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UNIVERSIDADE DO ALGARVE
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Declaração de autoria de trabalho

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Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(Pedro Miguel Viriato Letras)

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List of Abbreviations

A – Absorbance
AAE/ g DE – Ascorbic acid equivalents/ grams of dry extract
ALA – alfa (α) - linoleic acid
ANOVA – Analysis of variance
a_w **–** Water activity
CAD – Computer Assisted Design
CIE – Colour space International Comission on Illumination
Chla – Chlorophyll *a*
Chlb – Chlorophyll *b*
DDT – Dough development time
DMSO – Dimethyl sulfoxide
DPPH – 2,2-diphenyl-1-picryl-hydrazyl-hydrate
D65 – Standard illuminant CIE D65
EEAs – Essential amino acids
EFSA – European Food Safety Authority
EPA - eicosapentaenoic acid
FAO – Food and Agricultural Organization
FDA – Food and Drug Administration
FRAP – Ferric reducing ability of plasma
G' – Storage moduli
G'' – Loss moduli
GAE/ g DE – Gallic acid equivalents/ grams of dry extract
GLA – Gamma (γ)-linoleic acid
HCL – Hydrochloric acid
HPMC – Hydroxypropyl methylcellulose
LA – Linoleic acid
LDL – Low density lipoproteins
LVR – Linear viscoelastic region
SAOS – Small amplitude oscillatory shear
SLP – Selective Laser Printing
Spirulina – *Arthrospira platensis*
TPA – Texture Profile Analysis
TPTZ – Tripyridyltriazine
USD – United States of America Dollar
WHO – World Health Organization
3D – Three-dimensional

Abstract

3D food printing is a recent promising technology to break cultural barriers by introducing new food sources as microalgae, through innovative food shapes and textures, in a resource scarce world, unfeasible with the current intensive meat and agriculture industries. The present work intended to create an innovative gluten-free cereal snack nutritionally improved by incorporation of microalgae *Chlorella vulgaris* and *Arthrospira platensis* (Spirulina) using three-dimensional (3D) printing technology. Furthermore, design settings alterations were performed, registered, and presented in a poster, to explore the creative reach of this technology. From control and different percentage microalgae doughs (5-30%), the ones with most adequate rheology and texture properties for a correct printing process were selected and baked. Nutritional characterisation of the control and 5% microalgae-containing snacks was thus performed, including their total protein and fatty acid content, ashes, humidity, water activity, energy, and carbohydrates, as well as total phenolics, pigments and antioxidant activity. Physical traits of snacks including their colour and dimensions were also analysed. Control snacks presented a lighter and yellow colour compared to snacks containing *Chlorella* and Spirulina, which had higher green chromaticity, resulting from the natural colour of the biomass. Nutritional characterization revealed *Chlorella*- and Spirulina-containing snacks had both higher contents of protein and essential minerals than control snack. Overall, 5% Spirulina-containing snacks presented the most promising nutritional and sensory performance with higher antioxidant activity, mineral and protein content. 3D food printing is still limited to the built-in computer assisted design (CAD) software that printers provide. Incorporation of 5% Spirulina improved the nutritional characterization and consumer's perception of gluten-free products incorporating microalgae. Even so, microalgae incorporation in gluten-free foods using 3D printing requires further studying to allow its commercialization in the food market, while helping to provide consumers a more sustainable diet and respond to the current scarcity of food resources.

Keywords: 3D food printing, Microalgae, *Chlorella vulgaris*, *Arthrospira platensis*, *gluten-free*

Resumo

A impressão 3D de alimentos é uma tecnologia relativamente recente que promete quebrar barreiras tanto pela introdução de novos ingredientes, como as microalgas, como pelas formas e texturas completamente inovadoras. Este recurso toma particular importância dada a escassez de recursos que a humanidade enfrenta aliada aos efeitos advindos do aquecimento global e dos seus percursores, incluindo a agricultura e a exploração pecuária intensiva. No presente trabalho desenvolveu-se um snack cerealífero, isento de glúten e nutricionalmente melhorado através da incorporação de uma microalga, *Chlorella vulgaris*, ou uma cianobactéria, *Arthrospira platensis*, utilizando impressão tridimensional (3D). Vários snacks foram impressos com uma forma previamente otimizada (pata de pato), um design que se encontra incorporado no *software* de design assistido por computador (CAD) da impressora utilizada (Foodini – Natural Machines, Spain). A formulação das massas dos snacks controlo consistiu numa mistura de duas farinhas isentas de glúten (em igual proporção) - milho e arroz, sal, amido de milho e goma xantana (agente espessante), sendo a mistura homogeneizada antes de se adicionar os líquidos, azeite e água. No caso das massas contendo microalgas ou cianobactérias (incorporações de 5 a 30%), as farinhas e amido de milho foram proporcionalmente substituídas pela biomassa. Para aferir quais as melhores massas em termos de capacidade de impressão, todas as formulações foram submetidas a vários ensaios reológicos (varrimentos de tensão, frequência, tempo e ainda curvas de viscosidade) e de textura (teste TPA - adesividade, coesividade e firmeza). De acordo com os resultados obtidos foram selecionadas para as restantes análises as massas de controlo e com incorporação de 5% de biomassa, tanto de *Chlorella* como de Spirulina. Estas massas foram impressas com o design previamente mencionado e tratados termicamente num forno industrial de convecção a 180 ºC durante 6 min e 30 s. Adicionalmente, foram avaliadas diferentes condições de impressão que têm impacto na definição gráfica do produto final, nomeadamente, o fator de preenchimento (%), a altura (cm) da ponta do cartucho na primeira camada, a espessura das camadas, o tamanho da ponta do cartucho e a velocidade de impressão. Verificou-se que, embora exista a possibilidade de alterar definições de impressão, tais alterações afetam o design incorporado na impressora, e a criação de um design original sem erros, implicando um conhecimento profundo do funcionamento da impressora, cujo utilizador comum não possui. A cor dos snacks, que é um fator determinante da aceitação final do produto, foi

avaliada de acordo com o Colour Space International Comission on Illumination (CIE), avaliando-se a cor dos snacks antes e após o processo de cozimento. Os resultados foram expressos em termos de luminosidade (L*), cromaticidade verde (a*) e amarela (b*) e ainda diferença de cor (ΔE*) em relação ao controlo, como quanto ao efeito do processo de cozedura. De forma a realizar a caracterização nutricional e bioquímica dos snacks, estes foram triturados utilizando um processador industrial com um crivo de 0.5 mm. As dimensões dos snacks também foram analisadas em termos de peso, espessura, comprimento e largura. Também a atividade de água foi analisada à temperatura ambiente e em amostras independentes. O teor humidade dos snacks foi quantificado pelo método de referência, através de diferenças de peso inicial em amostras de cada formulação, submetidas a uma temperatura constante (105 ºC), até que variações de peso não se verificassem. Para determinar o conteúdo em cinzas, os snacks cozinhados foram incinerados até serem convertidos a um pó cinza-claro. A quantidade total de ácidos gordos foi determinada através de hidrólise, seguida de centrifugação, o que permitiu a separação por polaridade, e consequentemente, a extração dos lípidos (menos densos), que foram posteriormente secos durante 3 dias e pesados. A quantidade total de proteínas foi determinada através da quantificação do nitrogénio, utilizando o método DUMAS. A atividade antioxidante foi avaliada através de dois métodos: *ferric reducing ability of plasma* (FRAP) e 2,2-difenil-1-picril-hidrazil-hidrato **(**DPPH), sendo que os fenólicos totais foram também aferidos de acordo com Mohankumar *et al.* (2018), baseado na reação com o reagente de Folin-Ciocalteu. Por fim, também os pigmentos, incluindo clorofila *a*, *b* e carotenoides, foram quantificados. Todas as análises foram realizadas com triplicados de amostras independentes. Os snacks analisados foram submetidos à avaliação de um painel sensorial com provadores não treinados. Classificaram-se os atributos: cor, textura, aspeto, aroma, sabor e avaliação geral sob forma de escala hedónica de 7 pontos - de (1) desgosto muito a (7) gosto muito. A intenção de comprar foi também determinada utilizando uma escala de 7 pontos (de (1) nunca compraria a (7) compraria sempre). Os snacks que apresentaram melhores características de impressão continham entre 0 a 5% de incorporação de biomassa de microalgas, sendo que as restantes formulações, quer de *Chlorella* ou Spirulina apresentaram viscosidade pouco adequada para impressão. Para concentrações de biomassa mais elevadas, obtiveram-se designs com erros de impressão, sendo os mesmos mais acentuados para os níveis de incorporação mais elevados. As dimensões dos snacks não foram afetadas significativamente pela incorporação de microalgas em

comparação com snacks sem as mesmas. No entanto, verificou-se uma redução substancial da espessura, peso e restantes medidas após o processo de cozedura. Em termos de coloração, foi possível aferir que a incorporação de 5% de biomassa de *Chlorella vulgaris* levou ao escurecimento e aumento da cromaticidade verde em comparação aos snacks sem esta biomassa, que apresentavam coloração com maior luminosidade e maiores valores de cromaticidade amarela. Já os snacks incorporando 5% de *Arthrospira platensis*, revelaram uma coloração muito mais escura que as restantes e maior cromaticidade azul e verde no espaço de cor. Verificaram-se diferenças significativas ($p < 0.05$) entre as amostras com e sem microalga, no que diz respeito a parâmetros reológicos e de textura, sendo possível denotar um aumento de estruturação das massas com o aumento de incorporação de biomassa de microalgas, assim como um aumento da dureza dos snacks. A caracterização nutricional dos snacks possibilitou concluir que os snacks contendo 5% de biomassa de Spirulina apresentavam valores de proteína e minerais essenciais consideravelmente superiores aos restantes snacks, verificando-se o mesmo padrão relativamente à atividade antioxidante e pigmentos (clorofila *a* e carotenoides), com valores significativamente superiores ($p < 0.05$). O mesmo aperitivo (5% de biomassa de *Arthrospira platensis*) foi também o selecionado pelos pelo painel de avaliação sensorial, como sendo o mais apelativo em termos gerais, textura, sabor e aspeto. Como conclusão geral do trabalho realizado, verificou-se que a incorporação de biomassa microalgal permitiu a obtenção de snacks 3D sem glúten, mas com um limite máximo de incorporação de 5% (m/m). Esses snacks apresentaram uma melhoria a nível nutricional, nomeadamente do teor proteico, minerais essenciais e da atividade antioxidante, destacando-se o caso dos snacks com 5% de *Arthrospira platensis*, comparativamente com o controlo (sem qualquer incorporação de biomassa). A aceitação desses snacks por parte do painel de sensorial, permite perspetivar uma boa aceitação por parte dos consumidores e consequentemente prospetivar um potencial de comercialização. No entanto, a incorporação de microalgas em produtos isentos de glúten, requer ainda mais estudos, de forma que a sua introdução no mercado se torne uma realidade. Uma realidade que poderá ajudar a colmatar a previsível escassez de recursos alimentares e, em simultâneo, contribuir para uma alimentação mais sustentável, particularmente importante para pessoas que não podem consumir glúten.

Palavras-chave: Impressão de comida 3D, Microalgas, *Chlorella vulgaris*, *Arthrospira platensis*, *glúten*

Theme justification

As the world races towards an inevitable future where alternative food sources will need to be found in order to feed the world's population, the appearance, exploration, and implementation of new and alternative food sources is of utmost importance (Sun *et al.*, 2015; Dankar *et al.*, 2018). Moreover, there is a current trend among consumers to search for a healthier lifestyle that is much reflected on dietary products consumption choice (Khemiri *et al.*, 2020; Raymundo *et al.*, 2020). This leads to higher demand for gluten-free products, as their consumption is perceived by an increasing amount of people as healthy. Hence, innovation plays a critical role in the conception of new foods (Khemiri *et al.*, 2020; Raymundo *et al.*, 2020). *Chlorella vulgaris* and *Arthrospira platensis* are amongst non-traditional foods possessing a panoply of human health beneficial properties, from antioxidant activity to possessing polyunsaturated fatty acids and high protein content (Wieser, 2007; Graça *et al.*, 2018). Although past attempts of incorporating this easily grown resource have been conducted, few have succeed in capturing the consumer's interest mostly due to unappealing aspects related to the products' visual appearance or even taste (Raymundo *et al.*, 2020). However, choosing the type of food to be incorporated must contemplate the average dynamic everyday life of people seeking a healthier lifestyle in developed countries (Uribe-Wandurraga *et al.*, 2020b). Taking this into account, grab-and-go style snacks appeal to a wide consumers' age range, being also a practical choice. Moreover, with the common adult consuming about 40 packages of cookies per year, this is an opportunity for exploring gluten-free foods with additives like *Chlorella vulgaris* and *Arthrospira platensis* (Uribe-Wandurraga *et al.*, 2020a).

With 3D printing technology, the possibilities for the innovation of appealing foods, incorporating microalgae biomass are in some part limited to one's creativity, the food rheology properties, and the process itself (Sun *et al.*, 2015). Therefore, the present study is expected to contribute to the creation of gluten-free cereal snack with incorporation of microalgae in hopes to improve the life of consumers with an attractive healthy and practical meal option, while optimizing the manufacturing process that is non-consensual amongst the already existing 3D-printed cereal snacks.

The document presented is organized into two distinct parts:

PART I: Theoretical support for the work to be developed, which presents in a detailed way the main aspects that allowed the development of the experimental work.

PART II: Manuscript to be submitted for publication in an ISI scientific journal, Algal Research, summarizing the main results obtained.

Part of the work presented in this document, namely that which refers to the optimization of printing parameters, has already been presented in poster form, at the XV Encontro de Alimentos of the Portuguese Chemical Society (5-8 September) - https://xveqa.events.chemistry.pt, which took place in Madeira (**Annex I**).

PART I: Theoretical framework

1. 3D food printing

Additive manufacturing (AM) is a layer-by-layer material deposition method used with techniques in structure assemblage, most commonly known as 3D printing (Godoi *et al.*, 2016; Severini *et al.*, 2016; Liu *et al.*, 2017; Dankar *et al.*, 2018). Although initially focused on developing complex 3D structures with a variety of materials (metals, plastics) and subject areas (medicine, engineering, art), this technology has been adapted to gastronomy (Godoi *et al.*, 2016; Liu *et al.*, 2017). Through it, the manufacturing of breakthrough food designs with innovative textures, colours and shapes is possible with no prior culinary expertise, while incorporating traditional to non-traditional ingredients (insects, microalgae) that add nutritional value to foods (Godoi *et al.*, 2016; Severini *et al.*, 2016; Liu *et al.*, 2017; Dankar *et al.*, 2018). Moreover, the creation of foods based on individual nutritional requirements is possible by composition, density, or structure adjustment to the user's preference, overthrowing the often-wrong conception of a one-fits-all dietary plan (Sun *et al.*, 2015; Liu *et al.*, 2017; Dankar *et al.*, 2018). It can be useful to all age groups (namely people with chewing problems, athletes, and even children) by introducing visual captivating food shapes with nutritional value (Dankar *et al.*, 2018). Another advantage is related to the financial savings deriving from its use, with the replacement of complex procedures required by conventional means and frequently associated with human errors (Dankar *et al.*, 2018; Vieira *et al.*, 2020a). In terms of sustainability, AM benefits the environment with the use of underexplored food sources as microalgae, since these are usually easy to produce with very low greenhouse gas emissions in comparison to other protein

sources like meat, requiring also low maintenance (Liu *et al.*, 2017; Dankar *et al.*, 2018; Vieira *et al.*, 2020a). As food shortage is a growing issue due to the exponential global population growth, the latter alternative food sources in more appealing presentations takes special importance, as they improve consumer acceptance independently of cultural backgrounds (Sun *et al.*, 2015; Dankar *et al.*, 2018).

Depending on the nature of materials (liquid, powder, or cell cultures), different printing techniques might be adopted according to their suitability to the food layer deposition (Godoi *et al.*, 2016; Severini *et al.*, 2016). Layer-by-layer deposition is the basis technique where a product is designed in a computer assisted design (CAD) software or through downloaded 3D platforms (Liu *et al.*, 2017; Dankar *et al.*, 2018). Upon creation of the model, the design information is sent to the printer which can use four different techniques to print food: extrusion-based printing, selective sintering printing (SLP), binder jetting, and inkjet printing. Among these, extrusion-based and inkjet printing are the most adequate to liquid-based foods or soft materials, hence, the ones that our study contemplated using (Godoi *et al.*, 2016; Severini *et al.*, 2016; Liu *et al.*, 2017; Dankar *et al.*, 2018). Not until recently, extrusion-based printing has been explored in the creation of nutritional valuable foods, with most of the works performed seeking toppings and decorative sugar-based additives, lacking further purpose (Godoi *et al.*, 2016; Liu *et al.*, 2017). However, the most recent developed studies seem to explore a more nutrition aware and well thought approach to the development of novel 3D printed foods (Lille *et al.*, 2018; Uribe-Wandurraga *et al.*, 2020a; Viera *et al.*, 2020).

1.1. Extrusion-based printing

The knowledge rheology properties of the food materials is essential to understand and optimize the printing process and behaviour of the foods after processing (Liu *et al.*, 2017; Vieira *et al.*, 2020a). Compared to other techniques, extrusion-based printing is mainly used in doughs and purees which present viscoelastic behaviour (Liu *et al.*, 2017; Vieira *et al.*, 2020a). The rheology aspects inherent to these complex materials make them difficult to work with, since they have a predisposition to being deformed, limiting the development of intricate structures and sometimes require structuring support in their conception (Liu *et al.*, 2017; Dankar *et al.*, 2018). It is, therefore, critical to fully grasp the materials properties (firmness, adhesiveness, moisture content) and binding mechanisms upon deposition (Godoi *et al.*, 2016; Liu *et al.*, 2017). Equally important is the choice of the subtechniques to be applied (soft

material, melting and hydrogel-forming extrusions), each with its own purpose and binding mechanisms (Godoi *et al.*, 2016).

1.2. Extrusion-based subtechniques

Soft material extrusion is mainly used with purees and doughs with a binding mechanism based on the rheology properties of the constituents alone (Godoi *et al.*, 2016; Liu *et al.*, 2017 Vieira *et al.*, 2020a). Hydrogel-forming extrusion on the other hand, manufactures gelatinized products and is settled on ionic or enzymatic cross-linking (Godoi *et al.*, 2016). Finally, melting extrusion is solely used with chocolate until now (Godoi *et al.*, 2016; Lille *et al.*, 2018). However, hydrogel-forming mechanisms have different categories - chemical cross-linking, ionotropic cross-linking and complex coacervate formation - with only a few being reported in the making of 3D printed foods with hydrocolloids, with a much still needed progress in techniques with culinary purposes (Godoi *et al.*, 2016).

In screw-based extrusion, materials are allocated to the nozzle tip by a moving screw, resulting in a continuous extrusion (Liu *et al.*, 2017). The disadvantage associated to this is related to its inability to process materials presenting high viscosity and mechanical strength, leading to low resolution and layer deformation upon printing (Liu *et al.*, 2017). Air pressure-based extrusion, as the name suggests, uses air pressure to push low viscosity or liquid materials through the nozzle, not being adequate for cookie doughs (Godoi *et al.*, 2016; Liu *et al.*, 2017; Dankar *et al.*, 2018). On the other hand, syringe-based extrusion permits the printing of high resolution of high viscosity materials presenting a strong foundation (Liu *et al.*, 2017; Dankar *et al.*, 2018).

Inkjet printing is based on droplets accumulation of continuously deposited material and is usually used in food decoration (Godoi *et al.*, 2016; Liu *et al.*, 2017). Although slower, it allows a higher resolution and precision comparatively to other techniques (Godoi *et al.*, 2016). Even so, this printing method is usually used for printing 2D structures, typically in the development of low mechanical strength structures (Liu *et al.*, 2017; Dankar *et al.*, 2018). For these reasons, aside from syringe-based technique, both the latter and the remaining 3D techniques will not be further discussed, as they are not adequate for the printing of doughs, the bulk of the desired cereal snack. Worthwhile mentioning is that although an extrusion-based technique is the most adequate to our purpose, it has many downsides such as long fabrication time, low resolution and often difficulty in the manufacture of complex 3D structures (Sun *et*

al., 2015; Godoi *et al.*, 2016). Nevertheless, for a smooth printing process, extrusion printing settings should be adjusted.

