Universidade de Aveiro Departamento de Química
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Nuno Filipe Costa Afonso

Massas alimentícias enriquecidas com *Fucus vesiculosus*: avaliação nutricional e propriedades biológicas

Pastas enriched in *Fucus vesiculosus*: nutritional evaluation and biological properties

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Susana Maria de Almeida Cardoso, Investigadora Doutorada do Departamento de Química da Universidade de Aveiro, e coorientação do Doutor Artur Manuel Soares Silva, Professor Catedrático do Departamento de Química da Universidade de Aveiro.

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À minha tia-avó Fernanda, por tudo o que fez por mim

o júri

presidente

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palavras-chave ***Phaeophyta*, *Fucus vesiculosus*, massas fortificadas, composição nutricional, fitoquímicos, atividade antioxidante, bioacessibilidade, biodisponibilidade, minerais**

resumo Apesar da sua abundância natural, as algas ainda são consideradas um recurso pouco explorado, mas com muito interesse em diversos setores, nomeadamente alimentar e farmacêutico. Vários estudos que comparam as dietas de diferentes países, associam o consumo de algas marinhas ao bem-estar e vitalidade oriental contrariamente às sociedades mais ocidentais. Dentro das três classes de algas comestíveis, as pertencentes ao domínio *Phaeophyta* têm recebido muita atenção devido à sua abundância em polissacarídeos complexos, florotaninos, fucoxantina e iodo. Este trabalho reporta a composição nutricional (teor total de fibras, proteínas, cinzas e minerais) e o conteúdo em fitoquímicos (pigmentos e florotaninos) de uma massa alimentícia fortificada com 1% e 5.5% da alga castanha *Fucus vesiculosus*, antes e após o processo de cozimento. Paralelamente, foram investigadas algumas atividades biológicas, nomeadamente o potencial antioxidante e a capacidade de inibir três enzimas do metabolismo, α-amilase, α-glucosidase e lipase pancreática. Por fim, foi avaliada a bioacessibilidade e biodisponibilidade dos minerais. No geral, os resultados obtidos demonstram que, com cada incremento de alga, houve uma tendência em melhorar vários parâmetros. Para as massas com 5.5% de alga, os teores de fibras e cinzas aumentaram até 48% e 60%, respetivamente, enquanto que o teor de proteínas permaneceu inalterado. Confirmando ainda os níveis mais altos de cinzas em amostras enriquecidas, o teor de iodo aumentou para cerca de 91% da sua concentração inicial. Além disso, a *F. vesiculosus* contribuiu para um aumento dos fitoquímicos, nomeadamente de pigmentos como clorofila *a* (84%) e carotenoides (99%), e florotaninos (87%) nas massas. De uma forma geral, esses resultados estarão relacionados com a notória melhoria da atividade antioxidante das amostras. Após o cozimento, observou-se uma diminuição dos vários conteúdos mencionados anteriormente, nomeadamente proteínas, minerais e fitoquímicos, que, por sua vez, refletiram também uma perda da atividade antioxidante. A manutenção do teor de fibras após esse processamento térmico possivelmente contribuiu para inúmeros efeitos, como a retenção de proteínas, até certo ponto, mas também a retenção de minerais quando submetidos a uma digestão gastrointestinal simulada. Entre todos os minerais estudados, destacaram-se as frações de K e Mg, com as percentagens mais elevadas, 105% e 62%, para as bioacessíveis, e 56% e 24%, para as biodisponíveis, respetivamente. Adicionalmente salienta-se o iodo, cuja bioacessibilidade atingiu os 82%. Para este elemento, a biodisponibilidade foi apenas 4%, no entanto, dada a sua elevada quantidade na massa cozida, estima-se que 100 g de massa poderá disponibilizar aproximadamente 54% da sua dose diária recomendada. Assim, apesar dos desenvolvimentos positivos e da boa perceção relativa ao impacto da incorporação de algas e do processo de cozimento, ainda serão necessárias mais pesquisas para otimizar este e outros produtos, e abordar a viabilidade além dos ensaios *in vitro*.

keywords ***Phaeophyta*, *Fucus vesiculosus*, fortified pasta, nutritional composition, phytochemicals, antioxidant activity, bioaccessibility, bioavailability**

abstract Despite their worldwide abundancy, algae are still considered an under-exploited resource with a lot of interest in several industries, namely food and pharmaceuticals. Several reports comparing the diets of different countries, associate seaweed consumption with the oriental welfare and vitality contrarily to western societies. Within all three classes of edible seaweeds, those belonging to *Phaeophyta* have been receiving a lot of attention particularly due to their abundance in complex polysaccharides, phlorotannins, fucoxanthin and iodine. This work reports the nutritional composition (total fibre content, protein, ash and minerals) and phytochemical content (pigments and phlorotannins) of a pasta fortified with 1% and 5.5% of brown seaweed *Fucus vesiculosus*, prior and after the cooking process. In parallel the biological activities were evaluated namely antioxidant potential and capacity of inhibiting three metabolic enzymes, α-amylase, α-glucosidase and pancreatic lipase. Lastly, mineral bioaccessibility and bioavailability was accessed. Overall, the gathered results demonstrate that with each increasing seaweed concentration there is a tendency of improving several parameters. On a first approach, both fibres and ash contents increased up to 48% and 60%, respectively, while protein content remained unchanged. Further confirming the higher levels of ash on enriched samples, iodine content was augmented to around 91% of its initial concentration. Furthermore, *F. vesiculosus* contributed to a phytochemical increase, namely pigments, such as chlorophyll *a* (84%) and carotenoids (99%), and phlorotannins (87%) on the target pastas. Altogether, these results are most likely related to the improvement of the antioxidant capacity of the samples. Upon cooking, a general decrease on several contents was noted, namely protein, mineral and phytochemicals, which reflected a decline of the antioxidant property. The maintenance of fibre content upon this thermal processing possibly prompted to numerous effects, such as the retention of protein, to an extent, proving itself advantageous, but also the retention of minerals when submitted to simulated gastrointestinal digestion. Amongst all studied minerals, K and Mg fractions presented the highest percentages, 105% and 62% for the bioaccessible ones, and 56% and 24% for the bioavailable ones, respectively. Additionally, iodine stood out with a bioaccessible fraction of 82%. This element also had a bioavailability of 4%; however, it is estimated that the consumption of 100g of pasta could provide approximately 54% of its recommended daily dose. So, despite the positive developments and the good insight on the impact of both the seaweed incorporation and the cooking process, a lot of research is still required to optimize this product and to address the viability beyond *in vitro* assays.

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CHAPTER I

General Introduction

In a world of constant growth and change, a great deal of attention from the scientific community is being directed at marine environments, given they comprise approximately half of total global biodiversity.[1] This wide diversity of organisms is being recognized as a reservoir of structurally diverse and bioactive molecules, which can represent an enormous source of valuable compounds, such as sulphated polysaccharides, phlorotannins and diterpenes.[2,3] Despite this fact, much is still unknown regarding an inexhaustible list of marine organisms and their potential, among which is the algae class. Algae have been identified as an under-exploited plant resource with high potential not only as a bioresource in the food industry, but also in other industries, namely in cosmetics and pharmaceutics.[4,5]

Algae are photosynthetic marine organisms that belong to the *Eukaryota* Domain, comprising a total of 25-30,000 species worldwide of all forms and sizes, which range from unicellular microscopic organisms, also known as microalgae, to multicellular organisms of great size, or macroalgae.[6] As primary producers, they are fundamental organisms to the food chain of all aquatic ecosystems, producing oxygen and organic compounds serving as the basic trophic level or food for many other living beings.[7] Highlighting this importance is the fact that algae are amongst the most versatile organisms on Earth, with some algae living in complex habitats submitted to extreme conditions (for example, changes of salinity, temperature, nutrients, UVs irradiation).[6] This versatility results in the production of a great variety of secondary and biologically active metabolites, which cannot be found in other organisms. Importantly, and regarding this last subject, on top of being commonly found worldwide, which makes them an extremely accessible resource, other important aspects related to algae are their easy cultivation, since they do not compete with food crops for the use of arable land and fresh water resources, the fast growing cycles of the majority of species, and the possibility of controlling the production of some bioactive compounds by manipulation of the cultivation conditions.[8] In this way, algae can be considered as a good alternative to the chemical synthesis for these compounds.

Seaweeds, *i.e.*, marine macroalgae, are aquatic organisms that usually occupy coastal areas and can be divided into three major groups, namely *Rhodophytae* (red algae), *Chlorophytae* (green algae) and *Pheaophytae* (brown algae), depending on their nutrient and chemical composition.[9,10] Nowadays, seaweeds are used all around the world for direct consumption, having been included in Western cuisine as food or supplements, for the extraction of phycocolloids and bioactive components, and as biofertilizers.[11] Of particular

importance for this work are the macroalgae used for direct consumption, named edible seaweeds. Marine macroalgae are nutritionally very rich; apart from the low saturated to unsaturated fatty acid ratio and high concentration of complex polysaccharides, they are also well known for their natural richness in minerals, proteins and vitamins, as well as their high content of phytochemicals.[12] They have been an important part of the diet of many Far Eastern countries for centuries, such as Japan and Korea, and to a lesser extent in Europe and America.[13] Indeed, one of the main dietary differences between Eastern and Western societies is the higher seafood consumption, such as fish and marine algae, by the former.[14] Remarkably, this has led to stark differences in epidemiological studies between these societies, with higher seafood consumption being associated with a myriad of health benefits.[3,15–17] In fact, Japonese have the longest life expectancy in the world and the lowest rates of cardiovascular diseases, with these scores being correlated with their different dietary patterns, which include the regular consumption of macroalgae.[8] The aforementioned benefits are leading to an increased interest in the manufacture and consumption of high-value macroalgae-derived products in the Western culture, aiming to take advantage of their potential health benefits. In particular, *Pheaophytae* or brown algae present a high and diverse phytochemical content, making them a primary candidate for incorporation into foods among the three major groups of edible seaweeds.[3] It should be noted, however, that chemical composition of seaweed is highly dependent on factors such as natural habitat, maturity and, more importantly, the environmental conditions, *i.e.* the variations to which the natural habitat of algae might be subjected.[18] Therefore, they are presently regarded as the plant-based food of the future.[19] In order to further advance the knowledge on incorporation of brown seaweed and brown seaweed-derived high-value products in commonly found foods a review article was recently published (Afonso et al. 2019). This work comprehensively reviews the current literature on the benefits of brown seaweed and its compounds, and its translation into the manufacture of functional foods. Thus, the thesis work will focus on the evaluation of the chemical composition of a pasta fortified in brown seaweed *Fucus vesiculosus*, prior and after the cooking process, giving special attention to fucoxanthin, phlorotannins, fucoidan and mineral contents. In parallel, the biological activity will be accessed in terms of antioxidant potential and capacity on inhibiting three metabolism enzymes, while also considering aspects related to the bio-accessibility and bioavailability of target phytochemicals in the pasta matrix.

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CHAPTER II

Review. **Brown Macroalgae as Valuable Food Ingredients**

antioxidants

MDPI

Review

Brown Macroalgae as Valuable Food Ingredients

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Abstract: Due to the balanced nutritional value and abundance of bioactive compounds, seaweeds represent great candidates to be used as health-promoting ingredients by the food industry. In this field, Phaeophyta, i.e., brown macroalgae, have been receiving great attention particularly due to their abundance in complex polysaccharides, phlorotannins, fucoxanthin and iodine. In the past decade, brown algae and their extracts have been extensively studied, aiming at the development of well-accepted products with the simultaneous enhancement of nutritional value and/or shelf-life. However, the reports aiming at their bioactivity in in vivo models are still scarce and need additional exploration. Therefore, this manuscript revises the relevant literature data regarding the development of Phaeophyta-enriched food products, namely those focused on species considered as safe for human consumption in Europe. Hopefully, this will create awareness to the need of further studies in order to determine how those benefits can translate to human beings.

Keywords: *Phaeophyceae*; food fortification; algae; fibres; phlorotannins; fucoxanthin; minerals; iodine; nutrition; health-benefits; functional food

1. Introduction

Marine macroalgae i.e., seaweeds, have been well recognised for centuries by their importance in the diet of many Far Eastern countries, such as Japan and Korea [1,2]. They are nutritionally very wealthy, being claimed as a great source of complex polysaccharides, minerals, proteins and vitamins, as well as of several phycochemicals [3,4]. Actually, a regular seafood consumption, in which seaweeds are included, has been associated with a myriad of health benefits and a longer life expectancy [5,6] and these combined facts are leading to an increased interest in the manufacture and consumption of high-value macroalgae-derived products in Western cultures. Their consumption is also in line with the increasing awareness of consumers' perceptions towards organic products and of environmentally sustainable products. As a result, according to the Seafood Source report, the global seaweed market is expected to grow to USD 22.1 billion by 2024 [7].

Nowadays, amongst all three types of macroalgae (green, red and brown), brown algae are the most consumed species (66.5%), followed by red (33%) and green (5%) algae [8]. *Phaeophyceae* possess a high content of diverse phycochemicals and have been repeatedly claimed to exert important therapeutic properties, which turn them into great candidates to be used as bioactive agents in many industries, including the functional food market [9–11].

Europe has been recently highlighted as one of the most innovative regions regarding the use of seaweeds as a food ingredient with new products emerging on the European market increasing at exponential rates [12]. In fact, according to the Seafood Source report, the new products containing this new ingredient launched on the European market increased by 147% between 2011 and 2015, making Europe the most innovative region globally after Asia [13]. In this region, algae are considered as novel foods and a limited number of brown macroalgae species are considered to be safe for human consumption, namely *Fucus vesiculosus*, *Fucus serratus*, *Himanthalia elongata*, *Undaria pinnatifida*,

Ascophyllum nodosum, *Laminaria digitata*, *Laminaria saccharina*, *Laminaria japonica* and *Alaria esculenta* [14]. The present manuscript reviews the current knowledge on the incorporation of these brown seaweed species and brown seaweed-derived high-value products in common food products.

2. Chemical Particularities of Brown Macroalgae

The health-claims of *Phaeophyceae* are mainly associated with their abundance in specific nutrients and phycochemicals, particularly fibres, phlorotannins, fucoxanthin and minerals. However, their levels are greatly variable according to distinct factors, including the algae genera and species, maturity and the environmental conditions, i.e., the variations to which the natural habitat of algae might be subjected, namely season, temperature, salinity, oceanic currents, waves or even depth of immersion, as well as post-harvesting storage and processing conditions [9,10,15–18]. As such, this section describes their main structural characteristics as well as some of their most relevant bioactivities, highlighting their overall abundance in the targeted macroalgae of this review.

2.1. Polysaccharides

Brown macroalgae are known to produce different types of polysaccharides and/or fibres which, despite their variability, represent major components that can reach up to 70% of their dried weight (DW) [19]. In fact, previous reported data set the polysaccharide contents of relevant species, namely *L. japonica*, *F. vesiculosus*, *A. nodosum*, *Saccharina longicruis*, *U. pinnatifida* and *Sargassum vulgare* at 37.5%, 65.7%, 69.6%, 57.8%, 35.2% and 67.8% DW, respectively [20–23]. Amongst them, alginates, fucoidans and laminarins are the most representative ones.

Alginic acids or alginates, i.e., the salts of alginic acid, are the main polysaccharides in brown seaweeds [24], reaching up to 16.9% DW in *S. vulgare*, 20% DW in *S. longicruris*, 24% DW in *A. nodosum*, 32% DW in *Sargassum carpophyllum*, 40% DW in *Laminaria hyperborean* [25], 41% in *Sargassum siliquosum* and even to 59% DW in *F. vesiculosus* [26]. Within the cell wall, these polysaccharides are known to be partially responsible for the seaweed's flexibility [3] and therefore, expectedly, brown seaweeds grown under turbulent conditions usually have superior alginate contents than those of calm waters. In terms of structure, alginic acids or their corresponding extracted salts consist of α-L-guluronic acid (G) and β-D-mannuronic acid (M) (1→4)-linked residues arranged either in heteropolymeric (MG) and/or homopolymeric (M or G) blocks (Figure 1A–C). Regardless, the variations caused by diverse factors (e.g., algae species, seasonability, parts of the algae) are expected [16]. Noteworthy, alginates are considered one of the most important food colloids, with many applications in several industries such as foods, paper, pharmaceutical or cosmetics [27]. In fact, G-blocks in the presence of ions, such as Ca^{2+} form is the so-called egg-box, thus granting stiffness to the overall structure and conferring gel-forming properties to these polysaccharides [28]. Therefore, they are usually used as thickeners, gels, emulsifiers and stabilizers in order to improve quality parameters, especially in food grade products [29]. In addition to their wide applications, more recently, dietary alginates are being associated with positive health benefits in the gastrointestinal tract and appetite regulation, as well as antihypertensive and anti-diabetic effects [30]. Alginates are also considered great prebiotics as they were demonstrated to significantly promote the growth of several bacteria, including *Bifidobacterium bifidum*, *Bifidobacterium longum* and *Lactobacilli*, alongside with the increase of acetic acid, propionic acid and several short chain fatty acid metabolites, while decreasing deleterious metabolites, including faecal sulphide, phenol, *p*-cresol, indole, ammonia and skatole [31].

Fucoidans i.e., metabolites belonging to the fucans family, also have a structural role in brown algae, mostly preventing dehydration [3]. Their reported content in *Phaeophytae* is variable, ranging from approximately 6–8% DW in *L. japonica*, 3.2–16% DW in *U. pinnatifida*, and 3.4–25.7% DW in *F. vesiculosus* [10,32]. These polysaccharides are mainly composed of fucose and sulphate, although the presence of other types of monosaccharides (glucose, galactose, mannose, xylose and uronic acids), acetyl groups and proteins also occur [33]. Despite being molecules with high structural diversity, the representative backbone of fucoidans consists of (1→3)- and (1→4)-linked α-L-fucopyranose

residues, and these polysaccharides are commonly divided in two types, the first being characterized by long chains of (1→3)-linked α-L-fucopyranose residues (mainly present in *L. saccharina*, *L. digitata*, *C. okamuranus*, and *Chorda filum*) and the second consisting of alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues (characteristic from *A. nodosum* and *Fucus* spp.) (Figure 1D,E) [24,34].

Over the last years, extensive biological activities (e.g., antitumor, antioxidant, anticoagulant, antithrombotic, immunoregulatory, antiviral, anti-inflammatory among others) have been demonstrated with promising preclinical results, as recently reviewed [35]. As an example of in vivo studies, the effectiveness of a *F. vesiculosus* fucoidan injection towards oxidative stress in hyperoxaluric rats was demonstrated by Veena et al. [36] to be mediated by the stimulation of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Moreover, Huang et al. [37] reported that the ingestion of fucoidans isolated from *L. japonica* reduced the serum levels of total cholesterol, triglycerides, and low-density lipoprotein cholesterol in hyperlipidaemic rats, while increasing the enzymatic activity of lipoprotein lipase, hepatic lipase and lecithin cholesterol acyltransferase. In addition, their relevance in obesity and/or diabetes was also highlighted, in particular, by Xan et al. [38], who reported *F. vesiculosus* fucoidans' ability to inhibit α-glucosidase in vitro and to decrease the fasting blood glucose and glycosylated haemoglobin levels of db/db mice, as well as by Kim et al. [39], when administrating *U. pinnatifida* fucoidans to the same animal model. Although there is limited evidence to implicate a role of fucoidans in the gut microbiota, some works reported that fucoidans from different brown algae species greatly contributed for the increase in the growth of *Bifidobacterium*, *Lactobacillus* and *Ruminococcaceae*, either in mice or human faecal samples [31].

