DEFLAMIN, AN EDIBLE ANTI-INFLAMMATORY AND ANTICANCER PROTEIN ISOLATED FROM LEGUME SEEDS

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THESIS PRESENTED TO OBTAIN THE DOCTOR DEGREE IN **BIOLOGY**

2023

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JURY:

President: Doutor Jorge Manuel Rodrigues Ricardo da Silva, Professor Catedrático do Instituto Superior de Agronomia da Universidade de Lisboa.

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FINANCIAL INSTITUTIONS:

Fundação para a Ciência e Tecnologia (Portugal) – LEAF (UID/AGR/04129/2020), project (PTDC/BAA-AGR/2860811/2017) and PhD grant (SFRH/BD/132832/2017).

2023

Acknowledgments

Após o término desta jornada, quero deixar um sincero agradecimento a todas as pessoas que se cruzaram no meu caminho ao longo destes quatro anos.

Ao professor doutor Ricardo Boavida Ferreira pelos ensinamentos transmitidos.

À professora doutora Anabela Raymundo pela constante motivação, gargalhadas, paciência e conselhos importantíssimos para que esta tese fosse concluída.

À professora doutora Ana Lima por tudo.

A todos os colegas de laboratório.

À doutora Sandra Casimiro e professor doutor Luís Costa do IMM e a todo o grupo da professora doutora Norma Marroni. Muito obrigada por toda a parte experimental que tornou esta tese mais brilhante.

A todos os meus familiares e amigos.

“You have to believe in yourself,

That’s the secret.”

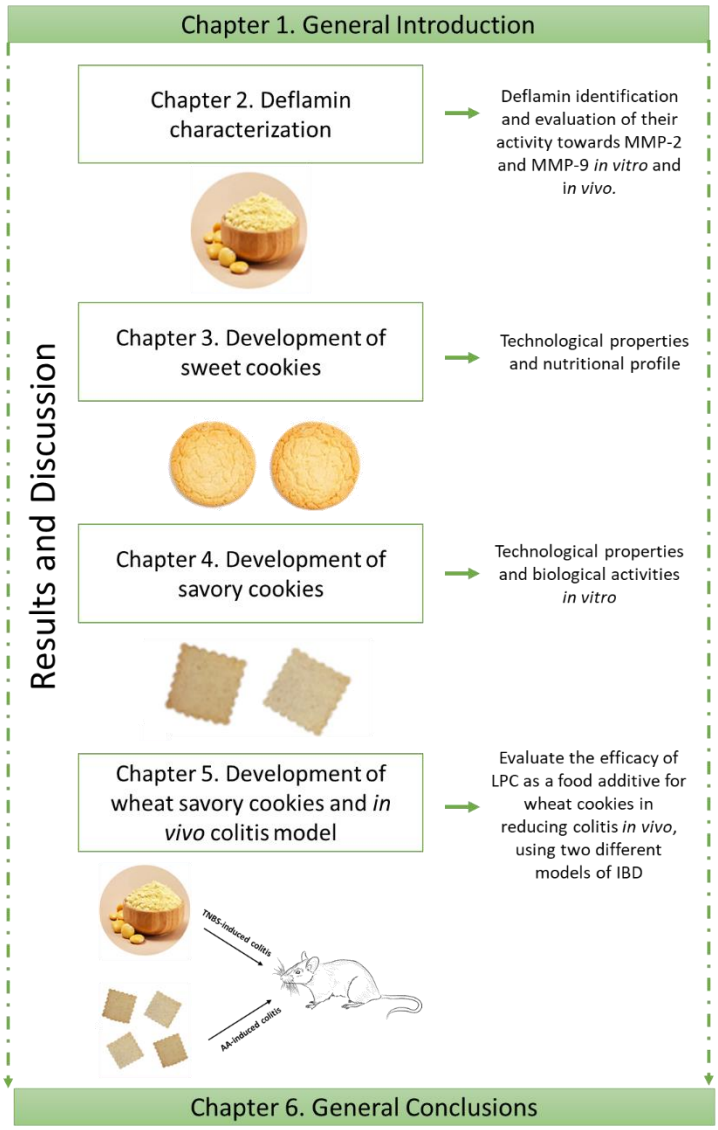
Charlie Chaplin

General framework of the PhD thesis

The present PhD thesis include the research work performed during last four the years (2018-2022) at the Linking Landscape, Environment, Agriculture and Food (LEAF) research center unit, Instituto Superior de Agronomia, University of Lisbon, supervised by Professor Ricardo Ferreira, Professor Anabela Raymundo and Professor Ana Lima. Part of the experimental laboratory work was performed at the Clinical and Translational Oncology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, supervised by Professor Sandra Casimiro and Professor Luís Costa, and at the Laboratory of Experimental Hepatology and Gastroenterology, Hospital de Clínicas de Porto Alegre, Brazil supervised by Professor Norma Marroni and colleagues.

This work was funded by the Portuguese Foundation for Science and Technology through the research unit of UID/AGR/04129/2020 (LEAF-Research Center), project (PTDC/BAA-AGR/28608/2017) and PhD grant (SFRH/BD/132832/2017).

This thesis is organized into 6 chapters, which correspond to 4 papers. The connection between the different chapters can be easily understood from the following diagram:



Abstract

Previous reports revealed a novel MMP-2 and -9 inhibitor in *Lupinus albus* seeds, named deflamin, with a strong potential as a nutraceutical for inflammatory bowel diseases (IBDs). In the present work our goal was 1) to better understand deflamin's mode of action and 2) to produce an effective delivery system for it to be incorporated in IBD functional diets.

Deflamin was found to be an oligomer comprising similar fragments from δ-conglutin and β-conglutin. It inhibited MMP-2 and -9 activities but not their gene expression and presented no cytotoxic effects. *In vivo*, it effectively reduced gelatinases activity *in situ*, hence minimizing the risk of secondary effects.

Based on a previous patented method we were able to develop a lupin protein concentrate (LPC) which was used as an additive to cookies (10g/100g dough). Sweet and savory, as well as different flours (wheat, rice, buckwheat, oat, kamut and spelt) were tested. Overall, the LPC was found to improve the organoleptic and physical properties of the cookies. The biochemical compositions of all cookies with LPC presented higher protein and a golden-brown coloring. Furthermore, the a_w and moisture content values suggested a high stability food product. Whilst the baking itself did not interfere with the gelatinase inhibitory activity in the cookies, the type of flour and the presence of sugar did, suggesting that the presence of carbohydrates can interfere with deflamin.

Our final goal was tested the efficacy of the LPC *in vivo*, either alone or as added to cookies using two different types of colitis. Results confirmed that the LPC alone was effective against TNBS-induced colitis, in a dose dependent-manner, reducing more than 50% the DAI scores and inhibiting MMP-9 activity. When added to cookies, the LPC induced a significant protective effect against acetic acid-induced colitis, reducing lesion, oxidative stress and DNA damage levels.

Overall, deflamin was shown to be an efficient and safe way to reduce IBDs symptoms, without prompting secondary effects. The developed LPC was found to be effective as a delivery system for deflamin and besides its activity towards MMP-9, the LPC further added a higher nutritional and antioxidant value to the already potential health benefits of deflamin. These findings can open new perspectives and novel approaches to tackle IBDS via functional diets.

Resumo

Estudos anteriores revelaram um novo inibidor das gelatinases MMP-2 e -9 presente em sementes de *Lupinus albus*, denominado deflamina, com elevado potencial como nutracêutico no combate de doenças inflamatórias intestinais (DIIs). No presente trabalho, o nosso objetivo foi 1) entender melhor o modo de ação da deflamina e 2) produzir um sistema de "entrega" eficaz para ser integrado em dietas funcionais para DII.

Descobriu-se que a deflamina é um oligómero com fragmentos de δ-conglutina e β-conglutina. Inibiu as atividades da MMP-2 e -9, mas não a sua expressão génica e não apresentou efeitos citotóxicos. *In vivo*, reduziu efetivamente a atividade das gelatinases *in situ*, minimizando o risco de efeitos secundários.

Com base num método patenteado anteriormente, desenvolvemos um concentrado de proteína de tremoço (LPC) que foi usado como aditivo em bolachas (10g/100g de massa). Foram testadas receitas doces e salgadas, bem como diferentes tipos de farinhas (trigo, arroz, trigo sarraceno, aveia, kamut e espelta). No geral, o LPC melhorou as propriedades organolépticas e físicas das bolachas. A composição bioquímica de todas as bolachas com LPC mostraram maior quantidade de proteína e uma coloração dourada. Adicionalmente, os valores de a_w e teor de humidade sugeriram um produto alimentício com elevada estabilidade. Enquanto a cozedura em si não interferiu na atividade inibitória das gelatinases nas bolachas, o tipo de farinha e a presença de açúcar sim, sugerindo que a presença de hidratos de carbono possa interferir com a atividade da deflamina.

O nosso objetivo final foi testar a eficácia do LPC *in vivo*, isolado ou adicionado a bolachas usando dois tipos diferentes de colite. Os resultados confirmaram que o LPC isolado foi eficaz contra a colite induzida por TNBS, de forma dose-dependente, reduzindo mais de 50% os scores DAI e inibindo a atividade da MMP-9. Quando adicionado às bolachas, o LPC induziu um efeito protetor contra a colite induzida por ácido acético, reduzindo a lesão, o stresse oxidativo e os níveis de danos no DNA.

No geral, a deflamina demonstrou ser eficiente e segura na redução dos sintomas das DIIs, sem provocar efeitos secundários. O LPC mostrou-se eficaz como sistema de "entrega" da deflamina e, além da sua atividade em relação à MMP-9, o LPC acrescentou um maior valor nutricional e antioxidante aos potenciais benefícios à saúde da deflamina. Essas descobertas podem abrir novas perspectivas e novas abordagens para combater as DII através de dietas funcionais.

Resumo alargado

As metaloproteinases de matriz 2 e 9 (MMP-2 e MMP-9) são consideradas importantes alvos clínicos em doenças inflamatórias e oncológicas. De facto, nas últimas décadas, houve uma elevada procura por inibidores eficazes dessas gelatinases. Estudos anteriores do nosso grupo revelaram um novo inibidor de MMP-2 e -9 (MMPI) em sementes de *Lupinus albus*, denominado deflamina, que parecia apresentar várias vantagens como nutracêutico para doenças inflamatórias intestinais (DIIs), reduzindo tanto a migração de cancro de cólon *in vitro*, bem como colite induzida *in vivo*. As DIIs são um dos ónus globais com um crescimento mais rápido entre as doenças crónicas, sem cura conhecida, tendo sido estipulado que dietas e alimentos funcionais podem ser a melhor forma de combater essas doenças. Assim, a descoberta de um novo composto bioativo como a deflamina tem um grande potencial para esse tipo de doenças. No entanto, embora o seu potencial como nutracêutico, a deflamina existe em pequenas quantidades nas sementes de tremoço, portanto, uma dieta de tremoço por si só não seria suficiente para melhorar as DIIs. Nesse contexto, o objetivo deste trabalho foi 1) caracterizar melhor a deflamina e entender o seu modo de ação e 2) produzir um sistema de "entrega" da deflamina eficaz para que possa ser incorporada em dietas-alvo para DII, mantendo a sua qualidade alimentar. Uma vez que o enriquecimento alimentar com compostos bioativos pode constituir uma das formas mais eficazes de fornecer compostos bioativos em dietas funcionais e uma vez que as proteínas de leguminosas já foram usadas com sucesso como aditivo alimentar de panificação, tentou-se produzir um concentrado proteico de tremoço (LPC) que pudesse ser usado como um aditivo alimentar em bolachas. Embora este LPC seja uma preparação alimentar em vez de um novo produto alimentar, deve conter deflamina bioativa suficiente para ser um alimento funcional eficaz contra DIIs. Nesse contexto, os objetivos específicos foram:

i. caracterizar completamente a deflamina e compreender melhor o seu modo de ação;

ii. desenvolver um concentrado proteico de tremoço (LPC) e testar o seu potencial tecnológico como aditivo alimentar em bolachas;

iii. testar a eficácia do LPC e de bolachas contendo LPC contra a MMP-9 usando modelos *in vivo* e *in vitro*.

Numa primeira abordagem, isolamos e caracterizamos a deflamina usando separações cromatográficas e eletroforéticas, bem como caracterização por Maldi-TOF e sequenciação. Descobriu-se que a deflamina é um oligómero composto por fragmentos semelhantes derivados de duas proteínas de armazenamento presente nas sementes de *Lupinus*: δ-conglutina e, em menor grau, β-conglutina, com peso molecular próximo de 50 KDa. Outros resultados mostraram que este MMPI inibiu a atividade de MMP-9 de maneira dose-dependente em células HT29, sem interferir a expressão génica nem o crescimento celular, aparentemente sem efeitos citotóxicos. No modelo de colite induzida por TNBS em murganhos, a deflamina manteve as suas bioatividades quando administrada por via oral (15 mg/kg) e reduziu significativamente os sintomas de colite, com uma inibição muito significativa das

atividades de MMP-2 e -9 nos cólons. No geral, estes resultados sugerem que a deflamina inibe diretamente a MMP-9, em vez da sua expressão, como é o caso da maioria dos MMPIs. Além disso, sendo resistente à digestão, conseguiu exercer a sua atividade no cólon, minimizando o risco de efeitos secundários e induzindo um efeito mais forte *in situ*.

O segundo objetivo foi desenvolver um método eficiente de incorporação da deflamina em bolachas, de maneira a incorporá-la em dietas preventivas/curativas de DII. Com base no método patenteado desenvolvido para isolar este MMPI, e considerando que a deflamina era resistente à desnaturação pelo calor, desenvolvemos um concentrado proteico de tremoço que consistia na extração em água de sementes de *Lupinus albus*, seguida de fervura a 100 ºC, remoção das proteínas por filtração e concentração por liofilização. O LPC produzido foi avaliado quanto ao seu perfil nutricional e foi utilizado como aditivo em bolachas, na proporção de 10 g de proteína/100 g de massa, tanto em bolachas doces quanto salgadas. Diferentes farinhas (trigo, arroz, trigo sarraceno, aveia, kamut e espelta) foram testadas e inicialmente foi testado o impacto do LPC nas propriedades físicas de massas e bolachas. No geral, o LPC foi desenvolvido para melhorar as propriedades organolépticas e físicas das bolachas com a vantagem de ser uma nova abordagem para adicionar proteína aos alimentos cozinhados a elevadas temperaturas. As composições bioquímicas de todas as bolachas com LPC apresentaram maiores teores de proteína e cinzas e a incorporação de LPC nas bolachas induziu uma coloração dourada específica, tornando-os mais apelativos. Além disso, os valores de a_w e teor de humidade foram muito baixos para todas as amostras, sugerindo um produto alimentício com elevada estabilidade, muito importante para o seu consumo.

Prosseguimos, assim, para a confirmação da atividade anti-MMP-9 da deflamina nas bolachas desenvolvidas, usando culturas de células HT29 e o ensaio da *DQ-gelatin*. Os resultados mostraram que enquanto a cozedura em si não interferiu na atividade inibitória da MMP-9, o tipo de farinha e a presença de açúcares sim. No geral, as únicas farinhas que não interferiram nas atividades do MMPI foram o trigo sarraceno e o trigo, enquanto todas as outras farinhas reduziram as atividades da deflamina. A presença de açúcar também reduziu a atividade do MMPI, porém somente após a cozedura. Esses resultados sugerem que a presença de hidratos de carbono pode interferir na atividade da deflamina, possivelmente devido a) a presença de β-conglutina que possui atividade lectina conhecida e b) uma interferência via reação de *Maillard*. Estes pressupostos também suportam o impacto que a matriz alimentar tem nas bioatividades dos compostos bioativos, principalmente quando usada como sistema de "entrega".

O objetivo final foi avaliar a eficácia do LPC em modelos de DII, como forma de validar a sua aplicação como aditivo alimentar para uso em dietas funcionais. Assim, a sua eficácia *in vivo* foi testada, isoladamente ou adicionado a bolachas de trigo, usando dois tipos diferentes de colite. Os resultados mostraram que, de facto, o LPC isolado foi eficaz contra a colite induzida por TNBS, de maneira dose-dependente, reduzindo mais de 50% os *scores* de DAI e inibindo concomitantemente a atividade de MMP-9. Quando adicionado às bolachas, as atividades do LPC foram mantidas e uma dieta de 4 dias com bolachas enriquecidas com LPC revelou um

efeito protetor significativo contra a colite induzida por ácido acético, havendo uma redução geral da lesão, stresse oxidativo e níveis de dano ao DNA. Além disso, se por um lado o LPC manteve as suas atividades de MMPI, por outro apresentou teores significativos de proteínas e hidratos de carbono (20,09 g/100 g e 62,05/100 g, respectivamente), bem como atividade antioxidante (FRAP: 351,19 mg AAE/10 mg e DPPH: 273,9 mg AAE/10 mg).

Assim, em geral, a deflamina mostrou ser uma maneira eficiente e segura de reduzir os sintomas das DIIs, sem provocar efeitos secundários. O LPC desenvolvido mostrou-se eficaz como sistema de “entrega” de deflamina, tanto como alimento funcional quanto como aditivo em bolachas. Além da sua atividade em relação à MMP-9, principalmente devido à sua concentração de deflamina, o LPC acrescentou ainda um elevado valor nutricional e antioxidante aos já potenciais benefícios da deflamina para a saúde humana. Tendo-se mostrado eficaz contra dois tipos diferentes de modelos de colite, o LPC parece ser um novo alimento funcional com elevado potencial para dietas preventivas/curativas contra DIIs. Esperamos que os resultados apresentados possam abrir novas perspectivas e novas abordagens para mudar o cenário atual das DIIs em todo o mundo.

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Chapter 3. Lupin Seed Protein Extract Can Efficiently Enrich the Physical Properties of Cookies Prepared with Alternative Flours

Figure 1. Texture parameters of control and lupin-enriched cookies prepared from five different flours. Solid bars represent the gluten-containing flours (oat, spelt and kamut) and the striped bars represent the gluten-free flours (rice and buckwheat): (**a**) the firmness; and (**b**) the cohesiveness. * represents $p < 0.05$ when compared with the corresponding control cookie.

Figure 2. Mechanical spectra of the control and the lupin-enriched doughs prepared with five different flours: (**a**) rice and spelt; (**b**) oat and kamut; and (**c**) buckwheat. Solid symbols represent G' (elastic modulus) and the dotted symbols represent G" (viscous modulus).

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Chapter 4. Technological Potential of a Lupin Protein Concentrate as a Nutraceutical Delivery System in Baked Cookies

Figure 1. Texture parameters of control and LPC cookies with alternative flours (buckwheat, rice, oat, kamut and spelt). (a) Firmness; (b) Adhesiveness; (c) Cohesiveness * represents $p < 0.05$ and ** represents $p < 0.001$ when compared with control cookie.

Figure 2. Mechanical spectra of control and LPC doughs prepared with alternative flours. (a) gluten-free flours, buckwheat, and rice, (b) oat; (c) gluten flours, kamut, and spelt. Close symbols represent G' (elastic modulus) and open symbols represent G" (viscous modulus).

Figure 3. Firmness of control and LPC cookies 24 h after baking of five different flours. Values are the average of six experiments ± SD. * represents $p < 0.05$ and ** represents $p < 0.001$ when compared with the corresponding control cookie.

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Chapter 5. Lupin protein concentrate as a novel functional food additive that can reduce colitis-induced inflammation and oxidative stress

Figure 1. Representative images of the effect of a 4-day lupin concentrate feed in TNBS-induced colitis in mice. LPC was administered orally in three different concentrations (0.1, 1 and 10 g/kg). Co: control group (healthy); Col: colitis group; Col + LPC: colitis group fed with LPC (at each concentration).

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Figure 3. Effect of administration of LPC cookies on rat with acetic acid-induced colitis. Anal sphincter pressure (**A**) and lipid peroxidation (**B**) were analyzed in both controls and diseased rats, as well as the activity of ROS-related enzymes superoxide dismutase (**C**) and glutathione peroxidase (**D**). Values are expressed as the mean ± standard error. Co: control; Co+LPCc: control + LPC cookie; Col: colitis; Col + Cc: colitis + control cookie; Col + LPCc: colitis + LPC cookie. * represents $p < 0.001$ when compared to the control and # $p < 0.001$ when compared to colitis.

Figure 4. Effect of administration of deflamin cookies on colon tissues. (**A**) Histopathological alterations in colitis in colon tissue. (**B**) Immunohistochemical detection of COX-2. (**C**) Immunohistochemical detection of TNF-α. Co: control; Co + LPCc: control + LPC cookie; Col: colitis; Col + Cc: colitis + control cookie; Col + LPCc: colitis + LPC cookie. All images are at a magnification of 100×.

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DQ-gelatin kit. Co: control group (healthy); Col: colitis group; Col + LPC: colitis group fed with LPC (at each concentration). Non-parametric data are expressed as the mean and respective maximum and minimum levels, and parametric data are expressed as medium ± SD. (* $p < 0.05$, ** $p < 0.001$ in relation to colitis and # $p < 0.05$ in relation to control).

Table 3. Expression of the positive pixels as the mean ± SD obtained for each colon tissue. Co: control; Co + LPCc: control + LPC cookie; Col: colitis; Col + Cc: colitis + control cookie; Col + LPCc: colitis + LPC cookie. * represents $p < 0.001$ when compared to colitis and # $p < 0.05$ when compared to control.

Table 4. Comet assay in peripheral blood of the studied groups. Co: control; Co + LPCc: control + LPC cookie; Col: colitis; Col + Cc: colitis + control cookie; Col + LPCc: colitis + LPC cookie. * $p < 0.001$ when compared to control and # $p < 0.001$ when compared to colitis.

List of Abbreviations

AA - Acid acetic

A_w - Water activity

BCO - Blad-containing oligomer

CAC - Colitis associated cancer

CD - Chron's disease

COX-2 - Cyclooxygenase-2

CRC - Colorectal cancer

DAI - Disease activity index

DPPH - 2,2-diphenyl-1-picrylhydrazyl

DQ-gelatin - Dye-quench gelatin

ECM - Extracellular matrix

EtOH - Ethanol

FDA - Food and Drug Administration

FPLC - Fast performance liquid chromatography

FRAP - Ferric reducing antioxidant power

GI - Gastrointestinal

GPx - Glutathione peroxidase

GRAS - Generally recognized as safe

GSH - Glutathione

HPLC - High performance liquid chromatography

IBD - Inflammatory bowel disease

IHC - Immunohistochemical

LPC – Lupin protein concentrate

LPC - Lupin protein extract

LPO - Lipoperoxidation

MALDI - Matrix-Assisted Laser Desorption Ionization

MMP - Matrix metalloproteinase

MMPI - Matrix metalloproteinase inhibitor

MS - Mass Spectrometry

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate buffer solution

ROS - Reactive oxygen species

SD - Standard deviation

SDS - Sodium dodecyl sulfate

SEC - Size exclusion chromatography

SOD - Superoxide dismutase

TBA - Thiobarbituric acid

TBARS - Thiobarbituric acid reactive substances

TCA - Trichloroacetic acid

TIMP - Tissue inhibitor metalloproteinase

TNBS - 2,4,6-trinitrobenzenesulfonic acid

TNF-α - Tumor necrosis factor alpha

TOF - Time-Of-Flight

TPC - Total phenolic compounds

UC - Ulcerative colitis

Chapter 1. Introduction and state of the art

1.1. The importance of bioactive foods for chronic diseases: the particular case of inflammatory bowel diseases

Today we live in the age of chronic diseases, justifying why bioactive compounds that exhibit health-promoting effects are receiving increasing attention. Since the strong link between dietary habits and health has been established, food, in addition to being a good source of nutrients with good sensory appeal, is also considered to be beneficial to the health and well-being of consumers [1].

Chronic diseases are conditions of long-term duration and may result from a combination of genetic, physiological, environmental, and behavioral factors [2]. The main types of chronic disease include cardiovascular diseases (which account for 17.9 million deaths globally every year), cancers (which are responsible for 9 million deaths annually), chronic respiratory diseases (3.9 million deaths per year), and diabetes (1.6 million deaths each year) [3]. In addition, mortality resulting from dementia more than doubled between 2000 and 2016, and it was the fifth leading cause of death worldwide in 2016 [3]. The increasing prevalence of these diseases is causing a huge economic impact on healthcare systems globally and is arousing the attention and interest of researchers and policymakers at all levels of governance and society. Strategies for managing these chronic conditions are usually multidimensional, and at the center of these approaches are nutritional and/or dietary interventions, regular physical activity, and lifestyle modifications [2]. The role of nutrition in chronic disease management is crucial as diet is a modifiable risk factor for most chronic conditions that exist either as single conditions or in comorbid states.

One type of chronic diseases are inflammatory bowel diseases (IBDs). IBDs are complex gastrointestinal tract disorders characterized by functional impairment of the gut wall, affecting the patient quality of life [4-6]. IBDs include ulcerative colitis (UC) and Crohn's disease (CD), which are generally associated with diarrhoea, nausea, abdominal pain, fatigue, rectal bleeding, weight loss and anxiety [4-6]. The natural course of IBDs is highly variable and their aetiology is still unknown [5]. IBDs are highly prevalent in Europe, affecting about 2.2 million people [4,6], and their incidence has dramatically increased worldwide over the past 50 years [4]. The link between inflammation and cancer is widely accepted: prolonged chronic inflammation induces oncogenic mutations, genomic instability, immune microenvironment changes, early tumour promotion, and angiogenesis [4-6]. These factors are likely related to the increased risk of colorectal cancer (CRC) in individuals suffering from IBD [7,8-10].

Currently, there is no effective therapy available that can completely cure IBD [5]. Available therapeutic options are incapable of targeting the underlying pathogenic mechanisms of IBD; instead, they are specifically designed to instigate and maintain the remission of the disease and help mitigate complications in patients (Figure 1) [4,5]. Aminosalicylates and corticosteroids are considered first-line therapies for IBD [5-7]. Both these drugs have shown efficacies in

ameliorating the severity and the symptoms of IBD through their abilities to downregulate the pro-inflammatory cytokines and signalling pathways [5,11,12]. Immunosuppressive agents, including azathioprine, 6-mercaptopurine, cyclosporine A, and antibiotics, which are mostly used as adjunct therapies, can decrease intestinal inflammation by suppressing the mucosal immune response [5,12]. A more recent and innovative approach is called “biological therapy,” where monoclonal antibodies, such as infliximab and adalimumap, are applied to downregulate the immune response pathways [11,12]. Despite providing some symptomatic and temporary relief, current drug therapies are described as inadequate due to their severe side effects [12]. Biological therapies, which are currently a mainstay for IBD treatment, are expensive and also associated with adverse health effects [12]. Therefore, the development of alternative IBD therapies using natural products that are highly effective, safe, and inexpensive is in great demand (Figure 1).

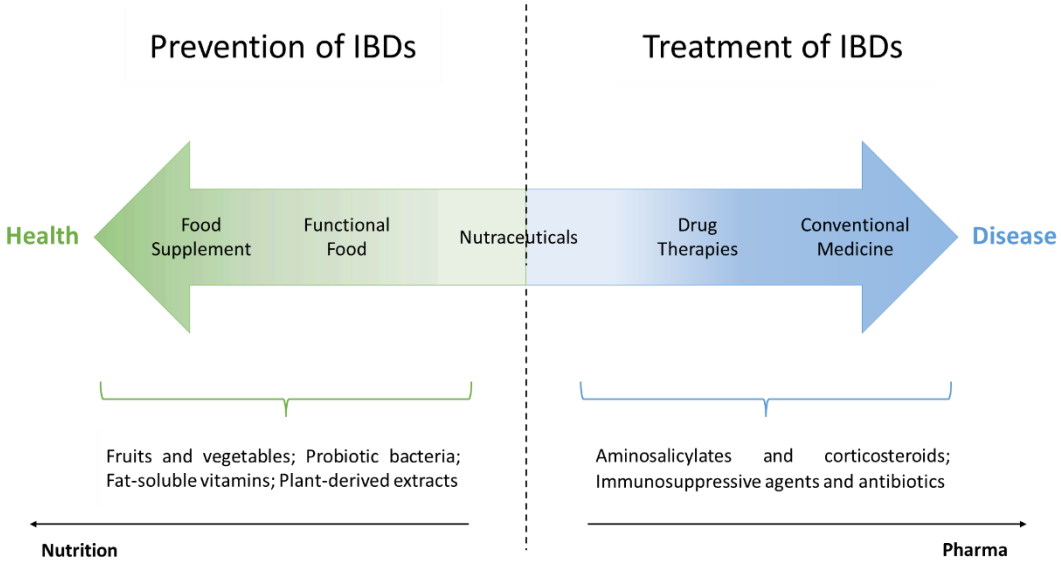

Figure 1. Nutrition-Pharma Interface.

There is a growing consensus that diet plays a critical role in the etiopathogenesis of IBD, and hence dietary therapy has a great implication on its treatment (Figure 2) [13]. Recent research evidence suggests that the supplementation of diet with fruits and vegetables, probiotic bacteria, dietary fibers, and fat-soluble vitamins can substantially reduce the symptoms of IBD, due to their anti-inflammatory bioactivities [14]. In contrast, as the high-fat and high-carbohydrate diets are apparently involved in the aetiology of IBD, reducing these foods could be an essential tool in the management of IBD [13-15].

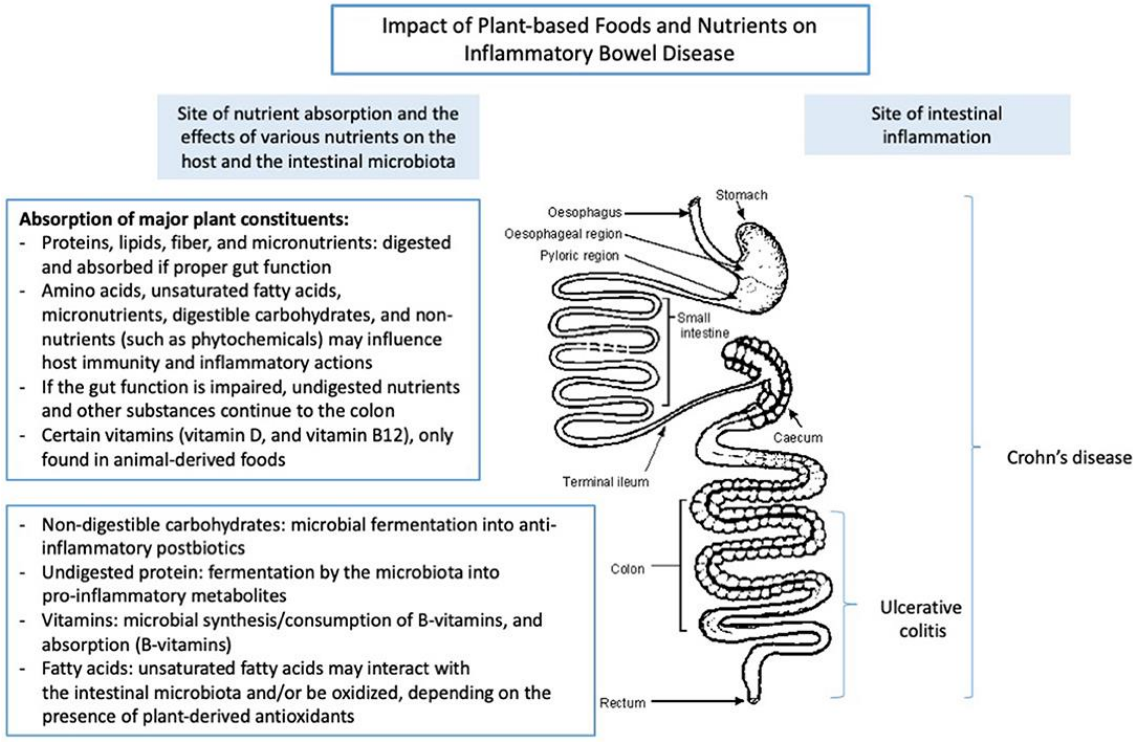

Figure 2. The impact of plant-based foods and nutrients on IBDs [16].

