,64].

Several researchers [14,15,60,65] have investigated the sequence of proteolysis events and elucidated the contributions of cereal and bacteria peptidases, that occur during the sourdough system (Figure 1).

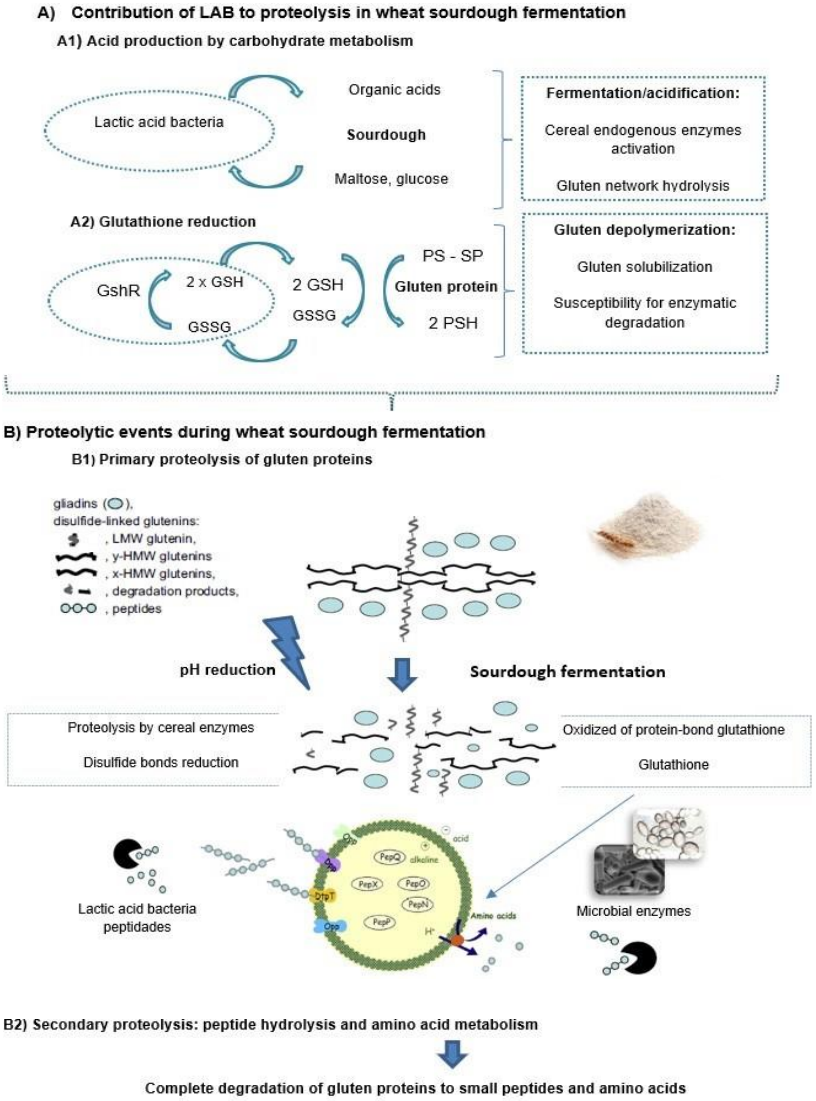

Figure 1. Representation of LAB contribution to gluten protein proteolysis during sourdough fermentation: **A**) Contribution of LAB in initial stages to environment acidification (A1), glutathione

reductase and related thiol-active enzymes (A2); **B**) Proteolytic events during sourdough fermentation based on primary proteolysis generated by the acidification and the reduction of disulfide linkages of gluten proteins by sourdough LAB, promoting the primary activity of cereal endogenous proteases (B1) and secondary proteolysis by intracellular peptidases of sourdough LAB, which complete the proteolysis of gluten proteins, liberating free amino acids (B2) (adapted from Ganzle et al. [14,15]; Gobbetti et al. [12]. The representation of gluten macro polymers is based on Wieser [57]. Abbreviations: GrhR, glutathione reductase, GSH and GSSG, reduced and oxidized glutathione, respectively, PSH and PS-SP, oxidized and reduce inter-or-intramolecular disulphide bridges in gluten proteins, respectively.