1.3. Extrusion settings

Depending on the material nature, nozzle size must be adjusted (Dankar *et al.*, 2018). Liu *et al.* (2017) tested the equation (1) that allows the setting of a critical nozzle height (h_c) in relation of the nozzle tip to the deposition plate:

$$h_c = \frac{V_d}{v_n D_n} \quad (1),$$

where h_c is the critical nozzle height, V_d the volume of slurries extruded out per unit time (cm^3s^{-1}), v_n the nozzle moving speed (mm s^{-1}), *Dn* the nozzle diameter (mm) and h_c is the optimal nozzle height (Liu *et al.*, 2017; Dankar *et al.*, 2018). Lower or higher nozzle heights than h_c, results in undesirable thicker lines and imprecise sections, respectively (Liu *et al.*, 2017). Thus, considering the available nozzle tip that we used has a diameter of 15 mm, printing speed must be adjusted to the level of resolution and softness intended, having in mind that higher resolution levels require higher time in the printing process (Liu *et al.*, 2017; Dankar *et al.*, 2018; Álvarez-Castillo *et al.*, 2021). The nozzle speed is critical to determine since it affects the final integrity and structure cohesiveness of the product (Severini *et al.*, 2016). Hence, Liu *et al.* (2017) suggested the derivation from equation (2), allowing calculation of the critical nozzle moving speed:

$$v_n = \frac{4Q}{\pi D_N^2} \quad (2),$$

Where v_n is the optimal nozzle speed (mm s^{-1}), Q is the material flow rate (cm^3s^{-1}) and D_N the nozzle diameter. Greater or lesser velocities than v_n results in a material with a diameter smaller or larger than that of the nozzle, respectively (Liu *et al.*, 2017).

Another important variable when performing extrusion-base printing is retraction, which is the contrary movement of the stepper motor possibly leading to slippage of dough out of the nozzle during no-printing movements (Dankar *et al.*, 2018). Additional care should be taken as retraction its augmented when flow of extrusion is increased, possibly causing internal structure faults when in an insufficient manner (Dankar *et al.*, 2018).

With all above variables taken accountable, it should be noticed that the printing process is also highly dependent on the food material and its rheologic properties. For this study, a green microalga, *Chlorella vulgaris*, and another microalga species, *Arthrospira platensis* were selected to enrich our gluten-free formulation based on reference literature.

2. Microalgae

Microalgae are photosynthetic microscopic organisms found in a variety of aquatic environments (both salt and freshwater), enduring a wide range of conditions (light, temperature, salinity, pH), and provide half of the earth's atmospheric oxygen supply while absorbing high amounts of carbon dioxide (Safi *et al.*, 2014; Khan *et al.*, 2018; Koyande *et al.*, 2019). They comprehend a very diverse group with more than 800 thousand species that can be explored for their bioactive compounds by the most varied industries (Koyande *et al.*, 2019). Although biotechnology has explored the potential for microalgae to areas as biofuels, pharmaceuticals, cosmetics or even in health procedures, recently due to climate change and resource scarcity related issues, they appear as a possible food source alternative (Safi *et al.*, 2014; Khan *et al.*, 2018; Koyande *et al.*, 2019). The interest in these organisms has surfaced much due to their nutritional composition, including high protein content, bioactive pigments (e.g., chlorophyll, *β*-carotene, phycobiliproteins and astaxanthin), high quantity of carbohydrates, lipids (namely important fatty acids as polyunsaturated fatty acids [PUFAs]), vitamins (A, B12, E), minerals (potassium, iron, magnesium, zinc) and polysaccharides (Safi *et al.*, 2014; Khan *et al.*, 2018; Koyande *et al.*, 2019).

2.1. Microalgae as a food source

Although scientific research upon these organisms beneficial properties is fairly recent, consumption of microalgae date back to the Aztecs, who frequently consumed biomass of these organisms (Safi *et al.*, 2014; Koyande *et al.*, 2019). Because of several reasons (climate change to fossil fuel reserves and drought), an increase in the consumption of microalgae (mostly of *Chlorella vulgaris* and *Arthrospira platensis*) has been registered in the past decades, either through supplements, pharmaceuticals or even cosmetics (Safi *et al.*, 2014; Batista *et al.*, 2017). 2017 Hunger Project estimated that 815 million out of the 7.6 billion people in the world do not have enough food to survive (Koyande *et al.*, 2019). With world population numbers exponentially rising,

meeting food and economic demands has become unfeasible both logistically and in terms of climate change (Vaz *et al.*, 2015; Koyande *et al.*, 2019). In this sense, alternative food sources as microalgae are being contemplated as an important nutrition source considering the high demand for other food sources such as meat and food crops (Khan *et al.*, 2018; Koyande *et al.*, 2019). Although they produce fairly equal amounts of protein, the protein yield is far higher in microalgae (4-15 tons/Ha/year) compared to those of terrestrial plant crops (0.6-1.2 tons/Ha/year for soybeans) (Koyande *et al.*, 2019). These advantages extend to the sustainability of the production methods. More specifically, while meat production consumes 100 times the amount of water that terrestrial crops require, microalgae can be produced without freshwater or arable land (Vaz *et al.*, 2016; Koyande *et al.*, 2019).

Additionally, with the industrialization, a quick-paced lifestyle associated with high caloric and unhealthy foods results in what we now know as a world epidemic, obesity (Khan *et al.*, 2018; Koyande *et al.*, 2019). Hence, the search for and the introduction of healthy and balanced foods is a priority, not only for the consumer who demands better nutritious foods, but also for the food industry, which needs to answer this demand and account for the composition of their products (Lucas *et al.*, 2018; Koyande *et al.*, 2019; Raymundo *et al.*, 2020). Thus, the search by companies for relatively unexplored food sources, like microalgae, with proven valuable biochemical composition has been on the rise (Lucas *et al.*, 2018; Koyande *et al.*, 2019; Raymundo *et al.*, 2020). Nowadays, microalgae are commercialized in tablets, powder biomass, liquids, and even mixed with foods such as candies, snacks or drinks (Batista *et al.*, 2017; Koyande *et al.*, 2019). The most recognised and used species comprise *Chlorella vulgaris*, *Arthrospira platensis* (both used in our study) and *Dunaliella salina*, mostly due to their attractive nutritional characterization but also for their approval by the Food and Agricultural Organization (FAO) and/or World Health Organization (WHO) as safe for consumption, in detriment of others lacking approval or not yet established in an already hard and novelty sceptic market (de Morais *et al.*, 2015; Koyande *et al.*, 2019; Khemiri *et al.*, 2020). Moreover, this decision took in consideration the fact that from the large variety of marine microalgae species available, only few are approved by EFSA as suitable for human consumption (*Tetraselmis chuii*, *Odontella aurita*, *Diancronema vlkianum*, *Dunaliella salina*) (Matos *et al.*, 2017; Mello-Sampayo *et al.*, 2017; Lafarga, 2019; Nunes *et al.*, 2020). Those which are suitable, still have a series of

mandatory conditions (incorporation in a restricted product category, maximum incorporation percentage) for their commercialization to be approved (Lafarga, 2019;).

Essential amino acids (EEAs) are not produced by the human body and thus need to be obtained from external sources like eggs, meat and dairy (Matos *et al.*, 2017; Koyande *et al.*, 2019). Nevertheless, such foods might not be available to all people, increasing the struggle to obtain these EEAs (Matos *et al.*, 2017; Koyande *et al.*, 2019). Microalgae (e.g., *Chlorella vulgaris* and *Arthrospira platensis*) are a good source of these compounds, because protein can be as high as 70% of the microalgal dry weight, being an alternative source of EEAs to malnourished populations lacking other food sources (Khan *et al.*, 2018; Koyande *et al.*, 2019).

2.1.1. *Chlorella vulgaris*

C.vulgaris is a green eukaryotic unicellular organism belonging to the phylum Chlorophyta, the first microalga discovered to have a well-defined nucleus by the Dutch researcher Martinus Beijerinck in 1890 (Safi *et al.*, 2014). Its fossil records on freshwater environments date back to the pre-Cambrian period (2.5 billion years ago) (Brasier *et al.*, 2002; Safi *et al.*, 2014). *Chlorella vulgaris* has a spherical cell varying from 2 to 10 µm of diameter with variable rigidity, depending on its growth phase (Safi *et al.*, 2014; Vieira *et al.*, 2020a). When mature, the cell wall microfibrillar layer increases its rigidity and the cell wall composition itself varies according to the environmental conditions (Safi *et al.*, 2014). This species reproduces asexually through a non-motile reproductive cell, the autospore, in optimal environmental conditions (Safi *et al.*, 2014). Interest in it first rose in the beginning of the 20th century due to its protein content, but it was not until the 1960s that, in Japan, its cultivation occurred at large scale for biomass consumption (Safi *et al.*, 2014; Khan *et al.*, 2018). Nowadays, *C. vulgaris* is commercialized in the most varied forms with a market value of over 138 million USD, its main producers being Taiwan, Germany and Japan (Safi *et al.*, 2014; Koyande *et al.*, 2019). Its rapid growth and resistance to unfavourable conditions and contaminants, makes this species an interesting microalga to produce at large scale (Safi *et al.*, 2014). Depending on the metabolites requirement, its growth conditions can be manipulated accordingly (Safi *et al.*, 2014). For example, under excess light and nutrient (nitrogen/phosphorus) limitation, this alga is known to accumulate lipids, in detriment of proteins, carbohydrates and pigments (Safi *et al.*, 2014). It can be cultivated in open pond systems, in closed photobioreactors and/or via

heterotrophic/mixotrophic growth (Safi *et al.*, 2014). Most frequently, these algae are harvested by centrifugation as it retrieves most of the biomass (95%) from water without damaging the cell wall (Safi *et al.*, 2014).

In *C. vulgaris*, proteins represent from 42 to 58% of its biomass dry weight and are involved in major cell functions, including its protection from hazards, maintenance, growth and repair (Gouveia *et al.*, 2007; Pulz and Gross, 2004; Safi *et al.*, 2014; Vieira *et al.*, 2020a). It is considered a superior source of both essential and non-essential amino acids by both WHO and FAO as well as a versatile food source due to its optimal emulsifying capacity from their proteins (Safi *et al.*, 2014). Depending on the growth conditions, lipids (glycolipids, waxes, hydrocarbons, phospholipids, and free fatty acids) can range between 5 and 68% of the dry weight in *C. vulgaris* biomass (Batista *et al.*, 2013; Safi *et al.*, 2014). Under favourable growth conditions, the levels of PUFAs like linoleic acid (LA) and eicosapentaenoic acid (EPA) increases (Safi *et al.*, 2014; Matos *et al.*, 2017). In this alga, starch (a carbohydrate polymer) is the predominant form of carbohydrates, serving as energy storage for cells (Safi *et al.*, 2014). Other energy sources such as the polysaccharide *β*-1-3-glucan, has proven health benefits as anti-tumoral, having also an effect on blood lipid reduction (Gouveia *et al.*, 2007; Safi *et al.*, 2014; Vieira *et al.*, 2020a). *C. vulgaris* pigments composition predominantly includes chlorophyll, but also accessory pigments and/or photoprotectors (Safi *et al.*, 2014). Examples are *β*-carotene, astaxanthin, canthaxanthin, lutein, chlorophyll *a*, chlorophyll *b* and pheophytin *a*, *b*, which have been recognised as having antioxidant activity and health beneficial properties such as cholesterol reduction and immune system fortification (Safi *et al.*, 2014; Matos *et al.*, 2017). In particular, lutein has been pointed as an important compound in disease prevention as it is capable of treating macular degeneration and has anti-cataract properties (Matos *et al.*, 2017).

2.1.2. *Arthrospira platensis* (Spirulina)

Spirulina belong to a seemingly simple group of microalgae which have complex evolutionary histories as long as 2.7 billion years (Safi *et al.*, 2014). Considered by WHO as a superfood due to their nutritionally dense features, *Arthrospira platensis* is a spiral multicellular microalga with exceptional health benefits, such as prevention and/or cure chronic diseases (cholesterol, blood sugar and pressure complications) (Desai and Sivakami, 2004; Pulz and Gross, 2004; Becker, 2007; Batista *et al.*, 2017; Koyande *et al.*, 2019; Vieira *et al.*, 2020a). Their high protein content (up to 70% dry

weight) surpasses other known commercialized products such as spinach or tofu. In addition, it is also rich in vitamins (A, B1, B12), PUFAs (oleic and linoleic acids), pigments with antioxidant activity [carotenoids (*β*-carotene, astaxanthin)] and minerals as iron, magnesium, manganese and potassium (Desai and Sivakami, 2004; Koyande *et al.*, 2019). Pigments as phycobiliproteins and carotenoids allow this blue-green microalgae to absorb light at several wavelengths and be protected against photo-oxidation and viruses (Khan *et al.*, 2018; Koyande *et al.*, 2019). These beneficial properties has led to the commercialization of this microalga in several forms including tablets, biomass, or even incorporated in foods (Khan *et al.*, 2018; Koyande *et al.*, 2019).

Because of its highly available bioactive and valuable components (pigments, polyunsaturated fatty acids, elevated protein levels and minerals), microalgae like *Chlorella vulgaris* and *Arthrospira platensis* are commonly used for food enrichment, many times determining the product properties (Batista *et al.*, 2017; Raymundo et al. 2019; Vieira *et al.*, 2020b). Hence, it is important to adjust microalgae incorporation according to the desired final characteristics of the product, since it may influence its colouration and colour stability over time due to carotenoid (and other pigments) content (Gouveia *et al.*, 2006; Raymundo *et al.*, 2019). Moreover, the high protein and polysaccharide content lead to structural modifications, due to interaction between molecules in the food matrices, as all of them may influence consumers' product acceptance (Raymundo *et al.*, 2019; Raymundo *et al.*, 2020). Thus, the general maximum incorporation into snack doughs for both *C. vulgaris* and *A. platensis* is around 6%, since higher values have been reported to affect snack characteristics such as firmness, which if in excess can be detrimental to sensory perception by the consumer (Raymundo *et al.*, 2018; Raymundo *et al.*, 2019). The final barrier to overcome is related to the poor European consumption of microalgae reflected on its low demand and associated strict legislation (Batista *et al.*, 2017). Nevertheless, since both *C. vulgaris* and *A. platensis* are recognised as safe for consumption by the European Food Safety Authority (EFSA), are the ones to be utilized in our study.

2.2. *Chlorella vulgaris* and *Arthrospira platensis* as nutraceuticals

Chlorella vulgaris and *Arthrospira platensis* importance lays on their potential to significantly benefit human's health due to their high content in bioactive compounds and overall good biochemical characterization (Liu *et al.*, 2016; Matos *et al.*, 2020).

Among the previously mentioned compounds, both contain carotenoids as *β*-carotene and astaxanthin, which are a part of a group of pigments with a wide range of known medicinal properties (de Morais *et al.*, 2015; Khan *et al.*, 2018). *β*-carotene, aside from reinforcing the immune system through free-radical scavenging, has also anticancer, and anti-degenerative diseases effects (Matos *et al.*, 2017; Khan *et al.*, 2018). Moreover, astaxanthin has strong-antioxidant activity that might prevent oxidative stress-related diseases and associated complications like diabetes, cancer, obesity, and aging (de Morais *et al.*, 2015; Khan *et al.*, 2018). Furthermore, humans have an inherent need for amino acids which they cannot normally synthetize, being only obtained from external sources (Khan *et al.*, 2018). Both *Chlorella* and Spirulina produce a series of EEAs (leucine, valine and isoleucine) and proteins with the potential to fulfil such need (Matos *et al.*, 2017; Khan *et al.*, 2018). An example is the presence in *Arthrospira platensis* of proteins as phycobiliproteins and phycoerythrin, which have proven antioxidant, anti-cancer, anti-inflammatory and anti-viral properties (Martelli *et al.*, 2014; de Morais *et al.*, 2015; Vaz *et al.*, 2016. Another example includes *Chlorella* and Spirulina natural bioavailability in vitamins. These compounds are very important, because micronutrients are vital for humans, as they perform the roles of co-enzymes or of active electron/proton carriers in the breakdown of macronutrients, with their absence leading to diseases such as beriberi, rickets or scurvy (Koyande *et al.*, 2019).

Among other important extrinsically synthetized compounds are PUFAs omega-3 (ω-3) and -6 (ω-6), since they ensure tissue integrity (Matos *et al.*, 2016; Khan *et al.*, 2018). *C. vulgaris* is an important source of the ω3-PUFAs; LA, α-linoleic acid (ALA) and γ-linoleic acid (GLA) (Matos *et al.*, 2016; Kratzer and Murkovic, 2021). *A. platensis* has also been found to be rich in fatty acids, such as sitosterol, GLA and LA, the last two ω6-PUFA (Matos *et al.*, 2016; Khan *et al.*, 2018; Kratzer and Murkovic, 2021) When in sufficient amount, GLA has been associated with low density lipoproteins (LDL) reduction, anti-inflammatory and cancer cell apoptosis (Matos *et al.*, 2016). Both EPA and GLA are present in these microalgae and have been correlated with prevention of cardiovascular diseases and an increase in anti-inflammatory responses (Matos *et al.*, 2020; Khan *et al.*, 2018).

In addition, it is consensual that the phenolic content and carotenoids on these microalgae species significantly contributes to their antioxidant activities (Matos *et al.*, 2020). *Arthrospira platensis* has the ability through antioxidants to prevent oxidation of foods (Matos *et al.*, 2020). Its content in polysaccharides and phytopigments like

chlorophyll, carotenoids and phycocyanin are responsible for compromising HeLa (cervical cancer human cell line) cell proliferation through cytotoxicity (Matos *et al.*, 2020). Anticancer activity has also been found to have a great potential in the case of *Chlorella vulgaris* (Matos *et al.*, 2020). *Chlorella vulgaris* antioxidant activity and eyesight health improving properties has been associated with their content in lutein, α- and *β*-carotene and vitamins C and E (Matos *et al.*, 2017; Matos *et al.*, 2020; Kratzer and Murkovic, 2021). In sum, both *Chlorella* and Spirulina are referred to as good nutraceuticals through food incorporation due to their easy digestion and chemical profile, namely low fibre, high protein/carbohydrate and essential fatty acid content (Matos *et al.*, 2016; Matos *et al.*, 2017; Matos *et al.*, 2020; Kratzer and Murkovic, 2021).

