Impact of ethanol treatment on the technological characteristics, nutritional composition, and bioactivity of gluten-free breads produced with different microalgae

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ABSTRACT

Consumption of gluten-free products has increased considerably in recent years. However, the substitution of gluten has been a challenge (Wang et al., 2017). On the other hand, microalgae can be considered one of the most promising functional food sources, as they have the potential to be a sustainable solution, but there are still improvements to become a regular source of food (Torres-Tiji et al., 2020). In the present research, microalgal biomasses were subjected to ethanol extraction to obtain less pronounced colours and flavours, with the purpose to increase consumer acceptance. The incorporation of microalgae in food can lead to changes in the rheology, texture, sensory properties, and nutritional composition (Nunes et al., 2020a; Nunes et al., 2020b). The objective of this study was to compare the impact of adding raw and ethanol treated *Tetraselmis chuii*, *Chlorella vulgaris* and *Nannochloropsis gaditana* in dough structure and technological aptitude, nutritional composition, and bioactivity of gluten-free bread.

The technological performance of the doughs was studied according to the rheological properties (torque, water absorption, development time, stability, softening, creep and recovery, frequency sweep, and viscosity). Firmness, cohesiveness, colour, and volume was also evaluated. The nutritional and chemical composition was evaluated based on the AOAC methods (proteins, lipids, carbohydrates, ashes, moisture, and minerals), and the bioactivity by determination of the total phenolic compounds, antioxidant activity and pigments (chlorophyll-a, chlorophyll-b, and carotenoids). For the sensory analysis, only the control and breads with incorporation of *Chlorella vulgaris* were tested.

The obtained results evidence that the treatment with ethanol is an interesting option to incorporate microalgae in food. Improvements in terms of bread texture, volume and sensory acceptance accompanied by an enriched nutritional composition were observed. This finding indicates that ethanol treatment might be a viable strategy for producing gluten-free bread of high nutritional value with greater consumer acceptance.

Keywords: gluten-free bread, microalgae, ethanol treatment, bioactivity, nutrition

RESUMO

O consumo de produtos sem glúten aumentou consideravelmente nos últimos anos, mas a substituição do glúten tem sido um desafio (Wang et al., 2017). Em relação às microalgas, estas podem ser consideradas uma das fontes alimentares funcionais mais promissoras, pois têm potencial para ser uma solução sustentável, mas ainda existem melhorias para se tornarem uma fonte regular de alimentos (Torres-Tiji et al., 2020). Neste estudo, as biomassas de microalgas foram submetidas a extração com etanol para obtenção de cores e sabores menos pronunciados, com o objetivo de aumentar a aceitação pelo consumidor. A incorporação de microalgas nos alimentos pode levar a mudanças na reologia, textura, propriedades sensoriais e composição nutricional (Nunes et al., 2020a; Nunes et al., 2020b). Assim, o objetivo foi comparar o impacto da adição de *Tetraselmis chuii*, *Chlorella vulgaris* e *Nannochloropsis gaditana*, ao natural e tratadas com etanol, na estrutura e aptidão tecnológica, composição nutricional e bioatividade de pães sem glúten.

O desempenho tecnológico das massas foi estudado de acordo com as propriedades reológicas. A firmeza, a coesividade, a cor e o volume também foram avaliados. As composições nutricional e química foram avaliadas com base nos métodos AOAC (proteínas, lipídios, carboidratos, cinzas, humidade e minerais), e a bioatividade pela determinação dos compostos fenólicos totais, atividade antioxidante e pigmentos (clorofila-a, clorofila-b e carotenóides). Para a análise sensorial, foram avaliados apenas o pão controlo e pães com incorporação de *Chlorella vulgaris*.

Os resultados obtidos evidenciaram que o tratamento com etanol é uma opção interessante para incorporar microalgas nos alimentos. Foram observadas melhorias em termos de textura, volume e aceitação sensorial dos pães, acompanhadas de uma composição nutricional enriquecida. Esta conclusão indica que o tratamento com etanol pode ser uma estratégia viável para a produção de pães sem glúten de alto valor nutricional e com maior aceitação pelo consumidor.

Palavras-chave: pão isento de glúten, microalgas, tratamento com etanol, bioatividade, nutrição

RESUMO ALARGADO

O tema abordado nesta dissertação foi desenvolvido no âmbito do projeto Algae to Future (Research Council of Norway's BIONÆR Programme project no: 267872/E50 A2F), que é financiado pelo Conselho de Pesquisa da Noruega e tem como objetivo estudar o potencial das microalgas para produzir proteínas de alta qualidade, ácidos gordos polinsaturados e hidratos de carbono de baixo carbono, e também como ingredientes saudáveis para alimentação no futuro. Algae to Future estabelece uma base para a produção industrial de microalgas na Noruega, utilizando recursos naturais e subprodutos de fontes existentes para a agricultura, aquicultura e indústria de processamento (Borgvang, 2021). Este projeto conta com a participação de 26 parceiros internacionais, incluindo a Universidade de Lisboa, que é responsável pelo desenvolvimento de pães de trigo e pães sem glúten com incorporação de microalgas.

O consumo de produtos isentos de glúten, principalmente pão, tem vindo a aumentar consideravelmente nos últimos anos, o que não se deve apenas ao aumento de doentes celíacos, mas também ao aumento do número de consumidores que não foram diagnosticados com doença celíaca, mas estão a eliminar o glúten da dieta. No entanto, a substituição do glúten nos produtos da panificação é um desafio, pois não existe nenhuma matéria-prima ou ingrediente capaz de substituir completamente o glúten em termos de construtor estrutural (Wang et al., 2017). Desta forma, os hidrocolóides são frequentemente usados como agentes espessantes, ligando-se às moléculas de água e aumentando a viscosidade da massa. Assim, é possível obter um produto final, neste caso o pão, com melhor volume, textura e com a qualidade pretendida (Mir et al., 2016). No presente estudo, o hidrocolóide utilizado foi a hidroxipropilmetilcelulose (HPMC), que é um polímero solúvel em água com propriedades únicas, sendo um componente importante e um dos mais utilizados no fabrico de pão isento de glúten, devido aos seus efeitos promissores na qualidade do produto final (Hager & Arendt, 2013; Mir et al., 2016).

Em relação às microalgas, estas podem ser consideradas uma das fontes alimentares funcionais mais promissoras, pois têm o potencial de ser uma solução sustentável para alimentos. No entanto, ainda existem melhorias a serem feitas antes que as microalgas se tornem uma fonte regular de alimentos (Torres-Tiji et al., 2020). As microalgas são recursos proteicos excecionais com potencial para se tornarem um alimento básico para os consumidores em todo o planeta, mas que têm um elevado impacto nas características sensoriais (odor e sabor) dos produtos onde são incorporadas, o que limita o seu nível de incorporação (Nunes et al., 2020b). Para além disto, são também ricas em ácidos gordos, principalmente ómega-3 e em diversos compostos bioativos, como é o caso dos polifenóis e carotenoides (Pina-Pérez et al., 2017). A incorporação de microalgas em alimentos pode levar a mudanças nas propriedades reológicas, textura, bioatividade, propriedades sensoriais e na composição nutricional (Nunes et al., 2020a; Nunes et al., 2020b).

No presente estudo, a biomassa das microalgas *Tetraselmis chuii*, *Chlorella vulgaris* e *Nannochloropsis gaditana* foi submetida a extração com etanol de modo a atenuar a sua cor, aroma e sabor. Procedeu-se à sua incorporação em pães com cores e sabores menos pronunciados, com o objetivo de aumentar a aceitação pelo consumidor e permitir um aumento dos níveis de incorporação.

No entanto, é importante também avaliar se este tratamento com etanol não eliminou completamente alguns componentes essenciais que poderiam melhorar a qualidade do pão sem glúten, principalmente ao nível da composição nutricional e bioatividade. Entre esses componentes estão, por exemplo, os minerais, as cinzas, os compostos fenólicos e os antioxidantes. Posto isto, o objetivo deste estudo foi comparar o impacto da incorporação de 4% de biomassa das microalgas *Tetraselmis chuii*, *Chlorella vulgaris* e *Nannochloropsis gaditana*, submetida a tratamento com etanol e não tratada, na estrutura e aptidão tecnológica da massa, composição nutricional e bioatividade de pães sem glúten.

A formulação da massa controlo foi elaborada a partir de uma formulação já otimizada num estudo anterior (Nunes et al., 2020b) com uma mistura de farinha de trigo sarraceno, farinha de arroz, fécula de batata, levedura, açúcar, sal, hidroxipropilmetilcelulose, óleo de girassol e água destilada. Para a formulação dos pães com incorporação de microalgas (tratadas com etanol e não tratadas), a quantidade destes ingredientes foi ajustada de acordo com as características das mesmas, sendo que, por exemplo, foi necessário adicionar menor quantidade de água aos pães com incorporação de microalgas não tratadas. Durante o processo de fabrico, em primeiro lugar, a água foi aquecida até 37ºC num termoprocessador, de forma a obter a temperatura ideal para a ativação da levedura. De seguida foi adicionada a levedura e o açúcar para esta ser ativada, e por fim foi adicionada a mistura dos restantes ingredientes sólidos com o óleo de girassol. Ao fim de 10 minutos no termoprocessador a massa foi colocada numa forma retangular para posterior fermentação e cozedura.

Desta forma, o desempenho tecnológico das massas isentas de glúten foi estudado de acordo com as propriedades reológicas no Microdough-Lab, onde foram avaliados os seguintes parâmetros: torque, absorção de água, tempo de desenvolvimento, estabilidade e suavidade. Esta avaliação das massas continuou no reómetro onde foram realizados testes de varrimento de tensão e de varrimento de frequência, sendo também avaliada a viscosidade. Também foi caracterizada a textura, mais especificamente a firmeza e coesividade, a cor e o volume dos pães. A avaliação da composição nutricional foi realizada com base nos métodos AOAC (proteínas, lipídios, hidratos de carbono, cinzas, humidade e minerais), e a bioatividade através da determinação dos compostos fenólicos totais (Folin-Ciocalteu), atividade antioxidante (pelos métodos DPPH e FRAP) e a quantidade de pigmentos totais (clorofila-a, clorofila-b e carotenoides).

No final, para estudar a aceitabilidade e intenção de compra dos pães isentos de glúten pelos consumidores, foi realizada uma prova de análise sensorial por um painel de 33 provadores não treinados. Devido à situação pandémica vivida nos últimos meses, esta prova de análise sensorial teve que ser ajustada pois, para além das provas realizadas na sala de provas do Instituto Superior de Agronomia, foi necessário realizar algumas provas em casa. As amostras foram identificadas e embaladas corretamente, e os provadores tiveram disponíveis todas as indicações necessárias para a correta realização da prova. Apenas os pães controlo e com incorporação de *Chlorella vulgaris*, não tratada e tratada com etanol foram analisados, pois, entre as três microalgas utilizadas, esta é a única aprovada pela Autoridade Europeia de Segurança Alimentar (EFSA) para consumo humano.

Os resultados obtidos evidenciaram que o tratamento com etanol é uma opção interessante para incorporar microalgas nos alimentos. Este tratamento permitiu a produção de pães sem glúten com cor e sabor mais agradáveis e com melhor aceitação sensorial, acompanhada de uma composição nutricional enriquecida. Verificou-se que o conteúdo de lípidos foi superior nos pães com microalgas tratadas com etanol, o que indica que este tratamento pode ser uma forma de inserir as microalgas na alimentação humana. Isto porque percebeu-se que estas tiveram um impacto positivo nos pães sem glúten e muitos dos componentes essenciais não foram eliminados, como é o caso dos compostos fenólicos, minerais e ácidos gordos. Para além disto, também foram observadas melhorias em termos de textura e volume dos pães, quer pela incorporação de microalgas tratadas com etanol, quer no caso das microalgas não tratadas. O pré-tratamento das microalgas com etanol melhora em grande medida as propriedades sensoriais dos pães sem glúten, sendo que, de um modo geral, os consumidores preferiram o pão com incorporação de *Chlorella vulgaris* tratada com etanol. Isto indica que este tratamento ou outro tratamento semelhante visando a eliminação dos constituintes solúveis do etanol e consequentemente o enriquecimento das proteínas das algas, pode ser uma estratégia viável para a produção de pães sem glúten de alto valor nutricional com maior aceitação pelo consumidor.

Palavras-chave: pão isento de glúten, microalgas, tratamento com etanol, bioatividade, nutrição

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a* – hue red/ green

A2F – Algae to Future

Abs – Absorbance

ANOVA – Analysis of variance

b* – hue yellow/ blue

CD – Celiac disease

CvR – *Chlorella vulgaris* raw

CvT – *Chlorella vulgaris* ethanol treated

DDT – Dough development time

DHA – Docosahexaenoic acid

DPPH – 2,2-difenil-1-picrilhidrazil

EFSA – European Food Safety Authority

EPA – Eicosapentaenoic acid

FDA – Food and Drug Administration

FRAP – Ferric Reducing Ability of Plasma

G' – Elastic modulus

G'' – Viscous modulus

GF – Gluten-free

GFB – Gluten-free bread

HDL – High-density lipoprotein

HPMC – Hydroxypropyl methylcellulose

L* – Luminosity

LDL – Low-density lipoprotein

NgR – *Nanocloropsis gaditana* raw

NgT – *Nanocloropsis gaditana* ethanol treated

RDV – Recommended Daily Values

TcR – *Tetraselmis chuii* raw

TcT – *Tetraselmis chui* ethanol treated

TPA – Texture Profile Analysis

WA – Water absorption

1. INTRODUCTION

The present work was developed to obtain a master's degree in Food Engineering within the scope of the project “Algae to Future (A2F)”, supported by the Research Council of Norway, that addresses the potential of microalgae to produce high-quality proteins, polyunsaturated fatty acids, and low-carbon carbohydrates, as healthy ingredients for food in the future. This Research Council establishes a basis for the industrial production of microalgae in Norway, using natural resources and by-products from existing sources for agriculture, aquaculture, and processing industry (Borgvang, 2021). This project has the involvement of 26 international partners, including the University of Lisbon, that is responsible for the development of wheat breads and gluten-free breads (GFB) with the incorporation of microalgae (Nunes et al., 2020a; Nunes et al., 2020b), involving several tasks from the characterization of the biomass produced in the project, through the use of GFB formulations, to its potential and impact on health and final validation in sensory analysis.

The objective of this dissertation is to evaluate the impact of adding microalgae *Tetraselmis chuii, Chlorella vulgaris* and *Nannochloropsis gaditana* in dough structure and technological aptitude, nutritional composition, and bioactivity of GFB. The microalgae were tested at 4% (w/w) of incorporation, and the breads selected based on the results obtained were analysed using a consumer panel, to assess acceptability and buying intention. In addition to this project being important because it is GFB and essential to provide more variety of foodstuffs suitable for celiac patients, it is also innovative due to the incorporation of microalgae in the bread dough.

Microalgae biomass as food ingredients are sustainable, as it involves a reduced carbon footprint in its production process and has several interesting compounds such as antioxidants, phenolic compounds, proteins, and fatty acids. Microalgae can be considered one of the most promising functional food sources (Nunes et al., 2020b), as they have the potential to be a sustainable solution for food, but further development is needed to insert them into conventional food production. Although microalgae present several strengths for sustainability, there are still improvements to be made before this becomes a regular source of food, such as the development of agricultural products, production scale, evaluating and perhaps increasing nutrient content, optimizing yields, and developing organoleptic characteristics enhanced so that they are attractive to the palate (Torres-Tiji et al., 2020). Microalgae are exceptional protein resources with the potential to become a staple food for consumers across the planet. When considering microalgae as the food of the future, it is also important to consider that there is a technological limit to the incorporation of microalgae, resulting from its impact on the food structure, which can be followed by a change in behaviour rheology. The introduction of microalgae biomass impart changes in the structure of food, but also in its colour and flavour. Consumers are very sensitive to changes in sensory characteristics, which induces limitations in the level of incorporation of microalgae in food (Nunes et al., 2020b).

The microalgae used were *Tetraselmis chuii*, *Chlorella vulgaris* and *Nannochloropsis gaditana*, and both have identical characteristics, yet they differ in others. *Tetraselmis chuii* has a high protein content, which is an important requirement for use in breads with a specific nutritional profile (Nunes et al.,

2020b). *Chlorella* species are often marketed as "healthy foods" and are being promoted to functional foods, used to prevent, or cure some diseases. And finally, *Nannochloropsis gaditana* is considered very promising, because it can be used for industrial applications due to its ability to accumulate proteins, lipids, and high levels of polyunsaturated fatty acids (Khemiri et al., 2020).

Therefore, it would be interesting to incorporate these microalgae in food, in this case, in GFB. Bread is a staple food with specific characteristics in terms of developing the elastic structure of the dough. As gluten confers unique rheological properties to products cooked with yeast, the absence of gluten is a major technological drawback. That said, the addition of microalgae with a high protein content may be important for the development of GFB and for the nutritional benefits to be achieved, which is particularly important in GFB, as celiac have nutritional deficiencies due to their absorption limitations (Nunes et al., 2020b).

The market for gluten-free (GF) products is growing and improving its quality even more (Capriles et al., 2016). Consumption of GF products, particularly bread, has increased considerably in recent times, which is not only due to the increase in Celiac Disease (CD), but also to the increase in the number of consumers who have not been diagnosed with CD, but are eliminating gluten from their diet (Nunes et al., 2020b). CD is an autoimmune disease and its worldwide one of the most common lifelong ones of the small intestine (Mir et al., 2016). This growth of consumption is mainly since consumers avoid gluten because they believe that GF products are a healthier option and that a GF diet is an effective way to lose weight, although there is no scientific evidence of that (Capriles et al., 2016). At present of GF diet is the only option to eliminate all the symptoms of celiac patients, it becomes essential to develop functional food that provide all necessary nutritional levels.

The lack of gluten has a critical effect on the rheology of the bread dough (compromised dough elasticity), process formulation and sensory quality of the final product. The substitution of gluten in bread-making is a challenge, as there is no raw material or ingredient capable of completely replacing gluten in terms of structural builder. Only the combination of different ingredients and their interactions, with appropriate technologies, can improve the quality of GFB (Martins et al., 2020). The technological challenge increases according to the dependence of the properties of the products in gluten, which is considerable in the manufacture of breads, which are the most studied products among all GF products (Capriles et al., 2016). Hydrocolloids are often used as a thickening agent, binding water, and increasing the viscosity of the dough, for better volume, texture, and final bread quality (Wang et al., 2017). In this study, hydroxypropyl methylcellulose (HPMC) was used as a thickening agent to replace gluten.

The relevance of this study lays on the need of providing a wider GFB variety suitable for celiac patients and brings innovation through the incorporation of microalgae in the bread dough.

Part of the results presented in the present dissertation were included in the following abstract that was presented to the scientific congress – XV Congresso de Química dos Alimentos (https://xveqa.events.chemistry.pt):

- Qazi, M. W., Sousa, I. G., Nunes, M. C., Raymundo, A. (2021). The effect of the microalgae *Chlorella vulgaris*, *Tetraselmis chuii* and *Nannochloropsis gaditana* on technological aptitude, nutritional composition, and bioactivity of gluten-free breads. (Funchal, Portugal, 5-8 September). Flash Oral Communication (FCO-08).

In addition, the following paper is currently being submitted to an international ISI journal:

- Qazi, M. W., Sousa, I. G., Nunes, M. C., Raymundo, A. (2021). Improving the nutritional and sensory properties of gluten-free bread using different species of microalgae: impact of ethanol bleaching. Submitted to Food Structure.

2. THEORETICAL FRAMEWORK

Due to the existence of several diseases and food intolerances, it is important to be constantly concerned about adequate food alternatives and develop innovative foods, with attractive characteristics to consumers and according with food trends. Thus, the incorporation of microalgae can be an excellent option since they are sustainable and have an interesting nutritional composition. Microalgae present beneficial properties to human health, from antioxidant activity to having polyunsaturated fatty acids and high content of proteins.

Before explaining all the methods used and results obtained, it is necessary to make a theoretical framework. Thus, bibliographic research was carried out on the importance of developing foods that can be consumed by celiac patients. In addition, it is also intended to understand the impact of the incorporation of microalgae in GFB, so it was necessary to research more about the components that these have in its constitution.

2.1. GLUTEN

Gluten is a set of insoluble proteins found in cereals such as oats, wheat, rye, and barley, and it has an indispensable function in the growth, development, and maintenance of the organism. It is composed of gliadins and glutenins, which are the main constituents of wheat, and is one of the most important ingredients in baking, being responsible for the viscoelastic behaviour of the dough (Martins et al., 2020). Gliadin corresponds to 70% of the ethanol-soluble protein fraction of wheat flour and is essentially present in wheat grain extracts, while glutenin is the protein fraction that cannot be extracted with water, dilute salt solutions and 70% ethanol. Both are important for the formation of the network and the quality of the final product. Although the exact structure and interactions of this protein network are still under debate, it is widely accepted that gliadin has a viscosity-increasing effect, while the elastic properties of the network and the wheat flour dough come predominantly from the glutenin fraction (El Khoury et al., 2018). The gluten matrix plays an important role in the extensibility, elongation resistance, mixing tolerance and gas holding capacity of the dough. In this way, it allows to obtain a high quality bread structure and has unique networking properties, which are important for bakery products (El Khoury et al., 2018; Martins et al., 2020). GF dough is unable to form a network of proteins similar to gluten (Martins et al., 2020).

The problem arises when food intolerance occurs, because in some people gluten causes an unusual reaction when it meets the small intestinal mucosa, since gluten is a major factor in inducing CD, although genetic factors are likely to play an important role in the onset of the disease (Lindfors et al., 2019).

As the popularity of the GF diet is increasing, the consumer demand continues to influence the food market and product labelling standards. Thus, in 2013, European Union Regulation No. 609/2013 established rules on composition and labelling requirements for GF products, ensuring that gluten intolerant individuals are informed about the difference between naturally GF foods and foods which are

produced or processed to eliminate/ reduce the gluten content. The Food and Drug Administration (FDA) has also ruled that products labelled "gluten-free" cannot exceed the 20 parts per million limits. As some products may contain hidden gluten, product labels and ingredient lists need to be carefully inspected, so for traditional foods with gluten, such as baked products, there are currently a wide variety of GF options available that use GF cereals (El Khoury et al., 2018).

