Development of sustainable energetic and nutritious balls based on chestnut and apple flours enriched in macroalgae from the Portuguese coast

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Dissertation for obtaining the degree of Master of

Food Science and Engineering

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2021

Acknowledgements

Firstly, I would like to give a general thanks to all the people who helped me and provided a good environment during all the months of development of the thesis.

To the ISA (Instituto Superior de Agronomia), for contributing to my academic path during these 5 years and providing an incredible view every day.

To my guiding professors, Anabela Raymundo and Cristiana Nunes, who always gave me the right way to build all this work, for the enthusiasm during its course and for all the trust they placed in me. To the professor Anabela Raymundo, for each challenge she proposes and for all the passion she dedicates in all the work. To professor Cristiana Nunes, for all the time she spent to help me in the use of each equipment and for the help in understanding what the best solution for each problem.

To professor Catarina Prista, for supplying one of the macroalgae (*Porphyra dioica*) and for helping to decode the spice to be used. To the latter, I also thank Sónia Oliveira.

To Joana Sales and Joana Soares, for their help with the chemical methods and supply of material.

To my sister, for always being available to help me and for her constant interest in my work.

To my parents, grandmother and remaining family for accompanying me in all the stages and for always believing in me.

To my colleagues at the Instituto Superior de Agronomia, who were always there with all their support.

And to my friends Ândria Viegas, Joana Oliveira, Alexandra Luís who were always present throughout these 5 years of the course. And to my friend Francisco Brum, who accompanied me during my master's degree.

Abstract

Macroalgae are increasingly being integrated into food, where their incorporation has contributed to an increase in its nutritional composition, namely mineral values and fibre (Gomez-Zavaglia et al., 2019; Milledge et al., 2016). This increased interest in their consumption is related to the health benefits they provide, resulting from the high number of bioactive compounds, as well as high content in polysaccharides and protein (Fradinho et al., 2019; Paiva et al., 2014).

The present study aimed to develop innovative nutritious balls based on chestnut and apple flours, incorporating three macroalgae from the Portuguese coast: *Porphyra dioica* and *Gracilaria gracilis* (red macroalgae), and *Ulva rigida* (green macroalgal), at different concentrations (1, 3, 5 and 10% w/w). The impact of the addition of macroalgae on the rheology, nutrition, bioactivity, sensory properties (colour, smell, taste, texture and general acceptance) of the nutritious balls was studied, and the maximum level of incorporation, to understand up to what algae content the consumer accepts the product.

The instrumental texture was analysed by Texture Profile Analysis (TPA), and small amplitude oscillatory shear (SAOS) measurements were carried out in a controlled stress rheometer. Colour was instrumentally measured using a colorimeter (CIELAB* system). The nutritional and chemical composition was evaluated based on the AOAC methods (lipids, ash, moisture), protein content by DUMAs methodology, and mineral profile using an ICP-OES equipment. Bioactivity was accessed by the determination of the total phenolic compounds (*Folin-Ciocalteu*), antioxidant activity (DPPH and FRAP assays).

The obtained results showed that all the three macroalgae can be used as natural innovative and sustainable ingredients to nutritionally enrich snacks using high incorporation levels, presenting a high number of nutritional claims, namely in terms of mineral content. However, the global appreciation showed higher scores for control (4.10) than for 3% and 5% *Gracilaria gracilis* nutritious balls, 3.85 and 3.63, respectively.

Keywords: Macroalgae, nutritious balls, texture, bioactivity, sensorial evaluation.

Resumo

As macroalgas são cada vez mais integradas nos alimentos, contribuindo para um aumento da sua composição nutricional, nomeadamente valores minerais e fibras (Gomez-Zavaglia et al., 2019; Milledge et al., 2016). Este interesse crescente no seu consumo deriva dos benefícios que proporcionam na saúde, resultantes do elevado número de compostos bioativos, bem como do elevado teor em polissacáridos e proteínas (Fradinho et al., 2019; Paiva et al., 2014).

O presente estudo teve como objetivo desenvolver bolinhas nutritivas inovadoras à base de farinhas de castanhas e maçãs, incorporando três macroalgas da costa portuguesa: *Porphyra dioica*, *Gracilaria gracilis* (macroalgas vermelhas), e *Ulva rigida* (macroalga verde), em diferentes concentrações (1, 3, 5 e 10% m/m). Foi estudado o impacto da adição de macroalgas na reologia, nutrição, bioatividade, propriedades sensoriais (cor, cheiro, sabor, textura e aceitação geral) das bolinhas nutritivas, e o nível máximo de incorporação, para compreender até que teor de algas o consumidor aceita o produto.

A textura foi analisada por análise de Perfil de Textura (TPA), e foram efetuadas medições de cisalhamento oscilatório de pequena amplitude (SAOS) num reómetro de tensão controlada. A cor foi medida utilizando um colorímetro (sistema CIELAB*). A composição nutricional e química foi avaliada com base nos métodos AOAC (lípidos, cinzas, humidade), conteúdo proteico pela metodologia DUMAs, e perfil mineral utilizando o equipamento ICP-OES. A bioatividade foi analisada pela determinação dos compostos fenólicos totais (*Folin-Ciocalteu*), atividade antioxidante (ensaios DPPH e FRAP).

Os resultados obtidos mostraram que as três macroalgas podem ser utilizadas como ingredientes naturais inovadores e sustentáveis para enriquecer nutricionalmente os aperitivos, utilizando elevados níveis de incorporação, apresentando um elevado número de alegações nutricionais, nomeadamente em termos de conteúdo mineral. No entanto, a apreciação global mostrou pontuações mais elevadas para controlo (4,10) do que para 3% e 5% de bolinhas nutritivas *Gracilaria gracilis*, 3,85 e 3,63, respetivamente.

Palavras chave: Macroalgas, bolinhas nutritivas, textura, bioatividade, avaliação sensorial

Resumo alargado

As macroalgas estão cada vez mais a ser usadas nos alimentos, onde a sua incorporação contribui para o aumento do valor nutricional, nomeadamente dos minerais e fibras (Ahmed et al., 2021), valores estes muito superiores aos dos vegetais terrestres. Este interesse crescente no seu consumo está relacionado com os benefícios para a saúde que estas proporcionam, como o elevado número de compostos bioativos presentes na sua composição, e o seu elevado teor em proteínas e polissacáridos (na *Ulva rigida*, o polissacárido presente é a ulvana, na *Porphyra dioica*, a galactana, e na *Gracilaria gracilis*, o agar, tendo este último um efeito mais gelificante). Um dos seus benefícios, é ser uma fonte de PUFA (ácidos gordos polinsaturados), como é o caso do ómega-3 (ω3) e ómega 6 (ω6). Por estas razões, as macroalgas apresentam grande potencial para serem utilizadas como ingredientes funcionais em diferentes matrizes alimentares (Cikos et al., 2017).

O presente trabalho tem como objetivo o desenvolvimento de bolinhas nutritivas, que funcionem como snack, à base de farinhas de castanha e maçã, incorporando três macroalgas da costa portuguesa: *Porphyra dioica (Erva Patinha) e Gracilaria gracilis* (*Cabelo de velha*) (macroalgas vermelhas), e *Ulva rigida (Alface do mar)* (macroalga verde), em diferentes concentrações (1, 3, 5 e 10 m/m). Foi estudado o impacto da adição das macroalgas no comportamento reológico e na textura, valor nutricional, bioatividade e propriedades sensoriais (cor, cheiro, sabor, textura e aceitação geral) das bolinhas nutritivas, bem como o nível máximo de incorporação, para compreender até que teor de incorporação o consumidor aceita o produto.

As bolinhas nutritivas são compostas por farinhas de castanha e maçã, obtidas a partir de subprodutos da indústria frutícola, correspondendo ao aproveitamento de frutos de pequeno calibre que não cumprem os requisitos comerciais para comercialização em fresco. Para a preparação das bolinhas, a farinha de castanha, a água e o pó de psyllium são aquecidos a 80° C, com o intuito de promover a gelatinização do amido. Após a mistura ter arrefecido a 45° C, a farinha de maçã, o xarope de ácer, a canela, o cardamomo e a casca de laranja são adicionados e triturados, com o intuito de acentuar o seu sabor. Após a pasta formada ser arrefecida até à temperatura ambiente, esta é colocada em moldes de semiesferas de 15 mm e armazenada no frigorífico. Para as formulações enriquecidas com macroalgas, estas foram adicionadas juntamente com a farinha de castanha, psyllium e água. O psyllium (hidrocolóide) foi utilizado como agente gelificante para melhorar a textura e estabilidade, visto a farinha de castanha ser isenta de glúten. O xarope de ácer e a farinha de maçã são usados como adoçantes. Para além de adicionar doçura e ser uma fonte de frutose, a farinha de maçã contém compostos bioativos tais como vitamina C, vitamina E, ácidos orgânicos (ácidos tartárico, málico e cítrico), compostos fenólicos (flavonoides), minerais e fibras (pectinas,

celuloses, hemiceluloses e lenhina) (Feliciano et al., 2010). A variedade “Bravo de Esmofe”, está entre as maçãs com maior capacidade antioxidante, levando a uma redução do risco de desenvolvimento de células tumorais e outras doenças (Serra et al., 2010). A castanha é rica em aminoácidos (lisina, treonina, ácido aspártico e ácido glutâmico), fibra, vitamina B e E (Torres et al., 2014). Segundo alguns autores, a sua adição aumenta a estabilidade e força do gel (Torres et al., 2014).

A textura foi analisada instrumentalmente por Análise de Perfil de Textura (TPA) e os testes reológicos oscilatórios (SAOS) foram realizados num reómetro de tensão controlada, sendo avaliado a viscoelasticidade linear. A cor foi medida instrumentalmente num colorímetro (sistema CIELAB*). A composição química e nutricional foi avaliada com base nos métodos AOAC (lípidos, cinzas, humidade), o conteúdo proteico pela metodologia DUMAs, e o perfil mineral utilizando o equipamento ICP-OES. A bioatividade foi determinada com base nos compostos fenólicos totais (Folin-Ciocalteu), e na atividade antioxidante pelas metodologias DPPH e FRAP.

Os resultados da textura mostraram uma diminuição da firmeza das bolinhas nutritivas em relação ao controlo (9,72 N) quando são adicionadas as macroalgas *Ulva rigida* e *Porphyra dioica*. No entanto, no caso da *Ulva rigida* este efeito é significativo ($p < 0,05$) apenas para 5% (5,72 N) e 10% (5,21 N) de incorporação. O mesmo não se observou com a *Gracilaria gracilis* (8,14 N para 10%), o que estará relacionado com o seu conteúdo no agente gelificante agar. Os resultados obtidos em termos de caracterização reológica estão de acordo com os de textura, tendo demonstrado que o impacto das algas em estudo na estrutura viscoelástica dos produtos gelificados é reduzido. Apesar disso, no caso da *Ulva rigida* e *Porphyra dioica* verifica-se uma redução significativa no G’ a 1Hz para os níveis mais elevados de adição de alga quando comparado com 1% e 3%.

Com a incorporação das algas, o valor de a_w aumenta em comparação com o controlo. A introdução das macroalgas não teve grande impacto no pH. O enriquecimento das bolinhas gelificadas com macroalgas não deverá ter impacto na vida útil do produto, uma vez que os valores de atividade da água do controlo já são elevados, tratando-se de um produto que requer refrigeração.

A nível do conteúdo em minerais, a introdução das algas proporcionou o seu aumento, sendo possível melhorar as alegações nutricionais a utilizar (European commission, 2011). Em vez da alegação “fonte de ferro” do controlo, é possível usar “alto teor em ferro” nos produtos com as três algas. As bolinhas energéticas com *Gracilaria gracilis* 5%, também têm a alegação “alto teor em potássio” e a alegação “sem gorduras” (inferior a 0,5 g de lípidos por 100 g de

produto), ao contrário do controlo e dos restantes produtos com 5% de macroalga, que têm alegação “baixo teor em gordura”, tendo estas menos de 3 g de lípidos por 100 g.

Não se verificaram diferenças em termos da atividade antioxidante recorrendo às duas metodologias usadas, DPPH e FRAP. Também com o método de *Folin-Ciocalteu* para determinação dos compostos fenólicos totais não se verificaram diferenças significativas ($p > 0,05$). O facto de não haver grande impacto nestes parâmetros quando são incorporadas as algas, estará relacionado com a riqueza de compostos fenólicos das farinhas que são utilizadas na sua preparação (farinha de maçã e farinha de castanha), que são a base da formulação controlo.

Nos testes de análise sensorial dos snacks com 3% e 5% de *Gracilaria gracilis* e do controlo, a classificação do atributo apreciação global obteve pontuação mais elevada para a formulação controlo (4,10) do que para as bolas nutritivas com 3% e 5% de *Gracilaria gracilis*, 3,85 e 3,63, respetivamente. Contudo, a amostra *Gracilaria gracilis 3%,* obteve melhor pontuação para a intenção de compra. O parâmetro sensorial menos apreciado das bolinhas energéticas foi a sua coloração, por terem tonalidades escuras. Entre as duas amostras *Gracilaria gracilis 3% e 5%* a diferença instrumental de cor corresponde ao $\Delta E<5$ (2,94), que não será detetado pelo olho humano (Castellar et al., 2006). Contudo, em relação ao controlo, a diferença total de cor ΔE, é de 8,21 e 11,03, para *Gracilaria gracilis 3%* e *5%*, respetivamente, tendo os provadores atribuído uma cotação mais elevada ao produto com menor teor de alga.

Os resultados obtidos evidenciaram que as três macroalgas estudadas podem ser utilizadas como ingredientes naturais inovadores e sustentáveis para enriquecer nutricionalmente os snacks, nomeadamente as bolinhas energéticas, utilizando elevados níveis de incorporação, resultando num maior número de alegações nutricionais, nomeadamente em termos de conteúdo mineral e teor de gordura.

Palavras chave: Macroalgas, PUFA, bolinhas energéticas, compostos bioativos, análise sensorial

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List of abbreviations

aa – Amino acid

TPA - Texture Profile Analysis

AOAC - Association of Official Analytical Chemists

ICP-OES - Inductively coupled plasma optical emission spectrometry

DPPH - 2,2-diphenyl-1-picryl-hydrazyl

FRAP - Ferric Reducing Antioxidant power

L* - Luminosity

a* - Scale of red/green tones

b* - Scale of yellow/blue tones

IMTA - Integrated multi-trophic aquaculture system

GHG - Greenhouse gas

PUFA - Polyunsaturated fatty acids

ALA - α-Linoleic Acid

EPA - eicosapentaenoic acid

DHA - Docosahexaenoic acid

BMI - Body mass index

HTA - Hexadecatrienoic acid

ROS - reactive oxygen species

RSA - Radical Scavenging Activity

a_w – Water activity

1. Introduction

Macroalgae are increasingly being introduced into the human diet, as biomass in the hydrocolloids industry (Bixler & Porse, 2011), for food texture improvement and for providing increased shelf life (Brownlee et al., 2011). They are also used in the medical and cosmetic fields (Ahmed et al., 2021). They have a good potential to be introduced into foods, due to the high number of bioactive compounds present, such as proteins and polysaccharides (Fradinho et al., 2019).