2.3. Antioxidant activity of microalgal biomass

Providing a good supplementation through the mentioned nutraceuticals has an even higher importance, since the human body continuously produces free radicals or reactive oxygen species (ROS) due to oxidative stress caused by smoking, exposure to sunlight and other hazards (Khan *et al.*, 2018; Koyande *et al.*, 2019). Antioxidants are produced to scavage these free radicals and prevent damage of important biomolecules such as DNA, proteins and membrane lipids (Khan *et al.*, 2018; Koyande *et al.*, 2019). Whenever endogenous antioxidants are not able to counteract ROS production, oxidative stress occurs, leading to cell damage that can contribute to the onset of diseases such as diabetes, auto-immune disorders and Alzheimer's disease (Khan *et al.*, 2018; Koyande *et al.*, 2019). Oxidation is a problem to humans in general, and to the food industry in particular, since peroxidation reduces shelf life and nutritional value of foods (Khan *et al.*, 2018). However, oxidative stress prevention can be supported by external input of antioxidant sources such as carotenoids, flavonoids, vitamins (C and E) and fatty acids (ω-3 and ω-6) (Khan *et al.*, 2018; Koyande *et al.*, 2019). Although synthetic antioxidants may have been an alternative to naturally found antioxidative compounds in the past, their potential harmful effect on human health led the food industry to start looking for naturally derived antioxidants as the ones found on microalgae, which provide a higher bioactive and effective antioxidant supply (Khan *et al.*, 2018; Koyande *et al.*, 2019). While green microalgae have chlorophyll and carotenoids as their main light-harvesting pigments, red and blue algae have other

pigments, such as phycobiliproteins, that range in colour from blue to red with various benefits as neuroprotective, anti-inflammatory and anti-viral properties (Koyande *et al.*, 2019). Carotenoids, such as astaxanthin and *β*-carotene, are isoprenoid structured lipophilic pigments found in photosynthetic organisms as *Chlorella* and Spirulina and higher plants, which have known health benefits that have been linked to their antioxidant activity and their ability to prevent, for example, cardiovascular and atherosclerosis (Matos *et al.*, 2017; Koyande *et al.*, 2019). Nevertheless, carotenoids are not the only metabolites derived from microalgae to contribute for their antioxidant activity (Khan *et al.*, 2018). For example, it has been shown that chlorophylls, aside from showing antioxidant activity, have anticarcinogenic, antimutagenic and anticarcinogenic preventive effects (Koyande *et al.*, 2019). Although this activity may have been proven in past studies, their determination is still necessary, and may be done through several methods including the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radicals scavenging assay and ferric reducing ability of plasma (FRAP) (Khan *et al.*, 2018). Considering all the above-mentioned beneficial characteristics associated to both *Chlorella vulgaris* and *Arthrospira platensis* incorporation in foods, we consider them to be the most adequate in the manufacture of gluten-free snacks.

3. Gluten-free products

Gluten is the insoluble residual mass resultant of washing starch granules and water-soluble constituents from wheat dough (Wieser, 2007). This viscoelastic mass commonly present in wheat, rye, and barley cereals is mainly composed of proteins (up to 85%) and lipids (up to 10%), with the remainder consisting of starch and non-starch carbohydrates (Wieser, 2007; Xu *et al.*, 2020). The proteins are divided into gliadins and glutenins, which through covalent and non-covalent bonds determine dough viscosity, water absorption capacity, cohesivity, elasticity, stress endurance and gas retention during fermentation and baking, performing a major role in wheat products texture (Wieser, 2007; Mariotti *et al.*, 2009; Xu *et al.*, 2020).

Although gluten presents a series of advantages to several cereal products, it can have serious hazardous consequences to celiac disease patients and those with gluten associated intolerances (Wieser, 2007; Xu *et al.*, 2020; Raymundo *et al.*, 2020). Celiac disease is an auto-immune system disorder leading to inflammation prompted by gluten in the upper small intestine, resulting in diarrhoea, fatigue, and anaemia, with the solely

solution being a lifelong gluten-free diet, allowing mucosal recovery (Segura and Rosell, 2011; Xu *et al.*, 2020).

Nevertheless, the seemingly easy to produce gluten-free products are actually an enormous challenge as gluten ensures structure formation during dough development and settling, conferring a viscoelastic network when hydrated and trapping carbon dioxide during yeast fermentation/chemical leavening (Xu *et al.*, 2020). Hence, it is common for products lacking gluten to be unpleasant and difficult to fabricate (Xu *et al.*, 2020). There are often reports of liquid batters, weird textures, doughs that are difficult to handle, and more importantly, often resulting in poor nutrition composition with low protein levels and high quantity of carbohydrates, sugars, and fat (Xu *et al.*, 2020).

Gluten-free food is defined by the U.S Food and Drug Administration (FDA) as either completely gluten-free or lacking any gluten-containing grain (wheat), derived from gluten-containing grain without gluten removal processing (wheat flour) and derived from a gluten containing grain with gluten removal processing (wheat starch), with limits of gluten presence in the latter at 20 ppm (Xu *et al.*, 2020). Conversely, the European Union (EC No 41/2009) defines gluten-free foods as not exceeding the 20-ppm mark (Xu *et al.*, 2020). Amid gluten-free products, bread and cookies show up as the most consumed cereal-based gluten-free foods, with cookies and crackers reported as more consumed than bread in people with celiac disease (Xu *et al.*, 2020). Much of these use gluten-free flours such as rice, sorghum, buckwheat, and chickpea that are combined with others like maize and potato starch (Nunes *et al.*, 2020; Xu *et al.*, 2020; Raymundo *et al.*, 2020). Moreover, they are usually composed of high levels of sugar and lipids, not adding a real nutritional value to the consumer, which can prove to be critical since there is more awareness on the healthy choices by the consumers but also in celiac patients who normally have additional pathologies (Xu *et al.*, 2020; Raymundo *et al.*, 2020). However, snacks have the convenience of not being as affected by gluten structure compared to other products, facilitating the incorporation of bioactive ingredients such as microalgae and other flours (Raymundo *et al.*, 2019). Even so, snack dough is a very complex and diverse system, consisting of suspensions of starch particles in gluten proteins, where minor interactions regulate the overall rheology properties, the post-processing and final quality of the snack (Raymundo *et al.*, 2018). Besides fat, the main factor disturbing the snack foundation and texture is the moisture

and water mobility, which is affected by the interaction between hydroxyl matrix groups (Raymundo *et al.*, 2018).

Nunes *et al.* (2020) created a poorly accepted gluten-free bread which incorporated *Tetraselmis chui* microalgal biomass but provided promising results for higher levels of microalgae incorporation in terms of antioxidant activity and bread properties (volume and softness) (Nunes *et al.*, 2020; Raymundo *et al.*, 2020). Martins *et al.* (2020) used also acorn flour to create a bread, which improved the texture and viscoelasticity of the fermented dough. Although still unpliable, this allowed for a better understanding of the dough rheologic properties (Raymundo *et al.*, 2020). In addition to acorn, other pseudocereal flours like carob fruit and plantain can be considered to be incorporated as they have proven to improve both nutrition and sensory experience (Arribas *et al.*, 2019; Xu *et al.*, 2020). Buckwheat is a pseudo-cereal containing a positive amino-acid profile, vitamins, and fibre that proved to confer favourable elastic behaviour and nutritional value (protein and fibre) to snack doughs (Nunes *et al.*, 2020; Xu *et al.*, 2020). For example, lysine (essential aminoacids) is one of the deficiencies in cereals that can be filled by incorporating some of the mentioned pseudocereals (Mariotti *et al.*, 2009; Xu *et al.*, 2020). Since flour and further ingredient choice take into consideration the importance of an uncharted sustainable by-product, while promoting an equally vital circular/viable economy to a resource scarce reality, its choice should contemplate the rheologic behaviour of doughs with each composition, with rice, buckwheat, amaranth, acorn, and carob fruit being the ones mostly explored and preferred. Therefore, some of these will be short-listed to be tested in our study (Martins *et al.*, 2020; Xu *et al.*, 2020; Raymundo *et al.*, 2020).

Additionally, sugar has an important role in the integrity and texture of the dough, with alternatives to refined and processed sugars being an important aspect to consider due to the growing awareness of consumers to health-related issues deriving from elevated sugar levels (Xu *et al.*, 2020). With this being said, artificial sweeteners like allulose, stevia or neotame must be contemplated due to their low caloric values compared to natural occurring sugars like fructose maize syrup, while preserving sucrose functionalities (Xu *et al.*, 2020). However, these are still fairly unexplored sugar sources, and to some extent, not well perceived by some consumers due to safety related issues and insecurities (Xu *et al.*, 2020).

To confer thickness as well as moisture to the dough, gums including xanthan gum, agarose or hydroxypropyl methylcellulose (HPMC) have been used as a

thickening agents, with xanthan gum proving to provide good stability on buckwheat snacks (Martins *et al.*, 2020; Xu *et al.*, 2020). Therefore, our study should contemplate the use of the latter, as thickness is an especially important aspect in the design of gluten-free goods (Xu *et al.*, 2020). Lastly, water and salt should be incorporated into the dough of the snack, as both are important for the final products' behaviour.

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PART II: Manuscript to be submitted for publication in an ISI scientific journal Algal Research

3D printed gluten-free cereal snack with incorporation of Spirulina (*Arthrospira platensis*) and/or *Chlorella vulgaris*

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Abstract

3D food printing is a recent technology promising to break cultural barriers by introducing new food sources as microalgae, through innovative food shapes and textures, in a resource-scarce world whose sustainability is at stake because of the current intensive production of meat and other agriculture products. The present work intends to create an innovative gluten-free cereal snack nutritionally improved by the incorporation of *Chlorella vulgaris* and *Arthrospira platensis* biomass using, 3D printing technology. Upon testing doughs without (control) or with different microalgal incorporation percentages (from 5 to 30%), the ones showing the most adequate rheology and texture properties for a correct printing process were selected and baked. Nutritional characterization of the control and 5% snacks was performed, in terms of total protein and fat content, ashes, humidity, water activity, energy, and carbohydrates, as well as total phenolics, pigments and antioxidant activity. Physical traits of snacks including their colour and size were also analysed. Control snacks presented a lighter and yellow colour compared to snacks containing *Chlorella* and Spirulina, which had higher green chromaticity. Nutritional characterization revealed that snacks containing *Chlorella* and Spirulina had both higher protein and essential minerals content. Overall, 5% Spirulina snacks presented the best nutritional and sensory performance, with higher antioxidant activity, mineral and protein contents. These Spirulina-snacks deserved a positive sensory appreciation from consumers.

1. Introduction

The current world human demographic explosion is incompatible in terms of food sources currently available, being the exploration of alternatives a mandatory goal in a near future (Sun *et al.*, 2015; Dankar *et al.*, 2018). As the environment is affected by the current main protein sources for human consumption, like meat, alternatives as the microalgae *Chlorella vulgaris* and *Arthrospira platensis* biomass, have been explored

due to their exceptionally good nutritional characterization including highly available bioactive molecules (pigments, polyunsaturated fatty acids, high protein levels and minerals) (Batista *et al.*, 2017; Raymundo *et al.*, 2019; Vieira *et al.*, 2020). However, challenges surface since cultural background, sensorial perception and people's routines play a major part in the food industry, and overall food acceptance among consumers (Sun *et al.*, 2015; Dankar *et al.*, 2018). Considering the dynamic lifestyle of consumers, followed by the growing demand for healthier products, particularly gluten-free foods, grab-and-go gluten-free snacks are considered to be an interesting healthy and practical food (Godoi *et al.*, 2016; Severini *et al.*, 2016; Liu *et al.*, 2017; Dankar *et al.*, 2018; Uribe-Wandurraga *et al.*, 2020b). Though, microalgae incorporation in foods (*Chlorella vulgaris* and *Arthrospira platensis*, particularly) is an already known concept; their high protein and polysaccharide content, smell, flavour and colour affects the structure of foods as well as consumer's perception (Raymundo *et al.*, 2018; Raymundo *et al.*, 2019). Despite this, the proven benefits, as well as being among the few microalgae recognised as safe for consumption by European Food Safety Authority (EFSA), lead us to explore its potential through 3D food printing in gluten-free cereal snacks. Showing up as a breakthrough technology that has promised to change consumer's perception on food sensorial experiences by introducing innovative shapes and textures, 3D printing has recently been growing in popularity among several stakeholders of the food industry (Dankar *et al.*, 2018). Besides introducing the possibility of creating complex and personalized designs without any expertise, it has the potential to establish new ground for non-traditional food sources as microalgae biomass and insects (Godoi *et al.*, 2016; Severini *et al.*, 2016; Liu *et al.*, 2017; Dankar *et al.*, 2018). Additionally, we will delve into 3D printing potential to become a reliable technology by studying the optimization of printing settings on computer assisted design (CAD) software and their consequences. As it stands, gluten-free products still face numerous challenges related to structure, viscoelastic behaviour, and their overall unpleasant sensory traits (Xu *et al.*, 2020). Moreover, it is still common to find commercialized gluten-free snacks with poor nutritional value, due to their high sugar and lipid contents (Xu *et al.*, 2020; Raymundo *et al.*, 2020). Although few studies have explored the incorporation of microalgae into snacks, their scrutiny as an additive in gluten-free snacks is still scarce, and so this study seeks to introduce a creative alternative to already commercialized gluten-free snacks by exploring 3D technology, through printing gluten-free cereal snacks, nutritionally improved by the incorporation of microalgal (*Chlorella vulgaris*

and *Arthrospira platensis*) biomass. It will involve the production feasibility assessment of snacks incorporating from 5 to 30% (w/w) microalgal biomass, through a series of analysis, including: i) nutritional characterization (protein, fatty acids, ashes, water activity, humidity, carbohydrates, energy); ii) rheology tests (stress, frequency, and time sweep tests as well as viscosity); iii) texture of doughs and snacks (Texture Profile Analysis and penetration tests); iv) antioxidant activity measured by the ferric reducing ability of plasma (FRAP) and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) methods and total phenolic content; v) pigment characterization; vi) assessment of the consumer's perception on the final product through a sensory evaluation.

2. Materials and methods

2.1. Doughs mixing and 3D printing

Control snack's formulation (*w/w*) was adapted from original previously tested formulation (unpublished results – Oliveira *et al.*, 2021) through a trial-and-error procedure by replacing oat flour with a 50/50 ratio of all-purpose corn and rice flours (Ceifeira, L1507/21; L3506/21) (30% of the formulation), 1% table salt (Auchan, L73624574), 0.2% xanthan gum (Sosa, L180920), 23.8% of corn starch (Espiga, L020305), 5% olive oil (Condestável, L019054) and 40% deionized water. All solid ingredients were first homogenized using a spatula in a circular motion, adding the liquids afterward, homogenizing them for 2 min, using the same technique to obtain a cohesive dough. *Chlorella vulgaris* (Allmicroalgae – Natural Products, L201950311) and Spirulina (Allmicroalgae – Natural Products, PSS0720) biomass was added, with contents from 5 to 30% (*w/w*), to doughs, by replacing the corresponding quantity of corn and rice flours, maintaining the same proportions. To allow residual stress to relax, doughs were covered and left to rest for 15 minutes at room temperature (based on previously performed time sweep tests, in which most of the doughs required 900 seconds to structure). Afterwards, these doughs were printed in a built-in CAD duck foot shape design using a commercially available 3D food printer (Foodini, Natural Machines, Spain) with a 1.5 mm nozzle at 20 $\pm$ 1 ºC. This shape has already been used in previous works (Álvarez-Castilho *et al.*, 2021) and allows for a high print detail to assess the respective graphic quality.

Printing settings were pre-defined by built-in CAD software and kept throughout the experiment. Pre-printing involved discharge of a considerable amount of dough due to pre-defined printer settings which guaranteed printability of the food material. In

each batch, five and a half snacks were printed in a layer-by-layer deposition technique, forming snacks with four 1.4 mm thick layers (**Figure 1**), totalizing a total time of six minutes. Snacks were then baked in a forced-air convention oven (Ariana, Italy) for 6 min and 30 s at 180 ± 5 ºC. Then, snacks were cooled down at room temperature for 15 min, and vacuum sealed in clear plastic bags. A total of 30 g of each snack formula were ground to powder using an industrial electric mill operating for 30 s with a 0.5 mm sieve at 7000 rpm and 22 ± 10 ºC. These were preserved in 50 mL falcon tubes and kept at -25 ± 5 ºC until biochemical analysis.

To assess the effect of altering pre-defined built-in designs settigns of the 3D food printer, a standard 5% *Chlorella vulgaris* dough was prepared and printed with an originally designed 4 layered Christmas tree shaped snacks (**Figure 2 – A-D**). Printing settings used in the standard design were altered, including first layer nozzle height from 1.4 to 2.8 and 0.7 mm, printing speed from 2500 to 1500 and 3500 mm/min, fill factor from 1% to 0% and 2%, layer thickness from 1.4 to 0.7 and 2.8 mm and nozzle size from 15 to 8 and 40 mm.

2.2. Snacks' raw and cooked dimensions

Snacks characteristics including height (thickness), width, length (cm) and weight (g) were measured with a calliper rule (Milomex, Z22855F) and a digital scale (Sartorius, ENTRIS623 - 1S, Germany). An amount of random 10 individual snacks from each formulation were measured before and after the baking process.

2.3. Nutritional characterization

For biochemical composition determination, snacks were ground to powdered samples, and analysed in triplicate. Humidity was measured using metal melting pots from which any organic matter had previously been removed upon incineration using a muffle furnace (SNOL, AACC 08-01, Lithuania) at 500 ± 1 ºC for 1 h. After cooling (200 ± 1 ºC), these were placed in a vacuum sealed desiccator for temperature cooldown and weighed in a digital scale (Denver Instrument Company, TC-403). From each ground formulation, triplicates of 2 g were weighed and placed in an oven (BINDER, ED56, Germany) at 105 ± 1 ºC. Samples were weighed until there were no weight variations. Total ashes content was measured by incineration using a muffle for four hours at 550 ± 1 ºC. Ashes were cooled in a desiccator and weighed in a digital scale (Denver Instrument Company, TC-403). Water activity (a_w) was assessed through

a water activity meter (Rot9ronic, Hygropalm, Switzerland) containing a sensor at controlled room temperature of 20 ± 1 ºC, using triplicates of each formula.

Total protein content of samples was evaluated in triplicates using a DUMAS equipment (VELP SCIENTIFICA, NDA 702, Italy), that evaluate the nitrogen content of the sample, through combustion method, allowing determination of protein content as % N × 6.25 (conversion factor).

Total fat content of each formulation was determined by hydrolysis as described by Doan *et al.* (2011). Triplicates of 100 mg of each formulation were added to a mixture of methanol (CH_3OH), chloroform ($CHCl_3$), and hydrochloric acid (HCl) was added in a ratio of 10:1:1.5, respectively. The mixture was extracted with *n*-hexane ($CH_3(CH_2)_4CH_3$)/chloroform ($CHCl_3$) (4:1 v/v), taken to a vortex for 2 min and centrifuged (HERMLE, Z383 K, Germany) 10 minutes at 20 ºC at 9600 *g*. The supernatant resultant from the centrifugation (fat fraction) was removed into previously weighed glass tubes. Tubes were placed inside an oven at 50 ± 5 ºC, for 3 days and weighed subsequently. The difference between the initial and the final weight of the tubes results in the total fatty acid content of those samples.

The total carbohydrate content of samples was determined by difference and energy (Kcal/100 g) was determined through the conversion factors as indicated in Annex XIV of Regulation (EU) No. 1169/2011.

Mineral profile (contents of Na, K, Ca, Mg, P, S, Fe, Cu, Zn, Mn, B, Pb, Cr, Ni and Cd) of each snack, as well as the biomass of *Chlorella vulgaris* and *Arthrospira platensis*, was determined in triplicates of 500 mg, using an Inductively Coupled Plasma Optical-Emission Spectrometry (5800 ICP-EOS, USA - Thermo Scientific™ iCap Series 7000; Thermo Fisher Scientific, Waltham, MA, USA) according to Martins *et al.* (2020). It was initiated by performing an acid digestion through the addition of 12 mL of hydrochloric acid (HCl) and 4 mL of nitric acid ($HCNO_3$) (ratio 3:1) to each sample. The mixture was let to cool down for a 24 h period and upon reaching room temperature, it was filtered and diluted to 50 mL with distilled water (Martins *et al.*, 2020).

2.4. Antioxidants

The methods for evaluating the antioxidant activity (FRAP and DPPH) and the content of total phenolic compounds require a previous extraction process. Initially, 2 g of each powdered formulation were dissolved in 10 mL of ethanol (96%) and

centrifuged for 10 min at 9600 *g*. These extracts were filtered through 0.2 µm syringe-connected (Braun, inject, Germany) filters (NY) and the ethanol was evaporated under vacuum by using a rotatory evaporator (BÜCHI, N-490, Switzerland). Dried extracts were dissolved in 20 g of dimethyl sulfoxide (DMSO) (C_2H_6OS), obtaining stock solutions at a concentration of 20 mg/mL that were stored at 4 ºC until the determination of antioxidant activity, total phenolic content, and pigment characterization.