Taking all factors into consideration, this proteolytic system is probably mediated by the following key enzymes and metabolic activities: the acidification by sourdough LAB shifts the dough pH to around 6.0 to 3.5-4.0, matching the ideal environmental conditions to the main enzymes in primary proteolysis, the cereal aspartic (dormant grains) and cysteine proteinases (in case of germinated grains) [12,14,52]. In addition, the acidification promoted by LAB contributes to reducing the disulfide linkages of glutenins, leading to gluten depolymerization, consequently increasing their solubility, making them more susceptible to enzymatic degradation [28].

Furthermore, the glutenins macropolymer is strongly affected by reducing agents, where the glutathione is the most important, since it undergoes thiol-exchange reactions with gluten proteins, decreasing the intermolecular disulfide cross-linking [66]. LAB also express glutathione reductase during growth in sourdough, reducing extracellular oxidized glutathione (GSSG) to reduced glutathione (GSH) [15,67,68].

This primary cereal endogenous proteolytic activity of gluten proteins generates different sized polypeptides [12,14]. Intracellular peptidases of sourdough LAB will probably complete the proteolysis of these end-products by a secondary proteolytic activity: through a complex system of ABC and ATP transporters, namely Opp (oligopeptide permease), DtpT (Di- and tripeptide permease) and Dpp (Dipeptide permease), the polypeptides will, across the cytoplasmatic membrane of LAB, liberate amino acids and microbial metabolites to the extracellular environment [12,69].

According to De Angelis et al. [69], the secondary proteolysis is mediated by the combined activity of five peptidases (PepN-aminopeptidase type N, PepO-endopeptidase, PEP-prolyl endopeptidyl peptidase, PepX- X-prolyl dipeptidyl aminopeptidase and PepQ—Prolinase) in which, all together were responsible for the degradation of the 33-mer peptide (the most important CD-immunogenic peptide from α-gliadin sequence within gluten) within 14 h of incubation.

The concentration of free amino acids will increase, which, in turn, will suffer additional catabolic reactions by the same microorganisms [12–15,65,70]. Therefore, the sourdough LAB system has been considered a potential bioprocess to improve the nutritional and bioactive properties of bakery goods. The synthesis of health-promoting metabolites through the native cereal proteolysis represents an interesting tool to the biological fortification of the bread, by essential amino acids, bioactive and antioxidant peptides [14,70].

4.4. Contribution of Sourdough Fermentation to Nutritional, Functional, and Human Health-Promoting Benefits

Sourdough-like fermentation, carried out by LAB within its microbial consortium, has been largely reported as a powerful tool to enhance nutritional and functional properties in flours [12,16,71] and wheat-based foods like the bread [17,72].

The combined effect of the cereal grain germination and microbial fermentation have been well-recognized for the biological fortification of bakery goods, including gluten-free bread, by increasing the content of essential free amino acids (FAA) and considerable improvements in protein digestibility and nutritional indexes [71,73].

LAB use polypeptides to meet their demand for complex nitrogen [14], and the analysis of peptide and amino acid levels in wheat sourdoughs indicated that they uptake these nitrogen sources to grow in the sourdough system [25].

Recent studies [25,26,74,75] have shown a considerable increase in total FAA and improvements in the in vitro protein digestibility, via protein proteolysis and polypeptides solubilization by sourdough fermentation. Among FAA, a considerable increase in y-aminobutyric acid (GABA), a non-protein amino acid that is primarily produced from the decarboxylation of L-glutamic acid, was found. GABA possesses well-known physiological functions such as neurotransmission, induction of hypotension, diuretic, and tranquillizer effects [76–78].

These LAB proteolytic systems have been recognized as a potential tool not only to improve the nutritional and functional benefits of the baking goods [75,79] but also with considerable positive effects on health [12,15]. Specific strains of LAB, possessing different enzymatic activities, are recognized as a suitable approach to generate several bioactive peptides associated with different biological roles on human health [80], which generally increase during food fermentation by LAB [81].

Recent developments focused on the accumulation of (bioactive) peptides and amino acid metabolites in dough and bread from sourdough LAB fermentation, with antioxidant, anti-inflammatory properties, and cancer-preventing activities, being associated with LAB peptidase activities [82]. The ability of sourdough LAB activities to generate bioactive peptides through proteolysis of native cereal proteins has been well demonstrated [28,83,84].