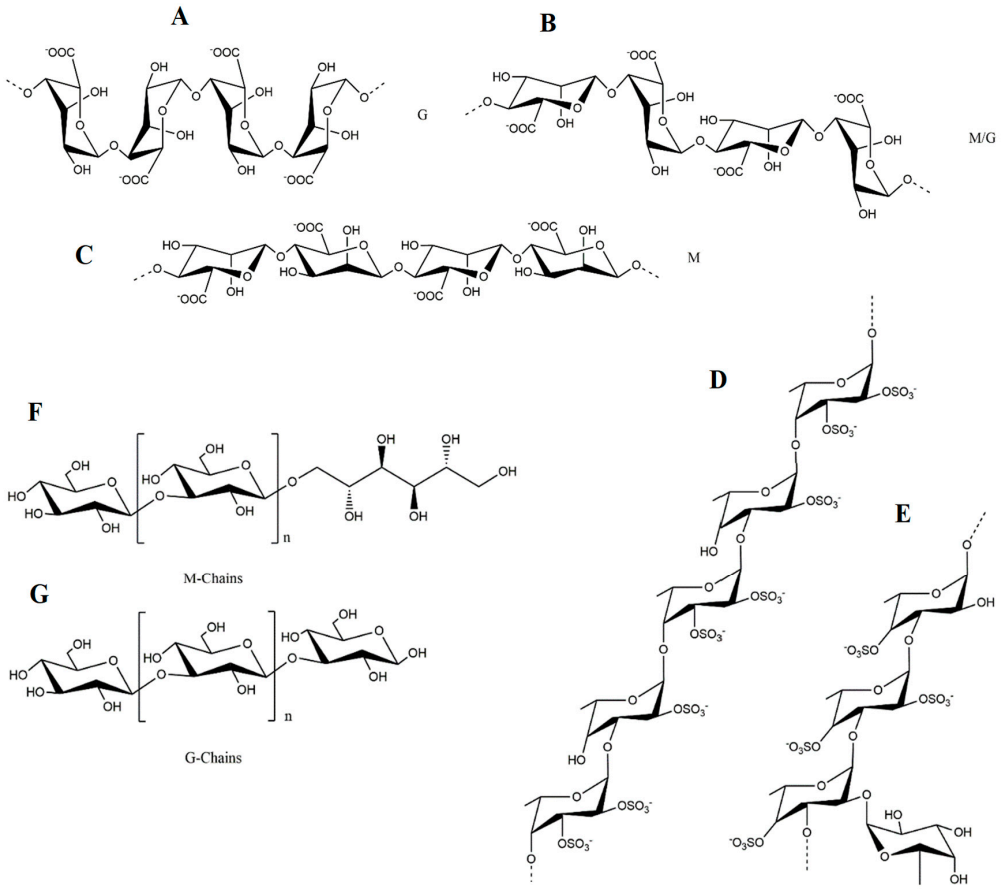

Figure 1. The structure of representative polysaccharides found in brown algae: (**A**–**C**) alginic acids; (**D**–**E**) fucoidans from *A. nodusum*/*F. vesiculosus* and *S. latissima*, respectively; (**F**–**G**) laminarins M and G chains.

Laminarins, also named laminarans or leucosins, on the other hand, belong to the glucan family and serve as reserve metabolites in brown algae [40]. These are commonly found in the fronds of *Laminaria* and *Saccharina* macroalgae and, to a lesser extent, in *Ascophyllum*, *Fucus* and *Undaria* species [41]. In general, they are relatively small polysaccharides composed of β-(1→3)-linked glucose monomers, containing large amounts of sugars and a low fraction of uronic acids (Figure 1F,G) [42]. Depending on the type of sugar at the reducing end, they are classified in two distinct types, specifically the M chains, which have a terminal 1-O-substituted D-mannitol, and the G chains, possessing a terminal glucose [16]. The content of these polysaccharides is also season-dependent, since seaweeds show no production or very less amounts in the winter and maximum production during summer and autumn [23]. As previously stated, *Laminariales* are known to produce high amounts of laminarins, with contents reaching up to 35% DW, particularly in *L. saccharina* and *L. digitata* [40]. Other reported values of laminarins content comprise those of *A. esculenta*, *U. pinnatifida*, *A. nodosum* and *F. serratus* (11.1%, 3%, 4.5% and up to 19% DW, respectively) [22–24,40].

The bioactivities of laminarins are scarcely exploited, but still they are considered as fibres and therefore can be partially or totally fermented by the endogenous intestinal microflora. This was demonstrated by Devillé et al. [43], when comparing the results from in vitro digestibility tests, where no hydrolysis of this fibre occurred, to those of in vivo tests, for which no traces of laminarin were detected in the faeces of fed Winstar rats after ingestion.

It should be noted that oligosaccharides from brown macroalgae polysaccharides may also exhibit interesting bioactivities, which can differ from those of the original polysaccharides. In this topic, alginate oligosaccharides have been claimed to possess radical scavenging activities with the great potential for application in the food industry [44], and even promising effects on neuro-inflammation, promoting microglial phagocytosis. This could be of great relevance for their application as a nutraceutical agent for neurodegenerative diseases, such as Alzheimer's disease [45]. In turn, in vivo experiments on renovascular hypertensive rats revealed that fucoidan oligosaccharides exhibited anti-hypertensive effects comparable to those of captopril, i.e., an approved drug used for the treatment of hypertension [46].

2.2. Phlorotannins

Phlorotannins are phenolic compounds almost exclusive to *Phaeophytae* and also represent their main phenolic pool. In brown seaweeds, they are associated with a myriad of functions, ranging from structural cell wall components, to biosyinthetic percursors and defensive mediators against natural enemies, acting as herbivore deterrents, inhibitors of digestion and agents against bacteria [11]. Phlorotannins are known to accumulate mostly in physodes (i.e., specialized membrane-bound vesicles of the cell cytoplasm), with levels that might represent up to 25% of seaweed's DW, despite variations which occur depending on distinct factors [47]. For example, the higher levels of phlorotannins in *Fucus* spp. are associated with high salinity waters and solar exposure during summer [10].

Being part of the tannins group, phlorotannins present a polymeric structure derived from several phloroglucinol (1,3,5-trihydroxybenzene) units and possess a high number of hydroxy groups, thus conferring them solubility in water [48]. Depending on the linkage between phloroglucinol monomer units, a wide range of compounds with different molecular weights can be obtained [49], which overall, are divided in four categories for each type of linkage: Fuhalols and phlorethols based on ether linkage, fucols based on C-C linkage, fucophlorethols for a combination of the previous ones, and, finally, eckols and carmalols, based on dibenzodioxin linkage (Figure 2).

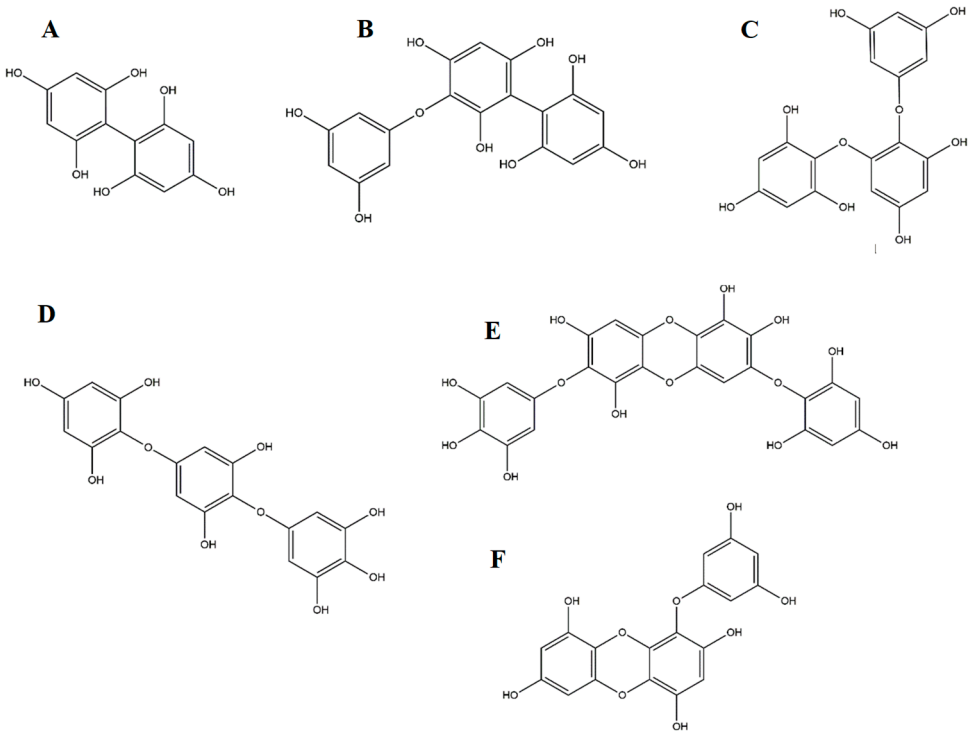

Figure 2. Some representative phlorotannins from brown seaweeds: (**A**) Fucol; (**B**) Fucophlorethol; (**C**) Phlorethol; (**D**) Fuhalol; (**E**) Carmalol; (**F**) Eckol.

Phenolic extracts from brown seaweeds have been demonstrated to exhibit various biological activities, including antioxidant, antidiabetic, anti-inflammatory and others [11,50,51]. In this regard, O'Sullivan et al. [52] observed the augment of glutathione levels in Caco-2 cell models when incubated with *A. nodosum*, *F. vesiculosus* and *F. serratus* phlorotannins extracts, while also highlighting the protective effects of the latter on the same model pretreated with H_2O_2. In vivo experiments have even demonstrated that the oral administration of 200 mg/kg/day of *F. vesiculosus* polyhenol-rich extracts over 4 weeks to Sprague-Dawley rats could increase the blood plasma reducing power, paraoxonase/arylesterase 1 (PON-1) activity and $O_2^{\bullet-}$ scavenging activity by 29%, 33% and 25%, respectively [53]. Likewise, the antidiabetic properties of *A. nodosum* and *F. vesiculosus* phenolic-rich extract were observed in vivo as the postprandial blood glucose levels and insulin peak decreased 90% and 40%, respectively, on rats under hyperglycemic diets supplemented with 7.5 mg/kg compared to the unsupplemented group [54]. In fact, the ingestion of 500 mg of this mixture containing *A. nodosum* and *F. vesiculosus* 30 min prior to the consumption of carbohydrates was shown to reduce the insulin incremental area of the curve and an increase in insulin sensitivity in a human clinical trial [55]. Human trials have also been carried out to evaluate the potential antiobesity effect of polyphenolic-rich extracts of *A. nodosum* (100 mg/day for 8 weeks). Although the treatment did not exhibit any significant benefits (no significant changes in C-reactive protein, antioxidant status or inflammatory cytokines), with the exception of a modest decrease of the DNA damage in the obese group, several phlorotannin metabolites were detected in the subjects plasma and urine, indicating that these compounds are metabolised and absorbed into the systemic circulation [56]. These observations are in line with those reported by Corona et al. [57] who also described the appearance of phlorotannin metabolites in urine and plasma collected from humans after consuming a capsule of *A. nodosum* extract containing about 100 mg of polyphenols.

2.3. Fucoxanthin

In opposition to red and green macroalgae, *Phaophytae* are characterized by the presence of the carotenoid fucoxanthin, which is responsible for their specific coloration. Fucoxanthin is a xanthophyll belonging to the tetraterpenoid family with a structure consisting of an unusual allenic bond and a 5,6-monoepoxide in its molecule (Figure 3). The content of this pigment is highly variable amongst different species, as well as dependent on extrinsic factors, with a large range being even described within the same species. The reported levels comprise in 171 mg/kg (*Fucus spiralis*), 224 mg/kg (*Fucus distichus*), 364 mg/kg (*Fucus evanescens*), 172–660 mg/kg (*A. nodosum*), 178–468 mg/kg (*Laminaria* spp.) [41,58].

Recently, this xanthophyll has earned particular attention mainly because of its promising effects in terms of antidiabetic, anti-obesity and antioxidant activities [59,60], with claims being supported by in vivo studies. For instance, the administration of *U. pinnatifida* lipids rich in fucoxanthin to male diabetic mice were associated with insulin resistance amelioration and the reduction of blood glucose levels [61]. Moreover, fucoxanthin isolated from the same macraolgae species was also shown to inhibit the differentiation of 3T3-L1 preadipocytes into adipocytes by down-regulating peroxisome proliferator-activated receptor gamma (PPARγ) [62]. Furthermore, a diet based on *U. pinnatifida* fucoxanthin was capable of inducing uncoupling protein 1 (UCP1) expression in white adipose tissue (WAT) of obese mice. When added as a supplement to rats fed with a high-fat diet, it prompted a decrement of the mRNA expression of significant enzymes associated with lipid metabolism, such as fatty acid synthase, acyl-CoA cholesterol acyltransferase, hepatic acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, hydroxy-3-methylglutaryl coenzyme A and SREBP-1C [63,64].

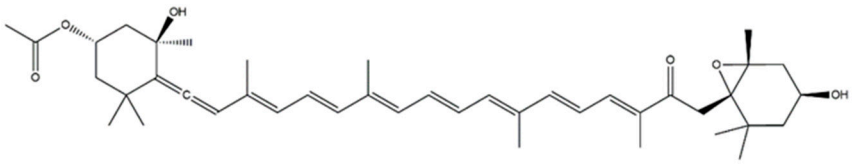

Figure 3. Structure of fucoxanthin.

2.4. Minerals

Due to their structural and physiological features, brown macroalgae are recognized for their superior ability to accumulate minerals. Although the content of minerals like calcium, magnesium, phosphorus, potassium, sodium and iron is usually high within the macroalgae matrix, one of the standout aspects, comparatively to plants in general, are both their low Na/K ratios and high iodine levels [4]. In fact, it is well accepeted that low Na/K ratios are an important aspect for good maintenance of cardiovascular health [65]. Therefore, according to the World Health Organization (WHO), the recommended value for this should be close to one, so consumption of food products with this proportion or below should be considered for healthy cardiovascular purposes [66]. In fact, several studies point to a Na/K ratio ranging between 0.3 and 1.5 in brown seaweeds, with particular interest for *Laminaria* spp. (0.3–0.4) from Spain origins, wich are significantly lower than diverse food products, such as cheddar cheese (8.7), olives (43.6), and sausages (4.9) [4,67–70]. Additionally, *Phaeophyceae* seaweeds, due to the rich composition in alginates and sulphated polysaccharides coupled with the presence of haloperoxidases in the cell walls, allow the accumulation of iodine to more than 30,000 times over its concentration in the surrounding environment which is even higher than any edible plant [71]. The major contents of iodine were documented for *L. digitata*, *A. nodosum*, *H. elogata* and *U. pinnatifida* exhibiting concentrations of 70, 18.2, 10.7 and 3.9 mg/100 g wet weight, respectively [72]. Moreover, other studies also highlight the particular affinity of Laminarales to accumulate iodine, particularly *L. digitata,* in which values are known to reach 9014 and 8122 mg/kg DW, in spring and autumn, respectively [23].

3. Use of Brown Macroalgae as Food Ingredient

Being considered as a rich and balanced source of nutrients and bioactive compounds, consumers and food industries have a growing interest to introduce macroalgae, including *Phaeophytae*, into the dietary habits of the western countries, with new products already being launched in the markets at high rates in Europe. The usage of brown species as food ingredients has, however, to overcome huge challenges, that go from the guarantee of enough biomass to sustain the market development, to the gain of consistent knowledge of their physicochemical features, as well as understanding the extension of their impact when used as ingredients in foods. This section highlights some of the developed foods in the field of seaweed-fortified products, categorized by the respective incorporated algae species, considering the authorized seaweeds for human consumption in France/Europe [14], and finalising with the influence this incorporation has on the foods' chemical, functional and structural behaviour.

3.1. *Fucus vesiculosus*

F. vesiculosus has found application as a functional ingredient in many different food matrices, mostly as a source of phlorotannins and antioxidant compounds, aiming to prevent food spoilage resultant from oxidative deterioration (Table 1). Fish and fish-derived products are one of the main matrices where several studies with this seaweed have been conducted. In this context, Dellarosa et al. [73] reported that neither aqueous nor 80% ethanol extracts from *F. vesiculosus* had significant effects on the lipid oxidation of fish cakes enriched with omega-3 polyunsaturated fatty acids, throughout a 28-days refrigerate storage. Nevertheless, the authors showed that no off flavour was detected in any samples tested, with low scores of rancid odour and flavour being registered in the sensory analysis. On the other hand, some studies conducted on cod fish muscle and/or protein indicated that the incorporation of *F. vesiculosus* extracts could indeed prevent the lipid peroxidation events and even improve some of their sensorial aspects. In fact, the effects of the incorporation of 1% and 2% of the antioxidant dietary fibre extracted from *F. vesiculosus* into minced horse mackerel revealed a significant reduction of the fish mince lipid oxidation throughout the 5 months of storage at –20 °C. These factors reduced the total drip after thawing and cooking the horse mackerel mince up to 3 months of frozen storage, a fact that could be due to the water holding capacities of the fibre. Furthermore, although the addition of 2% (but not 1%) of antioxidant dietary fibre caused changes in the fish mince flavour compared to the control, these were actually considered positive by the sensory panellists [74].

In a different approach, Wang et al. observed that some oligomeric phlorotannin sub-fractions obtained by Sephadex LH-20 chromatography from an 80% ethanol extract of *F. vesiculosus* were able to completely inhibit the haemoglobin-catalysed lipid oxidation in both washed cod muscle and cod protein isolates systems, during an 8-day period of ice storage. Moreover, with a concentration of 300 mg/kg, the effectiveness of these phlorotannins sub-fractions were comparable to that of 100 mg/kg propyl gallate, i.e., a highly effective synthetic antioxidant in muscle foods, thus evidencing the great potential of oligomeric phlorotannins to be exploited as natural antioxidants in fish and fish-derived products [75]. Similar results were further reported by Jónsdóttir et al. [76], who observed an inhibition of the lipid oxidation in haemoglobin-fortified washed cod mince system after incorporating 300 mg phloroglucinol equivalents/kg of an ethyl acetate fraction obtained from an 80% ethanol extract of *F. vesiculosus*. Other authors also demonstrated that the incorporation of a *F. vesiculosus* phlorotannin-rich fraction (obtained with 80% ethanol and further purified with ethyl acetate) into cod protein hydrolysates, not only prevented the lipid oxidation reactions during storage, but also increased their final antioxidant activity [77,78] and could even improve the bitter, soap, fish oil and rancidity taste of the final protein hydrolysates [77].

Table 1. Selected studies reporting the effects of the incorporation of *F. vesiculosus* or isolates as ingredients in different food matrices.