Bioactive natural compounds and functional foods have been a major focus of research throughout the last decade as potential therapies for IBD, and many research groups have demonstrated positive and outstanding results. Plant-derived extracts, antioxidants, phytochemicals, polyunsaturated fatty acids, and dietary peptides have demonstrated strong anti-inflammatory effects against IBD due to their modulatory actions on pro- and anti-inflammatory cytokines and signalling pathways [16-20]. Therefore, the available evidence suggests that the alternative therapies based on diets and functional foods may be the future of IBD management [14]. Under this context, many patients find diet to be an appealing alternative to conventional drug therapies. There are numerous links between inflammatory bowel diseases and diet, ranging from clinical evidence of frequent multifactorial nutritional deficiencies in IBD patients, to efficacy of nutrition therapy either as sole therapy or as adjunctive therapy [13,14,21]. In support, IBD patients frequently report those specific foods cause aggravation of their symptoms. However, scientific evidence to support specific dietary advice is currently lacking and dietary counselling is almost limited in clinical practice [21].

The concept of food possessing medicinal properties is not new and has been around for thousands of years. Nowadays, the term functional foods are used to describe foods or a part of foods that provide physiological benefits beyond basic nutritional functions, including maintenance of gut health [22]. However, there is no clear definition for functional foods. Furthermore, the Food and Drug Administration (FDA) does not have a statutory definition for functional foods and therefore does not regulate them [23]. Foods are typically considered functional if they contain one or more bioactive components such as nutrients (vitamins,

minerals, protein, etc.) or non-nutrients (phytochemicals including polyphenols, prebiotic dietary fibers, etc.) that affect one or more physiological functions in the body to improve well-being and health, reduce disease risk, and/or improve disease outcomes [22]. Strictly speaking, this definition comprises all common foods we ingests. Depending on which definition of functional foods is being considered or followed, functional food definitions may also include nutraceuticals, dietary supplements, and medical foods. Definitions for these terms also vary depending on the source.

The term nutraceutical was coined from "nutrition" and "pharmaceutical". As functional foods, this term has no regulatory definition [24]. Nutraceuticals are specific bioactive compounds naturally present in foods and may be isolated, modified, and/or chemically (or otherwise) synthesized and are typically administered in the form of a dietary supplement in different forms, such as a capsule, tablet, powder, solution, or potion, which are not generally ingested in association with the food and which have demonstrated physiological benefits and/or provide protection against chronic diseases [22,25,26]. The main difference between functional foods and nutraceuticals is that functional foods are food, typically containing bioactive compounds such as β-carotene and *trans*-resveratrol, whereas nutraceuticals are the bioactive compounds originally found in natural foods, but later introduced, after partial or full purification, in fortified foods, dietary supplements, or herbal products [22,26]. Nutraceuticals include a wide range of compounds such as bioactive peptides, phenolic compounds, carotenoids, lipids, vitamins, etc. [27]. However, except for the compounds that exert their function in the gut, the effectiveness of nutraceuticals in providing therapeutic or physiologic benefits depends significantly on preserving their bioavailability, defined as the fraction of an ingested compound that is absorbed and available for physiological functions (i.e., reaches the systemic circulation in an active form) [27]. Although several food bioactive compounds have been pointed as effective against IBD, their targets are often not enough to control the disease and their effectiveness in realistic scenarios is usually much lower than that observed in laboratorial models [16,28-30].

1.2. The inhibition of matrix metalloproteinases as novel therapeutic targets for IBD

Diseases that are caused by an excessive breakdown of extracellular components are typically influenced by the activity of matrix metalloproteinases (MMPs). So, a deregulated expression of these proteases and a disproportion between MMPs and tissue inhibitors of MMPs (TIMPs – natural inhibitors present in the body) can lead to the emergence of several diseases and pathologies. MMPs are a family of zinc-dependent endoproteases responsible for degrading and altering each component of the extracellular matrix (ECM) [31,32]. They are responsible for important physiological processes such as tissue repair, wound healing, hair follicle growth, organogenesis, embryogenesis and angiogenesis [31,32]. However, some pathologies are also related to MMP activity, such as inflammatory and auto-immune disorders, atherosclerosis and osteoporosis [31,32]. Being responsible for the degradation of ECM components, they also aid in metastization and carcinogenesis [33]. At present, over 20 different MMPs are known, each one characterized for its affinity to a specific substrate and by its own structure [31-33]. They also vary in their function and distribution [31-33]. These endoproteases play a decisive role

regarding the control of the cellular microenvironment [33], responding to stimuli from different sources. In addition, MMPs proteolytically activate or degrade a variety of non-matrix substrates, including chemokines, cytokines and growth factors [33].

It is widely known that MMPs are associated with several pathologies, and for this reason, they have become an important therapeutic target. The discovery and development of both natural and synthetic MMP inhibitors (MMPI) has been an attractive research field for several years, but many efforts of developing synthetic inhibitors failed, as they did not pass the clinical trial phase, due to several complications, such as severe adverse side effects [34,35]. Also, many of the compounds tested were non-specific and acted in a generalised way throughout the organism [35]. The ECM has further functions besides providing support to the cell, such as the alteration of several cell components, with MMPs being responsible for these biological activities [34]. These crucial functions of the MMPs must be considered, and their inhibitors may have to be targeted specifically, in order not to disrupt the beneficial functions of these proteases.

As mentioned above, MMPs were grouped according to their domain structure and substrate specificity into gelatinases, collagenases, stromelysins, matrilysin, and membrane-type MMPs (Figure 3) [34,35]. Some of the newly developed MMPIs target gelatinases (MMP-2 and MMP-9), as these assume a crucial role in the development of several diseases, such as cardiovascular diseases, tumorigenesis, metastasis and inflammation-derived diseases, such as rheumatoid arthritis and inflammatory bowel diseases [34-36].

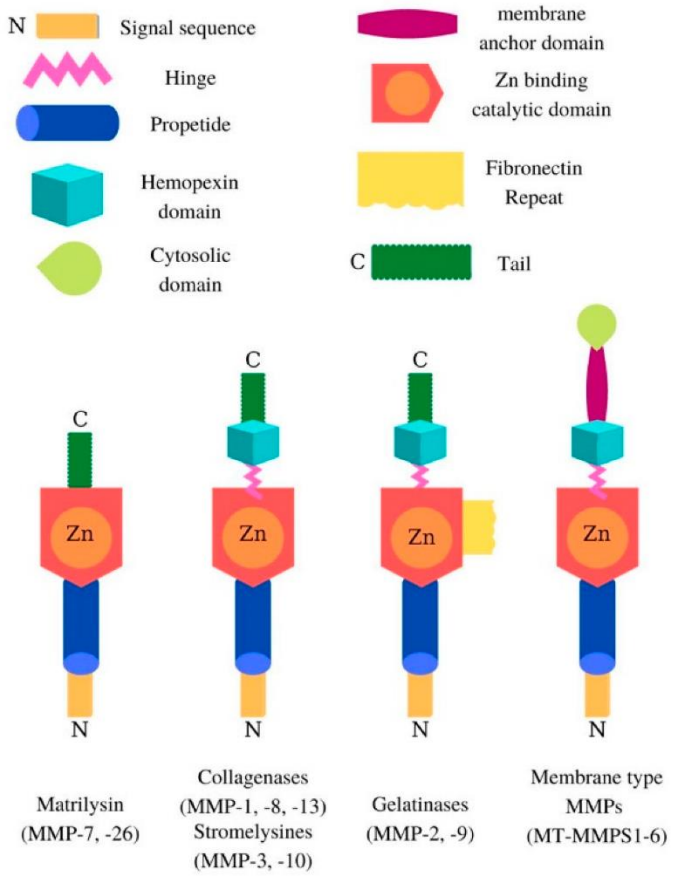

Figure 3. Modular organization of the different MMPs [36].

MMP-2 and -9 are, respectively, 72 and 92 kDa proteases which distinguish themselves structurally from other MMP families by the fact that they comprise, within their catalytic region, an extra fibronectin domain, with three fibronectin type II-like repeats [37]. They are therefore able to break a vast number of substrates, such as gelatin, collagen types IV and V, elastin, laminin, fibronectin, and proteoglycans [34,37]. Even though gelatinases have similar substrate specificity, they differ in tissue specificity. According to Bai *et al*. [37], MMP-2 is primarily produced by stromal cells, including fibroblasts, myofibroblasts, and endothelial cells, while MMP-9 is essentially produced by neutrophils and to a lesser extent by eosinophils, monocytes, macrophages, lymphocytes, and epithelial cells [37-39], being strongly induced in response to a variety of inflammatory pathologies. In fact, intestinal mucosal MMP-2 and -9 activities have been reported to be upregulated in humans with IBDs, in both CD and UC. MMP-2 contributes to ECM remodelling and the degradation of basal membrane type IV collagen, leading to intestinal ulceration, epithelial damage, and/or fistula formation [37,39]. In contrast, MMP-9 plays a crucial role in the induction of intestinal inflammation and wound healing. These promotes neutrophil migration, increases paracellular permeability, and reduces the adhesion complex integrity of the epithelium [37,39].

A major problem associated to unspecific inhibition of MMP-9 is that this enzyme is involved not only in various diseases but also in remodelling and scar tissue. Therefore, its complete inhibition causes several complications in the human organism [40,41].

The treatment of inflammatory and cancer disorders by MMP-9 inhibition involves the use of synthetic molecules, such as doxycycline and tetracycline, as drugs able to interact with a great number of targets [42]. Unfortunately, high toxicity, low selectivity, and a wide range of undesired side-effects are associated with their administration, including brain–blood barrier cross and the generation of toxic molecules after metabolic processing; they are often inadequate in long-term therapies or high dose requirements [39,43].

1.3. Food bioactive compounds as MMP inhibitors in IBD

In recent years, a distinct strategy in the search for novel MMP-9 inhibitors is to ‘look’ for functional foods and nutraceuticals that are already present in our regular diets. Plant seeds have long been known to contain compounds, such as phenolics and proteins, with a variety of potential bioactivities, such as the ability to inhibit MMPs. Many seeds have also been reported to contain MMPIs, such as grape [44], soybean [45], and dried longan (*Euphoria longana* Lam.) [46]. Lunasin is a 43-amino acid residue polypeptide identified and isolated from soybean seeds, and several studies suggest that this polypeptide exerts anti-inflammatory effects on human macrophages via inhibition of the Akt/NF-κB pathway [45]. However, some plant compounds have some problems like chemical inactivation by denaturation, destruction during the digestive process, or destruction during boiling (e.g. during cooking or food processing) [47]. Major challenges with the production of certain MMPIs like lunasin are the high commercial price due to its costly and time-consuming synthesis process [45]. Also, several different factors can compromise the bioavailability of these inhibitors, including an insufficient gastric residence time, low absportion and/or solubility within the gastrointestinal (GI) tract and instability during

food processing/storage or in GI tract [1]. In fact, most nutraceuticals are unlikely to provide the intended bioactive properties without the use of an appropriate delivery system [1].

1.4. Lupin as a source of bioactive MMP inhibitors against IBDs

Pulses, defined as the edible seeds of legumes, have been part of human and animal nutrition for centuries [48,49]. They stand out from other plant foods for being prime sources of protein and fibre [48]. Nutritionally they provide fiber, protein, low glycemic index carbohydrate, B vitamins and several minerals such as iron, copper, magnesium, manganese, zinc, and phosphorous [49]. Legumes are practically free from saturated fat [49]. The greatest value of this crop lies in the high protein and oil contents, although these may vary from species to species. Each 100 g of white lupin seeds contains approximately 32 g protein, 16 g crude fiber, 6 g carbohydrates and 6 g crude fat [50]. Along with being a highly nutritious food, evidence shows that legumes contain several compounds that possess specific bioactivities which may play an important role in the prevention and management of a number of health conditions, such as type 2 diabetes, hypertension, hyperlipidemia and weight management [51-53].

Previous studies performed in our group [54] tested the proteins from several edible legume seeds to detect if any inhibitory activity against MMP-9 activity was present. The species tested were legumes that are present in several well-known diets, especially in the Mediterranean diet. Those studies concluded that some of these species exhibited inhibitory action against MMP-9 activity, evident in the total protein extract as well as in isolated protein fractions [54]. Also, the study showed that, especially in the albumin fraction, inhibitory potential was present. Nonetheless, not all the tested legume species exhibited a very high activity, and for the species which produced the most promising results, the activity was present at significantly different levels, with *Lupinus albus* standing out amongst all specimens [54]. Subsequent *in vitro* assays were performed which demonstrated that the MMPIs found in these seeds had little effect on cell growth but had a lot of potential when it came to inhibit cell invasion. Once again, *L. albus* stood out, exhibiting the greatest inhibitory potential against MMP-9, which is known to be implicated in the process of cell invasion [54]. As such, this lupin was further tested and a polypeptide oligomer present in this species responsible for this strong MMPI activity was subsequently isolated.

This small polypeptide oligomer was named deflamin. Preliminary studies suggested it has high anti-inflammatory and anti-carcinogenic potential as a potent MMPI. Apart from the high inhibition of gelatinase activity shown, deflamin has other characteristics that makes it ideal to be used as a nutraceutical. Besides being water-soluble, deflamin can withstand high temperatures and low pH values, and previous results suggest it can survive digestion and is non-toxic to colon cells. These features suggest that this MMPI holds great potential to become a nutraceutical or functional food against IBDs. Nonetheless, the nature, sequence and characterization of this MMPI remain to be performed.

1.5. Deflamin as a potential food additive for functional diets

Because deflamin exists in small amounts in lupin seeds, a diet on lupin seeds *per se* would not suffice to exert the necessary effects. In fact, this is one of the most challenging problems associated to foodborne anti-inflammatory compounds [55]. Quite often they never reach clinical or even pre-clinical trials because their production is hampered by the cost of their synthetic production, or there are no cost-effective methods for obtaining gram quantities of their highly purified forms [56]. Due to its particular features and nature, our previous results suggest that deflamin could be feasible to develop a method to obtain a lupin extract or concentrate, suitable for consumption, that could contain physiologically bioactive amounts of deflamin. A method that could be up-scalable and cost-effective, whilst still being considered as generally recognized as safe (GRAS). This would be advantageous also because it would allow us to surpass all the constraints and challenges of producing a novel nutraceutical [57].

Since in recent years there has been an increasing interest in the use of lupin seeds as functional ingredients, the production of a lupin food additive containing enough deflamin to be used as a treatment or prevention of IBD would have additional advantages as a novel food product that could increase sustainability and production of lupins [58]. This crop is an important, low-cost and low-maintenance Mediterranean staple food that has been largely neglected [58]. Evidently there are major challenges when dealing with the incorporation of nutraceuticals in food products, mostly related to their chemical instability during food processing/storage (e.g. sensibility to light, oxygen, heat) or within GI tract (e.g. easily degraded by enzymes and/or pH), their low water solubility and low bioavailability [1]. All these factors can affect the functionality of the nutraceutical, and consequently, their health benefits may not be evident even when ingested in high amounts [1,56]. Furthermore, when directly incorporated in food products, nutraceuticals with unpleasant sensory properties or undesirable interactions with other food components, may negatively affecting the organoleptic properties of foods (appearance, texture, taste), as well as their stability, bioavailability or shelf-life [1]. Nonetheless, when used as food additives or substitutes, such as flours, and even as egg and butter substitutes, lupin proteins have been shown to have some important functional properties such as emulsifying and foaming capacity [59], and confer additional protein and nutrient contents to existing foods, making them an interesting alternative for existing ingredients [59]. They also have the advantage of being able to be incorporated in gluten-free products, an increasingly important feature in novel food product formulation [60]. In fact, lupin flour has been incorporated into several foods such as bread, cookies, and pasta [51,60,61]. Cookies and crackers have become one of the most popularly consumed snacks due to their low manufacturing cost, availability, high nutrient density, long shelf-life and potential to be supplemented with a wide variety of nutraceuticals [62,63].

1.6. Objectives

Considering the previous framework, the main goals of this work were to:

1) better characterize deflamin, namely its amino acid composition, molecular mass, among others,

2) develop a staple food or lupin enriched concentrate that may still be considered GRAS, acting as a vehicle for the ingestion of deflamin in preventive/functional diets,

3) use this deflamin-enriched concentrate as a food additive to staple foods, particularly baked cookies, which may act as a delivery system and as a way to produce novel lupin-based food,

4) test its effectiveness *in vivo* on two different colitis models of IBD.

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Chapter 2. *Lupinus albus* Protein Components Inhibit MMP-2 and MMP-9 Gelatinolytic Activity *In vitro* and *In vivo*

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Status: *International Journal of Molecular Sciences*, 2021, 22(24), 13286.

DOI: 10.3390/ijms222413286

Abstract: Matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) are regarded as important clinical targets due to their nodal-point role in inflammatory and oncological diseases. Here, we aimed at isolating and characterizing am MMP-2 and-9 inhibitor (MMPI) from *Lupinus albus* and at assessing its efficacy *in vitro* and *in vivo*. The protein was isolated using chromatographic and 2-D electrophoretic procedures and sequenced by using MALDI-TOF TOF and MS/MS analysis. *In vitro* MMP-2 and 9 inhibitions were determined on colon adenocarcinoma (HT29) cells, as well as by measuring the expression levels of genes related to these enzymes. Inhibitory activities were also confirmed *in vivo* using a model of experimental TNBS-induced colitis in mice, with oral administrations of 15 mg/kg. After chromatographic and electrophoretic isolation, the *L. albus* MMP-9 inhibitor was found to comprise a large fragment from δ-conglutin and, to a lower extent, small fragments of β-conglutin. *In vitro* studies showed that the MMPI successfully inhibited MMP-9 activity in a dose-dependent manner in colon cancer cells, with an IC50 of 10 µg/mL without impairing gene expression nor cell growth. *In vivo* studies showed that the MMPI maintained its bioactivities when administered orally and significantly reduced colitis symptoms, along with a very significant inhibition of MMP-2 and -9 activities. Overall, results reveal a novel type of MMPI in lupin that is edible, proteinaceous in nature and soluble in water, and effective *in vivo*, suggesting a high potential application as a nutraceutical or a functional food in pathologies related to abnormally high MMP-9 activity in the digestive system.

Keywords: *Lupinus albus* protein; MMP-9; MMP-2; gelatinases; MMP inhibitor; nutraceutical; gastrointestinal diseases

2.1. Introduction

With today's rise in chronic diseases, functional foods containing bioactive compounds that exhibit health-promoting effects are receiving increasing attention and have become a major trend for both consumers and the food industry [1]. This is particularly true for gastrointestinal diseases, such as cancer and inflammatory bowel diseases (IBDs), which are among some of the most diet-linked pathologies [2,3]. Under this context, the activity of molecules such as matrix metalloproteinases (MMPs), particularly gelatinases MMP-9 and MMP-2, has been recognized as major key players in inflammation [3,4] and in oncologic processes such as tumorigenesis, cell adhesion, and metastasis [5–7]. A considerably large body of evidence from pre-clinical and clinical tests shows that MMP-2 and MMP-9 inhibition can reduce both colorectal cancer (CRC) [3,8] and IBDs such as ulcerative colitis [5,8–10]. These findings have turned gelatinase inhibitors into very desirable pharmacological targets; however, previous efforts to target gelatinases MMP-2 and MMP-9 using broad-selective spectrum or semi-selective inhibitors (MMPIs) 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 [11–13]. One method to overcome these constraints would be to discover non-toxic, more specific MMPIs capable of acting directly in loco, without the side effects reported in previous inhibitors [12,13]. Although this has yet to be discovered, a substantial amount of research has turned towards the discovery of plant-food

derived MMPIs that may hold such features. Legume seeds are a good example, as they contain high levels of polyphenols, saponins, and protease inhibitors that have been reported to exhibit anticancer activities [14]. In fact, the considerably high number of reports on plant and food-based MMPIs concerns mostly secondary metabolites, such as flavonoids, alkaloids, and phenolic compounds [15]. However, these compounds present some constraints, since most of them can be destroyed during the digestive process and others are considered to be anti-nutrients, even exerting cytotoxicity at high levels. In our previous study, we analyzed and compared aqueous protein extracts from the seeds of eight different legume species that are usually consumed in Mediterranean diets and demonstrated that they inhibit MMPs and cancer cell migration [16]. Particularly, the water-soluble fraction of *Lupinus albus* seeds exhibited strong inhibitory activity on MMP-2 and MMP-9 in HT29 cells [17]. Reverse zymography revealed that this activity could be related to small polypeptides/proteins that seemed to be larger than other low molecular mass MMPIs also found in legume seeds, such as the Bowman–Birk or Kunitz inhibitors [18]. Recently, an up-scalable sequential method for producing a low molecular weight fraction for MMPI activity, using denaturing and precipitation steps, was developed [19] and patented (PCT International Patent Application No. PCT/EP2017/075020), demonstrating that MMPI can withstand heat and acid denaturation and suggesting a high potential as a nutraceutical in preventive diets. However, the nature and mode of action of these MMPIs remained to be characterized, particularly with respect to its activity validated *in vivo* after digestion.

Furthermore, although there is much evidence suggesting that several compounds from legume seeds can prevent cancer [20], with anticancer and anti-metastatic activities demonstrated in various animal models [14], only a few of them have been effectively isolated for nutraceutical purposes, and those that have, such as lunasin from soybean, exhibited no direct effect on MMPs [21]. Hence, compared to the latter, these new polypeptides from *L. albus* could be among the very few effective protein MMPIs found in legume seeds and may display various advantages as well as ease in isolation in larger amounts. Therefore, acknowledging that isolation and characterization of these polypeptides/proteins may open novel possibilities in the field of inflammatory diseases and cancer prevention, the goal of the present study is to identify and characterize these MMPIs from *L. albus* seeds and to evaluate their activities towards MMP-2 and MMP-9 *in vitro* and *in vivo*.

2.2. Results and Discussion

2.2.1. Isolation of the L. albus MMPI

In order to isolate the protein fractions found to be responsible for MMP-9 inhibition, the soluble protein pool of *L. albus* was fractionated by size-exclusion chromatography (SEC) and the individual fractions subsequently tested for inhibitory activity upon commercial MMP-9. Figure 1 shows the SEC protein profile obtained for the *L. albus* total soluble protein, with the corresponding polypeptide profiles of the collected fractions (1 to 6) analyzed by electrophoresis and their respective MMP-9 inhibitory activities as assessed by the Dye-quenched (DQ)-gelatin assay.

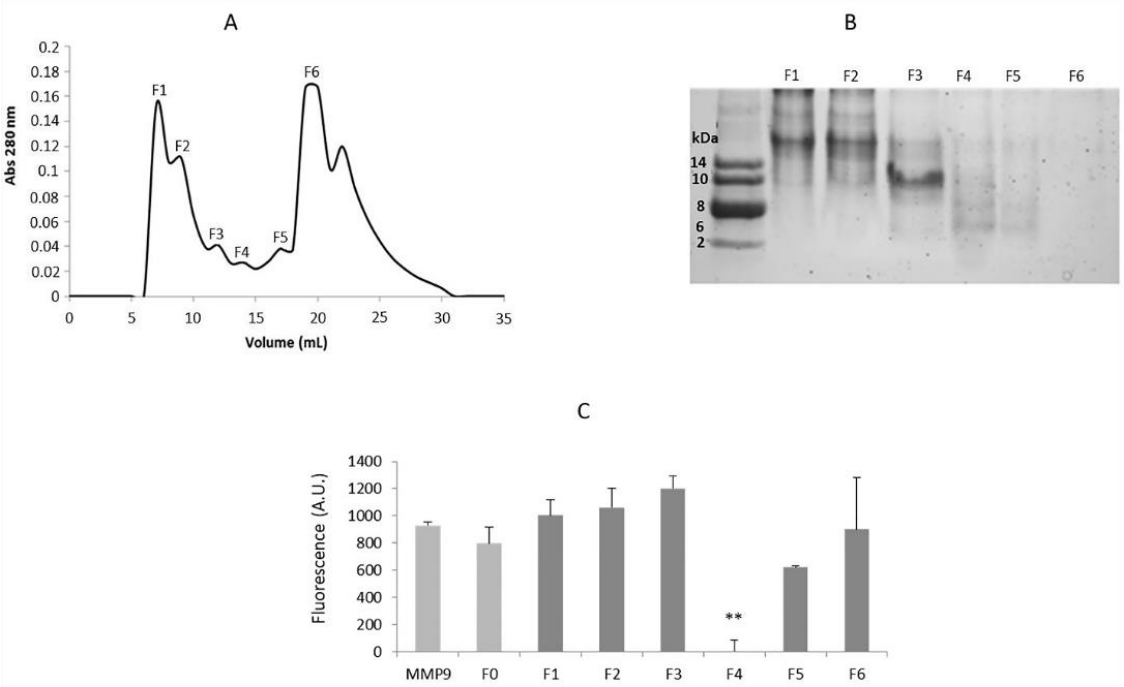

Figure 1. *L. albus* protein profile and corresponding MMP-9 inhibitory activity. (**A**) Size exclusion chromatography (SEC) in a Superdex 75 column of *L. albus* cotyledon total protein extracts. (**B**) Protein peaks were collected as fractions 1 to 6 and analyzed for polypeptide composition by SDS-PAGE. (**C**) MMP-9 proteolytic activity of fractions 1 to 6 obtained by SEC, as quantified by the DQ-gelatin method. Results are expressed in arbitrary units of fluorescence and represent an average of three replicates ± SD. ** $p < 0.001$ when compared to control.

As observed in Figure 1, throughout the protein profile shown in Figure 1A, only fraction F4 presented significant MMP-9 inhibition ($p < 0.001$) and corresponded to a low molecular mass fraction (<20 kDa), which is in accordance with the study previously presented by Lima et al. [17], where reverse zymography results suggested that the MMPI protein/polypeptide had a molecular mass lower than 20 kDa.

2.2.2. Nature and Composition of the L. albus MMPI

The SEC fraction 4 (Figure 1A) was further studied. Figure 2 shows the isolation and mass characterization of the *L. albus* MMPI comprising three steps: I—Isolation through high performance liquid chromatography (HPLC); II—1D and 2D electrophoretic separation of the selected peak in reducing and non-reducing conditions; and III—mass determination using SEC and MALDI-TOF MS.

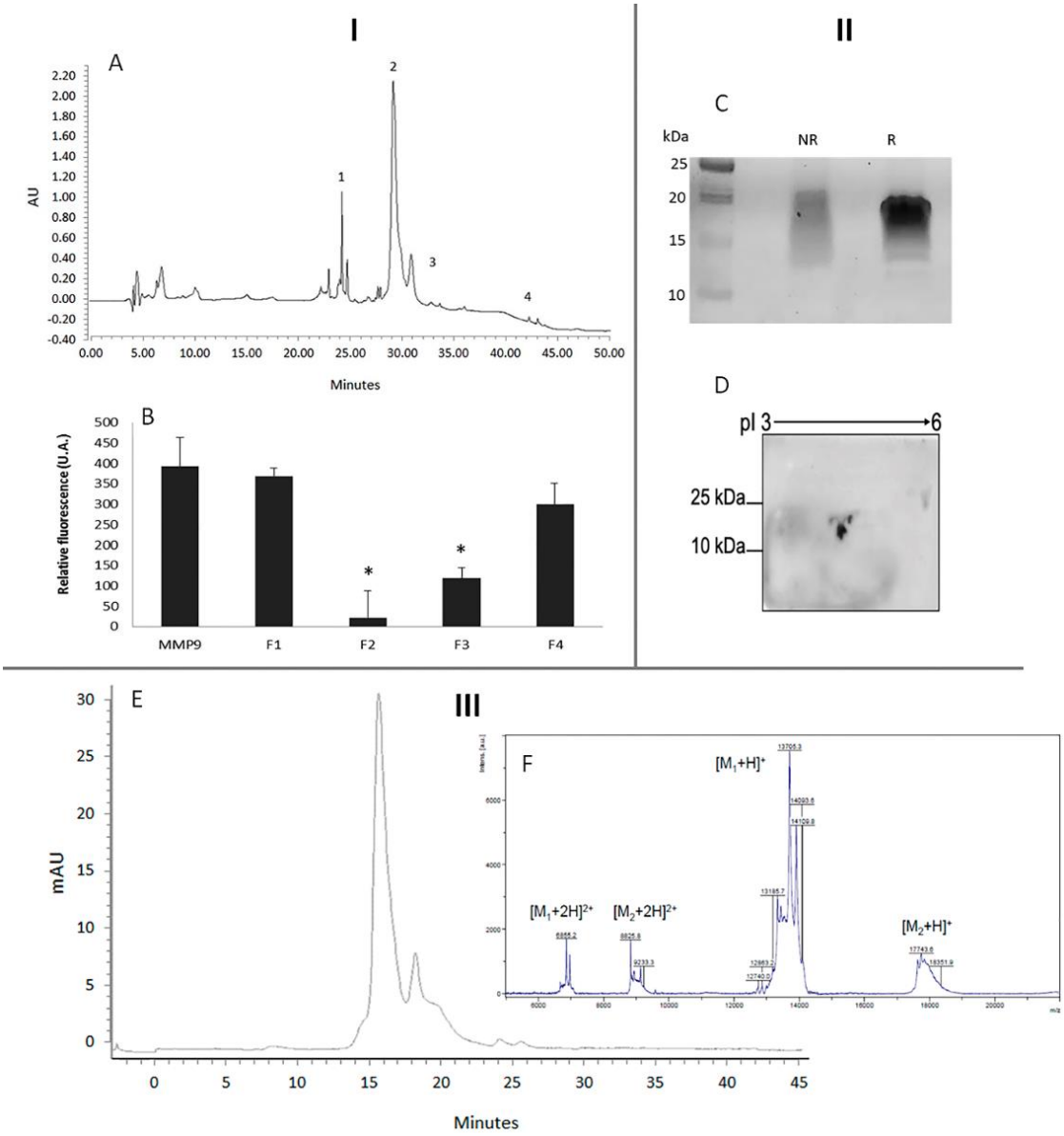

Figure 2. Characterization of the *L. albus* MMP-9 inhibitory protein. I: HPLC separation and peak purification of fraction 4 obtained by SEC (Figure 1A): (**A**) Reverse phase HPLC chromatographic profile of the MMP-9 inhibitory protein fraction 4 previously isolated from *L. albus* cotyledons by gel filtration. The main peaks obtained were collected as fractions 1 to 4. (**B**) MMP-9 proteolytic activity of fractions 1 to 4 in A, as quantified by the DQ-gelatin method. The results are expressed in arbitrary units of fluorescence and represent an average of three replicates ± SD. II: Electrophoretic analyses: (**C**) The polypeptide profile of fraction 2 in A was analyzed by denaturing electrophoresis performed under non-reducing (NR) and reducing (R) conditions. Representative image of the polypeptide composition of isolated MMPI from *L. albus* cotyledons separated by SDS-PAGE. (**D**) Two-dimensional electrophoretic separation of the isolated MMPI using 2D-GE IPG pH 3 to 6, 7 cm long, followed by SDS-PAGE 17.5% (*w/v*) acrylamide. III: Mass

determination: (**E**) Size exclusion chromatography of the isolated MMPI (fraction 2 in A) performed under non-denaturing conditions, showing its mass. (**F**) Intact protein analysis of the isolated MMPI by MALDI TOF MS.