Their distribution depends on physical (temperature, light, storms), chemical (pH, pollution and salinity) and biological (parasites, herbivores) factors (Baweja et al., 2016). Macroalgae production is done in semi-intensive systems, producing 43 ton of green macroalgae and 2 ton of red macroalgae. In Portugal, production in aquaculture, increased by 2.5% from 2018 to 2019 (INE, 2021).

Within the scope of the master's thesis in Food Engineering, gelled snacks (nutritional balls) based on chestnut and apple flours were developed from the incorporation of three macroalgae from the Portuguese coast: *Porphyra dioica* and *Gracilaria gracilis* (red macroalgae), and *Ulva rigida* (green macroalgal), used at different concentrations (1%, 3%, 5% and 10% w/w). These algae are marketed by the company Algaplus which is dedicated to the sustainable production of seaweed.

The objective of this research is to study the impact of macroalgae addition on texture, rheology, nutrition, bioactivity and sensory properties of the nutritious balls, as well as to determine the maximum incorporation level, in terms of technological functionality and sensorial profile. The latter is of great importance to understand up to what percentage of incorporation the consumer accepts the product. These analyses were carried out at the ISA - Instituto Superior de Agronomia, Universidade de Lisboa. Initially, an instrumental evaluation of the gelled balls texture (TPA) and rheology (SAOS measurements) was performed. Then its colour, a_w and pH were studied, as well as its nutritional analysis (moisture, ash, protein, lipids, carbohydrates). Mineral composition was studied by ICP-OES. Bioactivity was determined through the measurement of the antioxidant capacity (DPPH and FRAP assays) and total phenolic compounds (*Folin-Ciocalteu*). Finally, a sensory analysis was performed for the balls with *Gracilaria gracilis* to understand how the incorporation of macroalgae was accepted in terms of sensory attributes and purchase intention.

The snack produced meets the 2021 food trends "Feed the mind", "Quality redefined" and "United by food" (Portugal foods, 2021). The 1st trend "Feed the mind" relates to the fact that consumers are increasingly concerned about their health and well-being and opt for products that provide this. "Quality redefined" - Quality redefined, focuses on food safety and nutrition of products, and companies can consciously raise the price of the product, according to the quality of the product. Finally, "United by food" - United by food, being a snack, is a product aimed at sharing and conviviality.

Although the market is not yet vast, there is increasing acceptance of products incorporating macroalgae. However, the innovation of these products is still a great challenge, and the strategy is based on the appeal of nutritional and health benefits.

The work carried out gave rise to the publication of a panel communication in the Portuguese conference XV *Encontro de Química dos Alimentos* (Madeira, September 2021), which can be found in annex A, and its abstract in Annex B. The presentation of an abstract at the Dare2change congress (Porto, November 2021) (By-products from the fruit production and macroalgae from the Portuguese coast: a sustainable partnership to develop nutritious balls), which is presented in Annex C.

Sousa, F., Nunes, M. C., & Raymundo, A. (2021). Development of a sustainable energy and nutritious balls based on chestnut and apple flours enriched in macroalgae from the Portuguese coast. In *Book of abstracts XVEQA* (Madeira; p. 235). Panel communication PC-A20. https://xveqa.events.chemistry.pt/.

Sousa, F., Nunes, M. C., & Raymundo, A. (November 2021). By-products from the fruit production and macroalgae from the Portuguese coast: a sustainable partnership to develop nutritious balls. (Porto, Portugal, 18-19 November)

2. Theoretical framework

2.1. Macroalgae

Macroalgae are considered a natural, renewable, multicellular marine resource, present in salt water and a source of biologically active compounds (Alga+, s.d.) that play an important role in supporting the biodiversity of the sea. They are found among the rocks, visible at low tide (Pimenta, 2010) and have the ability to grow in varied conditions, namely at different pH (Amosu et al., 2014; García-Poza et al., 2020). They are composed of leaf, stem and root, and nutrients are absorbed throughout the algae (Philpott & Bradford, 2006).

The pigments that enhance the presence of colour in algae are chlorophyll in green algae, phycoerythrin in red algae and fucoxanthin in brown algae (Osório et al., 2020).

Increasing interest in the consumption of seaweed and seaweed-inserted products is related to their health potential and the bioactive compounds found in them.

They accumulate essential minerals that contribute to human nutrition and the functioning of the human organism, guaranteeing many of their beneficial effects on health, such as Potassium (K), Iodine (I), Iron (Fe), Zinc (Zn), Sodium (Na) and Magnesium (Mg), presenting much higher values than terrestrial vegetables. The mineral composition varies according to the growth cycle of the algae (Ross et al., 2008). Because it has a high quantity of minerals, it is a good food for vegetarians.

Their composition of sulphated polysaccharides, i.e., the structural polysaccharides of the cell wall, varies between 50% and 60%. Green algae contain ulvans and red algae galactans (Ciancia et al., 2020).

They are also a source of vitamins, notably vitamin B12, which is not found in vegetables and fights the effects of ageing and anaemia. Rich in polysaccharides, antioxidants and dietary fibre, namely soluble fibre, this being a barrier to starch digestion, and low in lipids (Gofii et al., 2000).

Generally, the protein present in seaweed is higher than the vegetable sources (wheat, rice or beans) but lower than the animal protein sources (milk or meat) (Holdt & Kraan, 2011).

The lipids are present in small amounts, however the polyunsaturated fatty acids present, such as ω3 (omega 3) and ω6 (omega 6), act as antioxidants, helping in the prevention of various diseases, such as diabetes and cardiovascular diseases (Mendis, 2011). These are concentrated in the galactolipid fractions.

Phenolic compounds are structural molecules of cell walls, and have antioxidant, antibacterial, anticancer function and help in the reproductive role of algae (Machu et al., 2015). Their high content in phenolic compounds being one of the most valuable properties of algae. Their chemical and nutritional properties, vary according to the species, habitat, environmental conditions, temperature and salinity of the water (Mohamed et al., 2012).

Despite the nutritional richness, macroalgae also accumulate toxic metals (Arsenic, Cadmium, Copper, Mercury and Lead), and there is no European Union legislation, regarding the limit of toxic elements in macroalgae and the negative effects on health (Circuncisão et al., 2018). According to Besada et al. (2009), the concentration of metals is related to pH, salinity, temperature, oxygen concentration and the properties of algae.

To reduce the Sodium (Na) content and increase the intake of Potassium (K) and other elements that are not present in foods containing NaCl (Sodium Chloride), its use is recommended as a salt substitute.

When added in products they will improve the structure, their nutritional value and give colour to the product, creating healthier and innovative products, for their nutritional richness and low lipid content (Różyło et al., 2017). Because they have high content of biologically active compounds, they have great potential to be used in food applications (Fradinho et al., 2019).

2.1.1. Mode of production

Macroalgae can be harvested from their natural habitat, or produced by aquaculture, with the latter having a higher percentage (94%).

Algaplus uses the IMTA system (integrated multi-trophic aquaculture system) on land, which contains biological certification. This method minimizes environmental impacts (excessive use of resources and increased discharge of effluents in coastal areas), ensuring the sustainability of aquaculture, enhancing the production of macroalgae, through the use of nutrients and reuse of organic and inorganic compounds of this technique (Fróis, 2016; Patarra, 2019).

Figure 1 represents how macroalgae are produced at the Alga+ company. In short, the seawater from the coastal lagoon flows into the fish ponds, which will be pumped into the macroalgae cultivation ponds through a filtration system. The nutrients present in the water will be used for their production. In other words, it makes the synergy between the fish (need the food supply) with the bivalves (organic matter), which filter the suspended matter and the algae (inorganic matter) absorbing the nutrients that are generated by the metabolism of the fish and bivalves, creating a balanced system (Ning et al., 2016).

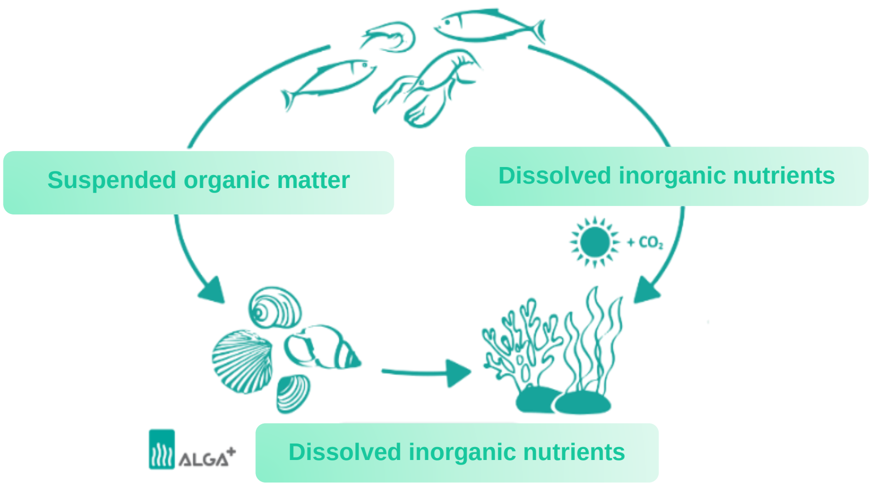


Figure 1: Integrated multi-trophic aquaculture system. Source: https://www.algaplus.pt/modo-de-producao/

2.1.2. Defence mechanisms

Algae are a food source for fish, sea urchins, gastropods and crabs and affect their development. They use chemical, morphological and microbial defence strategies, which vary according to the predator and the algae's characteristics.

To get around their predators and avoid them, they can grow in places that are difficult for them to access, most algae grow in crevices. Another technique, is the production of new tissues that are more palatable while their predators are inactive (Duffy & Hay, 1990).

As a chemical defence, algae use the secondary metabolites present in their constitution (terpenes, aromatic compounds, acetogenins), to ward off pathogenic agents. The red macroalgae are the ones that present the greatest diversity of secondary metabolites, having the greatest power of chemical defence. Terpenes and acetogenins are the metabolites that act most in defence (Baweja et al., 2016; Blunt et al., 2014; Maschek & Baker, 2008).

Parasite death occurs because algae form surface biofilms through microbial colonisation, altering hydrodynamics through significant physiological health pressures, leading to corrosion and destruction of underlying surfaces (Baweja et al., 2016).

2.1.3. Impact on health

Macroalgae are important in the prevention of chronic diseases, such as cancer and cardiovascular diseases, due to all the components present in them, contributing benefits such as the fact that they have antioxidant, antiviral and anticancer properties, reducing the incidence of these diseases.

Their low lipid content has relevance, especially in cardiovascular diseases and in lowering blood pressure, because they are a source of polyunsaturated fatty acids (PUFA), such as ω3, which has as metabolites, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Horrocks & Yeo, 1999; Manerba et al., 2010; Shahidi & Wanasundara, 1998), α with α eicosapentaenoic acid (ALA) being the precursor of these two fatty acids (Rajapakse & Kim, 2011).

According to Yoshizawa et al. (1995), *Porphyra dioica* is among the algae with the greatest ability to decrease carcinogenic effects, because it has *in vivo* and *in vitro* activity and because it is among the richest algae in iodine, ranging from 0.1 to 20 µg/d, and can exceed the upper tolerable limits of 600 µg/d (EFSA) and 1100 µg/d (World Health Organization), starting to be used as a treatment for cancer, in Korea (EFSA, 2014; Kwon & Nam, 2006).

A study from Spain, concludes that low iodine intake is directly proportional to increased risk of breast cancer only in postmenopausal women. In contrast, seaweed consumption was associated with increased risk of prostate cancer (Serra Majem et al., 1988). In Korean women, it was tested and confirmed that the intake of seaweed foods contains sufficient calcium for the prevention of osteoporosis (Lim et al., 2015).

Macroalgae are a great source of iodine, however it can be harmful for people who have thyroid problems. Iodine is a micronutrient necessary for the synthesis of the thyroid hormones, Tri-iodothyronine and Thyroxine (Cherry et al., 2019; Michikawa et al., 2012). In the case of hypothyroidism, the intake of seaweed is recommended because in addition to preventing the appearance of goitre, it helps to balance the thyroid dysfunction. For cases of hyperthyroidism, combating the risk of negative health consequences for these people (Aakre et al., 2020). That is, people with this dysfunction should eat a diet low in iodine, to lower the high production of thyroid hormones.

According to Brownlee et al. (2011), products that have seaweed incorporation reduce the rate of post prandial glucose and lipid absorption, producing nutritional health benefits for the consumer, having the effect of minimising the risks of obesity and diabetes.

2.1.4. Effect of climate change

Changes in the ocean affect the development of algae and their ecosystem because they are vulnerable to any changes in the ocean.

One of the major changes is the impact of greenhouse gases (GHGs) that originate through anthropogenic activities, leading to increased sea temperatures and climate change, leading to ocean acidification, where there is an increase in hydrogen ions (H^+) and a reduction in carbonate ions (CO_3^{2-}) (Harley et al., 2012). Both are caused by global warming and increased levels of CO_2 (carbon dioxide). Acidification results from the decrease in pH that is caused by the absorption of CO_2 levels in the oceans.

GHG are also generated by agriculture, where a large part is generated by ruminants, through the release of methane (CH_4) into the atmosphere being produced by enteric fermentation (digestive process where carbohydrates are converted into simpler molecules by microorganisms, being absorbed into your bloodstream), therefore it is called for less meat consumption, to minimize climate change.

It was found that algae, namely *Asparagopsis taxiformis* (red macroalgae) can reduce up to 82% of CH_4 emissions from cattle, being able to produce meat in a more sustainable way (Roque et al., 2020). The study focused on adding this alga to the feed of 21 cattle through a snack that controlled methane through their respiration and it was concluded that although the increased weight was similar to the other cattle without incorporation of this snack, the cattle under study expelled less CH_4 into the atmosphere, persisting throughout the study (Kinley et al., 2020). This fact is related to the inhibition of the enzyme present in the digestive system of cows, which produces CH_4. This inhibition is done by the algae present, which has the ability to synthesise the halogenated analogs of CH_4, such as bromoform and dibromochloromethane (Paul et al., 2006). In terms of differences in meat flavour, they were not noticed, which was also verified in the study of Roque et al., 2019, where *Asparagopsis armata* (red macroalgae) was incorporated into the diet of dairy cows, with the intention of the removal of 50% of CH_4. The great impasse is the scarce production of *Asparagopsis spp*, and it cannot be used on a large scale in farming.

2.1.5. *Ulva rigida*

Ulva rigida belongs to the green algae group, is known as "sea lettuce" and belongs to the phylum Chlorophyta (Figure 2). They are found in the calm water pools between tides.