2.4.1. DPPH

A calibration curve was done using ascorbic acid, diluting it from a stock solution (1 mg/mL) with distilled water down to several concentrations (0, 10, 25, 50, 75, 100, 150, 200 and 250 µg/mL) (Brand-Williams *et al.*, 1995; Sánchez-Moreno *et al.*, 1998). Triplicates of each standard solution were prepared by adding 3.9 mL of DPPH ($C_{18}H_{12}N_5O_6$) (60 µmol/L) to 0.1 mL of each dilution, incubating for 1 hour in a dark environment (Brand-Williams *et al.*, 1995; Sánchez-Moreno *et al.*, 1998). Upon incubation, methanol (CH_3OH) was used as blank on the spectrophotometer (Agilent Technologies, Cary 60 UV-Vis, USA), reading the absorbance of the calibration curve at 515 nm (Brand-Williams *et al.*, 1995; Sánchez-Moreno *et al.*, 1998). A negative control was performed by replacing the extract with water (Brand-Williams *et al.*, 1995; Sánchez-Moreno *et al.*, 1998).

Extracts analysis first involved the preparation of a DPPH solution by dissolving 4.8 mg of DPPH in 200 mL of methanol (Brand-Williams *et al.*, 1995; Sánchez-Moreno *et al.*, 1998). Triplicates were made, each containing 3.9 mL of DPPH solution, 0.1 mL of each extract and 0.1 mL of distilled water (Brand-Williams *et al.*, 1995; Sánchez-Moreno *et al.*, 1998). These were incubated in a dark environment for 1 hour and the absorbances were read again at 515 nm, using methanol as blank (Brand-Williams *et al.*, 1995; Sánchez-Moreno *et al.*, 1998). Since ascorbic acid equivalents were used for this procedure, the interpretation of results was done using a linear regression of the calibration curve, its parameters being used for calculation of ascorbic acid equivalents (mg AAE/ g DE) on the different extracts (Brand-Williams *et al.*, 1995; Sánchez-Moreno *et al.*, 1998).

2.4.2. FRAP

Antioxidant activity determination by FRAP assay required the preparation of several solutions, including 40 mM HCl, 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) ($C_{18}H_{12}N_6$), ferric chloride ($FeCl_3$), and acetate (0.3 M) buffer (pH=3.6). FRAP reagent was obtained by mixing the solutions TPTZ, ferric chloride and the sodium acetate buffer in a proportion of 1:1:10, respectively (Benzie and Strain, 1996). Several dilutions (0, 10, 25, 50 and 75 µg/mL) of an ascorbic acid stock solution (1 mg/mL), using distilled water, were made to obtain a calibration curve; 90 µL of each ascorbic acid dilution were pipetted, to which 270 µL of distilled water and 2.7 mL of FRAP reagent were added; the solutions were then homogenized and incubated in a water bath (Thermo Scientific, 2871, USA), at 37 ºC during 30 minutes (Benzie and Strain, 1996). Simultaneously, 90 µL triplicates of each snack extract were prepared by adding 270 µL of distilled water and 2.7 mL of FRAP solution, homogenized using a vortex and incubated in a water bath under the same conditions (Benzie and Strain, 1996). Upon time completion, absorbances of these solutions were read at 595 nm with distilled water used as blank (Benzie and Strain, 1996). As negative control, water instead of the extracts was used. To calculate the ascorbic acid equivalent values from the absorbance values, the calibration curve parameters obtained from its linear regression were used (Benzie and Strain, 1996).

2.5. Total phenolic compounds

Total phenolic content of samples was assessed through Mohankumar *et al.* (2018) procedure, based on the extracted obtained as described in 2.6. 150 µL of snack extract to which 150 µL of a Folin-Ciocalteau solution (12%) and to 2.4 mL of distilled water were added and then mixed with 300 µL of sodium carbonate (Na_2CO_3) solution (10%) after 5 min. All tubes were incubated in a dark environment at room temperature for a 2h period. Upon incubation, tubes were read on the spectrophotometer at 725 nm, using distilled water as blank. Negative controls were also prepared by replacing extract with distilled water. Expression of results was made as gallic acid [$C_6H_2(OH)_3CO_2H$] equivalents (mg GAE) per g of dry extract.

2.6. Pigments

Pigment characterization was performed by adding 3.8 mL of ethanol (96%) to 200 µL of snack extract. The mixture was incubated for 30 min in a dark environment and the absorbance (A) was read at 470, 648 and 664 nm, which corresponded to carotenoids, chlorophyll *a* (Chla) and chlorophyll *b* (Chlb), respectively. Ethanol was used as blank. Values were determined using equations 1, 2 and 3 (Lichtenthaler and Buschmann, 2001).

$$\text{Chla (µg/mL)} = 13.36 \times A_{664} - 5.19 \times A_{648} \quad (1),$$

$$\text{Chlb (µg/mL)} = 27.43 \times A_{648} - 8.12 \times A_{664} \quad (2),$$

$$\text{Carotenoids (µg/mL)} = (1000 \times A_{470} - 2.13 \times \text{Chla} - 97.64 \times \text{Chlb})/209 \quad (3),$$

2.7. Rheology and texture

Rheology measurements were based on small amplitude oscillatory shear (SAOS), using a controlled stress rheometer Haake MARS III (Thermo Fisher Scientific, Waltham, MA, USA), coupled with a UTC Peltier. Measurements were performed under controlled temperature 20 ± 0.5 ºC, using a 20 mm diameter serrated plate-plate. One tablespoon of each dough was placed on the lower plate and compressed at 0.6 mm/min between the two plates to the set gap at 1 mm (previously optimised for this type of material). Any excess dough was removed from the plates and liquid paraffin was added around the samples to prevent moisture loss. Dough was allowed to rest for 5 min before performing frequency sweep tests, ranging from 0.1 to 100 Hz, within the linear viscoelastic region (LVR). This region was previously determined, through stress sweep tests and a stress of 7 Pa was applied.

Dough development/fermentation time (DDT), considered as the time at which maximum torque is reached) (s) was also assessed through time sweep tests, performed on doughs immediately after mixing, at 5 ºC, during 1 hour, to obtain an equilibrium of the viscoelastic functions (Graça *et al.*, 2018; Khemiri *et al.*, 2020).

Viscosity measurements were also performed on each dough sample by using the same apparatus. Measurements were performed in triplicate for each formulation (after the 15 min doughs were allowed to rest). Furthermore, estimated viscosity was determined through data adjustment of a Williamson-Cross improvised model. The Cross equation (4) can be used to describe both high and low shear rate regions of

pseudoplastic solutions, which includes both the zero-shear viscosity, η_0, and the limiting viscosity at infinite shear rate, η_∞ (Kasapis and Bannikova, 2017):

$$\frac{\eta_0-\eta_\infty}{\eta-\eta_\infty}=(k\dot{\gamma})^m \quad (4)$$

where $\dot{\gamma}$ is the shear rate ($d\gamma/dt$), k is the consistency coefficient (s) and m is the flow index (dimensionless).

The Williamson model (5) is a derivation of the Cross model, considering that $\eta_\infty = 0$ (Álvarez-Castillo *et al.*, 2021):

$$\eta=\frac{\eta_0}{1+(k\dot{\gamma})^m} \quad (5)$$

Texture profile analysis (TPA) tests were performed 9 times on each formulation dough, allowing the determination of texture parameters as the maximum resistance to penetration reflected as firmness (N), adhesiveness (-N.s) and cohesiveness by using a texture analyser TA.XTplus (Stable Microsystems, Surrey, UK) with a 5 kg load cell and a cylindrical 10 mm diameter acrylic probe at room temperature (20 ± 1 ºC).

Snacks were also evaluated through penetration tests, that allowed determination of hardness (N) and brittleness (mm), using the same apparatus to evaluate doughs, but using a 2 mm cylindrical stainless-steel probe, at controlled room temperature (20 ± 1 ºC). Hardness was determined as the force peak (N) in the force *versus* time texturogram, being the required force to penetrate the snack (Batista *et al.*, 2019). Brittleness was determined as the distance to maximum force on the curve of the bite (Bourne *et al.*, 2002). This distance is an indicative of the speed at which snacks crumble, and consequently, an indicative of the snack brittleness (Bourne *et al.*, 2002).

2.8. Colour

Dough and snack colour was measured using a Minolta CR-400 (Japan) colorimeter with standard illuminant D65 at a visual angle of 2º. Results were expressed according to CIE system colour space defined by the International Commission of Illustration, L* defines the luminosity of a sample lightness (0 to 100%), a*, redness to greenness (60 to -60, positive to negative, respectively), and b*, yellowness to blueness (60 to -60, positive to negative, respectively) (https://www.konicaminolta.com/instruments/knowledge/color/part1/07.html). All samples were measured under the same light conditions using a white standard tile (L*

= 86.70, a* = 0.32, b* = 0.34) under artificial fluorescent light at room temperature. Ten replicates for each formulation (3 measurements per dough/cracker) were performed. Results were analysed in colour space and differences in L*, a*, and b* relatively to the control were also measured, as well as the total colour difference from control formulation, as follows:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (6).$$

The latter equation was also used to determine differences between the colour of the dough and the baked snack.

2.9. Sensory evaluation

Sensory analysis was performed in a standardized sensory analysis room, according to standard EN ISO 8589: 2007 procedure. The panel was composed of a total of 33 untrained panellists (10 males and 23 females, ages ranging between <18 and up 65 years old). To avoid fatigue of panellists but also due to the snacks' characteristics (texture, flavour, scent), only control and snacks incorporating 5% of *Chlorella vulgaris* and *Arthrospira platensis* were evaluated. The panel rated each snack in terms of colour, appearance, aroma, texture, flavour, global assessment and buying intent. These parameters were rated in accordance with a 7-point hedonic scale from like very much (7) to dislike very much (7), except for buying intent, which was also assessed in 7-point hedonic scale but from would certainly buy (7) to would never buy (1). Additionally, the members of the panel replied to other questions on their perception on foods incorporating microalgae, 3D food printing, snack and 3D technology used with microalgae incorporation.

2.10. Statistical analysis

Several assumption tests (Bartlett's test, Levene's tests, Kolmogorof-Smirnof test and Shapiro-Wilk test) were performed to verify the distribution/homogeneity of variances, applying the one-way analysis of variance (ANOVA)/t-test when the assumptions were met. Non-parametric tests (Kruskal-wallis) were also applied when the parametric tests requirements failed. Post-comparison tests including Tukey-HSD and Dunn's test were also applied to identify differences between groups of variables, for parametric and non-parametric data, respectively. XLSTAT (Addinsoft, France) was used for statistical analysis with a significance level of 95% ($p < 0.05$).

3. Results & Discussion

3.1. Printability

One of the perks of working with 3D food printers is the ability to create innovative designs. By introducing unpopular food sources, the use of appealing visual shapes can increase its acceptance among consumers, potentially breaking culture barriers towards a consumption of a certain food such as microalga biomass (Liu *et al.*, 2017; Dankar *et al.*, 2018). However, it is clear that there is still room for a considerable amount of research upon this subject, as not all designs are adequate for all food sources and many are dependent on the product's formula as well as of the pre-defined design settings. To obtain a successful printing process, the dough material must present specific characteristics in order to have a flawless concept that comes to life with 3D printing (Godoi *et al.*, 2016; Lille *et al.*, 2018; Vieira *et al.*, 2020). In this sense, the desirable feature includes materials which are easily extruded through the cartridge nozzle while maintaining their shape at the end of the print (Godoi *et al.*, 2016; Lille *et al.*, 2018; Vieira *et al.*, 2020).

Beforehand, it was concluded in terms of the dough recipe that the reduction in water was necessary to construct a firm dough incorporating corn and rice flours, which were less dense than the oatmeal flour originally designed in Oliveira's formula. In terms of the actual printing process and in concordance with the rheology results obtained, all doughs including control (without microalgae biomass), control without xanthan gum, and doughs containing 5, 10, 15 and 30 % *Chlorella* or Spirulina biomass were tested. Printing of duck foot shaped doughs occurred smoothly for control and 5% microalgae incorporation levels (**Figure 3.1**). An incorporation of 10% microalgae biomass into doughs led to occasionally faulty printed snacks containing errors including layer misplacement and deficient shapes of layers. Moreover, although 15% algal doughs were printable, as these presented a more viscous behaviour, the first snack was often printed with excess dough, originally destined to be left on the waste deposit. Even so, it was observed that control and 5 to 15% concentration doughs had an adequate printability process, mostly without major errors. As they presented a non-Newtonian shear thinning behaviour, dough easily flowed through the cartridge nozzle and maintained a solid shape. However, the extreme viscosity of 30% algal doughs made them unprintable, always resulting in an unsuccessful and continuous excessive extrusion effort by the machine to extrude dough into the waste deposit. In this sense, it

was observed that higher microalgal biomass incorporation led to failures associated with the printing process, in some cases, resulting in a faulty design with wrong shapes or deposition of excessive dough (retraction). A possible solution could be the addition of a plasticizer in order to decrease the viscosity of such doughs. Nevertheless, past results indicated that higher levels of glycerol lead to doughs with more elastic behaviour and those incorporating smaller values are harder to print, becoming hard (Verbeek and Berg, 2010; Álvarez-Castillo *et al.*, 2021). Hence, this might explain the printability of the doughs of the present study, not only due to the increasing protein and polysaccharide content in snacks containing higher microalgal biomass percentages but also due to their consequently lesser content in glycerol, which was proportionally reduced along with the flour content as algal biomass was added (Uribe-Wandurraga *et al.*, 2020a; Álvarez-Castillo *et al.*, 2021).

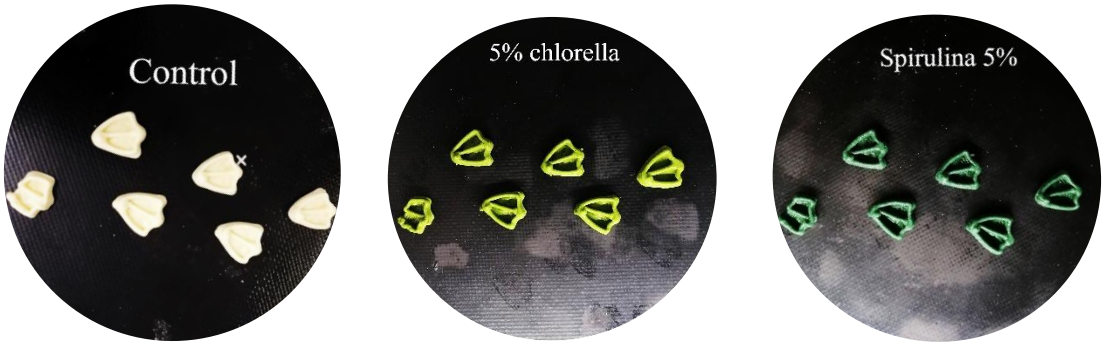

Figure 3.1. *Example of 3D printed control, 5% Chlorella and 5% Spirulina snacks before baking.*

Additionally, dough without xanthan gum was not printable due to its over excessive elastic behaviour and consequently fell through the nozzle tip as it lacked structure (**Figure 3.2)** (Liu *et al.*, 2017; Álvarez-Castillo *et al.*, 2021).

Figure 3.2. *Control dough printing attempt (left) and respective residue with excess water.*

In addition to the duck foot shape snacks, other shapes were created to observe the feasability and effectiveness of the printing process on original designs. Forms created with the inherent printer CAD program are displayed below (**Figure 3.3 – A-F**). Visual

traits obtained with different shapes reveal that 3D printing is still somewhat limited to the already present pre-designed concepts which passed rigorous testing procedures. Even so, new 3D models were developed for an Euronews report on microalgae, displaying designs like christmas trees (**Figure 3.3 – A**), dense maze cookies (**Figure 3.3 – B**), rose cookies (**Figure 3.3 – C**), ISA logo (**Figure 3.3 – D**), to UFO shapes (**Figure 3.3 – E**), and even Queen Elizabeth II (**Figure 3.3 – F**), in an attempt to highlight 3D food printing potential in exploring new food sources. Furthermore, it is possible to conclude that there is still progress to be made before 3D food printing becomes more user friendly. This technology requires prior knowledge in the order of the mechanism settings to correctly print costumable designs with finer details.

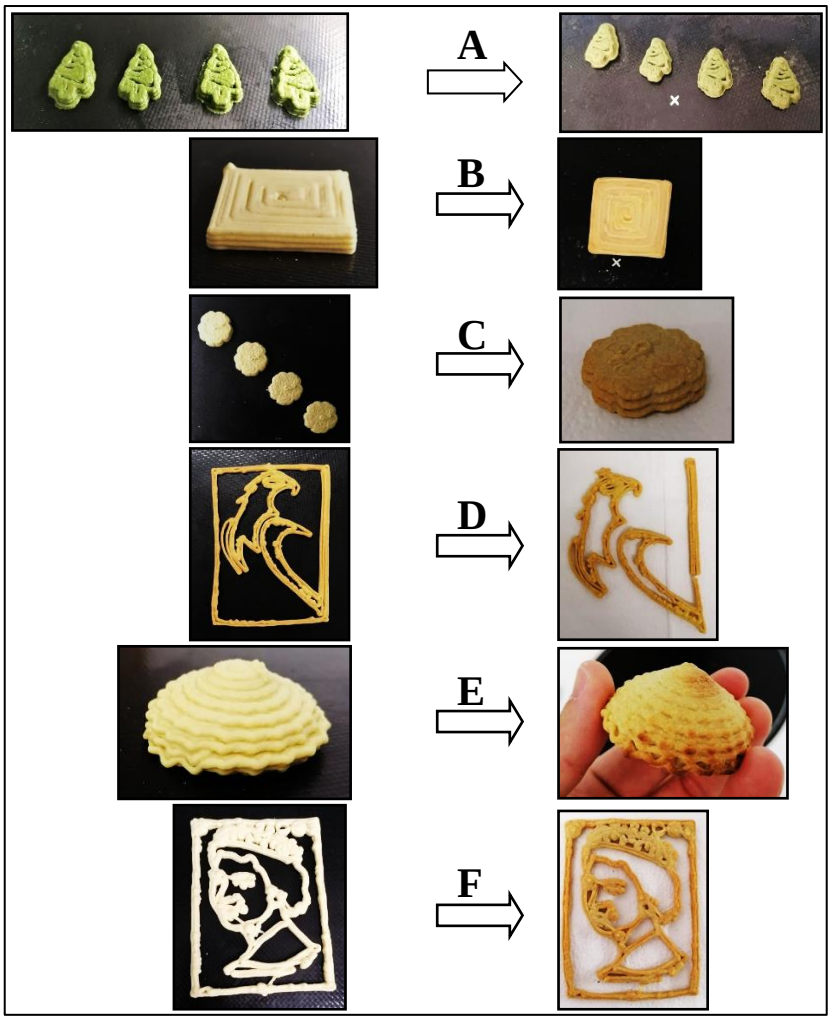

Figure 3.3 – A-F. *Original Christmas tree, dense maze cookie, rose cookie, ISA logo, UFO and Queen Elizabeth II design printed and cooked, respectively.*

The number of layers, thickness and speed of which they are printed all affect the final aspect of the product. For example, although the outside of the UFO shaped snack began to present burnt areas during baking (**Figure 3.3 – E**), its interior was still raw.

Other aspect that needs to be taken into consideration are the ingredients composing the formulations, since these affect the detail and structure of the product. Another imporant aspect is related to the post-processing procedure, either baking, frying, freezing, all of which are processes that differently affect the final design quality of the food.