2.2. CELIAC DISEASE

CD is derived from lifelong gluten intolerance in individuals who are genetically predisposed. This disease is recognized as an autoimmune disease, being a disorder of the gastrointestinal tract in which gluten ingestion leads to atrophy the small intestine mucosa by an immunologically mediated mechanism (Naqash et al., 2017). CD is one of the most common lifelong diseases worldwide of small intestine villi that absorbs nutrients due to an immune reaction to gluten resulting in mucosal damage and generalized nutrients malabsorption (Mir et al., 2016). The elimination of gluten from the diet is the only way to prevent the caused injuries and allows the body to recover. However, whenever there is consumption of gluten, the inflammations return, and the symptoms reappear (Naqash et al., 2017).

Although this disease mainly affected children, the diagnose has been increasingly common in adults. However, even if there is clearly a hereditary predisposition, the reason why only a few people develop CD is still not fully understood. That said, it is possible to state that CD is originated from the interaction of environmental, genetic, and immunological factors (Nascimento et al., 2012). Patients have severe disruption of the mucous layer of the small intestine, with several symptoms such as weight loss, diarrhoea, and abdominal cramps. Inflammation of the small intestine negatively affects the absorption of some vitamins and minerals (iron, magnesium, calcium and zinc), therefore, it is mandatory for patients with CD to adhere to a GF dietary supplement with nutrients (Javaria et al., 2016). CD is characterized by a wide variety of symptoms, the most common of which vary according to age. They emerge usually in childhood, being able to or not affect in adolescence, coming to reappear later at 30 or 40 years old. Therefore, celiac patients can be divided into two groups, according to the symptoms they present:

- Classic symptoms (usually children) – such as, chronic diarrhoea, abdominal distension, abdominal cramps, slimming or anorexia, malnutrition e growth retardation.
- Non-classic symptoms (usually adults) – such as, ferropenic anaemia, abdominal distension, oedemas, infertility and recurrent miscarriages, neurological and psychiatric changes (Nascimento et al., 2012).

Several studies, in Portuguese and European scope, have identified that only 1% of the population has CD. In response to gluten ingestion, patients show inflammation characterized by loss of absorptive villi and crypt hyperplasia (**Figure 1**). Symptoms of gluten sensitivity disappear after gluten is removed from the diet, currently only the GF diet as an effective treatment for these individuals. However, the

development of GF products for celiac patients is not only a pressing need, but also a demanding job (Naqash et al., 2017).

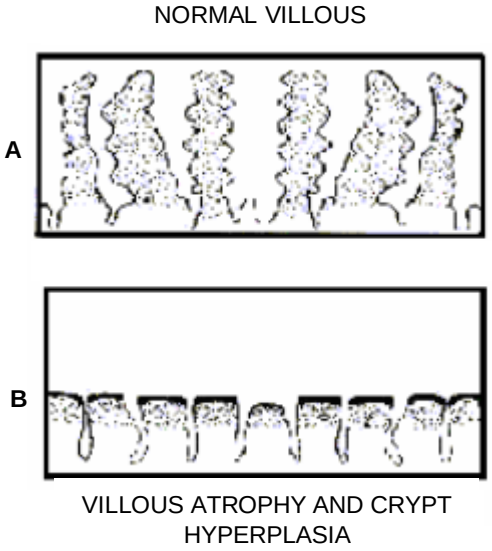


FIGURE 1 - Representation of a normal villous (A) comparing to a villous of a celiac patient (A). Source: adapted from Acelbra, 2021.

For individuals with CD and gluten sensitivity, the GF food market segment is fundamental for their diet. However, there is still difficulty to find GF products due to high prices in the market, compared to regular products, limited variety and poor availability and sensory properties. Thus, improving the nutritional quality of GF products remains an important task for research and development (Capriles et al., 2016).

Over time, regular clinical surveillance of the celiac patients is suggested, with monitoring of clinical/nutritional and laboratory parameters, appropriate to everyone. Current international Codex Alimentarius Commission has established a maximum content of prolamins (20mg/kg) allowed for a product to be considered GF (FAO, 2008) and safe for celiac. In this way, it is essential to ensure a varied and balanced diet, not restricting itself to products labelled as “gluten-free”. All GF foods should have the symbol shown in **Figure 2**, established by the certifying entity BIOTRAB. BIOTRAB has a GF program that, after carrying out an entire audit process, grants a Certificate of Conformity which indicates that the company has a food quality and safety system that allows the APC seal – BIOTRAB, safe food without gluten (BIOTRAB, 2021).

FIGURE 2 - Symbol used on labelling to refer to gluten-free products. Source: adapted from APC, 2021 (https://www.celiacos.org.pt/como-certificar-o-seu-estabelecimento/).

Taking all this information into account, one is sure that it is increasingly important to develop foods that can be consumed by celiac patients and that are, at the same time, attractive to the consumers.

2.3. GLUTEN-FREE BREAD

Bread is one of the most consumed foods and for some populations that remains an important source of energy as it is mainly consumed as the main source of calories in the diet. Gluten is one of the most important ingredients in bakery industry, being responsible for the viscoelastic behaviour of the dough. It has an important role in the extensibility, resistance to elongation, tolerance to the mixture and capacity of gas retention of the dough. GF dough is unable to form a protein network like gluten, so there are difficulties in producing GFB. Removing gluten creates a less cohesive and elastic dough so, the substitution of gluten in baking is a challenge, as there is no other ingredient capable of completely replace gluten, in terms of a structural builder. However, through the combination of different ingredients and their interactions, and with the use of appropriate technologies, it is possible to improve the quality of GFB (Naqash et al., 2017). The production of high-quality GFB made from different ingredients than wheat flour bread represents an important technological challenge. Also, it is why it is necessary to develop GFB with consumer acceptance (Mir et al., 2016). In production of GFB, it is difficult to replicate the aroma, texture, and flavour similar to traditional bread with gluten, since these are devoid of inherent texture formation events and aroma compounds produced in gluten-based products. Also, deficient gas retention and the resulting low volume of bread are the main challenges confronted, and the lack of gluten also leads to a liquid, which in turn results in crumbled baked bread, poor colour, and post-baked quality defects (Naqash et al., 2017). Therefore, bread is one of the most challenging food products when making GF alternatives (El Khoury et al., 2018).

In addition, GF products are often consumed by people who have had the opportunity to taste gluten-containing foods, so they already have product requirements and expectations in terms of texture, structure, flavour, and overall quality. Different ingredients and processing strategies are applied focusing on the texture, structure, and volume of GF bakery products (El Khoury et al., 2018). There has been an increasing need to overcome the problems related to the exclusion of gluten from breads and at the same time, to meet the expectations of celiac patients. Also, GFB are characterized by having poor crumb and crust characteristics, as well as a bad feeling and taste in the mouth. Since these products contain starch, they are deficient in other nutrients. Defects commonly found with GFB arise

due to inefficient expansion and gas retention during yeast, resulting in reduced volume bread having stiffer crumb. Such products also do not exhibit the rheological, textural, and cooking properties of the exclusive quality of products containing gluten. The viscoelastic properties of GFB have been improved by the addition of hydrocolloids (Naqash et al., 2017).

Next, it will be explained why it is so important to use hydrocolloids in the manufacture of GFB, as well as their main characteristics, and the three phases of the bread's manufacturing process.

2.3.1. FLOURS AND STARCH

In recent years, several products have been used in manufacturing process of GFB to replace flours that contain gluten, such as buckwheat and rice flour. Usually, these flours are used together with other additives and techniques in order to improve the physicochemical properties, the acceptability by the consumer and the shelf life of GF doughs and breads. Starches, on the other hand, play an important role in baking processes as, during the baking of bread, starch granules gelatinize and have the ability to retain air bubbles, facilitating gas retention during the fermentation stage (Wang et al., 2017).

In this study, buckwheat and rice flours were used. Buckwheat flour is rich in amino acids and has a higher amount of fibre, lysine, manganese, phosphorus, copper, and magnesium than other cereals. The main component of this flour is the starch, accumulated in the endosperm. Over time, buckwheat flour has been used for its potential therapeutic actions, as its consumption is often associated with lower cholesterol levels and glycaemic control in diabetes and obesity (Motta, 2015). Rice flour has functional properties to improve texture and whiteness, and its main components are proteins and starch. It has characteristic rheological properties that can play an important role in processing control, food texture estimation and thermal processing (Hur et al., 2011).

Potato starch was also used as ingredient in the GFB which has a high content of phosphorus esterified with some of the glucose units of the amylopectin (Neeraj et al., 2020). This starch increases gel strength more than any other native starch (Hur et al., 2011). Potato starch is unique compared to cereal starches, since it has larger granule size and purity, longer amylose and amylopectin chain length, presence of phosphate ester groups on amylopectin, ability to exchange certain cations with corresponding effects on viscosity behaviour, ability to form a thick viscoelastic gel. Potato starch had certain limitations such as low shear resistance, thermal resistance, thermal decomposition, and high tendency towards retrogradation. This behaviour limits its use in some industrial food applications (Neeraj et al., 2020).

2.3.2. HYDROCOLLOIDS

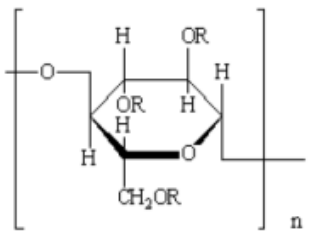
The quality of GFB is mainly influenced by the nature, content, and properties of hydrocolloids, which increase viscosity, flocculation, and coalescence of the dough. However, the effect of hydrocolloids changes depending on the other ingredients used during the production of GFB (Mir et

al., 2016). Hydrocolloids are compounds with a high molecular weight, containing a hydrophilic chain, often capable of binding firmly to large amounts of water and, thus, prolonging the shelf life of the product. They are applied as ingredients in the food industry to improve the texture and flavour mainly of bread (Padalino et al., 2016). They consist on a series of water-soluble polysaccharides with varied chemical structures, providing a wide range of functional properties that make them suitable for different applications in the bread industry, and are used as structuring agents to replicate the viscoelastic properties of gluten (Mir et al., 2016). When hydrocolloids interact with water during the bread-making process, they produce a gel network that increases the viscosity of the dough and strengthens the limits of expanding cells, increasing the gas holding capacity during baking, improving bread volume, structure, texture, and appearance, producing GFB with very high baking properties and quality (Padalino et al., 2016). Investigations into GF products, especially bread, have focused on improving technological parameters, including crumb volume and firmness, in addition to sensory perception (Mir et al., 2016).

In addition, hydrocolloids may be the easiest way to increase the dietary fibre content in GF bakery products (Padalino et al., 2016), and have also been used to improve texture, due to its ability to make a gel in small quantities that provide high consistency at room temperature, to increase moisture retention and to improve the overall quality properties of bread (Mir et al., 2016).

2.3.2.1. HYDROXYPROPYL METHYLCELLULOSE

HPMC (**Figure 3**) is a methyl cellulose modified by treatment with alkali and propylene oxide by which a small number of 2-hydroxypropyl groups are attached through ether links to the anhydro glucose units of the cellulose (FAO, 2011). It is a water-soluble polymer with unique properties about its hydration characteristic in solution as well as during temperature changes. During gelation, HPMC is able to form stronger hydrophobic bonds with other HPMC chains, resulting in stronger gel networks at higher temperatures (Hager & Arendt, 2013). In addition, the water level was also varied as it is well known that hydrocolloids can bind large amounts of water due to their high water-binding capacity, which can decrease starch gelatinization due to competition for available water (Hager & Arendt, 2013; Padalino et al., 2016). HPMC is an important component and one of the most used in GFB, due to its promising effects on the quality of the final product (Mir et al., 2016). HPMC is normally used as a thickener, in GFB, agglutinating the water, and increasing the viscosity of the GF dough.



*R = H or CH_3 or $CH_2CHOHCH_3$

FIGURE 3 - Chemical structure of hydroxypropyl methylcellulose (HPMC). Source: adapted from Hager & Arendt, 2013.

In European Union Commission Regulation No. 432/2012, it is established that HPMC has two health claims: claim 814 and 815. In the first it is mentioned that the consumption of this hydrocolloid, for foods containing 4g per quantified portion, together with the meal contributes to a smaller increase in blood glucose after that meal. Already in claim 815 it is mentioned that this hydrocolloid, for foods that provide a daily intake of 5g of it, contributes to the maintenance of normal levels of cholesterol in the blood.

2.3.3. MANUFACTURING PROCESS

The manufacturing process of GFB has three principal phases: mixture, fermentation, and baking. It starts with the mixture of all the ingredients which allows for the development of the dough. Following, during fermentation, the gas produced by yeast causes expansion of the gas cells previously incorporated into the dough during the mixing phase. The stability and the growth of gas bubbles generated determines the volume of the bread loaf and the texture. This phase is very important since any modification during the fermentation phase can modify the bread's crumb structure. In the next stage, the bread is baked in an oven and the dough is heated progressively from the outside towards the centre (Masure et al., 2016).

2.4. MICROALGAE

Microalgae have been consumed for thousands of years, in many and varied cultures (Torres-Tiji et al., 2020), its commercial cultivation started five decades ago, and its commercial application was first introduced in Japan in the 1960s (Mobin & Alam, 2017). They are microscopic autotrophic photosynthetic organisms of a single cell naturally found in the marine environment (Mobin & Alam, 2017). They are found almost everywhere on the planet and, compared to other plants, show higher growth rates and are responsible for producing atmospheric oxygen - more than 50% of the planet's oxygen (Mendonça, 2017).

Microalgae have a similar photosynthetic mechanism, but benefit from easier access to oxygen, water, and nutrients, as they are submerged in an aqueous medium. This advantage, together with its simplicity and the fact that they do not have non-photosynthetic fabrics, favours the efficiency of the conversion of solar energy into biomass (Mendonça, 2017). They produce complex compounds, such as lipids, carbohydrates, and proteins, using simple substances located in their surroundings. Most microalgae are "plant-like" photosynthetic microorganisms, but without the distinct types of cells and organs that terrestrial plants have and use carbon from the air for energy production. They also produce useful bio-products, such as carotenoids (especially β-carotene), astaxanthin, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), bioactive and functional pigments, natural food colouring, polysaccharides, and antioxidants (Mobin & Alam, 2017).

The microalgae sector is expanding, and the demand for them has been increasingly accentuated by large companies in the food industry looking to use innovative, natural, and sustainable raw materials.

This market still presents some challenges due to the lack of knowledge of the properties of microalgae and their use in different applications (Lima, 2019). Microalgae are considered notable, but a natural source that has not been explored for healthy eating. Several species of microalgae are identified as rich in carbohydrates, proteins, lipids, and nutritionally valuable components (Sathasivam et al., 2019). Therefore, it is possible to say that microalgae are inserted in the general trends of the food industry, as they are sustainable, functional, and totally natural.

Currently, most of the commercialized microalgae products are available in the markets as a natural food, in the form of tablets, capsules, and liquids. Microalgae have been credited with improving the immune system, lipid metabolism, intestinal function, and resistance to stress. Many studies have suggested algae as a potential source of dietary supplement or a substitute for conventional protein sources such as soy meal and fishmeal (Sathasivam et al., 2019).

In this project, were incorporated the microalgae *Tetraselmis chuii, Chlorella vulgaris* and *Nannochloropsis gaditana* in the structure of GFB. *Chlorella vulgaris* has been on the market as a food and food ingredient since 1997, so its access to the food market is not subject to Regulation (EC) No. 258/97 for Novel Foods. *Tetraselmis chuii* is approved for human consumption, in accordance with Implementing Regulation (EU) No. 2470/2017, but not in bakery products. However, *Nannochloropsis gaditana* is not yet approved for human consumption. The microalgae related to the A2F project are produced in autotrophy, undergo cell disruption in a bead-mill and are lyophilized.

2.4.1. *TETRASELMIS CHUII*

Tetraselmis chuii (**Figure 4**) is a green, single-celled, mobile microalgae, with an ellipsoidal shape that reproduces by longitudinal fission (Butcher, 1959). This microalgae lives in a marine environment and has high content of chlorophyll-a and chlorophyll-b, and a lipid profile rich in omega-3, making it suitable for food formulations (Allmicroalgae, 2021).

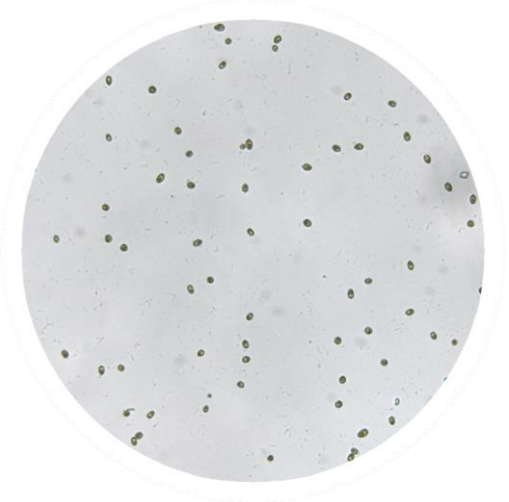


FIGURE 4 - *Tetraselmis chuii* observed under the microscope. Source: Allmicroalgae, 2021 (https://www.allmicroalgae.com/pt-pt/microalgas/).

Tetraselmis chuii is approved by EFSA (Regulation (EU) No. 2470/2017) and has a high protein content, which is an important requirement for use in breads with a specific nutritional profile, as is the case of GFB (Nunes et al., 2020b). However, this microalgae cannot be incorporated into bread, but only in sauces, special salts, condiments and food supplements (**Table 1**).

TABLE 1 - Specified category of food where can be incorporated *Tetraselmis chuii* and corresponding maximum levels. Source: adapted from Regulation (EU) No. 2470/2017.

	Specified category of food	**Maximum levels**	
Lyophilized from the microalgae *Tetraselmis chuii*	Sauces	20% or 250 mg/day	The designation of the novel food to be used on the labeling of foodstuffs containing it must be "freeze-dried from microalgae *Tetraselmis chuii*" or "lyophilisate from microalgae T. *chuii*". Food supplements containing lyophilized *Tetraselmis chuii* microalgae must bear the following statement "contains negligible amounts of iodine".
	Special salts	1%	
	Condiments	250 mg/day	
	Food supplements as defined in Directive 2002/46/EC	250 mg/day	

2.4.2. *CHLORELLA VULGARIS*

Chlorella species are single-celled and contain the green photosynthetic pigments, chlorophyll-a and chlorophyll-b, in the chloroplast. They multiply quickly requiring only CO_2, water, sunlight, and a small amount of minerals. These microalgae are unicellular, spherical in shape and photoautotrophic green microalgae without flagella. Grow commercially in photobioreactors, large circular tanks and mixed open tanks with paddle wheels or open circular tanks. The most used outdoor culture systems are circular tanks and ponds. It is harvested by centrifugation or auto flocculation, and after harvesting the biomass is pulverized or drum dried, and the powder sold directly or used to make tablets (Mobin & Alam, 2017). *Chlorella* is also known as healthy foods for humans and used for nutrient-rich foods for aquatic animals (Sathasivam et al., 2019) and are being promoted to functional foods, used to prevent, or cure common or acute illnesses such as Alzheimer's disease and cancer (Mobin & Alam, 2017). *Chlorella* has health benefits, such as assisting disorders like gastric ulcers, constipation, anaemia, hypertension, diabetes, infant malnutrition, and neurosis. In addition, *Chlorella* is also important as a source of natural pigments, specifically carotenoids, and can be used as a natural colouring agent (Fradique et al., 2010).

Chlorella vulgaris (**Figure 5**) is a non-motile reproductive cell that reproduces asexually, and one of the most common microalgae in the world. This specie is a sweet water microalgae and is able to accumulate important amounts of lipids (Safi et al., 2014) and has been used as an alternative medicine and is known as a traditional food in the Orient (Fradique et al., 2010). *Chlorella vulgaris* has a high content of protein, containing essential amino acids, of chlorophyll and vitamins and minerals such as

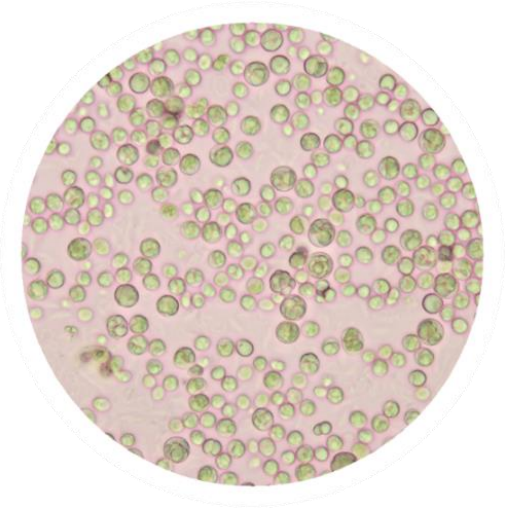


FIGURE 5 - *Chlorella vulgaris* observed under the microscope. Source: Allmicroalgae, 2021 (https://www.allmicroalgae.com/pt-pt/microalgas/).

2.4.3. *NANNOCHLOROPSIS GADITANA*

Nannochloropsis gaditana (**Figure 6**) is one of six known species of the genus *Nannochloropsis*, found mainly in marine ecosystems, but it can also occur in fresh and brackish water. This microalgae is considered very promising because it can be used for industrial applications due to its ability to accumulate proteins, and high levels of polyunsaturated fatty acids. Therefore, there is a growing interest in using it as a functional ingredient for human nutrition (Khemiri et al., 2020).

FIGURE 6 - *Nannochloropsis gaditana* observed under the microscope. Source: Allmicroalgae, 2021 (https://www.allmicroalgae.com/pt-pt/microalgas/).