Separation of the SEC fraction 4 (Figure 1) by RP-HPLC chromatography yielded different peaks, with different effects on MMP-9, with peak 2 presenting the highest inhibitory activity, as demonstrated by Figure 2B (Figure 2—I). This peak (RP-HPLC fraction 2) was further analyzed by 1D electrophoresis, under reducing and non-reducing to determine the potential presence of disulphide bonds and 2D electrophoresis. The results are shown in Figure 2C,D (Figure 2—II) and show the presence of different polypeptide bands in both conditions, suggesting an oligomer with the presence of disulphide bonds—possibly one or more interchain disulphide bonds—as judged by the difference in polypeptide patterns between lanes NR and R in Figure 2C, where the MMPI band does not present marked differences in mass when exposed to reducing or non-reducing conditions. It is noteworthy to observe that the protein band mass presented here is around 20 kDa, which seems to differ from Figure 1B which suggests a protein band of around 14 kDa; however, tricine-SDS PAGE would present a higher resolution of the low molecular weight bands, which can explain the apparent difference in masses.

For mass determination, MALDI-TOF analysis was also performed, and SEC chromatography was also used in order to identify its molecular mass (Figure 2—III). The MALDI-TOF analysis of the selected MMPI fraction is shown in Figure 2F and shows the presence of two groups of approximately 13 and 17 kDa mass fragments composed of several homologous fragments with slightly different masses, which agrees with 2D analysis (Figure 2D). On the other hand, SEC mass determination showed two major peaks with a mass around 20 and 60 kDa, which could suggest the presence of an oligomer. These features make this MMPI different from the previous protein inhibitors found in legume seeds.

The two spots of the MMPI fraction obtained by 2D electrophoresis were named AL1 and AL 2, and they were collected and sequenced by MS analysis. The sequence results for both spots are described below in Figure 3.

Figure 3. Amino-acid sequence of the *L. albus* MMPI. Mass spectrometry analysis of the isolated MMPI spots obtained from 2D analysis, AL 1, and AL2, respectively, as demonstrated on the top right. The matched peptides are shown in red.

MS analysis subsequently demonstrated that both spots obtained from the isolated MMPI fraction (AL1 and AL2) of *L. albus* had similar compositions and comprised the same mixture of a β-conglutin fragment and δ-conglutin large chain in both spots. Since under RP-HPLC separation conditions, both β-conglutin and δ-conglutin could be denatured, and the presence of fragments of these two storage proteins is not surprising. Moreover, if we consider the microheterogeneity that characterizes legume seed storage proteins and, in particular, the extremely complex post-translation modifications that β-conglutin undergoes from it(s) precursor(s), the fragment of β-conglutin fragment is also unsurprising [22]. The fact that both groups of fragments seem to be indissociable by chromatographic and electrophoretic procedures can suggest the presence of an oligomer, although, as far as it has been described by the literature, β-conglutins and δ-conglutins are two different proteins that are evolutionary distant. Nonetheless, it is important to consider that the first initial protein fraction by SEC was not under denaturing conditions and had the predicted molecular mass whilst presenting MMPI activity, and the same was found through reverse zymography in Lima et al. [17].

On one hand, if we consider these storage protein's features, the most likely hypothesis is that the *Lupinus* MMPI is δ-conglutin, because (a) it is the protein present in larger amounts in the MMPI fraction and (b) it holds several features that would render it a good MMPI. Belonging to the 2S sulphur-rich albumin family [23], δ-conglutin is a monomeric protein that comprises two small polypeptide chains linked by two interchain disulfide bonds: a smaller polypeptide

chain, which consists of 37 amino acid residues resulting in a molecular mass of 4.4 kDa, and a larger polypeptide chain containing 75 amino acid residues with a molecular mass of 8.8 kDa [24]. The larger polypeptide chain contains two intrachain disulfide bridges and one free sulfhydryl group [24]. This could tentatively explain the slight difference in apparent molecular mass detected between R-PAGE and NR-SDS-PAGE of the isolated MMPI (Figure 2C). This protein presents specific inherent unique features among the proteins from *L. albus*: In addition to its high cysteine content, it exhibits low absorbance at 280 nm. As far as the physiological role of δ-conglutin is concerned, a storage function has been proposed for this class of proteins. However, structural similarity with the plant cereal inhibitor family, which includes bi-functional trypsin/alpha-amylase inhibitors, may suggest a defense function for this protein in addition to its storage role [25] and might corroborate its role as MMPI. Furthermore, the presence of free sulfhydryl groups in δ-conglutin could be related to a high degree of affinity towards the Zn^{2+}-containing active site in MMPs and could explain its mode of inhibition. Indeed, one way to isolate δ-conglutins is by Zn precipitation [25]. Since MMP enzymes are zinc-dependent proteases, it is likely that the *L. albus* MMPI mechanism of action involves the MMP's zinc core. This could, therefore, suggest that 2s-albumins present in the seeds of several other species, such as legumes, could have the same ability to reduce MMPs. This hypothesis is substantiated by the previous results obtained in Lima et al. [17] where similar MMPI activities, in similar molecular weight albumin fractions, were found in other legume species.

On the other hand, some features of β-conglutin might also substantiate the hypothesis that it also could play a role in MMP-9 binding. Albeit being a trimeric protein devoid of disulphide bridges in which the monomers consist of a very large number of polypeptides, glycosylated or not, ranging from 16 to over 70 kDa, a large number of post-translational proteolytic processing sites give rise to the abundance of 7S mature polypeptides observed [26]. According to Duranti, Cucchetti, and Cerletti [27], the full post-germination degradation of this protein strongly supports a storage function for β-conglutin. Nonetheless, such an extensive range of post-translation modifications is known to generate small peptides that do not end up in the composition of mature β-conglutin but have functional activities in the seed. For example, BCO (Blad-containing oligomer) is one oligomer of β-conglutin polypeptide fragments known for its potent bioactivities against fungi. It is a stable breakdown product of β-conglutin proteolysis following germination and accumulates in *Lupinus* cotyledons from days four until 12 to 14 following the onset of germination. Its major and bioactive polypeptide, Blad, is an abundant, transient polypeptide chain of 20.4 kDa, almost exactly coincident with the first cupin domain of β-conglutin precursor and displaying lectin-like activity [28]. Being highly reactive and containing two bioactive cupin domains, it is possible that there are many fragments of β-conglutin precursors (e.g., fragments of its second cupin domain) with specific uncharted activities yet to be discovered. Previous investigations in our study revealed, however, that the MMPI from *L. albus* has, unlike Blad, neither constitute antifungal nor bactericide activities (results not shown), and the sequence of the β-conglutin fragments comprising MMPI does not match that of Blad.

Another interesting fact to consider is that α-conglutins, β-conglutins and γ-conglutins (but not δ-conglutin) have been demonstrated to precipitate with Ca^{2+} and Mg^{2+} in an electrostatically dependent manner [29]. Indeed, our previous studies showed that the MMPI

isolated from *L. albus* fragments can be separated with the addition of Ca^{2+} and Mg^{2+} and upon this separation both fractions lose their ability to inhibit MMP-9, but when combined once again, they regain their full MMPI inhibitory potential. This suggests that both fragments (β-conglutins and δ-conglutin) may have the potential for MMP-9 inhibition and might be more active when combined in the same fraction.

Whether a novel oligomer or not, this is still, as far as we are concerned, the first report of a direct MMP-9 inhibitory activity from legume storage proteins, rendering them different from the previous protein protease inhibitors such as serine protease Bowman–Birk inhibitors and Kunitz-type inhibitors [30] and opening up novel perspectives on the role of legumes in cancer and inflammatory diseases' prevention.

In order to evaluate if this MMPI fraction from *L. albus* is suitable for preventive/curative diets, we tested it against MMP-2 and MMP-9 using *in vitro* and *in vivo* models.

2.2.3. The L. albus MMPI Inhibits Both MMP-2 and MMP-9 In vitro, in a Dose-Dependent Manner, without Impairing Cell Viability or Gene Expression

Figure 4 shows the *in vitro* effect of the *L. albus* MMPI on HT29 cells, namely on total and specific gelatinase activity (Figure 4A,B), cell proliferation (Figure 4C), and MMP-2 and MMP-9 gene expression (Figure 4D). A set of four different concentrations of the isolated MMPI (100, 50, 10, and 5 µg/mL) was tested *in vitro* for total MMP-2 and MMP-9 inhibition using the DQ-gelatin method, as displayed in Figure 4A.

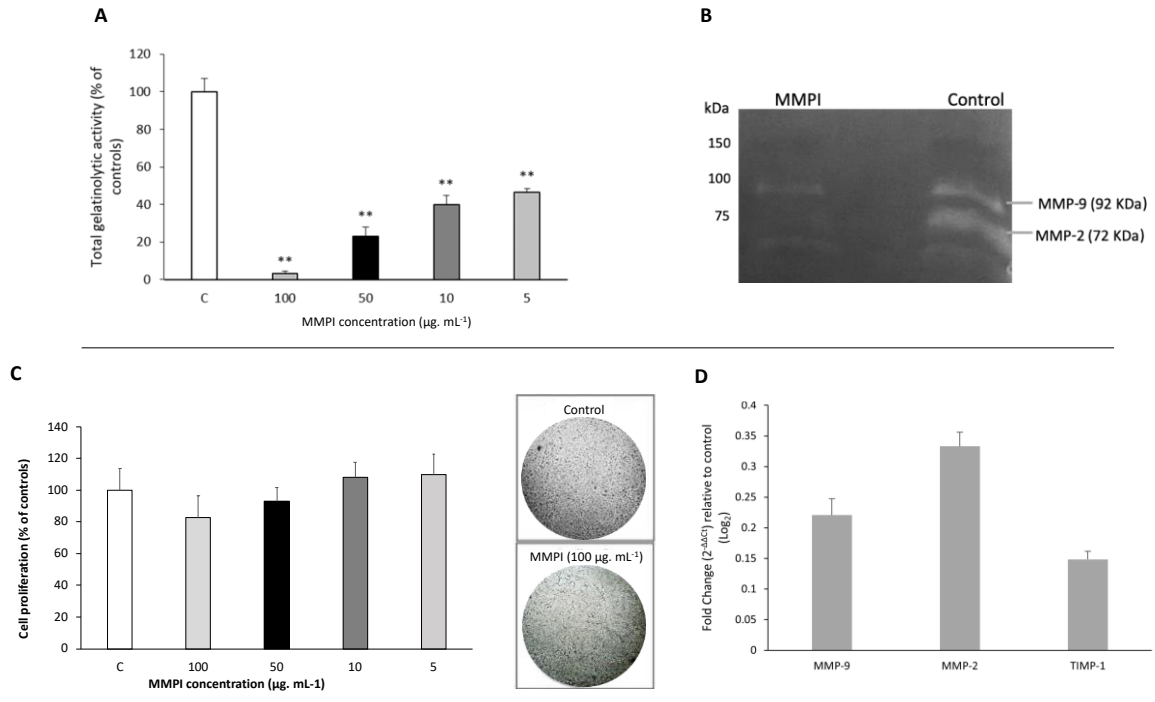

Figure 4. *L. albus* MMPI effect on MMP-9 and MMP-2 activity, cell proliferation, and gene expression in HT29 cells. (**A**) Dose-effect of the isolated *L. albus* MMPI on total gelatinolytic activity. The isolated MMPI was added at concentrations of 100, 50, 10, and 5 µg/mL and gelatinolytic activity was measured by the DQ fluorogenic assay. Gelatinase activities are expressed as percentage of controls. (**B**) Representative image of the zymographic profiles of specific MMP-9 and MMP-2 activities. White bands are consistent with higher gelatinolytic activities. HT29 cells were exposed to 50 µg/mL of the MMPI, and extracellular extracts were loaded on 12.5% (*w*/*v* acrylamide) polyacrylamide gels co-polymerized with 1% (*w*/*v*) gelatin.

(**C**) HT29 cell growth after 48-hour exposure to different concentrations of the *L. albus* MMPI and representative picture of HT29 cells morphology in controls and in the highest MMPI concentration. Cells were grown in the presence of 100, 50, 10, and 5 µg protein/mL and stained with MTT. Values are expressed as a percentage of the control. (**D**) *L. albus* MMPI influence on MMP-2, MMP-9, and TIMP-1 gene expression. Cells were grown in the presence of 50 µg protein/mL and transcripts were quantified by real-time PCR (RT-qPCR). Relative gene expression values are presented as $\log^2$-fold-change values in relation with the control conditions, using as reference gene Beta-actin. Data were normalized in relation to controls and the graphic is expressed as $2^{-\Delta\Delta Ct}$ values. All values represent the averages of at least three replicate experiments (n = 3) ± SD in each assay. ** $p < 0.001$ when compared to controls.

Figure 4A shows that the isolated *L. albus* MMPI is able to significantly reduce the activities of both MMP-2 and MMP-9 in HT29 cells, as observed in Figure 2B, which agrees with the results obtained in Lima et al. [17]. Moreover, it very significantly inhibited total gelatinase proteolytic activity ($p < 0.001$) in a dose-dependent manner, as tested by the DQ-gelatin assay (Figure 4A), with the highest concentration of 100 µg/mL inducing a gelatinase reduction greater than 90%. IC50 values determined for gelatinolytic inhibition were 10 µg/mL, which, when compared to other foodborne MMP inhibitors, for example, lunasine in soy [21] or some well-known MMPI phenolic compounds such as curcumine [31] or trans-resveratrol [32], *L. albus* MMPI appears to be more potent. Figure 4C illustrates HT29 cell viability in the presence of different concentrations of the MMPI isolated from *L. albus* (100, 50, 10, and 5 µg/mL) determined after MTT staining (which can only be metabolized by living cells). Results show that the isolated MMPI did not induce a significant reduction ($p > 0.001$) in cell growth and viability when compared to controls. Furthermore, there were no visible cytotoxic effects.

It is well known that many MMP-inhibitors act upon specific signaling pathways that participate in genetic targets involved in the specific cancer under study. For example, curcumin, a well-known food-borne MMP-9 inhibitor, acts by downregulating MMP-2 and MMP-9 expression [33] and NF-κB activity [34]; trans-resveratrol inhibits MMP-induced differentiation via the p38 kinase and JNK pathways in HTB94 chondrosarcoma cells [34, 35]; and lunasin from soy downregulates MMP-2 and MMP-9 expression via FAK/Akt/ERK and NF-κB signalling pathways [21]. Although DQ-gelatin assays show a direct inhibition of MMP-9 by the lupin MMPI, we further set out to test whether it had any influence on the expression of MMP-2 and MMP-9 genes as well as their tissue inhibitor TIMP-1 in HT29 cells. Results are present in Figure 4D. The fold change in the target gene is normalized to β-actin (control gene) (ΔCT) and then reported to a control sample (untreated) (ΔΔCT). Then, the fold change from the sample to untreated conditions is calculated ($2^{-\Delta\Delta Ct}$), allowing us to know how many times the gene is more expressed than the control. Using this method, we plotted the graphic with the $2^{-\Delta\Delta Ct}$ values. When upregulated in relation to the control situation, the values are in the positive side of the Y-axis and when downregulated, they are in the negative part of the axis. Hence the represented results show a tendency to upregulate MMP-2 and MMP-9, although only MMP-2 was significantly more expressed. Since gelatin zymography clearly shows that both enzymes are being inhibited, this result could show a tendency to upstream increase MMP-9 and MMP-2 expression as natural feedback to counter-regulate their lack of activity. TIMP-1 gene expression values, on the other hand, were not significantly altered when compared to controls

and would not pose any physiological significance. These findings might, therefore, support the notion that the isolated *L. albus* MMPI certainly does not reduce MMP expression but acts directedly on both gelatinases (which agrees with our earlier results in Figure 1). This could be of significant importance as an MMPI that acts directly upon MMP-2 and MMP-9 and that has no significant effects on gene expression and cell viability is more desirable because it could yield fewer secondary effects, particularly in the case of gut-related cancer pathologies where it could act in situ.

2.2.4. The L. albus MMPI Inhibits MMP-2 and MMP-9 In vivo and Reduces Colitis Injuries

In order to ascertain the effects of the isolated MMPI *in vivo* and considering that MMPIs are known as IBD reducing agents [5,8–10], we tested its effect on mice with TNBS-induced colitis by using oral administrations 3 h after colitis induction. Table 1 shows the effect of the isolated MMPI on colon length (cm) and on the extent of intestine injury (cm).

Table 1. Morphological and functional observations in clean and transversely opened colons after harvest. Effect of the *L. albus* MMPI administration on the length of colon (cm). Sham group (n = 6), TNBS group (n = 10); TNBS + MMPI p.o. (15 mg/kg; n = 9). The severity scoring of diarrhea is as follows: normal = 0; lightly soft stools = 1; soft stools = 2; liquid stools = 3. $^{\#}$ $p < 0.05$ vs. Control; * $p < 0.05$ vs. TNBS.

	Length of colons	**Size of lesion**	**Presence/consistency of diarrhea**
Control	14.5 ± 0.082	0	0
TNBS + EtOH 50%	11.8 ± 0.19$^{\#}$	3.6 ± 0.14$^{\#}$	3$^{\#}$
TNBS + MMPI p.o.	14.8 ± 0.33*	2.44 ± 0.84	1.13 ± 0.35*

Results show, as expected, that the animals in the control and ethanol groups exhibited no macroscopical signs of colon injury and presented no mortality, whilst intracolonic injection of TNBS resulted in a very significant ($p < 0.05$) decrease in colon length and an increase in the extent of visible injury (ulcer formation). However, p.o. administration of the *L. albus* MMPI resulted in an overall reduction in colon inflammation, with a significant attenuation of colon length reduction ($p < 0.05$), a significant reduction in the extent of visible ulcer formation ($p < 0.05$), and a significant reduction in the degree and severity of diarrhea ($p < 0.05$). Hence, overall, these results do not only corroborate the anti-inflammatory activities of the MMPI *in vivo* but also demonstrate that it is able to maintain its biological activity throughout the digestive process.

The activity of MMP-2 and MMP-9 in the observed colons is depicted in gelatin zymography Figure 5A and in the respective densitometric analysis (Figure 5B).

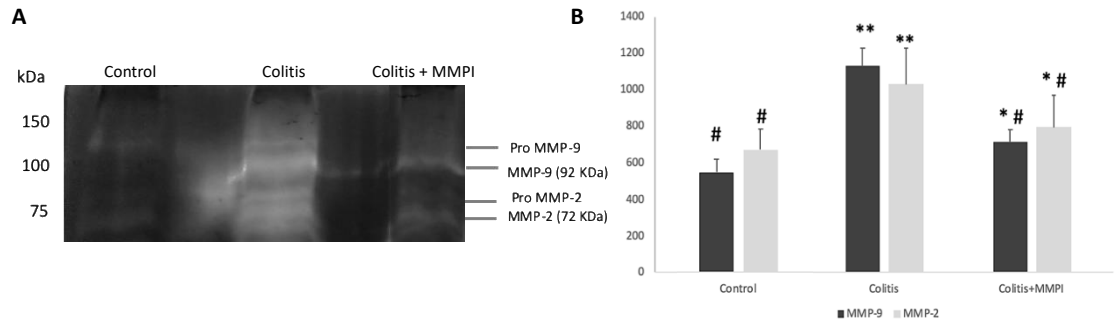

Figure 5. Effect of the *L. albus* MMPI administration on the colon tissue gelatinase activities of MMP-2 and MMP-9 from colitis-induced mice. (**A**): Representative image of the zymographic profiles of MMP-9 and MMP-2 activities of the colons. Protein extracts of the colon were loaded on 12.5% (*w*/*v* acrylamide) polyacrylamide gels co-polymerized with 1% (*w*/*v*) gelatin. (**B**): Densitometric analysis of the gelatinolytic activity of MMP-9 and MMP-2 obtained in the zymographies. Control group (n = 6); Colitis group (n = 10); Colitis + MMPI =colon from animals treated with *L. albus* MMPI (15 mg.kg^{-1}, n = 9, p.o.). Results are average of at least three replicates. $^{\#}$ $p < 0.05$ vs. Colitis; * $p < 0.05$ vs. Control and ** $p < 0.001$ vs. Control.

Results demonstrated that, as expected, TNBS-induced colitis significantly increased MMP-9 and MMP-2 activities ($p < 0.05$), demonstrated by the high intensity of the white bands, in both the active as well as in the inactive forms of the enzymes, whereas in controls a low activity of the active forms of MMP-2 and MMP-9 was observed (Figure 5A). With *L. albus* MMPI oral administration, there was an evident significant reduction in MMP-2 and MMP-9 activities ($p < 0.05$), but particularly in their active form, when compared to the TNBS group. These results are consistent with the data obtained from Table 1 and corroborate that oral administration of the *L. albus* MMPI inhibited the colitis-induced rise in gelatinolytic activities observed in animal models, leveling them to physiological and morphological levels closer to those observed in healthy controls. Furthermore, the fact that the isolated MMPI maintained its biological activity throughout the digestive process in oral treatments substantiates its potential use in a dietary approach.

2.2.5. L. albus MMPI May Present the Features of an Ideal MMP-9 Inhibitor for Gut-Related Diseases

The discovery that gelatinases are highly upregulated in several pathological processes has spurred the development of MMP inhibitors, whilst rendering them attractive targets for therapeutic intervention. However, a long history of unsuccessful clinical trials has demonstrated limited clinical utility for MMPIs because of several setbacks: generalized size-effects, dose-limiting toxicity, and severe side effects. One method to surpass these setbacks would be to use MMPIs capable of acting directly in loco, particularly in gut-related pathologies where the ingestion of specific MMP-2 and MMP-9 inhibitors could act directly within the digestive tract without producing any side effects. However, some major limitations should still

be surpassed, such as chemical destruction during the digestive process and absorption to the blood flow, thus inducing generalized effects. The findings presented in this study seem to suggest that novel *Lupinus* MMPI may hold the features of an ideal MMPI for dietary approaches against gut-related ailments, naturally excluding people who suffer from allergy to lupin's conglutins.

Previous results show that the fragments produced by this method are highly soluble in water and resistant to boiling heat, making it feasible to isolate them in larger quantities [19].

Our present results show that *L. albus* MMPI resists digestion and it is effective in low amounts while presenting no toxicity (hence, being suitable to use in dietary approaches) and it apparently inhibits gelatinases directly; hence, it is a good candidate for in situ approaches, particularly in the case of gastrointestinal pathologies related to aberrant gelatinase activities, such as inflammatory bowel diseases, as has been described for previous MMP inhibitors [3,8]. Although much work must be performed still, these features may prove further useful as a nutraceutical or in functional foods in the prevention and treatment of a very wide array of diseases related to MMP-9 activity.

2.3. Materials and Methods

2.3.1. Protein Isolation and Identification

Dry, mature seeds of *Lupinus albus* L. (lupin) were used in this study. The total soluble protein from approximately 100 g ± 0.1 g of dry lupin cotyledons (i.e., seed without embryo and tegument) was extracted by using 50 mM of Tris-HCl buffer, pH 7.5 (1:10, *w*/*v*). The homogenate was centrifuged at 13,500 *g* for 30 min at 4 °C.

2.3.2. Fast Protein Liquid Chromatography

Protein samples were fractionated by Fast Protein Liquid Chromatography (FPLC; GE Healthcare Life Sciences) size-exclusion chromatography (SEC) and injected into a Superdex TM 75 HR 10/30 column (GE Healthcare Life Sciences) and equilibrated with degassed 100 mM Tris-HCl buffer, pH 7.5, at a flow rate of 0.5 mL/min. Protein peaks eluted from the column were detected at 280 nm. All collected fractions were tested for their inhibitory activity on MMP-9 by using a DQ-gelatin assay (please see methods below).

For molecular mass estimation, the ÄKTA Pure chromatography system with the UV detector (GE Healthcare Life Sciences) was used. Gel filtration was performed on a Superose 12 HR 10/30 column (GE Healthcare Life Sciences) equilibrated with 100 mM Tris HCl buffer, pH 7.5, containing 50 mM NaCl. Proteins were eluted at 0.5 $mL.min^{-1}$ and the protein fractions were desalted twice on NAP-10 columns (GE Healthcare Life Sciences), lyophilized, and stored at −20 °C until required. The calibration curve was prepared with blue dextran, bovine serum albumin, chymotrypsin, and cytochrome c. All chromatographic steps were conducted at room temperature.

2.3.3. High-Performance Liquid Chromatography

The MMPI fraction isolated by FPLC-SEC and identified by the DQ-gelatin assay was fractionated in a High-Performance Liquid Chromatography (HPLC) device (Waters 2695

Separations Module) equipped with a Waters 2998 Photodiode Array Detector. Protein samples were separated in a C18 reverse phase column, Zorbax 300SB 5 µm, 250 mm × 4.6 mm. The elution was made with eluent A (0.1 % *v*/*v* Trifluoroacetic Acid (TFA)) and solvent B (acetonitrile in 0.1 % *v*/*v* TFA). Peak detection was made at 214 nm and 280 nm.

2.3.4. 1-D Electrophoresis

SEC protein fractions were separated by Tricine-SDS-PAGE in 12.5 polyacrylamide gels. Electrophoresis was performed in a vertical system, the Anode buffer was Tris-HCl, 200 mM, pH 8.9, and the cathode was 100 mM Tris, 100 mM Tricine, and 1% SDS (*m*/*v*). Samples were lyophilized and resuspended in sample buffer of 1% SDS (*m*/*v*), 8 M Urea, 1% (*v*/*v*), 1% (*v*/*v*) 2-mercaptoethanol, and 0.01 M Tris (adjusted to pH 6.8 with phosphoric acid). Samples were incubated at 60 °C for 15 min, and electrophoresis was performed at 200 V and 20 mA per gel. Protein bands were detected with Comassie G Staining, following standard procedures.
The HPLC isolated MMPI (50 µg/mL) was loaded onto a 17.5% (*w*/*v* acrylamide) polyacrylamide gel with denaturing, reducing buffer (100 mM Tris-HCl buffer, pH 6.8, containing 100 mM β-mercaptoethanol, 2% *w*/*v* SDS, 15% *v*/*v* glycerol, and 0.006% *w*/*v* m-cresol purple) or denaturing, non-reducing buffer (100 mM Tris-HCl buffer, pH 6.8, containing 2% *w*/*v* SDS, 15% *v*/*v* glycerol, and 0.006% *w*/*v* m-cresol purple). One-dimensional electrophoresis was carried out following the method described by Lima et al. [17].

2.3.5. 2-D Electrophoresis

The 2D-gel electrophoresis and spot digestions were carried out as described previously [36]. Isoelectric focusing IPG-Strips pH 3 to 6, 7 cm long and 17.5% (*w*/*v*) acrylamide SDS-PAGE gels were used.

2.3.6. MALDI-TOF TOF

The intact isolated MMPI was analyzed by MALDI TOF MS using an Ultraflex II MALDI-TOF TOF Bruker-Daltonics equipped with a LIFT cell and N2 laser. The mass spectrometer was operated with positive polarity in linear mode and spectra were acquired in the range of *m*/*z* 5000–20,000. A total of 1000 spectra were acquired at each spot position at a laser frequency of 50 Hz. External calibration was performed using a protein calibration standard I from Bruker: [M + H]+ of insulin (5734.51 *m*/*z*), ubiquitin I (8565.76 *m*/*z*), cytochrome c (12,360.97 *m*/*z*), and myoglobin (16,952.30 *m*/*z*); [M + 2H]2+ of cytochrome c (6180.99 *m*/*z*) and myoglobin (8476.65 *m*/*z*).

2.3.7. MS/MS Analysis

Prior to ESI MS/MS analysis, all samples were diluted with 100 µL of 0.1% (*v*/*v*) aqueous formic acid containing 3% (*v*/*v*) acetonitrile before loading onto an EASY-nLC II equipped with an EASY-column, 2 cm, ID 100 µm, 5 µm, C18-A1 (Thermo Fisher Scientific) and an EASY-column, 10 cm, ID 75 µm, 3 µm, C18-A2 (Thermo Fisher Scientific). Chromatographic separation was carried out using a multistep linear gradient at 300 nL/min (mobile phase A: aqueous formic acid 0.1% *v*/*v*; mobile phase B: 90% *v*/*v* acetonitrile and 0.1% *v*/*v* formic acid),

0–90 min linear gradient from 0% to 35% of mobile phase B, 90–115 min linear gradient from 35% to 90% of mobile phase B, and 115–120 min isocratic flow at 90% of mobile phase B.

Selected isolated peaks were analyzed on a 5600 TripleTOF mass spectrometer (ABSciex®) in information-dependent acquisition (IDA) mode. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column ChromXPTM C18CL reverse phase column (300 µm ID × 15 cm length, 3 µm particles, 120 Å pore size, Eksigent®) at 5 µL/min. Peptides were eluted into the mass spectrometer with a multistep gradient: 0–2 min linear gradient from 5 to 10%, 2–45 min linear gradient from 10% to 30% and, and 45–46 min to 35% of acetonitrile in 0.1% (*v*/*v*) TFA. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray™ Source, AB Sciex) with a 50 µm internal diameter stainless steel emitter (New Objective). Protein identification was obtained using Protein Pilot™ software (v 5.0, ABSciex®).

2.3.8. MMP-9 and MMP-2 Catalytic Activities

2.3.8.1. DQ-Gelatin Assay

The fluorogenic substrate DQ-gelatin assay was performed as described by Lima et al. [17].

2.3.8.2. Gelatin Zymography

In order to determine specific metalloproteinase activities, gelatin-zymography was performed according to standard methods [17,37], with the following modifications: SDS-polyacrylamide gels (12.5% *w*/*v* acrylamide) were copolymerized with 1% (*w*/*v*) gelatin. HT29 cancer cell culture supernatants treated with a non-reducing buffer containing 62.6 mM Tris-HCl pH 6.8, 2% (*w*/*v*) SDS, 10% (*v*/*v*) glycerol, and 0.01% (*w*/*v*) bromophenol blue were loaded into each well of the SDS-gel. Electrophoresis was carried out as described before [17] in a 12.5% (*w*/*v*) acrylamide resolving gel and a 5% (*w*/*v*) acrylamide stacking gel, performed in a vertical electrophoresis unit at 200 V and 20 mA per gel. After electrophoresis, gels were washed three times in 2.5% (*v*/*v*) Triton X-100 for 60 min each and were incubated 48 h with a specific buffer solution (50 mM Tris-HCl pH 7.4, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, and 0.01% *w*/*v* sodium azide), stained with Coomassie Brilliant Blue G-250 as described by Lima et al. [17]. White bands visible against a blue background marked the gelatinase activity of each proteinase [37]. Protein band intensities were determined by densitometry, as described previously [17].

2.3.9. In vitro Colon Cancer Cell Assays

3.9.1. HT29 Cell Cultures

The human colon adenocarcinoma cell line, HT29 (ECACC 85061109), obtained from a 44-year-old Caucasian female was used. HT29 cells were maintained according to Lima et al. [17].

2.3.9.2. Cell Viability Assay

HT29 cultured cells were seeded in 96-well plates (2×10^4 cells/well) and MMPI sample was added to the growth medium at different concentrations (100, 50, 10, and 5 µg/mL) concentration and incubated for 48 h. The extracellular medium was collected, and the wells washed with PBS to remove unattached cells. Cell proliferation and viability was determined

using the standard MTT assay as described before, with few alterations [17]. Briefly, 50 µL of serum-free media and 50 µL of MTT solution were added into each well and incubated at 37 °C for 3 h. After incubation, 100 µL of DMSO was added into each well. Absorbance was read at 590 nm.