Compared to other green algae, it is richer in protein, about 20-26% (Cruz-Suárez et al., 2009; Fujiwara-Arasaki et al., 1984), fibre, minerals (Fe, Ca, Mg, Mn, Na) (Ergün et al., 2009) and vitamins (A, B1, B2, C) (Carl et al., 2014; Silva et al., 2015; Vázquez-Rodríguez & Amaya-Guerra, 2016). The characteristic amino acids of this algae are Leucine, Phenylalanine, Valine, Aspartic acid and Glutamic acid (Lordan et al., 2011). Glutamic and aspartic acid are responsible for the characteristic taste of the seaweed (MacArtain et al., 2007).

Despite its low lipid content, it is rich in ω3 PUFAs, namely ALA, EPA and DHA (Pereira et al., 2012) and ω6, such as hexadecatrienoic acid (HTA). In addition to PUFAs, fibre and antioxidants, such as polyphenols and flavonoids, are important elements in reducing blood glucose and triglyceride levels (Mezghani et al., 2016; Vázquez-Rodríguez & Amaya-Guerra, 2016).

Vitamin C is found in greater quantities in green algae than in other types of algae, making the ingestion of a product containing this algae a reinforcement of the immune defence system and the activation of the intestinal absorption of iron (García-Casal et al., 2007; Lahaye & Ray, 1996). It stands out for containing higher amounts of magnesium and iron than other green algae (Jatmiko et al., 2019; Pereira, 2011), however the sodium and potassium contents are lower than those of red algae (Neto et al., 2018).

The sulphated polysaccharide present in green algae is Ulvan which has the role of inhibiting cellulase activity protecting it from the attack of marine bacteria, and is composed of rhamnose, xylose, glucuronic acid and iduronic acid. The amount of each chemical compound depends on its extraction method, seasonality and type of algae (Lahaye & Ray, 1996).

The interest of this alga is related to the bioactive activity of ulvans, as this encompasses anti-tumour, anticoagulant and antioxidant activity (Alves et al., 2013; Wang et al., 2014), and for being a source of fibre and source of prebiotics that are not digested by the gastrointestinal tract (Silva et al., 2013). When incorporated into energy-reduced foods, it helps in weight control, cholesterol reduction and prevention of gastrointestinal diseases (Carvalho et al., 2009).

Green macroalgae are named because they have chlorophyll type "a" and "b" and carotenoids associated with their green colouring. They use starch as an energy reserve substance (McHugh, 2003) . Its reproduction is by vegetative propagation or by fragmentation. It has an isomorphic biphasic life cycle, where the sporophyte and gametophyte phases are similar.

Figure 2: Ulva Rigida. Source: https://www.algaplus.pt/especies-em-cultivo/ulva-rigida/

2.1.6. *Porphyra dioica*

Porphyra dioica is a macroalgae representative of the red algae group (Rhodophyta) and although most red algae are filamentous, this one is parenchymatous, as it is formed by laminae that derive from cells interconnected by primary and secondary cell connections, originating two-dimensional tissues (Baweja et al., 2016).

Despite its preference for shallow cold waters (Alga+, n.d.), it can also remain in deeper waters due to one of its pigments present, ficobilin, due to its absorption of light of other wavelengths and enhances the appearance of the thallus, as it is a water-soluble pigment. The other pigment present is chlorophyll "a".

The phycoerythrin present, being water soluble, changes the colouration of these algae when they die, to green, leaving as dominant pigments, the chlorophylls. This pigment encompasses antioxidant properties, which may help in the treatment of neurodegenerative diseases that are caused by oxidative stress (González et al., 1999; Pádula & Boiteux, 1999). It gained economic importance because it has nutritional benefits and is one of the richest algae in protein, aminoacids (isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, alanine, glycine, glutamic acid and valine) and vitamins (A, C, B (Niacin and folic acid)). Its taste is due to the presence of three of the amino acids (aa) present: alanine, glutamic acid and glycine (non-essential aa) (McHugh, 2003).

It is better known as "Atlantic Nori" (Figure 3), this alga is used in the preparation of sushi, in order to provide flavour. However, it can also be used in crackers, soups, rice, bread, salads, snacks, among others (Mahadevan, 2015).

Being a red alga, it has higher mineral content (Iron, Zinc, Sodium, Potassium and Calcium), compared to green algae, conferring greater potential for product development (Venkatraman & Mehta, 2019) except for Sodium and Magnesium which is higher in *Ulva Rigida*. In a higher percentage in *Porphyra dioica* is zinc (Pereira, 2011).

The protein content varies according to the season, but this is one of the seaweeds that contains the highest protein content, which can be around 47% protein by dry weight (Fujiwara-Arasaki et al., 1984), compared with soya (Fleurence, 1999) making the product made with this seaweed more nutritionally interesting.

It’s composed of sulphated polysaccharides, such as galactans, which have bioactive properties, including anti-tumour activity and anticoagulant activity (Holdt & Kraan, 2011). In addition to these polysaccharides, *Porphyra dioica* also has a storage polysaccharide, floridian starch, which isn’t digested by the human body and is called dietary fibre. One of its applications beyond food is the extraction of hydrocolloids: such as agar and κ-carrageenan, which are present as polysaccharides in these algae, acting as prebiotics, having positive effects on the intestinal microbiota, helping to control pathogenic bacteria (Pina-Pérez et al., 2017).

Figure 3: Porphyra dioica. Source: https://www.algaplus.pt/especies-em-cultivo/porphyras-dioica

2.1.7. *Gracilaria gracilis*

Like *Porphyra dioica*, *Gracilaria gracilis* is a macroalgae representative of the red algae group (Rhodophyta), with uniaxial structure and cartilaginous texture. It lives in sandy substrate, never exposed, always covered by water (Alga+, n.d.), being fixed to the substrate by a fixation organ, having the shape of tangled human hair, thus its generic name "Ogonori" (Figure 4).

It stands out for its composition being rich in protein, carbohydrates, polysaccharides, polyphenols, antioxidants and PUFAs.

It is rich in polysaccharides, namely the hydrocolloids present, such as agar (Porse & Rudolph, 2017), calling it an agarophyte, having high syneresis power (water outflow during gelation). Agar consists of agarose, which gives the gelling power and agaropectin, which has thickening power (Lee et al., 2017). According to Lahaye et al. (1986) and Usov. (2011), the presence of agar in the algae cell wall, in addition to presenting resistance to pathogens, protects it against salinity and high temperatures and includes antiviral, antitumour, antimicrobial, antioxidant and anticancer properties (Gioele et al., 2017; Holdt & Kraan, 2011; Mazumder et al., 2002). The polyphenols present act as reducing free radical attack on tissues, minimizing oxidative stress.

According to Francavilla et al. (2013), this seaweed is rich in ω3-type PUFA (linolenic acid, eicosapentanoic acid, eicosatrienoic acid and docosahexanoic acid) and ω6-type PUFA (arachidonic acid and linoleic acid).

Being a red alga, the pigments present are chlorophyll a, carotenoids, and phycobiliproteins, namely phycoerythrin (Francavilla et al., 2013). According to Peixoto et al. (2019) these can replace artificial dyes, using them in the food industry.

Due to the presence of agar, this alga is increasingly being integrated into food products (Armisen, 1995), especially in Japanese cuisine (Peng et al., 2009).

Figure 4: Gracilaria Gracilis. Source: https://www.algaplus.pt/especies-em-cultivo/graciliaria-gracilis/

2.1.8. Use of seaweed and high-fibre foods in cereal-based products

According to Brownlee et al. (2011), products that have algae incorporation reduce the rate of post prandial glucose and lipid absorption, producing nutritional health benefits for the consumer, having the effect of minimising the risks of obesity and diabetes, and having high content of biologically active compounds. Macroalgae present great potential to be used in food applications (Fradinho et al., 2019).

Over the years, many studies have been conducted to evaluate the incorporation of both macroalgae and microalgae in cereal-based products such as cereal bars, snacks, pasta and bread.

a) Cereal bars

A study by Udayangani et al, 2017, aimed at incorporating the green algae *Ulva Lactuca* in cereal bars with different percentages of incorporation, observed that the one with higher macroalgae level (10%) had higher protein content, performed using AOAC methods. The addition of macroalgae contributed to a higher nutritional and antioxidant evaluation, which provide protection against reactive oxygen species (ROS), which would lead to health problems. Despite the benefits, this bar was not as well accepted as the other two bars with a lower amount of algae.

Spirulina, a blue-green microalgae, was added to cereal bars and it was found that its incorporation greatly increased the protein composition of the bars, compared to the control bar (Lucas et al., 2020). This increase was also verified in the study of Batista et al. (2017), where they developed biscuits with 2% and 6% incorporation of *Spirulina* and in Rodríguez De Marco et al. (2014), where *Spirulina* was incorporated in pasta concluding that its addition added almost twice as much protein compared to the control sample.

Regarding its ash content, the incorporation of microalgae increased this content, which provided a higher mineral content, such as Santos et al. (2016), where *spirulina* was incorporated into a smoothie prepared for the elderly and in Carvalho et al. (2017), introducing Spirulina in dietary supplements for athletes.

Concerning the colour of the cereal bars with *Spirulina*, its addition caused the colour parameters to decrease, being related to the pigments present in this microalgae and the concentration used (Lucas et al., 2020). The incorporation of 6% was the one that obtained a more intense colouration. These values are in agreement with the study of Lucas et al. (2018), where they incorporated this microalgae in snacks.

The hardness of the cereal bars did not change with the introduction of the microalgae, according to the study of Batista et al. (2017).

Being the toasted rice bran an excellent source of fibre, Garcia et al. (2012), used it to introduce in three cereal bars, concluding that its addition decreased the water activity (a_w), the strength of the rupture and its colour became darker, i.e., the greater amount of toasted rice bran, the darker the bar was. The bar with higher amount of bran (20%), presented higher amount of energy, due to the toasted wheat bran, contain higher fat content. At the nutritional level, its lipid content and fibre content was higher in the bars with higher percentage of toasted rice bran, which meets the results of Lobato et al. (2012), where bars were developed with soy protein, and the bars with higher fibre were those that had greater resistance to cutting, i.e. greater hardness, this fact happens due to compression that is caused by the presence of fibres.

The acerola is a fruit that is not so well known, however it has a high content of fibre, phenolic compounds, minerals and vitamin C, according to Marques et al. (2013). Therefore, in the study by Marques et al. (2015), acerola seed flour (ASF) and acerola pomace flour (ABF) were integrated into five cereal bars. Three of these bars stood out by scoring better at the preference level according to texture, appearance and taste, which were CB4 (12.5% ASF), CB5 (12.5% ABF) and finally CB1 (control bar), which only contained oats. CB4 and CB5, were the ones with lower triglyceride content and high level of fibre, being a nutritious bar. Concluding that the presence of ASF and ABF caused the fibre levels to rise, being a health benefit by reducing cardiovascular diseases and their presence also influenced the texture compared to CB1, being CB4 and CB5 more resistant to shear and higher hardness, this increase in resistance is due to compression caused by the presence of fibres and the difference in terms of grain size of oats, ASF and ABF.

b) <u>Bread</u>

The search for a healthier bread that provides more benefits for our health and well-being is increasingly present in our daily lives. Some studies with the introduction of macroalgae have been carried out to understand the benefits and even what rate of incorporation could be used.

To modify the rate of digestibility of the starch of white bread by an *in vitro* digestion system, two macroalgae (*Wakame and Chondrus crispus*) were incorporated and it was concluded that by integrating these algae, their rate of digestibility and degree of hydrolysis decreased, with the *Chondrus* algae showing more pronounced responses. Through these results, it can be stated that the introduction of algae is beneficial for glycaemic control, due to the promotion of a decrease in the rate of digestibility of the starch in white bread (Goñi et al., 2002).

In the development of bread, the incorporation of seven macroalgae and carob shells was tested. As in previous studies and in Cofrades et al. (2008), the ash content increased with its introduction, increasing its mineral content. When replacing wheat flour with carob pods and macroalgae, there was a significant increase in phenol content compared to the control bread. The bread with the macroalgae *Himanthalia elongata* was the one that obtained the highest values, not only in phenol contents, but it was also the one that improved the antioxidant activity of the bread, due to its greater antioxidant capacity compared to the other algae under study (Rico et al., 2018).

Finally, another study of the incorporation of two macroalgae, *Ascophyllum nodosum* (brown macroalga) and *Chondrus crispus* (red macroalga) in wholemeal bread, with the aim of understanding up to what percentage of incorporation (2%, 4%, 6%, 8%) the consumer accepts. As already expected, from previous studies and the mineral wealth of this seaweed, its ash content increased significantly, as did its dietary fibre, with the breads made by the red seaweed obtaining higher values than the one made by the brown seaweed. The protein levels showed differences between the two seaweeds. The breads made by *Chondrus crispus* obtained higher protein content as its incorporation was increased, which was not the case with *Ascophyllum nodosum*, since as its incorporation was increased, its protein content decreased (Lamont & McSweeney, 2021). These values are in agreement with Rupérez & Saura-Calixto, 2001, they studied the physicochemical properties of algae, and concluded that the protein value of brown algae ranges from 6.9 to 16.0% and of red algae from 20.9% to 29.8%.

Concerning sensory analysis, the panel of tasters agreed that the breads with 2% and 4% incorporation have a mild taste and would be able to keep them in their diet, as for the 6% and 8% breads were not as well accepted, due to a stronger, dry and grainy taste. The 2% bread was the most appreciated (Lamont & McSweeney, 2021). In the study of Mamat et al. (2018) the 2% incorporation of Kappaphycus *alvarezii (*red algae) in muffins was the best accepted.

c) <u>Corn snacks</u>

Contrary to the previously mentioned study by Lucas et al. (2020), in creating corn snacks with the incorporation of brown macroalgae, *Sirophysalis trinodis* and *Polycladia myrica*, at 2% and

4% incorporation, their addition did not significantly increase their protein content compared to the control sample, in a study of Etemadian et al. (2018).

However, the phenolic composition was higher in snacks with 4% *Polycladia myrica*, followed by snacks with 4% *Sirophysalis trinodis*, according to the study of Prabhasankar et al. (2009), about the introduction of *Undaria Pinnatifida* algae powder in pasta.

d) <u>Pasta</u>

In the study of Prabhasankar, Ganesan, Bhaskar, et al. (2009), *Sargassum marginatum* (brown macroalgae) was incorporated into pasta to improve its nutritional quality and it was found that the antioxidant capacity was higher in cooked pasta than in raw pasta, which may be related to the release of compounds that are responsible for eliminating radicals during cooking and which increased as a greater percentage of seaweed was incorporated. The addition of seaweed promoted an increased phenolic content in the dough. They also reported that incorporating more than 2.5% seaweed, cooking loss occurred due to the higher percentage of seaweed (5%), weakening the gluten network. These results were in line with the microstructure study done by Jyotsna et al. (2004). To prevent this from happening, the incorporation of algae up to 2.5% was the most feasible.