Rizzello et al. [85] showed a remarkable increase in the concentration of the anticancer peptide lunasin, a fragment of the larger 2S-albumins, the most widely studied peptide for its anticancer activities, by the fermentation of whole wheat flours with sourdough LAB. Therefore, LAB is recognized as the most useful microorganisms for bioactive peptides production in fermented foods [86,87]. Regarding these studies, the interest for the selection of LAB strains, as starter cultures, to the manufacture of healthier leavened baked goods is increasing [74,88,89].

Sourdough fermentation is also considered an effective tool for starch degradation and carbohydrates metabolism [15]. The presence of low pH values of around 3.5–4.0, promoted by LAB acidification activity [12,87] and the combination of protein-rich foods like yoghurt [90,91] and fibre sources [92] demonstrated to be a potential system to reduce the glycemic index of the baking goods.

Fois and coworkers [93] showed that the application of the sourdough system can decrease substantially the glycemic index to lower values (GI < 55) while improving the quality and shelf-life of fresh pasta. Similar results were reported by Wang et al., [24] on the influence of in situ dextran produced by *Weissella confusa*, during sourdough fermentation, on technological and nutritional properties of whole-grain pearl millet bread.

Additionally, considerable reduction (about 80%) of glycemic index on experimental sourdough bread, compared to baker's yeast bread, was achieved by Nionelli et al. [94]. This effect was attributed to biological acidification by LAB, which is one of the main factors that decrease starch hydrolysis rate and index [71]. Lactic acid, which is an organic acid synthesized by LAB activity, was identified as one of the main causes to reduce starch digestibility in the human body. Lactic acid seems to affect starch digestion by lowering the α-amylase enzymatic activity [53]. Furthermore, the chemical changes promoted upon sourdough fermentation may impact negatively on starch gelatinization performance it can increase the levels of resistant starch, which is not enzymatically digestible, therefore, with no impact on the glycemic index [95,96].

LAB enzymatic activities can contribute to increasing the soluble fibres and solubilization of the insoluble fibre fraction, correlated with the delay of starch digestion and absorption rates impacting on glycemic and insulinemic responses decrease [26–97]. Moreover, the generated peptides from native protein proteolysis, amino acids as well as

free phenolic compounds, which are liberated during sourdough fermentation, seem to have a crucial role in glucose metabolism regulation, consequently, lowering the GI [18,95].

Furthermore, sourdough fermentation can decrease the content of bound phenolic compounds increasing their bioavailability, which has been correlated with in vitro antioxidant capacity improvement [95]. Recently, Wang et al. [24] and Jiang et al. [25] reported that fermentation enhanced the solubilization of bound phenolic compounds with a consequent increase of the soluble compounds' fraction, possibly correlated with the higher DPPH radical scavenging activity achieved. These findings were consistent with those reported by Shumoy et al. [98], who emphasized that such increments might be a result of microbial acidification, and consequent activation of endogenous cereal enzymes and production of hydrolytic enzymes of LAB, during fermentation. Since the free phenolic compounds are more bioavailable the health benefits are potentially boosted [24]. In line with these findings, Bei et al. [99] found that the improvement of phenolic composition and their bioactivity can not only contribute to the antioxidant capacity but also inhibit the α-amylase and α-glucosidases performance, affecting its activity on starch hydrolysis. Additionally, digestive enzyme inhibitors from some polyphenol compounds are found to be promising approaches to help maintain a low GI diet, especially for starch-rich foods [24,100,101].

It is well-known that phytic acid is an abundant anti-nutritional factor, mainly located in the bran fraction of the whole grain's flours, strongly reducing the mineral bioavailability, due to the chelating complexes formed with mineral and trace elements. Nevertheless, it has been demonstrated that the fermentation process with LAB can efficiently degrade the phytate complex thanks to the activation of endogenous and microbial phytases [102], and successfully overcame its detrimental effect on the mineral availability [16,19,26].

Other bioactive compounds, such as anticancer, anti-inflammatory, and immunomodulatory peptides have largely been found on innovative sourdough-based products [72,79,103].