Functional Food	Functional Ingredient	Results	Ref.
Fish cakes	*F. vesiculosus* extracts: 100% H_2O, 80% EtOH	No off-flavours and lower rancid odour and flavour None of the extracts had influence on lipid oxidation nor quality of the products	[73]
Cod muscle and protein isolates	*F. vesiculosus* 80% EtOH extract and further fractions (EtOAc + Sephadex LH-20)	↓ Lipid oxidation in both fish muscle and protein isolates 300 mg/kg of the oligomeric phlorotannin fractions exhibited an effect comparable to that of 100 mg/kg propyl gallate	[75]
Cod mince	EtOAc fraction of *F. vesiculosus* 80% EtOH extract	↓ Lipid oxidation in fish muscle	[76]
Cod protein hydrolysates	EtOAc fraction of *F. vesiculosus* 80% EtOH extract	↓ Lipid hydroperoxide and TBARS formation during protein hydrolyzation ↑ Antioxidant activity of the final protein hydrolysates	[78]
Cod protein hydrolysates	EtOAc fraction of *F. vesiculosus* 80% EtOH extract	↓ Lipid oxidation during protein hydrolysates freeze drying ↑ Antioxidant activity of the final protein hydrolysates Improved sensorial aspects (bitter, soap, fish oil and rancidity taste)	[77]
Minced horse mackerel	*F. vesiculosus* antioxidant dietary fibre	↓ Lipid oxidation during 5 months of storage at −20 °C ↓ Total dripping after thawing and cooking after up to 3 months of frozen storage Improved fish mince flavour	[74]
Granola bars enriched with fish oil emulsion	*F. vesiculosus* 100% H_2O, 70% acetone and 80% EtOH extracts	↓ Oxidation products after storage ↓ Iron-lipid interactions Acetone and EtOH extracts provided additional lipid oxidation protection ↑ Phenolic content, radical scavenging activity and interfacial affinity of phenolic compounds Possible tocopherol regeneration	[79]
Granola bars enriched with fish oil emulsion	*F. vesiculosus* 100% H_2O, 70% acetone and 80% EtOH extracts	↓ Lipid oxidation during storage ↑ Effectiveness for lower concentrations of EtOH and acetone extracts ↑ Phenolic content, radical scavenging activity and interfacial affinity of phenolic compounds Possible tocopherol regeneration	[80]
Fish-oil-enriched milk and mayonnaise	*F. vesiculosus:* EtOAc fraction from an 80% EtOH extract, 100% H_2O	↑ Lipid stability and ↓ oxidation of EPA and DHA and subsequent secondary degradation products in both foods—H_2O extract at 2.0 g/100 g exerted higher inhibitory effects on mayonnaise's peroxide formation.	[81]
Fish-oil-enriched mayonnaise	*F. vesiculosus* 100% H_2O, 70% acetone, and 80% EtOH extracts	Dose-dependent inhibition of lipid oxidation exhibited by EtOH and acetone extracts H_2O extract increased peroxide formation	[82]
Pork liver pâté	*F. vesiculosus* commercial extract	Decrease in lightness values after storage Redness and yellowness maintained after storage Protection against oxidation comparable to BHT samples ↓ Total volatile compounds	[83]

Table 1. *Cont.*

Functional Food	Functional Ingredient	Results	Ref.
Pork patties	*F. vesiculosus* 50% EtOH extracts	↓ TBARS slightly Did not improve colour, surface discoloration or odour attributes No significant differences between seaweed and control samples in sensory analysis	
Milk	*F. vesiculosus* 60% EtOH extracts	↑ Milk lipid stability and shelf-life characteristics Appearance of greenish colour and fishy taste Overall sensory attributes were worsened	[84]
Yoghurts	*F. vesiculosus* 60% EtOH extracts	No influence on chemical and microbiological characteristics ↑ Yogurts lipid stability and shelf-life characteristics Overall sensory attributes were worsened	[85]
Pasteurized apple beverage	*F. vesiculosus* fucoidan extract	Dose-, time- and temperature-dependent bacteriostatic and bactericidal effects against *L. monocytogenes* and *S. typhimurium* *S. typhimurium* showed higher sensitivity to the extract	[86]
Bread	*F. vesiculosus* powder	↑ Dough viscosity and wheat dough consistency ↓ Porosity ↑ Density, crumb firmness and green colour of crust 4% seaweed powder was considered optimal	[87]

↑: increased; ↓: decreased; BHT: 2,6-di-*tert*-butyl-4-methylphenol; DHA: docosahexaenoic acid; EPA: eicosapentanoic acid; EtOAc: ethyl acetate; EtOH: ethanol; TBARS: Thiobarbituric acid reactive substances.

The fortification of food matrices with fish oils rich in n-3 long chain polyunsaturated fatty acids has been in high demand during recent years due to increasing consumer awareness of the beneficial effects of docosahexaenoic and eicosapentaenoic acids (DHA and EPA, respectively). However, this usually decreases the foods' oxidative stability, leading to the development of undesirable off-flavours and consequent shelf-life reduction [88]. In this field, *F. vesiculosus* extracts were found to be highly promising. According to Karadağ et al., [79] the introduction of 0.5 and 1 g/100 g of both *F. vesiculosus* ethanol and acetone extracts into fish oil-enriched granola bars effectively improved their lipid stability, contributing to an increase of the foods' phenolic content, radical scavenging activity, interfacial affinity of phenolics and eventual regeneration of tocopherol, which consequently cause the reduction of the iron-lipid interactions as well as the lipid oxidation during the storage period. These results agree with previous data demonstrating that addition of both ethanol and acetone *F. vesiculosus* extracts to granola bars enriched with multi-layered fish oil emulsion contributed to the reduction of the formation of primary and secondary oxidation products over the period of storage at 20 °C [80]. Enhancement of lipid stability was also described in two other fish oil-fortified food matrices, namely mayonnaise and milk, after incorporation of 1.0–2.0 g/100 g of an ethyl acetate fraction, obtained from *F. vesiculosus* 80% ethanol extract (rich in phenolics and carotenoids) [81], as well as in fish oil-fortified mayonnaise added with 1.5–2.0 g/kg of both acetone and ethanol extracts of this seaweed species [82]. Interestingly, in the particular case of fish oil-fortified mayonnaise, Hermund et al. [81] found that, despite its lower content of phenolics and carotenoids, *F. vesiculosus* water extracts, at high concentrations, could prevent the peroxides formation more effectively than the ethyl acetate fraction, much likely due to its higher metal chelating capacity resultant from the presence of polysaccharides or other highly polar compounds with strong metal chelating capacities. This outcome was, however, refuted in a latter study that reported an increased peroxide formation in fish oil-enriched mayonnaise also incorporated with *F. vesiculosus* water extracts [82]. The disparity found between these two works might be related to the differences in the trace metal contents of the aqueous extracts performed in each study since the

former had much lower iron content than the latter, which might be responsible for the induction of lipid oxidation in the food matrix.

Recently, the fortification of canola oil with 500 ppm of *F. vesiculosus* water extract was reported to reduce approximately 70% of the peroxides formation and 50% of the thiobarbituric acid reactive substances (TBARS) value compared to the control samples, both under accelerated storage conditions (60 °C). This confirms that this extract may in fact hold the potential to be exploited as a food antioxidant agent. Indeed, under similar conditions, butylated hydroxytoluene (BHT) (at 50 ppm) only inhibited peroxides formation and TBARS by 25% and 20%, respectively, thus showing that seaweed extracts could be used as a potential substitute for synthetic antioxidants. In the same line, in a different food matrix, namely low-fat pork liver pâté, the incorporation of 500 mg/kg of a commercial antioxidant extract of *F. vesiculosus* was also shown to be as effective as 50 ppm of BHT at inhibiting the formation of primary and secondary oxidation products over 180 days under storage at 4 °C, as well as in the maintenance of the redness and yellowness which were lowered in the control samples [83]. On the other hand, the fortification of pork patties with *F. vesiculosus* 50% ethanol extracts (250–1000 mg/kg) showed low performances on samples oxidative stability, with modest inhibitory effects on TBARS, compared to the control samples, but very far from that exhibited by BHT. Additionally, regardless the good acceptability in the sensory analysis, the incorporation of these *F. vesiculosus* extracts failed to improve colour, surface discoloration or odour attributes [89]. Therefore, further studies are necessary to conclude whether extracts of this seaweed are suitable for the application as oxidation inhibitors for the long-term storage of meat products.

Further aiming lipid stabilization in dairies, O'Sullivan et al. [84,85] tested the incorporation of 0.25% and 0.5% (*w*/*w*) of 60% and 40% ethanol extracts from *F. vesiculosus* into milk and yogurt, respectively. Indeed, both products showed a significant reduction of lipid oxidation alongside with improvements on their shelf-life characteristics. However, neither were well accepted in the sensory analysis, even for the lower concentrations, as the panellists reported an unpleasant green/yellowish colour and a fishy taste.

Although the majority of the studies carried out with this seaweed species were focused on their antioxidant activity and capacity to enhance foods' lipid stability, other authors have tried the incorporation of *F. vesiculosus* with different purposes. In a recent work, the incorporation of *F. vesiculosus* fucoidans into a new functional pasteurized apple beverage was found to be useful for controlling the growth of an undesirable microorganism, since strong bacteriostatic and bactericidal effects against *Listeria monocytogenes* and *Salmonella typhimuium* were observed in a dose-, time- and temperature-dependent manner [86]. On the other hand, Arufe et al. [87] studied the influence of the addition of different concentrations (2–8% *w*/*w*) of *F. vesiculosus* seaweed powder into wheat flour to the final rheological properties of the dough, such as the density and crumb texture. The authors found that for concentrations above 4%, the addition of *F. vesiculosus* powder caused the increase of the elongational dough viscosity and consequent decrease of its porosity, as well as the increase in the bread density, crumb firmness and appearance of a green colour. Therefore, 4% of *F. vesiculosus* powder would be the maximum amount that could be added to the bread without impairing its properties.

3.2. Himanthalia elongata

H. elongata has also been object of many studies comprising the development of seaweed-enriched foods, which, in addition to the improvement stability and/or shelf-life extension, also aimed to provide enhanced nutritional properties to the foods. In this field, many works reporting *H. elongata* fortified-foods were carried out on meat and meat-based products (Table 2). One of the most exploited attributes of this seaweed species is perhaps its wealthy mineral composition, which makes *H. elongata* a good candidate to be used as a salt replacer, contributing to the reduction of salt consumption and related health complications typical of western high-NaCl diets. It also increases the consumption of other elements, such as calcium potassium or iodine, which are usually lacking or below recommended levels in regular diets [4].

Table 2. Selected studies reporting the effects of the incorporation of *H. elongata* or isolates as ingredients in different food matrices.

Functional Food	Functional Ingredient	Results	Ref.
Poultry steaks	3% dry matter *H. elongata*	↑ Purge loss slightly ↓ Cooking loss ↑ Levels of total viable counts, lactic acid bacteria, tyramine and spermidine No important changes observed during chilled storage Positive overall acceptance by a sensory panel	[90]
Pork gel/emulsion systems	2.5% and 5% dry matter *H. elongata*	↑ Water and fat binding properties ↑ Hardness and chewiness of cooked products ↓ Springiness and cohesiveness	[1]
Low-salt pork emulsion systems	5.6% dry matter *H. elongata*	↑ Content of n-3 PUFA ↓ n-6/n-3 PUFA ratio ↓ Thrombogenic index ↑ Concentrations of K, Ca, Mg and Mn	[91]
Pork meat batter	3.4% powder *H. elongata*	↑ Water/oil retention capacity, hardness and elastic modulus. Thermal denaturation of protein fraction was prevented by seaweed alginates Nutritional enhancement	[92]
Restructured meat	5% powder *H. elongata*	Effects in rats: ↓ Total cholesterol ↑ CYP7A1, GPx, SOD, GR expression ↓ CAT expression	[93]
Restructured meat	5% powder *H. elongata*	↓ HSL and FAS and ↑ ACC ($p < 0.05$) expression on rats fed with seaweed fortified meat comparing with rats under hypercholesterolemic diet	[94]
Frankfurters	3.3% *H. elongata* powder	↑ Cooking loss ↓ Emulsion stability Combination of ingredients provided healthier meat products with lower fat and salt contents Worsened physicochemical and sensory characteristics	[95]
Beef patties	10–40% (*w*/*w*) *H. elongata*	↓ Cooking loss ↑ Tenderness, dietary fibre levels, TPC and antioxidant activity ↓ Microbiological counts and lipid oxidation Patties with 40% seaweed had the highest overall acceptability	[96]
Bread sticks	2.93–17.07% *H. elongata* powder	Highest concentration had higher phycochemical constituents, acceptable edible texture and overall colour	[97]
Bread	8% (*w*/*w*) *H. elongata*	↑ TPC ↑ Antioxidant activity in DPPH•, ORAC and TEAC	[98]
Yoghurt and Quark	0.25–1% dehydrated *H. elongata*	Alterations in all yoghurt attributes except for buttery odour, and acid and salty flavours Alterations in all quark attributes except yogurt odour, acid flavour and sweet flavour. Sensory characteristics worsened	[99]

↑: increased; ↓: decreased; ACC: acetyl CoA carboxylase; CAT: Catalase; CYP7A1: liver cytochrome P450 7A1; DPPH•: 2,2-diphenyl-1-picrylhydrazyl radical; FAS: fatty acid synthase; GPx: Glutathione peroxidase; GR: Glutathione reductase; HSL: hormone-sensitive lipase; ORAC: oxygen radical absorbance capacity; PUFA: polyunsaturated fatty acids; SOD: superoxide dismutase; TEAC: trolox equivalent antioxidant capacity; TPC: Total phenolic content.

Many of these studies were carried out by the group of Jiménez-Colmenero et al., who have developed several meat products in which the content of sodium chloride was partially replaced by different species of edible seaweeds, including *H. elongata*. Among the seaweed-containing formulations, frankfurters, restructured meats and meat emulsions were shown to have at least 50 to 75% less NaCl

than their conventional recipes [1,90,91,95,100,101]. Apart from the NaCl replacement, the fortification of frankfurters and meat emulsions with *H. elongata* also contributed to the increase of K content and subsequent reduction of the Na/K ratio from 3 to values below 1 (i.e., close to those recommended by WHO for maintaining a healthy cardiovascular condition). Additionally, the Ca, Mg and Mn contents in these two meat products increased to >1000%, >300% and >700%, respectively, compared with the conventional formulas, alongside with their water and fat binding properties [1,101]. Other effects resultant from *H. elongata* fortification in these matrices included the reduced cooking loss and increase in the Kramer shear force in restructured poultry meat [90]; increased water and oil retention in pork meat batter [92]; increased dietary fibre content in frankfurters [101]; and increased phenolic content and antioxidant activity in meat emulsions [91]. Overall, these products were well-accepted in the sensory analysis, with exception of frankfurters that were reported unpleasant mainly due to the increase of the dryness feeling and seaweed-like taste.

Cox and Abu-Ghannam [96] also reported that *H. elongata*-fortified beef patties (10–40% *w*/*w*) were very well accepted in the sensory analysis, particularly those with 40% of seaweed, getting even better scores than the control samples. This was mainly due to the improvements on the samples' texture and overall mouthfeel, which resulted from the decrease in the cooking loss (associated to the incremented fibre content) and the increase in tenderness for more than 50%. Furthermore, a significant enhancement of the phenolic content and antioxidant activity (in a dose-dependent manner), as well as a lowered microbiological count and lipid oxidation before the chilling stage and after 30 days of storage, were observed in all patties containing seaweed. In fact, at the end of the experiment, the samples containing above 20% of *H. elongata*, showed no bacterial growth at all, as well as considerably low levels of the lipid oxidation marker.

In vivo studies on rat models revealed that the introduction of restructured pork meat enriched with 5% *H. elongata* (RPS) in the animals' hypercholesterolemic diet significantly lowered the serum cholesterol levels that were augmented in the group under a non-RPS supplemented hypercholesterolemic diet. Moreover, a significant increase in SOD and GPx, alongside with a decrease of glutathione reductase (GR) expressions, were observed in both groups under hypercholesterolemic and regular RPS-supplemented diets, although increased glutathione reductase activity was also verified. Interestingly, the combined cholesterol and seaweed diet predisposed an increase in the expression of GR, SOD and liver cytochrome P450 7A1 (CYP7A1), i.e., a gene that encodes for the enzyme responsible for the elimination of cholesterol through the production of bile acids, but a decrease in the expression of CAT and GPx, suggesting a possible blocking effect of the hypercholesterolemic agent induced by seaweed incorporation [93]. In a similar study, rats under RPS-supplemented hypercholesterolemic diets, not only exhibited lower plasma cholesterol levels but also lower liver apoptosis markers, namely cellular cycle DNA, caspase-3 and cytochrome c [102]. Supporting these results, González-Torres et al. [94] confirmed that the administration of *H. elongata*-fortified restructured pork meat (at 5%) to rats under cholesterol-rich diets, partially blocked the hypercholesterolemic effect of the dietary pattern while changing the lipogenic/lipolytic enzyme expression (decreasing hormone-sensitive lipase and fatty acid synthase while increasing acetyl CoA carboxylase expressions compared with subjects under hypercholesterolemic diet) and reducing the wasting effect of hypercholesterolemia on adipose tissue in rats.

Apart from meat products, *H. elongata* powder has also been used to enrich breadsticks in order to enhance their nutritional properties. From the 10 formulations tested (with seaweed concentrations of 2.63 to 17.07% *w*/*w*), the highest was reported to have the most significant influence on the chemical properties of breadsticks. Furthermore, this sample also had higher levels of total dietary fibre, while the total phenolic content and antiradical activity were maximized at 138.25 mg GAE/100 g dry basis and 61.01%, respectively, maintaining an acceptable edible texture and colour of the samples. Therefore, since no significant difference was seen between the control and seaweed enriched breadsticks in terms of sensory analysis, this product could have great acceptability, especially to non-seaweed consumers [97]. The augmented phenolic content as well as the enhanced antioxidant activity were

also described on functional breads developed with 8% of *H. elongata* flour [98]. On the other hand, an attempt to supplement yogurt and quark with dehydrated *H. elongata* (0.25–1% *w*/*w*) turned out to negatively affect almost all the sensory parameters analysed, which makes this seaweed not very suitable for application in these two dairies, at least in these conditions [99].

3.3. *Undaria pinnatifida*

Similar to *H. elongata*, the applications of *U. pinnatifida* as functional ingredients have mostly been reported in meat and meat-derived products (Table 3). For instance, the incorporation of *U. pinnatifida* (1–4%) into pork beef patties increased their ash content as well as their juiciness due to the lower cooking losses compared to the control [103]. In a similar approach, the reformulation of low-salt (0.5%) and low-fat (<10%) beef patties by the addition of 3% of *U. pinnatifida* and partial or total replacement of pork backfat with olive oil-in-water emulsion, significantly affected the frozen storage characteristics of the products. This presented enhancements in terms of technological, sensory and nutritional properties, as well as improvements in their physiological benefits. These reformulated patties demonstrated less thawing and cooking losses, and were texturally softer than the samples without seaweed, most likely due to the microstructural changes caused by the formation of alginate chains.

Table 3. Selected studies reporting the effects of the incorporation of *U. pinnatifida* or isolates as ingredients in different food matrices.

Functional Food	Functional Ingredient	Results	Ref.
Beef patties	3% dry matter *U. pinnatifida*	↑ Binding properties and cooking retention values of, fat, fatty acids and ash Replacement of animal fat with olive-in-water emulsion and/or seaweed was reportedly healthier. ↓ Thawing and↑ softer texture Changes on the microstructure due to formation of alginate chains Overall acceptable products and fit for consumption	[104,105]
Chicken breast	200 mg/kg *U. pinnatifida*	↑ Redness and yellowness ↓ Lipid oxidation in chilling storage and after cooking Overall appearance and shelf-life were enhanced	[106]
Pork gel/emulsion systems	2.5% and 5% dry matter *U. pinnatifida*	↑ Water and fat binding properties ↑ Hardness and chewiness of cooked products ↓ Springiness and cohesiveness	[1]
Low-salt pork emulsion systems	5.6% dry matter *U. pinnatifida*	↑ Content of n-3 PUFA ↓ n-6/n-3 PUFA ratio ↑ Concentrations of K, Ca, Mg and Mn ↑ Antioxidant capacity	[91]
Pasta	100:0, 95:5, 90:10, 80:20 and 70:30 (semolina/*U. pinnatifida*; *w*/*w*)	10% *U. pinnatifida* was the most acceptable ↑ Amino acid, fatty acid profile and nutritional value of the product Fucoxanthin was not affected by pasta making and cooking step	[107]
Yoghurt and Quark	0.25–1% dehydrated *U. pinnatifida*	↑ Seaweed flavour with ↓ flavour quality for 0.5% seaweed Alterations in all yoghurt attributes except for buttery odour, and acid and salty flavours Alterations in all quark attributes except yogurt odour, and acid and sweet flavours. Sensory characteristics worsened	[99]
Bread	8% (w:w) *U. pinnatifida*:wheat flour	↑ TPC, ↑ Antioxidant activity in DPPH$^{\bullet}$, ORAC and TEAC	[98]

↑: increased; ↓: decreased; DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical; ORAC: oxygen radical absorbance capacity; PUFA: polyunsaturated fatty acids; TEAC: trolox equivalent antioxidant capacity; TPC: Total phenolic content.