In conclusion, the addition of seaweed and fibre foods add nutritional quality and is a beneficial point in the addition of any product.

3. Material and methods

3.1. Materials

For the preparation of the gelled and nutritious balls, three species of macroalgae was used in dehydrated state, namely *Porphyra dioica* (Atlantic nori), *Ulva Rigida* (Sea Lettuce) and *Gracilaria gracilis* (Ogonori), which are commercialised by the Portuguese company Algaplus (Portugal).

The other ingredients used were chestnut flour (Terrius, Portugal), apple flour (Terrius, Portugal), psyllium (Iswari, Portugal), tap water, cinnamon (Margão, Portugal), cardamom powder (Dietimport, Portugal) and fresh orange zest.

Psyllium is used in the composition of these nutritious balls as a gelling agent, in order to reinforce the gel structure since chestnut and apple flours are gluten-free.

3.2. Methods

3.2.1. Preparation of the nutritious balls

Figure 5 shows how the nutritious balls were prepared. The apple flour and *Porphyra dioica* were crushed in the Vorwerk TM31 Thermomix at maximum speed for 10s. Since the apple flour is very moist, it had to be ground to make it easier to use, along with the other ingredients. The *Porphyra dioica* was the only macroalgae that needed to be crushed because it was sold "dry, of larger granulometry", whereas the other algae were already powdered. The ingredients were weighed on the Kern ABJ-NM/ABS-N scales.

Before preparing these nutritious balls, preliminary steps were taken to select the best combination of aromas/spices.

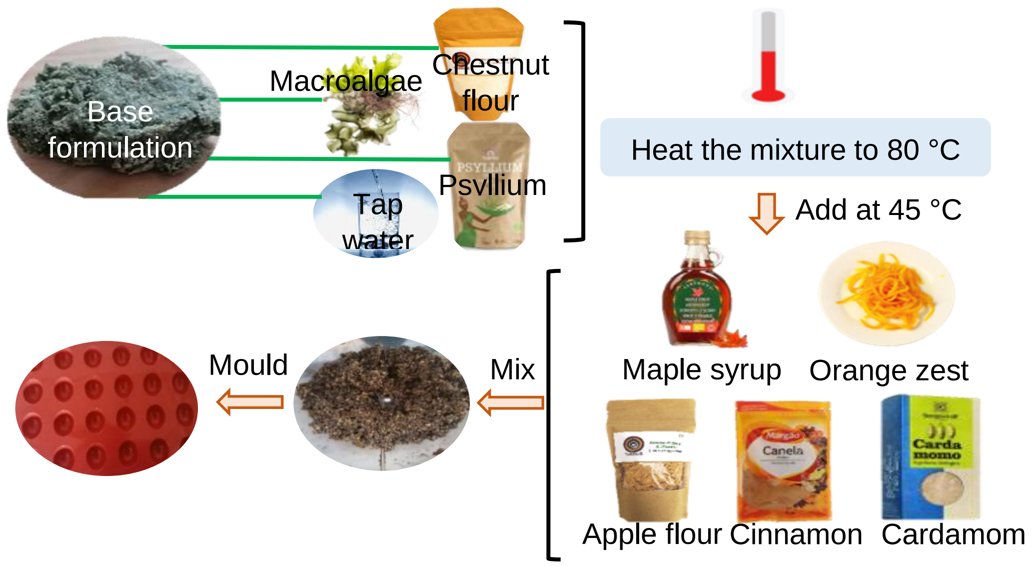


Figure 5: Preparation of the nutritious balls

Table 1 shows the formulations that were used for the control nutritious balls (F1) and for the nutritious balls with 1% (F2), 3% (F3), 5% (F4) and 10% (F5) of macroalgae.

Table 1: Formulations of nutritious balls

Ingredients	F1 (g/100 g)	F2 (g/100 g)	F3 (g/100 g)	F4 (g/100 g)	F5 (g/100 g)
Chestnut flour	20	19	17	15	10
Apple flour	12	12	12	12	12
Psyllium	7	7	7	7	7
Water	50	50	50	50	50
Macroalgae	0	1	3	5	10
Maple syrup	5	5	5	5	5
Cinnamon	2	2	2	2	2
Cardamom	1	1	1	1	1
Orange zest	3	3	3	3	3

3.2.2. Texture evaluation

Texture measurements were performed using a texturometer (TAX-T2i, Stable Micro Systems, U.K.) (Figure 6), with a 5 kg load cell. Texture profile analysis (TPA) test was performed, simulating two consecutive bites, with short space between each (5 s), to sample recovery between the two cycles of the penetration test. The cylindrical probe P/10L (10 mm) was used at a speed of 1 mm/s and 6 mm penetration distance. At least eight repetitions were used for each sample.

The texture parameters presented are firmness, that indicates the maximum force in the first cycle (N), and cohesiveness, that indicates the reaction to deformation, being the ratio of the work done in the second cycle by the work done in the first cycle (dimensionless).

Figure 6: Texturometer TAX-T2i. Source: https://extralab.com.br/produtos/stable-micro-systems

3.2.3. Linear rheological behaviour

To evaluate the linear viscoelastic behaviour of the gelled snacks, a Haake - Mars III (Thermo Scientific, Germany) controlled stress rheometer (Figure 7) was used, using the serrated parallel plates PP20 - 20 mm, with a gap between plates of 1.5 mm and a temperature of 20ºC. Samples were allowed to stabilize during 10 min and covered with liquid paraffin to prevent evaporation.

For each sample, a stress sweep test was carried out, to determine the linear viscoelastic zone and to choose the stress to be used in the frequency sweep test. For the control sample and the sample with 1% macroalgae, the frequency test was performed at 80 Pa and for the 3%, 5% and 10% samples were done at 20 Pa. For each sample at least three repetitions were made.

Figure 7: Haake - Mars III Controlled stress Rheometer. Source: http://www.rheologysolutions.com/thermo-scientific-haake-mars-iii/

3.2.4. Colour

Colour is a parameter with great relevance that affects the consumer's perception of food quality and evaluated by them when buying (Hunt & Pointer, 2011).

Colour analysis was performed using a Minolta CR350 colorimeter (Japan), based on the CIELAB system of colour coordinates (L*, a* and b*), with 8 repetitions per sample.

CIELAB system is based on a three-dimensional space in which each colour is represented by a single point in that space (Granato & Masson, 2010). L* is the luminosity, a* the scale of red/green tones and b* the scale of yellow/blue tones. To evaluate the colour difference, the total colour difference ΔE* is used, using equation 1.

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{Equation 1}$$

3.2.5. Water activity determination

For food to be optimally preserved with microbial control, it is important to study a_w to ensure that food remains safe to consumption. When a_w is high, strategies are implemented to ensure that it remains a safe food (Sandulachi, 2010).

For water activity (a_w) measurement, triplicates were taken for each sample at 20ºC using the HygroPalm Retronic a_w meter.

3.2.6. pH analysis

For the pH determination, the Basic 20 pH apparatus (Crison, Spain) was used, through the solids electrode and calibrated with pH4 and pH7 buffer solutions. Triplicate measurements of each sample were done.

3.2.7. Nutritional characterisation

For the nutritional assessment of the snacks, moisture, lipids, and ash were analysed by AOAC (Association of Official Analytical Chemists) methods, the protein was determined by the DUMAs methodology and minerals by using the methodology of Leitão et al. (2021).

3.2.7.1. Determination of moisture content

For the determination of the moisture content, the samples were dried at 105ºC for approximately 5 days, according to AOAC method Harris & Marshall. (2017). At last, triplicates were taken. The moisture content was calculated using equation 2.

$$Moisture\ content\ (\%) = \frac{m_{sample} - m_{dry\ sample}}{m_{sample}} \times 100 \quad \text{Equation 2}$$

3.2.7.2. Determination of protein content

Protein evaluation was done using the DUMAS methodology (Elementar, Germany), using the methodology of Buckee G.K. (2002), weighing between 100 to 110 mg of sample. They were made triplicates of each sample.

3.2.7.3. Determination of ash content

For ash analysis, 3 g of each sample was placed in a muffle furnace at 550 ºC for 5 h (Harris & Marshall, 2017).

3.2.7.4. Determination of lipid content

Total lipid content was determined according to Doan et al. (2011). 100 mg of sample was weighed into an erlenmeyer flask and 7 mL of Methanol-Chloroform-HCl (10:10:1.5) was added and placed on a hot plate at 90°C for 2 h with stirring. After this waiting time, the liquid is transferred into Falcon tubes. In the erlenmeyer flask, 3 mL of the Hexane-chloroform solution (4:1) is placed in order to remove all the sample, where it is again poured into the flacon tube, and this is stirred in the vortex for 2 min. It is then placed in the centrifuge at 7000 rpm for 10 min at 20ºC. Finally, the supernatant is removed into a test tube and the process is repeated. Another 3 ml of Hexane-chloroform solution is added, stirred for 2 min, centrifuged for 10 min, the supernatant is again removed and left to dry in an oven at 45°C. The percentage of lipids is calculated using the Equation 3.

$$Lipid\ content\ (\%) = \frac{m_{dry\ sample}}{m_{erlenmeyer\ sample}} \times 100 \quad \text{Equation 3}$$

3.2.7.5. Mineral analysis

The evaluation of minerals was based on the Leitão et al. (2021). From the dry sample obtained by determining the moisture content, 0.5 g of this sample was weighed in triplicate and placed in falcon tubes. In each tube was added, 12 mL hydrochloric acid (HCl) and 4 mL of nitric acid (HNO_3) which were left to digest overnight. Then it was placed in the extractor, one day, with 2 blanks and the lids half open. Finally, it was poured into test tubes and 10 mL of distilled water was added to make the reading in the ICP-OES. This equipment is advantageous because it makes a simultaneous multi elemental analysis, speed and high precision (Morgano et al., 1999).

3.2.8. Total phenolic compounds and antioxidant capacity

The preparation of the extraction was based on methods previously optimised by Barreira et al. (2014) and Reis et al. (2012). Firstly, it was made sample extraction, weighing 2 g of each sample, dissolving it in 10 mL of ethanol and homogenizing for 2 min at 8000 rpm using a Ultraturrax T-25. Then it was stirred at 150 rpm for 5h at 20ºC. After this, a centrifugation is carried out at 6000 rpm for 10 min and the supernatant was reserved at 4°C. The whole process was repeated, the two supernatants were mixed together, which were filtered with a syringe. Afterwards, the balloons were weighed and placed in the rotavapor to dry the samples. When dry, they were weighed to be added the reagent DMSO. For the nutritious balls were used 20 mg/mL of concentration and for the macroalgae were used 5 mg/mL, because macroalgae have a very strong antioxidant power (Almeida et al., 2021; Kosanić et al., 2015).

3.2.8.1. Antioxidant capacity

For the determination of the antioxidant capacity, two spectrophotometric methods were used, DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Fe^{3+} - Ferric Reducing Antioxidant Power), which are based on electron transfer reactions for the reduction of oxidants. It’s important to do more than one method, because each method measures different compounds. The equipment used was the spectrophotometer "Cary series UV-Vis spectrophotometer, Agilent technologies".

DPPH: For 100 µL of sample, 3.9 mL of daily DPPH solution was added, and this solution was stirred in vortex and reserved in the dark for 40 min at room temperature. After this time, the absorbance was read at 515 nm, all the readings were performed in triplicate (Brand-Williams et al., 1995). The calibration line was made through the concentration of Trolox (µmol/L) and the %RSA (Radical Scavenging Activity).

FRAP: For this method, 90 µL of sample, 270 µL of distilled water and 2.7 mL of FRAP (TPTZ+$FeCl_3$+acetate buffer; with ratio 1:1:10) was added. It was then stirred in vortex and placed in a water bath at 37°C. After this waiting time, the absorbances were read at 595 nm (Benzie & Strain, 1996). The calibration curve is obtained by relating the absorbance with the concentration of Trolox (µmol/L).

3.2.8.2. Total phenolic compounds

For the determination of total phenolic compounds, 150 µL of sample was combined with 140 µL of *Folin-Ciocalteu* and 2.4 mL of deionized water. It was then stirred in the vortex, leaving it to stand for 3 min. Then, 300 µL Na_2CO_3 was added, which was again stirred on the vortex. Finally, it rested for 2h in the dark at room temperature. Its absorbance was read at 725 nm (Mohankumar et al., 2018).This calibration curve was made by relating absorbance with gallic acid concentration (mg/L).

3.2.9. Sensory analysis

Sensory analysis was carried out on the nutritious balls with *Gracilaria gracilis* and aimed to understand which percentage of incorporation is better accepted by the consumer. This was the macroalgal chosen for the sensory analysis because it contains a more structuring gelling agent than the other macroalgae. The macroalgal levels tested were 3% and 5%, in comparison to control, since 1% is less interesting in terms of nutrition properties and 10% resulted in a product with an undesirable texture. The test was carried out in a sensory room, using 40 untrained panellists aged between 15 and 61, 30% male and 70% female.

3.2.10. Statistical analysis

Statistical analysis of the results was performed in the OriginPro8 program (Origin lab incorporation USA), where ANOVA and Tukey's test was applied for a significance level of 95% ($p < 0.05$).

4. Results and discussion

In Figure 8 the nutritious balls are represented for the different incorporation levels and the three different algae.

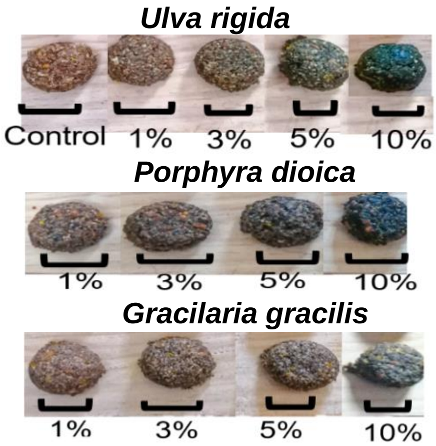


Figure 8: Energy and nutritious balls developed with different types of macroalgae (0%, 1%, 3%, 5% and 10% w/w)

4.1. Texture

Figure 9 and Figure 10 show the firmness and cohesiveness values of the nutritious gelled balls based on chestnut and apple flours with different levels of macroalgae.