2.4.4. NUTRITIONAL COMPOSITION

For microalgae to be considered a potential source of food, it is important to consider their nutritional composition. This varies significantly between species and within the same species, according to the growing environment (temperature and light) (Torres-Tiji et al., 2020). They consist of different compounds such as proteins, fatty acids, carotenoids, and other compounds of high economic interest, offering different uses, such as in the form of raw materials. They gather the necessary capacities to survive based only on sunlight through photosynthesis, later accumulating the micronutrients they need to develop in a healthy way, which give them varied biochemical profiles. These micronutrients present in microalgae, of high quality and totally natural, are increasingly sought as sustainable sources of food.

Microalgae are the primary sources of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the high fibre content they present makes them important in weight loss, as they promote a greater feeling of satiety. They are one of the most economical and interesting sources of antioxidants, as they have primary carotenoids that are dispersed within chloroplasts and chlorophylls. Microalgae have phytochemicals, such as flavonoids, phenolic acids, and carotenoids, with relevant biological potential for health. However, the reduced amount in which they are consumed, and the price are still factors that limit their use as a primary source of protein and micronutrients, and the environmental, seasonal, processing and confection factors can influence and vary the nutritional composition of these (Lima, 2019). There are other nutrients that have a positive impact on human health that can be supplied by microalgae, such as antioxidants (Torres-Tiji et al., 2020). Microalgae are known to contain a significant amount of nutrients that play important roles in cell life, they include simple natural food colouring and nutrients that exhibit a high level of biological activity (Nunes et al., 2020a). In addition, the consumption of some microalgae has been correlated with health benefits, including cardiovascular, immunomodulation, anti-aging, and anti-cancer (Torres-Tiji et al., 2020).

2.4.4.1. PROTEINS AND ESSENTIAL AMINO ACIDS

Protein is a crucial element in the human diet, providing most of the nitrogen that humans need (Torres-Tiji et al., 2020). They are essential macronutrients responsible for the overall growth of an individual, they are made up of long chains of amino acids, essential and non-essential, linked by peptide bonds. Essential amino acids (EAAs) are not synthesized in the human body and need to be consumed externally as food items (Pina-Pérez et al., 2017). Some microalgae have a very high percentage of protein, which composition is much richer in EAAs compared to common plant proteins (Torres-Tiji et al., 2020).

In general, protein of plant origin is of a lower quality than protein of animal origin, and one of the main factors that determine this quality is whether a source of protein contains all EAAs (Torres-Tiji et al., 2020). For the population that follows a vegetarian and vegan diet, there are very few options since most plant-derived proteins do not have a complete EAAs profile. Microalgae, on the other hand, are an excellent source of EAAs (Pina-Pérez et al., 2017).

2.4.4.2. LIPIDS AND FATTY ACIDS

Lipids are an indispensable component of cells and are precursors to many essential molecules and, as such, an adequate intake of them is crucial for the human diet. As well as EAA, there are some lipids that are essential, including linolenic acid and linoleic acid (Torres-Tiji et al., 2020). Microalgae are responsible for the production of essential fatty acids (EFAs), especially long chain polyunsaturated fatty acids (PUFAs), like linolenic acid, EPA e DHA. In addition, there are certain lipids that have been shown to have a positive impact on human health, the most important of which are DHA and EPA. DHA is a structural fatty acid important for the correct development of brain and eyes in babies and has been shown to support cardiovascular health development in adults (Pina-Pérez et al., 2017), and EPA is produced in large quantities in marine green algae and the production of this fatty acid can be increased by reducing the temperature and increasing salinity (Sathasivam et al., 2019). Certain microalgae can accumulate up to 30% to 40% of the total fatty acids produced as EPA, and other species can accumulate about 50% of the cell's total lipids as DHA. Therefore, algae can provide healthy fatty acids, which humans need in the diet (Torres-Tiji et al., 2020). Currently, fish and fish oil are the common source for obtaining PUFAs, but application as a food additive is limited due to the possible accumulation of toxins, fish odour, unpleasant taste, poor oxidative stability, and the presence of mixed fatty acids. The higher concentration of PUFAs in fish can be caused by the consumption of microalgae, which is a reason to consider microalgae as a potential source of PUFAs (Sathasivam et al., 2019). PUFAs play an important role in cellular and tissue metabolism, including regulation of membrane fluidity, electron, and oxygen transport, as well as thermal adaptation (Funk, 2001). Unsaturated fatty acids affect hyperlipidaemia, reducing cholesterol and triglyceride levels, and thus reduce the risk of heart disease and atherosclerosis (Sathasivam et al., 2019).

2.4.4.3. VITAMINS AND MINERALS

Most vitamins and minerals are not synthesized by animals, but produced by plants, and supplied to humans and animals through their diet. Like traditional plant foods, microalgae are very rich in vitamins and minerals, and their consumption has been correlated with health benefits, including, but not limited to, cardiovascular, health, immunomodulation, anti-aging and anticancer (Torres-Tiji et al., 2020). Microalgae are sources of vitamins such as vitamin A, B-complex vitamins, vitamin C and vitamin E, and minerals such as iodine (I), iron (Fe), potassium (K), calcium (Ca), and sodium (Na) (Lima, 2019).

2.4.4.4. CAROTENOIDS

Carotenoids are lipophilic pigments that occur in higher plants, microalgae, and non-photosynthetic organisms. Most carotenoids have therapeutic value, including anti-inflammatory activities that are largely attributed to their strong antioxidant effect which is used to protect organisms from oxidative stress. Carotenoid synthesis is aided using algae, which serve mainly as accessory pigments in photosynthesis (Sathasivam et al., 2019).

β-carotene is considered one of the most important carotenoids because it has an active form of pro-vitamin A, an additive for multivitamin preparations and healthy food products. The high light intensity, high salinity, extreme temperatures, and nutrient supplements are playing a significant role in

increasing the production of β-carotene. Algae rich in β-carotene inhibit low-density lipoprotein (LDL) oxidation and influences plasma triglycerides, cholesterol, and high-density lipoprotein (HDL) levels, inhibit LDL oxidation in diabetic patients and may be important in delaying the development of atherosclerosis (Sathasivam et al., 2019).

2.4.4.5. ANTIOXIDANTS

Microalgal biomass is considered a multi-component antioxidant system, which is generally more effective due to the interactions between different antioxidant components (Sathasivam et al., 2019). One of the most valuable nutritional properties of algae is related to its high content of polyphenols, carotenoids, and flavonoids, which are antioxidants. Antioxidants act to protect the human organism from damage by reactive oxygen species, which can lead to health disorders such as cancer, diabetes mellitus, neurodegenerative and inflammatory diseases (Koyande et al., 2019). Antioxidants are very powerful tools to combat oxidative stress and thus improve the health status of the general population. Phenols constitute the largest group of metabolites identified in algae species (Pina-Pérez et al., 2017).

2.4.5. CELL-WALL DISRUPTION

Once microalgae are incorporated into staple foods, they can have a substantial effect on health, with great benefits achieved when consumed regularly. However, cell wall integrity can significantly limit nutrient availability, as the structures of many microalgal species are covered by multiple layers of resistant cells that limit the release of cell wall constituents. The cell wall represents a natural barrier that results in low bioavailability of intracellular molecules (Nunes et al., 2020a). Controlled cell wall disruption has an important impact on the bioavailability of microalgal content. The mechanisms by which these bio actives are released from cells and altered during food processing and the ultimate bioactivity of these substances with powerful health benefits are important questions to be answered. Cell-wall disruption has been described as a spectrum, starting with minor damage and the release of biomolecules to complete cell disruption. Many methods of cell disruption are available, but disruption remains challenging as it depends on the structure and size of the cell wall and shape of the microalgae. Usually, physical methods are used to break the cell wall, as they avoid chemical contamination and preserve most of the functionality of intracellular biomolecules. Per example, there are studies (Duarte, 2018; Nunes et al., 2020a) that have verified that the use of *Chlorella vulgaris* as a food ingredient can be a promising method to enrich staple foods, such as bread, with bioactive compounds. The possibility of adding a microalgae biomass to foods depends on the type and intensity of processing, food system and interactions with other food molecules. Therefore, the rupture of the cell wall was applied to promote a controlled release of active bio composites (Nunes et al., 2020b).

2.5. IMPROVEMENTS IN BREAD'S COLOUR AND FLAVOUR

Generally, GFB presents poorer colour and flavour characteristics than wheat flour bread so, to improve these characteristics, studies have been carried out with the incorporation of underexplored flours (e.g., acorn) (Martins et al., 2020). However, the incorporation of microalgae in food, namely in bread, did not have much acceptance by consumers. Therefore, in the last years, several innovative studies have been developed to incorporate microalgae into food, namely pasta (Fradique et al., 2010), cookies (Batista et al., 2017), GFB (Duarte, 2018; Fernandes, 2019; Vasco, 2019) and wheat flour bread (Graça et al., 2018; Nunes et al., 2020a; Qazi et al., 2021a). However, the green colour of microalgae can adversely affect consumers perception about quality (Lafarga et al., 2019). As verified in studies carried out by Nunes et al. (2020b) and Khemiri et al. (2020), the characteristic green colour and intense flavour of algae tend to have a lower evaluation in sensory analysis. Hence, it is very important to develop strategies to increase consumer acceptance for the microalgae enriched foods.

Several studies have been carried out with microalgae in order to understand the best way to incorporate them into food. Chacón-Lee and González-Mariño (2010) suggested that the strong flavour of microalgae could be masked by exotic-flavoured spices. Other great solution can be the ethanol treatment of microalgal biomass. Qazi et al. (2021a) studied this treatment as a feasible strategy to address the sensory challenges that hinder incorporation of algae into foods, in order to eliminate the components responsible for the less appreciated colour and flavour of the bread. These authors concluded that with ethanol treatment, in addition to an increase in protein and dietary fibre content, there were also improvements in colour compared to breads with untreated microalgae. Therefore, it is important to further deepen the treatment of microalgae with ethanol so that they can be inserted into GFB, improving its colour and flavour.

3. MATERIALS AND METHODS

The study carried out was divided into several phases, considering the objectives to be achieved. Initially, the experimental part was based on studying the best formulation for the preparation of GF dough and bread. The addition of microalgae was studied at incorporation levels of 4% (w/w), in relation to the total of flours for a base of 14% humidity. The bread's crumb was developed according to a formulation already optimized in previous studies (Nunes et al., 2020 and Khemiri et al., 2020). The optimization of the water absorption (WA) level, of each formulation, was evaluated using Microdough-Lab. Afterwards, the physical characterization of the doughs was carried out using dynamic rheological tests (Rheometer Mars III, Haake) – stress sweep, frequency sweep and viscosity tests. The instrumental determination of colour, a fundamental attribute for the acceptability of the products, was performed using a colorimeter Minolta CR400, using the CIELAB system. The technological aptitude of the breads was evaluated through Texture Profile Analysis (TPA) (TA-XTplus Texturometer, Stable Micro Systems) and determination of volume and moisture. The TPA carried out over the days of bread conservation allows to obtain texturograms of force (N) vs. time (s) and determine variations in the firmness and cohesiveness values of the samples.

Then, the nutritional characterization of the breads was carried out by determining the protein content (DUMAS), lipids (hydrolysis followed by n-hexane extraction), ashes (incineration), moisture (kiln drying) and carbohydrates (calculation), adopting AOAC reference methods. At this stage, the bioactivity study of GFB extracts with microalgae incorporation was also carried out, with determination of total phenolic compounds (TPC) (Folin Ciocalteu), pigments (chlorophyll-a, chlorophyll-b and carotenoids) and antioxidant activity (DPPH and FRAP methods).

Finally, the breads with physical, nutritional and bioactivity characteristics with the greatest potential in terms of the market were subjected to a sensory analysis test, using a panel of untrained consumers, in order to identify which breads with microalgae had the best sensorial profile.

3.1. RAW MATERIALS

All ingredients used were selected according to market cost and GF guarantee. GFB samples were produced with buckwheat flour (*Próvida*, Mem Martins, Portugal), rice flour (*Espiga*, Alcains, Portugal), potato starch (Globo, Seixal, Portugal), dehydrated yeast (*Fermipan*, Setúbal, Portugal), sugar (*Continente*, Matosinhos, Portugal), salt (*Continente*, Matosinhos, Portugal), HPMC (*WellenceTM 321*, Dow, Germany), sunflower oil (*Fula*, Algés, Portugal) and distilled water, according to a previously optimized formulation by Nunes et al., 2020b.

Microalgae biomass (*Tetraselmis chuii*, *Chlorella vulgaris* and *Nannochloropsis gaditana*) were produced by the A2F partners in Norway. The treated microalgae were produced by NOFIMA, Norwegian Institute for Food, Fisheries and Aquaculture Research, Ås, Norway, that performed the ethanol treatment of the three microalgae. Briefly, the raw microalgae species (TcR, CvR and NgR) were received by NOFIMA in freeze-dried fine particles powdered form with about 5 to 7% of moisture.

Then, the microalgal biomasses were treated with 96% (v/v) ethanol, placed in a cellulose thimble using a Soxhlet extractor apparatus (Adams & Chittenden Scientific Glass, Berkeley, USA), according to the method described by Qazi et al. (2021a). It was determined that the microalgae have a high protein content and an important content of bioactive compounds. They have between 40-62% protein, 0-22% lipids, 6-17% ashes and 8-22% dietary fibres (**Table 2**).

TABLE 2 - Macronutrients (g/100g) in the microalgal biomasses of *Tetraselmis chuii* raw (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Cholorella vulgaris* raw (CvR), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* raw (NgR) and *Nannochloropsis gaditana* ethanol treated (NgT). Source: Qazi et al. (2021b)

Macronutrients	Proteins	Lipids	Ash	Dietary fibers
***Tetraselmis chuii* raw (TcR)**	42.1 ± 0.1	13.8	16.0 ± 0.1	8.9 ± 0.8
***Tetraselmis chuii* treated (TcT)**	59.5 ± 0.2	0.3	16.7 ± 0.1	15.1 ± 2.2
***Chlorella vulgaris* raw (CvR)**	47.8 ± 1.1	15.7	6.7 ± 0.6	13.8 ± 0.5
***Chlorella vulgaris* treated (CvT)**	58.8 ± 0.3	0.6	8.2 ± 0.0	19.0 ± 0.6
***Nannochloropsis gaditana* raw (NgR)**	43.3 ± 1.5	21.4	7.0 ± 1.1	12.2 ± 0.6
***Nannochloropsis gaditana* treated (NgT)**	61.7 ± 2.8	0.4	7.4 ± 0.1	21.8 ± 0.8

3.2. BAKING OF THE GLUTEN-FREE BREADS

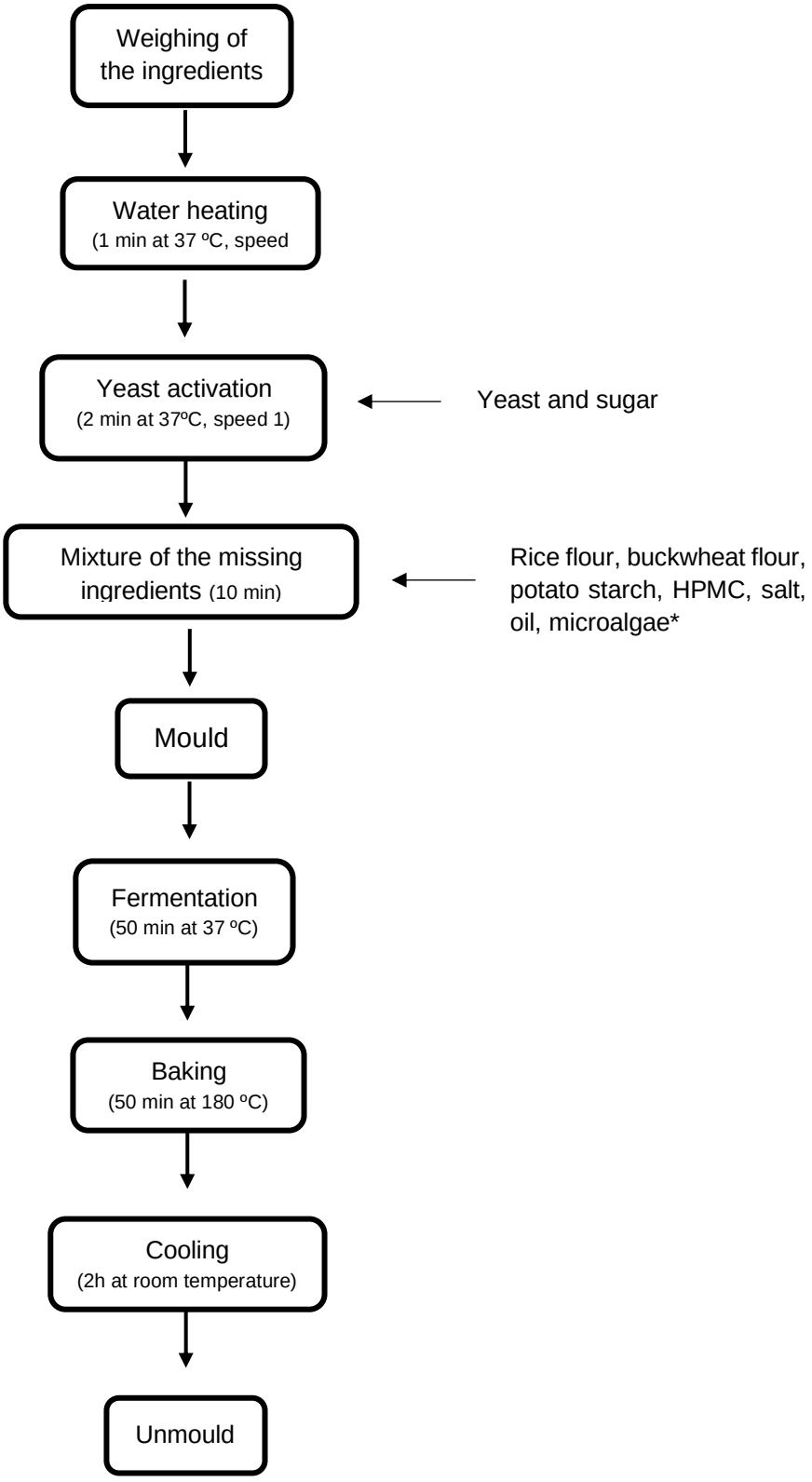
GFB were prepared according to a previously optimized method by Nunes et al. (2020b). As previously stated, the breads were prepared using rice flour, buckwheat flour, potato starch, microalgal biomass, hydroxypropyl methylcellulose (HPMC), dehydrated yeast, sugar, salt, sunflower oil, and distilled water. The amount of water added to each formulation was adjusted according to the results obtained in the mixing curves.

In **Table 3** there are described the amount of each ingredient used in the formulation of the GFB. Only the control bread does not have microalgae in its formulation. The amount of water added to each formulation was estimated according to tests carried out at Microdough-Lab, namely in the values of WA.

TABLE 3 - Description of the ingredients used in the formulation of gluten-free breads, control and with 4% incorporation of microalgae. Control is dough without microalgal biomass and GFB with 4% of *Tetraselmis chuii* (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Cholorella vulgaris* (CvR), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* (NgR) and *Nannochloropsis gaditana* ethanol treated (NgT).

Ingredients (g/100g)	Control	TcT, CvT and NgT	TcR, CvR and NgR
Buckwheat flour	46	44.2	44.2
Rice flour	31	29.8	29.8
Potato starch	23	22.1	22.1
Microalgae	-	4	4
HPMC (in relation to flour)	4.6	4.6	4.6
Salt (in relation to flours)	1.8	1.8	1.8
Sugar (in relation to flours)	2.8	2.8	2.8
Yeast (in relation to flours)	2.8	2.8	2.8
Sunflower oil (in relation to flour)	5.5	5.5	5.5
Water (14% moisture basis)	82.1	82.1	80.6

For the GF dough preparation (300 g/batch), and to standardize the kneading conditions, the ingredients were mixed in a thermoprocessing equipment (Bimby-Vorwerk, Carnaxide, Portugal), initially to heat the water for 1 minute at 37 ºC, at speed 1, and then to activate the yeast, by adding yeast and sugar for 2 minutes at 37 °C, at speed 1. Then, the remaining ingredients were added and mixed for 10 minutes in a dough mixing program (wheat ear symbol). The 300 g of GF dough were placed in a rectangular container (dimension 25.5x12.0x6.5 cm) and left to ferment for 50 minutes at 37 °C in an electric oven (Arianna XLT133, Unox, Cadoneghe Italy). The baking was carried in Johnson A60 oven (Johnson & Johnson, New Brunswick, NJ, USA) at 180 °C for 50 minutes. A container with water was placed in the oven to ensure a moist environment and to prevent premature crusting of the bread. The process flowchart is represented in **Figure 7**.



*Only control bread does not have incorporation of microalgae.

FIGURE 7 - Flowchart of the gluten-free bread manufacturing process.

After cooling until room temperature for two hours, the breads were sliced, and physical analysis were performed: weight, volume, crumb and crust colour, moisture and texture were evaluated. Finally, the breads were packed in polyethylene bags and stored at room temperature for a period of 48 hours. Two breads of which formulation were prepared (n=2).

For the chemical and nutritional analysis, the breads were dried in the oven, at about 50 °C for 5 to 6 hours, and then crushed to create the bread powder.

3.3. MIXING BEHAVIOUR OF THE GLUTEN-FREE DOUGHS

Microdough-Lab 28000 developed by Perten Instruments was used to investigate differences in formulation performance and determine the ideal WA capacity for each bread content (control and breads with 4% w/w of *Tetraselmis chuii* (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Chlorella vulgaris* (CvR), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* (NgR), *Nannochloropsis gaditana* ethanol treated (NgT)). This equipment is a small-scale dough mixer (4 g), and it is an analysis system with the action of mixing arms to determine the quality and processing characteristics of the flour and dough. The use of only four grams of the sample is ideal for the use of limited and valuable samples (Perten Instruments, 2017). This device assists in determining the characteristics of a flour, such as the amount of water absorbed to achieve the ideal consistency of the dough, the requirement for mixing time and the stability of the dough. The standard test from Microdough-Lab is for wheat flour and it aims to reach a maximum torque of 100 mN.m, while for GF dough the value is generally much lower. Due to this, preliminary experiments were conducted at a laboratory scale to identify the best formula that would attend as control with a sustainable dough consistency and acceptable bread texture, and the WA was adjusted to all the GFB formulations, to obtain a maximum peak torque around 70 ± 7 mN.m.