Moreover, the incorporation of *U. pinnatifida* in the patties' formulation did not hamper their lipid oxidation or microbiological counts, and although the content of Na and K were twice as high as the

control samples, the Na/K ratio were still close to 1. Likewise, magnesium and calcium levels were higher in seaweed-fortified samples, corresponding three and six-fold, respectively, to those of the conventional recipe. Interestingly, although a different flavour was pointed out in the sensory analysis, panellists generally described the reformulated patties to be more pleasant and palatable than the control [104]. This reformulation with *U. pinnatifida* also resulted in significant improvements in several parameters on cooked patties, namely in the binding properties and retention values of moisture, ash and particularly fat and fatty acids, the latter parameter being usually the most affected by the cooking process. This means that the incorporation of this seaweed in the patties greatly interfere with the fat and energy content of these food matrices, as well as their fatty acids profile [105]. Identical results were reported on low-salt gel/emulsion meat systems added with 2.5–5% of *U. pinnatifida*, which exhibited better firmness and chewiness due to improvements of the water and fat-biding properties [1]. The incorporation of 5.6% of this species in such systems was also reported to contribute to the increment of the products' phenolic content and antioxidant properties, as well as to improve their mineral profile, increasing the K, Mg, Ca and Mn contents while decreasing the Na content, thus consequently reducing the Na/K ratio from 3.5 in the control samples, to approximately 1. Contrastingly, despite the potential beneficial health effects, increasing the algae was considered a non-satisfactory strategy to achieve healthier lipid meat formulations, since it could affect the food's sensory properties and their lipid content was very low [91]. In turn, Sasaki et al. [106] observed that the addition of 200 mg/kg fucoxanthin extract from *U. pinnatifida* to raw ground chicken breast meat did not prevent the lipid oxidation during their freeze storage period (1 or 6 days). However, it did inhibit TBARS formation of cooked samples stored under the same conditions and improved the products' overall appearance, indicating that fucoxanthin could prevent the oxidation in these products and effectively extend their shelf-life.

Apart from the nutritional stability of the foods, the incorporation of *U. pinnatifida* into foods have also been demonstrated to have great beneficial effects in distinct parameters with impact in the cardiovascular system. According to Moreira et al. [108], the administration of *U. pinnatifida*-fortified restructured pork meat to Wistar rats under a cholesterol-rich diet, not only caused the lowering of the plasma redox index by increasing total and reduced glutathione together with the GR and SOD activity, but also contributed to the decrease of the caspase-3 activity and therefore, hypercholesterolemic-induced apoptotic response of their hepatocytes [102].

Only few studies have focused the use of *U. pinnatifida* in products other than meat. Nevertheless, Prabhasankar et al. [107] reported significantly higher phenolic content and antioxidant activity in the aqueous extracts of uncooked pasta containing different concentrations of *U. pinnatifida* (5–30% *w*/*w*) compared to the controls. Although the cooking process caused a loss in these two parameters, they were still significantly higher on seaweed-added pasta compared to the values observed in the conventional pasta. Importantly, the heat processes involved in pasta preparation and cooking did not damaged fucoxanthin. The seaweed incorporation also contributed to the improvement of the pasta amino acid and fatty acid profiles, as well as the increase of bioactive compounds. The pasta incorporated with 10% seaweed, which demonstrated the highest radical scavenging activities, was also the most well accepted in the sensory analysis. The augmented phenolic content and antioxidant activity were also described on functional breads developed with 8% of *U. pinnatifida* flour, although other seaweeds, such as *H. elongata* exhibited better results [98].

The incorporation of *U. pinnatifida*, up to 15% in cottage cheeses, was reported to cause a dose-dependent increment of their Ca, Fe and Mg. However, the textural quality was best for cheeses containing 9% of seaweed [109]. On the other hand, Nuñez and Picon [99] found that, among the 5 different seaweeds used to incorporate in yogurts and quark cheese, dehydrated *U. pinnatifida* at 0.5% (*w*/*w*) was the formulation that showed the highest seaweed flavour and the lowest flavour quality in both dairies, worsening almost all of their sensory aspects and making this seaweed unattractive for application in such dairies. To overcome this disadvantage, it would be interesting to explore alternative approaches, such as the application of seaweed in flavoured dairies, the application of

algae extracts instead of whole algae or the encapsulation of algae or extracts thereof, in order to assess whether these or other strategies could mask the negative impacts that *U. pinnatifida* has on the sensory aspects of these dairies.

3.4. Ascophyllum Nodosum

Although *A. nodosum* has not been much studied as a functional ingredient for incorporation in foods, some authors have reported promising results in this field (Table 4). For instance, Dierick et al. [110] found that, feeding pigs with 20 g of *A. nodosum*/kg of feed over 21 days caused the levels of iodine in muscle and internal organs to increase 2.7 and 6.8 times, respectively, compared to the pigs fed under a regular diet. This could be a viable approach to increase the daily intake of this mineral which is usually deficient in several European countries [4]. Alternatively, *A. nodosum* extracts applied to low-fat pork liver pâtés (500 mg/kg) was described to increase the protein content by approximately 4% compared to the control samples, without interfering with the chemical composition or microbial characteristics of the samples, throughout 180 days of storage at 4 °C. Furthermore, at the end of the experiment, the oxidative parameters on seaweed-added samples were comparable to those of BHT-added samples, both showing a similar degree of protection against oxidation as well as a significant reduction of volatile compounds after storage [83].

Table 4. Selected studies reporting the effects of the incorporation of *A. nodosum* or isolates as ingredients in different food matrices.

Functional Food	Functional Ingredient	Results	Ref.
Pork	20 g *A. nodosum* /kg feed	↑ I content in piglet's muscles and internal organs	[110]
Pork liver paté	*A. nodosum* extract at 500 mg/kg	↑ Protein content ↑ Redness and yellowness after storage Degree of protection against oxidation comparable to BHT samples ↓ Total volatile compounds	[83]
Milk	*A. nodosum* (100% H_2O and 80% EtOH) extracts (0.25 and 0.5 (*w*/*w*))	↓ TBARS formation ↑ Radical scavenging and ferrous-ion-chelating activities before and after digestion Supplementation on Caco-2 cells did not affect cellular antioxidant status EtOH extracts had greenish colour and overall sensory attributes were worsened	[84]
Yoghurts	*A. nodosum* (100% H_2O and 80% EtOH) extracts (0.25 and 0.5 (*w*/*w*))	No influence on chemical characteristics Yoghurts had antioxidant activity before and after digestion Supplementation on Caco-2 cells did not affect cellular antioxidant status Overall sensory attributes were worsened	[85]
Bread	1–4% *A. nodosum* per 400 g loaf	All samples sensorially accepted ↓ Energy intake after 4 h Glucose and cholesterol blood levels not affected	[111]

↑: increased; ↓: decreased; BHT: butylated hydroxytoluene; EtOH: ethanol; TBARS: thiobarbituric acid reactive substances.

On another perspective, *A. nodosum* extracts have proven to be effective in the inhibition of lipid oxidation and the improvement of antioxidant activity in dairies. Indeed, the incorporation of either aqueous or 80% ethanol extracts (0.25% and 0.5%) of this species in milk significantly decreased the TBARS formation and increased the radical scavenging and ferrous-ion-chelating activities either before or after in vitro digestion. However, this did not affect the cellular antioxidant activity or protect against DNA damage in human colon adenocarcinoma Caco-2 cells, suggesting that the fortification with *A. nodosum* extracts could improve certain milk qualities and shelf-life characteristics, but not

provide significant biological activity. Interestingly, despite fortified-milk with aqueous extract had good acceptability in the sensory analysis, those formulated with 80% ethanol extract was pointed to have a fishy taste and off flavour, thus having low acceptability by the panellists. Nevertheless, this issue could potentially be addressed by using food flavourings or through micro-encapsulation to camouflage the undesirable flavours [84]. A new set of studies on fortified yogurts with the same *A. nodosum* extracts also revealed the increment of the radical scavenging activity before and after in vitro digestion, which was shown not to affect parameters, such as the product's acidity, microbiology or whey separation. However, as previously stated, the biological activity on cellular models was absent and the sensorial analysis was positive for *A. nodosum* aqueous extracts but not for the 80% ethanol extracts [85]. On another approach, Hall et al. [111] reported that the addition of *A. nodosum* (1–4%) in bread significantly reduced the energy intake after a test meal in a single blind cross trial. Moreover, the same was verified after 24 h of seaweed-enriched bread consumption and no differences were observed in blood glucose and cholesterol levels. The authors highlighted, however, the need of a long-term interventional study to establish the real potential of *A. nodosum*-enriched bread energy intake, in addition to the metabolism of glucose and lipids.

3.5. Laminaria *sp.*

Laminaria is one of the most economically important algae genus since it comprises 31 species, being most widely exploited worldwide as raw materials for alginates production [112]. On the other hand, the studies focusing the use of these seaweeds as functional ingredients in foods are quite limited (Table 5). Nevertheless, due to their high content in iodine, some authors have investigated the use of *Laminaria* sp. as animal feed aiming to increase the iodine content in their muscle before slaughter. Indeed, the work carried out by Schmid et al. [113] demonstrated that feeding chars (*Salvelinus* sp.) with *L. digitata*-fortified fish meal (0.8%) over nine months, contributed to an increase of their total iodine content in approximately four times the levels found in the control fishes. Similar observations were described in other species, such as gilthead seabream (*Sparus aurata*) and rainbow trout, which revealed an increased iodine content in their fillets after *L. digitata* was introduced in their meals as well [114,115]. An identical experiment carried out with pigs also revealed that the supplementation of *L. digitata* in the animal's feed over 3 months resulted in an accumulation of 45% more I in muscle tissue and up to 213% in other internal organs compared to the pigs under a normal diet [116]. In a different approach, four group of pigs were assigned to different diets 35 days pre-slaughter in order to test whether alterations of their diets would affect bacterial count, lipid peroxidation and total antioxidant capacity of fresh meat during storage. Interestingly, the meat excised from the group fed with the *Laminaria* sp.-supplemented diet exhibited the best overall results, showing the highest antioxidant activity, the lowest lipid peroxidation and microbial counts, suggesting that feeding the animals with seaweeds might have a significant impact on the quality and shelf-life of their meat [117].

Alternatively, Moroney et al. [118] tested whether the incorporation of different concentrations (0.01%, 0.1% and 0.5% *w*/*w*) of *L. digitata* extract, containing laminaran and fucoidan in chopped pork patties would affect their quality and shelf-life period. The results showed that the surface redness of fortified raw patties, upon 14 days under modified atmosphere packages at 4 °C, decreased compared to the control samples, which led to a slight decrease of their quality parameters. Fortification with the extract at 0.5% caused a notable reduction of lipid oxidation in the cooked samples, but the formulated product was not very well accepted in the sensory analysis. A similar work was later conducted with fresh and cooked pork homogenates and commercial horse heart oxymyoglobin incorporated with *L. digitata*-extracted fucoidan, laminaran and a mixture of both. Although fucoidan showed the strongest radical scavenging activity, cooking and digestion of the samples caused a significant decrease of the antioxidant potential in the samples added with this fibre, which could possibly be attributed to its more acidic nature. Interestingly, despite this, polysaccharide was found to reduce lipid oxidation and also was responsible for catalysing the oxidation of oxymyoglobin. Notably, when the digested samples containing the mixture of laminaran and fucoidan were evaluated for their bioaccessibility in

a Caco-2 cell model, a decrease in radical scavenging activity of 44.2% and 36.6% was observed after 4 and 20 h of incubation, indicating a theoretical uptake of these polysaccharides. These results highlight the potential use of seaweed extracts as functional ingredients in pork with the advantage of possibly improving the human antioxidant defences [42].

Table 5. Selected studies reporting the effects of the incorporation of *Laminaria* sp. or isolates as ingredients in different food matrices.

Functional Food	Functional Ingredient	Results	Ref.
Chars	*Laminaria digitata* (0.8% in fish meal)	↑ 4 times the I content in fish muscle	[113]
Gilthead seabream	*Laminaria digitata* (10% in fish meal)	↑ I content in fish fillets	[114]
Rainbow trout	*Laminaria digitata* (0.65% in fish meal)	↑ I content in fish fillets	[115]
Pork	*Laminaria digitata* (1.16 and 1.86 g/kg feed)	↑ I content in pigs' muscles by 45% and internal organs by 213%	[116]
Pork	*Laminaria digitata* (5.32 kg/t feed)	↑ Antioxidant activity ↓ Lipid oxidations ↓ Microbial counts	[117]
Pork patties	0.01%, 0.1% and 0.5% (*w*/*w*) of 9.3% laminarin and 7.8% fucoidan from *L. digitata*	↑ Lipid antioxidant activity for L/F extract (0.5%) No effect in colour, lipid oxidation, texture or sensorial acceptance when adding L/F extract	[118]
Pork homogenates	3 and 6 mg/mL of laminaran, fucoidan and both from *L. digitata*	L had no antioxidant activity The L/F extract had higher antioxidant activity than F, after cooking and post digestion of minced pork. DPPH$^{\bullet}$ antioxidant activity lower in Caco-2 cell model with L/F extracts Seaweed extracts containing F had higher antioxidant activity of the functional cooked meat products.	[42]
Sausages	1–4% *L. japonica* powder	No changes in moisture, protein, and fat contents ↓ Lightness and redness values ↓ Cooking loss ↑ Emulsion stability, hardness, gumminess, and chewiness 1% seaweed had highest overall acceptability	[119]
Pork/chicken patties	*Laminaria japonica* (replacement od 2.25 g of pork/chicken for an equal amount of seaweed)	↓ Increased in postprandial glucose blood levels; ↓ TC and LDL-C	[120]
Yoghurt	*Laminaria* spp. (0.2% or 0.5% *w*/*w*)	↑ I, Ca, K, Na, Mg, and Fe	[121]

↑: increased; ↓: decreased; DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical; F: fucoidan; L: Laminarin; LDL-C: low density lipoprotein; L/F: Laminarin and Fucoidan; TC: total cholesterol.

In addition to *L. digitata*, other species of this genus have been reported for their positive effects as functional ingredients in foods. This is the case of *Laminaria japonica*, which was incorporated (1–4% *w*/*w*) in breakfast sausages contributing to a significant dose-dependent increase of their ash content, as well as to the improvements on the emulsion stability and textural parameters such as hardness, gumminess and chewiness. Moreover, the seaweed addition lowered samples' pH, lightness, redness and yellowness, and lowered cooking and water losses, particularly in samples added with 4%. Nevertheless, despite the higher benefits that were observed for higher seaweed

powder concentrations, the sensory evaluations determined that the 1% *L. japonica* sausage had the highest overall acceptability [119]. In addition, the incorporation of *L. japonica* in chicken or pork patties was inclusively demonstrated to have positive effects in the post-plasma glucose and lipids profiles in borderline-hyperlipidaemic adults voluntaries. The consumption of fortified-patties with 2.25 g of this species not only lowered the increased post-prandial serum glucose levels compared to the control group, but also the total cholesterol and low density lipoprotein concentrations, while maintaining the same levels of high density lipoprotein [120].

In an alternative to meat products, a new probiotic yogurt containing different concentrations of *Laminaria* sp. was developed with the aim of increasing its iodine content. Indeed, contrarily to the conventional yogurt, the fortified formulation contained not only high levels of I (average of 570 µg I/100 g), but also considerably incremented amounts of Ca, K, Na, Mg, and Fe [121], overall improving their mineral profile.

4. Future Considerations

Previous studies focusing brown seaweed-fortified products have demonstrated that in general, macroalgae can improve the nutritional value of the food products, either by incrementing levels of dietary fibres and/or minerals or their lipidic profiles. Thus, fortified foods with seaweed and/or seaweed extracts come out as possible nutritional alternatives to the original formulations. Moreover, the reported information seems to be solid regarding the fact that the fortification of foods with brown seaweeds and/or their extracts in general have positive impacts on both their oxidative stability and microbial inhibition effects. However, discrepant results are reported regarding the technological properties of the fortified products, namely on the stability of the food's structure. Hence, there is the need to guarantee the compatibility of seaweeds and the overall food matrix, which is not only a result of the seaweed itself, but of the combination of the seaweed with the proper ingredients. In addition, the incorporation of seaweeds in foods frequently comprises their sensorial attributes due to colour changes and the appearance of off-flavours. Nevertheless, some strategies such as fermentation, enzymatic processing or encapsulation of seaweeds or their extracts have already shown interesting effects at cloaking seaweeds' negative sensorial characteristics while maintaining their nutritional properties and stability of bioactive components [122–124]. Nevertheless, further research in this field is necessary to understand if the cost-benefit of the application of such techniques is viable on a larger scale.

Being a crucial factor in nutrition, the bioavailability of relevant nutrients and/or phytochemicals is another critical issue that will require much attention in the following years. This is highly dependent on food components and on individual gastrointestinal conditions. Alginic acid, fucoidans and laminarans are considered dietary fibres, meaning that they may be fermented by colon microflora, therefore surviving the majority of the digestion [125]. The rest of the compounds seem, however, to be absorbed at earlier stages. For instance, in vitro studies suggested that dietary fucoxanthin is metabolized to fucoxanthinol and amarouciaxanthin A [126,127]. In fact, daily administered dietary fucoxanthin (*L. japonica* and *U. pinnatifida* origin) was shown to accumulate as amarouciaxanthin A and fucoxanthinol in several mice tissues [128,129]. In humans, however, the plasma concentrations of fucoxanthin metabolites before and after 1-week dietary interventions with *U. pinnatifida* were shown to be either low (fucoxanthinol) or non-existent (fucoxanthin and amarouciaxanthin A), although a higher subject group would be required in order to confirm these results [130].

As for phlorotannins, to the authors knowledge, only one bioavailability study was made using this particular compound from any of the seaweeds of interest to this review. Recently, in a work developed by Corona et al. (2016), a food-grade phlorotannin extract from *A. nodosum* was submitted to in vitro and in vivo assays, the latter involving the oral administration of a 100 mg capsule with the same extract [57]. The in vitro digestion and fermentation allowed for the identification of 11 compounds including hydroxytrifuhalol A, a C-O-C dimer of phloroglucinol, diphlorethol/difucol and 7-hydroxyeckol, some of which were also detected in the urine and plasma of human participants,

thus confirming their absorption into the blood circulation. Moreover, although brown seaweeds are considered a great source of iodine, there is limited information regarding its bioavailability. Domínguez-Gonzaléz et al. [131] found out that despite the high in vitro bioaccessibility of iodine from *U. pinnatifida* and *S. japonica*, only a small percentage was bioavailable using dialysis membranes and an even lower in a biological system model consisting of two major cell types present in the intestine. Nevertheless, more favourable results were demonstrated when iodine-insufficient women were supplemented with encapsulated *A. nodosum*, since one third of the ingested iodine was found to be bioavailable [132].

Hence, it is clear that, not only is there a lack of information regarding the bioavailability of nutrients/phycochemicals in seaweeds and seaweeds-fortified foods in general, but also the relationship between seaweed-fortified products and their potential functionality remains almost unexploited. Indeed, evidences of biological effects of seaweeds-fortified products were barely proven in cellular models and even more rarely in in vivo trials and hence, must still be made to assure the conformity of the results. According to Plaza et al. [133], the principal guideline to follow in the design of a new functional food is to increase as much as possible the benefit/risk ratio, by increasing the benefit to the maximum and reducing the risk to the minimum, considering toxicity studies, for example. Increasing the benefit implies looking for a physiological wide effect, assuring the existing bioavailability and that the mentioned bioavailability is going to be kept along all the useful life of the food. Therefore, since the in vivo biological activity of phycochemicals depends on their bioavailability, in the future, it would be interesting to further access how important properties claimed for brown algae can transpose to human beings through seaweed fortified products. In order to reduce the risk, it is necessary to carry out toxicity studies, to use the functional ingredient in minimal effective doses and to use as functional ingredients, the products naturally found in foods or natural sources.

5. Conclusions

In conclusion, seaweeds are very valuable food sources with reportedly high nutritional value and high in bioactive compounds. In this regard, brown macroalgae are particularly known to accumulate specific metabolites, such as the polysaccharides alginic acids, laminarans and fucoidans, the phenolic compounds phlorotannins, the carotenoid fucoxanthin and exceptionally high levels of iodine with simultaneous low Na/K ratios, which overall confer them great potential to be used as functional ingredients. Thus far, most of the recent studies' main objectives focusing on testing the incorporation of macroalgae in foods, namely those considered as safe for consumption in Europe i.e., *F. vesiculosus, F. serratus, H. elongata*, *U. pinnatifida*, *A. nodosum*, *L. digitata, L. saccharina, L. japonica* and *A. esculenta*, were the increment of the product's shelf-life and the sensory properties culminating in the potential commercialization. From an economic point of view, this is rather important, but in terms of the benefits they could bring to the consumer, there is a lot of work to be done. Therefore, in order to effectively exploit this very promising raw material, the focus should be on their bioavailability, especially in humans.