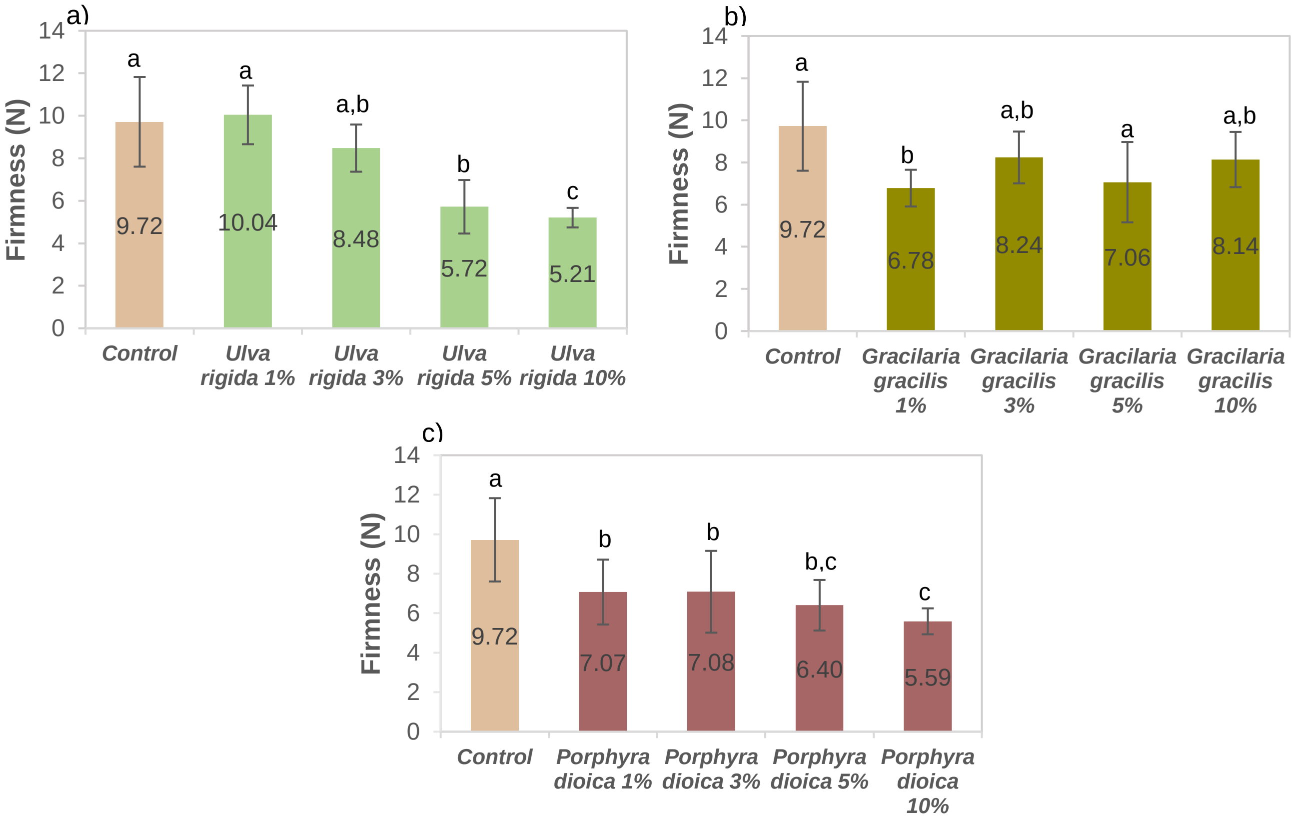


Figure 9: Effect of different levels and species of seaweed on firmness of the gelled snacks a) Control and Ulva rigida (1, 3, 5 and 10%); b) Control and Gracilaria gracilis (1, 3, 5 and 10%); c) Control and Porphyra dioica (1, 3, 5 and 10%)

Different letters in the same column mean significant differences between samples ($p < 0.05$)

From Figure 9, it is possible to observe that firmness decreases with *Ulva rigida* and *Porphyra dioica* addition, but for *Ulva rigida* this effect is significant ($p < 0.05$) only with 5% and 10% incorporation. This wasn't observed in *Gracilaria gracilis*, attributed to its high content in agar gelling agent (Torres et al., 2014).

The firmness of the gelled balls is due to the chestnut starch gelatinisation and the gelation of psyllium (Raymundo et al., 2014). When the chestnut flour is replaced by the macroalgae there is a decrease in firmness, especially for higher levels of incorporation. For *Gracilaria gracilis*, this effect does not occur probably due to the presence of the agar gelling agent (Gioele et al., 2017). The polysaccharides present in *Ulva rigida* and *Porphyra dioica* do not have the same structuring effect as starch or agar.

In the study of Menezes et al. (2015), the biomass of two green macroalgae (*Cladophora spp* and *Ulva spp*) was incorporated into conventional bread and obtained texture values similar to those of the gelled balls when *Gracilaria gracilis* was incorporated. Thus, these authors also verified the reduced impact of this microalgae incorporation on bread texture.

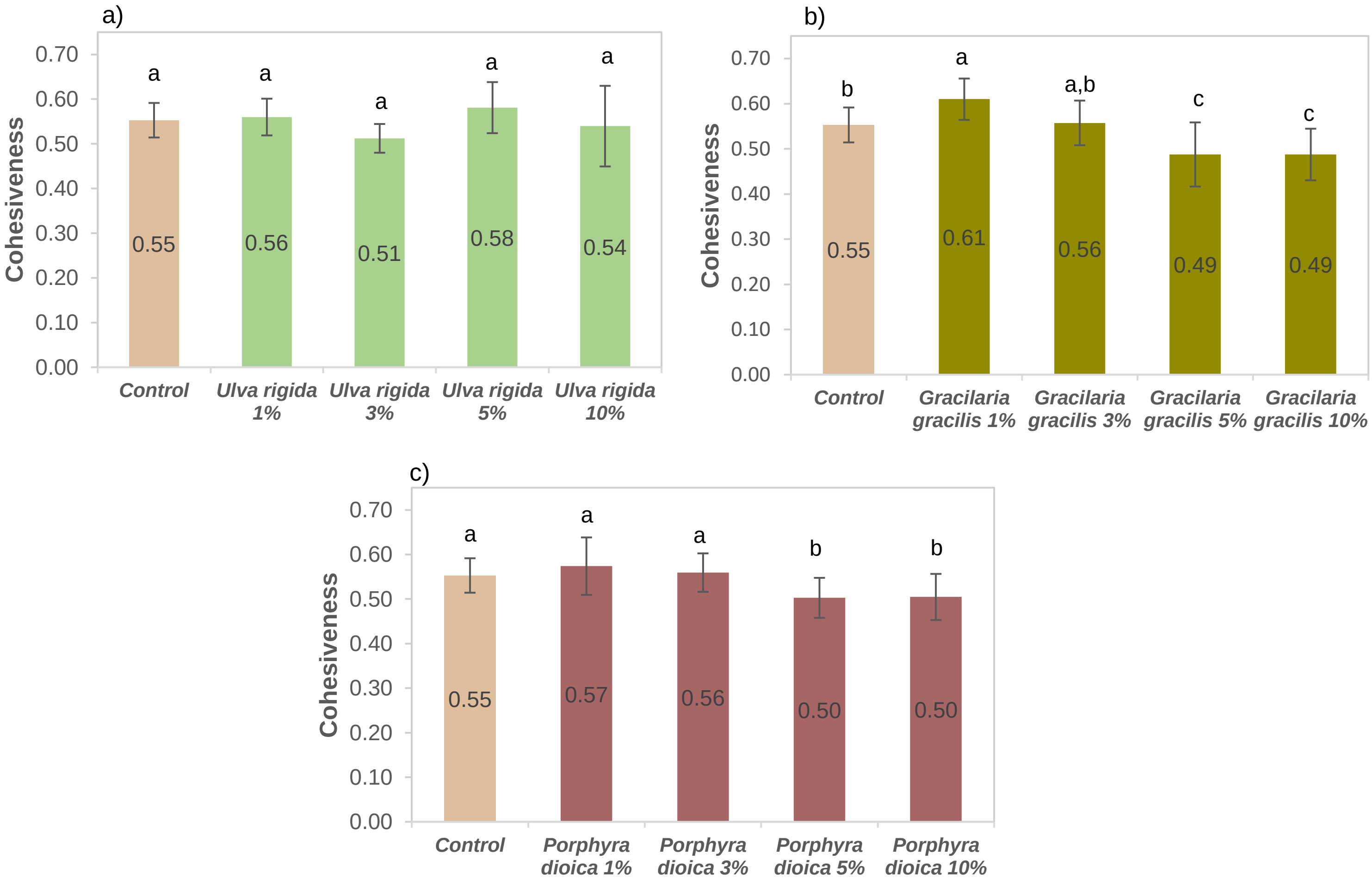


Figure 10: Effect on cohesiveness with different incorporations and different types of seaweed a) Control and Ulva rigida (1, 3, 5 and 10%); b) Control and Gracilaria gracilis (1, 3, 5 and 10%); c) Control and Porphyra dioica (1, 3, 5 and 10%)

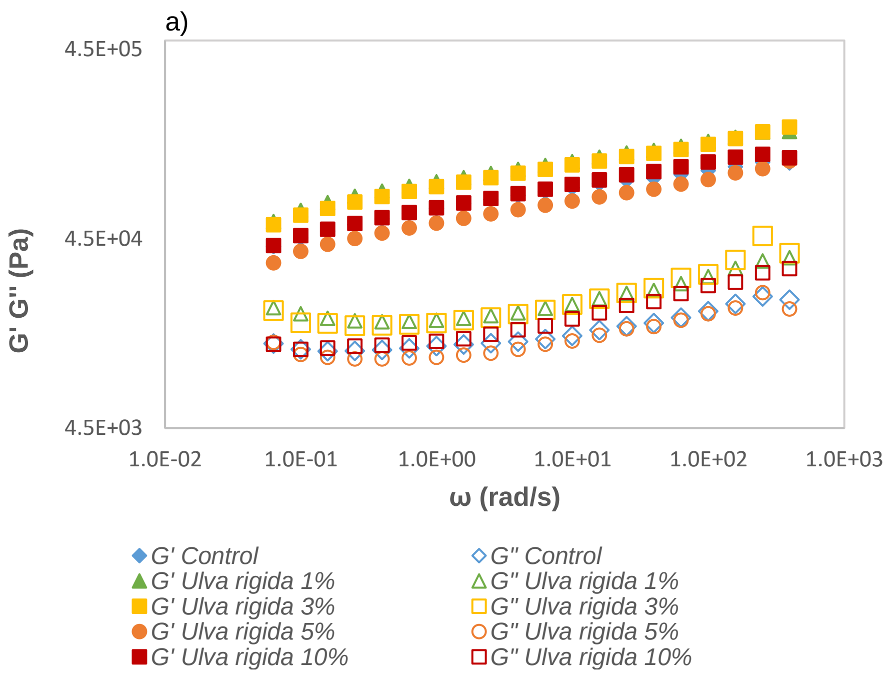
Different letters in the same column mean significant differences between samples ($p < 0.05$)

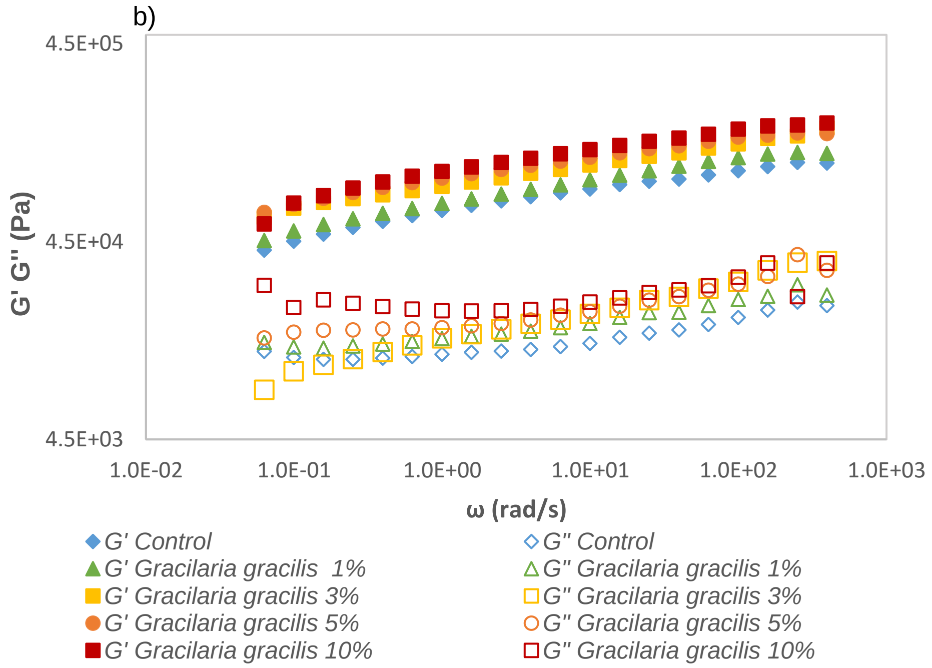
The snacks cohesiveness is shown in Figure 10, no significant differences ($p > 0.05$) between the control and the gelled balls with *Ulva rigida* macroalgal were obtained.

For *Gracilaria gracilis* and *Porphyra dioica*, Figure 10b) and c), there are significant differences ($p < 0.05$) between the control and the gelled balls with 5 and 10% macroalgae. This is in agreement with the study of Rodríguez De Marco et al. (2014), where significant differences between the control and the wheat bread dough with a higher percentage (10% e 20%) of microalgae were observed.

4.2. Rheological behaviour

The mechanical spectra of the gelled balls with *Ulva rigida*, *Gracilaria gracilis* and *Porphyra dioica*, obtained within the viscoelastic linear region, are shown in Figure 11.





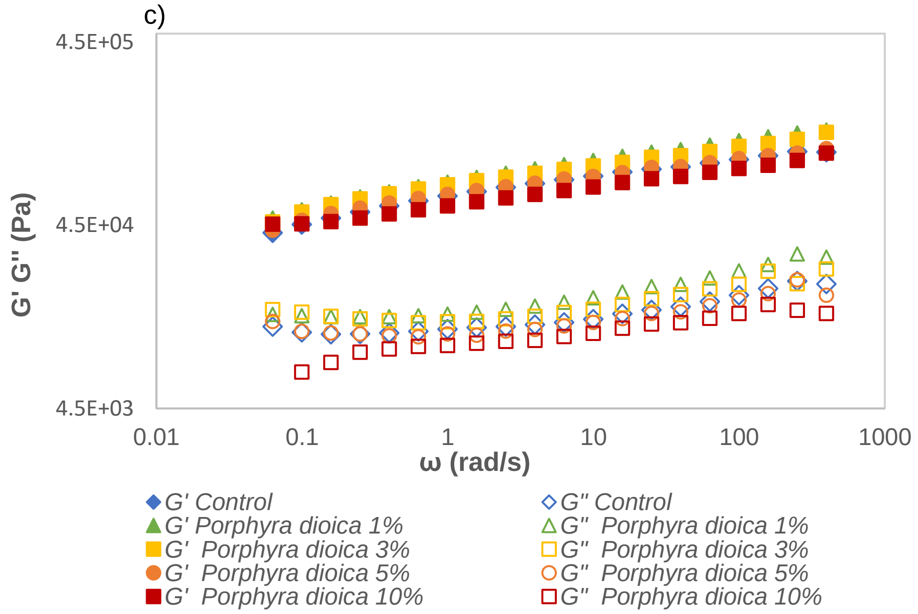


Figure 11: Mechanical spectra with different incorporations and different types of seaweed a) Control and Ulva rigida (1, 3, 5 and 10%); b) Control and Gracilaria gracilis (1, 3, 5 and 10%); c) Control and Porphyra dioica (1, 3, 5 and 10%)

Different letters in the same column mean significant differences between samples ($p < 0.05$)

For all the mechanical spectra obtained, the elastic modulus (G') is always higher than the loss modulus (G''), and both moduli are frequency dependent, correspondent to a weak-gel like behaviour. Similar behaviour was found by different authors, Grossmann et al. (2019) studied heat-induced gel formation of a protein-rich extract from the microalga *Chlorella sorokiniana*. The same was also verified in Mancebo et al. (2015), where the rheological properties of gluten-free pasta with HPMC, psyllium and different levels of water were studied, and a higher value of G' compared to G'' was verified. The same was found in the study of Fradinho et al. (2019), where the introduction of brown seaweed in gluten-free pasta was technologically and nutritionally evaluated.