The flours were mixed with distilled water at a constant temperature of 30 °C and speed of 63 rpm for 20 minutes, and the resistance to mixing is measured as torque, which were presented as a graph of torque (mN.m) *versus* time (min).

In **Table 4** is the description of the ingredients and respetive amount used in the formulation of the GF doughs used in Microdough-Lab, for the determination of mixing curves and all the parameters associated.

TABLE 4 - Description of the ingredients and respective amount used in the formulation of gluten-free dough for the determination of mixing behaviour (control – dough without microalgal biomass; with microalgae – dough with incorporation of ethanol treated and raw microalgae).

Ingredients (g/4g)	Control	With microalgae
Buckwheat flour	1.831	1.758
Rice flour	1.234	1.184
Potato starch	0.915	0.879
HPMC	0.184	0.184
Algae	-	0.157
Salt	0.072	0.072

Samples were evaluated in relation to torque, WA, dough development time (DDT), stability and softening. To use this method, it is important to note that the WA is the percentage of water absorbed by the mixture of flours. DDT is the variable that represents the development time of the dough, and it relates to the protein content, the quality of the flour and the test conditions. In turn, the stability corresponds to the tolerance of the flour to the mixture, and the softening is the difference of torque in time corresponding to the DDT and the final torque. The peak corresponds to the accumulated mechanical energy applied to the dough during the development time (Perten Instruments, 2017).

3.4. VISCOELASTIC BEHAVIOUR OF THE GLUTEN-FREE DOUGHS

Rheology is the science that studies the deformation and flow of materials when subjected to a certain stress, being that for solid materials we are facing a deformation and for liquid materials before a flow (Sousa, 2001). It is important in Food Science and Engineering, with numerous application areas in the food industry such as determining ingredient functionality in product development, shelf-life testing or intermediate or final product quality control (Batista, 2013). The bakery doughs are a target of study of rheology, presenting a viscoelastic behaviour.

The viscoelastic properties of the dough were determined from the stress sweep tests, for the determination of the linear viscoelastic range, and frequency sweep tests to determine the mechanical spectrum. These tests were performed only on the breads used in the sensory analysis test (CvR, CvT and control).

The viscoelastic behaviour of the GF doughs was used a rheometer Haake Mars III (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a UTC Peltier and fitted with a serrated parallel plate system with 20 mm diameter (PP20) and 1 mm distance between plates (previously optimized for this type of material). The GF doughs were tested using parallel plate geometry and the dough surface exposed was coated with paraffin oil to prevent drying and allowed to rest at 5 ± 1 °C for 10 min before testing at controlled room temperature (20 ± 1 ºC). First, the stress sweep test was performed to

determine the linear viscoelastic region (LVER) of each dough, so that the frequency sweep test is applied at a constant stress, within that region. In the next tests performed, a new portion of dough was used since the structure was broken in the while measuring the LVER. For the frequency scanning test shear stress of 10 Pa (previously determined LVER), under the same conditions, while varying the frequency from 0.1 to 100 Hz. The shear viscosity of the GF dough was also determined using a new portion of dough that rest for 3 min at 5 °C before testing at 20 °C during 15 min. The viscosity test was performed from 0.1 to 1000 1/s. The determinations were made at least in triplicate for each GF dough (n=3).

3.5. EVALUATION OF THE BREAD VOLUME

The bread volume was determined following the rapeseed displacement method AACC 10-05.01. This standard corresponds to the seed displacement method rapeseed for volume measurement. The bread was placed inside a box (dimension 52x20x10 cm), and it was filled to the surface with rapeseed. Afterwards, the volume of seeds that were inside the box was measured. Measurements were taken in duplicate for each formulation and the average values obtained were presented. Through this method it is possible to determine the volume of bread, which is calculated subtracting from the volume of the box, the volume of seeds needed to make up the total volume of the box with the bread.

3.6. COLOUR EVALUATION

The colour of the bread crust and crumb was measured using a colorimeter Minolta CR-400 (Japan). The results were expressed according the CIELAB system, using the following parameters: L*, that corresponds to lightness (values increase from 0 to 100%); a*, greenness to redness (−60 to 60, respectively); and b*, blueness to yellowness (−60 to 60, respectively). All measurements were conducted on the baking day, at room temperature, about 2 hours after baking. The measurements were replicated six times in the crust and in the crumb for each formulation, under artificial fluorescent light using a white standard tile (L* = 93.15, a* = −0.57 and b* = 3.98).

The total colour difference between breads containing microalgal biomass and the control sample was calculated using the following equation:

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

3.7. TEXTURE EVALUATION

Texture is the property that reflects a set of other properties and attributes of materials, being one of the most important sensory aspects of food and is very important in GFB. The texture of the bread crumb was characterized using a TA-XTplus texturometer controlled stress (*Stable Micro Systems*,

United Kingdom) equipped with a 5 kg load cell and cylindrical probe diameter 10 mm that was allowed to penetrate the manually sliced ~20 mm slice of the bread. The texturometer is an instrument that simulates the action of chewing in the human mouth, through an empirical test. This equipment has a dynamometer - force meter, which uses a probe (cylindric probe with 10mm, 14 mm of distance, 5 seconds of waiting time and 1 mm/s of crosshead speed) to contact the sample and deforms the food, by means of compression, penetration, or traction (Friedman et al., 1963).

The test used was TPA that was developed with the objective of studying the mechanical properties of foods and how these properties are related to texture sensory perceived. In this test, the conditions that food is subjected during the chewing process are replicated, with two penetrations or compressions, with a time interval between them, to simulate the action of two bites on the food (two bite test). The results obtained are expressed in a graph - texturogram, where is represented the force (N) versus time (s) (Sousa, 2001). Depending on the food product to be evaluated, certain parameters need to be considered. In the case of bakery products, the properties to be considered are the hardness or firmness and cohesiveness. Firmness is maximum force during the first compression cycle (force 2), and cohesiveness corresponds to the area of the positive force in the second compression divided by the area during the first compression, that means resistance to a second deformation in relation to resistance to a first deformation (Friedman et al., 1963).

Through TPA tests, firmness and cohesiveness were calculated, and obtained the texture of the GFB crumb, 2, 26 and 48 hours after baking, at controlled room temperature (20 ± 1 °C). Measurements were made twice on three slices of the same loaf (n = 6). The results presented are an average of two independent baking trials.

3.8. NUTRITIONAL PROPERTIES

The biochemical composition of the breads was determined in terms of moisture, ash, minerals, proteins, carbohydrates, and lipids. All the analyses were performed at least in duplicate.

The moisture of bread and flours was measured gravimetrically through an automatic moisture analyser PMB 202 (Adam Equipment, Oxford, NJ, USA) at 130 °C to a constant weight of sample. In case of the bread, the moisture was measured three times, one on the first slice and two on the second.

The rest of nutrients and bioactive compounds were measured in dried breads (< 3% moisture) grinded to powdered form. Total ashes content was determined by incineration in a muffle furnace (AACC 08–01, SNOL) at 550 ± 1 ºC for 24 hours.

Total protein content was estimated in duplicates of 100 mg of sample by combustion method (Kirsten, 1979) using Vario EL elemental analyser (Elementar, Langenselbold, Germany), by using DUMAS method. This method calculated the nitrogen (N) content, and the protein content was estimated by a conversion factor of 6.25 (%N x 6.25).

Lipid content was determined by hydrolysis as described by Doan et al. (2011), followed by n-hexane extraction. Three replicas of 100 mg of each formulation were added to a mixture of methanol, chloroform, and hydrochloric acid (ratio of 10:1:1.5, respectively). Briefly, this mixture was extracted with n-hexane/chloroform (4:1 v/v), taken to a vortex for 2 minutes and centrifuged (HERMLE, Z383 K, Germany) for 7000 rpm during 10 minutes at about 15 ºC. The resulting supernatant was removed and placed in a glass tube, weighed beforehand. The total fat content was quantified gravimetrically, therefore, these tubes were placed inside an oven at 50 ºC for two or three days, depending on the sample. After being removed from the oven, the tubes were weighed, and the difference between the final tube weight and the weight of the empty glass tube corresponds to the lipid content of each formulation.

The total carbohydrate content of the samples was reported by the difference between the protein, lipid, ash, and the moisture contents of the breads. The amount of minerals (K, Ca, Mg, P, S, Fe, Cu, Zn, and Mn) was estimated using an Inductively Coupled Plasma Optical-Emission Spectrometry (5800 ICP-OES, USA) Thermo ScientificTM iCap Series 7000 (Thermo Fisher Scientific, Waltham, MA, USA) following the method described by Martins et al. (2020).

3.9. EVALUATION OF THE BIOACTIVITY

To characterize the bioactivity of the GFB total phenolic compounds, pigments, and antioxidant capacity (determined by DPPH and FRAP methods) were carried out. In this way, one started with the extraction by adding 10 ml of ethanol (96% v/v) in 2 g of bread powder and then homogenization per three minutes at 8000 rpm at a homogenizer T25 basic, IKA Labortechnik. The next step was to centrifuge at 7000 rpm for ten minutes. This process was repeated twice and then the samples were filtered through 0.2 µm syringe-connected filters (Braun, inject, Germany). After filtration, the solvent was evaporated under vacuum, in a rotavapor (BÜCHI, N-490, Switzerland). Finally, dried extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain 20 mg/mL stock solution, that were stored at 4 °C until the experiments were conducted.

3.9.1. TOTAL PHENOLIC COMPOUNDS

The TPC of bread extract was evaluated using the method adapted from Mohankumar et al. (2018). The bread extract (150 µL) was added to 150 µL of Folin–Ciocalteu reagent (12%) and 2.4 ml of distilled water and then mixed with 300 µL of sodium carbonate (Na_2CO_3) solution (10%) after five minutes. The mixtures were incubated in the dark at room temperature for two hours, and then the absorbance was measured at 725 nm. The distilled water was used as blank. For the standard curve, gallic acid was used instead of extract, and this was defined with six concentration points (10, 25, 50, 100, 150 and 200). The TPC was reported as milligrams of gallic acid equivalents (mg GAE) per g of extract and corresponded to the mean value of triplicate tests.

3.9.2. ANTIOXIDANT ACTIVITY

The scavenging effect of bread extracts was determined using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) methodology, adapted from Brand-Williams et al. (1995). Extraction solutions with a volume of 100 µL each were added to 3.9 ml of the DPPH solution (60 µmol/L) in methanol, and the mixture was diluted with 100 µL of distilled water. In the control, the extract was substituted with the same volume of solvent, and in the blank probe, only water (3.9 ml) and the extract (100 µL) were mixed. After one hour in the dark at room temperature, the absorbance was measured at 515 nm, on the spectrophotometer (Agilent Technologies, Cary 60 UV-Vis, USA). The mean values of the antioxidant capacity were reported as mg of vitamin C equivalents per g of dry extract.

The reducing power of the bread extracts was determined using the ferric ion reducing antioxidant power (FRAP). The bread extract or ascorbic acid (90 µL) was added to 2.7 ml of FRAP reagent and 270 µL of distilled water. The absorbance was measured at 595 nm after 30 minutes of incubation in 37 ºC. The mean values of reducing power were reported as mg of ascorbic acid equivalents (AAE) per g of dry extract.

For the standard curve, both DPPH and FRAP, ascorbic acid (1 mg/ml) was used instead of extract, and this was defined with six concentration points.

3.9.3. PIGMENTS

The determination of total pigments (chlorophyll a, chlorophyll b and carotenoids) was done by adding 2.85 ml of ethanol (96% v/v) to 150 µL of bread extract (20 mg/ml). The ethanol was used as blank. After 30 minutes in the dark, the absorbance of the samples was measured at 470, 648 and 664 nm, for the determination of carotenoids, chlorophyll-a (Chla) and chlorophyll-b (Chlb), respectively. The values were determined using the following equations (Maadane et al., 2015):

$$Chla = 13.36 \times A_{664} - 5.19 \times A_{648} \quad (2)$$

$$Chlb = 27.43 \times A_{648} - 8.12 \times A_{664} \quad (3)$$

$$Carotenoids = (1000 \times A_{470} - 1.63 \times Chla - 104.96 \times Chlb)/221 \quad (4)$$

3.10. SENSORY EVALUATION

For the sensory evaluation, GFB with incorporation of *Chlorella vulgaris*, ethanol treated (CvT) and raw (CvR), were selected, as well as the control. Only breads with these microalgae were chosen because it is the only one approved by EFSA, for human consumption, in addition to greater interest in terms of structure and volume. The three breads were evaluated about their attributes of general appearance, colour, aroma, flavour, texture, global appreciation and buying intention. For this purpose, a taste panel evaluation form (**Annexes I and II**) was elaborated using a hedonic rating scale for the evaluation of each of the attributes. This scale ranges from "very unpleasant" (1) to "very pleasant" (5)

and, in the case of the intention to buy, from " would never buy" (1) to "would always buy" (5). The sensory analysis was carried out by 33 untrained tasters, and due to the pandemic situation in our country, 11 were carried out in the ISA test room and the remaining 12 were carried out in delivery mode. In this way, two versions of the taste panel evaluation form were prepared so that those who performed it at home had all the necessary instructions. All samples were identified with a code consisting of a number and two letters, and the samples were presented, using a random order, to the tasters as shown in **Figure 8**.

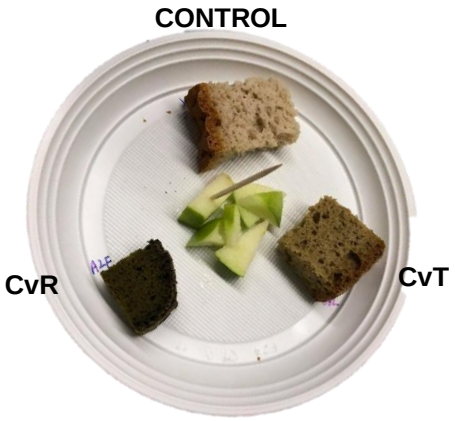


FIGURE 8 - Presentation of samples for the sensory analysis test.

3.11. STATISTICAL ANALYSIS

The statistical treatment of the experimental data was done using the software Minitab 17. Analysis of variance (ANOVA) was performed using the Tukey test for a significance level of 95% ($p < 0.05$), to assess the existence of significant differences between the mean values of the different parameters analysed.

4. RESULTS AND DISCUSSION

4.1. MIXING PROPERTIES

Usually, GF doughs have a completely different composition and structure than wheat flour doughs because they are adhesiveness and have poor mixing properties. Based on this consideration and to assess the effect of microalgal biomass incorporation on dough properties, preliminary tests were carried out to identify the best formulation with a sustainable dough consistency and acceptable bread texture. Although this Microdough-Lab test was developed for wheat dough, whose peak torque is around 100 mN.m for an ideal consistency, it is not possible to use this value in GF doughs.

To evaluate the mixing behaviour of all GFB formulations, WA was fixed around 75% because it was the value that allowed reaching the intended torque peak. For this, the ideal WA value (amount of water needed to reach the desired peak torque) was evaluated for the different formulations of breads with microalgal biomass incorporation and control to reach a peak of 70 ± 7 mN.m. As the dough was developed, its resistance to kneading was measured as torque, as shown in **Figure 9** in a graph of torque (mN.m) *versus* time (s). From the mixing curves it is possible to obtain different rheological parameters (**Table 5**). The mixing curves showed a high initial torque while the water was hydrating the flours, followed by its decrease. The mixing tolerance followed over 20 minutes (standard method) was different for the control and for the microalgae biomass replaced doughs.

It was verified that the GF doughs with incorporation of microalgae required higher WA to reach the desired torque peak value, with the exception of breads with TcR. In some cases, the increase in WA value was significant ($p < 0.05$), such as CvR (75.9 ± 0.3%) and NgT (76.0 ± 0.0%). The increase of WA in formulations incorporated with microalgae, when compared to the control, will be related to the extra presence of proteins from the microalgae (Graça et al., 2018). Thus, it was found that a higher protein content results in greater WA, which may indicate that the microalgae cells need more water to reach the desired consistency.

The doughs prepared with ethanol treated microalgae (TcT, CvT and NgT) presented a more stable torque over time. After statistical analysis (ANOVA) it is verified that the addition of different microalgae has no impact on the torque peak, DDT, and stability parameters ($p < 0.05$). This means that the rheological properties of the dough during the kneading operation do not differ significantly, demonstrating that the addition of 4% microalgal biomass has no significant impact. However, different doughs showed different degrees of softening. The TcR replaced dough presented the highest dough softening (18.7 ± 4.2 mN.m), followed by the control (14.8 ± 3.3 mN.m). The ethanol treated microalgal biomasses seemed to result in more stiff doughs compared to the corresponding raw biomasses, TcR, CvR and NgR forms. The only exception is *Nannochloropsis gaditana* because the NgR (10.8 ± 2.9 mN.m) was stiffer than NgT (12.7 ± 4.5 mN.m). This effect was significant ($p < 0.05$) between the TcR (18.7 ± 4.2 mN.m) and corresponding TcT (9.5 ± 2.3 mN.m) replaced doughs, but the same does not happen with the other microalgae. Differences in stability were not significant ($p > 0.05$) in any of the

given combinations of the GF doughs, which values are between 0.5 ± 0.1 min (control) and 1.4 ± 1.8 min (TcT).

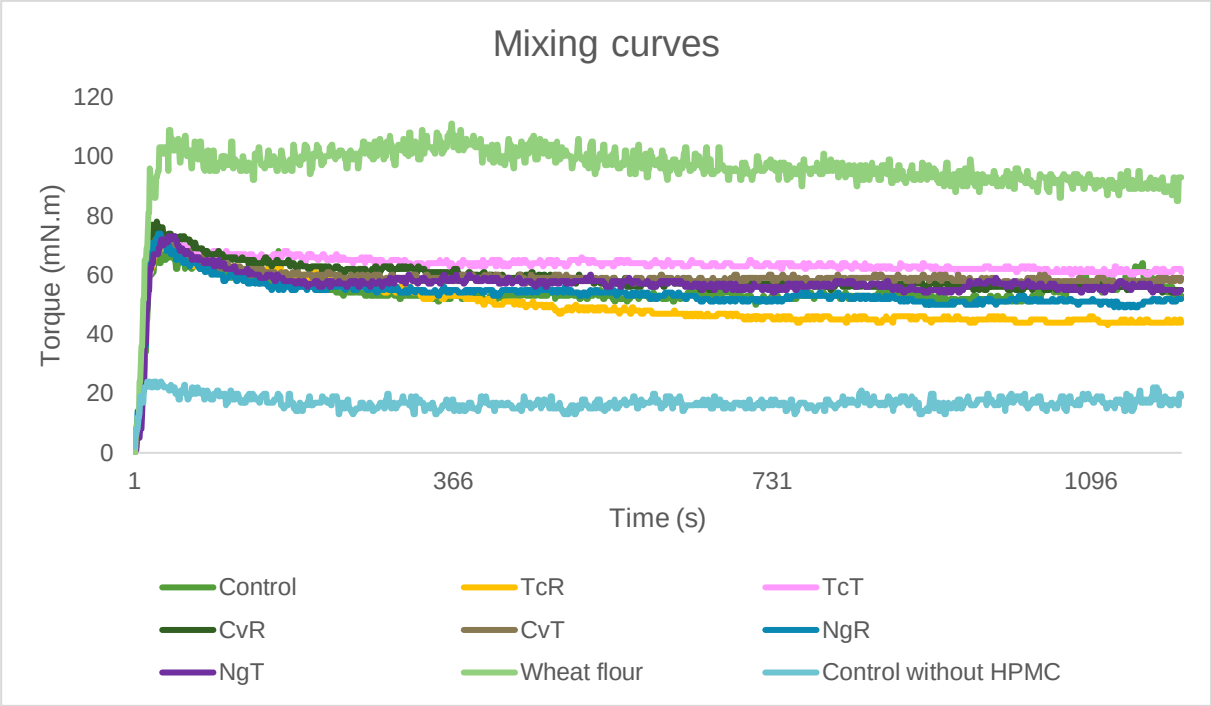


FIGURE 9 - Representation of mixing curves acquired from the control and the gluten-free breads with 4% replacement of *Tetraselmis chuii* (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Chlorella vulgaris* (CvR), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* (NgR) and *Nannochloropsis gaditana* ethanol treated (NgT).

TABLE 5 - Technological parameters obtained from mixing curves (Microdough-Lab) of the control and the microalgal biomass enriched gluten-free breads. Values are an average (n=3) ± standard deviation. Different letters in the same column shows significant difference (p=0.05).

Sample	Torque peak (mN.m)	WA (%)	DDT (min)	Stability (min)	Softening (mN.m)
Control	64.4^{a} ± 2.6	75.5^{bc} ± 0.0	0.8^{a} ± 0.1	0.5^{a} ± 0.1	14.8^{ab} ± 3.3
TcR	65.7^{a} ± 4.0	75.3^{c} ± 0.3	0.9^{a} ± 0.1	0.5^{a} ± 0.1	18.7^{a} ± 4.2
TcT	74.0^{a} ± 5.6	75.8^{ab} ± 0.3	0.9^{a} ± 0.1	1.4^{a} ± 1.8	9.5^{b} ± 2.3
CvR	74.0^{a} ± 3.7	75.9^{a} ± 0.3	0.8^{a} ± 0.0	0.4^{a} ± 0.0	16.3^{ab} ± 3.1
CvT	66.2^{a} ± 5.5	75.8^{ab} ± 0.0	0.8^{a} ± 0.1	0.4^{a} ± 0.0	11.2^{ab} ± 3.0
NgR	63.8^{a} ± 7.3	75.5^{bc} ± 0.0	0.8^{a} ± 0.1	0.4^{a} ± 0.0	10.6^{ab} ± 2.9
NgT	73.0^{a} ± 4.0	76.0^{a} ± 0.0	0.8^{a} ± 0.0	0.4^{a} ± 0.0	12.7^{ab} ± 4.5
Control without HPMC	23.3 ± 1.2	75.5 ± 0.0	0.7 ± 0.0	1.6 ± 0.7	8.5 ± 3.5
Wheat flour	105.0 ± 2.0	55.6 ± 0.2	7.1 ± 1.4	1.4 ± 1.4	18.5 ± 7.8

A control formulation (without HPMC) was also tested to compare and being able to confirm that it is an essential ingredient to GFB preparation. As shown in **Figure 9**, compared to the GF dough with HPMC (control), the dough without HPMC has a much lower torque, higher stability, and lower softening. In relation to dough prepared only with wheat flour, as expected, we obtain a peak of torque around 100 mN.m, and the DDT was much higher comparing with the GF doughs (7.1 ± 1.4 min). This test only served to verify if we obtained the expected values for the wheat flour and the objective was successfully reached.