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CHAPTER III

Experimental Work. **Dichotomy between the nutritional and biological improvement of pastas enriched with *Fucus vesiculosus* and the effects of the cooking process**

1. Objectives

Taken all the current knowledge gathered by Afonso et. al (2019)[1] and considering the increasing interest on the consumption of seaweed and seaweed derived products this work focused on the analysis of brown seaweed, *F. vesiculosus*, enriched pastas and had four main goals, a) to explore the nutritional composition, namely for fibres, protein, ash and minerals, and phytochemical content, regarding phlorotannins and pigments; b) to evaluate the biological properties of the product in terms of antioxidant activity and capacity to inhibit three key enzymes of the GI metabolism; c) to understand the impact of the cooking process of pastas on the aforementioned characteristics; and d) to comprehend the bioaccessibility and bioavailability of certain compounds upon simulated in vitro GI digestion of cooked samples.

2. Material and Methods

2.1. Pasta

Pasta samples from the variety *Tagliatelle*, namely the controls and those fortified with *F. vesiculosus* (1% and 5.5% dry weight), were manufactured by the team of researchers belonging to the Centre for Natural Resources Environment and Society (CERNAS) of the Agrarian School of the Institute Polytechnic of Coimbra, under the project HEPA (Healthier Eating of Pasta with Algae). The preparation of pasta with seaweed followed the common process for pasta production: mixture of semolina flour from wheat with seaweed flour, kneading, extrusion, moulding and cutting, and drying. Control samples have 100% semolina flour.

2.2. Reagents

All reagents were of analytical grade or of the highest available purity. Acetone, ethanol, methanol, *n*-hexane, ethyl acetate, acetonitrile HPLC grade, dimethylsulfoxide (DMSO), hydrochloric acid, glacial acetic acid, sodium chloride, sodium hydroxide, potassium hydroxide, sodium and potassium tartarate, tris-HCl, N-(1-naphthyl)-ethylenediamine dihydrochloride and starch were acquired from Fisher (Pittsburgh, PA, USA). The enzymes α-glucosidase from Saccharomyces cerevisiae (EC No.–3.2.1.20), α-amylase from porcine pancreas (EC No.–3.2.1.1) and lipase from porcine pancreas (EC No.–

3.1.1.3) together with 2,4-dimethoxybenzaldehyde (DMBA), 4-nitrophenyl α-D-glucopyranoside (PNPG), piperazine-N,N'-bis(2-ethanesulfonicacid) disodium salt (PIPES), 4-nitrophenyl butyrate (PNPB), tetramethylammonium hydroxide (TMAH), β-NADH, phenazine methosulfate (PMS) and formic acid were purchased from Sigma (St. Louis, MO, USA). Sodium di-hydrogen phosphate, phosphoric acid and potassium di-hydrogen phosphate were acquired from Panreac (Barcelona, Spain). Dinitrosalycilic acid, nitroblue tetrazolium chloride (NBT), sodium nitroprusside (SNP), sulphanilamide and acarbose were purchased from Acros Organics (Hampton, NH, USA), calcium chloride from ChemLab (Eernegem, Belgium) and orlistat from AlfaAesar (Ward Hill, MA, USA). Ultrapure water was obtained in an 18.2 MΩ cm, Milli-Q system.

2.3. Methods

2.3.1. Sampling

2.3.1.1. Raw and Cooked Pasta

Raw pasta samples, control (uCONT), 1% *F. vesiculosus* (uFUC1%) and 5.5% *F. vesiculosus* (uFUC5.5%) were grounded and stored in plastic samplers in the dark until further use.

The cooking procedure of pasta was adapted from Prabhasankar et al (2009a)[2] and the advised preparation method for *Tagliatelle* pasta. Briefly, 10 g of each pasta sample were added to 100 mL of ultrapure boiling water (≈100ºC) for 7 minutes, after which the material was withdrawn and the volume of collected gruel (drained liquid) was measured. After cooking, samples were stored at -80 °C for 24 hours and then lyophilized for 3 days. All samples, control (cCONT), 1% *F. vesiculosus* (cFUC1%) and 5.5% *F. vesiculosus* (cFUC5.5%) were then grounded, weighed and stored at room temperature and in the dark on plastic samplers until further use.

2.3.1.2. Preparation of extracts

For each sample, cooked and uncooked, 1 g of dried pasta powder was loaded into glass flasks covered with aluminium foil and dispersed in 99 mL of 70% acetone solution mixed with 1 mL of acetic acid. This mixture was incubated for 16 hours at room temperature under constant agitation. The mixture was then centrifuged at approximately 400 rpm for 15

minutes at 4 °C and the supernatant was filtered through a G4 glass filter. Afterwards the extract was concentrated in a rotary evaporator to about 2.5 mL in 70% acetone solution and stored at −20 °C until further analysis (2,4-dimethoxybenzaldehyde (DMBA) colorimetric assay for phlorotannin quantification). The same process was repeated with a slight deviation at the end in which instead of concentrating to 2.5 mL in 70% acetone solution, the solvent used was DMSO and was used for antioxidant assays (ABTS discolouring assay and SO and NO radical scavenging assays).

2.3.2. Total dietary fibre content

Total dietary fibre contents were estimated according to the enzymatic gravimetric method AOAC 991.43, using the Total Dietary Fiber Assay kit from Megazyme (Bray, UK). Briefly, 1 g of each sample was weighed into tall-form beakers and 50 mL of phosphate buffer 0.08M (1.40 g of Na phosphate anhydrate and 9.68 g of Na phosphate monobasic monohydrate, in 1 L of distilled water at pH 6.0±0.1) was added. Next, 50 µL of heat-stable amylase were dispersed in the solution and the beakers were incubated for 15 minutes at approximately 100°C, with agitation every 5 minutes. After cooling the solutions to room temperature, the pH was adjusted to 7.5±0.1 and 100 µL of protease solution were added. All beakers were then incubated for 30 minutes under constant agitation at 60°C and then cooled again to room temperature. pH was later adjusted to 4.5±0.2 and a new aliquot of 200 µL of aminoglucosidase was added. The mixture was again incubated under constant agitation for 30 minutes at 60°C and afterwards mixed with 280 mL of pre-heated EtOH 95% at 60°C. After 60 minutes at room temperature all fibres were vacuum filtered using previously prepared crucibles with celite beds and the beakers were washed with three 20 mL portions of EtOH 78%, two 10 mL portions of EtOH 95% and two 10 mL portions of acetone. All crucibles were dried overnight inside an air oven at 105°C and the obtained residue was homogenised with a mortar and pestle. A small amount was analysed for protein content and the rest was incinerated in a muffle furnace for 5 hours at 525°C. Total fibre content was then determined using the following equation:

$$\frac{m_{residue} - m_{ashes} - m_{protein}}{m_{sample(db)}} \times 100$$

where $m_{residue}$ was the mass in grams of the fibres on the crucible, m_{ashes} was the mass in grams of the total incinerated residue, $m_{protein}$ was the mass in grams of the total protein content and $m_{sample(db)}$ was the mass of weighed sample on a dry basis.

2.3.3. Ash and protein content

Ash content was determined by incineration of approximately 1 g of samples in a muffle furnace at 550 ◦C for 6 h and gravimetric quantification. Total protein content was estimated by determination of elemental nitrogen content by thermal conductivity using a TruSpec 630-200-200 CNHS analyser from LECO (St. Joseph, MI, USA), multiplied by the conversion factor of 5.83, which is specific for products based mainly on wheat, according to the Food and Agriculture Organization of the United Nations (FAO).[3]

2.3.4. Mineral content

For iodine, approximately 400 mg of pasta samples were weighed into high pressure Teflon vessels and digested with 10 mL of TMAH 25% in H_2O in a microwave system with an Ethos MicroSYNTH Microwave Labstation (Milestone Inc.). The microwave assisted extraction (MAE) occurred in two steps. In the first step, a ramp time of 5 min was used to increase the temperature from 20 to 175°C. A second step at 175 ◦C was applied for 10 min. After digestion, all solutions were transferred to volumetric flask and their volume was adjusted to 50 mL with ultrapure water. The remaining elements were digested with a similar process. Briefly, 400 mg of pasta samples were mixed with 4 mL of nitric acid in the same microwave system, at the aforementioned conditions. After the first digestion sequence, 500 µL of H_2O_2 were added and the process was repeated. Then, all samples were transferred to 50 mL volumetric flasks and their volume was adjusted with ultrapure water. All elements (K, Ca, Mg, Fe, Zn, Mn, Cu and I) were conveniently diluted and then quantified in a 7700 inductively coupled plasma mass spectrometer (ICP-MS) from Agilent Technologies, equipped with nickel sampler and skimmer cones and a collision/reaction cell.

2.3.5. Pigments

The extraction of pigments from uncooked and cooked samples was conducted with acetone 100%, in the proportion of 2:10 (weight: volume), during 1 minute in a Ultra-turrax

(Miccra KT). During this process all samples were covered in aluminum foil. Afterwards all extracts were centrifuged at approximately 600 rpm for 5 minutes at 4ºC. The supernatant was concentrated in a rotary evaporator to about 1.5 mL in 100% acetone solution, in a dark room. The resulting volume was then filtered through 0,45 μm (WhatmanTM) filters and 500 μL were kept in vials while the remaining volume was analyzed with UV spectrometry. The absorbance reading of pigments from the extract was conducted in a spectrophotometer (Shimadzu, UVmini-1240). Chlorophyll and carotenoid contents were determined through the equations described by Lichtenthaler (1987)[4] for acetone 100%, as follows:

$$C_a = 11{,}24\ A_{661{,}6} - 2{,}04\ A_{644{,}8};$$

$$C_b = 20{,}13\ A_{644{,}8} - 4{,}19\ A_{661{,}6};$$

$$C_{a+b} = 7{,}05\ A_{661{,}6} + 18{,}09\ A_{644{,}8};$$

$$\mathrm{C_c} = \frac{1000\mathrm{A_{470}} - 1{,}90\mathrm{C_a} - 63{,}14\mathrm{C_b}}{214}$$

where C_a was the concentration of chlorophyll *a*, C_b was the concentration of chlorophyll *b*, C_{a+b} was the concentration of chlorophyll *a* and *b* together, and C_c was the concentration of carotenoids, all expressed in mg per g of sample.

2.3.5.1. Total content of phlorotannins (DMBA assay)

The quantification of total phlorotannins methodology was carried out according to the 2,4-dimethoxybenzaldehyde (DMBA) colorimetric method adapted from Catarino et al. (2019)[5]. The working reagent was prepared, prior to use, by mixing equal volumes of the stock solutions of DMBA (2% m/v) and hydrochloric acid (6% v/v) prepared in glacial acetic acid. Afterwards, 250 μL of this solution was added to 50 μL of each acetone 70% extract in a 96-wells plate and the reaction was incubated in the dark, at room temperature. After 60 min, the absorbance was read at 515 nm on a ELX800 microplate reader and the phlorotannin content was determined by using a regression equation of the phloroglucinol linear calibration curve (0.06 - 0.1 mg/mL). The results were expressed as milligrams of phloroglucinol equivalents (PGE) per 100 grams of pasta.

2.3.6. Antioxidant activity

2.3.6.1. $ABTS^{\bullet+}$ discoloration assay

The $ABTS^{\bullet+}$ (2,2′-azino-bis 3-ethyl benzothiazoline-6-sulfuric acid) discoloration assay was performed according to Ferreira et al. (2019)[6] with slight modifications. The ABTS cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS aqueous solution with 2.42 mM potassium persulfate at room temperature in dark for 16 h. Then, the $ABTS^{\bullet+}$ solution was diluted with deionized water to an absorbance of 0,750-0,800 at 734 nm. 50µL of samples or standards in DMSO were added to 250 µL $ABTS^{\bullet+}$ solution in a 96-wells plate which was incubated for 20 minutes in the dark. Afterwards, the decrease of absorbance was recorded at 734 nm on a ELX800 microplate reader. Ascorbic acid dissolved in DMSO was used as positive control and the antioxidant activity was expressed as grams of ascorbic acid equivalents (AAE) per 100 grams of extract.

2.3.6.2. $SO^{\bullet}$ scavenging assay

The $SO^{\bullet}$ radicals were generated according to a described procedure by Valentão et al. (2001)[7] with slight modifications. In brief, on a 96-wells plate were added 75 µl of 0.2 mM nitroblue tetrazolium chloride (NBT) (3.3 mg of NBT in 20 mL of 20 mM phosphate buffer (2.72 g of sodium hidrogeno-phosphate in 1 L of deionized water at pH 7.4)), 0.3 mM 100 µl of β-NADH (2 mg of NADH in 10 mL of deionized water), 75 µl of pasta extracts in DMSO or standards and 50 µl of 0.15 µM of phenazine methosulfate (PMS) (3.06 mg of PMS in 1 mL of deionized water diluted ≈60x). All solutions were prepared daily. The reaction started with the addition of PMS and was conducted at room temperature for 5 minutes. Afterwards, the absorbance was recorded at 560 nm on a ELX800 microplate reader. Gallic acid dissolved in DMSO was used as a positive control and the antioxidant activity was expressed as grams of gallic acid equivalents (GAE) per 100 grams of extract.

2.3.6.3. $NO^{\bullet}$ scavenging assay

The production of nitric oxide (NO) was measured using a colorimetric reaction with the Griess reagent according to Francisco et al. (2011)[8], with slight modification. In brief, 100 µL of samples in DMSO or standards were added to a 96-wells plate, followed by 100 µL of 1.66 mM sodium nitroprusside (SNP) (5 mg of SNP dehydrate in 10 mL of 100 mM

potassium buffer (13.6 g of potassium phosphate in 1 L of deionized water at pH 7.4)) and an incubation time of 15 minutes under fluorescent light. Afterwards 100 µL of Griess reagent (0.1% of N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% (w/v) of sulphanilamide containing 2.5% of phosphoric acid) was added and the plate was stored in a dark room. The absorbance was recorded at 562 nm on a ELX800 microplate reader. Ascorbic acid dissolved in DMSO was used as positive control and the antioxidant activity was expressed as grams of ascorbic acid equivalents (AAE) per 100 grams of extract.

2.3.7. Enzymatic inhibition assays

2.3.7.1. α-amilase inhibition assay

Inhibition of α-amylase activity was measured according to Pereira et al. (2018)[9], with slight modifications. Briefly, DMSO extracts and seven different acarbose concentrations (0.1 – 0.001 mM) were dissolved in 20 mM sodium phosphate buffer with 6 mM of sodium chloride (1.78 g of hydrogen-phosphate and 175 mg of NaCl, in 500 mL of deionized water at pH 6.9). 200 µL of samples and controls were added to 400 µL of a 0.8% (w/v) starch solution in the same phosphate buffer (80 mg of starch in 5 mL of buffer at 100ºC and subsequently diluted with 5 mL of buffer after cooling down) and the mixture was incubated for 5 minutes at 37 ºC. The reaction was then started with the addition of 200 µL of α-amylase solution and after 5 minutes of incubation, 200 µL of the reaction mixture were collected and immediately mixed with 600 µL of DNS reagent (5 g of 3,5-dinitrosalicylic acid in boiling water, subsequently mixed with 150 g of potassium and sodium tartrate tetrahydrate and 100 mL of NaOH 2M, after cooling down, in 500 mL of distilled water) to stop the reaction. A second aliquot of 200 µL was further collected 15 minutes later and mixed with DNS reagent as well. All samples were boiled for 10 minutes and once they had cooled, 250 µL were transferred to the wells in a 96-well microplate for absorbance reading at 450 nm. Blank readings (no enzyme) were then subtracted from each well and the inhibitory effects towards α-amylase activity was calculated as follows:

$$\%\ \text{inhibition} = \frac{\Delta \text{Abs}_c - \Delta \text{Abs}_e}{\Delta \text{Abs}_c} \text{ x } 100$$

where ΔAbs_c was the variation in the absorbance of the control and ΔAbs_e was the variation in the absorbance of the extract. Acarbose was used as a positive control of inhibition.

2.3.7.2. α-glucosidase inhibition assay

Inhibition of α-glucosidase was measured according to the method previously described by Neto et al. (2018)[10]. In short, 50 µL of different DMSO extract concentrations (0-2 mg/mL, in 50 mM phosphate buffer pH 6.8) were mixed with 50 µL of 6 mM 4-nitrophenyl α-D-glucopyranoside (pNPG), dissolved in deionized water. The reaction was started with the addition of 100 µL of α-glucosidase solution and the absorbance was monitored at 405 nm every 60 s for 20 min at 37 ºC. Acarbose was used as positive control of inhibition.

2.3.7.3. Pancreatic lipase inhibition assay

Inhibition of lipase activity was measured following the procedure described by Pereira et al. (2018)[9]. The reaction mixture was prepared in a microtube by mixing 55 µL of five different concentrations of DMSO extract (0-2 mg/mL) dissolved in Tris buffer 100 mM (pH 7.0) with 467.5 µL of Tris-HCl (100 mM, pH 7.0, containing 5 mM of $CaCl_2$) and 16.5 µL of enzyme. The reaction was started by adding 11 µL of 20 mM 4-nitrophenyl butyrate diluted in DMSO. Final DMSO concentration in the reaction mixture did not exceed 2%. The reaction mixture was then quickly transferred to a 96-well plate and incubated for 35 min at 37 ºC while the absorbance was being measured every 60 s at 410 nm. Orlistat was used as a positive control of inhibition.

2.3.8. *In vitro* digestion procedures

The evaluation of the bioaccessibility and bioavailability of minerals was carried out using an *in vitro* gastrointestinal digestion system, as previously described by González et al (2017)[11]. Both protocols required two sequential phases, a gastric and an intestinal digestion of the cooked pasta samples.

2.3.8.1. Gastric, intestinal and PIPES solutions

All simulated gastric and intestinal solutions used both in bioaccessibility and bioavailability assays were prepared previously or during the assay whenever required. Pepsin solution 0.16 g/mL HCl 0.1 was prepared by dissolving 0.8 g of pepsin in 5 mL of 0.1 M HCl. Since pepsin activity can decrease over time, a solution was freshly prepared before each experiment. The intestinal solution involved the solubilization of 0.4 g of pancreatin and 2.5 g of bile salts in 100 mL of 0.1 M sodium bicarbonate. Both solutions were taken to the ultrasound until total or mostly total dissolution was achieved. Exclusive to the bioavailability assay, a solution of piperazine-N,N'-bis(2-ethanesulfonicacid) disodium salt (PIPES) 0.15 N was prepared by solubilization of 5.19 g of PIPES in 200 mL of ultra-pure water.

2.3.8.2. Bioaccessibility assay

Approximately 0.5 g of cooked pasta samples were weighed into erlenmeyer flasks and dispersed in 20 mL of ultrapure water. Following the adjustment of pH to 2.00±0.1, 0.15 g of a previously prepared pepsin solution 0.16 g/mL HCl 0.1 M was added to each recipient (approximately 140 µL depending on its density). The erlenmeyer flasks were then covered and the solution was incubated at 37°C with orbital-horizontal shaking at 150 rpm for 120 minutes. After the incubation time all solutions were cooled in an ice bath to stop enzymatic activity, pH was adjusted to 7.00±0.1 and 5 mL of the intestinal solution (pancreatin (0.4% m/v) and bile salts (2.5% m/v) in 0.1 M $NaHCO_3$) were added. The erlenmeyers were covered and a new incubation step with orbital-horizontal shaking and in the same conditions took place. Afterwards the solutions were cooled on an ice bath and the content was then transferred to falcon tubes. In order to separate the soluble (bioaccessible) phase and the residue, the digest was centrifuged at 6000 rpm for 30 minutes at 4°C. The supernatant was carefully removed, filtered through 0.45 µm (WhatmanTM) filters and stored at -20ºC before analysis. A blank, consisting of all the reagents excluding samples, was also obtained in parallel to control possible contamination. The bioaccessible fraction of our samples was determined through the following equation:

$$\frac{[\text{mineral}]_{\text{BAc}}}{[\text{mineral}]_{\text{sample}}} \text{x} 100$$

where BAc was the bioaccessible mineral content after *in vitro* digestion.