In order to carry out a more detailed comparison between the mechanical spectra of the balls obtained with the three macroalgae, the elastic modulus values (G') obtained for an oscillation frequency of 1 Hz were compared (Figure 12).

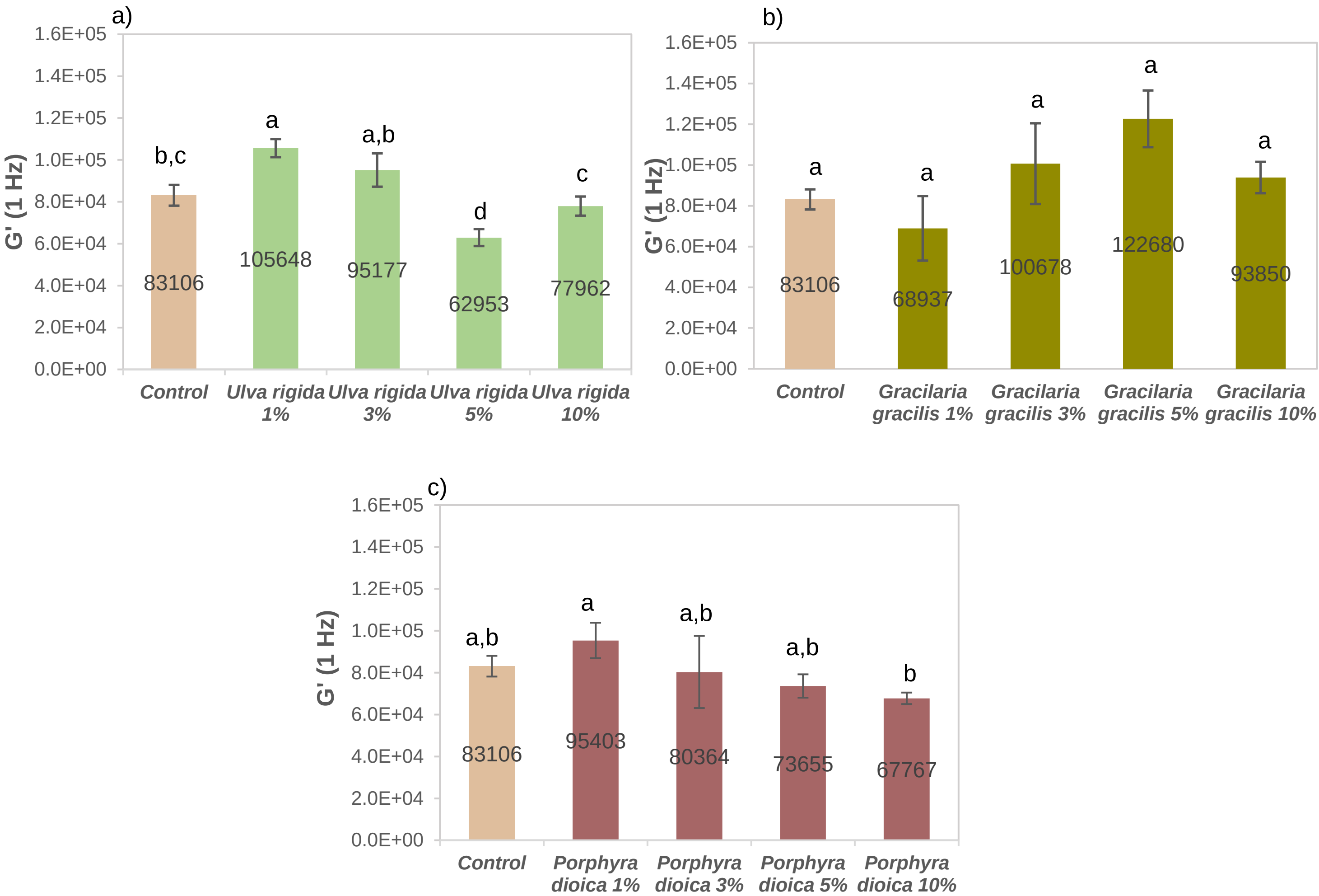


Figure 12: Elastic modulus (G') of nutritious balls a) control and Ulva rigida (1, 3, 5 and 10%); b) control and Gracilaria gracilis (1, 3, 5 and 10%); c) control and Porphyra dioica (1, 3, 5 and 10%)

Different letters in the same column mean significant differences between samples ($p < 0.05$)

From the Figure 12b) it can be seen that the use of *Gracilaria gracilis* in the gelled balls did not affect the viscoelasticity, since there are no significant differences ($p > 0.05$) between the G´ values at 1 Hz for the control and algae samples. In this case, the rheological results are in close agreement with the texture results.

The incorporation of the macroalgae *Ulva rigida* and *Porphyra dioica*, showed a decrease in their level of structuring, mainly for higher levels of incorporation. This fact is due to the polysaccharides present in these algae not having such a strong level of structuring as the starch, which is present in chestnut flour.

4.3. Colour

The CIELAB colour parameters (L*, a* and b*) and the total colour difference ∆E* of the nutritious balls with different levels of macroalgae are presented in Table 2.

Table 2: Colour parameters of nutritious balls

	L*	a*	b*	∆E* in relation to control	∆E* in relation to the previous sample
Control	$42.43 \pm 2.86^{a\ x\ A}$	$8.21 \pm 0.75^{a\ x\ A}$	$16.23 \pm 5.52^{a\ x\ A,B}$		
Ulva rigida 1%	36.86 ± 2.73^{b}	5.02 ± 1.40^{b}	11.22 ± 9.07^{a}	8.14	
Ulva rigida 3%	36.16 ± 2.51^{b}	3.50 ± 1.39^{c}	10.06 ± 7.2^{a}	9.98	2.05
Ulva rigida 5%	35.44 ± 2.82^{b}	3.12 ± 1.22^{c}	9.79 ± 7.63^{a}	10.78	0.85
Ulva rigida 10%	30.89 ± 1.74^{c}	0.01 ± 0.78^{d}	12.56 ± 1.56^{a}	14.63	6.17
Gracilaria gracilis 1%	37.37 ± 1.40^{y}	6.53 ± 0.54^{y}	18.19 ± 1.62^{z}	5.69	
Gracilaria gracilis 3%	35.01 ± 2.04^{z}	4.71 ± 0.73^{z}	16.10 ± 1.57^{z}	8.21	3.64
Gracilaria gracilis 5%	32.23 ± 1.12^{k}	4.09 ± 0.65^{z}	15.35 ± 1.77^{z}	11.03	2.94
Gracilaria gracilis 10%	29.38 ± 1.80^{w}	3.45 ± 0.87^{k}	12.14 ± 2.48^{y}	14.48	4.34
Porphyra dioica 1%	37.03 ± 2.60^{B}	6.99 ± 0.58^{B}	17.68 ± 1.41^{A}	5.73	
Porphyra dioica 3%	$34.87 \pm 1.36^{B,C}$	5.42 ± 0.94^{C}	$15.42 \pm 1.89^{A,B}$	8.10	3.49
Porphyra dioica 5%	33.51 ± 1.92^{C}	4.50 ± 0.55^{D}	13.49 ± 1.08^{B}	10.04	2.53
Porphyra dioica 10%	29.10 ± 2.21^{D}	3.36 ± 1.17^{E}	10.49 ± 2.13^{C}	15.30	5.46

Different letters in the same column mean significant differences between samples ($p < 0.05$)

When macroalgae are incorporated, the values of L* and a* decrease, as the study of Nunes et al. (2020), about *Tetraselmis chuii* microalgae in bread The control presents higher L*, correspondent to a lighter sample. The a* values decrease as macroalgae are incorporated, getting closer to the green tone. Concerning the b* coordinate, there are differences for 10% in *Porphyra dioica* and *Gracilaria gracilis*. For *Ulva rigida* there are no significant differences ($p > 0.05$).

Total colour difference in relation to the control sample ∆E*>5, which means that their colours are distinguishable by the human eye (Castellar et al., 2006).

Comparing samples with different algae levels, only 10% *Ulva rigida* and *Porphyra dioica* have ∆E*>5. However, according to Mokrzycki & Tatol. (2011), between 2 and 3.5, differences can be detected by the human eye. According to this, the differences in colour between *Gracilaria gracilis* 3% e 5% (2.94), and *Porphyra dioica* 3% e 5% (2.53), are detected.

4.4. a_w and pH

In Table 3 are represented the values obtained for pH and a_w of the nutritious balls. Generally, it can be observed that the a_w values increase with the incorporation of macroalgae. For *Ulva rigida* there are only significant differences ($p < 0.05$) with 5% and 10% incorporation.

Table 3: aw and pH of nutritious balls and macroalgae

		pH	a_w
Control		5.44 ± 0.06$^{a\ x\ A}$	0.915 ± 0.010$^{b\ y\ C}$
Ulva Rigida	**1%**	5.32±0.04^{b}	0.916 ± 0.014^{b}
	3%	5.32±0.02^{b}	0.923 ± 0.010^{b}
	5%	5.42 ± 0.07^{a}	0.945 ± 0.003^{a}
	10%	5.48 ± 0.08^{a}	0.946 ± 0.001^{a}
Gracilaria Gracilis	**1%**	5.32 ± 0.16^{x}	0.921 ± 0.003^{x,y}
	3%	5.60 ± 0.25^{x}	0.929 ± 0.007^{x}
	5%	5.56 ± 0.19^{x}	0.929 ± 0.005^{x}
	10%	6.08 ± 0.21^{y}	0.939 ± 0.006^{x}
Porphyra Dioica	**1%**	5.55 ± 0.19^{A}	0.928 ± 0.005^{B}
	3%	5.49 ± 0.21^{A}	0.931 ± 0.002^{B}
	5%	5.47 ± 0.14^{A}	0.942 ± 0.006^{A}
	10%	5.36 ± 0.11^{A}	0.944 ± 0.003^{A}

Different letters in the same column mean significant differences between samples ($p < 0.05$)

For the balls with *Gracilaria gracilis* incorporation there is a significant difference ($p < 0.05$) between the control and 1% algae, but there are not significant differences between the gelled snacks with different algal levels (1,3, 5 and 10%).

García-Segovia et al. (2017) also obtained higher aw values with addition of *Tetraselmis suecica, Isochrysis galbana, Tetraselmis suecica, Scenedesmus almeriensis, and Nannochloropsis gaditana* in bread (0.94-0.95). Being the gelled balls, a product without baking, it allows better preservation of the bioactive compounds.

Water activity (a_w) is the amount of free water available in food and is important for predicting microbial growth and choosing the right packaging (Mathlouthi, 2001). Because gelled balls have a very high a_w, there is a greater risk of spoiling and the proliferation of micro-organisms. According to Bozoglu & Erkmen. (2016), gram-negative bacteria grow at high a_w values, above 0.98, and if the gelled balls have aw values below 0.98, these bacteria will not be present. As the gelled balls have $0.91<a_w<0.95$, it provides the growth of yeast.

Food safety depends on a_w and pH. With high values in both parameters, microorganisms may develop. To reverse the situation, natural dehydration through the heat of the sun, mechanical drying through drying tunnels, or lyophilization through rapid freezing of food at a low temperature and then a relatively high-vacuum environment can be used (Bozoglu & Erkmen, 2016).

The most appropriate for this product, is to understand which packaging is suitable to ensure a longer shelf life.

With *Porphyra dioica* there are no significant differences ($p > 0.05$) between pH values, which is also the case with *Gracilaria gracilis*, except for the 10% incorporation, presenting a higher pH value. For *Ulva rigida*, there is a decrease with 1% and 3%, but control is not significantly different ($p > 0.05$) from 5% and 10% samples.

4.5. Nutritional composition

The nutritional composition (energy value, carbohydrates, protein, lipids, moisture and ash) of the nutritious balls (control, *Ulva rigida* 5%, *Gracilaria gracilis* 5%, *Porphyra dioica* 5%) and of the macroalgae are shown in Table 4.

Table 4: Nutritional analysis of nutritious balls and macroalgae

		Energy value (kcal/100 g)	Carbohydrates (g/100 g)*	Protein (g/100 g)	Lipids (g/100 g)	Moisture (g/100 g)	Ash (g/100 g)
Nutritious balls	**Control**	191.74	41.42	3.45 ± 0.061^{b}	1.36 ± 0.115^{a}	52.74 ± 0.764^{b}	1.03 ± 0.028^{d}
	***Ulva rigida* 5%**	164.82	35.15	3.19 ± 0.046^{b}	1.27 ± 0.225^{a}	58.56 ± 1.257^{a}	1.83 ± 0.004^{b}
	***Gracilaria gracilis* 5%**	197.07	43.93	4.22 ± 0.109^{a}	0.50 ± 0.001^{b}	49.36 ± 0.045^{c}	1.99 ± 0.06^{a}
	***Porphyra dioica* 5%**	172.61	36.11	3.98 ± 0.155^{a}	1.36 ± 0.151^{a}	57.14 ± 0.083^{a}	1.41 ± 0.042^{c}
Macroalgae	***Ulva rigida***	254.79	39.81	18.60 ± 0.127^{C}	$2.35 \pm 0.351^{A,B}$	12.01 ± 0.172^{A}	27.23 ± 0.09^{A}
	Gracilaria gracilis	287.07	40.45	26.02 ± 0.273^{B}	2.35 ± 0.206^{B}	6.55 ± 0.81^{B}	24.62 ± 0.758^{B}
	Porphyra dioica	298.78	37.36	30.15 ± 0.442^{A}	3.19 ± 0.193^{A}	12 ± 0.238^{A}	17.30 ± 0.286^{C}

***** Carbohydrates were calculated by difference

Different letters in the same column mean significant differences between samples ($p < 0.05$)

The protein values increase with algae addition for the macroalgae *Ulva rigida* and *Porphyra dioica*. The nutritious balls with 5% *Gracilaria gracilis* were those with the highest protein value and contain less lipid content. The macroalgae with the highest protein content is *Porphyra dioica*, however, this is the macroalgae with the highest lipid value. Probably there may have been errors in protein analysis for snacks. In the study of Holdt & Kraan (2011), about the bioactive compounds of various macroalgae, *Porphyra dioica* also had higher protein value.