4.2. RHEOLOGY PROPERTIES

Dynamic (oscillatory) tests on the rheometer measure elastic modulus (G′) and viscous modulus (G’’), and their contributions to viscoelastic behaviour. Dough is a highly viscoelastic material that rises during waterproofing, whereas a dough that is not elastic does not reach a good volume.

To determine the linear viscoelastic region (LVR), stress sweep tests were conducted in control and doughs with 4% (w/w) addition of *Chlorella vulgaris* raw and ethanol treated (CvR and CvT, respectively) (**Figure 10**) at shear stress 0.1 to 100000 Pa. The dough structure started to deviate at shear stress above 10 Pa for all the compositions. Therefore, a shear stress of 10 Pa was recognized as the LVR, and this value was used in frequency sweep test.

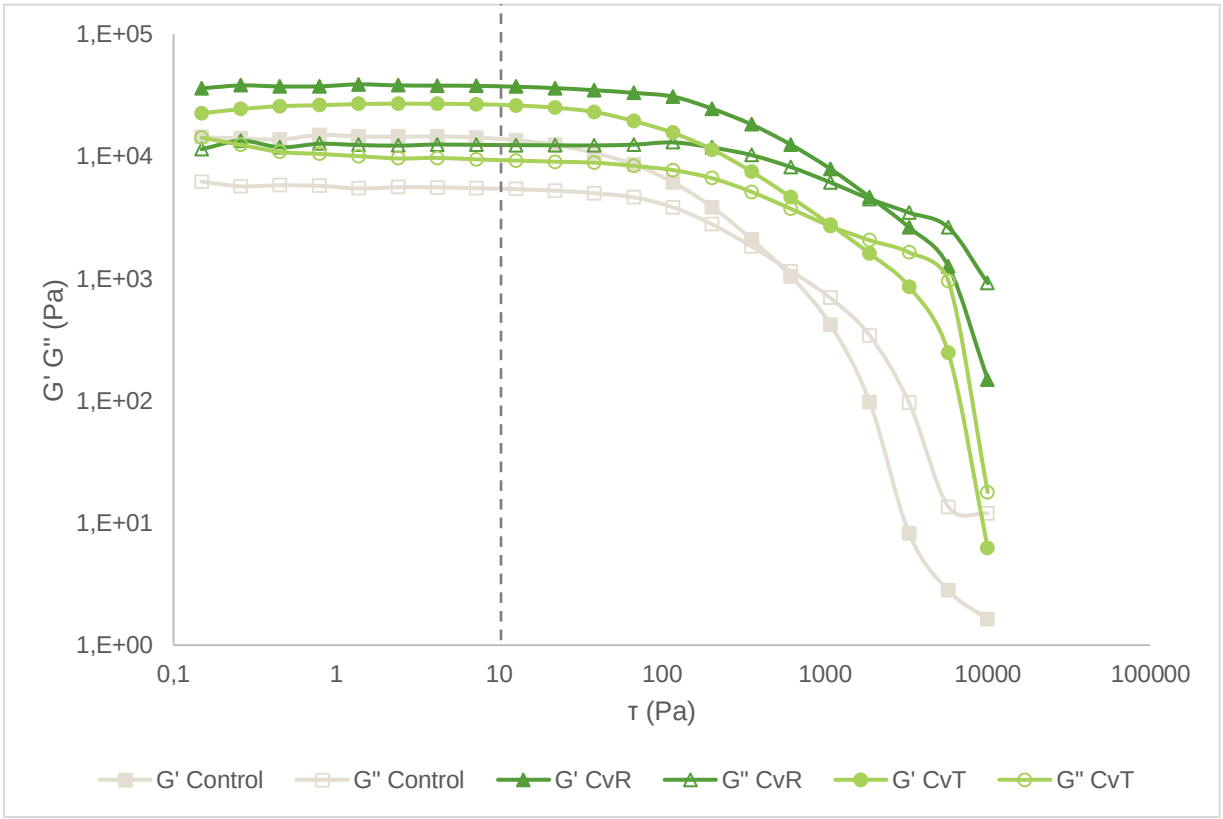


FIGURE 10 - Elastic (G’) and viscous modulus (G’’) (Pa) acquired through stress sweep tests of control and gluten-free doughs with 4% of *Chlorella vulgaris*, raw (CvR) and ethanol treated (CvT). Different symbols refer to different formulations, whereas filled and hollow symbols refer to G’ and G’’ of each formulation, respectively.

In **Figure 11**, it is possible to observe the obtained results of the frequency sweep tests carried out on the GF doughs. Analysing the mechanical spectra, all the GF doughs have a G' higher than G'', which allows one to say that the doughs present a behaviour similar to a weak-structured gel, although the viscoelastic modules present a spacing of less than a decade. In addition, a considerable dependence of the viscoelastic functions with frequency is also observed, being in agreement with the described weak gel like structure. A similar behaviours was found by Macedo et al. (2020) for wheat flour bread produced with whey powder. These authors also obtained a G' higher than G'' in all the samples, and the addition of whey exerted had a limited effect on the dynamic viscoelastic properties, since the results were quite similar. Graça et al. (2018) studied the impact of *Chlorella vulgaris* on the rheology of wheat flour dough and concluded that the microalgae have effects on dough properties. They obtained a similar pattern for the mechanical spectra, with higher G' than G'' values due a possible strengthening effect of the dough structure, by a reinforcement of the protein matrix resulted from the high protein content of microalgae.

All samples showed a similar level of structure, but the magnitudes of G′ and G'' increased with incorporation of *Chlorella vulgaris* raw (CvR), while CvT remained very similar to the control. Hereupon, the treatment with ethanol of microalgae influences the rheology of the dough, making it less elastic.

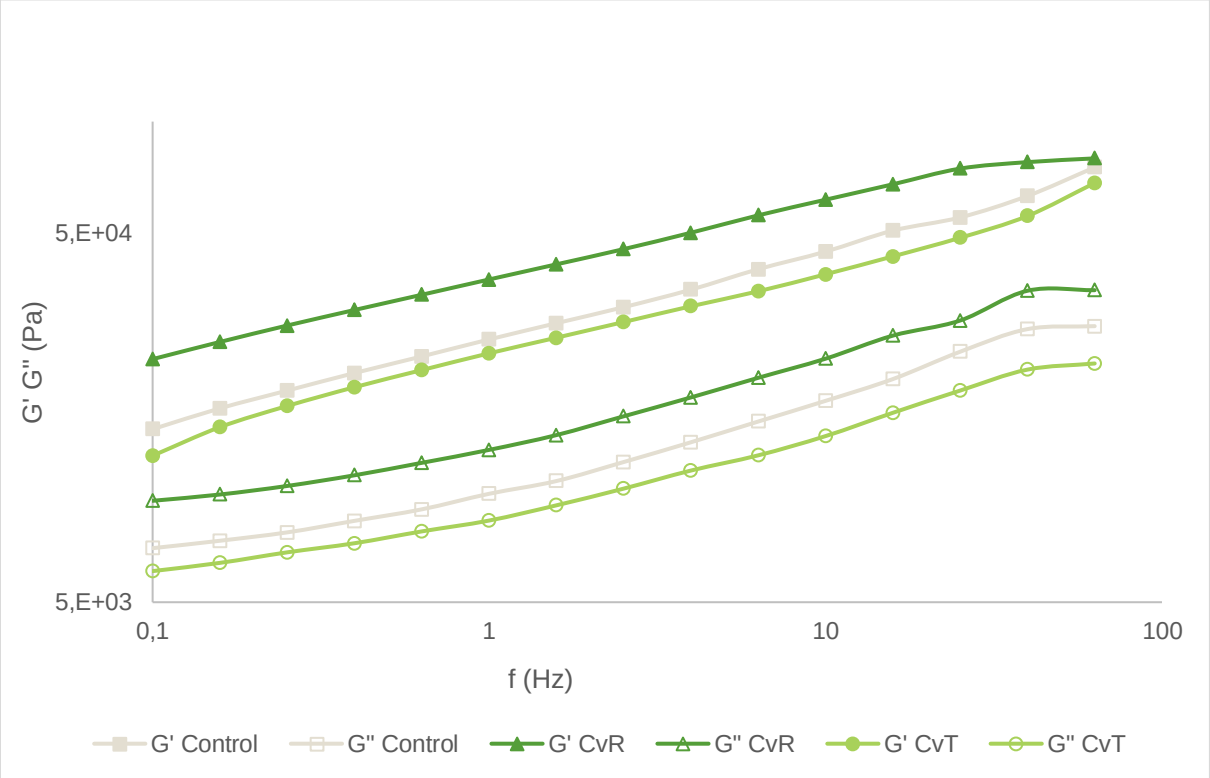


FIGURE 11 - Elastic (G') and viscous modulus (G'') (Pa) acquired through frequency sweep tests of the control and gluten-free doughs with 4% w/w incorporation of C*hlorella vulgaris* raw and ethanol treated (CvR and CvT, respectively). Different symbols refer to different formulations, whereas filled and hollow symbols refer to g' and g'' of each formulation, respectively.

In relation to viscosity tests, the flow behaviour of the different samples are presented in **Figure 12** as the viscosity (Pa.s) as a function of the shear rate (1/s). While shear rate increased the viscosity of all formulations decrease. All the GF doughs have similar results; however, it is possible to point out that with the increase of shear rate, viscosity of CvR has a higher decrease when compared with control and CvT. The power law was adjusted to the obtained results, in order to allow a more objective comparison of the results, through the parameters K - consistency index and n - flow index (**Table 6).** It can be observed with respect to n, there were no significant differences ($p > 0.05$) between the samples, but in the case of K it was found that CvR had a consistency index significantly different ($p < 0.05$) from the control.

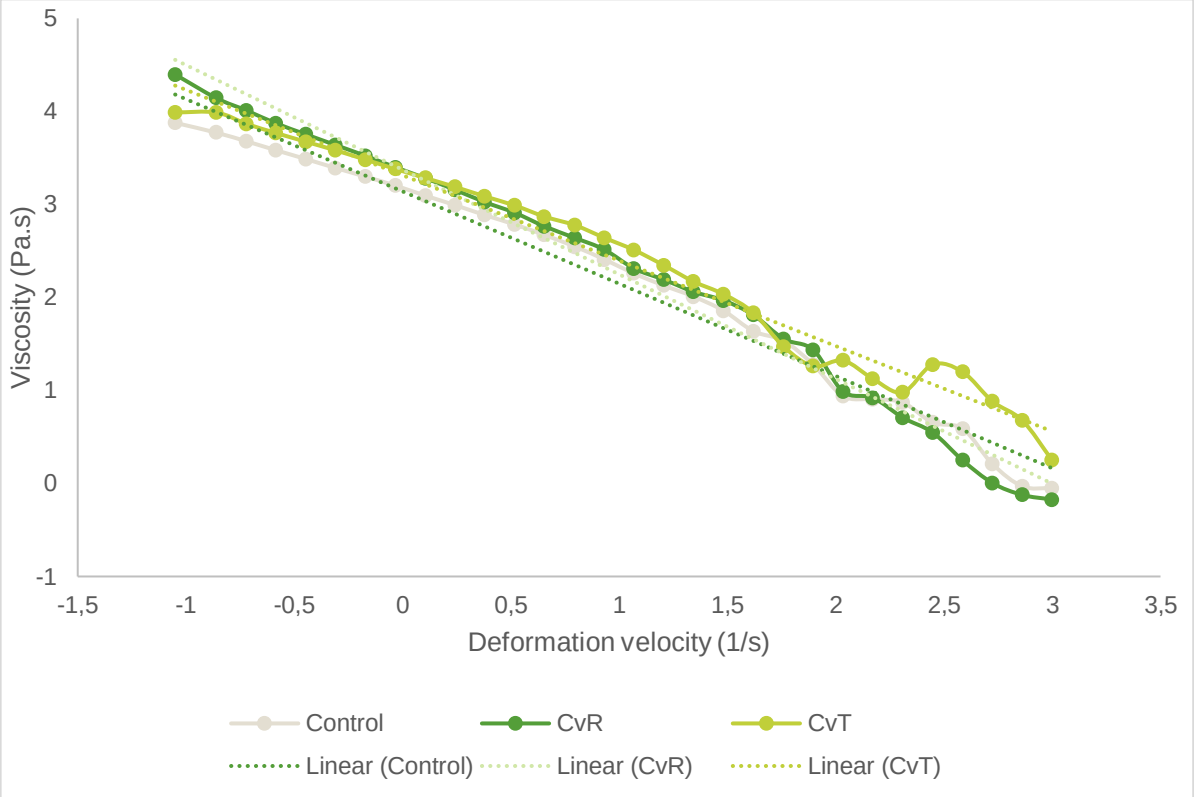


FIGURE 12 - Viscosity (Pa.s) of the control and gluten-free doughs with 4% w/w incorporation of *Chlorella vulgaris* raw and ethanol treated (CvR and CvT, respectively). Different symbols refer to different formulations.

TABLE 6 - Values of consistency index (K) and flow index (n) of the control and gluten-free doughs with incorporation of raw and ethanol treated *Chlorella vulgaris* (CvR and CvT, respectively). Same letters in columns correspond to non-significantly different values between formulations ($p < 0.05$).

	n	K
Control	0,088^{a}	1386,426^{b}
CvR	0,058^{a}	2114,494^{a}
CvT	0,049^{a}	1740,346^{ab}

It is possible to conclude that the viscosity of the doughs with microalgae is slightly higher comparing with the control. These results are in agreement with the results obtained in the texture evaluation and the mechanical spectra of the samples. As explained in the next point, the firmness of the breads with incorporation of microalgae was higher than the control. In relation of mechanical spectra, breads with CvR have an elastic and viscous modulus higher than the control, while CvT was slightly similar to control dough. This can be explained by the protein content since the breads were enriched with *Chlorella vulgaris*. In fact, the incorporation of proteins increases the level of dough structuring, making it firmer.

4.3. BREAD'S TEXTURE BEHAVIOUR

Texture behaviour of GFB was evaluated 2, 26, and 48 hours after baking, using a TPA test. The results were interpreted in terms of firmness (N), and cohesiveness (dimensionless), as shown in **Figures 13 and 14**, respectively. Over time, as expected, the firmness values increased in all samples. In the breads with incorporation of microalgae a significantly increased the crumb firmness ($p < 0.05$), comparing with control, was observed. So, the incorporation of microalgae had an impact on the texture. The control had the lowest firmness (1.43 ± 0.23 N, 2 hours after baking), while the GFB with 4% (w/w) of microalgae had higher firmness values, at 2, 26 and 48 hours after baking. The breads prepared with the TcR (4.21 ± 0.35 N) and CvR (5.94 ± 1.73 N) biomasses were significantly firmer compared to the control, 2 hours after baking. These values increased over time. Ethanol treated microalgal biomasses (TcT, CvT and NgT) generally lead to a decrease in bread crumb firmness compared to the corresponding raw biomasses (TcR, CvR and NgR). Therefore, 48 hours after baking, CvR as a firmness of 13.00 ± 4.74 N, while the CvT as 6.74 ± 2.41 N. NgT (1.80 ± 0.31 N, 2 hours after baking) was the GFB that came closest to the control, with no significant differences between them ($p > 0.05$).

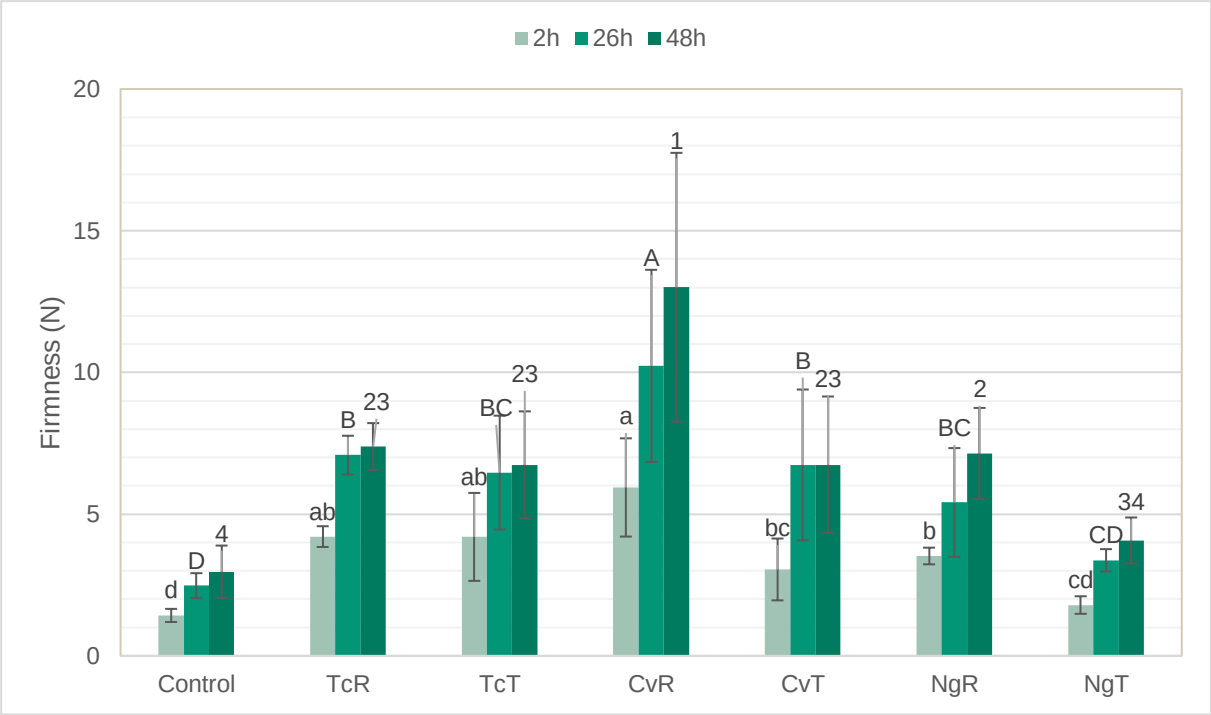


FIGURE 13 - Crumb firmness of control and gluten-free breads prepared with *Tetraselmis chuii* (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Chlorella vulgaris* (CvR), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* (NgR) and *Nannochloropsis gaditana* ethanol treated (NgT). Standard deviation is expressed on graphic error bars. Different small letters, capital letters and numbers for significant differences in storage interval (2, 26 and 48 hours after baking, respectively) of each sample ($p < 0.05$).

The increase observed in the firmness of the breads with incorporation of microalgae, compared to the control, shows the positive effect of microalgae in protecting the bread structure, and can be explained by the higher protein content they contain. In a study by Piteira et al. (2004), it was found that protein and carbohydrate molecules from microalgae can play an important role in the WA process, which promotes the increase in bread firmness. There are other studies that have shown that starch is involved in the bread aging process, that is, the increase in crumb firmness is related to starch retrogradation (Gary & Bemiller, 2003). And moisture can also influence aging, as the starch retrograde rate is directly proportional to the moisture content.

In bread texture evaluation, cohesiveness is an important texture property and should be considered as it is related to chewing, process that is replicated during the TPA test. This characterizes the extent to which the product recovers its deformation before its breakage (Matos & Rosell, 2012). The obtained results showed that the cohesiveness in all samples is quite similar, but exist significant differences, in some cases, two hours after baking ($p < 0.05$). This effect was significant ($p < 0.05$) in the bread made with biomass CvT compared to the corresponding CvR. In fact, 2 hours after baking, CvR has a cohesiveness of 0.56 ± 0.06 and CvT 0.66 ± 0.04. Over time, these values decrease in all formulations, which means that the breads lose cohesiveness. Only in breads with incorporation of *Chlorella vulgaris* ethanol treated (CvT) and *Nannochloropsis gaditana* ethanol treated (NgT) were significantly different from control ($p < 0.05$).