Additionally, in order to assess how altering printing settings affect the final structure and visual appearance of the product, an original Christmas tree design was printed with different printing settings. It was verified that altering the slightest values of the parameters, including the first layer nozzle height, the printing speed, the layer tickness or even the nozzle size can greatly affect the final aspect of the food (**Figure 3.4 – A-D**). Smaller or higher nozzle sizes may not be adequate for the pre-defined food design settings, as these nozzles are usually destined for other food materials (**Figure 3.4 – A & B**). Printing speeds may be adjusted to obtain a better time efficiency in the food production, however, there may be limits in terms of details achievement with higher printing speeds. First layer nozzle height greatly affects the structure of the food design (**Figure 3.4 – C**). These results sugest that when working with gluten-free cereal doughs, enriched with microalgae biomass, changing these parameters can result in variations on the printing time, design accuracy, food structure, and quantity of dough used (**Figure 3.4 – A-D**). Taken together, it can thus be stated that each food design should be thoroughly analysed to obtain the most sustainable, productive, and visually appealing food when using 3D food printing.

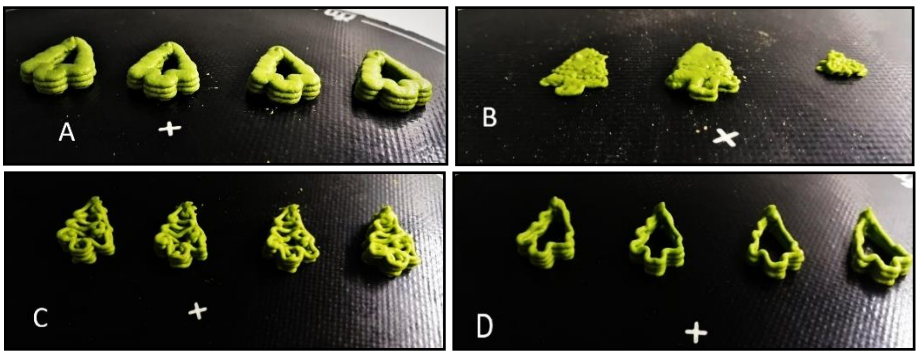

Figure 3.4 – A-D. *Printing outcomes of different 3D food printer settings. A and B higher and smaller nozzle sizes were used, respectively. C shows first layer nozzle height variation. D shows fill factor alterations.*

3.2. Dimensional analysis

In relation to size measurements performed on freshly printed snacks (**Table 3.1; Table 3.2**) it can be observed that these present a smaller size in terms of thickness (height) compared to reference values provided by the Foodini printer. There is a clear

significative ($p < 0.05$) thickening of snacks incorporating both 10% (0.574 ± 0.165 cm) and 15% *Chlorella* (0.607 ± 0.122 cm) biomass, comparatively to the control (0.522 ± 0.364 cm) (**Table 3.1**). In relation to width, length, and weight of the snacks, these were not significantly ($p > 0.05$) affected as result of any percentage of *Chlorella* biomass incorporation (5%-15%) (**Table 3.1**).

Table 3.1. *Control and,* Chlorella *snacks height (cm), width (cm), length (cm) and weight (g). Standard deviation is displayed with each value. Different letters represent significantly different statistical groups ($p < 0.05$) between control and snacks containing different microalgae concentrations (5%, 10% and 15%).*

	Control	***Chlorella* 5%**	***Chlorella* 10%**	***Chlorella* 15%**
Height (cm)	0.522 ± 0.364^{bc}	0.502 ± 0.190^{c}	0.574± 0.165^{ab}	0.607 ± 0.122^{a}
Width (cm)	1.935 ± 0.342^{a}	1.938 ± 0.533^{a}	1.938 ± 0.388^{a}	1.973 ± 0.389^{a}
Length (cm)	2.215 ± 0.655^{a}	2.257 ± 0.276^{a}	2.221 ± 0.238^{a}	2.222 ± 0.433^{a}
Weight (g)	0.985 ± 0.114^{a}	0.962 ± 0.036^{a}	0.999 ± 0.072^{a}	0.945 ± 0.071^{a}

The same parameters were evaluated towards the effect of Spirulina incorporation at different concentrations (5, 10 and 5% w/w). It was observed that there is a significant ($p < 0.05$) increase in thickness when incorporating 10 and 15% (0.621 ± 0.210 and 0.646 ± 0.306 cm, respectively) of this biomass compared to control snacks (**Table 2**). Much like when incorporating *Chlorella* biomass, none of the width, length and weight dimensions of the control snacks were significantly altered by the incorporation of Spirulina biomass (5%-15%) (**Table 2**).

Table 3.2. *Control and,* Chlorella *snacks height (cm), width (cm), length (cm) and weight (g). Standard deviation is displayed with each value. Different letters represent significantly different statistical groups ($p < 0.05$) between control and snacks containing different microalgae concentrations (5%, 10% and 15%).*

	Control	Spirulina 5%	Spirulina 10%	Spirulina 15%
Height (cm)	0.522 ± 0.364^{c}	0.598 ± 0.103^{bc}	0.621 ± 0.210^{ab}	0.646 ± 0.306^{a}
Width (cm)	1.935 ± 0.342^{a}	1.942 ± 0.463^{a}	1.948 ± 0.522^{a}	1.956 ± 0.557^{a}
Length (cm)	2.215 ± 0.655^{a}	2.210 ± 0.523^{a}	2.190 ± 0.492^{a}	2.230 ± 0.670^{a}
Weight (g)	0.985 ± 0.114^{a}	0.964 ± 0.056^{a}	0.939 ± 0.090^{a}	0.960 ± 0.117^{a}

These results suggest that the addition of *Chlorella* and *Spirulina* (up to 15%) did not significantly ($p > 0.05$) affect the length, width or, weight of the snacks comparatively to control (**Table 3.1; Table 3.2**). Regarding the thickness (height) of the snacks, it was observed that only with higher percentages as 15% *Chlorella* and 10% to 15% Spirulina incorporations led to increases in such parameter, comparatively to the control (**Table 3.1; Table 3.2**). This increase in the snacks thickness can be attributed to higher quantity of protein from the incorporation of microalgae biomass, which provides more structure to these snacks.

3.3. Nutritional characterization

There is an inherent step to prepare snacks from a raw dough, its post-processing, which in this case corresponded to a baking process. This process leads to changes in the chemical characteristics of the final product, in relation to the initial printed design (Dankar *et al.*, 2018; Vieira *et al.*, 2020). Hence, not only to guarantee an adequate nutritional characterization of the final product but also to ensure its safety upon consumption, nutritional characterization of the snacks included the assessment of minerals, total fatty acids, humidity, ashes, protein content, carbohydrates content (calculated by difference) and its energy (Kcal) (**Table 3.3)**. Among all produced snacks, control, 5% *Chlorella* and 5% Spirulina snacks were selected due to their rheology properties and higher potential acceptance by the consumer in light of their sensory characteristics (smell, taste, colour, texture), and thus, were subjected to the previously mentioned analysis.

Table 3.3. *Nutritional characterization including water activity (a_w), humidity (%), ashes (%), total fatty acids (%) and protein content (%) of control,* Chlorella*, and Spirulina snacks at 5% concentration. *Calculated by difference. Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups ($p < 0.05$).*

Samples	Control	*Chlorella* 5%	Spirulina 5%
Humidity (%)	12.514 ± 0.503^{a}	11.212 ± 0.215^{b}	10.538 ± 0.098^{b}
Ashes (%)	1.937 ± 0.143^{a}	2.254 ± 0.162^{a}	2.145 ± 0.142^{a}
Total fatty acids (%)	10.079 ± 1.671^{a}	7.025 ± 1.135^{a}	8.234 ± 0.799^{a}
Protein (%)	5.658 ± 0.051^{c}	7.856 ± 0.034^{b}	9.871 ± 0.184^{a}
Carbohydrates*	70.921	71.221	69.212
Energy (kcal/100g)	315.469	323.956	324.566

Snacks revealed a nutritional characterization with improved characteristics, when compared to the control formulations (**Table 3.3**). The incorporation of microalgae *Chlorella vulgaris* and *Arthrospira platensis* resulted in snacks with significantly ($p < 0.05$) smaller humidity (%) values (11.212 ± 0.215 and 10.538 ± 0.098, for *Chlorella* and Spirulina, respectively) than when compared to control snacks (12.514 ± 0.503 for control snacks). The higher water retention capability by the algal biomass explains the lower humidity found in snacks containing microalgae, since these biomasses are known to have high water absorption capability (Batista *et al.*, 2017; Graça *et al.*, 2018; Fradinho *et al.*, 2020). This is supported by the significantly ($p < 0.05$) higher protein content in the 5% Spirulina (9.871 ± 0.184) snacks compared to both 5% *Chlorella* (7.856 ± 0.034) and control (5.658 ± 0.051) snacks (**Table 3.3**). Furthermore, the decrease in humidity can be explained by the water loss associated with the baking process, leading to the decrease of humidity in the snacks. *Chlorella* 5% on its own also had significantly ($p < 0.05$) higher values of protein than control snacks. These protein levels can be attributed to the incorporation of algal biomass in the snacks' formula, causing a significant increase in the protein content as result of the naturally abundant protein content of these two microalgal species (Batista *et al.*, 2017; Batista *et al.*, 2019). These results are in agreement with other baked-products studies incorporating these microalgae, which indicate a similarly consequent protein increase (Batista *et al.*, 2017; Lucas *et al.*, 2018; Graça *et al.*, 2018; Uribe-Wandurraga *et al.*, 2020a). Spirulina snacks have higher protein content compared to those of the *Chlorella*

snacks, which can be explained by the higher protein content of the former compared to the latter microalga, which was also observed in previous studies (Batista *et al.*, 2017; Koyande *et al.*, 2019; Niccolai *et al.*, 2019). Moreover, it can be claimed that Spirulina 5% snacks of this study are a "source of protein", according to Regulation (EC) 1924/2006, since the protein content constitutes 12.2% of the total energy of the snack (Batista *et al.*, 2019; Niccolai *et al.*, 2019).

In respect to ashes and total fatty acids content, the incorporation of both microalgae did not significantly ($p > 0.05$) alter in the snacks tested. These ash contents are in concordance with past studies upon using equal levels of biomass incorporation (Batista *et al.*, 2017; Batista *et al.*, 2019).

The amount of carbohydrates (g/100g) (calculated by difference) found in the formulations assayed did not vary between snacks containing or lacking microalgal biomass but, still, lower values were found on snacks containing 5% Spirulina (69.212) compared to 5% *Chlorella* (71.221) or even control snacks (70.921) (**Table 3.3**). Carbohydrates values are the result of the sum of the remainder nutritional parameters (fatty acids, proteins, and ashes). Thus, the similar and high percentage of carbohydrates in snacks either 5% Spirulina, *Chlorella* 5% or control, could be attributed to the high carbohydrate content of corn flour and corn starch, but also to microalgae biomass, which has elevated carbohydrate content, as concluded by previous studies (Niccolai *et al.*, 2019).

Finally, energy levels (Kcal) were found to be higher in snacks containing 5% Spirulina (324.566 Kcal) than those containing 5% *Chlorella* (323.956) and control snacks (315.469) (**Table 3.3**). Total energy higher values in snacks containing either alga may be explained by their higher protein content, resulting in a higher energy input.

Water activity (a_w) is an important parameter to evaluate in snacks, particularly those containing low moisture like in this study, as it can affect their crispiness, the physical-chemical stability of foods and their sensory perception (Batista *et al.*, 2019; Vieira *et al.*, 2020). As a_w quantifies water availability for microbial, enzymatic, or chemical reactions, it is usually used for appraisal of microbial growth and chemical stability of foods (Vieira *et al.*, 2020). Water activity of control snacks (0.682 ± 0.005) had significantly higher values ($p < 0.05$) than those of *Chlorella* 5% (0.613 ± 0.003) and Spirulina 5% (0.640 ± 0.013). Since values under or equal to 0.8 and 0.6 hinder bacterial and mould/yeast growth, respectively, chemical stability and anti-microbial

activity of control snacks can be considered lower than any of the remaining snacks incorporating either *Chlorella* or Spirulina (Vieira *et al.*, 2020; Khemiri *et al.*, 2020). As snacks containing 5% Spirulina have significantly lower a_w than 5% *Chlorella*, a more potent anti-microbial activity of these snacks becomes evident. These results are in concordance with several studies enhancing the anti-microbial activity and the chemical stability when microalgae are incorporated into foods (Batista *et al.*, 2017; Batista *et al.*, 2019). Although these values do present improvements to control snacks, they are somewhat insufficient to assure their crispiness, since they surpass the 0.5 threshold mentioned by several authors (Batista *et al.*, 2019; Mota *et al.*, 2020), who suggest that the addition of microalgae containing high protein content causes an increase in a_w values. Nevertheless, despite that the results obtained presented somewhat insufficient values of a_w, these did not translate into real loss of the snack's crispiness, as confirmed by texture results. This suggests that crispiness might be affected by other variables, such as the ingredients of the formulation. All of these statements are specially important when considering shelf life of a product, since low a_w values may prolong the shelf life of a product (Batista *et al.*, 2019; Khemiri *et al.*, 2020).

Mineral composition of microalgae can be highly variable, even when the biomass of different strains belonging to the same species is applied to different products (Kratzer & Murkovic, 2021). As can be seen in **Table 3.4,** an increase in important minerals that are involved in a balanced nutrition in snacks containing either *Chlorella vulgaris* or *Arthrospira platensis*, compared to control snacks, was obtained. Specifically, there were significantly ($p < 0.05$) higher iron values in 5% Spirulina snacks (0.439 ± 0.042 mg/100g) compared to those of the control (0.125 ± 0.044 mg/100g) and *Chlorella* 5% (0.192 ± 0.036 mg/100g) snacks, which may be crucial for increasing physical performance at all ages (Sharma *et al.*, 2016; Khemiri *et al.*, 2020). Potassium, being associated to intracellular fluid balance, carbohydrates metabolism, protein synthesis and nerve impulses, is an important mineral (Safi *et al.*, 2014). Snacks incorporating 5% Spirulina (12.147 ± 0.523 mg/100g) and 5% *Chlorella* (8.871 ± 0.108 mg/100g) presented significantly ($p < 0.05$) higher potassium values than those found in control snacks (4.902 ± 0.888 mg/100g), with the potassium contents in Spirulina snacks clearly surpassing those of the remaining snacks (**Table 3.4**). It is also important to point out that *Chlorella* and Spirulina (2.136 ± 0.073 and 1.601 ± 0.014 mg/100g, respectively) presented significantly higher ($p < 0.05$) calcium content than that of the

control snack (0.382 ± 0.096 mg/100g) (**Table 3.4**), which can provide important for bone built on youngsters that lack access to higher calcium rich foods such as milk (Ross *et al.*, 2011; Khemiri *et al.*, 2020). Magnesium contents were found to be significantly ($p < 0.05$) higher in snacks containing Spirulina (4.030 ± 0.090 mg/100g) comparatively to those in the control (1.573 ± 0.029 mg/100g) (**Table 3.4**), which may also be important when considering that magnesium has the biological role of ensuring proper enzyme and metabolism function, as well as a normal nervous system activity (Safi *et al.*, 2014). Another equally important mineral is zinc, as it participates in a series of metabolic processes including synthesis of carbohydrates, lipids, and proteins (Safi *et al.*, 2014). In 5% *Chlorella* snacks (0.180 ± 0.002 mg/100g), zinc was found in significantly ($p < 0.05$) higher quantities compared to those of the control snack (0.097 ± 0.003 mg/100g). Since microalgae can accumulate contaminants in their cells, the levels of contaminants in the snacks were assessed for safety measures and was concluded to be similar for both of them. For example, neither lead (0.007 ± 0.003, 0.005 ± 0.002, 0.006 ± 0.002 mg/100g, for control, *Chlorella* 5%, and Spirulina 5%, respectively) nor other possible contaminant such as cadmium (0.000 ± 0.000, 0.000 ± 0.000, 0.000 ± 0.001 mg/100g, for control, *Chlorella* 5%, and Spirulina 5% snacks, respectively) presented any risk to the human health (**Table 3.4**), as these low values were situated within the recommended limits imposed by the European Commission Regulation (Nagajyoti, Lee and Sreekanth, 2010; Commission Regulation (EU) 488/2014, 2014; Commission Regulation (EU) 1005/2015, 2015). Taken together, the addition of microalgae can thus be essential in gluten-free products to improve the mineral content of snacks, as celiac patients are known to have issues related to mineral absorption (Nunes *et al.*, 2020; Khemiri *et al.*, 2020). Finally, it can concluded that the mineral content improvement resultant from the incorporation of these microalgae is in concordance with other studies incorporating the same species in baked products such as bread, cookies, and biscuits (Saharan and Jood, 2017; Khemiri *et al.*, 2020).

Table 3.4. *Mineral composition (mg/100g) of control,* Chlorella *and Spirulina snacks at 5% concentration. Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups ($p < 0.05$).*

Sample	Control	Chlorella 5%	Spirulina 5%
Na	54.349 ± 1.552^{a}	55.331 ± 1.106^{a}	57.053 ± 1.438^{a}
K	4.902 ± 0.888^{c}	$\mathbf{8.871 \pm 0.108^{b}}$	$\mathbf{12.147 \pm 0.523^{a}}$
Ca	0.382 ± 0.096^{c}	$\mathbf{2.136 \pm 0.073^{a}}$	$\mathbf{1.601 \pm 0.014^{b}}$
Mg	1.573 ± 0.029^{c}	2.321 ± 0.066^{bc}	$\mathbf{4.030 \pm 0.090^{a}}$
P	6.210 ± 0.152^{c}	$\mathbf{13.733 \pm 0.114^{a}}$	$\mathbf{12.521 \pm 0.245^{b}}$
S	5.022 ± 0.181^{c}	7.205 ± 0.068^{b}	9.834 ± 0.167^{a}
Fe	0.125 ± 0.044^{b}	0.192 ± 0.036^{b}	$\mathbf{0.439 \pm 0.042^{a}}$
Cu	0.039 ± 0.001^{ab}	0.041 ± 0.003^{a}	0.036 ± 0.003^{b}
Zn	0.097 ± 0.003^{b}	$\mathbf{0.180 \pm 0.002^{a}}$	0.065 ± 0.002^{c}
Mn	0.026 ± 0.001^{b}	0.055 ± 0.001^{a}	0.040 ± 0.001^{ab}
B	0.005 ± 0.000^{b}	0.010 ± 0.001^{a}	0.006 ± 0.001^{ab}
Pb	0.007 ± 0.003^{a}	0.005 ± 0.002^{a}	0.006 ± 0.002^{a}
Cr	0.0134 ± 0.003^{a}	0.012 ± 0.002^{a}	0.017 ± 0.006^{a}
Ni	0.007 ± 0.002^{a}	0.007 ± 0.002^{a}	0.009 ± 0.002^{a}
Cd	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	0.000 ± 0.001^{a}

3.4. Antioxidants

The antioxidant activity was assessed by two different methods including FRAP and DPPH assays. Past studies (Batista *et al.*, 2017) revealed higher antioxidant activity of green microalgae such as *Chlorella vulgaris*, justified by their higher content on chlorophyll *a* and *b*, compared to other microalgae. In this study, FRAP assay indicated that control snack (27.405 ± 0.929 mg AAE/g DE) presented a lower ($p < 0.05$) antioxidant activity compared to that of *Chlorella* 5% (72.736 ± 0.126 mg AAE/g DE), however, Spirulina (121.782 ± 0.131 mg AAE/g DE) snacks showed a significantly ($p > 0.05$) higher antioxidant activity than those of *Chlorella* or the control snack (**Figure 3.5**). This may be attributed to the natural antioxidant activity found on the Spirulina biomass described in many conducted studies on this microalga, in addition to the higher promptness of chlorophylls to be degraded when subjected to high temperatures (Lucas *et al.*, 2018; Koyande *et al.*, 2019; Batista *et al.*, 2019).