5. The role of Sourdough to Reduce FODMAPs Compounds

The sourdough system has also been exploited to produce low-FODMAPs products since it was demonstrated a high potential to lower the quantity of the most indigestible oligosaccharides (mainly fructans and α-GOS) to levels that can be tolerated by NCGS and IBS individuals [1]. Some of the research works done in FODMAPs reduction by sourdough fermentation are given in Table 3.

Table 3. Degradation of fructans and α-GOS from foods by sourdough fermentation.

Product/Subtract	Method Applied	FODMAP Reduction	Reference
Whole wheat bread	Fermentation of 4.5 h, 30 °C using bakery´s yeast (*Saccharomyces cerevisiae)*	90% of fructans and raffinose	Ziegler et al. [50]
Whole rye bread	Sourdough fermentation rye bread (not specified) Traditional bakery´s yeast rye bread	62% in fructans 32% in fructans	Andersson et al. [104]
Wheat bread	Bakey´s yeast fermentation of 180 min, 35 °C	40% in fructans	Gélinas et al. [105]
Whole wheat bread	Bakery´s yeats and *K. marxianus* fermentation of 180 min, 30 °C	95% in fructans	Struyf et al. [106]
Seed Beans flour (*Phaseolus vulgaris*)	Natural fermentation	100% Raffinose	Granito et al. [107]

Black Beans flour (*Phaseolus vulgaris*)	Fermentation by *Lactobacillus casei and Lactobacillus plantarum*	88.6% raffinose	Granito and Álvarez [108]
Soy milk (*Glycine max*)	Fermentation by *Lactobacillus rhamnosus* 6013	100% raffinose	Liu et al. [109]
Soy milk (*Glycine max)*	Fermentation by Kefir starter culture (Clerici Sacco)	100% raffinose	Bau et al. [110]
Soy milk (*Glycine max)*	Fermentation by *Lactobacillus acidophilus, Bifidobacterium animalis* and *Streptococcus thermophilus*	40% raffinose	Battistine et al. [111]
Faba bean flour (*Vicia faba*)	Fermentation by *Weissella cibaria*, Weissella confusa, Pediococcus pentosaceus Leuconostoc kimchi	100% raffinose, 84% verbascose	Rizzello [112]
Chickpea flour (*Cicer arietinum*), Sprouted Lentil flour (*Lens culinaris*)	*Fermentation by Lactobacillus rossiae, Lactobacillus plantarum and Lactobacillus sanfrancensis*	95% raffinose	Montemurro et al. [65]

LAB produces lactic and acetic acids lowering the dough pH, which allows the activation of a few specific enzymes, suitable to reduce the FODMAP compounds [113,46].

In addition, other important baking conditions cannot be excluded, as the proofing time, temperature, and the types of microorganism used in sourdough, to achieve a major impact to lower the FODMAPs levels in the final products [113,1].

However, the greater factor to reduce these compounds content below the "cutoff" line in sourdough bread is the proofing time [48,46].

Ziegler and coworkers [48] have demonstrated the importance of the long-fermentation time, showing that a proofing time of 4.5 h was suitable to reduce FODMAP content (fructans and raffinose) of around 90% in whole wheat bread and 77% in spelt bread. Additionally, the authors emphasized that a shorter proofing time of around 1 h lead to an increase in fructose since it is a result of the breakdown of fructans.

Like gluten sourdough degradation, the selection of specific bacteria strains is the key to producing a final bread with low-FODMAP compounds, and with no negative impact on optimal quality attributes (e.g., bread volume). Andersson and coworkers [105] have shown lower fructans content (62% decrease) in rye bread prepared by sourdough method than in traditional baker´s yeast-leavened rye bread (only 32% decrease).

This is most likely due to the action of bacterial hydrolytic enzymes, but it is also possible that endogenic enzymes are activated at the lower values of pH achieved in sourdough systems. Gelinas et al. [105] reported that around 40% of wheat fructans can be degraded during baking by *S. cerevisiae.* However, Struyf et al. [106] claimed that these levels of fructans reduction are not enough for individuals with functional gastrointestinal disorders by FODMAP compounds, as IBS patients. Struyf and coworkers [107] demonstrated that the combination of an inulinase-secreting yeast, *Kluyveromyces marxianus*, with *Saccharomyces cerevisiae* can reduce significantly, of around 90%, the fructans in dough made from whole wheat flour, whereas only 56% of fructans were degraded by *S. cerevisiae*.