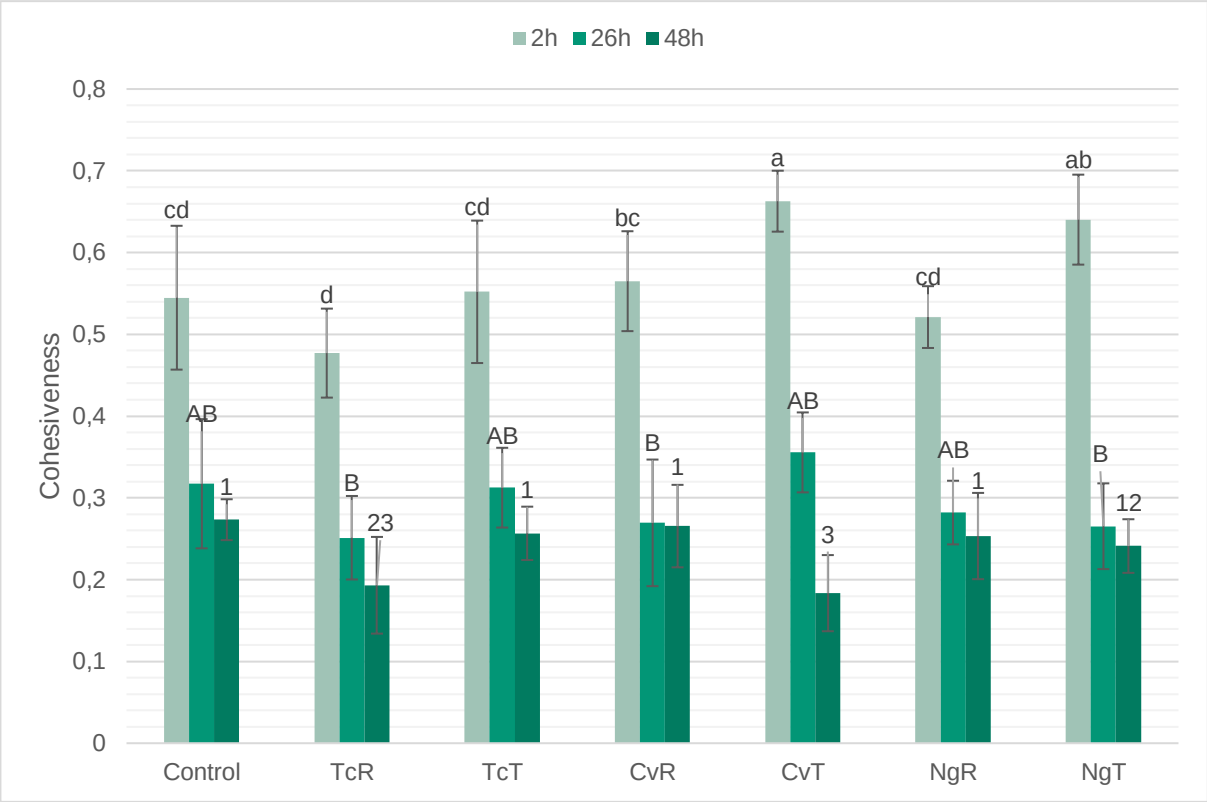


FIGURE 14 - Cohesiveness of control and gluten-free breads prepared with *Tetraselmis chuii* (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Chlorella vulgaris (*CvR), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* (NgR) and *Nannochloropsis gaditana* ethanol treated (NgT). Standard deviation is expressed on graphic error bars. Different small letters, capital letters and numbers for significant differences in storage interval (2, 26 and 48 hours after baking, respectively) of each sample ($p < 0.05$).

Although in this case the bread is GF, the results obtained in the present work are not consistent with previous studies analysing wheat bread that showed no effects of commercial *Chlorella vulgaris* on firmness at levels of incorporation of 1% to 5% (Graça et al., 2018) and by other microalgal species in 1.5% (García-Segovia et al., 2017). Also Khemiri et al. (2020) study the incorporation of 1% and 3% of *Chlamydomonas* sp. EL5 and *Nannochloropsis gaditana* L2 in GFB, and this resulted in an increase in firmness and a decrease in cohesiveness, compared with control. Only bread with 1% of *Nannochloropsis gaditana* L2 has higher cohesiveness than control. Nunes et al. (2020a) incorporated *Chlorella vulgaris* in wheat bread and in the results were observed a decrease in crumb firmness, significantly different from the control. The same did not happen in another study carried out by the same authors (Nunes et al., 2020b), where *Tetraselmis chuii* was incorporated in GFB. In this case, with the addition of 1, 2 and 4% of *Tetraselmis chuii* there was an increase in firmness and a decrease in cohesiveness, being this decrease significant in breads with 4% of microalgal biomass, as well as the results obtained in this dissertation.

Due to their poor texture GFB breaks easily so, the increase in firmness due to the addition of microalgae can be considered a positive result, as it made the bread stronger in terms of texture. This

can also be confirmed through sensory analysis, where consumer perceptions about these textural changes are evaluated, as will be shown next.

4.4. IMPACT OF MICROALGAE ON BREADS COLOUR

The effect of microalgae addition of on the colour parameters of the bread crumb and crust were evaluated and the results are in **Tables 7 and 8**, respectively. Generally, higher luminosity values (L*) indicates a lighter bread. Comparing with control, the L* values, in crumb, registered a significant decrease with the addition of microalgae, and in crust, that parameter increased significantly in all samples ($p < 0.05$), except CvT (48.95 ± 0.90) and NgT (51.21 ± 3.38). In crumb, as expected, this decrease is more significant in breads with incorporation of raw microalgae (38.55 ± 0.30 (TcR), 39.84 ± 0.29 (CvR) and 38.75 ± 4.39 (NgR)), and in crust, the increase is higher in GFB with ethanol treated microalgae (43.79 ± 7.70 (TcT), 48.95 ± 0.90 (CvT) and 51.21 ± 3.38 (NgT)). Regarding the variation in the colour of the bread crust, the samples enriched with microalgae showed the lowest values of L*, which was significantly associated with the level of addition. About the chromaticity parameters, a* and b*, it was observed that almost all formulations have a spatial location in the green (a* negative) and yellow (b* positive) zone. There is as an exception the chromaticity parameter a* in the crust because, despite being positive values, they are quite low. Analysing the a* in crumb it is verified that the value is higher for the control (2.62 ± 0.12) as well as CvT (1.84 ± 0.28) and NgT (2.85 ± 0.23), with no significant differences ($p > 0.05$) in these formulations. In crust, comparing with control (11.54 ± 0.36), a* decrease significantly in the GFB with microalgae ($p < 0.05$), this decrease being more significant in GFB with raw microalgae whose values change to around 1. In crumb, a significant reduction in lightness with more intense green (a* negative) and yellow (b* positive) colour was observed because of the incorporation of microalgae, in comparison with the control. Although CvR (10.97 ± 0.46) and TcT (12.02 ± 2.29) have higher value in b* parameter than control (12.05 ± 0.91). The incorporation of 4% (w/w) of ethanol treated and raw microalgal biomass led to a decrease in redness values (a* positive) which changed to green (a* negative), except for NgT (2.85 ± 0.23) where a slight increase in the value of this parameter was noticed. This could be because *Nannochloropsis gaditana* has less chlorophylls in its composition or that it was completely removed in the ethanol treatment.

TABLE 7 – Results obtained from the effect of the incorporation of microalgae on colour of the bread crumb. Same letters in columns correspond to non-significantly different values between formulations ($p < 0.05$).

Crumb				
	L*	a*	b*	ΔE*
Control	$68.12^{a} \pm 0.72$	$2.62^{a} \pm 0.12$	$12.05^{c} \pm 0.91$	-
TcR	$38.55^{d} \pm 0.30$	$-0.03^{c} \pm 0.21$	$13.87^{b} \pm 1.40$	29.74
TcT	$52.84^{c} \pm 5.23$	$-0.03^{c} \pm 0.08$	$12.02^{c} \pm 2.29$	15.50
CvR	$39.84^{d} \pm 0.29$	$-0.78^{d} \pm 0.16$	$10.97^{c} \pm 0.46$	28.50
CvT	$54.13^{c} \pm 0.77$	$1.84^{b} \pm 0.28$	$16.35^{a} \pm 0.22$	14.65
NgR	$38.75^{d} \pm 4.39$	$-0.56^{d} \pm 0.47$	$15.00^{ab} \pm 3.56$	29.69
NgT	$63.69^{b} \pm 0.33$	$2.85^{a} \pm 0.23$	$15.89^{a} \pm 0.23$	5.86

TABLE 8 – Results obtained from the effect of the incorporation of microalgae on colour of the bread crust. Same letters in columns correspond to non-significantly different values between formulations ($p < 0.05$).

Crust				
	L*	a*	b*	ΔE*
Control	$51.21^{a} \pm 1.88$	$11,54^{a} \pm 0,36$	$21,12^{a} \pm 1,82$	-
TcR	$40.32^{bc} \pm 2.12$	$1,30^{d} \pm 1,09$	$9,85^{d} \pm 4,0$	18.72
TcT	$43.79^{b} \pm 7.70$	$5,38^{c} \pm 0,83$	$14,20^{c} \pm 4,02$	11.87
CvR	$38.8^{c} \pm 0.50$	$1,01^{d} \pm 0,78$	$6,96^{e} \pm 0,63$	21.57
CvT	$48.95^{a} \pm 0.90$	$6,14^{c} \pm 0,65$	$17,33^{b} \pm 0,24$	6.97
NgR	$38.71^{c} \pm 6.03$	$1,34^{d} \pm 0,02$	$11,3^{d} \pm 3,67$	18.88
NgT	$51.21^{a} \pm 3.38$	$9,57^{b} \pm 1,47$	$20,22^{a} \pm 2,45$	2.16

From observing **Figure 15**, it is visible that the addition of microalgae has impact in the GFB colour. Total colour differences (ΔE*) (**Tables 7 and 8**) were evaluated in all bread samples and showed a significant increase ($\Delta E^* > 5$), due the incorporation of microalgae, which means that the differences in bread colour (crust and crumb) are enough to be detected by the human eye. In practically all the breads with microalgae were significant colour differences, compared to the control. The only exception was on the colour of the bread crust with incorporation of *Nannochloropsis gaditana* ethanol treated (NgT) since the colour difference was 2.16 ($\Delta E^* < 5$). Regarding colour variation, it can be concluded that the breads with raw microalgae present a greater difference in relation to the control (around 30 in case of crumb, and around 20 in case of crust).

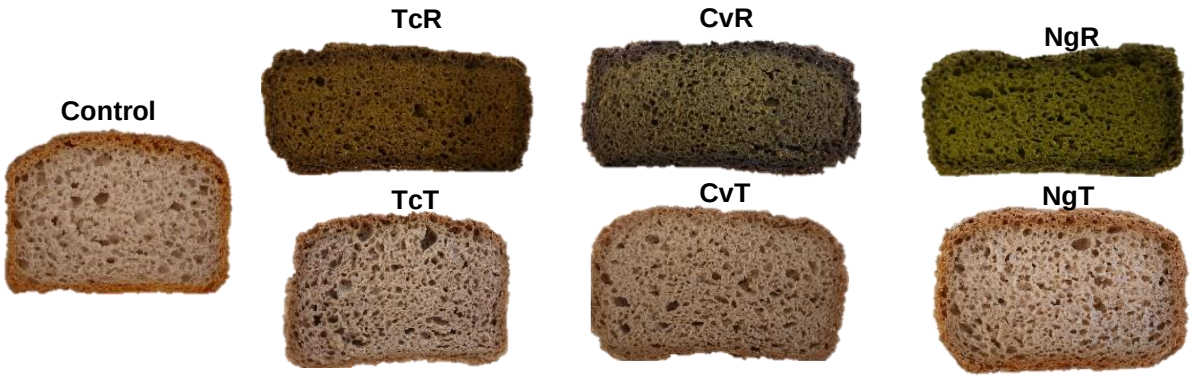


FIGURE 15 - General appearance of the gluten-free breads prepared with control and 4% of microalgae: *Tetraselmis chuii* (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Chlorella vulgaris* (CvR), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* (NgR) and *Nannochloropsis gaditana* ethanol treated (NgT).

The incorporation of microalgae has a great impact on the colour of the breads. Generally, the change in bread colour depends on the presence of pigments in the microalgae biomass, particularly chlorophyll content that characterizes green microalgae (Caporgno & Mathys, 2018). In fact, in GFB with ethanol treated microalgae, the browning that was observed in both the crumb and the crust, and which was accentuated by the degradation of microalgal pigments, can be considered a positive impact, as GFB are generally characterized by poor colour compared to breads that contain gluten (Martins et al., 2020).

The results obtained in this study were similar to those obtained by Vasco (2019), that developed GFB enriched with *Tetraselmis chuii* and verified that breads crumb with incorporation of microalgae appear in the green and yellow regions of colour space (a*negative and b* positive, respectively), and also as a lower luminosity than the control. Also, with incorporation of *Chlorella vulgaris* in GFB, Duarte (2018) obtained similar results. These authors also verified that the differences in breads crumb and crust colour were clearly detected by human eye.

4.5. VOLUME OF THE BREADS

Regarding the specific volume (ml/g) (**Figure 16**), the control bread has the highest volume comparing to the rest of the formulations. Both the raw and the ethanol treated algae biomass replacement decreased significantly ($p < 0.05$) the bread volume. The decrease in bread volume was less with ethanol treated biomass addition (TcT, CvT, NgT) against the corresponding (TcR, CvR and NgR) replacement.

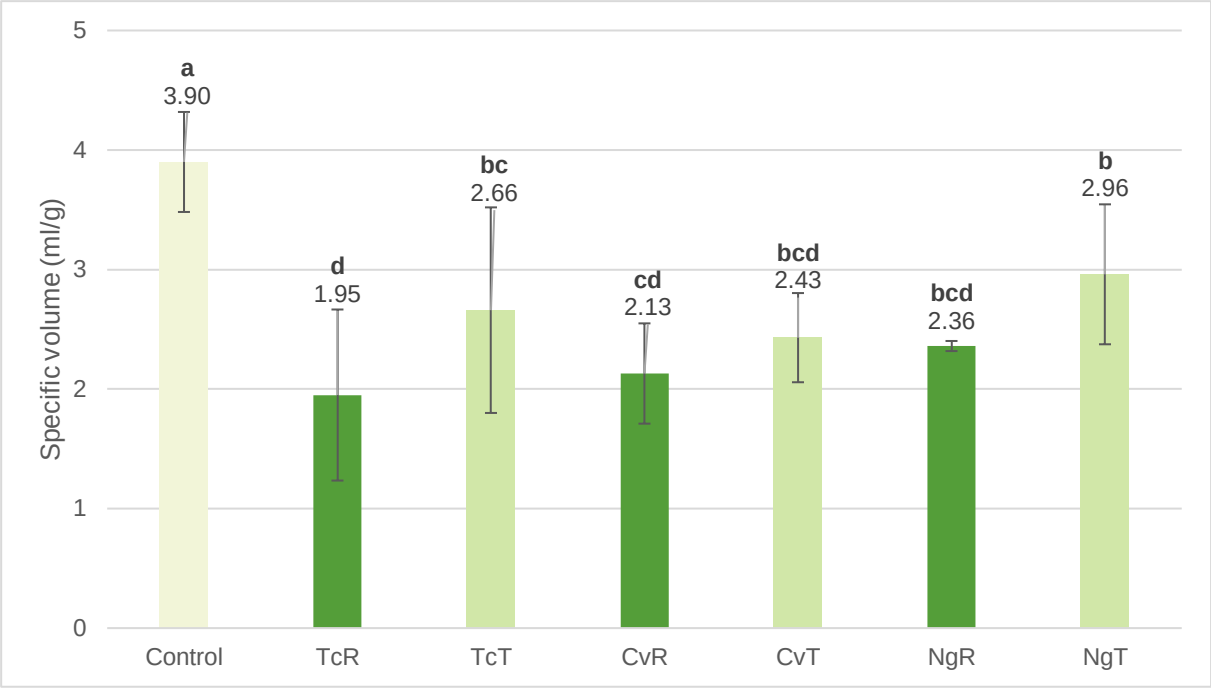


FIGURE 16 - Specific volume (ml/g) of control and gluten-free breads with incorporation of *Tetraselmis chuii* (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Chlorella vulgaris* (CvT), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* (NgR) and *Nannochloropsis gaditana* ethanol treated (NgT). Standard deviation is expressed on graphic error bars. Same letters in columns correspond to non-significantly different values between formulations ($p < 0.05$).

In general, the incorporation of microalgae induced a reduction in the specific volume of breads, as has already been seen in a study developed by Qazi et al. (2021a). Furthermore, it was found that this reduction in volume is less accentuated in the case of microalgae that were subjected to treatment with ethanol. Thus, this can be explained by the fact that ethanol causes a certain protein denaturation, which facilitates the creation of the structure, as it was shown by Nikolaidis et al. (2017). These authors concluded that the use of ethanol revealed causes a marked increase in the denaturation of the whey protein isolate.

Similar results were obtained by Qazi et al. (2021a), that studied the impact of ethanol treatment of microalgae in bread quality. There was a decrease in the volume of bread when incorporated *Tetraselmis chuii* and, although there was a slight increase in the specific volume when this microalgae suffered a ethanol treatment, these differences was not significant. In a study carried out by Figueira et al. (2011) in which 2, 3, 4 and 5% of *Spirulina plantesis* were added to GFB, it was found that the addition of up to 4% did not cause a significant difference in volume specific of breads. However, the addition of 5% reduced this volume, which indicates that the level of incorporation of microalgae can significantly influence the specific volume.

4.6. BREAD'S NUTRITIONAL AND TECHNOLOGICAL PROPERTIES

The nutritional properties of the GFB are described in **Table 9**. In relation to the moisture, the results obtained are between 43.27 ± 1.03 g/100g (CvR) and 45.53 ± 1.42 g/100g (NgT), and it was found that there are no significant differences between the breads ($p < 0.05$). This can be explained by the fact that the amount of water added to each bread formulation was previously adjusted in the Microdough-Lab, according to the characteristics of the doughs.

All the breads with microalgal biomass have more ashes content than the control (1.46 ± 0.00 g/100g). The breads with highest ashes levels are those with incorporation of *Tetraselmis chuii* (1.79 ± 0.01 g/100g (TcR) and 1.75 ± 0.04 g/100g (TcT)), and their values are significantly different from control and NgT (1.58 ± 0.03 g/100g), but not from the other formulations ($p > 0.05$). Compared to the control, the ash content increased in all the GFB regardless of the application of the ethanol treatment, since the minerals are not soluble in the ethanol. Despite that, we can say that the total ashes content is quite similar in all the GFB. As microalgae are an important source of minerals it would be expected that, by incorporating microalgae into GFB, the ash content would increase. Usually, the mineral content is characterized by the amount of ash, that is, the mineral components are converted into products whose weight is considered a measure of the ash content of the coal (Popov et al., 2011).

The lipid content recorded higher values in the raw biomasses (TcR, CvR and NgR). No difference in the lipid content was noticed in the bread's formulations ($p > 0.05$), due to ethanol treatment intended to eliminate the green pigments also practically removed all the lipids from the treated biomasses. The obtained results are between 3.02 ± 0.28 g/100g (control) and 4.03 ± 0.19 g/100g (NgR). The protein content significantly increased with the replacement of the 4% all the microalgal biomasses, due to the high protein content of the algae biomass. Higher protein enrichment was recorded by the treated than the raw biomasses, but the differences was no significant ($p > 0.05$). Similarly, the carbohydrates were nearly similar in all the formulations, which values are from 42.01 g/100g (NgT) to 44.04 g/100g (CvR).

According to the values in **Table 2**, corresponding to the nutritional composition of the microalgal biomass, it would be expected that GFB with TcT, CvT and NgT would have higher protein and ash contents, since the ethanol treated microalgae had more protein and ash content. In relation to proteins, the obtained results were in line with expectations. However, in relation to ash, it was only in the case of *Tetraselmis chuii* that TcT was found to have a higher amount than TcR. In the remaining samples, with the ethanol treatment, there was a decrease in the ash content.

TABLE 9 - Nutritional characterization (g/100g) of the gluten-free breads including moisture, ashes, lipids, proteins, and carbohydrates. Standard deviation is displayed with each value. Letters represent statistically significant differences between samples ($p < 0.05$).

Sample	Moisture	Ash	Lipids	Proteins	Carbohydrates
Control	$45.44^{a} \pm 1.25$	$1.46^{d} \pm 0.002$	$3.02^{a} \pm 0.28$	$6.23^{d} \pm 0.05$	43.85
TcR	$45.23^{a} \pm 0.57$	$1.75^{ab} \pm 0.04$	$3.52^{a} \pm 0.38$	$7.03^{c} \pm 0.05$	42.47
TcT	$44.20^{a} \pm 1.00$	$1.79^{a} \pm 0.01$	$3.45^{a} \pm 0.21$	$7.11^{bc} \pm 0.36$	43.45
CvR	$43.27^{a} \pm 1.03$	$1.63^{abc} \pm 0.08$	$3.62^{a} \pm 0.38$	$7.44^{ab} \pm 0.05$	44.04
CvT	$44.45^{a} \pm 0.85$	$1.60^{bcd} \pm 0.04$	$3.15^{a} \pm 0.79$	$7.67^{a} \pm 0.07$	43.13
NgR	$44.30^{a} \pm 0.95$	$1.60^{bcd} \pm 0.06$	$4.03^{a} \pm 0.19$	$7.07^{bc} \pm 0.08$	43.00
NgT	$45.53^{a} \pm 1.42$	$1.58^{cd} \pm 0.03$	$3.57^{a} \pm 0.63$	$7.31^{abc} \pm 0.02$	42.01

*Carbohydrates was estimated by difference.

Generally, GFB are characterized by their inadequate nutritional quality (Naqash et al., 2017). In this case, it is possible to conclude that several parameters were positive affected by enrichment with microalgae because the content of ash, and proteins increased, even with a low level of microalgae addiction.

Studies using microalgal biomass for food enrichment also have shown improvements in different nutritional parameters. Ak et al. (2016), in breads with incorporation of other microalgae, while ash and protein content of the breads with *Spirulina* were significantly higher than the control, no significant differences were observed in terms of moisture and lipid contents. Lucas et al. (2018), conclude that the incorporation of *Spirulina sp*. in snacks also enriched the nutritional composition with the increase of protein, and ash content. Other authors that studied the incorporation of different microalgae in GFB (Khemiri et al., 2020) also verified that the protein and ash content increased with that incorporation. In this last study, also the lipid content increased with the incorporation of 3% of *Chlamydomonas* sp. EL5 and *Nannochloropsis gaditana* L2.

The mineral profile was improved in all the GFB enriched with microalgal biomass, comparing with the control (**Table 10**). Breads with TcR and TcT were particularly high in calcium (Ca), and iron (Fe) contents. Potassium (K), magnesium (Mg), sulphur (S), copper (Cu), zinc (Zn), and manganese (Mn) increased nearly to similar extent, regardless of the type of algae biomass used. Besides that, only in case of Mg and Mn it is possible to achieve 15% of recommended daily value (RDV) (56.3 mg and 0.3 mg, respectively) since the results obtained were between 199.2 ± 3.9 mg/g (control) and 358.9 ± 6.9 mg/g (NgR) for Mg, and between 0.5 ± 0.0 mg/g (control) and 1.1 ± 0.0 mg/g (NgR). In general, cereals have high mineral content, except in Ca (Coultate, 2002). In the present study, Ca content significantly

increased ($p < 0.05$) by the *Tetraselmis chuii*, indicating that this microalgae provides an alternative for breads where high Ca content is required.