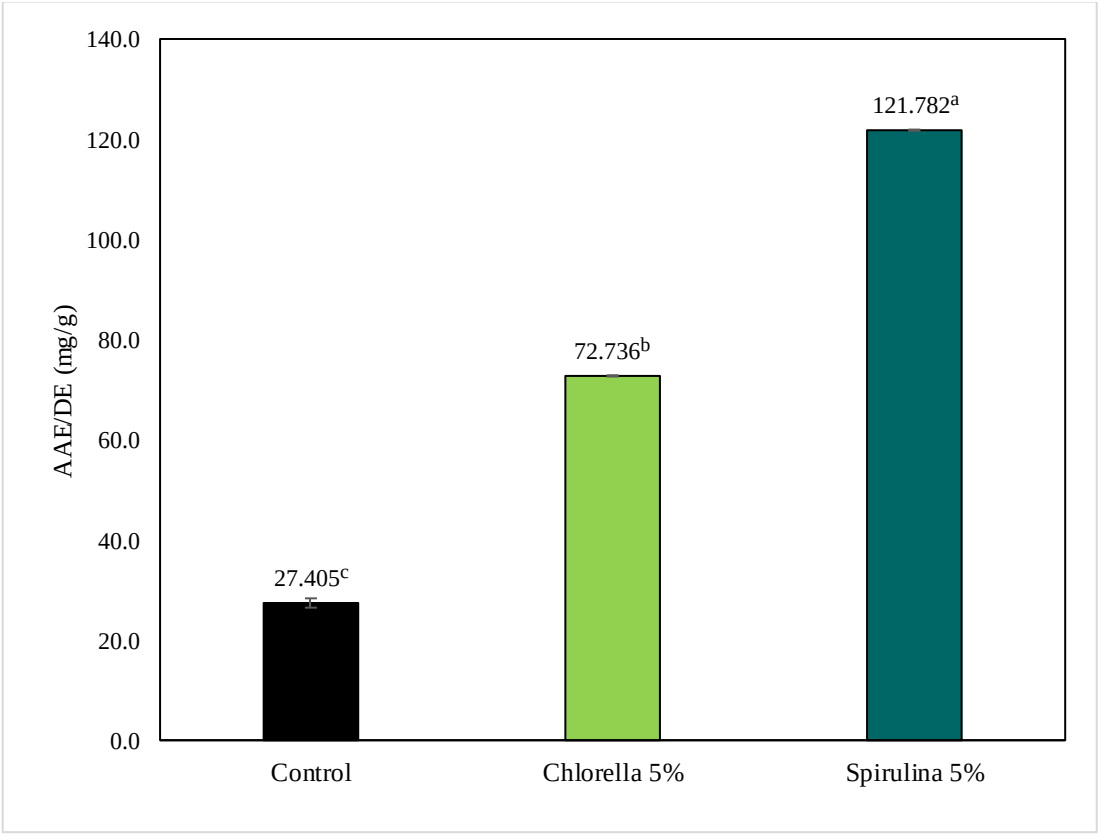

Figure 3.5. *Antioxidant activity (expressed as ascorbic acid equivalents mg/g dry weight) determined by FRAP of control,* Chlorella*, and Spirulina 5% concentrations snacks. Results are expressed as expressed as average ± standard deviation. Different letters represent statistically significant differences between groups ($p < 0.05$).*

DPPH results were in concordance with FRAP in terms of the antioxidant activity, as the snacks with higher ($p < 0.05$) activities were those containing Spirulina (26.598 ± 0.211 mg AAE/g DE, for Spirulina 5%) in comparison with *Chlorella* snacks (25.427 ± 0.019 mg AAE/g DE, for *Chlorella* 5%). Conversely, control snacks displayed the lowest antioxidant activity (22.749 ± 0.080 mg AAE/g DE) (**Figure 3.6**). This reaffirms the previous conclusion regarding the higher antioxidant activity of Spirulina 5% snacks as determined by the FRAP assay, and the superior antioxidant activity provided by the incorporation of Spirulina biomass observed in past studies (Saharan and Jood, 2017; Batista *et al.*, 2019). Ultimately, such elevated antioxidant activity could be attributed to the presence of phycobiliproteins, namely phycocyanin (El-Baky *et al.*, 2015; Saharan and Jood, 2017; Batista *et al.*, 2017; Batista *et al.*, 2019).

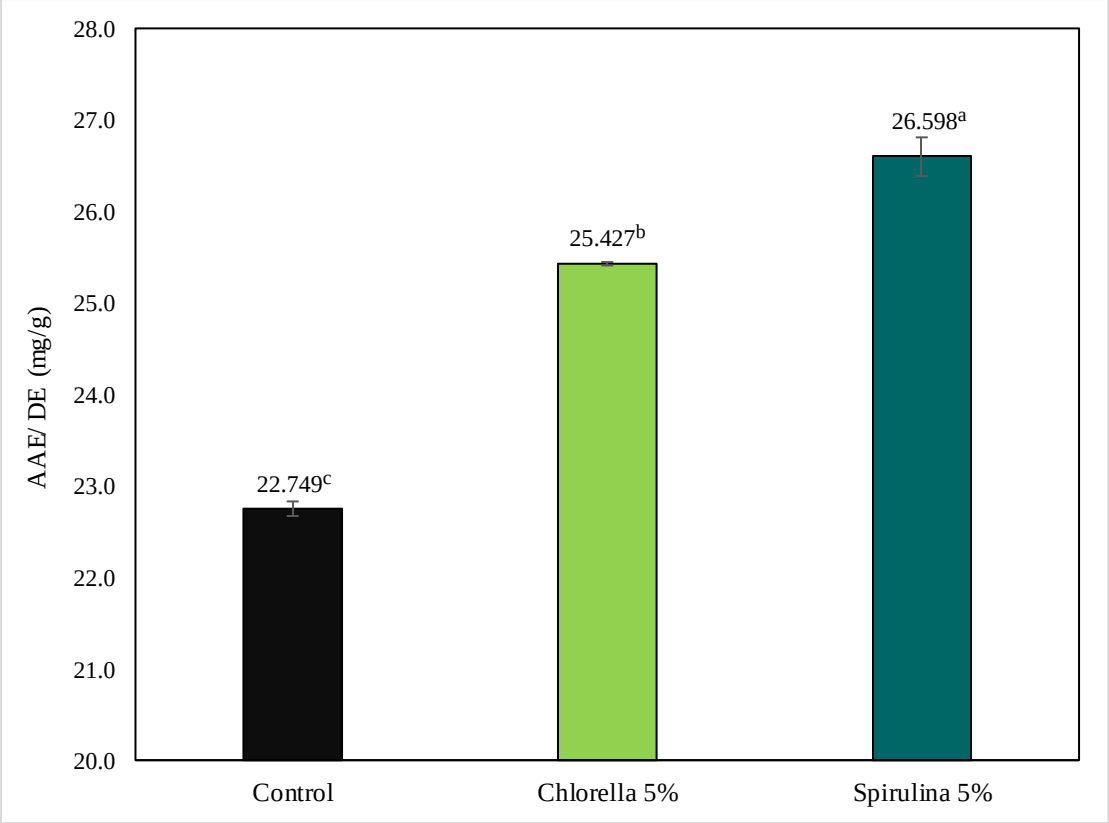

Figure 3.6. *Antioxidant activity (expressed as ascorbic acid equivalents mg/g dry weight) determined by DPPH of control,* Chlorella*, and Spirulina 5% concentrations snacks. Results are expressed as expressed as average ± standard deviation. Different letters represent statistically significant differences between groups (p < 0.05).*

3.5. Total phenolics

Phenolic compounds as phenols, tannins, lignins and phenolic acids are secondary metabolites considered to be a very important class of natural antioxidants. These were evaluated as a whole (total phenolics) in terms of total presence in the selected snacks (Vaz *et al.*, 2016; Batista *et al.*, 2017). Total phenolic content results revealed that formulations lacking algae biomass incorporation (control snack) had a lower total phenolic content (0.711± 0.010 mg GAE/g DE) than both *Chlorella* 5% (1.095 ± 0.003 mg GAE/g DE) and Spirulina 5% (1.431 ± 0.004 mg GAE/g DE) (**Figure 3.7**). These results are supported by previous studies, which demonstrated an increase in the total phenolic content in snacks incorporating Spirulina biomass comparatively to control and *Chlorella* foods (Batista *et al.*, 2017; Batista *et al.*, 2019). *Chlorella vulgaris*, however, presented lower values compared to those obtained in the latter study, which may be attributed to the use of different strains of this species.

Furthermore, as the total phenolic content of *Arthrospira platensis* is higher than that of *Chlorella*, this difference could also explain these results, as shown by most previous reports (Saharan and Jood, 2017; Batista *et al.*, 2017; Batista *et al.*, 2019;

Matos *et al.*, 2020). In this sense, past studies (Batista *et al.*, 2017; Batista *et al.*, 2019) indicate that Chlorophyta such as *Chlorella* undergo a higher phenolic loss due to degradation processes involving heat (baking), comparatively with Spirulina, explaining the higher phenolic content in snacks incorporated in the latter alga in this study (**Figure 3.7**). Other hypothesis explaining the total phenolic content of the different microalgae, may be related to their production methods, since they are purposefully manipulated to obtain a product with specific desirable attributes (Matos *et al.*, 2020). In this context, it may be that *Chlorella* biomass used in this study was cultivated in a manner which promoted the production of phenolics by the cells to a lesser extent when compared to Spirulina.

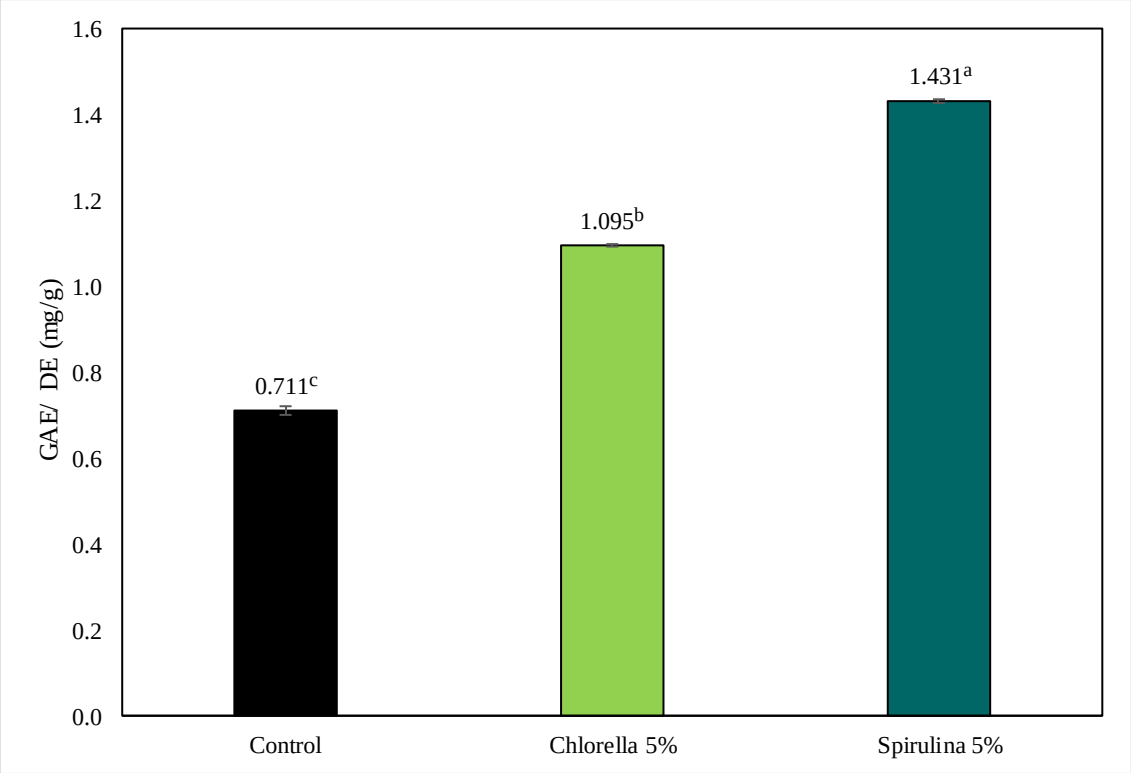

Figure 3.7. *Total phenolic content (expressed as gallic acid equivalents mg/ g dry weight) of control,* Chlorella, *and Spirulina 5% concentration snacks. Results are expressed as expressed as average ± standard deviation. Different letters represent statistically significant differences between groups ($p < 0.05$).*

3.6. Pigments

Pigment analysis revealed that control snacks contained far less ($p < 0.05$) chlorophyll *a* (0.0578 ± 0.001) and chlorophyll *b* (0.127 ± 0.003) compared to *Chlorella* 5% snacks values (1.122 ± 0.001 and 0.175 ± 0.001, for chlorophyll *a* and chlorophyll *b*, respectively) (**Figure 3.8**). Snacks containing 5% of Spirulina biomass presented significantly ($p < 0.05$) higher values of chlorophyll *a* and carotenoids (0.238

± 0.000 and 0.0345 ± 0.001, respectively) than the remainder, whereas chlorophyll *b* content was significantly ($p < 0.05$) lower than the other snacks (0.0821 ± 0.001). Despite pigment composition in *Chlorella* 5% snacks not being higher on chlorophyll *a* compared to Spirulina snacks, as it was expected since *Chlorella* vulgaris is a green alga, it still presented significantly higher chlorophyll *b* levels (**Figure 3.8**). Higher carotenoid content was also detected on Spirulina snacks. Although there are significant changes, this pigment characterization (**Figure 3.8**) of both snacks containing either *Chlorella vulgaris* or *Arthrospira platensis* is typical of these species, since *Chlorella* is known to have predominantly abundant values of chlorophyll *a* and *b*, whereas Spirulina has a higher carotenoid content (Safi *et al.*, 2014; Batista *et al.*, 2017).

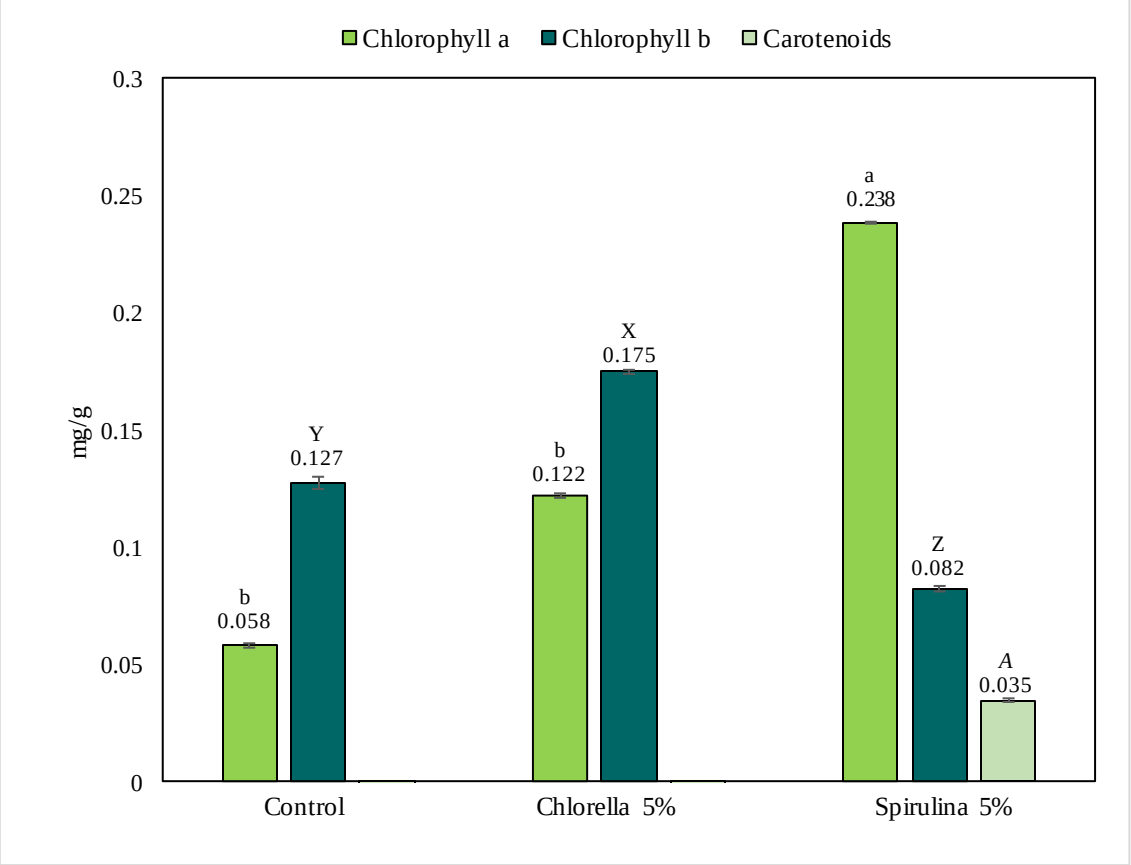

Figure 3.8. *Pigment characterization (mg/g dry weight) including chlorophyll a, b and carotenoid content of control,* Chlorella, *and Spirulina 5% concentrations snacks. Results are expressed as average ± standard deviation. Different letters represent statistically significant differences between groups ($p < 0.05$).*

3.7. Rheology behaviour

Dough's rheology properties influences its printability, being the assessment of parameters including their viscosity, the time to stabilize the dough structure and linear viscoelastic behaviour, key to determine how feasible these doughs are to print, their

shape retention upon material deposition and the final product quality (Uribe-Wandurraga *et al.*, 2020a). In this sense, G' and G'' are expressions of elastic and viscous behaviour of foods, respectively (Yang *et al.*, 2018; Vieira *et al.*, 2020). Elastic modulus has its importance since it indicates mechanical strength and shape retention capability of foods, whereas viscous modulus affects extrusion of dough (Yang *et al.*, 2018; Uribe-Wandurraga *et al.*, 2020a). Both values can be used to determine the behaviour of foods and conclude upon its printability depending on whether it is more or less elastic/viscous (Yang *et al.*, 2018; Uribe-Wandurraga *et al.*, 2020a; Uribe-Wandurraga *et al.*, 2020b). Frequency sweep tests (mechanical spectra) allowed to obtain relevant information about the degree of structuring of materials, which may be related to the stability of the systems and the physical characteristics of the final product. Upon determination of the LVR, frequency sweep assays were performed on the doughs.

***a)* Impact of xanthan gum on the dough rheology performance**

Xanthan gum is a widely used hydrocolloid in the food industry, functioning as a texture improver by increasing dough properties and retarding starch retrogradation (Xu *et al.*, 2020). It is usually added to gluten-free doughs seeking to refine rheology properties, facilitate hydration of dry ingredients and retain moisture of the final product (Xu *et al.*, 2020). In practical terms, it appears that the addition of xanthan gum makes it possible to obtain doughs with more interesting characteristics in terms of printability. A control formulation (no microalgae addition) was considered and 0.2% xanthan gum was incorporated. The effect of the addition of hydrocolloid on the viscoelastic behaviour of the dough was investigated (**Figure 3.9**).