2.3.10. Assessment of Gene Expression by Quantitative Real-Time PCR (RT-qPCR)

RNA Extraction and cDNA Synthesis

Total RNA was extracted from HT29 cells using the NZY Total RNA Isolation kit as described by the manufacturer. Quantification was carried out in a Synergy HT Multiplate Reader, with Gene5 software, using a Take3™ Multi-Volume Plate (Bio-Tek Instruments Inc., Winooski, VT, USA). For reverse transcription, the RevertAid reverse transcriptase priming with oligo-d(T) kit was used according to the manufacturer's recommendations (Thermo Scientific, Waltham, MA, USA).

For each gene studied (MMP-9, MMP-2, and TIMP1), a set of specific primers was designed and used to amplify HT29 cDNA resulting from the transcription of 2 µg of total RNA by using conventional PCR and gel agarose electrophoresis. When amplification was observed, confirming the expression, the transcripts were quantified by real-time PCR (RT-qPCR), which was performed in 20 µL reaction volumes composed cDNA derived from 2 µg RNA and 0.5 µM gene-specific primers (Online Resource 1) in SsoFast™ EvaGreen® Supermixes (Bio-Rad, Hercules, CA, USA) using an iQ5 Real-Time Thermal Cycler (BioRad, Hercules, CA, USA). Reaction conditions for cycling were as follows: 95 °C for 3 min followed by 40 cycles at 95 °C for 10 s, 61 °C for 25 s, and 72 °C for 30 s. Each analysis was performed in triplicate reactions of three biological replicates. The corresponding quantification cycles (Cq) were determined by the iQ5 optical system software (Bio-Rad, Hercules, CA, USA) and exported to a MS Excel spreadsheet (Microsoft Inc., Redmond, WA, USA) for further analysis. Cq values of each gene of interest were normalized with respect to actin (Act) [38].

2.3.11. In vivo Animal Model of Colitis

2.3.11.1. Animals

Male CD-1 mice, 25 to 30 g in weight and 5 to 6 weeks of age (Harlan Iberica, Barcelona, Spain), were housed in standard polypropylene cages with ad libitum access to food and water inside a controlled environment room kept at 22 °C ± 1 °C with a 12 h light and 12 h dark cycle at the Faculty of Pharmacy Central Animal Facility, University of Lisbon, Lisbon, Portugal.

2.3.11.2. Animal Care and Maintenance

Experiments were performed by agreeing to the most recent rules and recommendations for the care and processing of laboratory animals, namely to the presently adopted European Commission regulations (Directive 2010/63/EU). In addition, the studies were performed in agreement with the ARRIVE Guidelines for Reporting Animal Research. The Ethics Committee of the Faculty of Pharmacy (University of Lisbon) also endorsed the experimental protocol (0019/2018; date of approval: 27 February 2018).

2.3.11.3. Induction of Colitis

TNBS was instilled as an intracolonic single dose as previously described [39]. On induction day (day 0), mice were anesthetized with ketamine (100 mg/kg) and xilazine (10 mg/kg). Then, 100 µL of TNBS solution was administered with a catheter inserted 4.5 cm into the colon. Mice were kept for 30 min in a Trendelenburg position to avoid reflux. Four days after induction, mice were euthanized by cervical dislocation and necropsied. The colon was removed, freed from surrounding tissues, and opened longitudinally for observation and classification of diarrhea severity. Subsequently, the colon was washed with PBS for macroscopical observation of the tissue.

2.3.11.4. Experimental Groups

Animals were randomly allocated into four experimental groups as described below.

1. Control group (*n* = 6): Animals were subjected to the procedures described above except for intracolonic administration which comprised 100 µL of saline solution. During the 4 days of the protocol, the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.
2. Ethanol (EtOH) group (*n* = 6): Animals were subjected to the procedures described above except for intracolonic administration with 100 µL of 50% (*v*/*v*) ethanol. During the 4 days of the protocol, the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.
3. TNBS group (*n* = 10): Animals were administered with 100 µL of 2.5% (*w*/*v*) TNBS in 50% (*v*/*v*) ethanol. During the 4 days of the protocol, the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.
4. TNBS + Lupinus MMPI p.o. (*n* = 9): Animals were administered with 100 µL of 2.5% (*w*/*v*) TNBS in 50% (*v*/*v*) ethanol. During the 4 days of the protocol, the animals were administered orally with the isolated MMPI (15 mg/kg).

Oral administrations were performed daily by gastric gavage, starting from 4 h after the initial administration of TNBS.

2.3.11.5. Macroscopic Evaluation of Colitis Severity

After colon removal, a longitudinal incision was performed for observation of content and classification of diarrhea severity by an observer blinded regarding the experimental groups. Afterwards, the colon was rinsed with saline and analyzed with a surgical microscope for closer observation of the tissues. The colon length was then measured, as well as the extent of the injuries (if present).

2.3.11.6. Gelatin Zymography of Colon Extracts

Extraction of colon proteins was performed according to Castaneda et al. [40]. In order to determine specific metalloproteinase activities in colon protein supernatants, a gelatin-zymography was performed as described before [17] with the following modifications: SDS-polyacrylamide gels (12.5% *w*/*v* acrylamide) were copolymerized with 1% (*w*/*v*) gelatin. Colon polypeptide extracts previously treated with a denaturing, non-reducing buffer containing 62.6 mM Tris-HCl pH 6.8, 2% (*w*/*v*) SDS, 10% (*v*/*v*) glycerol, and 0.01% (*w*/*v*) bromophenol blue

were loaded into each well of the SDS-gel. Electrophoresis was carried out as described before (Section 3.8.2).

2.3.12. Statistical Analysis

All experiments were performed in triplicate in at least three independent times and the data are expressed as the mean ± standard deviation (SD). SigmaPlot software (version 12.5) was used for comparing different treatments, using one-way and two-way analysis of variance (ANOVA). Tukey's test was used to compare differences among groups and the statistical differences with *p* values lower than 0.05 were considered statistically significant.

2.4. Conclusions

Here, we have shown novel MMP-2 and MMP-9 inhibitory activity from protein components of lupine, which were found to be active *in vivo* when administered orally, whilst alleviating the symptoms of induced colitis in mice. These results open novel perspectives on the role of storage proteins in *Lupinus* and other legume seeds as possible nutraceutical or functional foods in the prevention and treatment of diseases related to MMP-9 activity.

2.5. References

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Chapter 3. Lupin Seed Protein Extract Can Efficiently Enrich the Physical Properties of Cookies Prepared with Alternative Flours

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Status: *Foods,* 2020, 9(8), 1064

DOI: 10.3390/foods9081064

Abstract: Legume proteins can be successfully used in bakery foods, like cookies, to obtain a protein-enriched product. A lupin extract (10 g/100 g) was added to gluten and gluten-free flours from different sources: rice, buckwheat, oat, kamut and spelt. The impact on the physical properties of the dough and cookies was evaluated for the different systems. Rice and buckwheat doughs were 20% firmer and 40% less cohesive than the others. The incorporation of lupin extract had a reduced impact on the shape parameters of the cookies, namely in terms of area and thickness. The texture differed over time and after eight weeks, the oat and buckwheat cookies enriched with lupin extract were significantly firmer than the cookies without lupin. The incorporation of lupin extract induced a certain golden-brown coloring on the cookies, making them more appealing: lightness (L*) values decreased, generally, for the cookies with lupin extract when compared to the controls. The a_w and moisture content values were very low for all samples, suggesting a high stability food product. Hence, the addition of lupin extract brought some technological changes in the dough and cookies in all the flours tested but improved the final product quality which aligns with the trends in the food industry.

Keywords: white lupin seeds extract; snack; protein; texture; viscoelastic behavior

3.1. Introduction

Current trends in the development of new food products identified by companies in consumer studies, such as Innova Market Insights, are gluten-free products, alternative vegetable proteins and snacks. In this context, the snack market is very prominent, with the demand for healthy snacks becoming increasingly relevant [1]. Cookies and crackers have become one of the most popularly consumed snacks due to their low manufacturing cost, availability, high nutrient density, long shelf-life and potential to be supplemented with a wide variety of nutraceuticals [2,3]. It is widely known that wheat cookies, commonly available in the market, lack good quality protein because of their deficiency in lysine. For this reason, the production of wheat cookies with various legume seeds has been proposed [4], to increase the protein content and improve an amino acid balance of the final product, due to the contribution of lysine by legumes and the contribution of methionine by cereals [5]. However, wheat gluten consisting of glutenins and gliadins cause severe intestinal inflammation in individuals suffering with celiac disease or other forms of gluten intolerance [6]. Hence, several alternative flours have an increase in demand, such as spelt and kamut, as is the case for species of the *Triticum* genus, but with a healthier nutritional profile than modern wheats, as they provide more nutraceutical compounds, vitamins and minerals [7,8].

A study performed under *in vitro* inflamed conditions reported that ancient *Triticum* grains (spelt and kamut) had a less inflammatory activity by decreasing IL-8 production, when compared to the modern grains [8]. Another study showed a better response to oxidative stress in rats fed with kamut bread than with wheat bread [9]. The available data suggest that ancient grains cause less inflammation than modern wheat grains, being an important and healthier alternative as food ingredients for the bakery industry.

Another alternative is oat (*Avena sativa*). The nutritional and health benefits presented by this grain are correlated with an increased intake of β-glucans, polyunsaturated fatty acids, and essential amino acids [10]. β-Glucans in wheat comprise ca. 1% of the seed, but 3 to 7% in oat seeds [11]. The potential use of β-glucans as a food ingredient in functional dietary fiber is increasing. Despite a relatively large quantity of globulins, the proportion of prolamins (like avenin) in oat is lower when compared to the wheat gliadins [12]. In addition, avenins are more easily digested than gliadins. Apparently, the lack of toxic epitopes in avenins compared to gliadins reduce their immunogenicity for celiac patients when compared to wheat prolamins [12]. Nevertheless, oat consumption is only recommended for celiac patients in remission since the contamination of oat by wheat, barley or rye is currently the main limitation for its use in a gluten-free diet [10]. "β-Glucans contribute to the maintenance of normal blood cholesterol levels" (Commission Regulation (EU) 432/2012 of 16 May 2012), is a health claim which highlights the improving health benefits of an oat-containing diet.

The gluten-free flours mostly used by the food industry are rice and buckwheat. Rice flour is a cheap product with a relatively low nutritional value when compared to other gluten-free flours, namely buckwheat, quinoa and maize [13]. The use of buckwheat flour has increased considerably due to the recognition by the consumers of its potential health benefits, presenting an increased commercial interest in the gluten-free market [14]. Nevertheless, several studies showed that many gluten-free foods are deficient in dietary fiber, micronutrients and protein [15,16]. Therefore, the combination of gluten-free flours with other health-promoting factors, such as proteins and bioactive peptides, has also received increased attention as potential functional foods [17].

Legume seeds are food ingredients with high nutritional quality and a low glycemic index when compared to cereal grains [18]. Moreover, legume proteins in the form of flour or concentrate constitute a good supplement for cereal-based foods, because legume and cereal proteins are complementary in their essential amino acids compositions [4,19]. In particular, white lupin (*Lupinus albus)* seeds have received attention as a source of bioactive proteins [20] and have been used as an additive to food products, in order to improve their functional and nutritional properties [19,21]. In 100 g, white lupin contains approximately 32 g protein, 16 g crude fiber, 6 g carbohydrates and 6 g crude fat [22]. Protein content in lupin is higher than in other legumes such as haricot bean, lentil and chickpea, which contain 28.8%, 26.7% and 24.8% protein, respectively [23,24]. For these properties, some researchers used *L. albus* to develop bakery products, such as bread [25], cookies [26] and pasta [21], and the ingestion of lupin-containing foods has been associated with the prevention of diabetes by the hypoglycemic effect, cardiovascular disease, and more recently, digestive tract diseases [20,21,27].

In the available literature, the major studies on lupin-containing foods have been made with flours prepared from the whole seed, therefore containing fat, oligosaccharides, protein and fiber. In addition, there are few or no studies comparing the incorporation of lupin protein concentrate (LPC) in wheat alternatives such as spelt, kamut, oat and gluten-free (rice and buckwheat) flours. In this sense, the main goal of this study was to evaluate the impact of the addition of LPC in the physical properties of sweet cookies prepared with flours (containing or not gluten) of different origins.

3.2. Materials and Methods

3.2.1. Preparation of Lupin Seed Protein Extract

Lupinus albus L. sweet seeds were purchased from Jouffray Drillaud, France. Approximately 100 g ± 0.1 g of dry lupin seeds were milled to a powder and extracted using milli-Q water (1:10, *w*/*v*). The extract was stirred overnight at 4 °C. The homogenate was filtered through a miracloth. The final sample was stored frozen a −80 °C overnight and lyophilized (Edwards, Crawley, UK. The final amount of LPC was 50 g [20] and its proximate composition was: 64.07% carbohydrates, 18.06% protein, 7.76% ash, 9.80% moisture and 0.32% lipids. This characterization was performed according to Batista et al. [28], except for the protein content which was quantified by the Bradford method [29].

3.2.2. Flour Composition

Rice flour (Ceifeira, lot L 3411/18, Lisbon, Portugal), oat flour (Próvida, lot 20190910, Lisbon, Portugal), spelt flour (Próvida, lot 20191112, Lisbon, Portugal), kamut khorasan flour (Próvida, lot 20190725, Lisbon, Portugal), buckwheat flour (Próvida, lot 20210630, Lisbon, Portugal) and other ingredients were purchased from a local market. The nutritional composition of the five flours used is summarized in Table 1.

Table 1. Nutritional composition of the five different flours used in the cookies' formulations (g/100 g of flour). Values were provided by the suppliers Ceifeira and Próvida.

	Rice	Oat	Spelt	Kamut	Buckwheat
Energy (kcal/100 g)	350	370	295	385	366
Protein	7.6	14.0	13.0	15.0	13.3
Total lipid	0.7	7.6	1.8	15.0	3.4
Of which saturated	0.3	1.3	0.3	0.6	0.7
Total carbohydrate	78.5	56.0	55.0	60.0	61.5
Of which sugars	0.1	1.1	6.8	2.0	2.0
Of which fiber	2.4	10.0	4.0	11.0	10.0
Salt	<0.1	0.01	0.02	0.03	0.08

3.2.3. Cookies Preparation

Cookies were prepared according to an optimized formulation [28,30], using the following ingredients (as g/100 g): flour (54), sugar (15), margarine (18), water (12) and baking powder (1). For all the samples, the same quantities of the ingredients were used, except for the flour, which was replaced by 10% (*w*/*w*) LPC in the case of lupin cookies. The procedures were similar for the different flours used and the sample without LPC incorporation was considered as a control sample for each corresponding flour. The amount of LPC to be incorporated in cookies was based on the previous studies [31,32].

Batches of 100 g were prepared, and the ingredients were mixed for 15 s at a speed of 4 in a food processor (Bimby, Vorwerk, Wuppertal, Germany). The sweet cookies were molded in a square mold and baked at 110 °C for 40 min in a forced-air convection oven (Unox, Italy). After

cooling for 30 min at room temperature, the cookies were stored in hermetic containers, at room temperature and protected from the light.

3.2.4. Dough Rheology

Rheological measurements were conducted using a controlled strain rheometer (Haake, Mars III, Thermo Fisher Scientific, Karlsruhe, Germany) at a constant temperature (25.0 °C ± 0.1 °C), controlled by a Peltier system. The rheometer was equipped with serrated parallel-plate geometry (20 mm diameter) to overcome the slip effect. The dough pieces were compressed with a 1.5 mm gap. Following the preparation, the dough was allowed to rest for 5 min before measuring. The stress and frequency sweeps were carried out at 25 °C. The stress sweep, with a constant frequency (1 Hz), was performed to identify the linear viscoelastic region. Frequency sweep tests were performed with a constant stress within the linear viscoelastic region and in a frequency range from 0.01 to 100 Hz to obtain the values of elastic modulus (G' (Pa)) and viscous modulus (G" (Pa)).

3.2.5. Dimensions

The dimensions of the cookies were evaluated using a digital caliper (Powerfix, Germany). The width and thickness of the ten cookies from each formulation were measured after 24 h of cookie preparation.

3.2.6. Color Analysis

The color of the cookie samples was measured using a Minolta CR-400 (Japan) colorimeter. The results were expressed in terms of L*, lightness (values increasing from 0 to 100); a*, redness to greenness (60 to −60 positive to negative values, respectively); and b*, yellowness to blueness (60 to −60 positive to negative values, respectively) according to the CIELab system. The total color difference between the sample cookies during the storage time (up to eight weeks) was determined using average L*, a* and b* values. The measurements were performed under similar light conditions using a white standard (L* = 94.61, a* = −0.53, and b* = 3.62), at room temperature, replicated eight times for each cookie sample (control and lupin-enriched cookies) and for week 0 (24 h after baking) and week 8.

The total colour difference between the control and the lupin-enriched cookies was obtained by Equation (1):

$$\Delta E^* = (\Delta L^{*\,2} + \Delta a^{*\,2} + \Delta b^{*\,2})^{1/2}$$

3.2.7. Texture Analysis

Instrumental texture analysis was conducted in a TA.XTplus (StableMicro Systems, Godalming, UK) texturometer. Texture measurements were performed at 20 °C ± 1 °C in a temperature-controlled room.

3.2.7.1. Dough Texture

Dough samples were submitted to texture profile analyses (TPAs), simulating the action of a double chewing. The dough was contained in a cylindrical flask of 2.5 cm in diameter and 4.5

cm in height. The TPAs were performed in a penetration mode using an acrilic cylindrical probe of 4 mm in diameter, 15 mm of penetration and 1 mm/s of crosshead speed. Firmness and cohesiveness were the two primary texture properties used to compare the doughs, as they were the ones with the greatest capacity to discriminate between the different samples. The firmness of the dough was considered to be the maximum force in the first cycle [33]. The cohesiveness describes how well a food retains its form between the first and second chew and it is a ratio between the work performed in the second and the first cycle [33]. These analyses were repeated eight times for each dough sample.

3.2.7.2. Cookie Texture

Cookie texture was evaluated with a penetration test, using a cylindrical probe of 2 mm in diameter, plunged 8 mm at 1 mm/s. Resistance to penetration was evaluated by the maximum peak shown on the texturogram which corresponds to the N value. These determinations were replicated at least eight times for each cookie sample (control and lupin-enriched dough) at week 0 (24 h after baking) and week 8.

3.2.8. Water Activity Determination

Cookie samples were analyzed for water activity (a_w). This was determined using a thermohygrometer (HygroPalm HP23-AW, Rotronic AG, Bassersdorf, Switzerland) at 20 °C ± 3 °C. The tests were performed during storage (24 h and 8 weeks after baking) by crushing the samples into little pieces. The cookies (control and with lupin) were assayed in triplicate.

3.2.9. Moisture Content

The moisture was determined gravimetrically following ISTISAN protocols (ISTISAN Report 1996/34, method B, page 7), using an incubator (Binder GmbH, Germany) at 105 °C until a constant weight was achieved.

3.2.10. Statistical Analyses

Experimental data were obtained at least in triplicate and were statistically analyzed using SigmaPlot (version 12.5). An analysis of variance (one-way ANOVA) was applied to evaluate the differences between samples at a significance level of 95% ($p < 0.05$). Tukey's test was used to compare the differences between groups. All the results are presented as the mean ± standard deviation (SD).

3.3. Results and Discussion

3.3.1. Physical Properties of the Dough

Figure 1 shows the firmness (a) and cohesiveness (b) of the control dough produced with five flours of different origins and with the corresponding doughs enriched with 10% (*w*/*w*) LPC. The doughs prepared with different types of flour have different texture properties (firmness and cohesiveness).

It should be noted that the control gluten-free dough without LPC (rice and buckwheat), are 20% firmer and 40% less cohesive than the others. This behavior should result from the different composition of these two flours (Table 1). In these cases, the structuring of the system is essentially achieved by the starch present, although the different types of proteins present

can also contribute to the reinforcement of this structure. Thus, the doughs obtained from these two flours have a greater resistance to penetration (high firmness), which is related to more compact doughs. The absence of the gluten matrix decreases the air retention capacity of the system [34], contributing to firmer doughs. At the same time, a reduction in the cohesiveness associated with a greater disaggregation is observed [35]. These characteristics are less positive in terms of the technological handling of these doughs.

In the case of rice, the high starch content is relevant, compared to other flours, which has an important impact on structure creation. Regarding the buckwheat, the type of proteins involved could also explain the increase in firmness and decrease in cohesiveness since its proteins are rich in lysine and arginine, unlike the other flours studied [36]. Complementary studies can be developed, in the future, in order to support this statement.

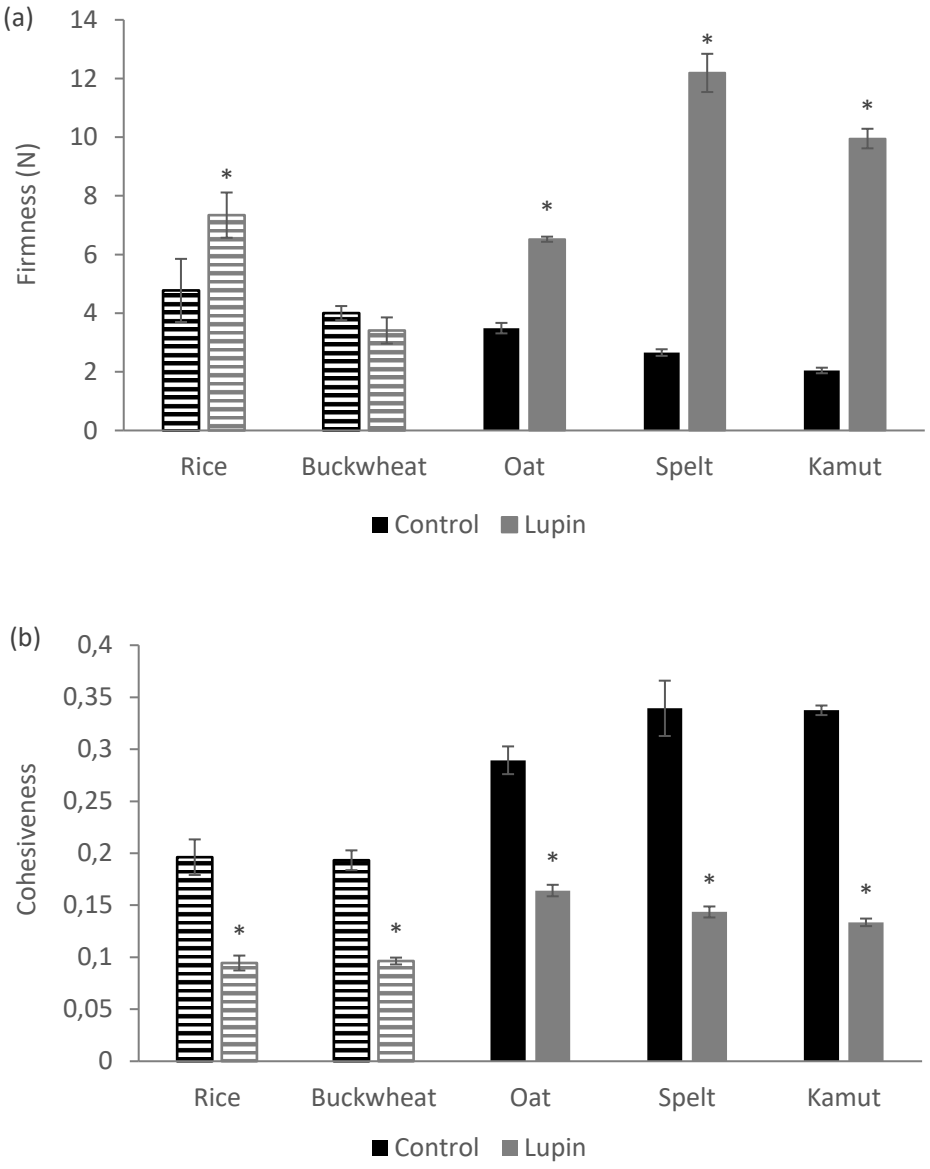

Figure 1. Texture parameters of control and lupin-enriched cookies prepared from five different flours. Solid bars represent the gluten-containing flours (oat, spelt and kamut) and the striped bars represent the gluten-free flours (rice and buckwheat): (**a**) the firmness; and (**b**) the cohesiveness. * represents $p < 0.05$ when compared with the corresponding control cookie.

When 10% (*w*/*w*) of the flours under study is replaced by LPC, a relevant impact on the texture characteristics of the dough is observed. In general, the incorporation of proteins contributes to an increase in dough firmness (Figure 1a) and a significant ($p < 0.05$) reduction of at least 50% in cohesiveness (Figure 1b). A similar behavior was observed by the other researchers upon the addition of potato peel to cakes [37], whey protein to cheese [38] and lupin flour to biscuits [26].

It is important to highlight the strong impact of LPC addition on the two gluten rich flours—spelt and kamut doughs are about four times firmer (from 2.66 N to 12.19 N, in the case of spelt and from 2.05 N to 9.95 N in the case of kamut) than the corresponding control. This should result from a strong interaction between the main macromolecules present in the system: (i) lupin proteins–flour starch; and (ii) lupin proteins–flour gluten proteins. This type of interactions is strongly dependent on the protein composition of the added protein fraction, as well as on the starch conformation [39]. More important than the total amount of macromolecules present in the dough, which is similar in all the cases, is the biochemical composition and conformation of these proteins and polysaccharides. A firmer dough should reflect a more effective entangled network developed among these macromolecules [39], which may be important in terms of the dough stability, but which translates to a less cohesive dough.

The relevant reinforcement on the structure observed for the kamut and spelt doughs, due to the incorporation of LPC, allows us to predict that there was a reinforcement in the gluten structure already present in the control doughs, resulting from a synergy between the gluten and the lupin proteins. The firmness increase and cohesiveness decrease resulting from the LPC incorporation has a relevant impact in technological terms: the doughs become more difficult to mold, meaning it may be necessary to optimize the cookie production process such as the optimization of the water absorption (e.g., MicrodoughLab procedure) that consists of the quantity of water needed to reach the optimal dough consistency [40].

The impact of LPC addition on the linear viscoelastic behavior of the cookie's dough prepared with five different flours can be observed in Figure 2. These results were obtained from small amplitude dynamic rheological measurements (small amplitude oscillatory system - SAOS) and are related to the degree of dough structuring, reflecting the level of molecular interactions that are established, especially among the macromolecules present.

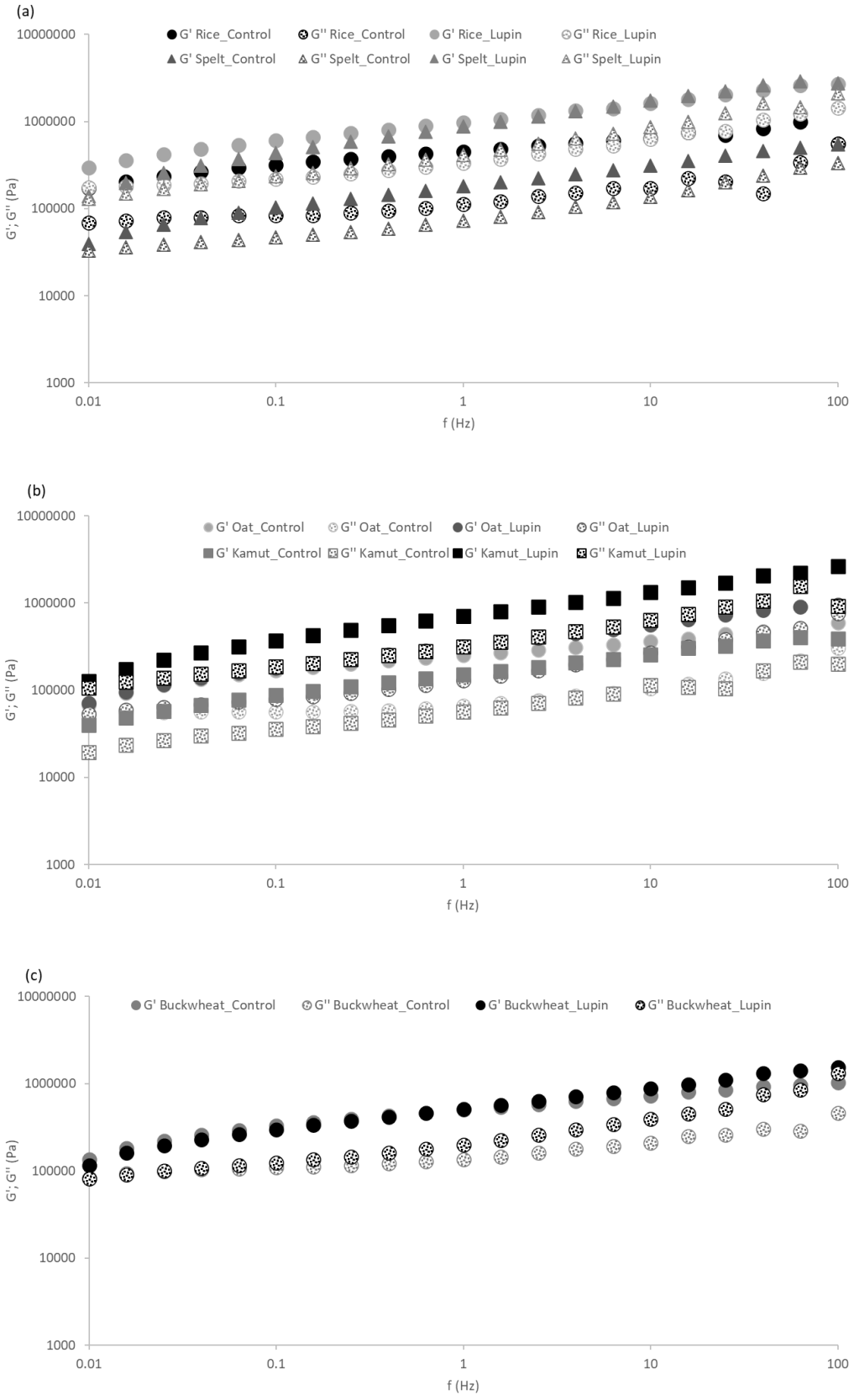

Figure 2. Mechanical spectra of the control and the lupin-enriched doughs prepared with five different flours: (**a**) rice and spelt; (**b**) oat and kamut; and (**c**) buckwheat. Solid symbols represent G' (elastic modulus) and the dotted symbols represent G" (viscous modulus).

The evolution of G' (storage modulus) and G" (loss modulus) over the frequency range tested reveal that both moduli slightly increased with increasing frequency. This weak gel-like rheological behavior is typical of cookie doughs [41] and other cereal dough products such as bread [42] and pasta [43].

The addition of LPC causes the reinforcement of the dough structure for all the flours studied, except for the buckwheat flour. This is evidenced by the higher values of G' and G" for the formulations enriched with LPC, compared to the standard flours. These results are in agreement with the texture results-also in terms of firmness, the buckwheat flour formulation was the only one without significant differences ($p > 0.05$) due to the addition of LPC.

To obtain a more detailed comparison among the linear viscoelastic behaviors of the different formulations, Table 2 shows the G' values obtained at 1 Hz (G' 1 Hz) from the three replicates of each test. It turns out that the G' 1 Hz values were significant higher in rice, spelt and kamut flours, when the lupin incorporation dough was compared with the control without LPC. The maximum value for G' was 8.3×10^5 Pa for the lupin-incorporated rice flour. However, the greatest increment measured in G' 1 Hz due to the addition of LPC was achieved for the kamut flour. In these cases, lupin incorporation increased the degree of dough structuring, which results from the formation of more complex three-dimensional structures among the macromolecules present in the systems, as previously discussed for the dough texture results.

Table 2. Values of G' when the frequency corresponds to 1 Hz. Values are the means of at least three experiments ± SD. * represents $p < 0.05$ when compared with the corresponding control cookie.