When a food has 15% RDV in its composition it is considered a source of the corresponding minerals, and if it has at least twice the content required for the nutrition claim, 30% of the RDV, it is considered that has high content of that mineral (European Community Regulation No. 1924/2006). It was possible to achieve 15% of RDV only in the case of Mg, P, Fe, and Mn in all the combinations of bread including the control. It is still possible to point out that we can consider that NgR is a source of K (358.9 ± 6.9 mg/g), in addition to having a high content of Fe (5.9 ± 0.1 mg/g), since its value is higher than 30% of RDV (4.2 mg/g). Also, in all the breads formulations were achieved 15% of RDV in case of Mn (0.3 mg/g), so, all the GFB were considered a source of Mn. Beyond this, the breads with microalgal biomass can still be considered rich in Mn as they have more than 30% of RDV (0.6 mg/g) in their composition.

TABLE 10 - Mineral composition (mg/g) of gluten-free breads and the recommended daily value (15 and 30%). Standard deviation is displayed with each value. Different letters represent statistically significant differences between samples ($p < 0.05$).

Sample	K	Ca	Mg	P	S	Fe	Cu	Zn	Mn
Control	$199.2^{c}\pm3.9$	$6.4^{e}\pm0.1$	$56.4^{d}\pm0.8$	$140.^{d}\pm2.3$	$70.0^{e}\pm0.3$	$2.6^{e}\pm0.2$	$0.1^{c}\pm 0.004$	$0.9^{d}\pm 0.02$	$0.5^{f}\pm0.01$
TcR	$223.7^{b}\pm2.5$	$67.5^{b}\pm2.3$	$68.6^{b}\pm1.7$	$164.6^{c}\pm0.4$	$104.3^{bc}\pm0.6$	$4.8^{bc}\pm0.5$	$0.2^{c}\pm0.007$	$1.0^{cd}\pm0.01$	$0.7^{d}\pm0.01$
TcT	$201.6^{c}\pm0.7$	$88.7^{a}\pm0.4$	$64.1^{c}\pm0.1$	$173.4^{bd}\pm0.6$	$103.8^{bc}\pm1.2$	$5.5^{ab}\pm0.3$	$0.1^{c}\pm0.001$	$1.0^{c}\pm0.01$	$0.8^{b}\pm0.001$
CvR	$218.6^{b}\pm1.8$	$8.0^{e}\pm0.3$	$62.6^{c}\pm0.1$	$166.5^{c}\pm1.9$	$94.7^{d}\pm0.5$	$2.9^{de}\pm0.1$	$0.2^{b}\pm0.002$	$0.9^{d}\pm0.003$	$0.6^{e}\pm0.004$
CvT	$220.4^{b}\pm3.1$	$9.2^{e}\pm0.2$	$69.8^{b}\pm1.2$	$183.4^{b}\pm3.9$	$108.9^{b}\pm1.4$	$3.8^{cd}\pm0.3$	$0.2^{b}\pm0.002$	$1.0^{c}\pm0.02$	$0.7^{cd}\pm0.01$
NgR	$358.9^{a}\pm6.9$	$17.9^{c}\pm0.3$	$111.9^{a}\pm0.5$	$287.5^{a}\pm6.6$	$166.7^{a}\pm4.9$	$5.9^{a}\pm0.1$	$0.2^{a}\pm0.003$	$1.8^{a}\pm0.02$	$1.1^{a}\pm0.008$
NgT	$202.3^{c}\pm4.6$	$14.0^{d}\pm0.2$	$68.7^{b}\pm0.5$	$173.8^{bc}\pm0.8$	$99.8^{cd}\pm0.6$	$2.8^{de}\pm0.03$	$0.1^{c}\pm0.01$	$1.1^{b}\pm0.01$	$0.8^{bc}\pm0.002$
15% of RDV* (mg)	300.0	120.0	56.3	105.0	NM	2.1	0.2	1.5	0.3
30% of RDV* (mg)	600.0	240.0	112.6	210.0	NM	4.2	0.4	3.0	0.6

*Recommended daily value (RDV) per European Community Regulation No.1924/2006, Directive No. 90/494 (CE).
NM – not mentioned

The presence of micronutrients is essential for health and functioning of the organism, and CD is one of the health conditions known to be associated with decreased mineral absorption. However, more than half of the world's population suffers from micronutrient deficiency, principally Fe and Ca. Some nutritionists consider that 50% of anaemia is due to insufficient dietary Fe intake, what is always associated with sensation weakness, delayed cognitive and memory deficits (Meenakshi et al., 2010). Therefore, the enrichment of the GFB with microalgae biomass presents a good alternative to the issue of insufficient intake of micronutrients (Khemiri et al., 2020). The nutritional value of the GFB usually increases with algal biomass as noticed in previous studies (Khemiri et al., 2020; Nunes et al., 2020b) using 4% microalgal biomass incorporation due to the rich nutritional value of the algae biomass. Martins et al. (2020) using a similar formulation of GFB, obtained generally higher values by enriching formulations with microalgae. In a study developed by Menezes et al. (2015) was used a mixture of macroalgae *Ulva* sp. and *Cladophora* sp. in wheat bread, to achieve an increase in protein content. Ashes and micronutrients content were significantly affected by the addition of microalgae, with the most positive impact being observed in Fe and Ca.

4.7. IMPACT OF MICROALGAE IN THE BREADS BIOACTIVITY

The results of TPC, pigments and antioxidant capacity tested following DPPH and FRAP methods, are represented in **Figures 17, 18 and 19 (graphs A and B)**, respectively. Compared with the control, the breads with raw microalgae (TcR, CvR and NgR) show higher bioactivity, but the same does not happen in case of the GFB with ethanol treated microalgae (TcT, CvT and NgT).

Phenolic compounds (simple phenol, flavonoids, tannins, and phenolic acids) are considered some of the most important classes of natural compounds with numerous health benefits, including antioxidant potential (Nunes et al., 2020a). GFB with raw microalgae (TcR, CvR and NgR) has the highest amount of TPC, which values are 5.47 ± 0.11 mg GAE /g, 4.38 ± 0.15 mg GAE /g and 4.87 ± 0.37 mg GAE /g, respectively. Treatment with ethanol significantly reduced the TPC level in all the corresponding bread with TcT, CvT and NgT ($p < 0.05$) and the values are 1.92 ± 0.23 mg GAE /g, 3.77 ± 0.04 mg GAE /g and 4.06 ± 0.25 mg GAE /g, respectively. Even the control (4.42 ± 0.22 mg GAE /g) has higher TPC than the ethanol treated microalgae breads, which means that the ethanol treatment removed phenolic compounds.

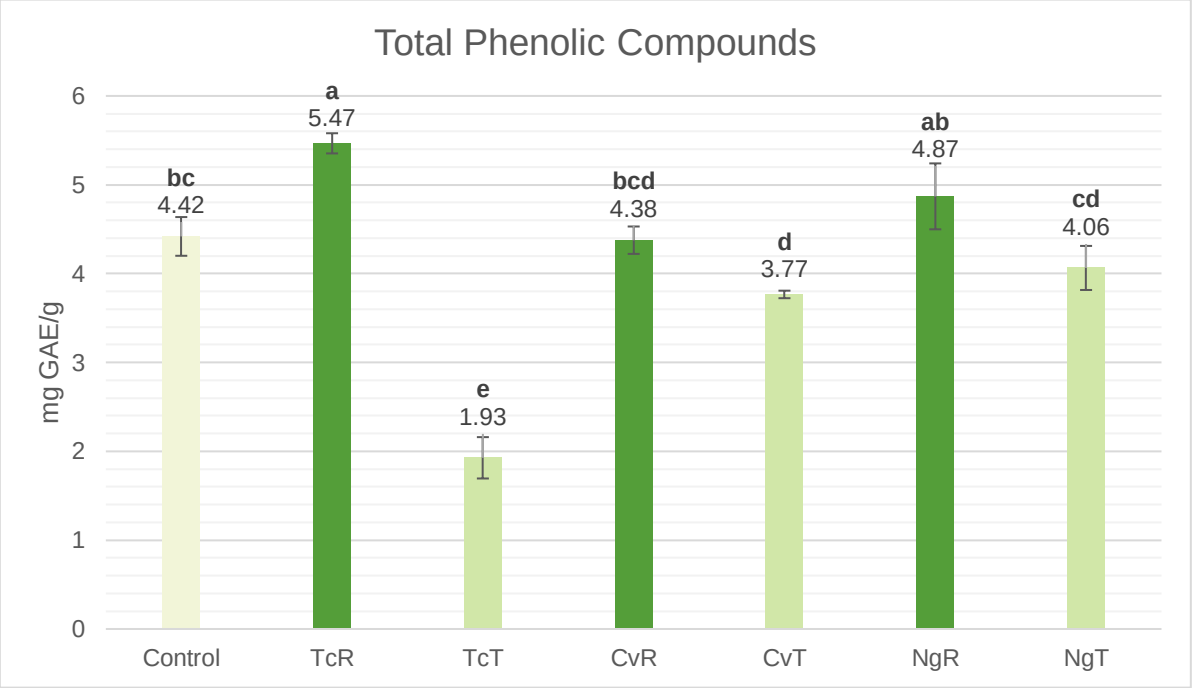


FIGURE 17 - Total phenolic content (mg gallic acid equivalents/g) of gluten-free breads enriched with 4% (w/w) of microalgal biomass in comparison with control bread. Error bars indicate the standard deviations of the repetitions (n = 3). Letters represent statistically significant differences between samples ($p < 0.05$).

Evidently, the treatment of microalgae with ethanol induces a reduction in TPC since they are not extracted by ethanol. This fact is more relevant in breads with *Tetraselmis chuii* (TcR and TcT). As verified by Nunes et al. (2020b), where was evaluated the impact of *Tetraselmis chuii* on bioactivity of GFB, the addition of microalgal biomass at 4% (w/w) also resulted in an increase of TPC.

The antioxidant activity of the GFB is in **Figure 18**. A significantly higher antioxidant activity was recorded in all the raw microalgae breads (TcR, CvR and NgR), compared with the treated microalgae (TcT, CvT and NgT) in most cases, regardless of the estimation method (FRAP or DPPH). The results obtained through the FRAP and DPPH methods were very similar. The breads with the highest antioxidant activity were those with incorporation of TcR, CvR and NgR, and the bread with the highest antioxidant activity was the one with TcR (5.82 ± 0.08 mg vitamin C equivalent /g DE (DPPH) and 5.38 ± 0.52 mg acid ascorbic equivalent /g DE (FRAP)). Either through the DPPH method or through the FRAP, it was verified that the ethanol treatment also caused a decrease in the antioxidant activity of bread. These values are identical to those of the control (4.19 ± 0.36 mg vitamin C equivalent /g DE (DPPH) and 3.64 ± 0.22 mg acid ascorbic equivalent /g DE (FRAP)), except for TcT (2.17 ± 0.22 mg vitamin C equivalent /g DE (DPPH) and 1.04 ± 0.12 mg acid ascorbic equivalent /g DE (FRAP)), since during the practical work one more extraction was performed than in the remaining samples ($p < 0.05$). Therefore, this error might have been the main reason for this situation.

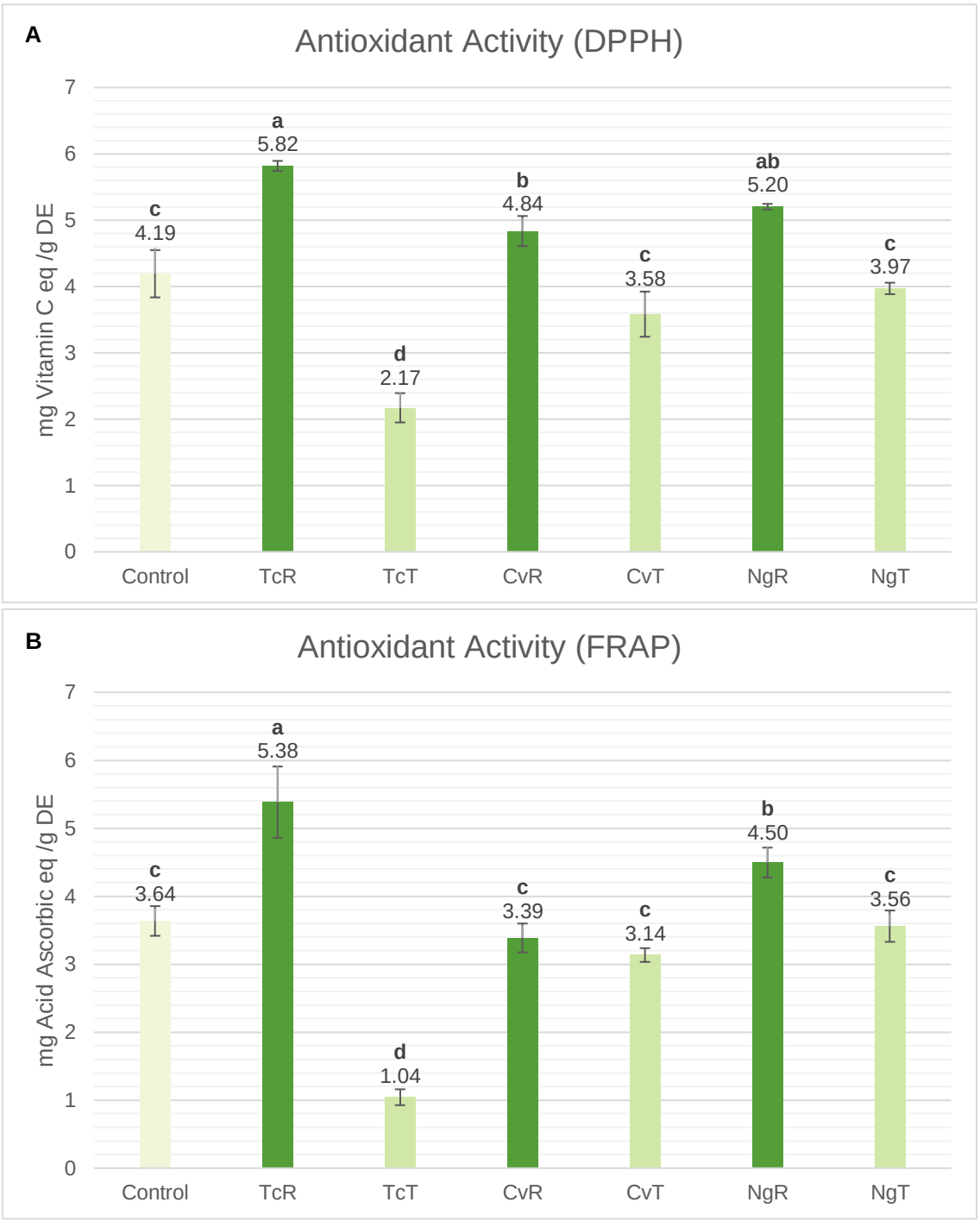


FIGURE 18 - Antioxidant capacity measured using the DPPH method (mg.g−1 ascorbic acid equivalents) and FRAP method (mg.g−1 ascorbic acid equivalents) of gluten-free breads enriched with 4% (w/w) of *Tetraselmis chuii* (TcR), *Tetraselmis chuii* ethanol treated (TcT), *Chlorella vulgaris* (CvT), *Chlorella vulgaris* ethanol treated (CvT), *Nannochloropsis gaditana* (NgR) and *Nannochloropsis gaditana* ethanol treated (NgT) in comparison with control bread. The given values represent average ± standard deviation (n=3), while different letters for a given parameter indicate significant difference (p> 0.05).

As shown in **Figure 19**, it was found that control bread and breads with incorporation of treated microalgae (TcT, CvT and NgT) did had no pigments in its composition. As expected, only the breads baked with raw microalgae (TcR, CvR and NgR) were dominated by the chlorophyll-a (2.24 ± 0.30 mg/g, 2.33 ± 0.00 mg/g and 2.05 ± 0.01 mg/g, respectively), while chlorophyll-b (0.81 ± 0.07 mg/g (TcR) and

0.20 ± 0.02 mg/g (CvR)), and carotenoids (0.30 ± 0.21 mg/g, 0.13 ± 0.03 mg/g and 0.51 ± 0.03 mg/g, respectively) were also present, but in lesser amount. Chlorophyll-a is the pigment present in bigger amount, being that NgR only has chlorophyll-a and carotenoids. Ethanol treatment completely removed the pigments (dark green colour), therefore, like the control, the breads baked with TcT, CvT and the NgT were entirely devoid of pigments. Therefore, ethanol can be considered an efficient solvent for a complete extraction of the pigments. The carotenoids content was significantly different in all breads ($p < 0.05$), being that NgR was the one with more content (0.51 ± 0.03 mg/g) (Qazi et al., 2021b).

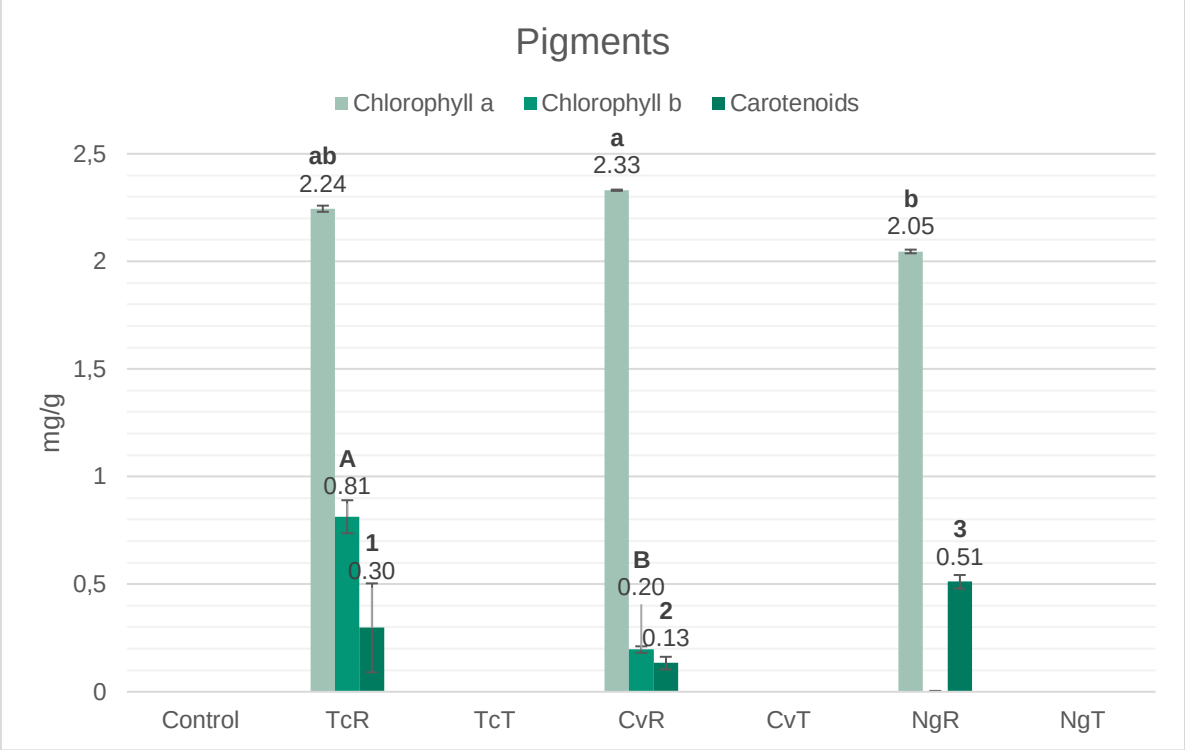


FIGURE 19 - Total pigments in gluten-free breads enriched with microalgae and the control. The given values represent average ± standard deviation (n=3). Significant differences in chlorophyll-a, chlorophyll-b and carotenoids are shown by small letters, capital letters and numbers, respectively ($p < 0.05$).

The fact that control has a high TPC and antioxidant activity is probably due to the presence of buckwheat flour in GFB formulation in the major quantity, being that buckwheat has been shown to have high polyphenols and antioxidant activity in bread previously by Verardo et al. (2018). In studies performed by other authors there was also observed an increase in antioxidant activity in samples with incorporation of microalgal biomass. Khemiri et al. (2020), verified that the addition of microalgal biomass also resulted in an increase in TPC, which was 0.18 mg/g of gallic acid equivalents in the control bread and 0.44 mg/g in the bread containing *Chlorella vulgaris* raw. Niccolai et al. (2019) obtained similar results in terms of TPC and antioxidant activity (DPPH method), while these content increased with the addition of 2, 6 and 10% of *Arthrospira platensis* biomass. Nunes et al. (2020a), studied the addition of the microalgal biomass and it resulted in an increase in the TPC. Compared with the control, the incorporation of the fresh microalgal biomass led to an increase in the antioxidant capacity of the breads. In their experience bread prepared with the commercial *Chlorella vulgaris* presented a lower TPC and was similar to the control. With incorporation of brown microalgae, in a study

developed by Różyło et al. (2017) were observed the increase of antioxidant activity. Usually, with the incorporation of green microalgae in GFB, the TPC values are lower than when are incorporated brown microalgae (Różyło et al., 2017).

4.8. SENSORY ANALYSIS

Sensory analysis is a science discipline used to measure, analyse, and interpret reactions of individuals to characteristics of foods and materials as perceived by the senses of eyesight, smell, taste, touch, and hearing. It can be applied in the analysis and development of new products, in product shelf-life tests, raw material or final product quality control or in market tests. There are factors dependent on the individual and factors related to the environment that can influence the sensory analysis test. Therefore, it is important that the tests are carried out under specific conditions, in addition to considering several factors of the taster (Esteves, 2014). Sensory food analysis is an important aspect of new product development and marketing, as it offers insights into consumer behaviour and quality assurance.

The sensory analysis test was performed on the control and the GFB with 4% (w/w) incorporation of *Chlorella vulgaris*, ethanol treated (CvT) and raw (CvR). **Figure 20** represents the average scores of the sensory parameters as evaluated by the non-celiac panel (n=33) whose age ranged from 19 to 73 years old.