According to Regulation (EU) No. 1924/2006, if at least 12%/20% of the energy value of the food is provided by protein, it can be considered as a source of protein/high protein content, respectively. It is low in lipid if it contains less than 3 g of lipid per 100 g, and “fat free” if it has less than 0.5 g of lipid per 100 g. According to this legislation, all macroalgae may have the claim “high protein content”, however the nutritious balls could not present nutritional claims related to protein.

Regarding the amount of lipids, there are significant decrease ($p < 0.05$) from control only for the snack with *Gracilaria gracilis*. The other nutritious balls, have low lipid content, such as the macroalgae *Ulva rigida* and *Porphyra dioica*.

In relation to control, the moisture value of the snacks increased with the addition of the macroalgae *Ulva rigida* and *Porphyra dioica* and decreased with the introduction of *Gracilaria gracilis*. The different moisture values of the gelled balls result from the moisture values of the algae.

The incorporation of macroalgae results in a higher ash content. The ash values obtained for macroalgae are in agreement with those for gelled balls. In the study of Holdt & Kraan (2011), about the bioactive compounds of algae, including the three macroalgae of the present study, similar values were obtained. *Ulva rigida* presented higher values than *Porphyra dioica*.

Comparing the values of macroalgae with vegetables, such as carrot (0.60 g/100 g), pumpkin (0.40 g/100 g) and asparagus (0.80 g/100 g) (INSA, 2010), it can be concluded that the algae have much higher values, correspondent to higher mineral content.

4.5.1. Mineral

Table 5: Mineral composition of nutritious balls and macroalgae

	Nutritious balls				**Macroalgae**		
	Control	***Ulva rigida* 5%**	***Gracilaria gracilis* 5%**	***Porphyra dioica* 5%**	***Ulva rigida***	***Gracilaria gracilis***	***Porphyra dioica***
Na	13.7±0.7^{d}	102.5±5.1^{b}	44.8±0.9^{c}	120±5.4^{a}	2331.8±13.7^{A}	741.6±11^{C}	2206.2±33.3^{B}
K	456.7±80.7^{b}	477.3±34.9^{b}	637.1±14.3^{a}	399.4±19.5^{b}	2177.3±28.3^{B}	4142.6±1.8^{A}	1699.9±4.4^{C}
Ca	67.5±2.9^{c}	87.6±2.3^{b}	108.6±6.0^{a}	62.6±1.1^{c}	717.2±17.3^{B}	1139.3±68.2^{A}	117.3±3.3^{C}
Mg	27.1±0.2^{c}	107.7±5.0^{a}	41.5±0.9^{b}	46.6±1.2^{b}	2766.6±11.3^{A}	326.7±6.5^{B}	317.2±9.6^{B}
P	45.9±4.08^{b}	42.3±1.82^{b}	61.5±1.37^{a}	44.8±0.61^{b}	240.3±3.48^{C}	429.9±19.28^{A}	276.7±1.58^{B}
S	61.1±23.06^{d}	290.1±9.25^{a}	199.5±12.26^{b}	148.6±2.22^{c}	6383.9±152.63^{A}	3282.1±171.83^{B}	3074.8±4.50^{B}
Fe	3.30±0.14^{d}	11.1±0.61^{b}	19.5±0.29^{a}	4.60±0.32^{c}	205.6±6.39^{B}	338.9±14.95^{A}	11.5±0.60^{C}
Cu	0.25±0.011^{a}	0.19±0.004^{c}	0.22±0.003^{b}	0.23±0.009^{a,b}	0.89±0.007^{B}	0.47±0.012^{C}	0.95±0.020^{A}
Zn	0.64±0.05^{b}	0.46±0.01^{c}	0.72±0.01^{a}	0.64±0.02^{b}	0.93±0.11^{C}	3.73±0.13^{B}	4.53±0.09^{A}
Mn	2.41±0.02^{b}	2.30±0.06^{b}	8.84±0.11^{a}	2.23±0.06^{b}	9.65±2.37^{B}	135.8±5.85^{A}	2.89±0.03^{B}
B	0.69±0.24^{b}	0.74±0.11^{b}	1.28±0.04^{a}	0.58±0.06^{b}	6.09±0.20^{B}	17.06±0.47^{A}	1.95±0.05^{C}

Different letters in the same column mean significant differences between samples ($p < 0.05$)

With the incorporation of algae at 5%, the nutritious balls showed a higher mineral content Table 5 compared to the control, achieving more nutritional claims.

According to Regulation (EU) No 1169/2011, for a product to be a mineral source it must meet 15 % of the nutrient reference values specified and 30% to be considered high mineral content.

The control could have the nutritional claim “high content of Manganese (Mn)”. The snack balls with 5% *Ulva rigida*, *Gracilaria gracilis,* or *Porphyra dioica* could have the claims “high content of Iron (Fe) and Manganese (Mn)”. The 5% *Gracilaria gracilis* could also have the claim “high content of potassium (K)”.

All the nutritious balls are “source of copper (Cu)”. Control is also a “source of K and Fe”. 5% *Ulva rigida* is a “source of Magnesium (Mg) and K” and 5% *Porphyra dioica* is a “source of K”.

The Na/K ratio is related to cardiovascular disease. According to the World Health Organisation (WHO), this ratio can be close to 1. The nutritional balls present a Na/K ratio below this value (1), contributing to a reduction of cardiovascular diseases (Blaustein et al., 2012). The high iron content is an advantage, since iron is one of the components responsible for the formation of haemoglobin and myoglobin, which transports oxygen in the blood (Herrmann & Geisel, 2002).

These results are in agreement with Fradinho et al. (2019) where macroalgae were incorporated into pasta, increasing the mineral composition.

In the study of Neto et al. (2018), the mineral component of *Ulva rigida* and *Gracilaria gracilis* was studied. They obtained similar values, with *Gracilaria gracilis* having a higher mineral value.

Table 6 shows the amount of heavy metals in the nutritious balls and macroalgae.

Table 6: Heavy metals of nutritious balls and macroalgae

	mg/100 g						
	Nutritious balls				Macroalgae		
	Control	*Ulva Rigida* 5%	*Gracilaria Gracilis* 5%	*Porphyra Dioica* 5%	*Ulva Rigida*	*Gracilaria Gracilis*	*Porphyra Dioica*
Pb	0.04±0.005[a]	0.04±0.008[a]	0.05±0.003[a]	0.03±0.004[a]	0.23±0.004[B]	0.37±0.015[A]	0.04±0.009[C]
Cr	0.041±0.003[b]	0.144±0.002[a]	0.129±0.023[a]	0.052±0.003[b]	2.802±0.025[A]	1.774±0.002[B]	0.073±0.006[C]
Ni	0.02±0.004[a]	0.004±0.004[b]	0.00038±0.00003[b]	0.007±0.007[b]	1.262±0.011[A]	0.352±0.117[B]	0.249±0.002[C]
Cd	0.003±0.0006[a]	0.003±0.0001[a]	0.004±0.0001[a]	0.004±0.0013[a]	0.010±0.0003[C]	0.014±0.0002[B]	0.022±0.0002[A]

Different letters in the same column mean significant differences between samples ($p < 0.05$)

The maximum contents of Cadmium (Cd) and Lead (Pb) are established in the Regulation Nº 1881/2006. It is verified for Pb that both the algae and nutritional balls are above the maximum content of 0.02 mg/100 g.

For Cd the values are below the maximum content (0.010 mg/100 g), with the exception of the macroalgae *Gracilaria gracilis* and *Porphyra dioica.*

Since algae presented a value higher than recommended, it was expected that also the gelled balls would present values above the supposed. A future application is the significant reduction of these values, which should be worked out with the production company. However, there may also have been an error in their quantification.

4.6. Total phenolic compounds and antioxidant capacity

Two methodologies were used (DPPH and FRAP assays) to measure the antioxidant activity. Total phenolic compounds (TPC) were determined by the *Folin-Ciocalteu* method (F-C). In the Table 7 are presented the antioxidant activity values of the nutritious balls and the three macroalgae.

Table 7: Total phenol content and antioxidant activity of the nutritious balls and the macroalgae

		DPPH (mg Trolox/g DE)	FRAP (mg Trolox/g DE)	TPC (mg GAE/g DE)
Nutritious balls	**Control**	2.15 ± 0.03[b]	5.53 ± 1.12[a]	2.23 ± 0.23[a,b]
	***Ulva rigida* 5%**	1.98 ± 0.12[b]	5.61 ± 0.73[a]	2.59 ± 0.39[a]
	***Gracilaria gracilis* 5%**	2.22 ± 0.06[b]	6.34 ± 0.25[a]	2.23 ± 0.11[a,b]
	***Porphyra dioica* 5%**	2.99 ± 0.23[a]	4.48 ± 0.46[a]	1.69 ± 0.04[b]
Macroalgae	***Ulva rigida***	5.35 ± 0.13[B]	32.60 ± 2.76[B]	5.24 ± 0.67[B]
	Gracilaria gracilis	13.0 ± 1.47[A]	42.82 ± 1.92[A]	8.87 ± 0.06[A]
	Porphyra dioica	4.98 ± 0.53[B]	8.64 ± 0.66[C]	2.28 ± 0.33[C]

Different letters in the same column mean significant differences between samples ($p < 0.05$)

With DPPH methodology, the snack with *Porphyra dioica* had a higher antioxidant capacity (2.99 mg Trolox/g DE) than the other snacks (control, *Ulva rigida* 5% and *Gracilaria gracilis* 5%). However, *Porphyra dioica* is not the algae with higher value, *Gracilaria gracilis* presented higher antioxidant capacity (DPPH and FRAP) and TPC. Therefore, it can be concluded that some experimental problems may occurred, and that there is no great impact of adding macroalgae in terms of DPPH scavenging power.

With the FRAP assay, despite the statistical difference between the seaweeds, there were no significant differences ($p > 0.05$) between the snacks.

Each methodology measures different compounds. DPPH, by decreasing the absorbance of the 2,2-diphenyl-1-picryl hydrazyl radical, measures its uptake. FRAP measures antioxidant capacity by reducing Fe^{3+} to Fe^{2+}. When reduced, it forms a coloured product (Gülçin, 2012). The difference between the DPPH and FRAP results is due to the different antioxidants present in algae. The antioxidants present in *Gracilaria gracilis* are ficobilins, lutein and fucoxanthin (Baweja et al., 2016) and *Porphyra dioica* is rich in vitamin B and fucoxanthin (Venkatraman & Mehta, 2019). In green algae, as *Ulva rigida*, what represents greater antioxidant power is the presence of lutein, vitamin E and β-carotene (Baweja et al., 2016).

For total phenolic content (TPC), *Porphyra dioica* snack had the lowest value (1.69 mg GAE/g DE), significantly lower ($p < 0.05$) than *Ulva rigida* snack (2.59 mg GAE/g DE), but there are no significative differences between all the other snacks. *Porphyra dioica* algal also presented a lower TPC (2.28 mg GAE/g DE), and *Gracilaria gracilis* had the highest value (8.87 mg GAE/g DE). In Monteiro et al. (2020) study, three different extraction methods were studied to obtain extracts of macroalgae and microalgae, which were evaluated for their phenolic content and antioxidant activity. In this study, also found higher values of TPC for the macroalgal *Gracilaria gracilis*.

Macroalgae induces no great increase in the antioxidant capacity of these snacks, due to the richness in phenolic compounds and other biomolecules of apple and chestnut flours. The apple flour, from the Bravo de Esmofe variety, has a superior antioxidant capacity than other apples, reducing the development of tumour cells (Vilas-boas et al., 2021). In these nutritious balls the chestnut flour was replaced by 5% macroalgae. The chestnut flour is rich in aminoacids (lysine, threonine, aspartic acid and glutamic acid), fibre, vitamin B and E. According to Massantini et al. (2021), where the chestnut quality progress was evaluated, it presented high values of TPC (0.92 mg GAE/g), representing almost half of the TPC value of the energy balls. However, the chestnut´s shell and integument are present in the integral chestnut flour that was used for the gelled balls, providing a high number of phenolics (Vasconcelos et al., 2010), and thus should present a higher TPC value than that obtained by Massantini et al. (2021). This should explain why 5% of substitution had no great effect on antioxidant capacity and TPC of the nutritious microalgae-balls when compared to control.

4.7. Sensory analysis

Figure 13 and Figure 14 show the results obtained from the sensorial analysis of 3% and 5% *Gracilaria gracilis* snacks, which aimed to understand which percentage of incorporation is better accepted by the consumer. Table 8 shows the scores obtained for each parameter, for better perception of the Figure 13.

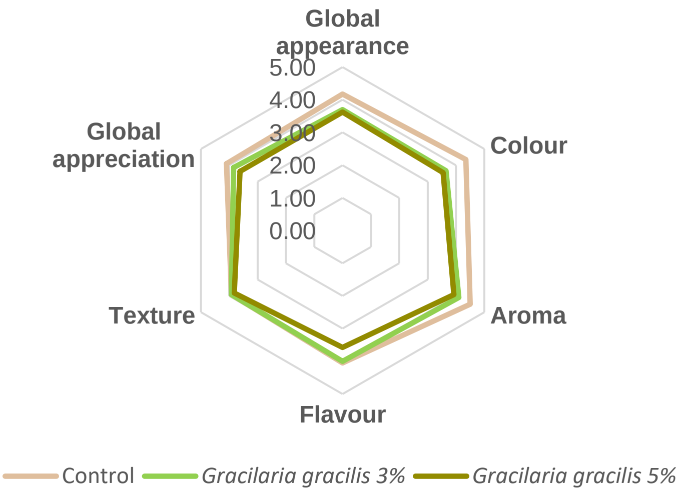


Figure 13: Attributes relating to the sensory analysis of nutritious balls

Table 8: Scores obtained for each parameter

	Control	*Gracilaria gracilis 3%*	*Gracilaria gracilis 5%*
Global appearance	4.18	3.70	3.63
Colour	4.35	3.65	3.55
Aroma	4.50	4.10	3.93
Flavour	4.05	4.00	3.58
Texture	3.93	3.93	3.83
Global appreciation	4.10	3.85	3.63

The highest scoring sample was the control, having a more prominent aroma (4.50), the least liked parameter was the texture, scoring less than 4 (3.93). However, sample with *Gracilaria gracilis* 3% got a similar scored on texture, flavour and aroma.

Sample with *Gracilaria gracilis* 5% was the least appreciated in all parameters. Its lowest value was relative to the colour. The scores obtained for colour attribute of *Gracilaria gracilis* 3% (3.65) and 5% (3.55) are similar.