A recent survey performed by Loponen and coworkers [114], showed that the strain belonging to *Lactobacillus crispatus*, was also capable to hydrolyze fructans. From this work, a potential problem emerged, since the fructose release by the fructans hydrolysis during sourdough can be converted to mannitol by heterofermentative LAB, which is alsoa FODMAP compound. However, Loponen and Ganzle, [50] suggested that the mannitol can also be reduced by the activity of specific *lactobacilli*.

As described above, the α-GOS are ubiquitous in plant seeds and a major source of anti-nutritional compounds mainly found in legumes [115]. Based on the increased role of legumes and pulses in modern diets, namely as a source of protein, there is a crucial interest to reduce this oligosaccharide in food.

Considering the abundance expression of the α- galactosidase in bacteria and fungi, sourdough fermentation can be an attractive alternative to degrade this antinutritional factor [107], and in part generate novel flavours [116] and textures [117].

Soybean is worldwide considered the commercial plant containing higher amounts of α-GOS. Nonetheless, the almost complete reduction of raffinose and stachyose from soy milk by specific LAB fermentation have been well reported in previous studies [109–111].

LAB fermentation has also been applied to entire seeds and pulses, and promising results were reported by Granito and Alvarez [108], in which, significant removal of around 90% in raffinose in whole soybeans by LAB fermentation was obtained. Several studies have reported a considerable degradation of α-GOS on faba bean fermentation, using different LAB [107,112]. Furthermore, Montemurro et al. [26], showed a significant reduction of raffinose (95%) in chickpeas and lentils by fermentation using different LAB.

Indeed, evidence has shown that sourdough baking reduces and converts FODMAPs in the rye and wheat flour, but the extent of FODMAP reduction is dependent on the nature of the fermentation organisms, the conditions of the fermentation process, as well as the raw material [50].

Despite the lack of support from clinical trials, sourdough-derived products are likely to play a significant role when developing healthier baking products for individuals with non-celiac clinical symptoms as patients who suffer from IBS [8].

6. Conclusions and Future Perspectives

This review highlighted the interesting scientific research work that has been done by several researchers, exploring the potential of sourdough LAB fermentation in the Food Science and Technology field, demonstrating its positive impact on Food Nutrition and Human Health.

As an overview, sourdough fermentation is considered the most traditional and effective tool to improve the nutritional and functional value of baking products, answering modern consumers′ demand for health-promoting products, representing a new opportunity for the food industry [26,70,118].

Taking all into consideration, future efforts should focus on the implementation and optimization of the sourdough LAB process at the industrial scale, as a promising bioprocess alternative to match consumer demand, responding well to the needs of modern consumers and healthy-market niches.

Additionally, considering the new era of food processing based on more natural and sustainable production and food waste reduction, sourdough fermentation can be an alternative approach to be exploited using whole grain flours, with great potential and impact on circular economy and the ecological footprint.

Therefore, future efforts should be focused on targeting the optimization of sourdough bioprocesses selecting specific lactic acid bacteria and yeasts, depending on the functional/nutritional characteristics of the raw material and those desired in the food product.

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Chapter 5. Overall conclusions and future perspectives

5.1. Overall conclusions

Whilst aligning the market trends with the purpose of the present work, this thesis was designed to attend to the consumer´s demand, particularly, to specific market niches with nutritional needs (children, elderly, celiac, and irritable bowel syndrome patients) and to the modern consumers seeking for more natural, clean label and sustainable functional foods for a healthier lifestyle.

The focus of the thesis was driven to the development of new functional staple food products, especially bread, including gluten-free bread and sourdough-wheat bread, with health-promoting and preventive diet benefits, by the incorporation of nutritional/functional and bioactive ingredient sources, such as plain yoghurt and curd cheese. These three studies complete a coherent piece of work constituting the subject of this dissertation. It was crucial and complementary to understand the functionality of the dairy proteins on bread matrix, as well as to exploit it to its fullest on the nutritional and bioactive properties of the end-products.