	G' 1 Hz (Pa)	
	Control	**Lupin**
Rice	$4.6 \times 10^5 \pm 9.1 \times 10^4$	$8.3 \times 10^5 \pm 1.3 \times 10^5$ *
Buckwheat	$5.0 \times 10^5 \pm 2.9 \times 10^4$	$5.2 \times 10^5 \pm 1.5 \times 10^4$
Oat	$2.5 \times 10^5 \pm 8.3 \times 10^4$	$2.9 \times 10^5 \pm 2.6 \times 10^4$
Spelt	$2.0 \times 10^5 \pm 5.1 \times 10^4$	$6.2 \times 10^5 \pm 9.6 \times 10^4$ *
Kamut	$1.6 \times 10^5 \pm 1.4 \times 10^4$	$7.6 \times 10^5 \pm 8.5 \times 10^4$ *

3.3.2. Physical Properties of Cookies

Characteristic dimensions of the LPC incorporation in five different flours are presented in Table 3. In general, the incorporation of LPC had significant differences ($p < 0.001$) in all the flours tested, except for oat flour. For spelt, kamut and buckwheat flours, the addition of LPC increased the area in relation to the control. However, the rice flour cookies were the only ones with a significant ($p < 0.001$) reduction in the cookie area. Therefore, the presence of gluten does not seem to affect the cookie area and no direct relationship can be established with the expansion of the structure. In relation to thickness, the two gluten-free flours (rice and buckwheat) showed a significant increase ($p < 0.05$) in the LPC-containing cookies, unlike the gluten flours, where it showed a generalized decrease. Similar studies were performed with wheat cookies and Jayasena and Nasar-Abbas [26] reported no effect in the cookie diameter and an increase in the cookie thickness with the presence of 10% (*w*/*w*) lupin flour.

Nevertheless, Bilgiçli and Levent [44] demonstrated no effect in the thickness in cookies containing lupin flour, whereas Tsen et al. [45] showed a reduction in the cookie diameter prepared with soy protein isolates.

Table 3. The dimensions of each cookie formulation with 10% (*w*/*w*) of lupin protein concentrate (LPC). Values are the averages of ten cookies ± SD. * represents $p < 0.05$ and ** represents $p < 0.001$ when compared with the corresponding control cookie.

Cookie Formulation		Area (cm^2)	Thickness (mm)
Rice	Control	15.63 ± 0.06	2.55 ± 0.14
	LPC	15.08 ± 0.12 **	2.94 ± 0.08 *
Buckwheat	Control	13.26 ± 0.11	2.62 ± 0.11
	LPC	16.05 ± 0.16 **	3.14 ± 0.10 *
Oat	Control	15.61 ± 0.10	3.25 ± 0.34
	LPC	15.44 ± 0.21	2.79 ± 0.12
Spelt	Control	13.00 ± 0.03	3.57 ± 0.10
	LPC	15.94 ± 0.11 **	3.35 ± 0.28
Kamut	Control	15.30 ± 0.07	2.94 ± 0.20
	LPC	16.69 ± 0.06 **	2.68 ± 0.16

In summary, even in cases where statistically significant results were obtained, all the structural alterations resulting from the addition of LPC could be neglected, as far as the magnitude was concerned (maximum 20% variation for the area and thickness of buckwheat cookies). This conclusion can be important in terms of technological performance and consumer acceptance.

The texture properties of foods are an important requirement for their acceptance by consumers, especially in what concerns crispy products, such as cookies [46]. In this sense, the impact of LPC addition to different types of cookies was evaluated in both the presence and absence of gluten. Firmness values (N) obtained in week 0 and eight weeks later are presented in Figure 3.

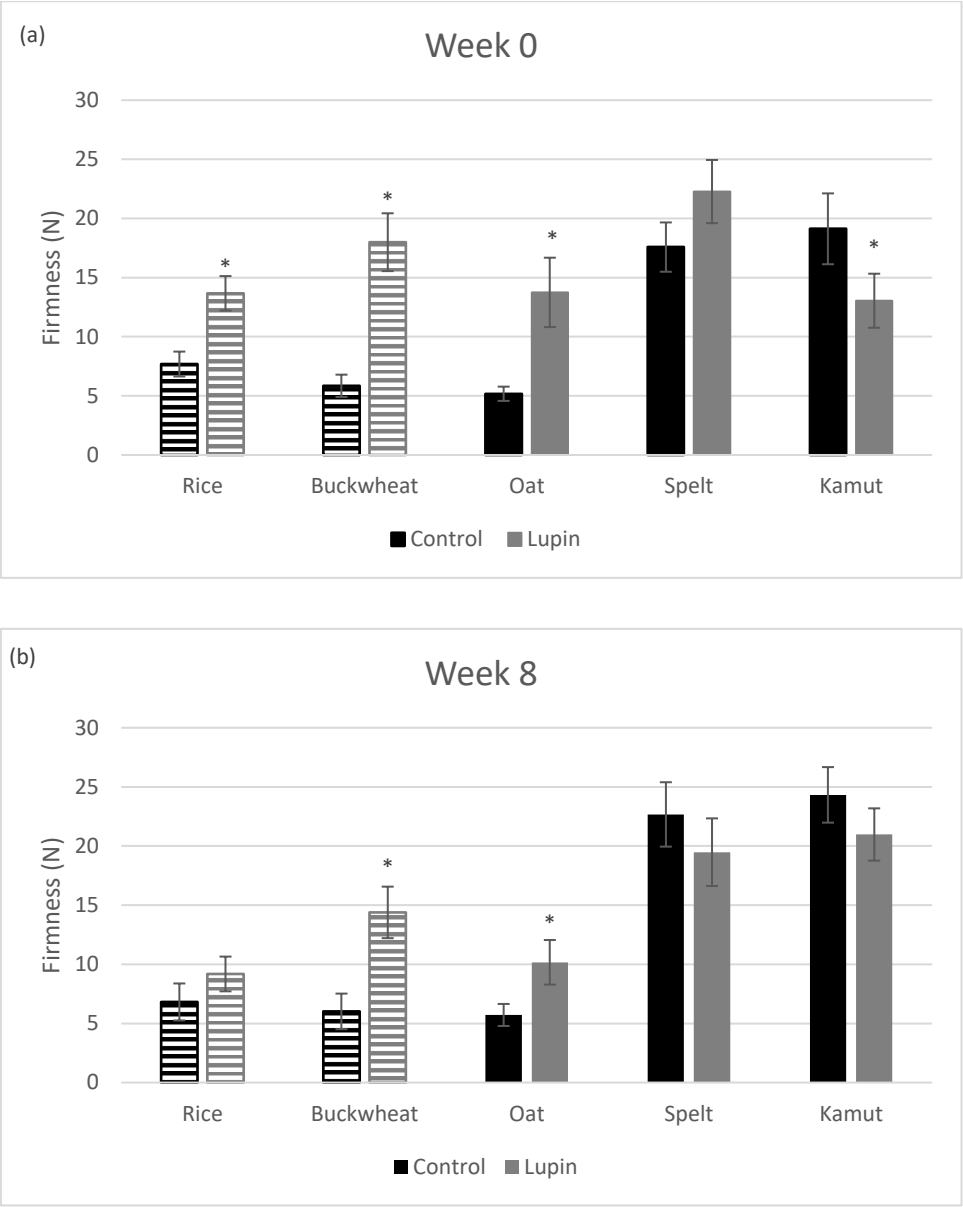

Figure 3. Firmness of the control and lupin-enriched cookies at week 0 (**a**) and week 8 (**b**). Solid bars represent the gluten-containing flours (oat, spelt and kamut) and the striped bars represent the gluten-free flours (rice and buckwheat). Values are the means of at least six experiments ± SD. * represents $p < 0.05$ when compared with the corresponding control cookie.

It is evident that the cookies prepared with the ancient grains and without LPC (spelt and kamut) were firmer than the other control cookies and this observation remained valid after storage (eight weeks). The changes induced by the LPC in the cookie structure differed over time, since at week 0 only the spelt flour had no significant difference ($p > 0.05$) when compared to the control; however, after eight weeks, the spelt, kamut and rice flours showed no significant differences ($p > 0.05$) when the cookie with LPC and the control were compared. Additionally, the oat and buckwheat flours were statistically different over time (eight weeks), meaning that the incorporation of lupin clearly modified the texture of the cookies, making them firmer. Hence, it cannot be stated that the differences between the five different flours on one hand and LPC addition on the other occurred due to the presence of gluten. Jayasena and Nasar-Abbas [26], Obeidat, Abdul-Hussain and Al Omari [47] and Bilgiçli and Levent [44] reported that cookie

hardness increased with the addition of lupin flour in the cookie formulation. This can also be stated for other types of legume seeds, such as chickpeas [48], green lentils and navy beans [4]. The different behavior observed between the doughs and the respective cookies is corroborated with other studies [41]. Indeed, macromolecular structures present in each flour undergo dramatic changes during heat treatment. In spelt and kamut flours, gluten is the main element that accounts for the structure; in oat, the main structural role is played by β-glucans, and in gluten-free flours (rice and buckwheat), the structure is mainly accounted for by starch. When the LPC (protein) is added, there is an overall structural rearrangement leading to distinct interactions among these macromolecules, as supported by our results. The interactions among macromolecules and the type of structures which arise are differentially affected by the heat treatment which takes place during cooking.

The impact of LPC addition on the color parameters of cookies is summarized in Table 4. The ΔE* values were calculated to compare the color variation in relation to the cookies without LPC. In the same table, the water activity (a_w) and moisture content (H) of the ten formulations studied are also indicated.

Table 4. Values of ΔE*, L*, a_w and the moisture content (H, % *w*/*w*) of the control and lupin-enriched cookies. Values are the means of at least three experiments ± SD, except ΔE* which is the difference between the control and lupin-enriched cookie colors. * represents $p < 0.05$ and ** represents $p < 0.001$ when compared with the corresponding control cookie.

		Rice	Buckwheat	Oat	Spelt		Kamut
ΔE*	Week 0		21.52	20.58	12.22	31.19	15.80
	Week 8		24.99	20.18	11.97	20.20	22.03
L*	Week 0	Control	80.40 ± 1.28	72.84 ± 0.81	71.27 ± 0.92	55.16 ± 3.04	73.43 ± 0.69
		Lupin	65.74 ± 2.73 **	56.78 ± 1.53 **	61.21 ± 1.47 **	64.68 ± 2.05 *	59.72 ± 1.11 **
	Week 8	Control	80.81 ± 1.06	69.37 ± 2.89	71.41 ± 0.97	78.43 ± 3.57	72.79 ± 1.70
		Lupin	62.10 ± 1.51 **	55.28 ± 3.02 *	63.11 ± 2.00 *	60.50 ± 2.21 *	53.41 ± 2.80 **
a_w	Week 0	Control	0.36 ± 0.02	0.21 ± 0.01	0.04 ± 0.02	0.09 ± 0.01	0.50 ± 0.03
		Lupin	0.59 ± 0.01 **	0.35 ± 0.01 **	0.09 ± 0.00 *	0.40 ± 0.02 **	0.39 ± 0.01 *
	Week 8	Control	0.17 ± 0.00	0.12 ± 0.01	0.12 ± 0.00	0.22 ± 0.00	0.15 ± 0.01
		Lupin	0.23 ± 0.01 **	0.37 ± 0.00**	0.33 ± 0.00 **	0.36 ± 0.00 **	0.35 ± 0.00 **
H (%)	Week 0	Control	3.42 ± 0.14	5.61 ± 0.61	1.04 ± 0.11	2.75 ± 0.22	1.97 ± 0.08
		Lupin	2.88 ± 0.11 *	3.88 ± 0.08 *	2.29 ± 0.16 **	4.33 ± 0.08 **	2.85 ± 0.06 **

The ΔE* values obtained were always higher than 5 for both time periods studied (week 0 and week 8), which means that the color difference between the lupin-enriched cookies and the control is visually distinguishable by the human eye. These differences result mainly from a general decrease in the lightness parameter (L*) in all lupin-containing cookie samples, resulting in a golden-brown color. These results agree with other studies, showing a decrease in cookie lightness with lupin flour at the same concentration level [44]. The results can be explained by the Maillard reaction, as proteins and sugars initiate a complex cascade of reactions during heating (higher than 100 °C), producing the darker color [49]. This darkening did not have a negative impact on the characteristics of the final product; on the contrary, the LPC cookies presented very appealing colors, as those supported by other studies [26,28].

Cookies are a relatively dry product with a low moisture content and water activity values. These parameters are crucial to predict both the stability and safety of the product, with great impact in conservation, particularly for the maintenance of a crispy texture [50]. Moisture content values of cookies with and without LPC are low (ranging from 1.04 to 5.61%),

comparing favorably with other studies on similar cookies and indicating a positive impact in terms of conservation [30].

The a_w values for lupin-enriched cookies at week 0 are significantly higher ($p < 0.05$ or $p < 0.001$) than those of the control cookies. After 8 weeks of storage, all the LPC cookies had similar (except for rice flour) a_w values, but significantly higher ($p < 0.001$) than the controls. Furthermore, all the samples were shown to have an a_w value of less than 0.5 (except lupin-enriched cookies with rice flour at week 0), which means that all cookie formulations (with and without LPC) had a low percentage of free water for microbial proliferation, leading to a high stability product [50]. Such low a_w values are essential to prevent microbial growth on the cookies. Uysal et al. [51] found an increase in a_w values with the incorporation of apple and lemon fiber in cookies. Batista et al. [28] also found an increase in a_w values, resulting from the incorporation of microalgae biomass with a high protein content. However, Fradinho et al. [30] found an opposite effect when Psyllium fiber was added to the cookies similar to those prepared in the present work, resulting from the high-water holding capacity of Psyllium. The differential capacity to retain the water of the molecules present in the formulation had a direct impact on the water activity of the final product. For the LPC cookies, the water holding capacity of the protein should be lower than that of the respective flour, justifying the increase in water activity.

Lupin is considered a potential functional food because of its protein content, dietary fiber and more recently discovered bioactivities [20,21] that need to be explored in food products in the near future.

3.4. Conclusions

Consumers are currently more cognizant about the environmental effects and nutritional benefits of foods. In this sense, lupin can be considered a suitable raw material for food production due to its nutritional and health-promoting properties.

Lupin protein concetrate (LPC) addition to gluten and gluten-free flours showed a high impact in dough structure, increasing the degree of structuring. This impact on dough texture had technological implications, resulting in a greater difficulty in the molding process, which can be optimized in terms of industrial processing. The lupin-enriched dough based on buckwheat flour was unique because it did not show significant differences ($p > 0.05$) when compared to the control dough, being technologically more stable and easier to work with. Regarding the physical properties of the final products, the cookies based on buckwheat and oat flours were always firmer than the corresponding control cookies. Rice and buckwheat flours supplemented with LPC produced cookies with a significant increase in thickness ($p < 0.05$), unlike in gluten flours (oat, spelt and kamut). These parameters are very important, since less thickness suggests more crispness, a highly desirable property appreciated by consumers. Supplementing flours with LPC improves color and decreases lightness, making cookies more pleasant to consumers. After eight weeks, the a_w values of all LPC-containing cookies were significantly higher ($p < 0.001$) than the controls, a characteristic which has a positive impact in conservation.

Overall, our results show that the cookies prepared with flours with or without gluten can be produced successfully by replacing 10% of the flour with LPC. Therefore, the inclusion of

10% (*w*/*w*) sweet lupin protein extract in formulations improves the nutritional value and quality of cookies.

3.5. References

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Chapter 4. Technological Potential of a Lupin Protein Concentrate as a Nutraceutical Delivery System in Baked Cookies

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Status: *Foods,* 2021, 10(8), 1929

DOI: 10.3390/foods10081929

Abstract: Previous reports have shown that lupin protein extracts (LPC) contain a polypeptide named deflamin with a potent matrix metalloproteinase (MMP)-9 inhibitory activity. The aim of our study was to develop an efficient delivery method for incorporating deflamin into cookies using different alternative flours. A lupin protein concentrate (10 g protein/100 g cookie dough) was added to gluten and gluten-free flours to produce savoury cookies, and its impacts on the physical properties of doughs and cookies, as well on the maintenance of deflamin's anti-MMP-9 activity, were analysed. The results showed that the biochemical compositions of all cookies with LPC presented higher protein and ash contents when compared to the control cookies. Rice, buckwheat and oat doughs were firmer than the others, whereas the addition of LPC to kamut and buckwheat flours made cookies significantly firmer than the controls. Additionally, strong interactions between LPC and several flours were observed, yielding different impacts on the MMP-9 bioactivity. Overall, the only flour that did not interfere with the desired nutraceutical activities was buckwheat, with 60% MMP-9 inhibitory activity and a concomitant reduction of colon cancer migration; hence, buckwheat flour was revealed to be a good vehicle to deliver bioactive deflamin, showing strong potential as a functional food to be used in preventive or curative approaches to gastrointestinal diseases.

Keywords: lupin extract; anti-MMP activity; cookies; physicochemical properties; HT29; MMP-9

4.1. Introduction

Consumers' rising concerns regarding health and well-being have resulted in increasing attention being given to bioactive food compounds that exhibit health-promoting effects. In fact, the diet-based prevention of chronic diseases, such as inflammation and cancer [1,2,3], has become a great focus of attention, based on the principle that preventing a disease is more effective than curing it [1,2,3]. In particular, the enhancement of the nutritional and health properties of food products through the incorporation of nutraceuticals has become a topic of great interest to both consumers and the food industry [1]; however, one of the great constraints related to the direct incorporation of nutraceuticals and bioactive compounds into food products is in identifying adequate materials and production techniques [3]. Indeed, the choice of the correct food matrixes is a key step, since it may have a strong impact on the bioactivity. Baked snacks such as crackers and biscuits are usually well accepted and consumed throughout the world, and can be excellent vehicles for nutraceutical and protein enrichment because of their wide consumption and long shelf life [4]. Previous studies from our group have incorporated a GRAS-safe lupin protein concentrate in sweet baked cookies, using gluten and gluten-free flours, which increased their nutrition potential [5]. Lupin seeds are well known to provide a diversity of essential nutrients, including carbohydrates, dietary fibre, protein, minerals and vitamins [6], which exhibit important biological activities, such as lower the glycaemic index and decreasing hypertension and cholesterol [6]. Amongst legumes, lupin has the highest protein content [7], providing it great potential for consumption. Furthermore, Lima et al. [8] showed that the protein extract from lupin can reduce cell migration in colon carcinoma cells through matrix metalloproteinase (MMP)-9 inhibition [8], mostly due to the presence of a protein oligomer nutraceutical named deflamin, which seems to be an excellent MMP-9 inhibitor [9]. Isolated

deflamin also reduced inflammation and the expression of inflammation mediators in colitis models *in vivo* [9]. From this point of view, the incorporation and maintenance of the nutraceutical activities of lupin in baked cookies could be used to produce functional foods for the prevention and amelioration of chronic diseases related to MMP-9, such as inflammatory bowel diseases and colon cancer [10,11]. The incorporation of a lupin protein extract instead of lupin flour also presents several advantages, including the elimination of potentially toxic compounds, such as digestive enzyme inhibitors and antinutritional compounds [12], guaranteeing the presence of sufficient amounts of active deflamin [9]. Nonetheless, previous results have demonstrated that the MMP-9 inhibitory activity of deflamin was reduced in the presence of sugar-containing dough; therefore, in the present study we tested whether the lupin protein concentrate could be used as a vehicle for the MMP-9-inhibitory compound deflamin in savoury baked cookies. The use of wheat flour substitutes as acceptable alternatives reduces calorie intake and increases the availability of healthier snacks on the market, in turn reducing inflammation [13,14]; therefore, we tested the incorporation of such flour substitutes in different types of matrices, such as gluten-containing flours from spelt and kamut, which have been reported to cause less inflammatory responses than *Triticum aestivum* [15], oat, rice and buckwheat flours, which are the most consumed gluten-free flours [16].

Overall, our main goal was to assess the impacts of the addition of lupin protein extract on the physicochemical properties and bioactivities of savoury cookies with and without gluten flours from different origins. Our main goal was to develop a staple food that could act as a vehicle for the ingestion of a deflamin-rich lupin concentrate in the form of baked cookies, without changing its status as a functional food, whilst implementing adaptation strategies to reduce the impacts of inflammatory and cancer diseases through cost-efficient and applicable dietary approaches.

4.2. Materials and Methods

4.2.1. Preparation of Lupin Seed Protein Extract

Lupinus albus seeds were obtained from Jouffray Drillaud, France. Protein extraction was performed according to Mota et al. [5] with slight alterations, using approximately 100 g ± 0.1 g of powdered *L. albus* seeds with 1:10 (*w*/*v*) milli-Q water. The extract was stirred overnight at 4 °C and was filtered using Miracloth. The homogenate was maintained in a 100 °C water bath for 30 min and then filtered again. The extract was kept frozen at −80 °C and lyophilized (Edwards, Crawley, UK). The final quantity obtained was 50 g of lupin protein concentrate (LPC) [8].

4.2.2. Flours Composition

The nutritional compositions of the gluten-containing flours, kamut and spelt; of the gluten-free flours buckwheat, rice and oat; and of the remaining ingredients, namely sunflower oil, salt and baking powder, were obtained as described by Mota et al. [5].

4.2.3. Cookies Preparation

Savoury cookies were prepared using 65.5% flour, 1% salt, 1.5% baking powder, 7.5% sunflower oil and 24.5% water, according to a previously optimized model formulation [17]. In all

tested samples, the ingredients were added in similar quantities, except for the flour, which in LPC cookies was substituted by 10% LPC, in contrast to controls, to which no LPC was added. The quantity of LPC contained in the cookies was established using previous data [5]. All ingredients were mixed in a food processor (Bimby, Vorwerk), where 100 g batches were mixed for 15 s at speed 4. The savoury cookies were moulded, baked and stored as described by Mota el at [5]. On the other hand, the sweet cookies used in this study were prepared according to Mota et al. [5], using 54% flour, 15% sugar, 18% margarine, 12% water and 1% baking powder.

4.2.4. Dough Rheology

Rheological measurements were performed according to Mota et al. [5], using a controlled stress rheometer (Haake MarsIII—Thermo Scientific, Karlsruhe, Germany) with a UTC–Peltier system. Frequency sweep tests were performed within the viscoelastic linear region, which was previously defined through a stress sweep test, at 1 Hz using a serrated parallel-plate geometry with a 20 mm diameter. Dough pieces were compacted with a 1.5 mm gap and the edge parts were coated with liquid paraffin to prevent moisture losses during tests. The temperature used for the stress and frequency sweeps was 25 °C.

4.2.5. Colour Analysis

The colour of the cookies samples was measured using a Minolta CR-400 (Japan) colorimeter. The method used was previously described by Mota et al. [5]. The measurements were performed under the same light conditions using a white standard (L* = 94.61, a* = −0.53, and b* = 3.62) at control temperature, replicated ten times for each sample (control and LPC) 24 h after baking.

Total colour differences between control and LPC cookies were assessed using Equation (1):

$$\Delta E^* = (\Delta L^{*\cdot 2} + \Delta a^{*\cdot 2} + \Delta b^{*\cdot 2})^{½} \quad (1)$$

4.2.6. Texture Analysis

Texture analysis was performed in a TA.Xtplus (StableMicro Systems, Godalming, UK) texturometer. The measurements were performed at 20 °C ± 1 °C, in a temperature-controlled room.

4.2.6.1. Dough Texture

For the dough texture profile analyses (TPA), samples were subjected a simulation of the action of double chewing using the two-bite test, according to the method previously described by Mota et al. [5]. Each analysis was repeated eight times.

4.2.6.2. Cookie Texture

Each cookie's texture was assessed with a penetration test as described earlier by Mota et al. [5]. These tests were reproduced at least eight times for each cookie (control and LPC) 24 h after baking.

4.2.7. Water Activity Determination

The water activity (a_w) was established using a thermohygrometer (HygroPalm HP23-AW, Rotronic AG, Bassersdorf, Switzerland) at 20 °C ± 3 °C. After 24 h of baking, the tests were performed by crushing the cookies, with each cookie (control and LPC) measured in triplicate.

4.2.8. Chemical Compositions of the Cookies

The approximate chemical compositions of the cookies were evaluated based on powdered samples. According to the AOAC 950.36 official method for baked products, the protein content was evaluated using the Kjeldahl method. The total nitrogen content was multiplied by 5.7 (conversion factor) to acquire the cookie crude protein according to Batista et al. [17]. Crude fat was measured using ether extraction according to AOAC 2003.05. A minimum of 1.5 g of each cookie (with and without LPC) was weighed into a 26 mm × 60 mm cellulose extraction thimble. The content of petroleum ether lipids was evaluated by Soxtec extraction (Soxtec System HT 1043/1046 extraction unit (Tecator AB, Höganäs, Sweden), with 15 min of boiling and 60 min of rinsing, followed by 15 min of drying. Finally, the lipid content was determined gravimetrically. Ash content, representing the inorganic fraction of the cookies, was measured by incineration at 550 °C in a muffle (AACC 08-01.01). Moisture content was determined according Mota et al. [5]. Total carbohydrates were calculated by difference.

4.2.9. Bioactivities of the Cookies

4.2.9.1. Protein Extraction

Approximately 10 g ± 0.1 g of cookie was milled in a coffee grinder (Taurus Aromatic, Oliana, Spain) and the resulting cookie powder was resuspended in 40 mL of 100 mM Tris–HCl buffer (pH 7.5, 1:4 *w*/*v*) and stirred for 4 h at 4 °C. Each sample was then centrifuged at 12,000× *g* for 30 min at 4 °C, then supernatants were collected and stored at −20 °C.

4.2.9.2. Specific MMP-9 Inhibition

To determine the MMP-9 inhibitory activity levels of different cookies, the samples were prepared as described in Section 2.9.1. The supernatants were stored at −20 °C. MMP-9 inhibition was tested using the DQ fluorogenic assay as previously described [8]. To compare the MMP-9 inhibitory activity levels of savoury cookies versus sweet cookies, we produced sweet cookies using the same flours, using the method already described by Mota et al. [5].

4.2.9.3. Wound Healing Assay

The human colon adenocarcinoma cell line HT29 (ECACC 85061109), obtained from a 44-year-old Caucasian female, was used in this study. HT29 cells were maintained as described by Lima et al. [8]. For cell migration evaluation, the wound healing assay was performed as described by Lima et al. [8]. In this test, a cell-free area is established in a confluent monolayer by eliminating the cells using a scratch. Then, the cells tend to migrate into the gap. The measurements of the migrated area are determined at 0 and 48 h. Each well contained cookie protein extract at a concentration of 100 µg/mL of total soluble protein.

4.2.10. Statistical Analyses

The experimental data were acquired in triplicate and were analysed using SigmaPlot (version 12.5), as described previously [5]. Analysis of variance (one-way ANOVA) was used to calculate the differences between samples $p < 0.05$ representing significance levels of 95% and $p < 0.001$ of 99.99%. Differences between treatments were compared using Tukey's test.

4.3. Results

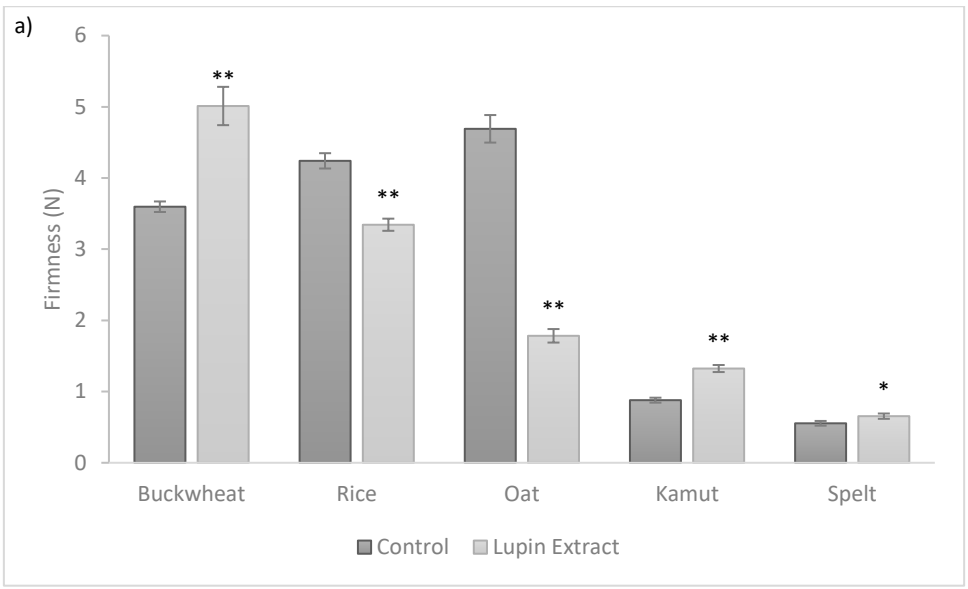
With the emergence of the nutraceutical industry, the development of novel candidates with high applicability, as well as being innovate and easy to manufacture industrially, has become a major goal; however, the delivery of such nutraceuticals in functional foods is often challenging. The food matrix is the first and one of the most important factors affecting the biological fate of a nutraceutical. The delivery systems should be congruent with the food matrix and should not influence the properties of the food product (in terms of aroma, taste, appearance and texture) [3]. For deflamin, its resistance to heat and its ability to be easily concentrated in the form of a lyophilized lupin protein powder extract meant that it could be easily introduced in baked cookies [18]. Nonetheless, our previous preliminary results suggested that the type of flour, and particularly the presence of sugar, had strong impacts on deflamin's activity.

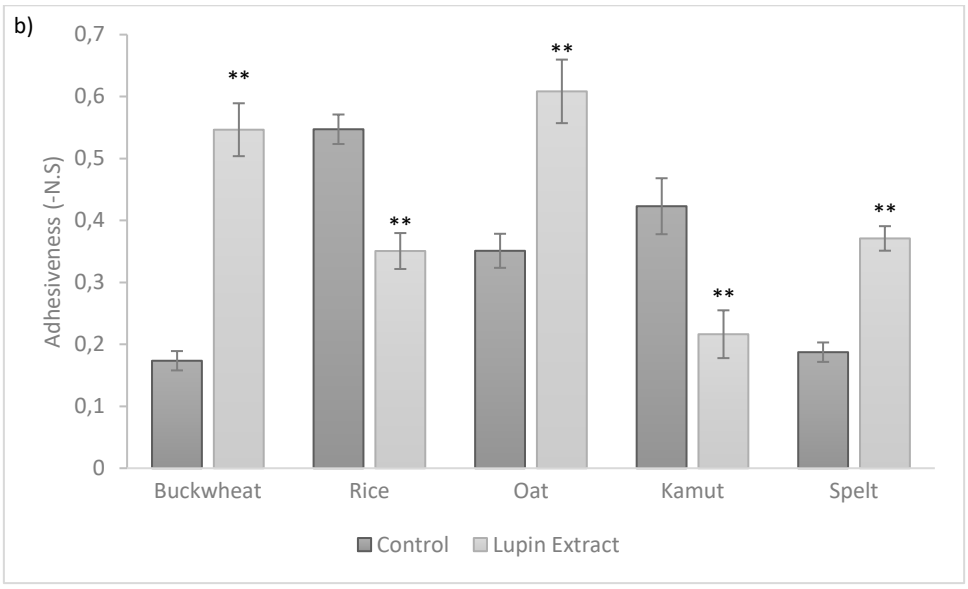
As such, the propose of the present work was to deliver the GRAS lupin protein concentrate previously developed in [5] in savoury baked cookies, aiming to maintain its effective anti-MMP-9 functional activities. The lupin protein concentrate (LPC) was, therefore, combined with different types of flours, with and without gluten, including spelt, kamut, oat, rice and buckwheat, with no addition of sugar.