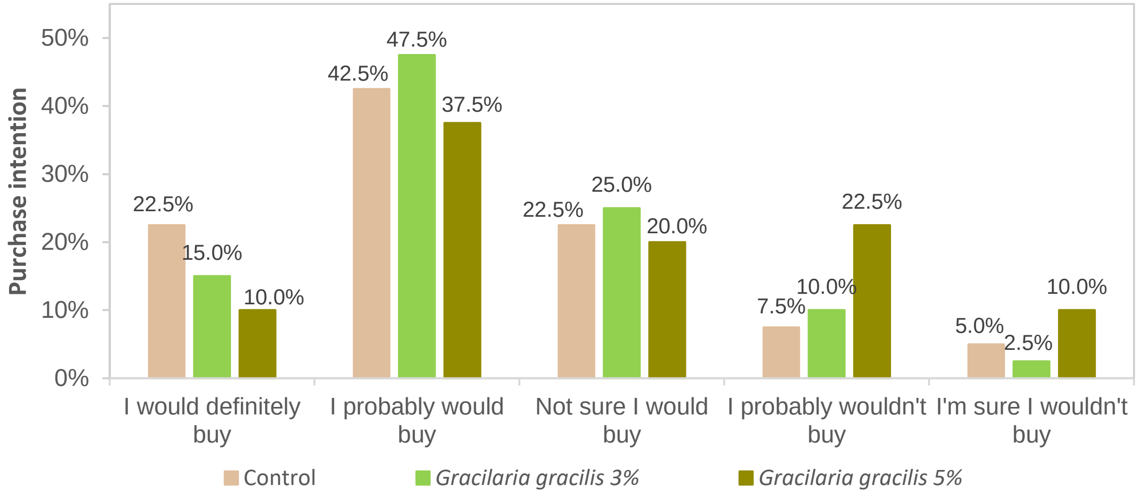


Figure 14: Purchase intention of nutritious balls

Concerning the purchase intention, the highest percentages obtained for all the macroalgae are "I probably would buy", being the highest value in *Gracilaria gracilis* 3% (47.5%).

The sample *Gracilaria gracilis* 5% is the one with the highest percentage value of "I probably wouldn't buy" (22.5%) and "I'm sure I wouldn't buy" (10%).

5. Conclusion

According to the presented results, it was concluded that the addition of macroalgae has impact on properties of the gelled snack balls, namely in terms of nutritional composition and mechanical behaviour.

The texture results showed that as the macroalgae *Porphyra dioica* and *Ulva rigida* are incorporated, snack firmness decreases, indicating that the polysaccharides present in the algae do not have the same structuring effect as starch, which is the main ingredient of the chestnut flour. With *Gracilaria gracilis*, this effect was not shown, due to the agar present in this macroalgae, which is a more structuring polysaccharide.

The rheology results are in close agreement with those of texture, with a significant reduction in G' for high levels (5% and 10%) of *Ulva rigida* and *Porphyra dioica*.

The nutritious balls with macroalgae presented a darker colouration than the control, and total colour differences between all the levels of algae and the control are detected by the human eye.

From the nutritional analysis, it was concluded that the incorporation of macroalgae led to a greater number of nutritional claims. On a mineral level, all the gelled balls are high in iron and the 5% *Gracilaria gracilis* also has high potassium content and the claim "fat-free" can be used.

In general, antioxidant capacity and total phenolic compounds did not increase with the incorporation of the studied macroalgae. This is explained by the richness in phenolic compounds and other biomolecules of apple and chestnut flours, which are already in the control formulation.

From the sensory analysis of *Gracilaria gracilis* snacks and the control sample, it was concluded that the snack product with the highest value in all parameters was the control, but similar scores were achieved by *Gracilaria gracilis* 3% for some attributes (4.10 for control and 3.85 for 3% *Gracilaria gracilis* snack for global appreciation). Moreover, in terms of purchase intention, *Gracilaria gracilis* 3% obtained the highest score.

The obtained results showed that the studied macroalgae can be used as natural innovative and sustainable ingredients to nutritionally enrich snacks using high incorporation levels (1% to 10%), presenting a high number of nutritional claims, namely in terms of mineral content.

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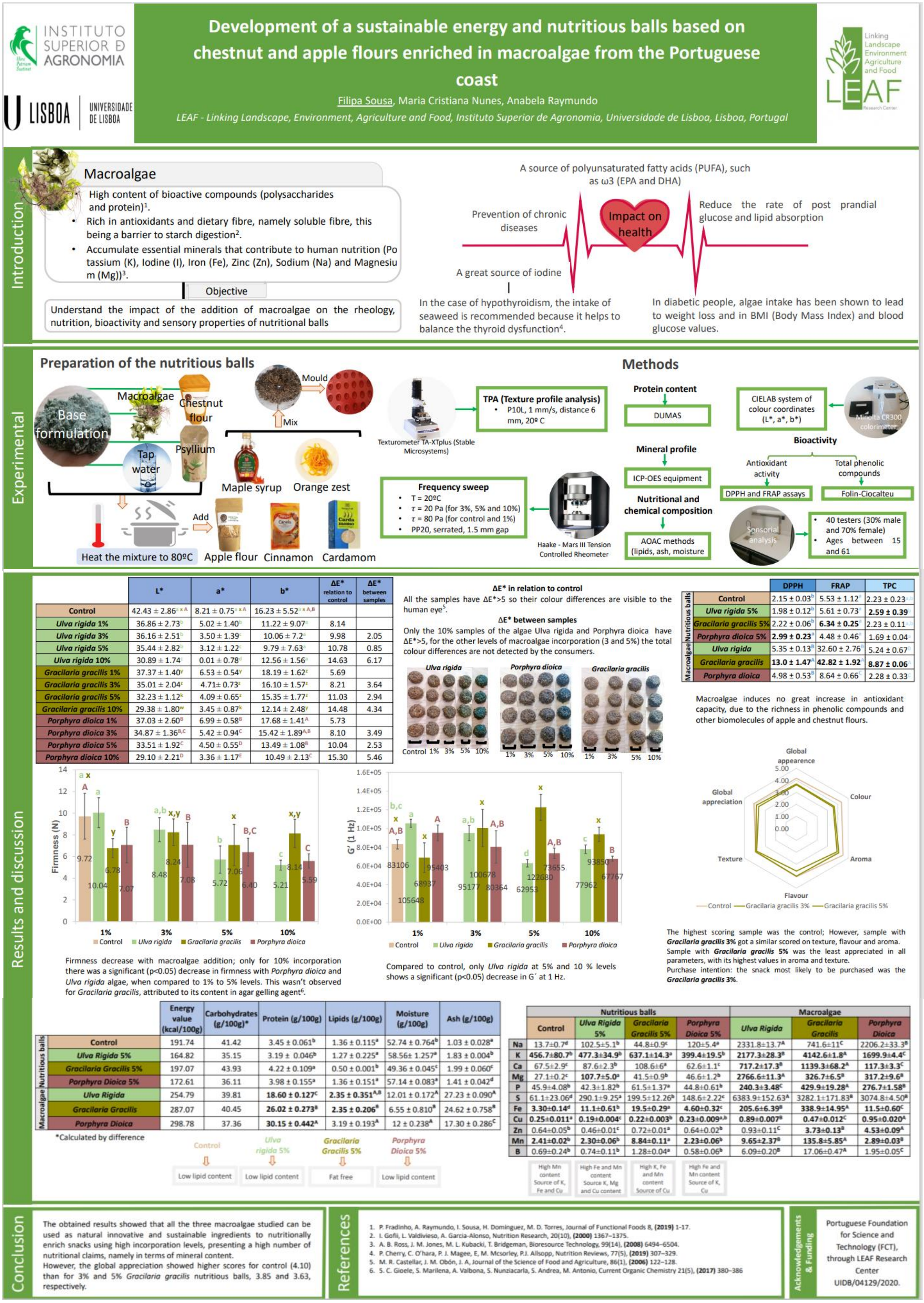
7. Annexes

Annex A - communication in panel



Annex B – Abstract XVEQA

XV ENCONTRO DE QUÍMICA DOS ALIMENTOS

PC-A20: Development of a sustainable energy and nutritious balls based on chestnut and apple flours enriched in macroalgae from the Portuguese coast

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Macroalgae are increasingly being integrated into food, where their incorporation has contributed to an increase in mineral values and fibre[1]. Azores is one of the Portuguese places where its consumption is common and its impact on human health is being studied[2]. This increased interest in their consumption is related to the health benefits they provide, resulting from the high number of bioactive compounds present in its composition, as well as high content in polysaccharides and protein[1-2]. They have great potential to be used as ingredients in different type of food products, despite some concerns related do their sensorial impact, namely flavour, odor and colour.

The present study aimed to develop an innovative gelled snack, in the form of energy and nutritious balls, incorporating three macroalgae from the Portuguese coast: *Porphyra dioica*, *Gracilaria gracilis* (red macroalgae) and *Ulva rigida* (green macroalgal), at different concentrations (1%, 3%, 5% and 10% w/w). The impact of the addition of macroalgae on the rheology, nutrition, bioactivity and sensory properties of the nutritious balls was studied, as well as the maximum level of incorporation, to understand up to what algae content the consumer accepts the product.

The energy balls were based on chestnut and apple flours, obtained from by-products of the fruit industry. These flours are obtained from small calibre fruits which do not comply all quality standards to be market as a fruit. The chestnut flour, the tap water and psyllium powder were heated to 80° C, to promote the starch gelatinization. After the mixture has cooled, the apple flour, maple syrup, cinnamon, cardamom powder, and orange peel are added. After cooling, the gel was placed in 15 mm semispheres moulds and stored in the refrigerator. For the formulations enriched with macroalgae, these were added along with the chestnut flour, psyllium, and water. Psyllium fibre was used as a gelling agent to improve texture and stability, and maple syrup as a natural sweetener, together with apple flour. Besides adding sweetness and being a source of fructose, apple flour contains bioactive compounds such as vitamin C, vitamin E, organic acids (tartaric, malic and citric acids), phenolic compounds (flavonoids), minerals and fibres (pectins, celluloses, hemicelluloses and lignin). Bravo variety is among the apples with the highest antioxidant capacity, leading to a reduction in the risk of developing tumour cells and other diseases[3]. The chestnut is rich in aminoacids (lysine, threonine, aspartic acid and glutamic acid), fibre, vitamin B and E. According to some authors, its addition increases the stability and strength of the gel[4].

The texture was analysed by Texture Profile Analysis (TPA) and small amplitude oscillatory shear (SAOS) measurements carried out in a controlled stress rheometer, to determine the impact of different macroalgae levels in the gel structure. The nutritional and chemical composition was evaluated based on the AOAC methods (lipids, carbohydrates, ash, moisture), and protein content by DUMAs methodology. Bioactivity was accessed by the determination of the total phenolic compounds (*Folin-Ciocalteu*), antioxidant activity (DPPH and FRAP assays), and mineral profile using an ICP-OES equipment. Colour was instrumentally measured using a colorimeter (CIELAB* system) and sensory attributes (colour, smell, taste, texture and general acceptance) of the different snacks were compared using an untrained panel of consumers.

The developed gelled snacks enriched in macroalgae (Figure 1) do not have any additive, assuming a clean label positioning, presenting an appealing green colour and a structure compatible with consumption on the go. In general, the texture results showed a decrease in the firmness of the gelled snack when macroalgae are added. However, only for 10% incorporation there was a significant (p <

0.05) decrease in firmness for *Porphyra Dioica* and *Ulva Rigida* algae, when compared to 1% to 5% levels. This was not observed for *Gracilaria* gracilis, attributed to its content in agar gelling agent[5].

The obtained results showed that all the three macroalgae studied can be used as natural innovative and sustainable ingredients to nutritionally enrich snacks using high incorporation levels, reaching high sensory scores in terms of global appreciation and buying intention. An improvement of the nutritional profile resulting from the macroalgae incorporation was achieved, namely in terms of mineral profile and antioxidant activity.

Figure 4: Snacks – Energy and nutritious balls developed with different types of macroalgae (0%, 1%, 3%, 5% and 10% w/w).

Keywords: Macroalgae, energy balls, snack, texture, bioactivity, sensorial evaluation.

Acknowledgements & Funding: Portuguese Foundation for Science and Technology (FCT), through LEAF Research Center UIDB/04129/2020.

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Annex C – Dare2change congress abstract

By-products from the fruit production and macroalgae from the Portuguese coast: a sustainable partnership to develop nutritious snack balls

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Macroalgae are increasingly integrated in foods due to the health benefits they provide, resulting from the high number of bioactive compounds present in their composition, as well as the high content of polysaccharides and proteins[1-2].

This study aimed to develop an innovative nutritious snack, incorporating three macroalgae from the Portuguese coast: *Porphyra dioica* and *Gracilaria gracilis* (red macroalgae) and *Ulva rigida* (green macroalgal), at different concentrations (1%, 3%, 5% and 10% w/w). The impact of the addition of macroalgae was studied in terms of TPA (Texture Profile Analysis) and rheology behaviour. A colorimeter was used for colour evaluation. Nutritional composition was evaluated by AOAC methods (lipids, ash, moisture) and protein content by DUMAs. Mineral profile, using ICP-OES, was also studied. Bioactivity was accessed by total phenolic compounds (*Folin-Ciocalteu*) and antioxidant activity (DPPH and FRAP assays). Sensory properties (colour, aroma, flavour, texture and general acceptance) were studied, as well as the maximum level of incorporation, to understand the acceptance of the algae in the product.

Healthy-balls are based on chestnut and apple flours, obtained from the by-products of small-calibre fruits that don't meet all the quality standards for market, contributing to the non-waste and sustainability of the product. The use of chestnut flour[3] and psyllium[4] promotes the strength and stability of the gel.

The texture results showed a decrease in the firmness of the gelled snack with *Ulva rigida* and *Porphyra dioica* addition, but for Ulva rigida this effect is significant ($p<0.05$) only with 5% and 10% incorporation. This wasn't observed for *Gracilaria gracilis*, attributed to its content in agar, a structuring polysaccharide[5].

The results showed that the three macroalgae can be used as innovative and sustainable ingredients to nutritionally enrich snacks using high levels of incorporation, with more nutritional claims, particularly in terms of mineral content.

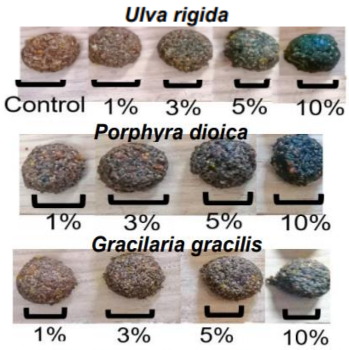

Figure 1: Nutritious snack balls developed with different types of macroalgae (0%, 1%, 3%, 5% and 10% w/w).

Keywords: Macroalgae, nutritious balls, snack, texture, bioactivity, sensorial evaluation.

Acknowledgments: Portuguese Foundation for Science and Technology (FCT), through LEAF Research Center UIDB/04129/2020.

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