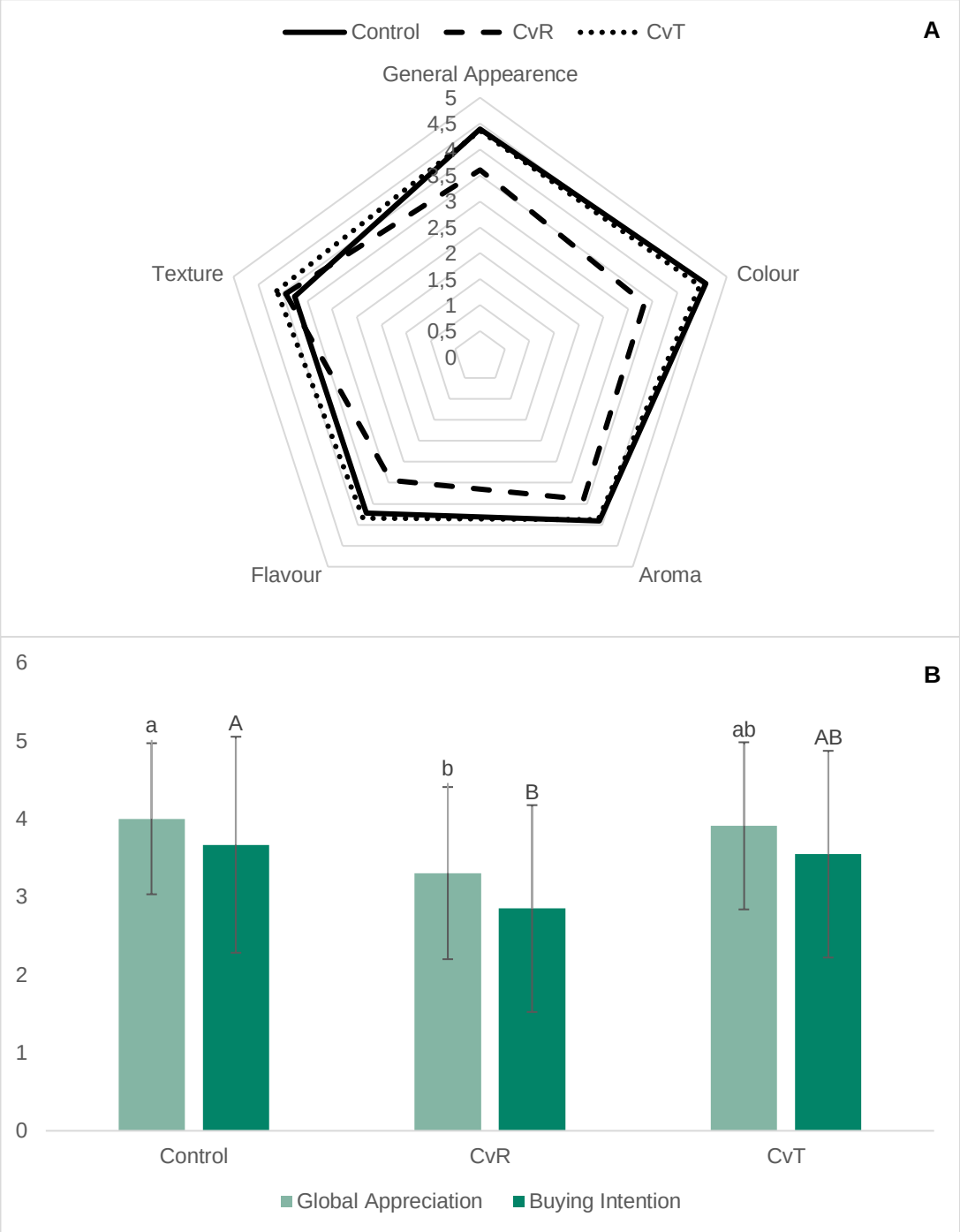


FIGURE 20 - Responses of the sensory analysis panel tasters (n = 33) regarding gluten-free breads enriched with 4% (w/w) *Chlorella vulgaris* (CvR) and *Chlorella vulgaris* ethanol treated (CvT), as well as the control sample. Standard deviation is displayed with each value as error bars. Letters represent statistically significant differences between samples ($p < 0.05$).

The control bread had a high classification in all parameters, which means that the formulation was very well accepted by the tasters. It was observed that bread with incorporation of 4% (w/w) CvT had a higher classification than bread with CvR, being the evaluation very similar to that of control. Texture was the parameter where there were practically no differences between all samples, and the evaluation obtained was around 4 (pleasant). Overall, the panellists showed a significantly higher "global appreciation" for the CvT and the control (both around 4 – pleasant) in comparison to the CvR replaced

GFB (around 3 – indifferent). The differences in “global appreciation” for the CvT and the control was non-significant ($p < 0.05$). Similar to the “global appreciation”, a high “buying intention” was shown by the consumers for the control and the CvT compared to the CvR replaced GFB. In general, the results obtained in sensorial evaluation revealed that the CvT was significantly closer to the control in practically all the evaluated attributes. CvR had lower evaluation (flavour, colour, and aroma), which is what usually obtained in breads with raw microalgal biomass.

The evaluation of CvT shows that it was appreciated in the sensory test as much as the control. This is due to the elimination of pigments from the biomass, and the characteristic aroma and flavour were also eliminated. It is also known that algae biomass in its native form contains sulphur compounds, which are responsible for the perception of the aroma of microalgae (Lafarga, 2019). These compounds are largely eliminated by treatment with ethanol, as shown by Qazi et al. (2021a).

If one separates the tasters in two groups according with the age rate (**Figure 21: A** and **B**, according 20 tasters with age between 19 and 39; **C** and **B**, according 13 tasters with age between 40 and 73), the only difference is that the younger tasters appreciate more the GFB with incorporation of *Chlorella vulgaris* raw than the older tasters. This fact is very clear on graph of “buying intention” (**Figure 21, B** and **D**) that, comparing with the older tasters, has a higher evaluation. The other parameters are very similar in all age rate.

In general, the sensory study showed that the treatment with ethanol or similar processing step where the colour of the algae biomass is eliminated, is a promising strategy in the introduction of foods with the addition of microalgae in the market.

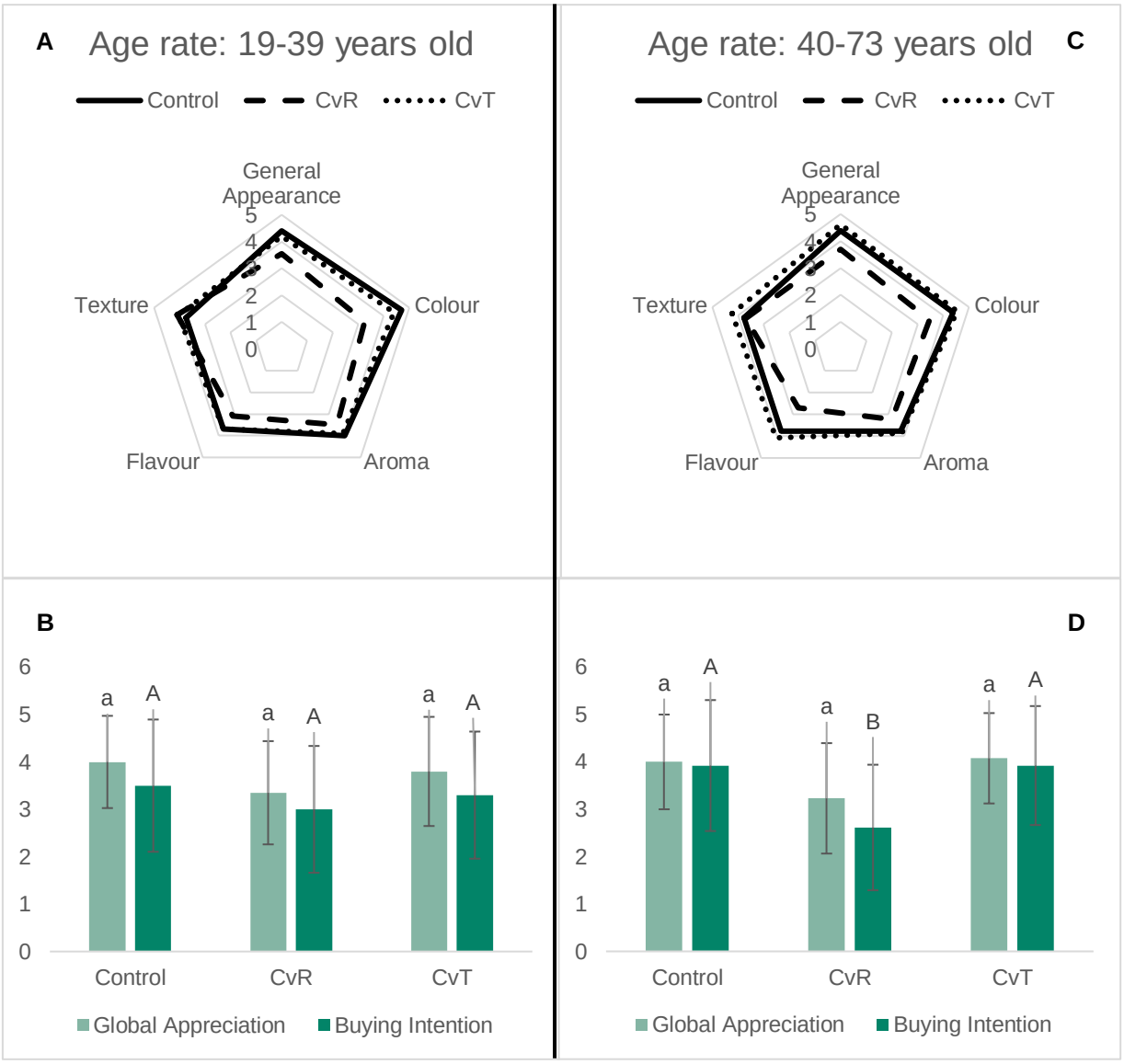


FIGURE 21 - Responses of the sensory analysis panel tasters (n = 33) according to age rate. A and B correspond to the tasters with age between 19 and 39 years old; C and D correspond to the tasters with age between 40 and 73 years old. Standard deviation is displayed with each value expressed as error bars. Letters represent statistically significant differences between samples ($p < 0.05$).

The tasters left some comments during the sensory analysis test. In general, they said that the breads have a very balanced flavour, and that control and CvT are close in terms of texture and overall profile, with more pleasant CvT in terms of colour. Some of the tasters also mentioned that, despite the intense flavour of the bread with incorporation of *Chlorella vulgaris* raw (CvR), the impact/ benefits that this seaweed could bring to health would lead them to buy the sample. Tasters said that CvR had a very intense aroma and flavour and that all three samples were good compared to commercial GFB. Furthermore, this GFB could be an interesting alternative for consumers interested in healthy products with an innovative taste and colour.

In conclusion, the results obtained in the test showed that the products would have an acceptance in the market, as, in general, all parameters were considered pleasant for the consumer. The CvR bread sample had the most impact due to its distinct aroma and colour, as the public consumers are not used

to this type of food product. Through the test it was possible to see that GFB with microalgae could be integrated into a market, but preferably with CvT. Despite being a different product than usual, the consumer bought it out of curiosity, to try it or for a specific occasion.

5. CONCLUSIONS

Based on the results obtained it is possible to conclude that microalgae can be used as an innovative ingredient to improve the nutritional properties and technological behaviour of bread, and the treatment with ethanol helps reduce the sensory issues commonly encountered with microalgae addition.

GFB is of great importance especially for patients with CD. A nutritious GFB can be produced by adding microalgal biomass such as *Tetraselmis chuii*, *Chlorella vulgaris* and *Nannochloropsis gaditana*. The sensory properties are generally compromised dominated by intense green colour and fish flavour. However, by treating the microalgae biomass with ethanol this problem can be reduced to a large degree. Microalgal biomass led to produce GFB with a significant structuring impact in terms of firmness and cohesiveness. However, the rheological parameters used to characterize dough properties did not provide any clear correlation with respect to baking performance. In some tests, differences were observed between breads produced with raw microalgae and microalgae that were subjected to ethanol treatment. Per example, GFB prepared with raw microalgae obtained a higher content of bioactive composites, that are very important to human health.

Gluten components properties are important for the technological purpose of bakery products. In the case of these GFB, the microalgae proteins, in purified form (ethanol treated), played the structure building role to some extent, normally performed by gluten. The incorporation of 4% w/w microalgae resulted in an increase of this content and of other important components (lipid and ashes). Thus, the use of microalgae as a protein source can be a beneficial alternative for humans, animals, and the environment since microalgae breads always had high protein content.

As expected, breads produced with incorporation of raw *Tetraselmis chuii*, *Chlorella vulgaris* and *Nannocloropsis gaditana* have a green coloration significantly different than control. This difference in colour was easily detected by human eye. The GFB prepared with *Chlorella vulgaris* biomass was used in sensory tests since at present this is the only approved specie by EFSA. The addition of microalgae biomass treated with ethanol had a positive impact on all sensory attributes evaluated, being very similar to the control.

In general, the breads with incorporation of *Nannochloropsis gaditana* were the ones with the best characteristics. Although the ethanol treatment reduced the bioactivity of the breads, in relation to TPC and antioxidant activity the results were very similar to the control and breads with this microalgae also presented an excellent volume. In addition to these positive points, beyond to the high amount of protein, *Nannochloropsis gaditana* ethanol treated biomass has the highest amount of fibre, which is also important to human health, even more in the case of individuals suffering from celiac disease.

In conclusion, the improvement in texture parameters of the GFB with incorporation of microalgae was probably caused by the presence of a substantial amount of protein in the microalgal biomass. Overall, the current study required that microalgae can be considered as a suitable ingredient in GFB, enhancing its structure and nutrition profile. In fact, the ethanol treatment of microalgae had a great impact in the acceptance by consumers, principally in the main objective which were the parameters of

colour and flavour. On the other hand, this treatment negatively affected the bioactivity of the breads (less TPC and antioxidants), despite having improved its characteristics about mineral and protein content, and volume.

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ANNEXES

I. TASTE PANEL EVALUATION FORM – ISA TESTING ROOM

Sensory Analysis Test

Gluten-free bread enriched with microalgae

Name (optional): _____ **Date** __/__/____

Age range: □ ≤ 19 □ 20-29 □ 30-39 □ 40-49 □ 50-59 □ ≥60 **Gender**: □ Male □ Female

We thank you for your participation in this sensory analysis test, which aims to assess the main sensory attributes of gluten-free bread with incorporation of microalgae.

If you are **allergic** or **intolerant** to any food, you must inform the person in charge in the room, before starting the test.

You should also inform the person responsible if:

- Smoked less than 1 hour ago
- Find yourself sick
- You are using a strong perfume
- Feel that there is a reason that could interfere with the sensory test

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Before starting the test, we kindly ask for your cooperation in filling out some questions, according to your consumption habits.

1. Do you consume bread regularly? □ Yes □ No

1.1. If so, how often? □ Two or more times a day □ Once a day □ A few days a week □ A few times a month □ Rarely

2. Are you a regular consumer of gluten-free foods? □ Yes □ No
3. Are you a consumer of gluten-free bread? □ Yes □ No
4. Do you usually buy breads made with different flours (wheat, rye and corn)? □ Yes □ No

4.1. If so, what are the main reasons why?
_____
_____

Procedure:

- Observe the product presented and make its assessment as to appearance, colour and aroma.
- Taste the product and evaluate it for taste and texture.
- Rate the product concerning its overall appreciation and purchase intention.
- Answer the following questions by ticking with X what you think is most appropriate.

GENERAL APPEARANCE	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

COLOUR	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

AROMA	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

FLAVOUR	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

TEXTURE	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

GLOBAL APPRECIATION	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

BUY INTENTION	Sample Code		
	W2A	A2F	S8L
Would always buy			
Would probably buy			
Don’t know			
Probably would not buy			
Would never buy			

Any other comments about the samples:

Thank you!

II. TASTE PANEL EVALUATION FORM – DELIVERY MODE

Sensory Analysis Test

Gluten-free bread enriched with microalgae

Name (optional): _____ **Date** __/__/____

Age range: □ ≤ 19 □ 20-29 □ 30-39 □ 40-49 □ 50-59 □ ≥60 **Gender**: □ Male □ Female

PLEASE PAY ATTENTION TO ALL INSTRUCTIONS.

We thank you for your participation in this sensory analysis test, which aims to assess the main sensory attributes of gluten-free bread with incorporation of microalgae.

Due to the current pandemic situation, the present test cannot be held in a test room suitable for the purpose, so find a calm and stimulus-free place (other people, food, television) to carry out your test.

Please do not comment on your opinions until everyone in your group / aggregate has finished the test.

If you are **allergic** or **intolerant** to any food, you must inform the person in charge in the room, before starting the test.

You should also inform the person responsible if:

- Smoked less than 1 hour ago
- Find yourself sick
- You are using a strong perfume
- Feel that there is a reason that could interfere with the sensory test

---

Before starting the test, we kindly ask for your cooperation in filling out some questions, according to your consumption habits.

5. Do you consume bread regularly? □ Yes □ No
5.1. If so, how often? □ Two or more times a day □ Once a day □ A few days a week □ A few times a month □ Rarely
6. Are you a regular consumer of gluten-free foods? □ Yes □ No
7. Are you a consumer of gluten-free bread? □ Yes □ No
8. Do you usually buy breads made with different flours (wheat, rye and corn)? □ Yes □ No
8.1. If so, what are the main reasons why?
_____
_____

Procedure:

- Observe the product presented and make its assessment as to appearance, colour and aroma.
- Taste the product and evaluate it for taste and texture.
- Rate the product concerning its overall appreciation and purchase intention.
- Answer the following questions by ticking with X what you think is most appropriate.

GENERAL APPEARANCE	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

COLOUR	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

AROMA	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

FLAVOUR	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

TEXTURE	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

GLOBAL APPRECIATION	Sample Code		
	W2A	A2F	S8L
Very pleasant			
Pleasant			
Indifferent			
Unpleasant			
Very unpleasant			

BUY INTENTION	Sample Code		
	W2A	A2F	S8L
Would always buy			
Would probably buy			
Don't know			
Probably would not buy			
Would never buy			

Any other comments about the samples:

Thank you!

III. ABSTRACT SUBMITTED FOR CONGRESS – XV CONGRESS OF FOOD CHEMISTRY

The effect of the microalgae *Chlorella vulgaris*, *Tetraselmis chuii* and *Nannochloropsis gaditana* on technological aptitude, nutritional composition, and bioactivity of gluten-free breads

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This study is included in the project "A2F - Algae to Future", supported by the Research Council of Norway, that addresses the potential of microalgae to produce high-quality proteins, polyunsaturated fatty acids, and low-carbon carbohydrates, as healthy ingredients for food in the future. It establishes a basis for the industrial production of microalgae in Norway, using natural resources and by-products from existing sources for agriculture, aquaculture, and processing industry.[1] This project has the involvement of 26 international partners, including the University of Lisbon, that is responsible for the development of wheat breads and gluten-free (GF) breads with the incorporation of microalgae.[2-3]

Consumption of gluten-free products, particularly bread, has increased considerably in recent years, which is not only due to the increase in celiac disease, but also to the increase in the number of consumers who have not been diagnosed with celiac disease, but are eliminating gluten from the diet. The substitution of gluten in bread-making is a challenge, as there is no raw material or ingredient capable of completely replacing gluten in terms of structural builder.[4] Hydrocolloids are often used as a thickening agent, binding water, and increasing the viscosity of the dough, for better volume, texture, and final bread quality.[5] In this study, hydroxypropyl methylcellulose (HPMC) was used.

Microalgae can be considered one of the most promising functional food sources, as they have the potential to be a sustainable solution for food, but there are still improvements to be made before microalgae become a regular source of food.[6] They are exceptional protein resources with the potential to become a staple food for consumers across the planet, but these are very sensitive to changes in sensory characteristics (odor and flavor), inducing limitations in the level of microalgae incorporation.[3]

In the present research, microalgae biomass were subjected to ethanol extraction to obtain less pronounced colors and flavors, with the purpose to increase consumer acceptance, and allowing an increase of the incorporation levels.

The incorporation of microalgae in food can lead to changes in the rheology, texture, sensory properties, and in the nutritional composition.[2-3] The objective of this study was to compare the impact of adding 4% (w/w) of raw and ethanol treated *Tetraselmis chuii*, *Nannochloropsis gaditana* and *Chlorella vulgaris*, produced by A2F partners, in dough structure and technological aptitude, nutritional composition, and bioactivity of GF breads.

The technological performance of the doughs was studied according to the rheological properties on the Microdough-Lab (torque, water absorption, development time, stability and softening) and on the rheometer (creep & recovery, and frequency sweep measurements). Firmness, color, and the volume of the breads was also evaluated. The nutritional and chemical composition was evaluated based on the AOAC methods (proteins, lipids, carbohydrates, ashes, moisture, and minerals), and the bioactivity by determination of the total phenolic compounds (*Folin-Ciocalteu*), antioxidant activity (DPPH and FRAP) and pigments (chlorophyll-a, chlorophyll-b, and carotenoids).

For the sensory analysis, by an untrained panel of 33 consumers, only the control and GF breads with incorporation of *Chlorella vulgaris* (raw and ethanol treated) were tested since it is the only microalgae approved by the European Food Safety Authority (EFSA) for human consumption.

The obtained results evidence that the treatment with ethanol is an interesting option to incorporate microalgae in food. This treatment allowed the production of GF breads with a more pleasant color and aroma, and with improved sensory acceptance accompanied by an enriched nutritional composition. Improvements in terms of bread texture and volume were also observed. Microalgae pretreatment with ethanol improves the GF breads sensory properties to a large extent. This finding indicates that ethanol treatment or similar aimed at elimination of ethanol soluble constituents and consequently enrichment of the algal proteins might be a feasible strategy for producing GF breads of high nutritional value with greater consumer acceptance.

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