4.3.1. Physical Characteristics of the Cookie Dough

We first set out to determine the impacts of the addition of LPC on the technological properties of the different doughs. The parameters obtained through TPA, such as the firmness, adhesiveness and cohesiveness, were the texture properties used to compare doughs, since they can distinguish variations between samples [19].

Figure 1 shows the firmness (a), adhesiveness (b) and cohesiveness (c) values for control and 10% (*w*/*w*) LPC doughs produced with five alternative flours. Compared to the control dough, the ancient grain doughs (kamut and spelt) were less firm and more cohesive, while the oat dough was firmer and less adhesive than the other doughs. These results are in agreement with Angiolini and Colar [20], who conducted a study on bread enriched with oat, buckwheat, spelt and kamut. The presence of the gluten protein matrix increases the air retention capacity of the structure [21], causing less firm doughs.


a)
Firmness (N)
6
5
4
3
2
1
0
**
**
**
**
*
Buckwheat
Rice
Oat
Kamut
Spelt
Control
Lupin Extract


b)
Adhesiveness (-N.S)
0,7
0,6
0,5
0,4
0,3
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0,1
0
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Buckwheat
Rice
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Kamut
Spelt
Control
Lupin Extract

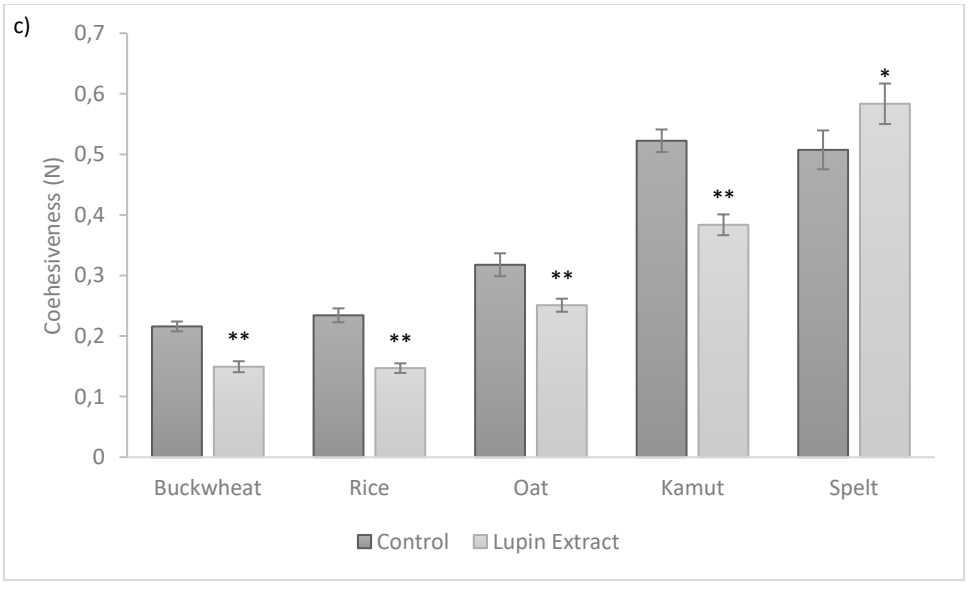
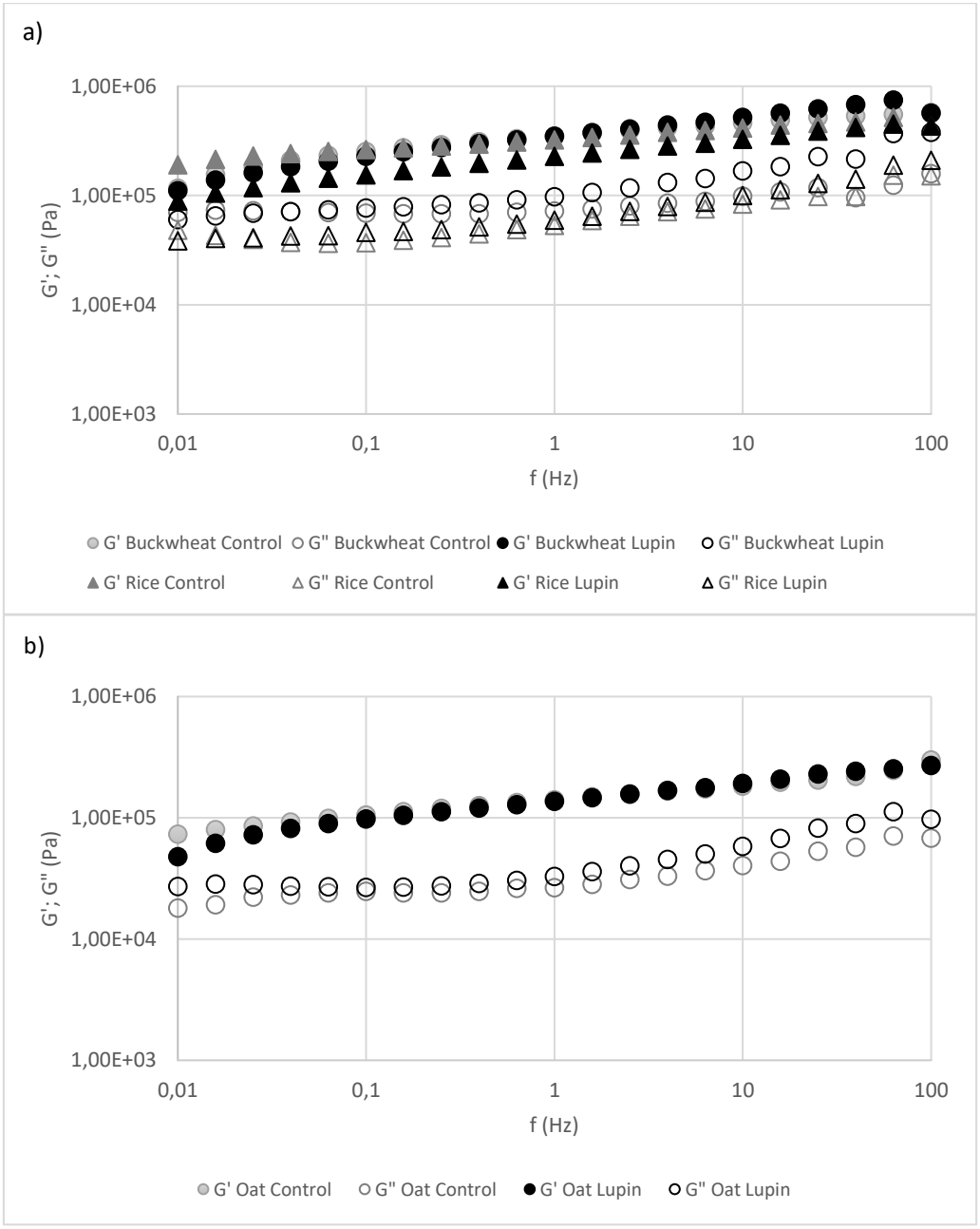

c)
Coehesiveness (N)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
**
**
**
**
*
Buckwheat
Rice
Oat
Kamut
Spelt
Control
Lupin Extract

Figure 4. Texture parameters of control and LPC cookies with alternative flours (buckwheat, rice, oat, kamut and spelt). (a) Firmness; (b) Adhesiveness; (c) Cohesiveness * represents $p < 0.05$ and ** represents $p < 0.001$ when compared with control cookie.

These results are in agreement with Angiolini and Colar [20], who conducted a study on bread enriched with oat, buckwheat, spelt and kamut. The presence of the gluten protein matrix increases the air retention capacity of the structure [21], causing less firm doughs.

The impact of the LPC on the cookies is in agreement with the results obtained by Mota et al. [5] for the incorporation of LPC in sweet cookies. All parameters tested showed significant differences ($p < 0.05$) between LPC and control cookies. In terms of firmness, the gluten-free flours, namely buckwheat and rice flours with LPC, showed values of 5 N and 3.3 N, respectively, being firmer when compared to the other alternative flours. The more adhesive doughs were those prepared from oat and buckwheat flours at 0.55 N and 0.6 N, respectively; however, regarding cohesiveness, the behaviour was opposite in comparison with firmness, because the LPC cookie with gluten-free flours were less cohesive and the ancient grain flours were more cohesive. Clearly, as expected, the gluten proteins played a crucial role in the matrixes of the cookies, as was reported in other studies [22,23]. On the other hand, the distinct behaviours observed amongst gluten-free flours likely resulted from: (i) different extensions of the interactions between LPC, proteins and polysaccharides from the gluten-free starch flour; (ii) the different hydration level of each flour. In fact, Boucheham et al. [24] demonstrated that legume seed flours have higher water absorption capacity (WHC) levels than cereal flours, resulting from the higher protein contents present in legume-derived ingredients. In fact, protein–water interactions are related to the WHC [25], meaning the addition of LPC should influence the hydration levels of flours and cause less cohesiveness in the corresponding doughs.

In Figure 2, the viscoelastic behaviours of the five alternative flours in cookie doughs with and without LPC are represented.


a)
1,00E+06
1,00E+05
1,00E+04
1,00E+03
G'; G" (Pa)
0,01
0,1
1
10
100
f (Hz)
G' Buckwheat Control
G" Buckwheat Control
G' Buckwheat Lupin
G" Buckwheat Lupin
G' Rice Control
G" Rice Control
G' Rice Lupin
G" Rice Lupin
b)
1,00E+06
1,00E+05
1,00E+04
1,00E+03
G'; G" (Pa)
0,01
0,1
1
10
100
f (Hz)
G' Oat Control
G" Oat Control
G' Oat Lupin
G" Oat Lupin

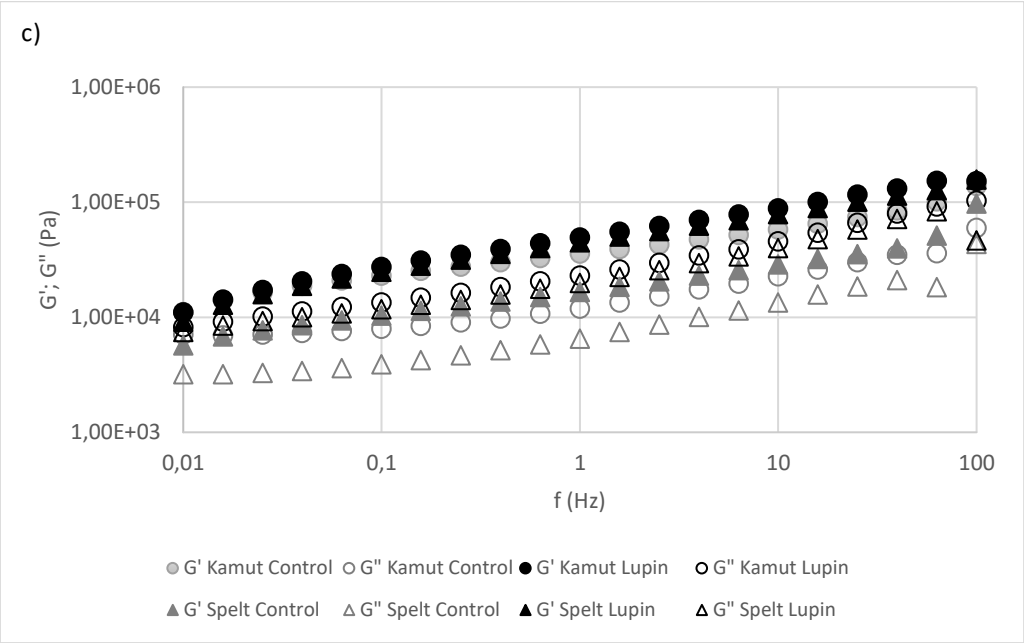


Figure 5. Mechanical spectra of control and LPC doughs prepared with alternative flours. (a) gluten-free flours, buckwheat, and rice, (b) oat; (c) gluten flours, kamut, and spelt. Close symbols represent G' (elastic modulus) and open symbols represent G" (viscous modulus).

The G' (storage modulus) and G'' (loss modulus) increased with increasing frequency range and revealed a weak gel-like rheological behaviour that is characteristic of cookie doughs and in agreement with the results demonstrated by Mota et al. [5] and Raymundo, Fradinho and Nunes [26]. Regardless of the nature of flour, the mechanical spectra were similar among samples with no drastic changes; consequently, the incorporation of LPC did not induce changes in the structures of the doughs. This trend was not observed for spelt flour, since the G' and G'' values for the LPC-containing dough increased when compared to the respective control dough. This behaviour, i.e., the increase of the viscoelastic functions for spelt flour as the result of the addition of LPC, likely resulted from the higher content of free sugars presented in this flour. The presence of a high sugar content has a huge impact on the water absorption [27] and consequently on the development of the structure development between LPC proteins and spelt proteins and polysaccharides. Similar behaviour was also noted by Sahagún and Gómez [28] after introducing 30% potato protein into corn cookie doughs and by Mota el al. [5] after the incorporation of 10% lupin protein into buckwheat flour to produce sweet cookies.

4.3.2. Physical Properties of Cookies

The physical properties of cookies frequently determine their attractiveness and desirability (or undesirability). As such, firmness (Figure 3), the colour parameters (Table 1) and water activity levels (Table 1) of all cookies with and without lupin extract were evaluated.

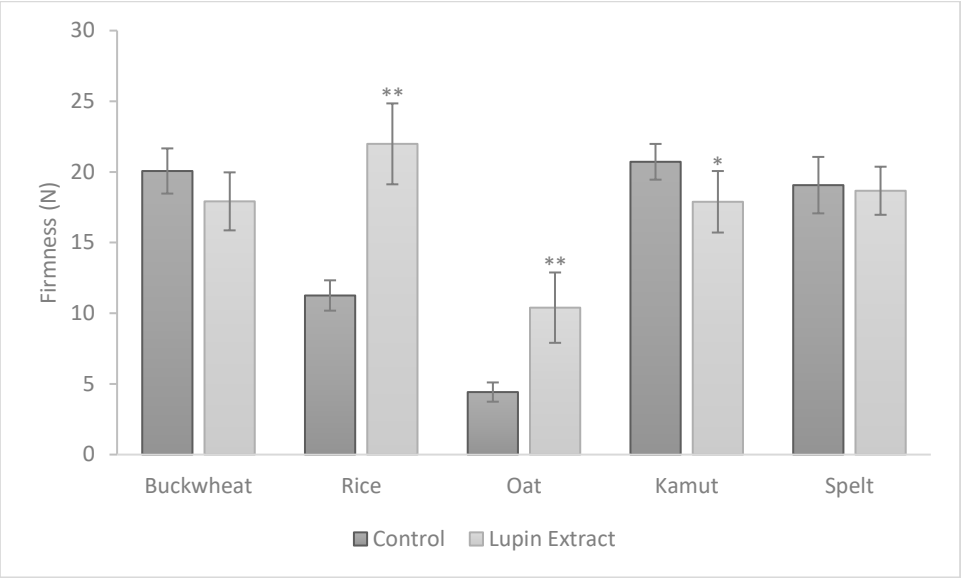


Figure 6. Firmness of control and LPC cookies 24 h after baking of five different flours. Values are the average of six experiments ± SD. * represents $p < 0.05$ and ** represents $p < 0.001$ when compared with the corresponding control cookie.

The texture results of cookies with 10% (w/w) LPC incorporation are presented in Figure 3. The variations observed at the level of the several studied doughs are reflected at the level of the baked cookie, clearly indicating differences in its firmness. Cookies prepared with oat and rice flours (without LPC) revealed less firmness in comparation to other flours. The impact of LPC were relevant in kamut flour ($p < 0.05$) and very significant in rice and oat flours ($p < 0.001$). Cookies with LPC and rice flour were firmer (22 N), whereas cookies with LPC and oat flour were less firm (10.4 N). These distinct behaviours can be related with macromolecule structure of the five flours due to absence or presence of gluten. The impact of LPC cause a structural rearrangement leading to diverse interactions amongst these macromolecules (starch and protein interactions) and altered by the heat treatment that exists during cooking. Furthermore, Raymundo, Fradinho and Nunes [24] showed the same behaviour differences between the doughs and the respective cookies.

The impact of LPC cookies on the colour is summarized in Figure 4 and table 1. The ΔE* values were determined to compare the colour variation of cookies without LPC. In the same table, colour parameters (L*, a*, and b*) and water activity (a_w) of all cookies are shown as well.

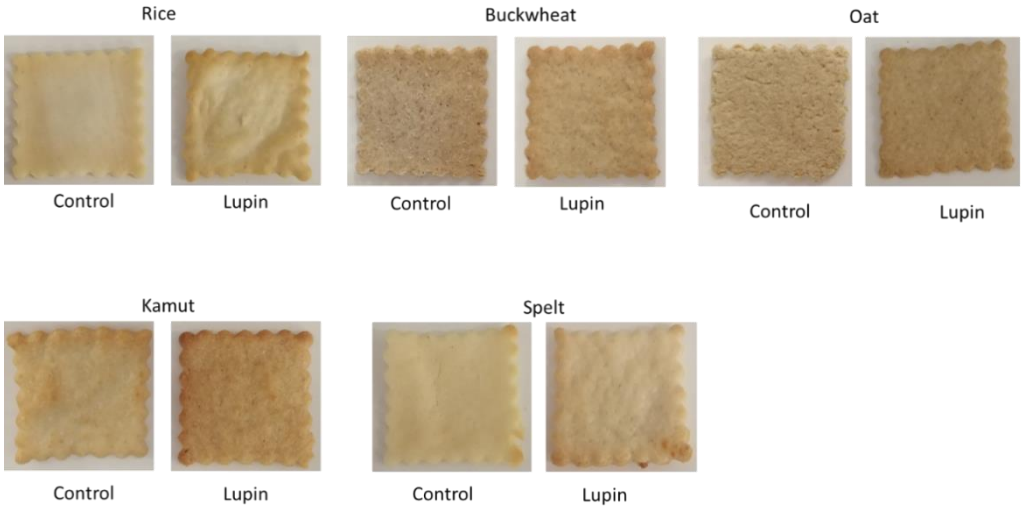


Figure 4. Representative images of control and LPC cookies 24 h after baking of five alternative flours.

Table 1. Values of ΔE*, L*, a*, b* and water activity (a_w) of control and LPC cookies. Values are the averages of at least five experiments ± SD, except ΔE* that is the difference among control and LPC cookies. * represents $p < 0.05$ and ** represents $p < 0.001$ when compared with the corresponding control cookie.

		Buckwheat	Rice	Oat	Kamut	Spelt
ΔE*		5.98	17.13	5.20	2.88	14.64
L*	Control	63.71 ± 1.94	78.63 ± 1.30	66.33 ± 2.72	68.93 ± 1.33	81.82 ± 2.08
	Lupin	60.75 ± 2.8*	71.04 ± 1.99**	65.76 ± 2.03	68.13 ± 1.96	71.86 ± 5.93**
a*	Control	7.70 ± 0.65	-1.04 ± 0.09	4.20 ± 0.49	5.64 ± 0.62	2.13 ± 0.36
	Lupin	5.51 ± 0.53**	4.01 ± 0.55**	6.53 ± 0.54**	8.75 ± 0.97**	9.45 ± 0.79**
b*	Control	26.08 ± 2.62	16.66 ± 1.93	25.94 ± 0.46	33.10 ± 2.04	28.15 ± 2.72
	Lupin	25.25 ± 1.22	29.36 ± 1.67**	30.21 ± 2.13**	34.25 ± 2.77	34.38 ± 1.28**
a_w	Control	0.09 ± 0.01	0.19 ± 0.01	0.07 ± 0.00	0.53 ± 0.01	0.57 ± 0.01
	Lupin	0.54 ± 0.02**	0.38 ± 0.00**	0.28 ± 0.01**	0.67 ± 0.00**	0.48 ± 0.00**

The colour variations between the control and LPC-enriched cookies expressed in terms of ΔE* values were greater than 5 for all flours, except for kamut, indicating that the colour differences amongst the LPC cookies and the controls were visually distinguishable by the human eye [29]. The golden-brown colour resulted from reductions in the lightness parameter (L*) in all LPC-containing cookies. Nevertheless, the highest ΔE* values were obtained for rice and spelt flours at 17.13 and 14.64, respectively. These results were likely due to the Maillard reaction, as amino acids and reducing sugars start a flow of reactions throughout heating (higher than 100 °C), generating darker colours [30]; additionally, these two flours in particular were whiter than the others. Despite being darker, the LPC cookies presented very appealing colours and their appearance did not suffer negatively, as supported by similar studies [5,26].

The a_w values for LPC cookies were significantly higher ($p < 0.001$) than those for the control cookies. Moreover, the incorporation of LPC increased the a_w values for all flours tested,

except for spelt flour, which was in agreement with the results shown by Mota et al. [5]. Furthermore, several studies observed increases in a_w values with the addition of apple fibre to cookies [31] and with the addition of microalgae with a high protein content [32]. For the lupin-enriched cookies, the increases in the protein content led to lower water-holding capacity values when compared to the respective control flours, supporting the higher a_w values. Furthermore, the a_w values remained at low levels and the addition of LPC did not modify the preservation characteristics of the food products.

4.3.3. Chemical Composition of Cookies

The proximate analysis of foods involves the determination of the principal components, namely ash (total minerals), lipids, protein, moisture and carbohydrates. Table 2 presents the approximate chemical compositions of the cookies prepared with lupin extract.

Table 2. Chemical compositions of five different cookies with and without LPC (%, dry weight). Results are expressed as averages ± standard deviation (n = 3). Note: * represents $p < 0.05$ and ** represents $p < 0.001$ when comparing LPC cookies with the respective controls.

		Total Ash (%)	Crude Fat (%)	Crude Protein (%)	Moisture (%)	Carbohydrates[#] (%)
Buckwheat	Control	4.15 ± 0.10	12.32 ± 0.06	10.14 ± 0.06	3.22 ± 0.05	70.18
	Lupin	4.78 ± 0.04**	10.89 ± 0.03**	13.85 ± 0.16**	3.99 ± 0.12**	67.75
Rice	Control	3.16 ± 0.13	7.45 ± 0.13	5.22 ± 0.06	6.65 ± 0.07	77.52
	Lupin	3.81 ± 0.10*	9.18 ± 0.26**	10.18 ± 0.25**	6.80 ± 0.11	70.03
Oat	Control	4.14 ± 0.18	14.45 ± 0.13	8.03 ± 0.06	6.22 ± 0.21	67.16
	Lupin	5.15 ± 0.09**	14.02 ± 0.04*	12.42 ± 0.06**	4.27 ± 0.20**	64.15
Kamut	Control	3.48 ± 0.13	9.96 ± 0.22	10.99 ± 0.09	10.06 ± 0.11	65.51
	Lupin	4.31 ± 0.26*	9.66 ± 0.12	14.53 ± 0.31**	5.29 ± 0.08**	66.21
Spelt	Control	3.42 ± 0.11	10.81 ± 0.06	8.60 ± 0.25	7.19 ± 0.05	69.98
	Lupin	4.54 ± 0.10**	11.74 ± 0.12**	12.60 ± 0.31**	4.60 ± 0.23**	66.52

Carbohydrates were determined by difference in the means of ash, fat, protein, and moisture.

Lupin-enriched cookie samples exhibited significantly higher ($p < 0.001$) protein contents than the control (Table 2), as expected. LPC cookies with buckwheat (13.85%) and kamut (14.53%) flours had the highest crude protein values, while the lowest value was verified in rice flour

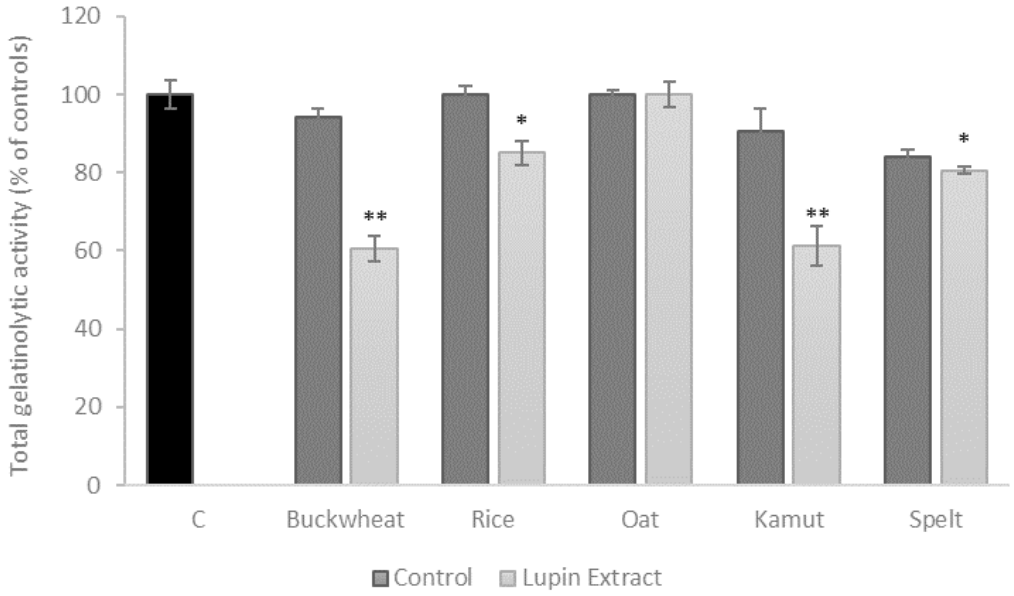
(10.18%). The enrichment of snacks and biscuits with different protein sources has received extensive attention from several authors; this is the case for the incorporation of microalgae, with studies revealing increases in protein content [32,33]. On the other hand, the ash contents were significantly higher ($p < 0.05$) for rice and kamut flours, with very significant values ($p < 0.001$) for buckwheat, oat and spelt flours when comparing LPC cookies with the respective controls. Alomari and Abdul-Hussain [34] and Bilgiçli and Levent [35] demonstrated the same increases in ash content in bread supplemented with different concentrations of lupin flour and with incorporation of lupin flour in wheat cookies, respectively.

Overall, the increases in the amounts of protein and in several physicochemical parameters corroborate that the addition of LPC can increase the quality of the savoury biscuits produced. The use of lupin may provide more advantages since this species is exceptional amongst legumes, having some of the highest quantities of digestible plant protein (38%) and dietary fiber (30%) and a particular low quantity of antinutritional compounds, meaning it does not need to be soaked or cooked [3]. Additionally, since sweet lupin consumption is known to decrease blood pressure, improve blood lipids and insulin sensitivity, and favourably alter the gut microbiome, there is increasing interest in this legume as an ingredient to improve the nutritional value of baked goods (particularly gluten-free products) [36,37].

4.3.4. Anti-MMP-9 Activities of Cookies

Since the main goal of the present work was the use of LPC as a delivery system for the previously detected nutraceutical MMPI properties of *L. albus,* we tested the inhibitory effects of all cookies against MMP-9 activity using the standard DQ gelatin assay (Figure 5).

a)



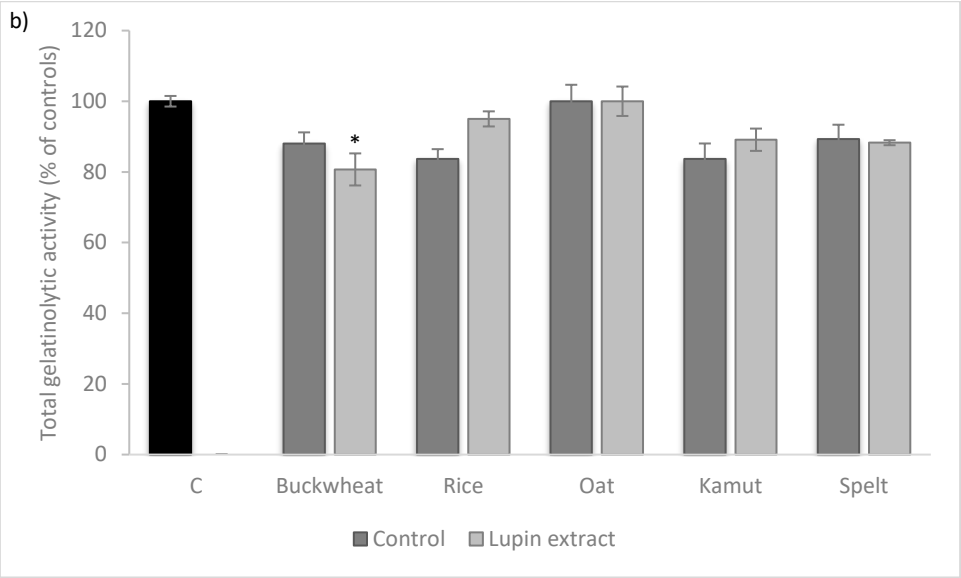


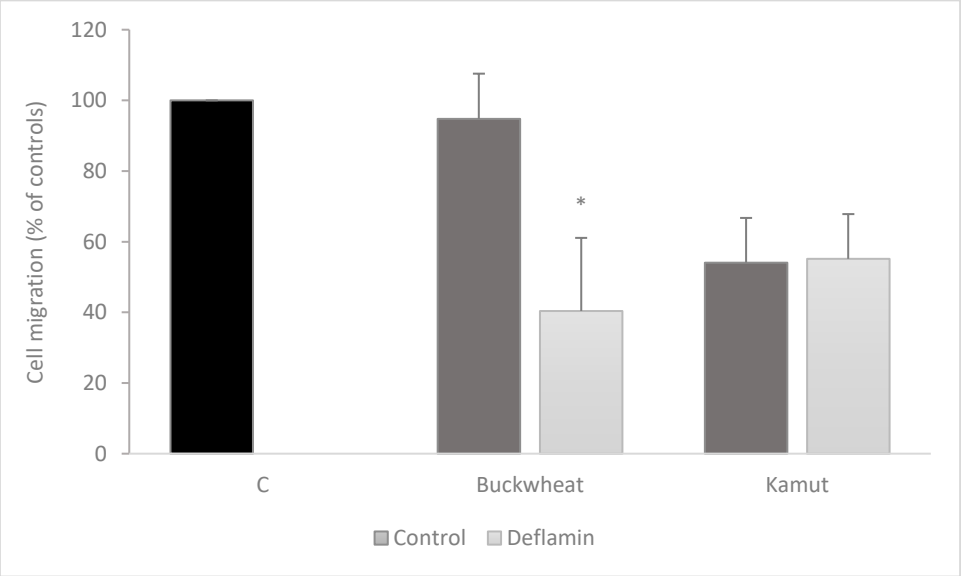
Figure 5. The effect of five different flours with and without LPC on MMP-9 activity. a) Savoury cookies; b) Sweet cookies. The positive control (C) does not inhibit MMP-9, resulting in 100% proteolytic activity. All samples were added at same volume (80 µL) and gelatinolytic activity was measured. Gelatinase activities are expressed as relative fluorescence as a % of controls (C) and represent the means of three replicate experiments (n = 3) ± SD. *$p < 0.05$ and **$p < 0.001$ when compared to cookie controls.

Although the MMPI activity was already shown to resist baking, the inhibitory activity against MMP-9 was very significantly altered in the presence of the different flours. Figure 5a shows that although all lupin-enriched savoury cookies, except for oat flour, were able to significantly inhibit the gelatinase proteolytic activity ($p < 0.05$) in slow levels (around 20%) the only physiological relevant inhibitions were observed by buckwheat and kamut flours with 39.6% and 38.9%, respectively. Although both flours have been reported as anti-inflammatory: Valli et al. [15] performed an *in vitro* study in HepG2 cells and reported that kamut bread had an anti-inflammatory activity when compared to other grains and the anti-inflammatory effects of buckwheat have been reported in *in vivo* models of intestinal inflammation by reducing colonic mucosa inflammation [35], comparison with the controls shows that the activity detected was not due to their intrinsic activity, but rather due to the effect of incorporating the LPC with anti-MMP-9 activity as previously reported by Lima et al. [8]. The reasons for the differences found in the different flours could be somehow related to the amount of starches and sugary molecules in each one. Indeed, MS sequencing of the oligomer that forms deflamin shows the presence of fragments of beta conglutin, which is well known for its lectin activities (data not shown). It is feasible that by binding to different sugars in different flours, the deflamin in the LPC may lose some of its activity. This is substantiated by the fact that when added to sweet cookies, the LPC loses its MMP-9 inhibitory activity (Figure 5b). Whichever case results here clearly show that in the case of deflamin, the biochemical composition of the food matrix is crucial for its optimal function.