In wheat-containing bread, the two dairy products studied, revealed to be promising baking ingredients to improve the overall technological properties of the bread, while resulting in pleasant bread quality attributes, in terms of appearance and taste, increased volume and softer crumb. A synergic protein-protein interaction between both animal (dairy proteins) and vegetable (gluten proteins) macromolecules was found and rheological evidenced, suggesting a reinforcement of the viscoelastic behaviour of the obtained bread dough matrix.

As for gluten-free bread manufacturing, both dairy products showed to be potential protein sources to substitute the gluten-like structure, contributing to build up a network capable of retaining the gas during leavening and baking, improving considerably the crumb softness and volume, with good appearance and taste features, resulting in products highly comparable to their wheat counterparts.

In both bread types developed, gluten-containing and gluten-free bread, the marked influence of yoghurt or curd cheese enrichments to lower the staling rate and to extend the shelf-life suggested a high potential to future applications on bakery products manufacturing, limiting the chemical preservatives addition, which is in line with current consumer trends for more natural and clean label foods.

It is worth noting that the enrichment of both bread type formulations with yoghurt and curd cheese showed promising advantages in terms of increased nutritional and functional quality, such as protein and minerals enrichment and reduced glycemic index, and health-promoting functionality such as phenolic compounds, antioxidant capacity and anti-inflammatory properties.

In the context of celiac and irritable bowel syndrome individuals, the gluten-free bread obtained can give an important nutritional contribution to these patients as part of a health-promoting and preventive daily diet, which is in line with the market trends of “tailored to fit” based on a personalized diet to those with nutritional added-needs.

Facing the demand for more natural functional foods, clean label, and made by sustainable processes, the application of sourdough biotechnology, using yoghurt as a starter, revealed to be a promising alternative to meet these requirements.

The yoghurt-enriched wheat sourdough bread led to a significant improvement on crumb texture delaying considerably the staling bread aging and hence extending the shelf life over the storage time. These features can constitute an advantageous approach to the manufacturing of more natural and clean label foods. As for nutritional properties, the yoghurt-sourdough fermentation stage promoted a notable increase of soluble phenolic profile, apparently correlated with the higher antioxidant capacity obtained. Considerable advantages in terms of functional properties were gained such as the in vitro starch and pre-digestion of the proteins, which resulted in intermediate-low glycemic index, higher protein digestibility, and availability of free essential amino acids. The yogurt-sourdough bread suggests fulfilling the demand for low-carb and glycemic response, and easily digestible foods, which is in line with food market trends of “Tailored to fit” and “Nutritional hacking”.

In summary, the incorporation of yogurt and curd cheese as baking ingredients is a promising strategy to formulate new protein-enriched bakery products with high-biological, easily digestible and a good source of minerals, creating added-value and health promoting functional products, with good quality and high consumer acceptance, which is appreciated by industry and consumers.

5.2. Future work prospects

For future work prospects, the obtained results of this thesis might be considered as a new window opportunity to the design of new research future lines, to prove the positive impact of dairy products addition on the glycemic index reduction and to improve the antioxidant and anti-inflammatory potential of foods.

Considering that the long-term fermentation is a potential bioprocessing tool to increase the bioavailability of bioactive compounds (e.g., bioactive peptides and encrypted phenolic compounds) improving the bioaccessibility of nutritional benefitting compounds on human health, the anti-inflammatory properties of the sourdough bread obtained, based on the inhibition of MMP-9 activity, should be further assessed. Furthermore, to confirm a possible anticancer activity by MMP-9 inhibition, its impact on cancer cell migration, using the wound healing assay, should also be evaluated.

It is worth noting that, since only a fraction of the nutrients is absorbed by the human body, and effectively contributing to the individual’s health, to prove the functionality of the developed products as health promoters on human body, the bioavailability of the interesting compounds (e.g., bioactive peptides from yoghurt native protein breakdown) should be further studied.

From the industrial point of view, it would be important to consider the industrial scaling-up production, taking the developed products to a commercial level. Therefore, a cost/benefit analysis should be performed as well as the establishment of collaborations with industrial partners to give value to the three types of developed products.