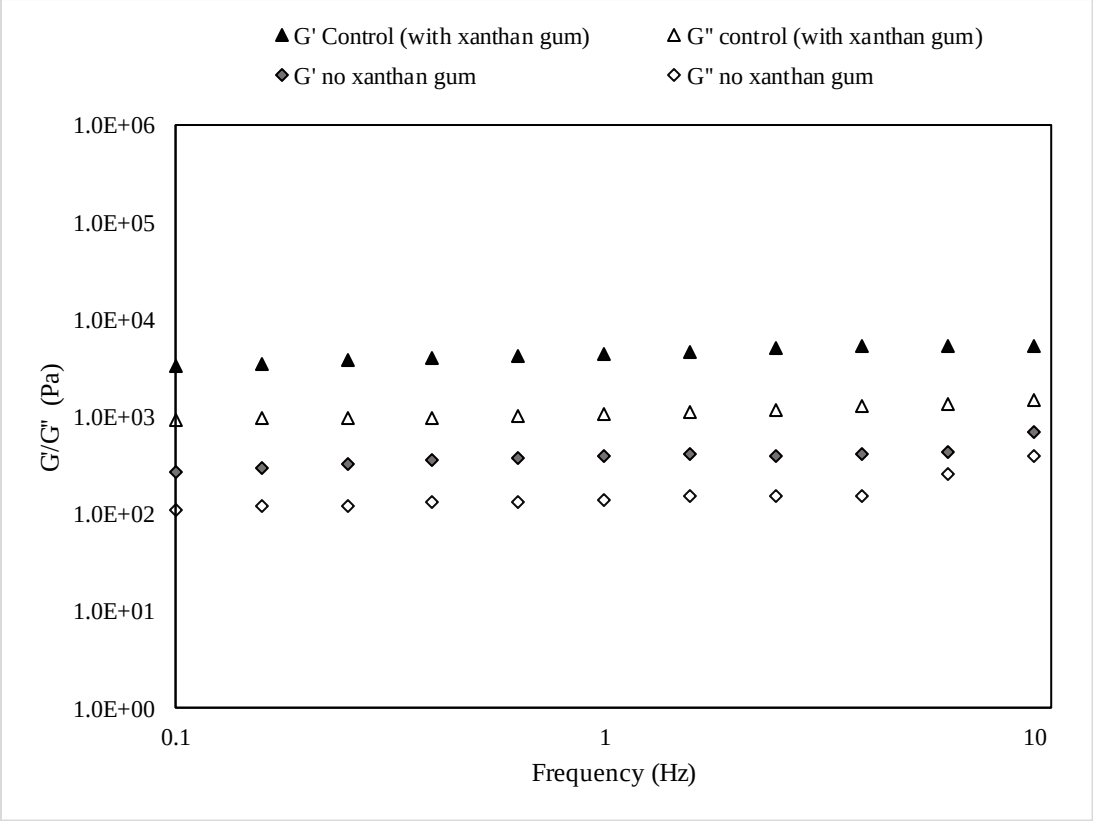

Figure 3.9. *Storage (G') and loss moduli (G'') (Pa) acquired through frequency sweep tests of control and control without xanthan gum doughs. Different symbols refer to different formulations, whereas filled and hollow symbols refer to G' and G'' of each formulation, respectively.*

From observing **Figure 3.9**, it becomes clear that both formulations have a similar behaviour: the elastic modulus (G') values are much higher than the viscous modulus (G") and both are little dependent on the applied oscillation frequency (0.1-10 Hz). This type of behaviour is typical of very structured systems and stable doughs, having already been found by other authors for doughs of the same type - in snacks (Álvarez-Castilho *et al.*, 2021), in biscuits (Mota *et al.*, 2020) and in bread (Graça *et al.*, 2018). Nevertheless, it can be stated that, although xanthan gum only constitutes 0.2% of the formulation, its presence is crucial to provide structure and stability over time since doughs not containing the hydrocolloid were excessively elastic, and thus, not printable (**Figure 3.2)**. This idea is reinforced by the results obtained in the comparison of the elastic modulus (G') for formulations with (4.29E+03 $\pm$ 3.60E+01 Pa) and without xanthan gum (5.26E+02 $\pm$ 3.30E+02 Pa) (**Table 3.5**), revealing that the incorporation of this hydrocolloid contributes to a significant ($p < 0.05$) increase in this rheological parameter, which has positive implications in terms of the dough's structure and consequently on its printing process.

Table 3.5. *Elastic modulus (G') values at 1 Hz, for dough with and without xanthan gum incorporation. Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups ($p < 0.05$).*

	G' (1 Hz) (Pa)
Dough with xanthan gum (0.2%)	4.29E+03 ± 3.60E+01[a]
Dough without xanthan gum	5.26E+02 ± 3.30E+02[b]

It is clearly observed that the addition of a small amount of xanthan gum (0.2%) allows a very relevant increase in the viscoelastic parameters (G' and G''), which translates into a greater degree of structuring of the dough. This change in viscoelastic behaviour produces a high impact in terms of printing, allowing to obtain more stable doughs, whose printing is easier and more detailed. This is further reinforced by the results obtained on time sweep tests.

Concerning control doughs (containing xanthan gum), time sweep revealed (**Figure 3.10**) enough structure over time, reaching a plateau after 3359 seconds of both viscoelastic parameters, G' (5095.26 Pa) and G'' (3359.10 Pa), whereas in control doughs lacking xanthan gum (7915.26 and 1113.34 Pa, respectively), we observe storage moduli (G') growth over time, never reaching a plateau state, that would signify a structured dough. These results indicate that in the absence of a gluten matrix, the solely structuring role performed by the combining proteins and polysaccharides present in corn and rice flours as well and on corn starch, are insufficient to provide a structure to doughs, hence, the addition of hydrocolloids is crucial for this purpose.

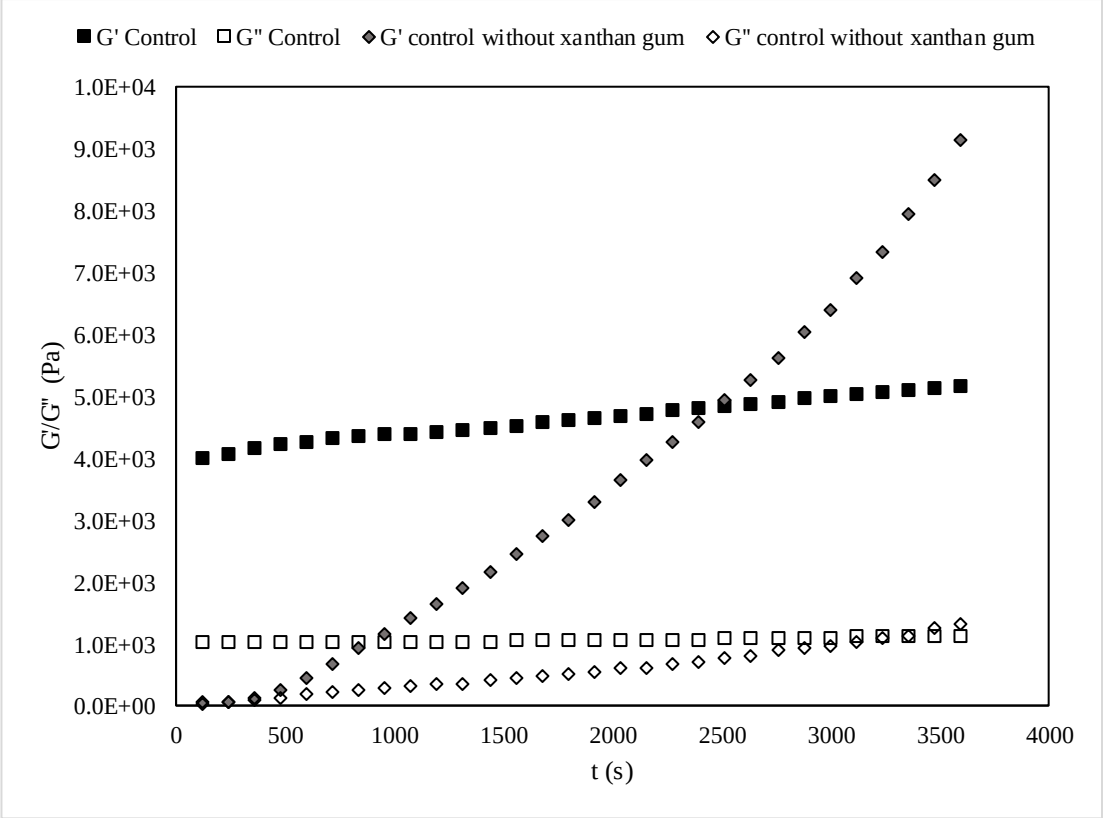

Figure 3.10. *Storage (G') and loss moduli (G'') (Pa) acquired through time sweep tests of control and control without xanthan gum doughs. Different symbols refer to different formulations, whereas filled and hollow symbols refer to G' and G'' of each formulation, respectively.*

Gums as xanthan gum are known to increase dough viscosity but also to improve their texture and sensory parameters (Preichardt *et al.*, 2011; Xu *et al.*, 2020). Studies showed that the addition of xanthan gum improves viscoelastic properties of doughs, including their firmness and viscosity (Preichardt *et al.*, 2011; Herranz *et al.*, 2016; Tebben, Shen and Li, 2018). Results of other viscoelastic parameters are further reinforced by the results obtained by the viscosity curves (**Figure 3.11**), where it is possible to perceive that control doughs containing xanthan gum had higher viscosity values than formulations lacking such ingredient, for all the range of the selected shear-rates.

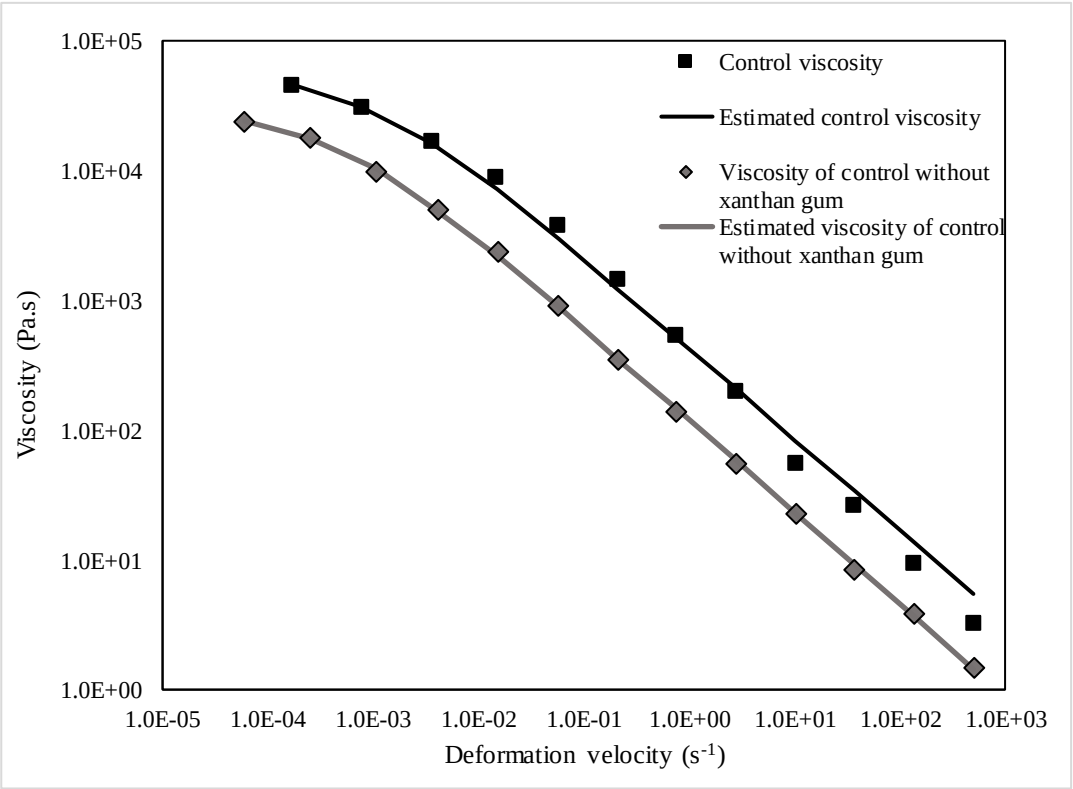

Figure 3.11. *Viscosity (Pa.s) acquired of control and control without xanthan gum doughs. Different symbols refer to different formulations. Viscosity values were adjusted to a Cross-Williamson model to obtain the estimated viscosity.*

Through the several rheology tests performed, the structuring role of the xanthan gum on gluten-free cereal doughs became evident, since in its absence, all results revealed doughs with high elasticity and with unstable rheology properties that are inadequate for a correct printing process.

The following table (**Table 3.6**) reflects the conclusions obtained in the previous graphic (**Figure 3.11**), where the zero-shear viscosity (η_0) increases with the incorporation of xanthan gum on gluten-free control doughs. This highlights the higher viscosity at rest and strength of the doughs containing xanthan gum. Furthermore, it is possible to see a lower consistency coefficient (k) in doughs containing xanthan gum, enhancing the higher viscosity of doughs containing the hydrocolloid xanthan gum. Finally, through the flow index (m), it is possible to observe an equal behaviour of doughs containing and lacking xanthan gum.

Table 3.6. *Cross-Williamson model parameters of control with and without xanthan gum used for determination and adjustment of estimated viscosity.* R^2 *is also shown.*

Samples	Cross-Williamson	η_0	*k*	*m*	R^2
Control	$y = \frac{6.14E+04}{(1+((1.22E+03\times\dot{\gamma})^{0.7}))}$	6.14E+04	1.22E+03	0.7	0.999
Control without xanthan gum	$y = \frac{3.02E+04}{(1+((2.24E+03\times\dot{\gamma})^{0.7}))}$	3.02E+04	2.24E+03	0.7	0.999

b) Impact of Chlorella vulgaris *on the dough rheology*

In terms of *Chlorella vulgaris* incorporation, values of both G' and G'' increased with the level of incorporation of microalgae in the dough (**Figure 3.12**). All the samples presented a similar pattern in terms of their mechanical spectra, with G' values being higher than the respective G'' in the whole range of frequency studied.

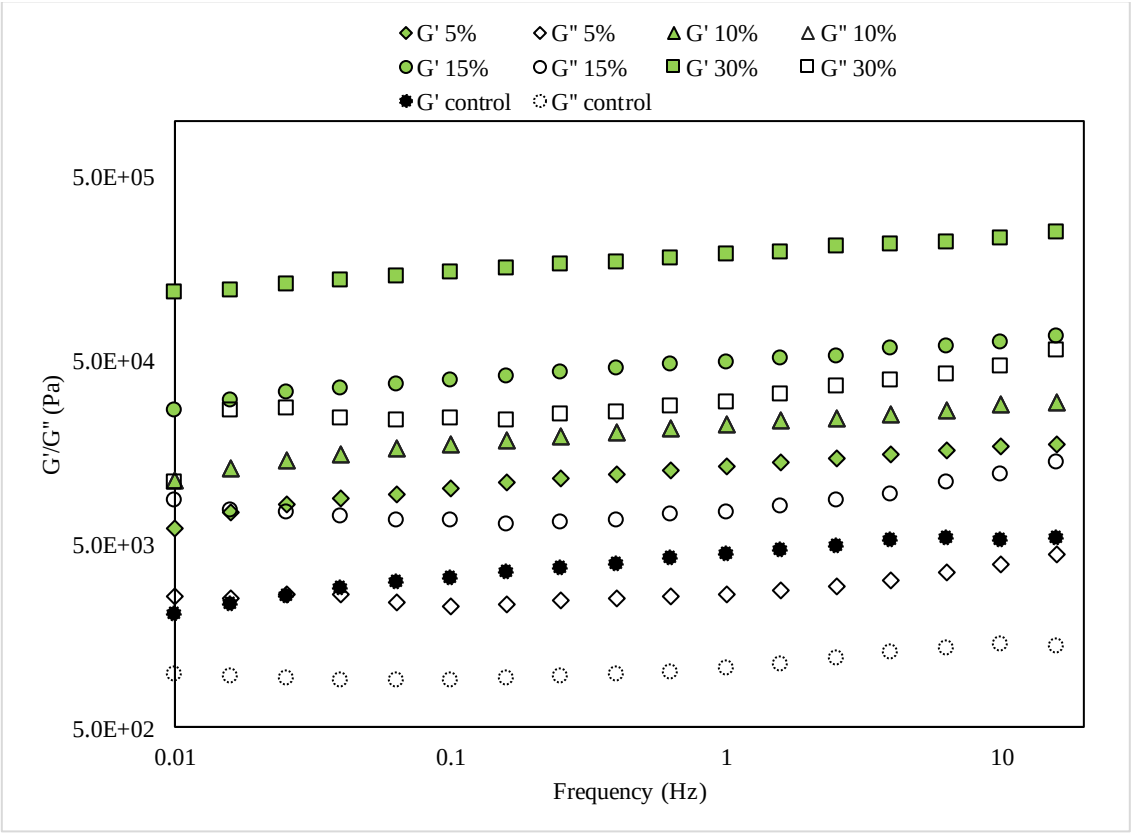

Figure 3.12*. Storage (G') and loss moduli (G'') (Pa) acquired through frequency sweep tests of different concentration (5%, 10%, 15% and 30%) of* Chlorella *doughs compared to control. Different symbols refer to different formulations, whereas filled and hollow symbols refer to G' and G'' of each formulation, respectively.*

It is also possible to see that doughs containing *Chlorella* (**Figure 3.12**) have higher values of G' compared to G'', which indicates that these doughs have a higher

mechanical strength and shape retention ability compared to control doughs, possibly due to their higher protein content (Graça *et al.*, 2018; Uribe-Wandurraga *et al.*, 2020a; Álvarez-Castillo *et al.*, 2021). This is explained by the increase in protein chain interactions, that restricts the dough's mobility, as well as the lack of plasticizing effect due to minor corn starch incorporation (Álvarez-Castillo *et al.*, 2021). On **Table 3.7** it is possible to compare the G' values obtained for each of the doughs, at a frequency of 1 Hz, in that a clear and significant ($p < 0.05$) increase in the elastic modulus is observed with increasing microalgal concentrations. These values suggest a weak gel-like rheology behaviour that is characteristic of cereal products, and significantly different ($p < 0.05$) than those found on control doughs, indicating that the microalga biomass addition contributed to their structuring (Mota *et al.*, 2020).

Table 3.7. *Impact of* Chlorella vulgaris *addition on the elastic modulus (G') values at 1 Hz for dough with different levels of incorporation (5%, 10%, 15% and 30%). Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups (p < 0.05).*

	G' (1 Hz) (Pa)
Control	4.29E+03 ± 3.60E+01^{d}
***Chlorella* 5%**	1.27E+04 ± 5.53E+02^{d}
***Chlorella* 10%**	2.63E+04 ± 3.96E+03^{c}
***Chlorella* 15%**	4.55E+04 ± 2.29E+03^{b}
***Chlorella* 30%**	1.87E+05 ± 5.45E+03^{a}

In terms of viscosity, *Chlorella* formulations revealed that this parameter increases with the addition of algal biomass, the 30% formulation showing the highest viscosity values (**Figure 3.13**).