4.3.5 Protein cookies extract inhibit colon cancer cell migration

Since our previous studies showed that the LPC had an ability to reduce colon cancer cell migration [8,9] we further set out to determine the effect that savoury cookies might have on cancer cells. Figure 6 shows cell migration patterns of HT29 cells after a 48h exposure to the cookie protein extracts, that showed very significant inhibitory activity of MMP-9 above, using the wound healing assay.

a)



b)

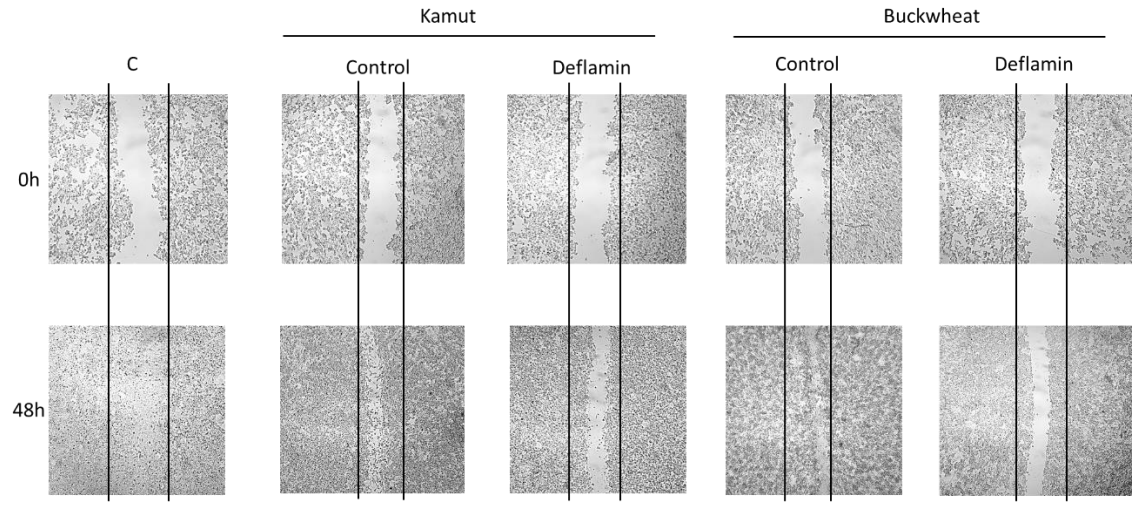


Figure 6. HT29 cell migration after exposure to cookies as determined by the wound healing assays. a) Relative migration rates. Values are the averages of three replicate tests ± SD and are expressed as a percentage of the wound closure in relation to 0 h. * represents $p < 0.05$ when compared to controls. b) Representative images of cell migration demonstrating the inhibitory effect of kamut and buckwheat cookies. Cells were exposed to 100 µg protein/mL.

Results clearly demonstrate that the buckwheat cookie with incorporation of lupin extract had a significant inhibitory effect on cell migration ($p < 0.05$), whereas in controls it did not, corroborating that the LPC maintained its activities. As for kamut cookies, both controls and

LPC-containing cookies reduced HT29 cell migration, suggesting that this was due to other components of this flour. Previous reports have shown that kamut can cause less inflammatory responses than wheat [15], however this is, to our knowledge, the first report of this type of activity in this cereal.

Overall, in the present study only the buckwheat flour demonstrated no significant effects upon the MMP inhibitor (MMPI) activity of our lupin extract, whilst maintaining its anti-inflammatory and anticancer potential. Buckwheat flour is considered a functional food and it is presumed that its proteins are responsible for health benefits, with Giménez-Bastida et al. [39] showing that buckwheat bread reduced the effects of TNF-α on migration and cell cycle in myofibroblasts, exhibiting anti-inflammatory activity [39]. Overall, the above results allow us to infer that LPC-containing cookies could have great potential as functional foods.

4.4. Conclusions

Cookies are important ready-to-eat baked snacks that are consumed globally. Regarding today's market demands, cookies are becoming increasingly popular as functional foods with the use of alternative flours, providing both added nutritional value and bioactivity. In the present study, an efficient delivery method for incorporating a bioactive lupin extract into cookies using different alternative flours was presented. Technologically, it was found that the incorporation of the lupin extract has an impact on the characteristics of the dough and the final product, depending on the type of flour. The biochemical compositions of all cookies with lupin extract presented higher protein and ash contents when compared to the control cookies. The only flour that did not interfere with the desired nutraceutical activities was buckwheat, because it showed higher bioactivity against MMP-9 activity whilst maintaining strong inhibition of colon cancer migration. Furthermore, lupin-enriched buckwheat cookies showed improved colour rendering their appearance more attractive to consumers; hence, buckwheat cookies were demonstrated to be a good vehicle to deliver a potent nutraceutical from lupin in preventive and curative diets, particularly in diets used to treat inflammation and cancer diseases of the gastrointestinal tract. In the near future, sensory analysis of the buckwheat cookies should be carried out to evaluate the acceptability of this new product by consumers.

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Chapter 5. Lupin protein concentrate as a novel functional food additive that can reduce colitis-induced inflammation and oxidative stress

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Status: *Nutrients,* 2022, 14(10), 2102

DOI: 10.3390/nu14102102

Abstract: Food fortification with bioactive compounds may constitute a way to ameliorate inflammatory bowel diseases (IBDs). Lupin seeds contain an oligomer named deflamin that can reduce IBD's symptoms via MMP-9 inhibition. Here, our goal was to develop a lupin protein concentrate (LPC) enriched in deflamin and to test its application as a food additive to be used as a functional food against colitis. The nutritional profile of the LPC was evaluated, and its efficacy *in vivo* was tested, either alone or as added to wheat cookies. The LPC presented high protein and carbohydrate contents (20.09 g/100 g and 62.05/100 g, respectively), as well as antioxidant activity (FRAP: 351.19 mg AAE/10 mg and DPPH: 273.9 mg AAE/10 mg). It was also effective against TNBS-induced colitis in a dose dependent-manner, reducing DAI scores by more than 50% and concomitantly inhibiting MMP-9 activity. When added to cookies, the LPC activities were maintained after baking, and a 4-day diet with LPC cookies induced a significant protective effect against acetic acid-induced colitis, overall bringing lesions, oxidative stress and DNA damage levels to values significantly similar to controls ($p < 0.001$). The results show that the LPC is an efficient way to deliver deflamin in IBD-targeted diets.

Keywords: colitis; HT29; IBD; lupin; antioxidant; MMP-9; cookies; functional food

5.1. Introduction

With the alarming growing incidence of chronic diseases worldwide, functional foods that exhibit health-promoting effects and can be safely used to reduce or prevent symptoms are receiving a lot of attention for certain types of pathologies such as gastrointestinal disorders, particularly inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn's disease (CD) [1]. These have long been recognized as a global burden, with prevalence rates of almost 250 cases per 100,000 persons, which are increasing worldwide in individuals of all ages [2]. Symptomatology includes mucosal inflammation, abdominal pain, diarrhea, bleeding and morphological changes in the colon such as epithelial erosion, edema formation and leukocyte infiltration (cryptitis) [3], which severely reduce the patients' life quality [4]. The only available pharmacological treatments for IBDs are anti-inflammatory or immunosuppressive drugs, which are not only insufficient and show low-to-no effect for temporary symptoms, but are also prone to severe side effects [2]. To add to this, they are also expensive, which is a barrier for many patients, particularly for long-term therapies [2]. Therefore, it is unsurprising that the use of natural products to fight IBDs, particularly functional foods, is gaining worldwide attention. Indeed, diet itself is increasingly recognized as one of the most important influencing factors [5,6]. Whilst a wide range of dietary factors have been studied and recognized as driving the onset of inflammation, with certain food items being considered important triggers for IBDs [5,6], functional food efficacy against these diseases has also been broadly studied over the last decade. One of the most studied compounds are fat-soluble vitamins, such as vitamin D, which modulate inflammatory responses via the regulation of proinflammatory gene expression, transcription factors and the activation of signaling cascades that mediate inflammatory responses [7]. Other antioxidant compounds like β-carotene and flavonoids play a key role in IBDs by limiting the production of free radicals in cells, inhibiting lipid oxidation or limiting the synthesis of proinflammatory cytokines [8]. Non-starch polysaccharides, classified as dietary

fiber, have also been studied extensively as therapeutics against inflammation and other immune-related problems. For instance, Lean et al. treated mice with fucoidans, a compound found in edible brown macroalgae, and observed a reduction in colitis symptoms, diarrhea and the relative weight of the colon, reducing inflammation and edema [9]. Additionally, research evidence suggests that other bioactive food compounds including peptides [10], polyphenols [11] and essential oils [12] exhibited anti-inflammatory and antioxidant effects in animal models of colitis. However, they rarely exist in sufficient amounts in foods to exert the necessary effect [13]. Therefore, the fortification of food products with these nutraceuticals may constitute a simple way of delivering them in sufficient amounts in diets. In fact, there has been an increasing research interest in the development of functional foods/beverages enriched with different nutraceuticals throughout recent years [1]. However, many nutraceuticals often exhibit several limitations, including low water solubility and stability, interactions with the food matrix, low bioavailability, poor absorption and even chemical transformation during digestion, which strongly limit their direct incorporation into food products [1]. To this end, we have previously developed a lupin protein concentrate (LPC) from *Lupinus albus* seeds that contains high amounts of a bioactive polypeptide oligomer named deflamin (patent WO/2018/060528), one of the few existent protein matrix metalloproteases (MMPs) inhibitors (MMPIs) derived from staple foods that effectively reduces gelatinolytic activity *in vivo* [14]. MMPs comprise a family of zinc-dependent endopeptidases with a well-recognized key role in intestinal inflammation [15], particularly gelatinase MMP-9, which has been shown to be up-regulated in human colitis and other IBDs and is positively correlated with disease severity [15]. It has been shown that MMP-9 inhibition can reduce IBD development [15], meaning that deflamin may hold great potential to be used in functional diets. When administered orally, isolated deflamin reduced colitis lesions, whilst inhibiting MMP-9 activity in mice [14]. In our previous reports, the deflamin-enriched LPC was added to low gluten and gluten-free cookies (10 g protein/100 g cookie dough) [16]. These studies showed that some of the flours used strongly interfered with deflamin activity. Although the LPC could be a very promising delivery system for functional foods against gastrointestinal diseases, it was never tested *in vivo*. Being a lupin concentrate per se, it should be expected that the other components of the LPC, such as polyphenols and other proteins, might assist the bioactivity of these cookies as well, particularly by reducing oxidative stress [17]. Evidence suggests that IBDs are associated with an imbalance between ROS and antioxidant activity, which generates oxidative stress as the result of either ROS overproduction or a decrease in antioxidant activity [18,19], meaning that the main key players in ROS scavenging are often identified as important clinical targets in IBD management [17–19]. Recent reports demonstrate that lupin is a promising source of antioxidant phenolics for functional food production, as well as an inductor of a healthier gut microbiome [20]. These observations highlight the importance to further pursue the potential of this LPC as a functional ingredient, and to test its potential *in vivo*, both as an MMP-9 inhibitor and as an oxidative stress reducer in IBDs. The present study aimed to evaluate the nutrient and antioxidant value of the LPC, to test its efficacy as a food additive for wheat cookies in reducing colitis *in vivo*, using two different models of IBD, and in inhibiting MMP-9 and other inflammatory pathways, including oxidative stress.

5.2. Materials and Methods

5.2.1. Biological Materials

The biological material used for the present study was the quiescent seeds of sweet lupin (*Lupinus albus* L.), cv Amiga (Jouffray-Drillaud, Cissé, France). The human colon adenocarcinoma cell line, HT29 (ECACC 85061109), obtained from a 44-year-old Caucasian female, was also used throughout this work.

5.2.2. Preparation of the Lupin Protein Concentrate (LPC)

Approximately 100 g ± 0.1 g of dry lupin seed protein was extracted using milli-Q water (1:10, *w*/*v*). The homogenate was filtered through miracloth (Ref: 475855, Merck Millipore, Massachusetts, USA). The filtered sample was subsequently boiled for 10 min and filtered again through miracloth. The protein sample was finally freeze-dried (Edwards Micro Modulyo, Crawley, UK) and stored at −80 °C.

5.2.3. Chemical Characterization of LPC

The approximate chemical composition of the lupin extract was calculated based on powdered samples. Total protein determination was evaluated by Dumas Nitrogen Analyser NDA 702 (Velp Scientifica, Usmate Velate, Italy) and the conversion factor used was 5.7. Crude fat was measured using ether extraction according to AOAC 2003.05 and Mota et al. [16]. Ash content, representing the inorganic fraction of the LPC, was measured by incineration at 550 °C in a muffle according to AACC 08-01.01. Moisture content was determined gravimetrically, according to Mota et al. [16]. Total carbohydrates were calculated as (100-[protein + fat + ash + water content]).

5.2.4. Antioxidant Activity of LPC

The total phenolic content (TPC) of the LPC was assessed following the method described by Batista and colleagues [21], with minor alterations. LPC phenolic compounds were extracted in methanol in a ratio of 1:5 (*w*/*v*). The homogenate was stirred for 1 h in the dark at 4 °C, followed by centrifugation (12.000× *g*, 4 °C, 30 min). The LPC extract or different concentrations of gallic acid (10 µL) were added to Folin–Ciocalteu reagent (Ref: F9252, Sigma-Aldrich, St. Louis, MO, USA) (100 µL at 0.1 M), thoroughly mixed with 80 µL of 7% (*w*/*v*) sodium carbonate, incubated in the dark for 15 min at room temperature and the absorbance measured at 630 nm. The scavenging effect of LPC was determined using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) methodology, and the LPC extract reducing power was evaluated by applying the ferric ion reducing antioxidant power (FRAP) method, both described by Batista et al. [21]. Tests were performed in triplicate for each antioxidant activity assay to guarantee reproducibility of the results.

5.2.5. In vivo Mouse Model of TNBS-Induced Colitis

Six-week-old male CD-1 mice were purchased from Charles River. 2,4,6-Trinitrobenzene sulfonic acid (TNBS, Ref: P2297, Sigma-Aldrich; 2.5% *w*/*v*) in 50% (*v*/*v*) ethanol was instilled as an intracolonic single dose, as previously described [14]. On the induction day (day 0), the mice

were anesthetized with ketamine 100 mg/kg + medetomidine 10 mg/kg. Then, 100 µL of TNBS solution was administered through a catheter, carefully inserted 4.5 cm into the colon. The mice were kept for 20 to 30 min in a Tredelenburg position to avoid reflux. The experimental groups included control animals (*n* = 3) without colitis induction, vehicle (Col; *n* = 5), orally administered LPC (0.1 g/kg; p.o. 1×day, for 4 days; LPC-1; *n* = 5), orally administered LPC (1 g/kg; p.o. 1×/day, for 4 days; LPC-2; *n* = 5) and orally administered LPC (10 g/kg; p.o. 1×/day, for 4 days; LPC-3; *n* = 5). Oral administration was performed by oral gavage, daily, starting at 4 h after colitis induction. At day 4, mice were sacrificed by an administration of 0.25 mg/kg BW sodium pentobarbital. At necropsy, blood was collected by cardiac puncture into centrifuge tubes, and serum was stored at −20 °C for further use. Colons were removed, measured in length and observed for diarrhea severity classification. An extraction of colon proteins was performed according to Castaneda et al. [22], with some modification. Briefly, snap-frozen samples of colon were homogenized with liquid nitrogen and extracted in ice-cold extraction buffer (2.5 mL/g tissue) containing 100 mM Tris-HCl, 100 mM NaCl, 100 mM $CaCl_2$ and 0.05% Briji 35 (pH 7.6). After 10 min on ice, colon protein extracts were centrifuged for 10 min at 13,000× *g* at 4 °C, the supernatants were collected and stored at −80°C until assayed.

5.2.5.1. Disease Activity Index (DAI)

To evaluate colitis severity, the disease activity index (DAI) was assessed at the end of the experiment (day 4). DAI was calculated based on clinical sign scoring, including weight loss and stool character, as described by Maheshwari, Balaraman, Sailor and Sen [23].

5.2.5.2. Total Gelatinolytic Activity

To evaluate MMP-9 activity in colon samples, MMP-9 inhibition was tested using the DQ-gelatin assay (Ref: E12055, Thermo-Fisher Scientific, Waltham, MA, USA), as previously described by Lima et al. [24]. All assays were performed in triplicate.

5.2.6. Cookie Preparation

5.2.6.1. Savoury Cookies

Savoury cookies were prepared using 61.5% wheat flour (T55), 1% salt, 1.5% baking powder, 7.5% sunflower oil and 28.5% water, according to a previously optimized model formulation [16,21]. Batches (100 g) were prepared and the ingredients mixed for 1 min on position 4 in a food processor (Bimby, Vorwerk, Wuppertal, Germany). The cookies were molded using 2, 4 and 6 positions on a pasta roller system (three times each position). A square mold was used to cut the laminated dough. The savoury cookies were baked in a forced-air convection oven (Unox, Cadoneghe, Italy) at 180 °C for 10 min and were dried in the chamber at 60 °C for 30 min. Finally, the cookies were cooled to room temperature for 30 min, stored in hermetic containers and protected from light.

5.2.6.2. Sweet Cookies

The sweet cookies were prepared using 57% wheat flour, 15% white sugar, 1% baking powder, 18% margarine and 9% water [25]. Batches (100 g) were prepared and the ingredients mixed

for 15 s at a speed of 4 in a food processor (Bimby, Vorwerk, Wuppertal, Germany). The sweet cookies were molded in a circular mold and baked at 110 °C for 40 min in a forced-air convection oven (Unox, Cadoneghe, Italy). After cooling for 30 min at room temperature, the cookies were stored in hermetic containers and protected from the light.

Ingredients were added in identical quantities in all cookie samples, except for the flour, which was substituted by 10% of lupin extract in the LPC cookies.

5.2.7. In vitro Colon Cancer Cell Assays

5.2.7.1. Testing LPC Bioactivity in Cookies

The total soluble protein content of each cookie sample (with and without LPC) was extracted with 100 mM Tris-HCl buffer pH 7.5, in a ratio of 1:4 (*w*/*v*). This solution was stirred overnight at 4 °C. Samples were centrifuged in a Beckman J2-21M/E centrifuge at 12.000× *g* for 30 min at 4 °C, and the supernatant was collected and stored at −20 °C.

5.2.7.2. Wound Healing Assay

HT29 cells were maintained in RPMI medium (Ref: R5886, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Ref: F9665, Sigma-Aldrich), 2 × 10^4 UI/mL penicillin and 20 mg/mL streptomycin at 37 °C, in a humidified atmosphere of 5% (*v*/*v*) CO_2. Cell migration was assessed by wound healing assay. HT29 cells (5 × 10^5 cells/well) were seeded in 24-well plates and allowed to reach 80% confluence. Simulated wounds were performed as described by Lima et al. [24]. Cells were washed twice with PBS to remove debris. Each well was filled with fresh media containing the cookie protein extracts (100 µg/mL). After 48 h, the invaded area was measured for each treatment and compared to the corresponding area at 0 h to determine the area covered de novo by the migrating cells. MMP-9 activity was determined as previously described (Section 2.5.2). In each experiment, both positive (without LPC) and negative (without enzyme) controls were included for all samples to correct possible proteolytic activities present in the LPC extract. All data were corrected by subtracting their corresponding negative controls.

5.2.8. In vivo Assays Using an AA-Induced Colitis Model

5.2.8.1. Colitis Induction and Experimental Groups

Male Wistar rats were housed t in the vivarium of Universidade Luterana do Brasil (ULBRA, Porto Alegre, Brazil) under a 12 h light/dark cycle and kept at 22 °C ± 2 °C with 55–60% humidity. Water and food were provided ad libitum. Animals weighing an average of 350 g each were divided in five groups: control (Co), control + LPC cookie (Co + LPCc), colitis (Col), colitis + control cookie (Col + Cc) and colitis + LPC cookie (Col + LPCc). The colitis induction model used was adapted from Hartmann et al. [26]. The animals were anesthetized intraperitoneally with a mixture of 50 mg/kg xylazine and 100 mg/kg ketamine hydrochloride. Colitis was induced by intracolonic administration of 4% acetic acid (AA), and the groups received 4 g of LPC cookie or control cookie orally, by gavage, 3 h after colitis induction, daily, for 3 days. Finally, the animals were euthanized by exsanguination under anesthesia and, after necropsy, a portion of the colon (8 cm) was removed. The colons were homogenized with 9 mL of phosphate buffer

(KCL 140 mM, phosphate 20 mM, pH 7.4) per gram of tissue. The protein concentration was determined according to the Bradford method [27].

5.2.8.2. Anal Sphincter Pressure Measurement

The anal sphincter pressure measurement was performed prior to euthanasia. For this procedure, the animals were anesthetized, and manometry was performed using a balloon catheter of water. The result is expressed in cm H_2O. Three pressure measurements were made for each animal [26].

5.2.8.3. Lipoperoxidation

Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS), measuring the amount of substances that react with thiobarbituric acid (TBA). All tissue samples were mixed with 10% trichloroacetic acid (TCA) and 0.67% TBA, heated at 100 °C for 15 min and cooled in ice. The samples were centrifuged at 1.500× *g* for 10 min and 4 °C. The absorbance was determined using a 96-well plate reader at 535 nm [26].

5.2.8.4. Activity of Antioxidant Enzymes

The activity of superoxide dismutase (SOD), defined by its ability to inhibit the reaction of superoxide radicals with adrenaline, was monitored spectrophotometrically at 560 nm. Results were expressed as USOD/mg protein [28]. The activity of glutathione peroxidase (GPx) was assessed by the NADPH oxidation rate in the presence of reduced glutathione and glutathione reductase. Sodium azide was added to inhibit catalase activity. GPx activity was measured spectrophotometrically at 340 nm and expressed as nmol/min/mg protein [28].

5.2.8.5. Alkaline Comet Assay

The comet assay was performed as described by Vercelino et al. [29]. The slides were neutralized, stained and analyzed according to Nadin et al. [30]. Images of 100 randomly selected cells were observed from each animal (n = 5 per group) and scored according to tail size in five classes, ranging from undamaged (100 × 0) to maximally damaged (100 × 4), resulting in a single DNA damage score for each animal and, consequently, for each group. The damage frequency (%) was calculated based on the number of cells with tail versus those with no tail.

5.2.8.6. Histopathological Analysis

After the death of the animals, their colons were removed and fixed in 10% buffered formalin for 24 h. Paraffin blocks were sliced with a rotary microtome to create 3 mm thick sections. The tissues were stained with hematoxylin and eosin. The slides were photographed using a NIKON Labophot binocular microscope at a magnification of 200×. The histological analysis was based on changes in the crypts and the presence of inflammation in the colon [26]. The expression of COX-2 and TNF-α proteins in intestinal tissues was determined by immunohistochemical analysis, as described by Hartmann et al. [26]. The slides were analyzed using a microscope equipped with a digital camera using Image-Plus software (Media Cybernetics, Bethesda, MD,

USA). The quantification of COX-2 and TNF-α expression was performed via digital analysis with ImageJ and involved counting the positive pixels stained by immunohistochemical analysis with the IHC profiler plugin.

5.2.9. Statistical Analysis

All experiments were performed with at least six replicates in three independent times, and the data are expressed as the mean ± standard deviation (SD) in the case of normal distributions, and as the mean and maximum and minimum levels when data did not follow a normal distribution. Data analysis was performed using the SigmaPlot software (version 12.5). In order to carry out the inferential analysis, and considering the fulfillment of the necessary criteria for the performance of parametric tests, the Kolmogorov Smirnov normality test was performed. In the cases where the samples did not follow a normal distribution, non-parametric tests were used.

For *in vitro* assays analysis, and given that the sample presented a normal distribution, a one-way analysis of variance (ANOVA) was performed using Tukey's test to compare the differences between groups, with P values less than 0.05 considered statistically significant.

For *in vivo* experiment analysis, a comparison between groups was performed by one-way analysis of variance, followed by the Student Newman–Keuls procedure. $p < 0.05$ was considered statistically significant.

5.3. Results and Discussion

5.3.1. Lupin Protein Concentrate (LPC) Holds Nutritional Value and Antioxidant Potential

In our previous works, a lupin protein concentrate (LPC) was developed which contained deflamin, a potent matrix metalloproteinase (MMP)-9 inhibitor that could efficiently reduce colon cancer cells' migration and MMP-9 activity *in vivo* and *in vitro* [14,24]. Although deflamin has been demonstrated to be a potential nutraceutical for IBDs [11], a diet rich in lupin seeds does not seem to be enough for it to exert sufficient activity in IBDs. In addition, some additional components of lupin such as fibers, phytate and protease inhibitors (among others) can hamper digestion and are unadvised for patients suffering from IBDs [5,6]. Under this context, an LPC could achieve significant importance as a bioactive food additive in functional foods, since it contains a higher concentration of deflamin, without most of its anti-nutritional factors [31]. Besides exerting a dose-dependent inhibition of MMP-9 *in vitro* [14], the LPC presents the extra advantage of being a prepared food, rather than a purified or isolated compound (nutraceutical of pharmacological), which allows it to be characterized as GRAS while permitting an easier application in functional diets [16]. As a lupin concentrate, it has the additional advantage of containing other beneficial health activities due to the presence of specific polyphenols and carbohydrates from lupin seeds which have been reported to reduce oxidative stress and improve the gut microbiome, both rather important factors in reducing or preventing IBDs [20]. Nonetheless, although LPC was tested against MMP activity [16], its nutritional profile and antioxidant activities remained to be studied. Under this context, we initially set out to evaluate the nutritional profile of the LPC, including the phenolic content, and also test its antioxidant potential. The results obtained are shown in Table 1. It is noteworthy to highlight that we used

sweet white lupin (*Lupinus albus*) cv. Amiga, whose seeds present little or no alkaloids, thus abolishing the toxicity when compared to bitter lupin seeds.

Table 1. Chemical composition and antioxidant capacity of the lupin protein concentrate (LPC). Data are expressed as the mean ± SD.

Component	Amount
Moisture (g/100 g)	10.31 ± 2.53
Total Ash (g/100 g)	7.25 ± 0.27
Crude Fat (g/100 g)	0.31 ± 0.05
Crude Protein (g/100 g)	20.09 ± 0.58
Carbohydrates * (g/100 g)	62.05
Total Polyphenols (mg/100 g)	35.19 ± 2.5
Antioxidant Activity	
FRAP (mg AAE/10 mg LPC)	351.19 ± 2.5
DPPH (mg AAE/ 10 mg LPC)	273.9 ± 2.03

* Carbohydrates were determined by differences in the mean ash, fat, protein and moisture contents.

Being a water-soluble protein concentrate, we expected to find that LPC is a low-fat food product with a high level of protein and water-soluble carbohydrates, as demonstrated in Table 1. Previous reports have shown that water soluble protein extracts from lupin, particularly after boiling, are free from phytate and lectins, which allows it to be more digestible [31].

The presence of a high amount of protein as well as water-soluble carbohydrates suggests a good application of the LPC as a nutritional food additive. Indeed, several authors have pointed lupin as a new and valuable source of protein to nutritionally supplement traditional foods [32], both in animal and human nutrition, and even as an alternative to soybean [32].

The presence of phenolic acids and flavonoids in lupin seeds has been reported in many studies, although when compared to other members of the lupin genus, *L. albus* has been demonstrated to have a lower amount of phenolic compounds (212.12 to 491.51 mg/100 g d.m. as gallic acid equivalents) [32]. In our measurements, the total amount of phenolic compounds was, expectedly, even lower, at around 35 g/100g d.m., since we can only obtain the water-soluble polyphenols in the LPC. Nonetheless, data show that the LPC still holds antioxidant capacity, as measured by the DPPH and the FRAP methods. The main therapeutic effect associated with the antioxidant activity of phenolics mainly protects an organism against the damaging effects of the active oxygen species and free radicals that initiate oxidative processes [32]; hence, these results corroborate that the potential of the LPC against IBDs as an antioxidant agent (besides the MMP-9 inhibition) should also be pursued.

5.3.2. LPC Reduces the Clinical Characteristics of Ulcerative Colitis, in a Dose-Dependent Manner

Since lupin is already widely used in food production, particularly as a technologically desirable additive in bakery products as well as in dietary and functional food products [32], it seems plausible that our LPC could be considered a promising new addition to the lupin food additives

already in the market. However, although previous works have shown that LPC contained anti-MMP-9 activity *in vitro*, its activity against IBDs remained to be tested *in vivo*. As previously reported, the effectiveness of nutraceuticals in providing therapeutic or physiologic benefits depends heavily on preserving their bioavailability [1], which may be compromised by several factors such as gastric residence time, low permeability and/or solubility within the gastrointestinal (GI) tract, and instability during food processing/storage or in the GI tract [1]. Although orally-fed deflamin was shown to be resistant to digestion and effectively reduced colitis in TNBS-induced colitis in mice [14], it is possible that the presence of other components in the protein concentrate could interfere with its bioactivity and resistance to digestion. In fact, it has been reported that most nutraceutical activities can be altered by their delivery system, often via interactions with other bioactive molecules [1]. Therefore, before using the LPC as a functional food additive, its dose-response was required to be tested to establish a proper daily dose for functional foods. Therefore, we tested the LPC *in vivo* in mice using three different concentrations, ranging from 0.1 to 10 g/kg, with the goal of (a) testing its efficacy *in vivo* and (b) determining the best LPC dose to be used in functional foods and diets. Figure 1 shows the representative images of each colon group, whilst Table 2 shows the disease activity index (DAI) scores, colon lengths observed as well as the MMP-9 activity in colons (Table 2) in mice with TNBS-induced colitis, both in control groups and in groups which were fed daily with three different LPC concentrations.

Figure 1. Representative images of the effect of a 4-day lupin concentrate feed in TNBS-induced colitis in mice. LPC was administered orally in three different concentrations (0.1, 1 and 10 g/kg). Co: control group (healthy); Col: colitis group; Col + LPC: colitis group fed with LPC (at each concentration).

Table 2. Effect of a 4-day lupin concentrate feed in TNBS-induced colitis in mice. Disease activity index (DAI) determined as the average of the score of weight loss and stool consistency; average colon length (cecum until rectum), represented in cm; MMP-9 activity measured by the DQ-gelatin kit. Co: control group (healthy); Col: colitis group; Col + LPC: colitis group fed with LPC (at each concentration). Non-parametric data are expressed as the mean and respective maximum and minimum levels, and parametric data are expressed as medium ± SD. (* $p < 0.05$, ** $p < 0.001$ in relation to colitis and # $p < 0.05$ in relation to control).