2.3.8.3. Bioavailability assay

The bioavailability procedure is similar to the bioaccessibility one with a small deviation. During the first incubation with pepsin at 37°C with orbital-horizontal shaking the dialysis membranes were cut to approximately above the height of the erlenmeyers and boiled in water for 20 minutes. Following the boiling time, all membranes were thoroughly washed with deionized water and with a tweezers the bottom was tied up in order to insert 15 mL of PIPES inside. The membrane was then placed inside the erlenmeyer and the digestion proceeded as described before, with the addition of 5 mL of the intestinal solution after pH adjustment to 7.00±0.1. After the second incubation, the content of the membrane was transferred to falcon tubes and stored at -20ºC before analysis. A blank, consisting of all the reagents excluding samples, was also obtained in parallel to control possible contamination. The bioavailable fraction of cooked samples was determined through the following equation:

$$\frac{[\text{mineral}]_{\text{BAv}}}{[\text{mineral}]_{\text{sample}}} \text{x} 100$$

where BAv was the bioavailable mineral content after *in vitro* digestion and dialysis.

2.4. Statistical analysis

Values were presented as mean ± standard deviation for all variables. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons post hoc test. Results were considered significantly different when $p < 0.05$. GraphPad Prism (version 8.0) was the software used.

3. Results and discussion

The impact of *F. vesiculosus* incorporation into raw pasta was accessed in terms of nutritional (total fibre content, protein, ash and minerals) and phytochemical (pigments and phlorotannins) contents, as well as regarding the biological potential, namely antioxidant activity and inhibitory effects towards key digestive enzymes. The same study was performed with cooked samples, in order to comprehend the effects of cooking on the target chemical and biological parameters. Lastly, cooked samples were also subjected to *in vitro* digestion to access their mineral bioaccessibility and bioavailability. The gathered results and their discussion are displayed in following subtopics.

3.1. Total fibre content (TFC)

TFC, comprising soluble and insoluble fibres, of uncooked and cooked pasta samples is represented in **Figure 1**. For increased levels of algae incorporation in the fortified pasta, the amount of total fibres was also improved, with uCONT, uFUC1% and uFUC5.5% reaching 3.30 ± 0.75, 4.94 ± 0.80 and 6.40 ± 0.41 g/100 g pasta, up to a 48% increase concerning the latter. However, when comparing fortified samples with controls only uCONT and uFUC5.5% depicted statistical differences. Upon cooking the pasta samples, TFC tendency was to increase (cCONT = 4.80 ± 0.10, cFUC1% = 5.42 ± 0.15 and cFUC5.5% = 6.87 ± 0.01 g/100 g pasta) when comparing to uncooked ones, however such differences were not statistically relevant. Nevertheless, this strongly suggested that the incorporation of *F. vesiculosus* into pasta increased its TFC, which was also not impaired by this type of heat processing. In fact, since *F. vesiculosus* is very well known for its high and rich content of both soluble and insoluble fibres such as alginic acids, fucoidans and laminarans (up to 59%, 25.7% and 7% DW, respectively), even with the small amount of seaweed found on the target samples, these results come out as expected.[12] Likewise, Prabhasankar et al. (2009b)[13] found out that fortification of pastas with 2.5% DW of *Sargassum marginatum* caused an increment of about four times on the fibre content. On a similar carbohydrate-based product (17.07% *H. elongata* enriched breadsticks), Cox and Abu-Ghannam (2013)[14] reported a TFC of 7.95%, representing an increase of 43.65% when compared with controls. In addition, in the work of Cofrades et al. (2008)[15], the authors found that the incorporation of 5% *H. elongata* to meat systems contributed to a TFC of 2.52%, further confirming that brown seaweed incorporation into food matrixes confers

higher TFC. In order to confirm which fibres are present, further studies should be made. The supposedly increase of TFC upon cooking was unexpected, however, it should be highlighted that the fibre quantification assay required an *in vitro* digestion process with enzymes in order to produce the fibres. During this process, cooking samples seemed easier to digest than uncooked ones, hence this small deviation could be accepted.

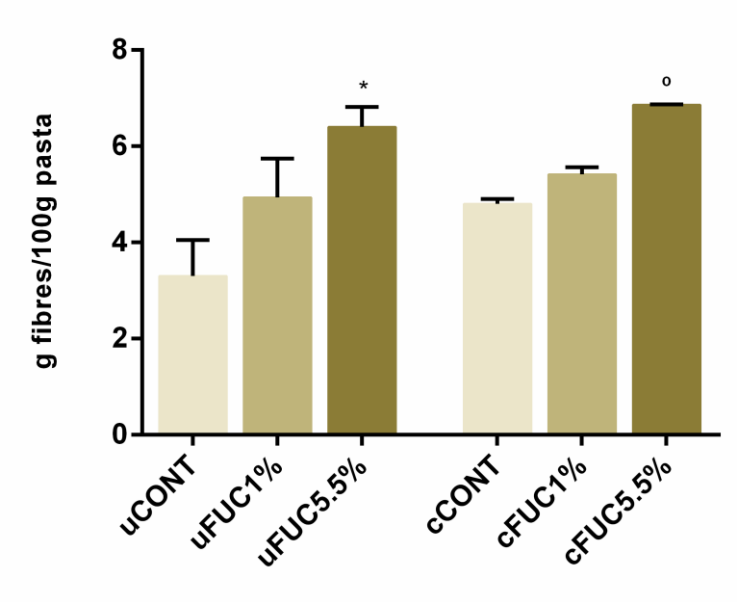

Figure 1. Effects of *F. vesiculosus* fortification in pasta and the impact of the cooking process on total fibre content levels. Values are expressed as mean ± standard deviation (*p < 0.05 *vs* uCONT; °p < 0.05 *vs* cCONT).

3.2. Protein content

Protein levels of *F. vesiculosus* enriched pasta and controls, before and after the cooking process, are shown in **Figure 2**. The increment of seaweed proportion on this product did not seem to affect its protein content since all three samples had closely the same amount of protein on a dry basis percentage (uCONT = 13.29 ± 0.01, uFUC1% = 13.42 ± 0.12 and uFUC5.5% = 13.41 ± 0.06 g/100 g pasta) and there was no significance between them. This strongly suggested that protein content of *F. vesiculosus* powder was the same as the pasta itself, so, by replacing one ingredient with the other this parameter did not shift. According to PortFIR[16], a national data base on food composition and nutritional profile from Instituto Nacional de Saúde Doutor Ricardo Jorge, a generic pasta composition comprehends on average 12.1 grams of protein per 100 grams of pasta which further

confirmed our results. It was demonstrated by Prabhasankar et al. (2009)[2] that a significant improvement of amino acid scores in 10% *U. pinnatifida* fortified pasta was achieved, meaning that, although brown macroalgae are relatively rich in amino acids, the amount of seaweed used on these target samples might not be enough to induce such increase in protein content. Upon cooking, each set of samples suffered a significant decline on their protein content (cCONT = 11.87 ± 0.13, cFUC1% = 11.96 ± 0.11 and cFUC5.5% = 12.39 ± 0.04 g/100 g pasta) comparatively to their uncooked counterparts. This suggested that protein content seems to be impaired by those conditions, which was somehow expected. Considering that protein denaturation often occurs at high temperatures, one can predict that this may trigger a loss of protein to the gruel possibly due to acquiring a more soluble conformation.[17] Additionally, due to the noted significant differences, samples enriched in 5.5% *F. vesiculosus* appeared to be better at holding these macromolecules than the remaining samples, hinting that this seaweed in foods might be important in maintaining protein profile after this type of heat processing. Surprisingly, according to Fernández-Martín et al. (2009)[18] the same was observed in pork meat batter with 3.4% *H. elongata* powder, and explained due to the presence of alginates which could prevent thermal denaturation of a considerable protein fraction. As highlighted on the previous topic, TFC of uFUC5.5% was increased more than other samples and was even maintained upon cooking. Given that this sample was also the one presenting significantly higher protein content upon cooking, one can hypothesise that it might also be related to the higher fibre content, more specifically due to alginates. It should be noted, however, that this is only an assumption which needs further confirmation.

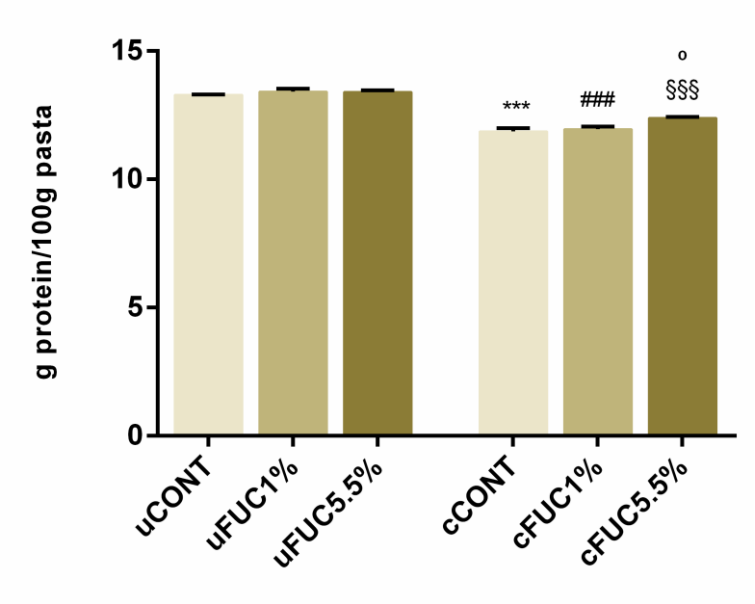

Figure 2. Effects of *F. vesiculosus* fortification in pasta and the impact of the cooking process on protein content. Values are expressed as mean ± standard deviation (***p < 0.001 *vs* uCONT; ###p < 0.001 *vs* uFUC1%; §§§p < 0.001 *vs* uFUC5.5%; °p < 0.05 *vs* cCONT).

3.3. Ash and mineral content

Ash content of *F. vesiculosus* enriched pasta and controls, before and upon cooking is shown in **Figure 3**. With growing seaweed concentration, the amount of ashes was significantly enhanced when comparing to control, although the difference was greater between uCONT and uFUC5.5%, presenting an increase of approximately 60%. Amongst fortified groups, uFUC1% and uFUC5.5%, there was also a significant increase in ash content, hence indicating that mineral content had been improved on the target samples. It was also observed that cooking the pasta resulted in considerable decreases when comparing each set of samples, uncooked and cooked. Considering this, one may assume that minerals profile was not only changed due to macroalgae fortification, as well as to cooking procedure. This was herein confirmed for iodine (**Figure 4**).

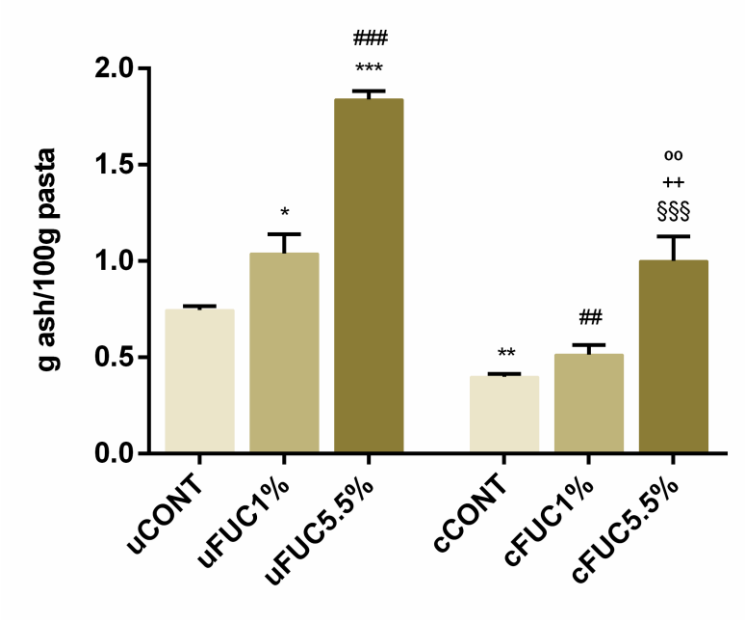

Figure 3. Effects of *F. vesiculosus* fortification in pasta and the impact of the cooking process on ash content. Values are expressed as mean ± standard deviation (*p < 0.05 *vs* uCONT; **p < 0.05 *vs* uCONT; ***p < 0.001 *vs* uCONT; ##p < 0.01 *vs* uFUC1%; ###p < 0.01 *vs* uFUC1%; §§§p < 0.001 *vs* uFUC5.5%; ^{oo}p < 0.01 *vs* cCONT; $^{++}$p < 0.01 *vs* cFUC1%).

In fact, iodine concentration rose with each increasing content of seaweed in pasta (uCONT = 0.22 ± 0.19, uFUC1% = 0.75 ± 0.17 and uFUC5.5% = 2.71 mg/100 g pasta) although between uCONT and uFUC1% that increment was not significant. Following the cooking process, iodine content decreased to concentrations of 0.18 ± 0.12, 0.66 and 1.97 ± 0,05 mg/100 g pasta, for cCONT, cFUC1% and cFUC5.5%, respectively. Although *F. vesiculosus* is particularly rich on iodine, therefore confirming the superior levels upon seaweed fortification, such results were not expected for control samples, since, on a nutritional basis, wheat flour accounts for low values of this mineral.[19] In fact, according to Haldimann et al. (2005) the average iodine content for wheat flour is 0.0035 mg/100 g DW, although, because it is a cereal and therefore retains minerals from the soil, a rich in iodine soil may explain the superior content within this control samples.[20]

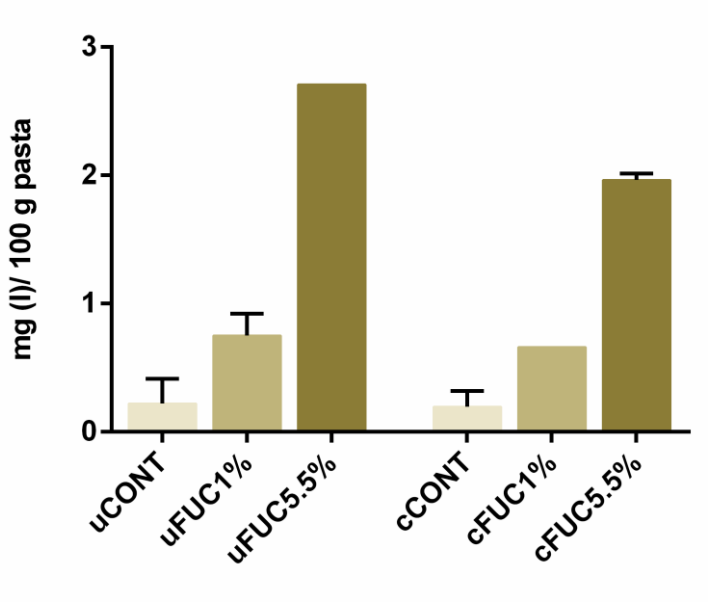

Figure 4. Effects of *F. vesiculosus* fortification in pasta and the impact of the cooking process on iodine levels. Values are expressed as mean ± standard deviation.

Due to technical/equipment difficulties, the monitoring of other minerals beyond iodine in all samples was not possible to execute, hence precluding a detailed comparison of the impact of the algae fortification and cooking process on minerals. In spite of this impediment, the mineral profile of cooked pastas was evaluated, namely for potassium, calcium, magnesium, iron, zinc, manganese and copper (**Figure 5 A** to **G**, respectively). Levels of the macro elements (K, Ca and Mg) reached values of 75.7 ± 13.6, 35.2 ± 13.5 and 29.2 ± 5.0 mg/ 100 g of pasta for cCONT, 87.9 ± 25.0, 56.5 ± 28.6 and 32.8 ± 11.6 mg/ 100 g of pasta for cFUC1%, and 174.9 ± 3.6, 138.2 ± 16.0 and 69.6 ± 3.1 mg/ 100 g of pasta for cFUC5.5%, respectively. Some of these results were verified to be statistically different such as between controls and samples fortified with 5.5% seaweed for all three macro elements and between both fortified samples for K and Mg. In sum, the concentration of these elements followed the order K < Ca < Mg for all our samples, suggesting that *F. vesiculosus* contributed to the higher proportion of these minerals; however, it should be noted that the cooking process might also influence their depletion from the food matrix. Concerning the micro elements (Fe, Zn, Mn and Cu), and also considering the results previously discussed for I, the observed concentrations followed the order Fe < Zn < Mn < Cu < I for cCONT with values of 1.31 ± 0.11 (Fe), 0.77 ± 0.27 (Zn), 0.49 ± 0.16 (Mn), 0.22 ± 0.04 (Cu) and 0.18 ± 0.12 (I) mg/ 100 g pasta. However, this order shifted in *F. vesiculosus* enriched

samples to Zn < Fe < I < Mn < Cu with values of 1.85 (Zn), 1.77 ± 0.23 (Fe), 0.66 (I), 0.56 ± 0.27 (Mn) and 0.23 ± 0.03 (Cu) for cFUC1%, and Fe < Zn < I < Mn < Cu with values of 5.46 ± 0.35 (Fe), 1.85 (Zn), 1.97 ± 0.05 (I), 1.70 ± 0.07 (Mn) for cFUC5.5%. Moreover, statistically different results were observed in Fe and Mn both between controls and 5.5% seaweed fortified samples and also between both fortified samples. Overall mineral content increased in the presence of *F. vesiculosus* in pastas; however, due to the lack of comparison between uncooked and cooked samples, whether it was maintained upon cooking or impaired by is something that should be further investigated.

The recommended daily allowance (RDA) for minerals is essential to understand possible benefits and risks resulting from the consumption of food products and was established for nutrition labelling of foodstuffs according to the Commission Directive 2008/100/EC.[21] In this regard, the contribution of the target samples to RDAs taking into consideration their mineral content present in 100 grams of pasta are displayed in **Table 1**. Overall, the results herein gathered allowed to conclude that all fortified samples were a great resource of minerals, contributing to higher RDAs when comparing to controls, with special emphasis on cFUC5.5% due to its higher content of seaweed. This sample accounted to approximately more than four times the RDA of Ca, Fe and Mn regarding cCONT, and even ten times more the RDA of I, up to an amount of 1310%. Even though this value reached extremely high proportions, also considering the controls which already met the RDA, one must consider the effects of digestion and absorption, later discussed.

Table 1. Contribution of cooked pastas to recommended daily allowances of the selected minerals (%).