	DAI Score		Colon Length (cm)		Total Gelatinolytic Activity (%)
	Mean ± SD	Min; Max	Mean ± SD	Min; Max	Mean ± SD
Co	0 ± 0	(0; 0)	11.4 ± 0.4	(10.9; 11.8)	0 ± 1.9
Col	3.4 ± 0.4	(2; 4)	7.2 ± 0.77) #	(6.5; 8.2	100 ± 1.5
Col + LPC (0.1 g/kg)	2.5 ± 0.6 *	(1; 3)	10.8 ± 2.2	(8.7; 12.8)	83.1 ± 1.7 *
Col + LPC (1 g/kg)	2.2 ± 0.3 *	(1; 3)	9.7 ± 1.3	(8.8; 10.9)	71.6 ± 1.3 **
Col + LPC (10 g/kg)	1.5 ± 0.2 **	(0; 3)	10.4 ± 0.4	(10.1; 10.9)	65.4 ± 1.2 **

The results show that the DAI scores were significantly enhanced in the colitis group when compared to the control (healthy) group, but were significantly and consistently lower in all three LPC concentrations (Table 2), in a dose dependent manner, being reduced by more than 50% in treatments with the 10 g/kg LPC administration when compared with the untreated colitis group. A similar impact of LPC was observed in colon lengths. As expected, the colon length in the TNBS group was significantly reduced in comparison with the healthy group (Figure 1, Table 2). With the LPC oral administrations, colon length was significantly higher, also in a dose dependent manner, being significantly higher in the groups treated with 1 and 10 g/kg LPC when compared to the colitis group (10.4 ± 0.2 and 7.23 ± 0.7 cm in the highest LPC concentration and controls, respectively) and overall more significantly similar to healthy individuals (11.2 ± 0.4 cm) ($p < 0.001$). Concerning the observed colon MMP-9 activity (Table 2), results also show a similar trend. Predictably, the untreated colitis group showed an elevated MMP-9 activity when compared to the control, which was consistently reduced with increasing doses of the LPC ($p < 0.001$). Whilst the lower dose of 0.1 g/kg of LPC reduced 20% of MMP-9 activity, the higher doses induced a reduction of 29% and 37% for 1 and 10 g/kg LPC, respectively. The results, therefore, suggest that the LPC contains enough activity to significantly reduce colitis and colitis-induced MMP-9 activity *in vivo* in dosages higher than 1 g/kg LPC per day to obtain the desired effects in reducing colitis lesions. In similar studies performed with persimmon, pennyroyal and spearmint phenolic extracts in TNBS-induced colitis in mice, an attenuation of histological features, inflammatory markers and reduction in lesion extent in the colon were also observed, corroborating the efficacy of these bioactive compounds [33–35]. It is, nonetheless, to the best of our knowledge, the first time that a protein concentrate has been shown to be effective against colitis.

5.3.3. LPC Bioactivity, When Used as a Food Additive, Is Influenced by the Presence of Sugar

In our previous work, we tested the deflamin activity stability of LPC in different types of flours. LPC (from a technological point of view, a concentration of 10 g protein/100 g cookie was selected as the best quantity) was added to gluten-containing and gluten-free flours (rice and buckwheat) to produce savoury cookies. The results obtained showed that most low-gluten and

gluten-free flours were not the best vehicle to deliver bioactive deflamin [16] since they reduced its activity. Only buckwheat flour showed a better stability of MMP-9 inhibitory activity [13]. Knowing that wheat is a preferred flavor in food markets, and considering that LPC has not been tested in this type of flour, in the present work we opted to incorporate LPC in wheat cookies in sufficient amounts to reach the desired daily dose: above 1 g LPC/kg of body weight. Our main goals were to develop a food product that was well accepted by consumers, cost-effective and with a structure allowing the incorporation of higher levels of LPC. Wheat flour, having better viscoelastic properties, could also facilitate the incorporation of greater amounts of LPC without impairing its technological features, in addition to being more cost-effective than gluten-free flours.

Savoury and sweet cookies were baked with the addition of LPC to the wheat flour, and their activities were tested both against MMP-9 and the migration rates of HT29 colon cancer cells. Both cooked and uncooked doughs were tested and the results are shown in Figure 2.

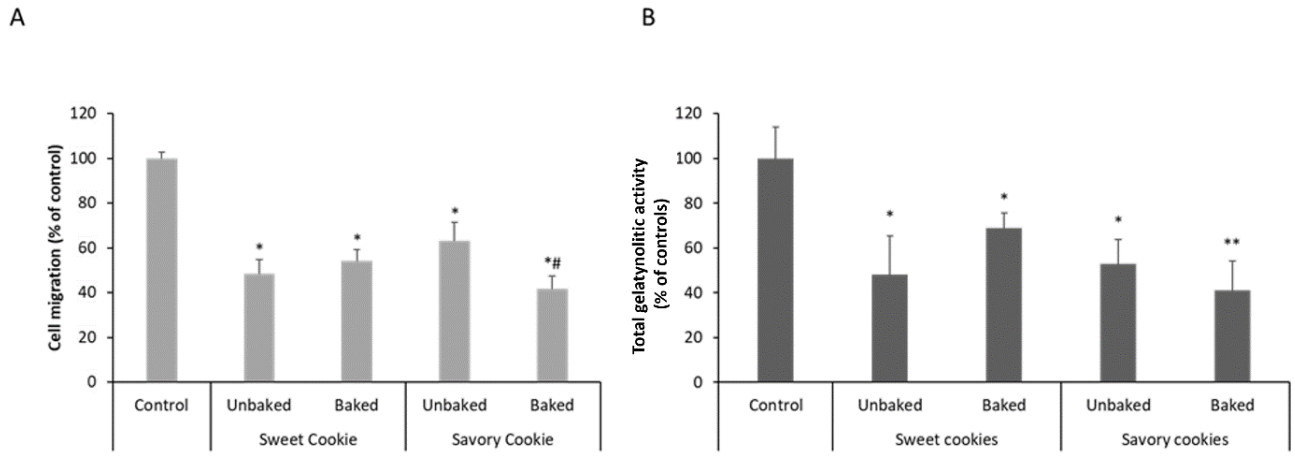

Figure 2. LPC bioactivity in wheat cookies against (**A**) colon cancer cell migration and (**B**) MMP-9 activity. Sweet and savoury cookies were tested, both baked and unbaked dough. Data are expressed as the means ± SD. (* $p < 0.05$, ** $p < 0.001$ when compared to control; # $p < 0.05$ when compared to unbaked savoury cookie).

Previous results [16] demonstrated that the nature of the flour could interfere with the activity of deflamin, but that the baking process itself would not. This may derive from the observation that deflamin is resistant to boiling (patent WO/2018/060528). According to Mota et al. [16], the amount and type of sugars present in different flours apparently interfere with the activity of deflamin in LPC. In the present study, the results show that the bioactivity of LPC was maintained with the wheat flour, both in inhibiting MMP-9 (Figure 2B) and in reducing colon cancer migration (Figure 2A). However, interestingly, results differed significantly ($p < 0.05$) between savoury and sweet biscuits after baking. Although both activities were maintained in unbaked dough, after baking the sweet cookies have significantly less inhibitory activity (Figure 2A,B), both in MMP-9 inhibition and in the ability to reduce cancer cell migration.

These results suggest that wheat flour per se does not interfere with LPC bioactivity, as previously demonstrated for gluten-free flours [16] but baking in the presence of sugar does. Previous experiments conducted on different types of flours suggested that different amounts of

starches and sugary molecules could interfere with deflamin activity when LPC was used as a food additive. This hypothesis is consistent with our results on deflamin MS sequencing [14], which showed, in purified deflamin preparations, the presence of β-conglutin fragments, which are known for their lectin-type sugar binding activities. Nonetheless, by comparing unbaked with baked doughs we can see that it was not the presence of sugar per se that reduced the LPC activity, but the baking process, which allows us to infer that perhaps a Maillard reaction, due to the high amount of proteins in LPC, may be responsible for inhibiting deflamin activities. This is also substantiated by the development of a golden-brown color observed in LPC-containing cookies, particularly in sweet cookies, which was also previously reported [16], suggesting the involvement of Maillard reactions. Overall, these results substantiate the importance of the biochemical composition of the food matrix when incorporating nutraceuticals in foods [1] and should be taken into consideration in future studies using LPC as a food additive. On this matter, Szwajgier et al. [36] observed that enriched bread with plant polyphenols can play a role in HT29 cells by the inhibition of the growth and viability of tumor cells. A similar study performed by Gawlik-Dziki and collaborators [37] showed that bread enriched with broccoli sprouts inhibited the proliferation of stomach cancer cells. Nonetheless, important factors such as digestion and bioavailability are also of vital importance for the efficacy of these bioactives and must be also evaluated using *in vivo* models.

5.3.4. LPC-Enriched Cookies Are Effective as Functional Foods against AA-Induced Colitis In vivo

Once the LPC-containing cookies were selected, we proceeded to test their anti-inflammatory activity *in vivo*. Since the concentration of 10 g protein/100 g cookie dough was preferred for yielding the best technological biophysical properties such as texture, LPC cookies had to be fed to animals in sufficient amounts so that at least a dose of 1 g LPC/kg of body weight per day would be assured. This is roughly the equivalent to 4 g of cookies per day, a higher dose than mice could take daily. Therefore, we used a rat colitis model instead, so that the dose per day could be enough to significantly reduce colitis. Since we aimed at testing not only the anti-inflammatory activity but also the antioxidant activity of LPS *in vivo*, we opted for an acetic acid (AA)-induced colitis. In this way, we could provide information on the effect of deflamin and of LPC on a model of IBD that is very similar to human acute IBD (ulcerative colitis) in terms of pathogenesis, histopathological features and inflammatory mediator profile, as opposed to the acute colitis of the TNBS model [38,39]. AA-induced colitis was found to cause non-transmural inflammation characterized by increased neutrophil infiltration into the intestinal tissue, vascular dilation, necrosis of mucosal and submucosal layers, edema and destruction of crypts [38,39]. Additionally, AA-induced colitis induces higher oxidative stress [26,38], allowing us to better observe any type of antioxidant potential as well. This was particularly important due to the results presented above on the antioxidant potential of LPC (Table 1), and because several studies have revealed that oxidative stress plays a critical role in the initiation and progression of IBDs [19,26,38,39].

For the reasons outlined above, we tested the effect that a daily dose of 4 g LPC cookies/kg of body weight had on the lesions and oxidative stress levels in rats with AA-induced colitis. Figure

3 shows the effect that the ingestion of LPC-containing cookies had on sphincter pressure (A) and lipid peroxidation (B), as well as on superoxide dismutase (C) and glutathione peroxidase activities (D).

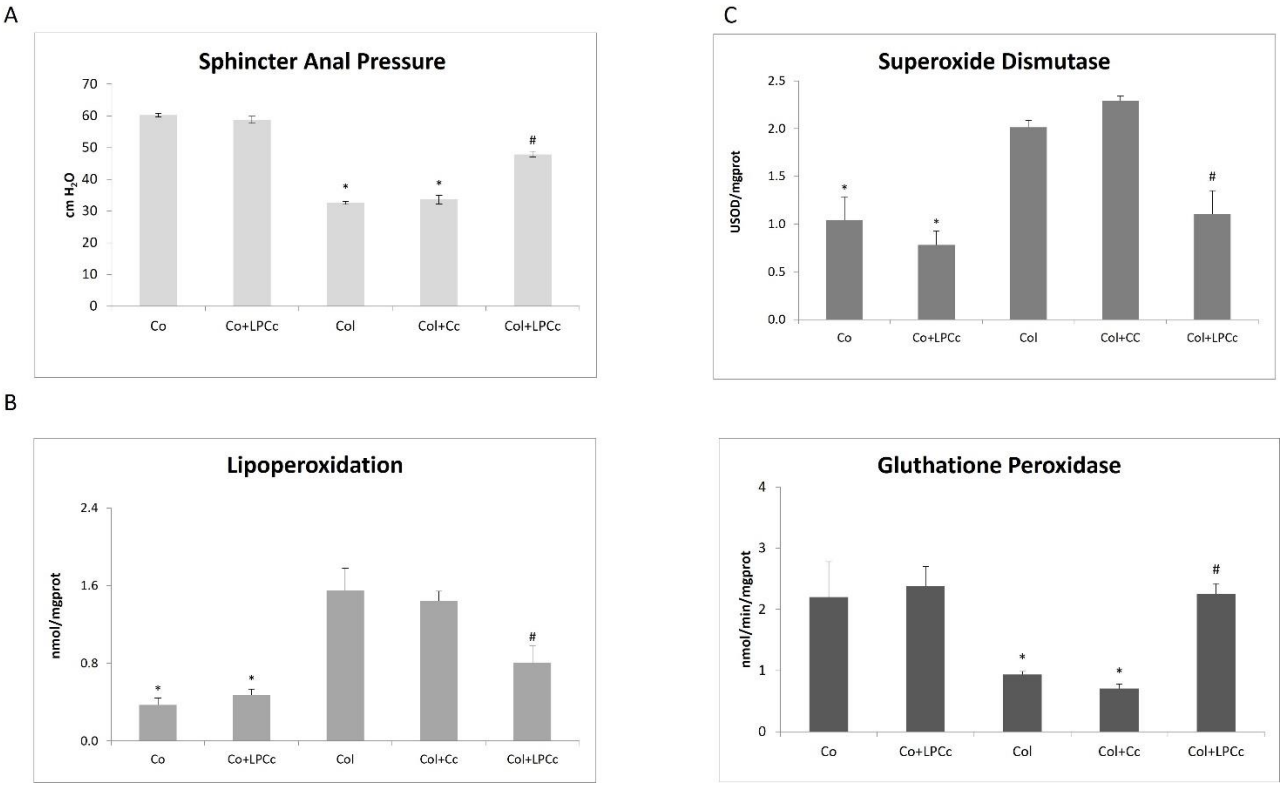

Figure 3. Effect of administration of LPC cookies on rat with acetic acid-induced colitis. Anal sphincter pressure (**A**) and lipid peroxidation (**B**) were analyzed in both controls and diseased rats, as well as the activity of ROS-related enzymes superoxide dismutase (**C**) and glutathione peroxidase (**D**). Values are expressed as the mean ± standard error. Co: control; Co+LPCc: control + LPC cookie; Col: colitis; Col + Cc: colitis + control cookie; Col + LPCc: colitis + LPC cookie. * represents $p < 0.001$ when compared to the control and # $p < 0.001$ when compared to colitis.

As a way to evaluate the extent of colitis and diarrhea, we measured the anal sphincter pressure using anorectal manometry (Figure 3A). As expected, the colitis group showed a significant decrease when compared to the other groups, due to the muscle relaxation caused by severe diarrhea. However, the animals fed with LPC-cookies showed a significant increase in the sphincter pressure values ($p < 0.001$). Noticeably, rats with colitis fed with the control cookies (with no LPC) had similar values when compared to the colitis group.

We also aimed to evaluate the impact that LPC could exert on the level of oxidative damage induced by colitis, as determined by the level of lipid peroxidation (LPO) in the colon tissues of the different animal groups (Figure 3B). As expected, there was a very significant increase in LPO levels in the colitis group. As observed for the anal pressure measurements, no differences between the colitis group and the Col+Cc group were observed, suggesting that there was no impact on LPO when the rats were fed with wheat cookies. However, a significant reduction in LPO ($p < 0.001$; Figure 3B) was detected in the LPC-cookies treatment group, which was reduced by 40% when compared to the colitis group. This significant reduction in oxidative

lesions, along with the reduction in anal sphincter pressure, suggests that there was a potent protective effect of LPC on colitis.

It has been well established that, in IBD patients, the production of reactive oxygen species (ROS) is increased [23,39], mostly because the infiltration of neutrophils leads to the production of superoxide anion (O_{2^-}) and initiates the production of various ROS that significantly contribute to the progression of tissue necrosis and mucosal dysfunction [17,19,39]. Several enzymes, such as SOD and GPx, prevent the accumulation of O_2 and hydrogen peroxide (H_2O_2) and are, therefore, considered the primary line of defense. We further analyzed the impact of LPC cookies on the activities of SOD and GPx, and results are depicted in Figure 3C,D, respectively. In our study, we observed a significant increase in SOD activity in the colitis group (Figure 3C), most likely to compensate for the damage caused by the action of acetic acid in the animals' intestines. The enzyme SOD has an essential role in cellular redox balance, promoting dismutation in an attempt to free radicals, protecting tissues against oxidative damage [28]. However, whilst the Col + Cc group presented similar levels ($p < 0.001$) of SOD activity when compared to the colitis groups, the treated group showed a significant decrease in SOD activity, even reaching levels that were significantly similar to the control (healthy) group. This trend was also consistent with the activity of GPx (Figure 3D), which was reduced in the colitis group as well as the Col+Cc treatments ($p < 0.001$), but was restored with the LPC cookie administration compared to the colitis group, once again reaching similar levels to those observed in healthy animals. Glutathione peroxidase (GPx) has a great physiological importance because it catalyzes the decomposition of inorganic peroxide and organic peroxides using glutathione (GSH) as a co-substrate, which is a key component protecting against damage by free radicals in the physiological system [40]. Several studies have shown that GPx is responsible for H_2O_2 detoxification when it is present at low concentrations [41], and the reduction of GPx activity in the intestines of animals with colitis is known to be blocked after the administration of antioxidants [41,42]. Similar results were demonstrated by Tahan et al. [43], who administered doses of melatonin in a colitis model in rats and also observed an increase in the enzyme GPx. This corroborates the results found in our study.

Overall, considering the oxidative stress levels, our results showed that the oral administration of LPC-containing cookies ameliorated the symptoms induced by colitis and consistently reduced the damage and enzymatic activities related to oxidative stress, restoring the levels of ROS-related enzymes to healthy levels. These results further substantiate that LPC, besides reducing MMP-9 activity, also affects the oxidative stress induced by colitis. More importantly, its activity was not reduced by the digestion process, which often occurs with nutraceuticals [44]. Previous reports showed that the activity of deflamin isolated from lupin seeds was maintained when it was orally administered in animal models of disease [14] and was able to significantly reduce colitis and gelatinase activity. However, this is the first report that shows the maintenance of deflamin activity in LPC after digestion, as well as of the anti-oxidative activity of LPC itself. It is important to note that deflamin per se does present noticeable antioxidant activity (data not shown). Hence, the LPC combines antioxidant activity with the MMP-9 inhibition by deflamin, elevating the potential of this food additive.

To better evaluate the protective effects of LPC in AA-induced colitis, we further assessed the impact of LPC cookies on the histological lesions induced by colitis, as well as on the expression of important biomarkers of inflammation, COX-2 and TNF-α. The results are depicted in Figure 4. Table 3 presents the expression of the positive pixels of each colon group.

Figure 4. Effect of administration of deflamin cookies on colon tissues. (**A**) Histopathological alterations in colitis in colon tissue. (**B**) Immunohistochemical detection of COX-2. (**C**) Immunohistochemical detection of TNF-α. Co: control; Co + LPCc: control + LPC cookie; Col: colitis; Col + Cc: colitis + control cookie; Col + LPCc: colitis + LPC cookie. All images are at a magnification of 100×.

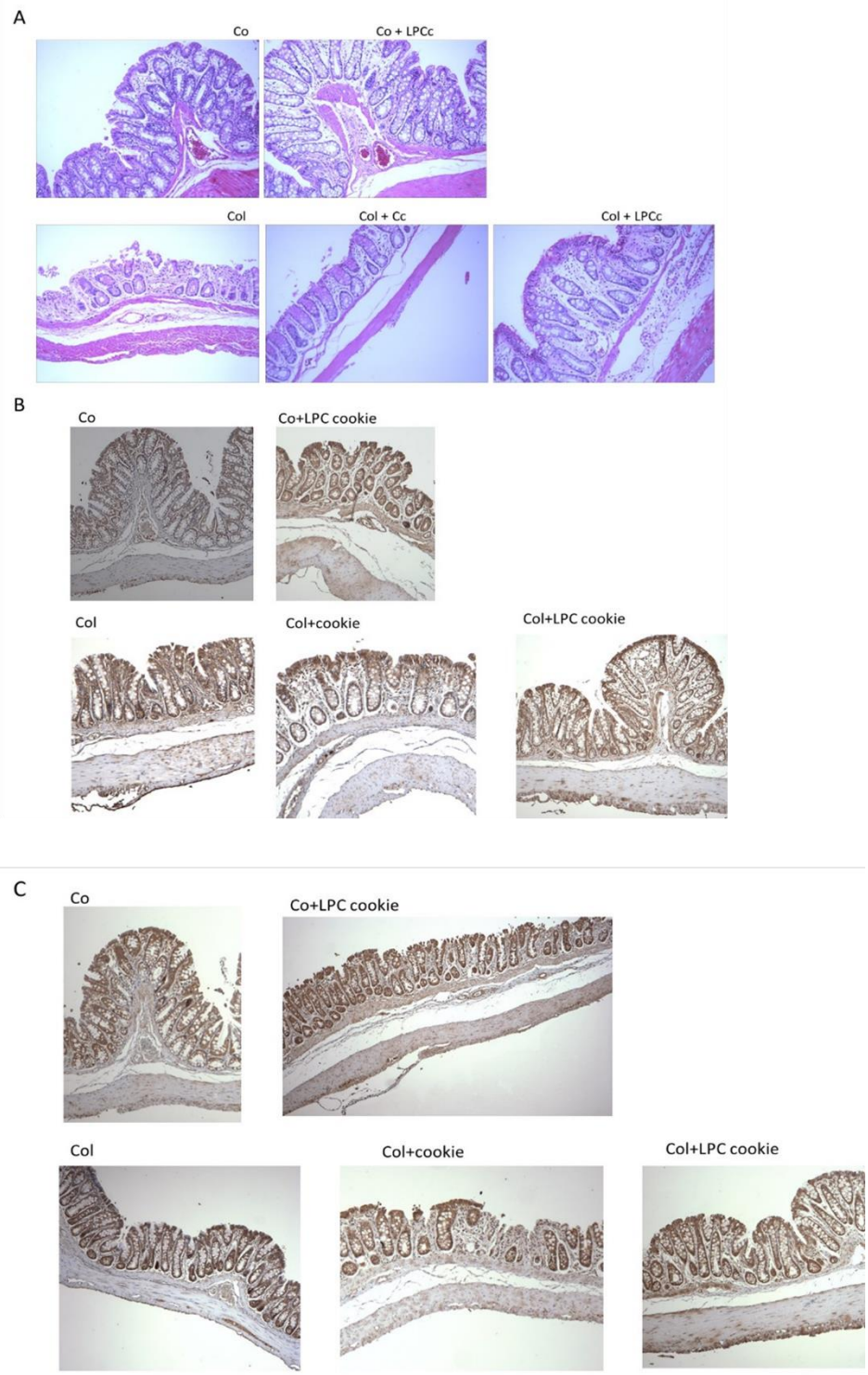

Table 3. Expression of the positive pixels as the mean ± SD obtained for each colon tissue. Co: control; Co + LPCc: control + LPC cookie; Col: colitis; Col + Cc: colitis + control cookie; Col + LPCc: colitis + LPC cookie. * represents $p < 0.001$ when compared to colitis and # $p < 0.05$ when compared to control.

	COX-2	**TNF-α**
Co	0.9 ± 0.3 *	0.7 ± 0.1 *
Co + LPCc	1.94 ± 1.5 *	2.4 ± 0.6 *,#
Col	11.7 ± 1.98 #	8.7 ± 1.6 #
Col + Cc	5.1 ± 2.1 *,#	2.5 ± 0.6 *,#
Col + LPCc	0.9 ± 0.2 *	1.2 ± 0.2 *,#

Figure 4A shows the histopathological alterations in colon tissue with colitis. Representative H&E-stained histological sections showed that the colons from both Co and Co + LPCc groups presented a healthy epithelium with well-organized crypts and no ulceration or tissue erosion. In contrast, the histopathological analysis showed macroscopic and microscopic alterations in the colitis group compared to control groups. The colitis group showed inflammatory lesions, characterized by loss of crypts, surface erosion and ulceration. Notably, in the Col + LPCc group, we observed a significant reduction in overall colon injury, suggesting, once again, a protective LPC effect on histopathological lesions in comparison with the untreated Col group. These results substantiate that LPC can attenuate the tissue changes induced by experimental colitis. This result was expected, as previous studies showed that deflamin could reduce the tissue lesions induced by experimental TNBS-induced colitis, corroborating the maintenance of its activity in LPC cookies. Nonetheless, these results could also be associated, at least in part, with an antioxidative effect. Indeed, studies using antioxidant compounds such as vitamin E and rivastigmine also showed a reduction in the damage caused by agents that are used to induce colitis [45,46]. Therefore, we further analyzed the expression of the biomarkers of inflammation, COX-2 and TNF-α, by IHC. We observed a significant increase ($p < 0.01$) in both biomarkers in colon tissue in the colitis group (Figure 4B,C and Table 3), as opposed to the control (healthy) groups, which did not show positive staining. Treatment with LPC-cookies reduced the expression of both these biomarkers in the treated groups ($p < 0.01$), corroborating the anti-inflammatory effect of LPC (Table 3).

Aiming at further testing the protective effects of LPC-cookies, we evaluated the level of DNA damage induced by colitis in all studied groups using a comet assay. Table 4 shows the results obtained for damage index and damage frequency in peripheral blood.

Table 4. Comet assay in peripheral blood of the studied groups. Co: control; Co + LPCc: control + LPC cookie; Col: colitis; Col + Cc: colitis + control cookie; Col + LPCc: colitis + LPC cookie. * $p < 0.001$ when compared to control and # $p < 0.001$ when compared to colitis.

Group	Damage Index	Damage Frequency
Co	17.0 ± 5.7 #	17.0 ± 5.7 #
Co + LPCc	21.4 ± 3.6 #	21.4 ± 3.6 #
Col	68.8 ± 7.9 *	57.5 ± 11.5 *
Col + Cc	40.3 ± 9.2 *#	35.3 ± 10.4 *#
Col + LPCc	17.4 ± 3.4 #	17.2 ± 3.3 #

The data showed a significant increase in baseline DNA damage in the colitis group when compared to the healthy groups, as measured by damage index and damage frequency (Table 4). As in previous assays, cookies supplemented with LPC significantly ($p < 0.001$) decreased the damage induced by colitis, restoring it to values similar to the controls, in both measured parameters. It is noteworthy to observe that in the healthy groups, there was no impact on DNA induced by the LPC cookies, hence substantiating the notion that LPC is a GRAS food product and can be safely used by consumers without any IBD symptoms. This is consistent with previous reports which showed that protein extracts from lupin, as well as isolated deflamin, did not exert any apparent cytotoxicity on HT29 cells [14,24].

5.3.5. Considerations on the LPC Cookies as Functional Foods

Currently, *Lupinus albus* is not only appreciated for its nutritional value but also for its functional properties in bakery and confectionary [47], and for human health. Ingestion of lupin-containing foods has been associated with the prevention of obesity, diabetes, eventually cardiovascular disease and, more recently, digestive tract disease [48,49]. The discovery of deflamin and its potential for IBDs has further enhanced the potential of lupine-based foods. Here, the development of a lupin protein concentrate that has nutritional value, antioxidant activity and also reduces colitis-inflammation whilst surviving digestion, and without exerting any toxic effects, can have a great potential for functional diets against IBDs. The incorporation of LPC in cookies has the added advantage of a longer shelf-life and the ability to serve as vehicle for supplementation with nutraceuticals [13,50]. Evidently, there are several limitations to these types of studies, namely the sample size, the duration of the study, lack of other types of IBD models and the fact that these are animal models and not clinical studies. Nonetheless, the efficacy of the LPC against two different types of colitis and the maintenance of the activity in the colons (*i.e.,* it survived digestion) allow us to speculate on its high potential for future human studies. Currently, there is no effective therapy available capable of treating IBDs and, to date, this is, to the best of our knowledge, the first functional food developed with realistic applications for IBDs. Further studies should, therefore, be pursued in order to bring these types of functional foods onto clinical studies, allowing the assessment of their potential against IBDs to be validated.

5.4. Conclusions

The lupin protein concentrate (LPC) was shown to be effective as a delivery system for deflamin, both as a functional food and as an additive to wheat cookies. Besides its activity towards MMP-9, the LPC further added a high nutritional and antioxidant value to the already potential health benefits of deflamin. Having been shown to be effective against two different types of colitis models, the LPC seems to be a potential functional food to be used in preventive/curative diets against IBDs.

5.5. References

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Chapter 6. Final Conclusions

Inflammatory bowel diseases (IBDs) have long been recognized as a global burden, with an increasing incidence worldwide in individuals of all ages. The only available pharmacological treatments for IBDs are anti-inflammatory or immunosuppressive drugs, which are insufficient, show low to no effect for temporary symptoms, and are prone to severe side effects. The fortification of food products with food bioactive compounds may constitute a simple way of delivering them in sufficient amounts in diets and exert anti-inflammatory and antioxidant effects in patients.

Previous reports revealed a novel bioactive polypeptide oligomer named deflamin that has the ability to reduce IBD development via MMP-9 inhibition and which holds great potential to be used in functional diets. However, deflamin occurs in low amounts in lupin seeds and therefore a diet of lupin alone would not suffice to exert its bioactive effects. In the present work, deflamin was not only characterized but a lupin protein concentrate (LPC) from *Lupinus albus* seeds that contains high amounts of this MMP-9 inhibitor (MMPI) was also developed. Whilst being considered as GRAS (generally regarded as safe) and possessing the ability to be added to food products, such as cookies, LPC opens a novel perspective on bioactive foods that can be used in functional diets against IBDs.

Overall, the MMPI was found to be an oligomer comprising fragments derived from two *Lupinus* seed storage proteins: δ-conglutin and, to a lower extent, β-conglutin. *In vitro* studies showed that this novel gelatinase inhibitor successfully impaired cancer cell invasion whilst reducing MMP-9 activity in a dose-dependent manner and with an EC50 of 10 µg/mL. *In vivo* studies showed that the MMPI maintained its activities throughout digestion and significantly reduced colitis lesions.

We also developed a LPC that presents high nutritional value, antioxidant activity and the ability to reduce MMP-9 *in vitro,* therefore showing great potential against IBDs. This LPC contains not only bioactive deflamin, but is also resistant to baking and digestion as well. When administered orally, it reduced colitis lesions in mice models of disease, in a dose-dependent manner.

Technologically, using different types of flour, LPC is also an excellent food additive to cookies. Its addition to gluten-containing and gluten-free flours showed a high impact in dough structure, increasing with several beneficial inputs. Supplementing flours with LPC improved color and decreased lightness, making cookies more pleasant to consumers. Hence, overall, our results show that cookies prepared with LPC-containing flours exhibit improved technological properties. The addition of the LPC also improved the nutritional value of cookies, enhancing protein content and antioxidant activity as well. Technologically, incorporation of the lupin extract has an impact on the characteristics of the dough and on the final product, depending on the type of flour used. Different types of flours and different matrices did not influence to a great extent deflamin activity in general, but the presence of starch and sugars reduced its MMPI activities. Wheat and buckwheat flours were the best flours to maintain deflamin activity.

Since our goal was to pursuit a practical and realist approach, a higher importance focused on *in vivo* results and on the effect of digestion on LPC activity. When administered orally, LPC-enriched wheat cookies were effective in reducing the physiological, genetic and oxidative lesions induced by two different *in vivo* models of colitis, restoring the lesions, in the treated groups, to levels similar to the healthy groups.

In short, our results show a novel functional food or food additive, with potential to tackle IBDs and exhibiting both functional and nutritional properties. They also brought to light novel information on the mode of action of deflamin and on its potential as a functional food or nutraceutical. Currently, there is no effective therapy available capable of treating IBDs, and up to this day, this is, to the best of our knowledge, the first functional food developed with realistic applications towards these diseases. We also showed that interactions between LPC and the food matrix influences deflamin activity, emphasizing the importance of evaluating these types of interactions when incorporating food bioactive compounds in food products. Hopefully, future works will take us one further step towards improving the dietary approaches to reduce or prevent the constraints of the global burden that are currently the inflammatory bowel diseases.