	K	Ca	Mg	Fe	Zn	Mn	Cu	I
cCONT	3.8	4.4	7.8	9.3	7.7	24.5	21.5	130.3
cFUC1%	4.4	7.1	8.8	12.7	18.5	28.2	22.7	441.3
cFUC5.5%	8.7	17.3	18.6	39	18.5	85.2	28.8	1310

Values are expressed as %, considering a portion of 100 g of pasta, and the RDAs according to the Commission Directive 2008/100/EC

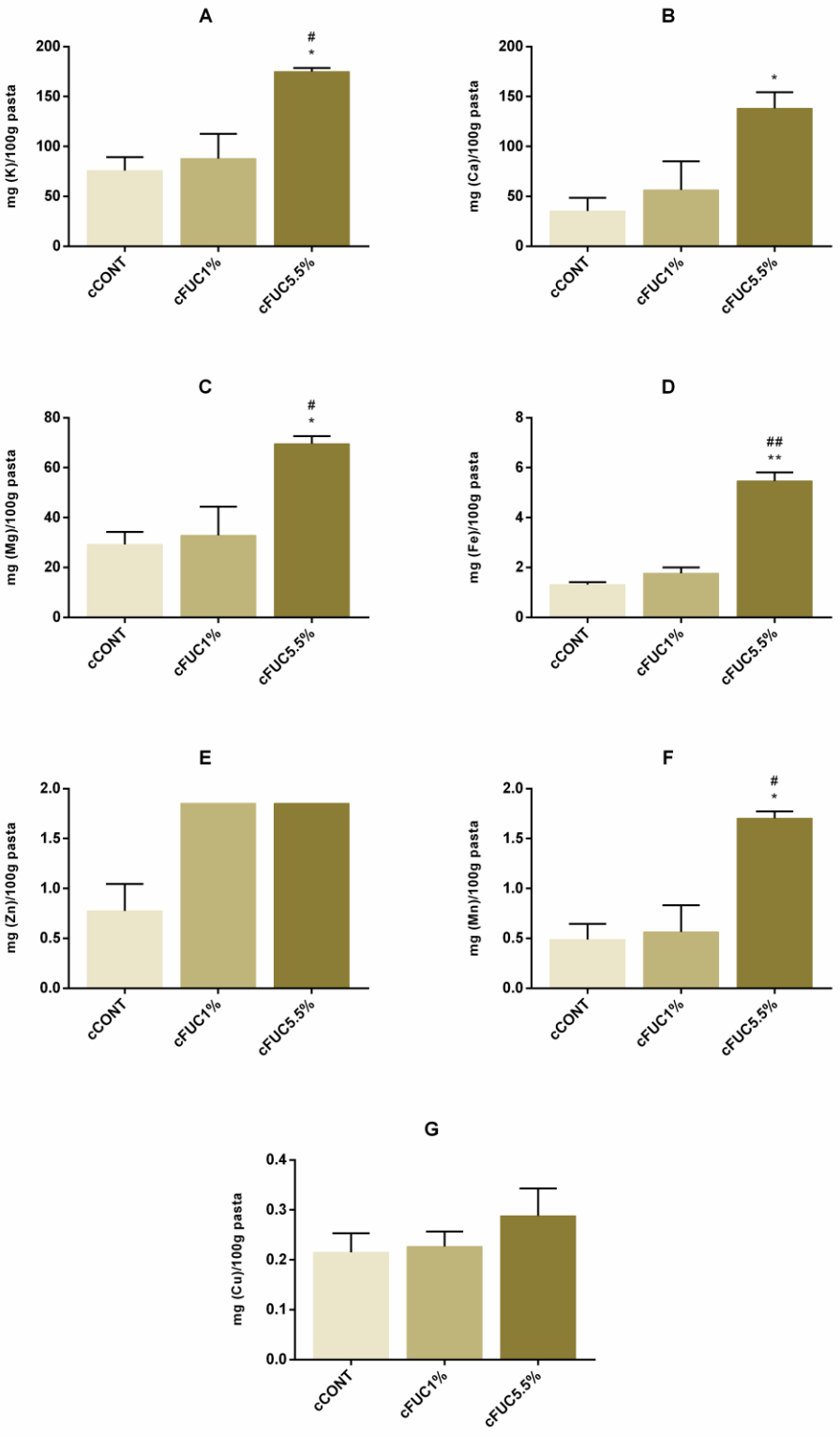

Figure 5. Mineral content of cooked pasta: potassium (A), calcium (B); magnesium (C), iron (D), zinc (E), manganese (F) and copper (G), expressed in mg/100g pasta. Values are expressed as mean ± standard deviation. (*p < 0.05 *vs* cCONT; **p < 0.01 *vs* cCONT; #p < 0.05 *vs* cFUC1%; ##p < 0.01 *vs* cFUC1%);

3.4. Phytochemical content

The contents of chlorophyll *a* and carotenoids in *F. vesiculosus* pasta samples and controls, before and after cooking are displayed in **Figure 6**. The incorporation resulted in higher amounts of chlorophyll *a* (uCONT = 15.8 ± 10.8, uFUC1% = 249.6 ± 37.0 and uFUC5.5% = 1840.0 ± 63.4 µg/100 g pasta) and carotenoids (uCONT = 27.9 ± 3.2, uFUC1% = 32.7 ± 4.0 and uFUC5.5% = 173.7 ± 7.0 µg/100 g pasta) with increasing concentrations of seaweed. Chlorophyll *a* was substantially increased between fortified groups and the control samples, and even between all fortified groups (**Figure 6A**), while regarding carotenoids, there were no differences between uCONT and uFUC1%, although statistical differences were noticed between uFUC5.5% and each of the other groups (**Figure 6B**). Overall, the gathered results indicated that seaweed incorporation into pasta predisposed to an increase of its pigment levels. In fact, these findings were expected since brown seaweeds are well known for possessing chlorophyll *a*, particularly *F. vesiculosus* ranging from 500-750 µg/g FW, of this compound.[22] Moreover, according to Silva et al. (2019)[23] this seaweed also presents a diverse carotenoid content such as fucoxanthin, β-carotene, and lutein, reaching proportions of 0.85 ± 0.06, 0.47 ± 0.04 and 0.18 ± 0.02 µg/mg DW of seaweed, respectively, which could also be associated to such increase with fortified samples. Finally, as anticipated, chlorophyll *b* was not detected since it is not found in brown seaweeds.[24]

Still in **Figure 6** is represented the impact of the cooking process upon the pigment content, in which were observed significant decreases when comparing to their uncooked counterparts. In this regard, chlorophyll *a* concentration decreased approximately 16%, 82% and 92%, for cCONT, cFUC1% and cFUC5.5%, respectively (cCONT = 17.1 ± 7.4, cFUC1% = 49.8 ± 10.4 and cFUC5.5% = 301.3 ± 19.4 µg/100 g). For carotenoids, whereas controls suffered no changes upon cooking, the remaining samples were reduced around 80% and 84%, for cFUC1% and cFUC5.5%, respectively (cCONT = 30.8 ± 5.8, cFUC1% = 5.7 ± 1.8 and cFUC5.5% = 13.7 ± 3.1 µg/100 g pasta). Similar findings for clorophyll *a* were observed by Erge et al. (2008)[25], who described that the thermal treatment at 100°C reduced the content of this pigment in green peas, and by Weemaes et al. (1999)[26] who highlighted the degradation of chlorophyll *a*, starting at 60°C in broccoli juice. Furthermore, as this pigment gets degraded and loses the Mg^{2+}, a new compound is generated, namely pheophytin which is grey coloured.

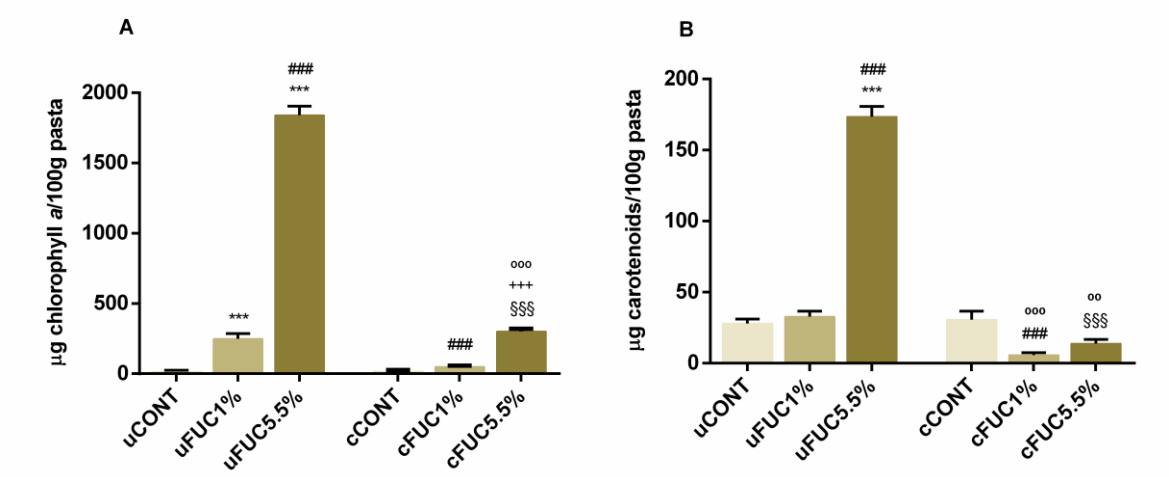

Figure 6. Effects of *F. vesiculosus* fortification in pasta and the impact of the cooking process on chlorophyll *a* (A) and carotenoid (B) content. Values are expressed as mean ± standard deviation (***p < 0.001 *vs* uCONT; $^{###}$p < 0.001 *vs* uFUC1%; §§§p < 0.001 *vs* uFUC5.5%; ^{ooo}p < 0.001 *vs* cCONT; ^{oo}p < 0.01 *vs* cCONT; $^{+++}$p < 0.001 *vs* cFUC1%);

Phlorotannin content of the target groups, expressed in mg PGE (phloroglucinol equivalents) per 100 g of pasta, is displayed in **Figure 7**. Consistent to what has been previously noted, with the increment of seaweed concentration there was also an increase of phenolic content when comparing to controls (uCONT = 0.15 ± 0.11, uFUC1% = 0.36 ± 0.14 and uFUC5.5% = 1.22 ± 0.27 mg PGE/100 g pasta), particularly of uFUC5.5% up to 87% improvement. Despite cooking seemingly decreased phlorotannin content (cCONT = 0.05 ± 0.03, cFUC1% = 0.26 ± 0.17 and cFUC5.5% = 1.02 ± 0.25), especially between samples with 5.5% *F. vesiculosus*, no significant differences were detected, suggesting that this process had no significant effect on this parameter. On a first approach, *F. vesiculosus* fortification in pasta increased its phlorotannin content, which was maintained upon cooking the target samples. To our knowledge there are no studies regarding phlorotannin content (measured by DMBA colorimetric assay) in functional foods, but instead total phenolic content (measured by Folin-Ciocalteau). Despite this, although brown seaweeds in general possess a wide range of phenolic compounds, phlorotannins have been reported as the only phenolic compounds in *F. vesiculosus* with a content reaching 12% DW[27] which explained the increase, even a small one. As a mean of comparation Rico et al. (2018)[28] found out that, in 8% *H. elongata* enriched bread, the phenolic content reached 1.86 µmol GAE/g of bread, presenting statistical differences when comparing to control. Further confirming that seaweeds produce functional products with higher phenolic content, it was observed by

Prabhasankar et al. (2009a)[2] a range of total phenolic content of 0.88-1.07 mg GAE/g pasta in 5-30% *U. pinnatifida* enriched pasta, which was followed by a significant decrease after the cooking process. Once again, the work of Prabhasankar et al. (2009b)[13] highlighted significant loss upon cooking in 5% *Sargassum marginatum* enriched pastas. In both these studies, the leaching of phenolic compounds to the gruel was considered and corroborated on its analysis, having presented higher values of phenolic compounds. According to Ferreira et al. (2019)[6], phlorotannins from *F. vesiculosus* can be extracted in water following optimum conditions such as 100°C during 5 minutes, closely the same conditions as the ones used during the cooking process of our samples. Having said that, there was the possibility of some phlorotannins passing from the pasta to the gruel, a hypothesis that should be further confirmed.

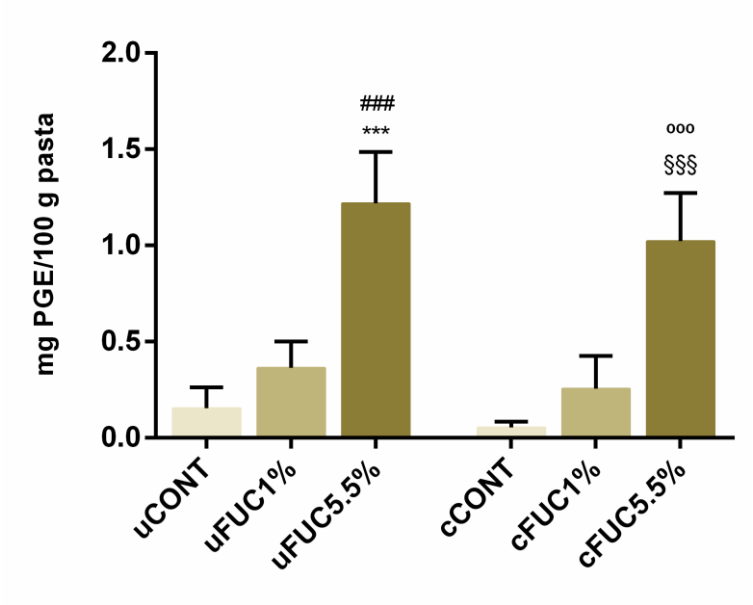

Figure 7. Effects of *F. vesiculosus* fortification in pasta and the impact of the cooking process on phlorotannin content. Values are expressed as mean ± standard deviation (***p < 0.001 *vs* uCONT; ###p < 0.001 *vs* uFUC1%); PGE, phloroglucinol equivalents;

3.5. *In vitro* antioxidant activity

The antioxidant activity of pastas before and after cooking was accessed through three *in vitro* antioxidant activity methods, namely $ABTS^{\bullet+}$, $SO^{\bullet}$ and $NO^{\bullet}$ scavenging, using the respective acetone 70% extracts dissolved in DMSO. Results are displayed in **Figure 8**.

The $ABTS^{\bullet+}$ assay is a widely used method for the assessment of the antioxidant capacities of natural products and is a useful reagent for investigating *in vitro* free radical

scavenging activities of compounds. As shown in **Figure 8A,** no significant changes were detected in the antioxidant activity of the extracts, although it tended to increase with the increment of seaweed (0.28 ± 0.14, 0.36 ± 0.25 and 0.57 ± 0.21 g AAE/100 g extract for uCONT, uFUC1% and uFUC5.5%, respectively). This indicateed that fortification with *F. vesisulosus* could possibly provide antioxidant properties to this product but might not be that high. Upon cooking there was an overall decrease on the $ABTS^{\bullet+}$ scavenging activity (0.13 ± 0.03, 0.17 ± 0.01 and 0.27 ± 0.07 g AAE/100 g extract for cCONT cFUC1% and cFUC5.5%, respectively) but only significant differences were detected between the samples with the highest concentration of *F. vesiculosus*, suggesting that antioxidant properties might be maintained upon cooking.

Differences regarding the antiradical properties among samples are more evident in **Figure 8B**, for the superoxide assay. Overall, significant increases between samples of fortified pasta and controls, and even between fortified samples (uCONT = 0.0003 ± 0.0005, uFUC1% = 0.033 ± 0.003 and uFUC5.5% = 0.103 ± 0.005 g GAE/100 g extract) were observed. Again, the cooking process caused a reduction of the superoxide scavenging activity, (cCONT = 0.0003 ± 0.0005, cFUC1% = 0.02 ± 0.01 and cFUC5.5% = 0.08 ± 0.01 g GAE/100 g extract), that decrease being also significant between fortified samples.

The nitric oxide scavenging assay evaluates the capacity to collect $NO^{\bullet}$ which is an extremely important free radical released during inflammation.[29] **Figure 8C** displays the antioxidant activity accessed through the $NO^{\bullet}$ assay, expressed in grams of AAE per 100 grams of extract, and an overall similar profile could be observed regarding the previous one. In fact, there was a significant increase of activity between fortified samples and controls and in between fortified samples with overall levels reaching 0.24 ± 0.01, 0.62 ± 0.05 and 1.03 ± 0.06 g AAE/100 g extract for uCONT, uFUC1% and uFUC5.5%, respectively. Contrarily to the previous assays, however, there were no significant changes whatsoever between uncooked and cooked samples (cCONT = 0.23 ± 0.03, cFUC1% = 0.52± 0.12 and cFUC5.5% = 0.91 ± 0.17 g AAE/100 g extract).

Contrarily to the herein gathered results, total antioxidant activity reported by Prabhasankar et al. (2009a)[2] was said to be significantly increased when comparing 5-30% *U. pinnatifida* (seaweed powder) fortified pasta samples (1.33-2.82 mg AAE/g pasta) with controls (0.61 mg AAE/g pasta). The same authors observed statistical differences between controls and 5% enriched samples, 7.79% and 9.29% scavenging activity, respectively

according to the $SO^{\bullet}$ assay. In both reports the antioxidant activity is clearly higher when comparing to the present work. Furthermore, on another case study, a significant increase in antioxidant activity was also confirmed by Cox and Abu-Ghannam (2013)[30] upon 10-40% dehydrated *H. elongata* incorporation into beef patties, starting at 30.23%. Hereupon, these differences in antioxidant capacities, especially when comparing those from this work, are most likely due to the difference in seaweed or in seaweed proportion. In fact, with distinct seaweeds one can find a wide range of different compounds that exhibit antioxidant properties like polysaccharides, pigments, or even vitamins, apart from the expected polyphenols.[31] On the other hand one can point out the differences between the analysed extracts, since with each solvent there is also a myriad of different compounds that could be selectively present. In this context, Wang et al. (2010)[32] observed that, although the *F. vesiculosus* extract with ethyl acetate (EtOAc), which was confirmed to be phlorotannin rich and therefore had the most total phenolic content between all extracts, had surprisingly lower antioxidant activity than a 80% EtOH extract. Thereby, fortified pastas may possess even more compounds capable of scavenging free radicals than the ones which are present in 70% acetone extracts. Further analysis should be made in order to identify and quantify these compounds.

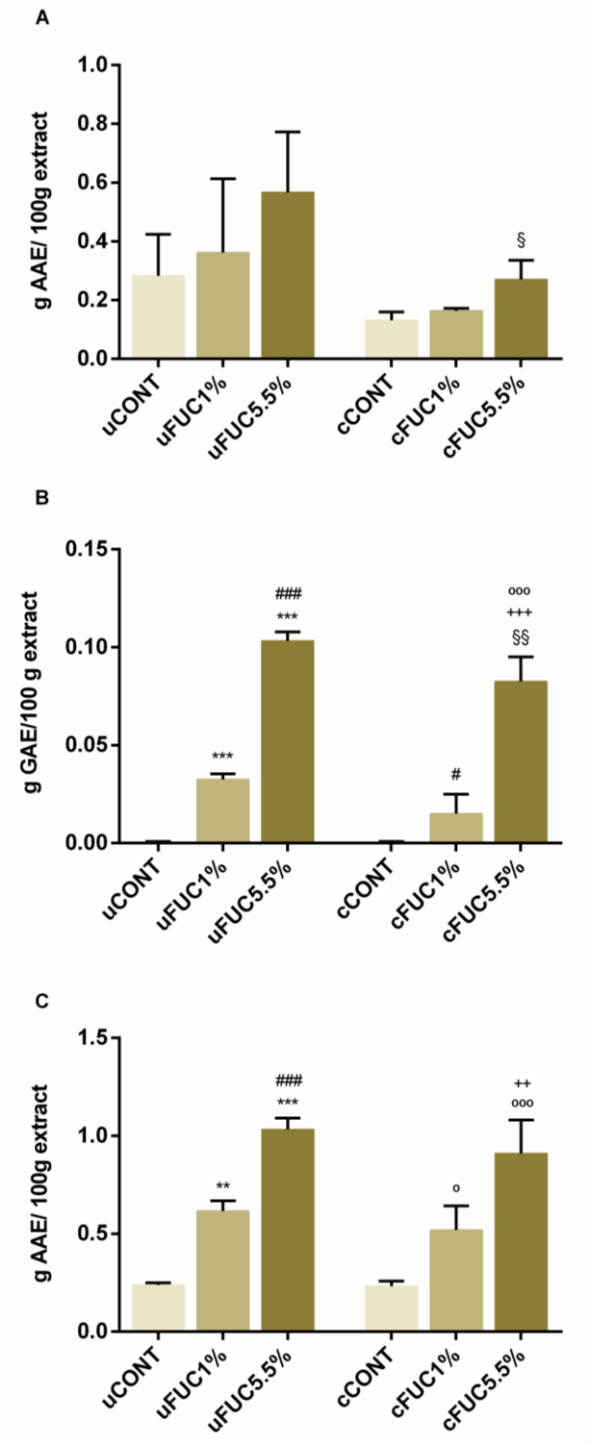

Figure 8. Effects of *F. vesiculosus* fortification in pasta and the impact of the cooking process on the antioxidant activity, measured in three different assays: ABTS$^{\bullet+}$ (A), SO$^{\bullet}$ (B) and NO$^{\bullet}$ (C) assays. Values are expressed as mean ± standard deviation (***p < 0.001 *vs* uCONT; **p < 0.01 *vs* uCONT; ###p < 0.001 *vs* uFUC1%; #p < 0.05 *vs* uFUC1%; §§p < 0.01 *vs* uFUC5.5%; §p < 0.05 *vs* uFUC5.5%; ^{ooo}p < 0.001 *vs* cCONT; ^{o}p < 0.05 *vs* cCONT; $^{+++}$p < 0.001 *vs* cFUC1%; $^{+}$p < 0.05 *vs* cFUC1%); AAE, ascorbic acid equivalents; GAE, gallic acid equivalents.

3.6. Enzyme inhibition effects

The inhibition activity towards key enzymes from lipid and carbohydrate metabolism, namely α-glucosidase, α-amylase and pancreatic lipase, was also assessed. **Figure 9** compares the ability to inhibit each of these enzymes for uFUC5.5%, their respective inhibitor and cCONT, for glucosidase.

As shown, α-glucosidase was inhibited with both controls and 5.5% *F. vesiculosus* fortified pastas (**Figure 9A**), suggesting that this seaweed is not responsible for such biological activity. However, when comparing to acarbose, the reference inhibitor, the target samples showed higher inhibitory power. Regarding the remaining enzyme inhibition assays, namely α-amylase (**Figure 9B**) and pancreatic lipase (**Figure 9C**), no significant inhibitory effect was observed up to the highest tested concentration (0.125 mg/mL and 0.05 mg/mL in both cases) while acarbose or orlistat could inhibited their activity by 50% at 0.002 mg/mL and 0.025 µg/mL, respectively, overall indicating that these samples may not possess such biological activity. As active participants in human sugar and fat metabolism, these enzymes have been the focus of studies aiming to prevent noncommunicable chronic diseases, such as diabetes and obesity, since their inhibition contributes to the well-being of the consumer. The fact that these samples are not able to inhibit those particular enzymes only means that they do not possess anti-obesity and anti-diabetic effects.

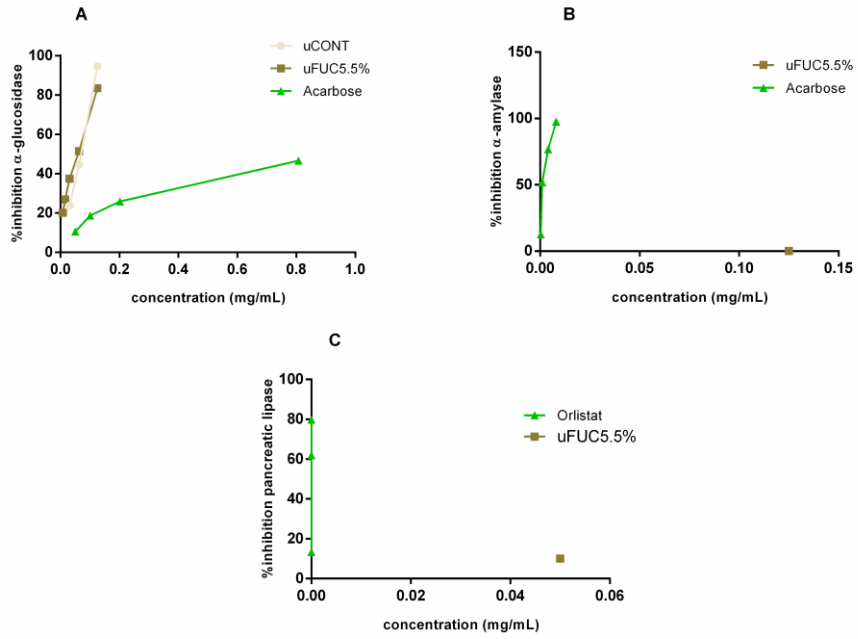

Figure 9. Effects of *F. vesiculosus* fortification in pasta on α-glucosidase, α-amylase and pancreatic lipase inhibition (%). Values are expressed as mean; acarbose (6.46 mg/mL) and orlistat (1 µg/mL) are the reference inhibitors.

3.7. Mineral bioaccessibility and bioavailability in cooked pasta

Considering the results of the nutritional analysis described throughout topics **3.1** to **3.4**, the use of *F. vesiculosus* as a food ingredient in the formulation of pasta was found to mainly result in increases of fibre, pigment and mineral content. Whereas fibres, by designation are "indigestible", therefore being resistant to the process of digestion and intestinal absorption, and pigment content was significantly decreased upon cooking, the bioaccessibility (i.e. the quantity or fraction released from the food matrix in the gastrointestinal tract which becomes available for absorption) and bioavailability (i.e. the fraction of ingested nutrient or compound that reaches the systemic circulation and is utilized) studies were directed to minerals. The obtained results, for each target samples, regarding the bioaccessible fractions (%BAc) and bioavailable fractions (%BAv) of minerals, namely potassium, calcium, magnesium, iron, zinc, manganese and iodine, are represented in **Figure 10A-G**. The %BAc of the 7 elements ranged from 10.2% to 62.1%, 10.2% to 69.8%, and 7.9% to 104.6%, while the %BAv ranged from 0.99% to 53.82%, 1.60% to 77.13%, and 2.97% to 55.9%, for cCONT, cFUC1% and cFUC5.5%, respectively. For 5.5% enriched samples, both the highest %BAc and %BAv were observed for K, followed by I and Mg, and for K, followed by Mg and Zn, respectively. It should be noteworthy that both K and Mg were the only two minerals with both high bioaccessibility and bioavailability. The lowest registered %BAc and %BAv were detected for Fe, followed by Ca and Mn, and Fe, followed by Ca and I. Intriguingly, iodine was one of the minerals with both the highest bioaccessibility and the lowest bioavailability fractions, further confirmed due to presenting statistical differences between them in all fortified samples (cFUC1% and cFUC5.5%). This microelement also displayed a significant increase in %BAc between both fortified samples, suggesting no interference with the pasta matrix. Bioaccessible and bioavailable fractions varied for each mineral in our cooked pasta samples, expectedly, in fact, since these processes were dependable on several factors including the chemical profile of the nutrient, its release kinetics from the food matrix, the interactions with other food components and ultimately the presence of suppressors and other cofactors.[33] On a first approach, according to Urbano and Goñi (2002)[34], the retention coefficients for elements like Ca, Mg, Zn, Fe, and K was lower than the control group, in rats fed with brown seaweed *U. pinnatifida,* which was explained by the possible interactions between minerals and dietary fibre. Indeed, Raes et al. (2014)[35] also reported that several

components in food matrices exhibit retention properties in minerals, such as phenolic compounds and phytic acid, which have both shown to reduce the bioavailability of Fe and Zn. It was previously highlighted the increase in TFC with each increasing seaweed concentration in our pastas, a profile also maintained upon cooking. This could possibly explain the decrease in %BAc and %BAv observed for both Fe and Zn when comparing to controls. It has been demonstrated that interactions with several anionic polysaccharides, such is the case of alginates may form insoluble complexes with minerals,[36] thus decreasing both their release from the food matrix and ultimately their bioavailability. Despite Ca having one of the highest contents in our cooked samples, the bioaccessible and bioavailable fractions were slightly low. This outcome was possibly due to the presence of alginates which formed the well-known egg-box that retained divalent cations, and impaired the bioavailability of this element.[37] In this regard, Bocanegra et al. (2003)[38] highlighted that apparent absorption of Ca in rats fed with *L. digitata* showed a decrease of 10% compared to their control counterparts, even when being supplemented with the highest amount of Ca. Moreover, the same work also demonstrated that Mg bioavailability did not seem to be affected by the consumption of the seaweed, thus suggesting low fibre interference in its bioavailability process. Since Mg bioavailability suffered no apparent alterations either from %BAc and %BAv, one can say the aforementioned study goes into agreement with our results. One standout fact is that, although I presented increasingly higher bioaccessibility, the bioavailability was reduced to almost 5%. Similar findings were reported by Hortas et al. (2011)[39], in which the *in vitro* bioavailability of I in two different brown seaweeds was said to below 5%, confirming that this element has relatively low bioavailability. Moreover, Domínguez-Gonzaléz et al. (2017)[11] confirmed that gastrointestinal digestion of I was considerably high (49–82%) but still less than 28% was bioavailable, having suggested possible interaction with seaweed compounds that predispose to the formation of soluble complexes with high molecular weight. Other types of interactions may occur with proteins, such as pepsin, pancreatin, or biliary extracts, that may also affect the bioavailability of I.[40] Despite the general low bioavailability of this mineral it was estimated that the consumption of 5.5% *F. vesiculosus* enriched pasta (≈ 100g) could provide to intestinal absorption approximately 81 µg of I, which corresponds to 54% of its RDA. In sum, of all these elements, Fe and Zn seemed to be the elements most affected by the food matrix, whereas K, Mg, Ca and Mn bioaccessibility did not change despite the overall increase in these

minerals with higher proportions of seaweed in pasta. The only elements which were not affected by the food matrix throughout the digestive process were I and Cu, although having demonstrated little biosorption.

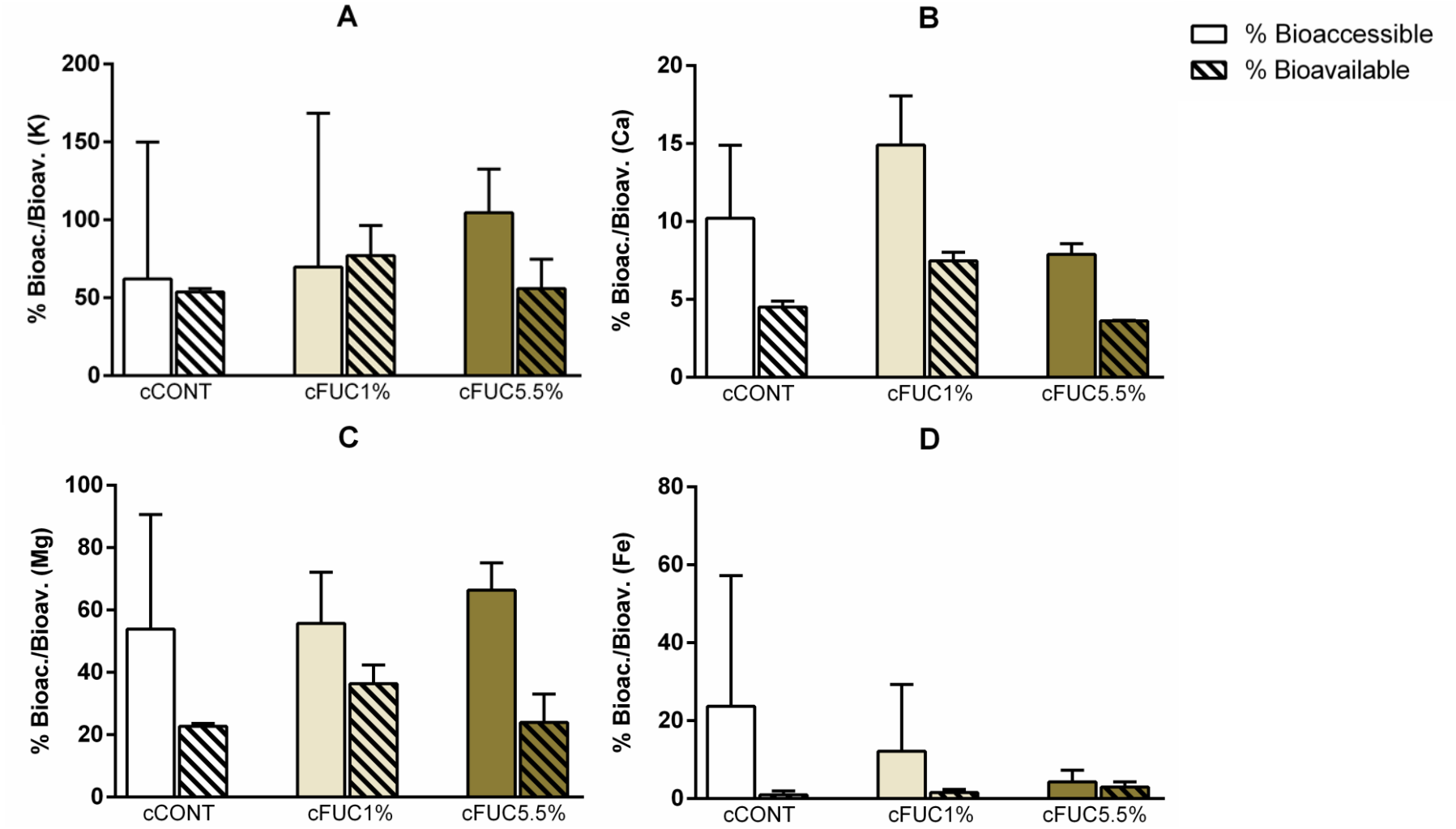

Figure 10. Mineral bioaccessibility and bioavailability fractions of cooked pasta samples: potassium (A), calcium (B), magnesium (C) and iron (D). Values are expressed as mean ± standard deviation.

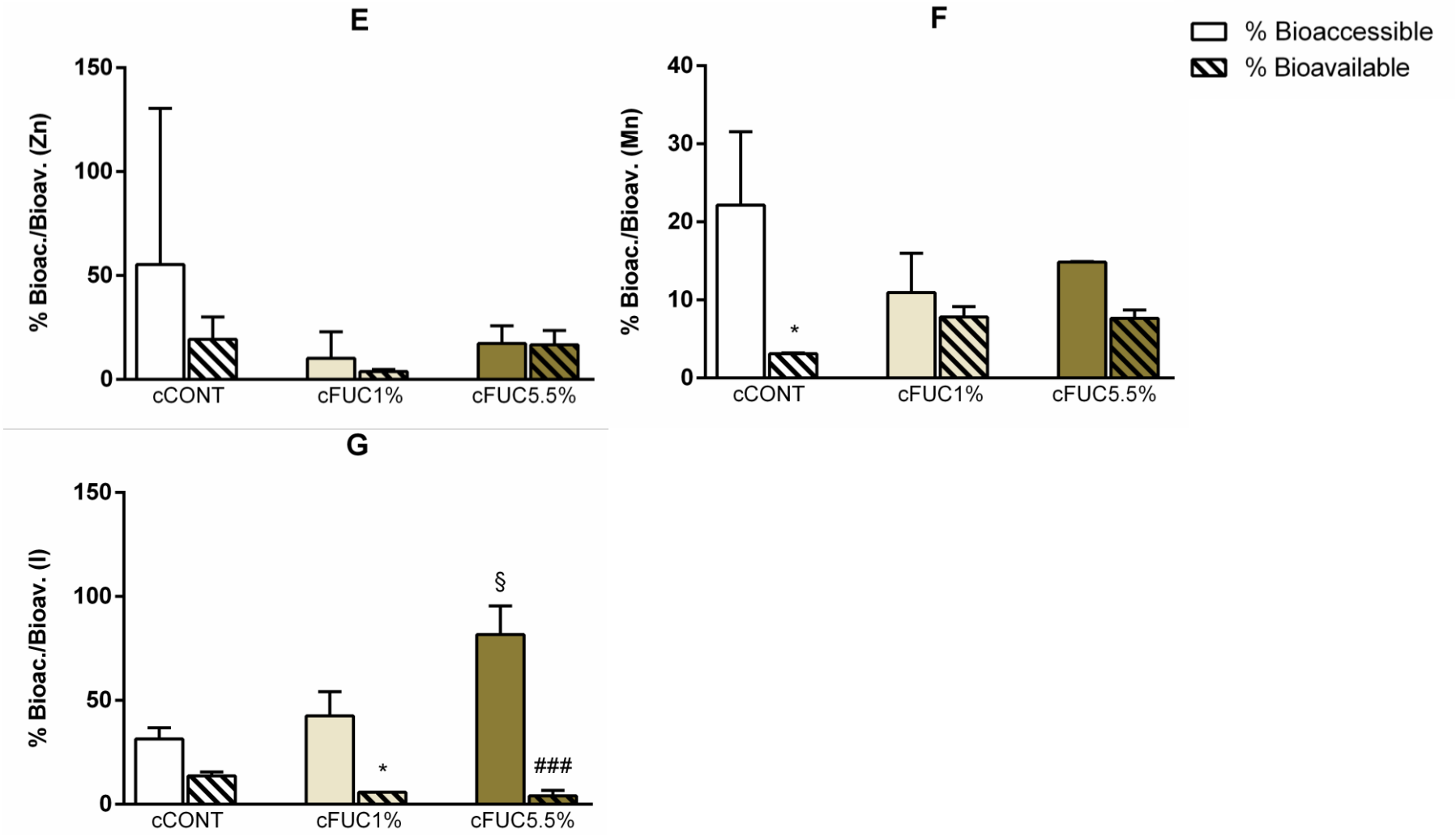

Figure 10. (continuation) Mineral bioaccessibility and bioavailability fractions of cooked pasta samples: zinc (E), manganese (F), and iodine (G). Values are expressed as mean ± standard deviation. (*$p < 0.05$ for BAc *vs* BAv (cCONT and cFUC1%) $^{###}p < 0.001$ for BAc *vs* BAv (cFUC5.5%); $^{§}p < 0.01$ for BAv (cFUC1%) *vs* BAv (cFUC5.5%)).

4. Conclusion

The purpose of the present study was to evaluate the chemical and nutritional composition of *F. vesiculosus* enriched pasta, prior and after the cooking process, exploring the biological activity in terms of antioxidant potential and capacity on inhibiting three metabolism enzymes, and understand the product's mineral bioavailability and bioaccessibility. Overall, the main objectives were achieved and the key findings were: a) fortification of pasta with *F. vesiculosus* extracts does, in fact, improve the mineral, phytochemical and fibre content, b) antioxidant activity is increased with each increment of *F. vesiculosus* in pasta, c) *F. vesiculosus* fortification into pastas does not contribute to the inhibition effects towards the studied metabolism enzymes, d) cooking of pasta changes on different degrees the previous observed compounds/effects and e) *in vitro* mineral bioaccessibility and bioavailability are mostly affected by the food matrix.

Edible seaweeds, and of particular interest to this study, *F. vesiculosus*, can be used in common foods as a precursor for nutritional enrichment. With each increasing seaweed concentration in the target pastas, both fibres and ash contents were improved, up to 48% and 60%, respectively, for the highest seaweed enriched sample, while protein content remained unchanged. Further confirming the higher levels of ash on enriched samples, iodine content was surprisingly raised to around 91% of its initial concentration. There was also a tendency of phytochemical increase, namely pigments, such as chlorophyll *a* (99%) and carotenoids (84%), and phlorotannins, the brown seaweeds' exclusive phenolic compounds (87%). Likewise, *F. vesiculosus* enrichment was related to the improvement of bioactive properties in terms of antioxidant capacity of pastas. Altogether, the gathered results indicate that up to 5.5% fortification with this algae, uncooked samples demonstrate promising effects both on the nutritional aspect and the antioxidant parameters. Upon cooking the target samples, there was a decrease of several contents, namely protein, mineral and phytochemicals, which was also related to a slight decrease of the aforementioned antioxidant properties. The maintenance of fibre content, however, possibly prompted to numerous particularities, such as the retention of protein upon thermal processing, to an extent, proving itself advantageous, but also the retention of minerals when submitted to simulated GI digestion. The overall mineral digestion was reflected on the bioaccessibility and bioavailability assays, where the food matrix was mainly held responsible for the release of these elements and impediment of absorption, particularly on the iodine.

The increasing awareness and demand for healthier products is already established. It is a growing field, both economic and scientific, that opens up a new line of research where foods enriched in seaweeds can prove themselves advantageous. The promising effects that marine macroalgae possess, particularly those highlighted throughout this work, contribute to significant improvements on the food product which in turn may lead to a healthier lifestyle for human beings. In fact, this experimental work further explored the effects of *F. vesiculosus* fortification and the impact of the cooking process, usually preceding the consumption. Data showed that, while seaweed contributes to the enrichment of pastas, the thermal processing associated to cooking impaired several parameters. So, despite the positive developments, a lot of research is still required to optimize this product and to address the viability beyond *in vitro* assays. Specifically, the focus should shift to human trials considering people from different backgrounds, genders, age and physiognomy.

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