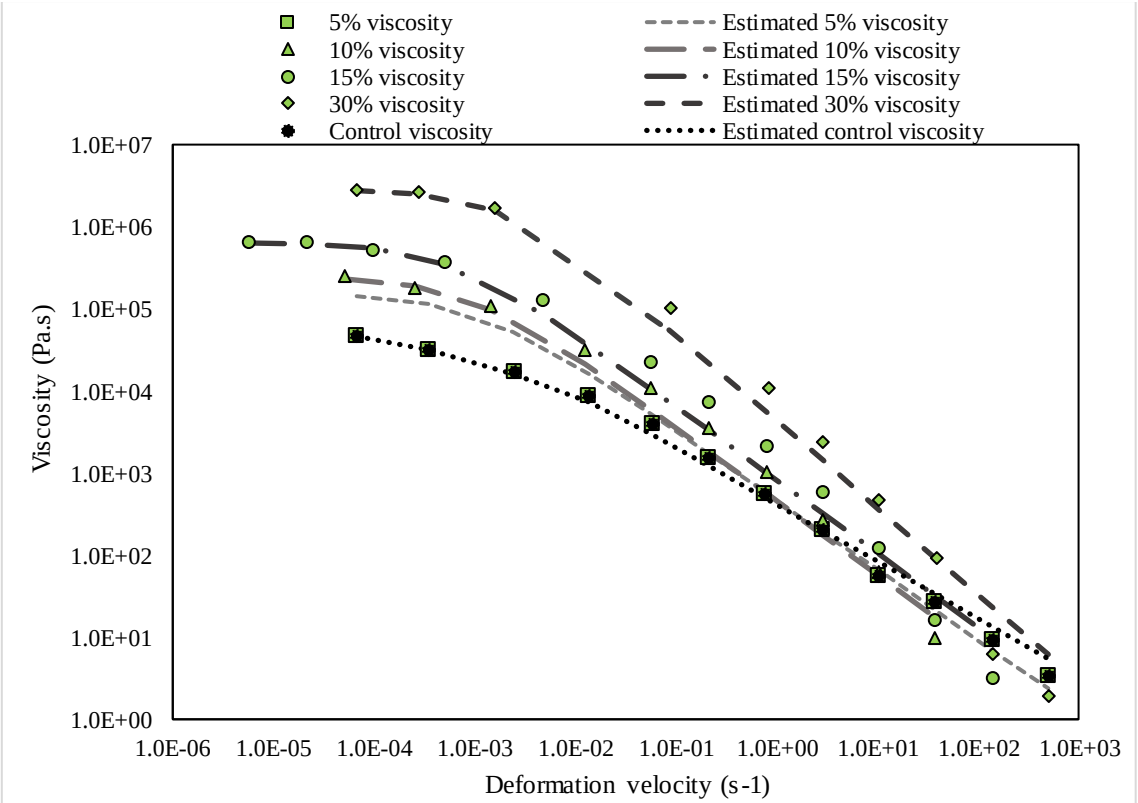

Figure 3.13. *Viscosity (Pa.s) acquired of different concentration* Chlorella *doughs (5%, 10%, 15% and 30%) compared to control. Different symbols refer to different formulations. Viscosity values were adjusted to a Cross-Williamson model to obtain the estimated viscosity.*

Table 3.8 reflects the viscosity behaviour of doughs containing *Chlorella* (**Figure 3.13**), where η_0 increases with the incorporation of *Chlorella* biomass comparatively to control doughs. This reflects the higher viscosity at rest and strength of the doughs containing algae biomass, that was equally observed in the graph (**Figure 3.13**). Finally, through *m*, it is possible to observe an increase in the values of this parameter with increasing microalgae incorporation, reflecting the difficulty associated with the handling of higher level of incorporation of microalgal biomass in doughs due to their high viscosity.

Table 3.8. *Control and* Chorella *(5%, 10%, 15% and 30%) Cross-Williamson model parameters used for determination and adjustment of estimated viscosity.* R^2 *is also shown.*

Samples	Cross-Williamson	η_0	*k*	*m*	R^2
Control	$y = \frac{6.14E+04}{(1+((1.22E+03\times\dot{\gamma})^{0.7}))}$	6.14E+04	1.22E+03	0.7	0.999
Chlorella 5%	$y = \frac{1.55E+05}{(1+((9.26E+02\times\dot{\gamma}))^{0.9})}$	1.56E+05	9.26E+02	0.85	0.998
Chlorella 10%	$y = \frac{2.48E+05}{(1+((1.09E+03\times\dot{\gamma})^{0.9}))}$	2.48E+05	1.10E+03	0.9	0.994
Chlorella 15%	$y = \frac{6.43E+05}{(1+((1.62E+03\times\dot{\gamma})^{0.9}))}$	6.43E+05	1.62E+03	0.9	0.995
Chlorella 30%	$y = \frac{2.83E+06}{(1+((4.92E+02\times\dot{\gamma})^{1.1}))}$	2.83E+06	4.92E+02	1.05	0.999

c) Impact of Spirulina on the dough rheology

Regarding doughs containing *Arthrospira platensis*, a similar behaviour was observed; the values of G' and G'' progressively increased with biomass incorporation (**Figure 3.14**). The pattern of the mechanical spectra is different from those obtained with *Chlorella*; there is a greater dependence of viscoelastic functions with frequency. This behaviour reflects a different type of structure, which may result from the presence of proteins with different structural characteristics.

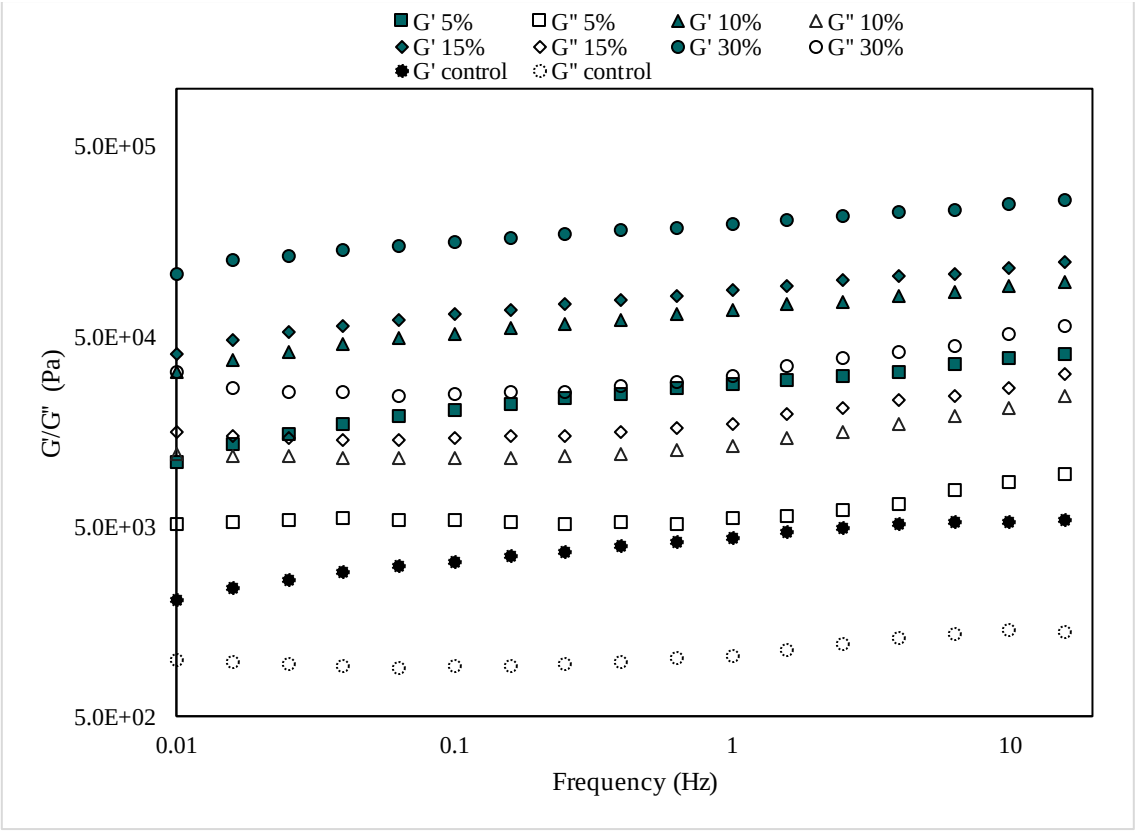

Figure 3.14. *Storage (G') and loss moduli (G'') (Pa) acquired through frequency sweep tests of different concentration (5%, 10%, 15% and 30%) of Spirulina doughs compared to control. Different symbols refer to different formulations, whereas filled and hollow symbols refer to G' and G'' of each formulation, respectively.*

In addition, from **Table 3.9**, it can be observed that the G' values (at 1 Hz) drastically increase with the increase in Spirulina content, which is reflected in the increase in the degree of structuring of the samples, due to the increase in microalgal protein in the system (Graça *et al.*, 2018). These values have orders of magnitude similar to those found for masses with *Chlorella*, although they were always higher for each of the levels of incorporation studied. This is also related with the higher content of protein of the Spirulina.

Table 3.9. *Impact of Spirulina addition on the elastic modulus (G') values at 1 Hz for dough with different levels of incorporation (5%, 10%, 15% and 30%). Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups ($p < 0.05$).*

	G' (1 Hz) (Pa)
Control	4.29E+03 ± 3.60E+01^{e}
Spirulina 5%	2.84E+04 ± 3.45E+03^{d}
Spirulina 10%	6.09E+04 ± 6.78E+03^{c}
Spirulina 15%	8.44E+04 ± 9.87E+03^{b}
Spirulina 30%	1.63E+05 ± 8.74E+03^{a}

Regarding Spirulina, dough viscosity presented a similar trend to that found on *Chlorella* doughs: the dough containing 30% displayed the highest viscosity, whereas the dough with only 5% microalgal biomass had the lowest viscosity (**Figure 3.15**).

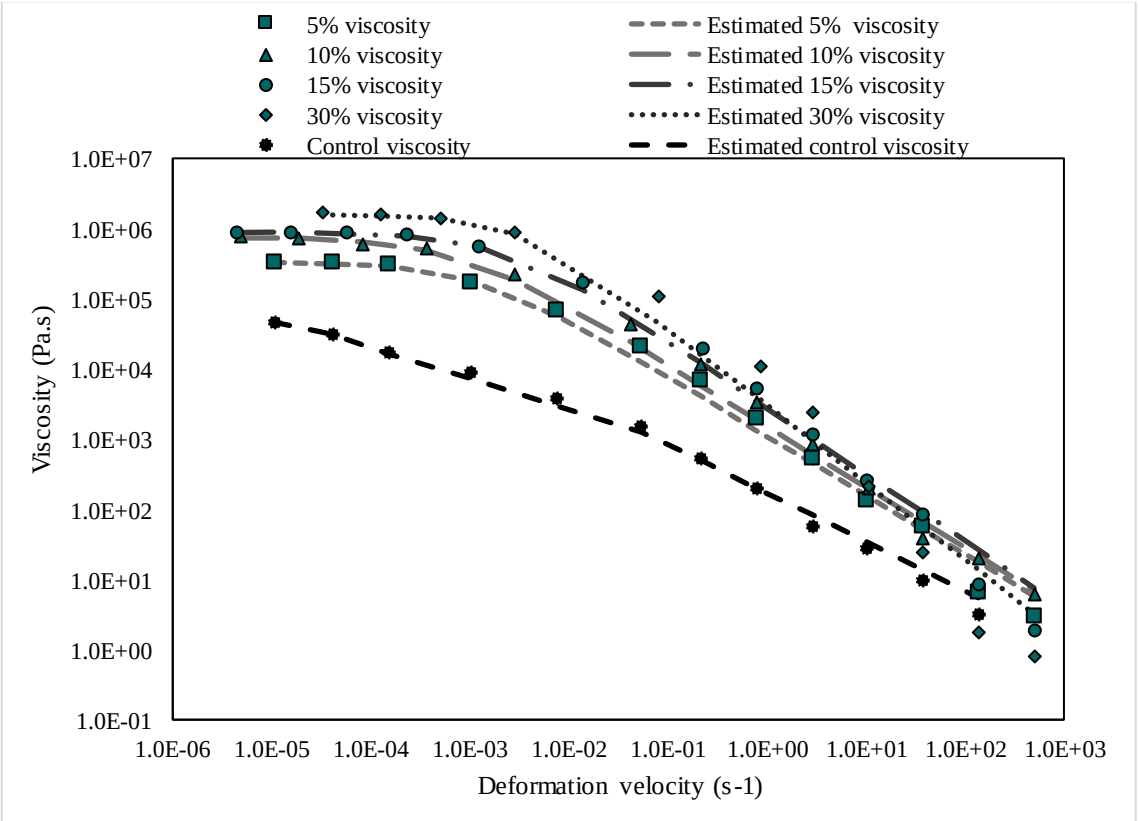

Figure 3.15. *Viscosity (Pa.s) acquired of different concentration Spirulina doughs (5%, 10%, 15% and 30%) compared to control. Different symbols refer to different formulations. Viscosity values were adjusted to a Cross-Williamson model to obtain the estimated viscosity.*

Table 3.10 reveals the estimated viscosity of doughs containing Spirulina (**Figure 3.15**), where it is clear that η_0 increases with incorporation of this microalga biomass comparatively to control doughs. This reflects the higher viscosity at rest and

strength of the doughs containing algae biomass, that was equally observed in the graph (**Figure 3.15**). Lastly, there is an increase of *m* values with increasing Spirulina incorporation, reflecting the same difficulty associated with the handling of higher percentage doughs as in the case of *Chlorella*, due to their high viscosity.

Table 3.10. *Control and Spirulina (5%, 10%, 15% and 30%) Cross-Williamson model parameters used for determination and adjustment of estimated viscosity.* R^2 *is also shown.*

Samples	Cross-Williamson	η_0	*k*	*m*	R^2
Control	$y = \frac{6.14E+04}{(1+((1.22E+03\times\dot{\gamma})^{0.7}))}$	6.14E+04	1.22E+03	0.7	0.999
Spirulina 5%	$y = \frac{3.35E+05}{(1+((8.45E+02\times\dot{\gamma}))^{0.8})}$	3.35E+05	8.45E+02	0.85	0.996
Spirulina 10%	$y = \frac{7.40E+05}{(1+((1.21E+03\times\dot{\gamma})^{0.9}))}$	7.41E+05	1.21E+03	0.87	0.994
Spirulina 15%	$y = \frac{8.79E+05}{(1+((4.30E+02\times\dot{\gamma})^{1.0}))}$	8.79E+05	4.31E+02	0.95	0.998
Spirulina 30%	$y = \frac{1.58E+06}{(1+((3.00E+02\times\dot{\gamma})^{1.1}))}$	1.58E+06	3.01E+02	1.10	0.998

As far as viscosity is measured on *Chlorella* doughs, we observed an increase in viscosity proportional to the incorporation of alga biomass (**Figure 3.13**), translating into doughs that were also progressively harder to extrude out of the cartridge nozzle and resulting in faultier printing outcomes for higher percentage doughs. This trend was equally identified on doughs containing Spirulina biomass (**Figure 3.15**), with doughs with higher incorporation percentages presenting higher viscosity values and, thus, leading to designs that were harder to print correctly. An increase in viscosity can be interpreted as a result of an increase in thickening agents such as hydrocolloids or ingredients with high protein and polysaccharide content, as microalgal biomass (Zhao *et al.*, 2019). Past studies revealed similar results where an increase in viscosity values was correlated with higher levels of microalgal biomass incorporation (Uribe-Wandurraga *et al.*, 2020a). Such result was attributed to the protein, polysaccharide, and fibre contents of the samples, since these molecules have a high-water retention capacity, consequently increasing viscosity (Uribe-Wandurraga *et al.*, 2020a).

3.8. Texture

3.8.1. Dough texture

The final product behaviour is very much dependent on several aspects that are related to the dough's characteristics, including its firmness, adhesiveness and cohesivity, all of which were evaluated through TPA tests (Vieira *et al.*, 2020). In relation to firmness, it was observed that significantly ($p < 0.05$) higher values were recorded on doughs with higher concentrations of microalgal biomass (5.103 $\pm$ 0.430 N, for *Chlorella* 30%) compared to the less concentrated doughs (0.235 $\pm$ 0.043 N, for *Chlorella* 5%) (**Figure 3.16 – A**). This increase was also seen in the case of *Chlorella* (0.389 $\pm$ 0.053 N and 0.708 $\pm$ 0.054 N, for *Chlorella* 10 and 15%, respectively) and Spirulina (0.996 $\pm$ 0.097 and 1.916 $\pm$ 0.128, for Spirulina 10 and 15%, respectively). Each of them had considerably higher firmness than the doughs containing a lower percentage (5%) of *Chlorella* and or no incorporation at all (control) (0.150 $\pm$ 0.016 N) (**Figure 3.16 – A**). These results are backed by former conclusions on this matter (Gouveia *et al.*, 2006; Gouveia *et al.*, 2007), which were attributed to the structuring effect that *Chlorella vulgaris* biomass had on doughs due to its elevated protein and carbohydrate content, causing a higher water absorption, structural reinforcement and, consequently, dough firmness (Gouveia *et al.*, 2007; Batista *et al.*, 2017; Raymundo *et al.*, 2018; Mota *et al.*, 2020; Khemiri *et al.*, 2020). Firmness increase can also be due to the interaction of the main macromolecules, including proteins as well as polysaccharides, such as starch, in the algal biomass, namely their biochemical propeties (Mota *et al.*, 2020). These macromolecular interactions occurring in the doughs can be correlated to the linear viscoelastic behaviour doughs presented in frequency sweeps (**Figure 3.9; 3.12 and 3.14**) and viscosity (**Figure 3.11**; 3.**13 and 3.15**) (Mota *et al.*, 2020). Dough's higher firmness in formulations containing 30% algal biomass relatively to the control and other percentage doughs, is in agreement with the inability of this dough to be printed. Moreover, these results are backed by Vieira *et al.* (2020), who mentioned that extreme high levels of firmness have led to inadequate products and doughs not suitable for 3D printing (**Figure 3.16 – A**).

In regard to adhesiveness, higher values are recorded with increasing incorporation of both *Chlorella vulgaris* and *Arthrospira platensis* (**Figure 3.16 – B**). With even the lowest incorporation of *Chlorella* (2.209 $\pm$ 0.190 N.s for *Chlorella* 5%) it is possible to observe (**Figure 3.16 – B**) a significant increase in adhesiveness of doughs

compared to that of the control (0.732 ± 0.203 N.s). With the exception of *Chlorella* 10% doughs, it is possible to see (**Figure 3.16 – B**) a sustained increase on the dough adhesiveness with increasing microalgae incorporation, since both 15% (2.286 ± 0.235 N.s) and 30% (14.111 ± 0.415 N.s) *Chlorella* doughs have significantly ($p < 0.05$) higher values in comparison to control and the remaining *Chlorella* doughs. In the case of doughs incorporating Spirulina biomass (**Figure 3.16 – B**), it is possible to conclude that the increase in adhesiveness is only significative when adding more than 5% biomass (2.109 ± 0.141 N.s for Spirulina 5%), since at this percentage, values were similar ($p > 0.05$) to those found on control doughs. Moreover, as in *Chlorella* doughs, it is visible (**Figure 3.16 – B**) that doughs incorporating very high levels of microalga biomass (30% Spirulina) are significantly ($p < 0.05$) more adhesive (13.419 ± 1.419 N.s, for 30% Spirulina) that doughs with lower percentages of incorporation such as 10% (4.426 ± 0.586 N.s, for 10% Spirulina). The increase in both firmness and adhesiveness of doughs incorporating 10% can be an important point to consider in future studies, since these are important parameters in gluten-free doughs, that normally lack attractive texture properties (Khemiri *et al.*, 2020).

Dough cohesiveness characterizes the extent to which the product recovers the deformation before it ruptures (Khemiri *et al.*, 2020). This parameter was not significantly altered ($p > 0.05$) by the addition of *Chlorella vulgaris* in comparison to control formulations (**Figure 3.16 – C**) (Khemiri *et al.*, 2020). Nevertheless, it is possible to identify a significant drop in values of this parameter in doughs incorporating 30% of microalga, which presented significantly ($p < 0.05$) lower cohesiveness (0.279 ± 0.020) than the remaining *Chlorella* doughs (0.720 ± 0.038, 0.688 ± 0.021 and 0.690 ± 0.048, for 5%, 10% and 15% *Chlorella* doughs, respectively) (**Figure 3.16 – C**). In an equal manner, cohesiveness of doughs incorporating Spirulina drastically decreased with high incorporation of biomass – 30% – having significantly lower ($p < 0.05$) cohesiveness (0.373 ± 0.040) comparatively to the control. This abrupt decrease in cohesiveness of 30% *Chlorella* and Spirulina doughs (**Figure 3.16 - C**) can be attributed the higher amount of protein of these doughs, compared to the remainder, leading sometimes to the collapse of cohesiveness of doughs as result of the interaction of the different molecules and not enough water quantity. These lower cohesiveness values were observed in doughs that were harder to handle (30%), confirming results obtained in past studies (Mota *et al.*, 2020).

There were also doughs which were not printable, namely 30% doughs, fact which was inherently related to texture parameters as adhesiveness and firmness (**Figure 3.16 –A-C**), which resulted in the unfeasibility to promote a good printing process. In these doughs we witnessed high values of firmness (**Figure 3.16 – A**) and adhesiveness (**Figure 3.16 – B**) comparatively to control and less percentage doughs, which contradicts Álvarez-Castillo *et al.* (2021) conclusions about low adhesiveness hindering correct printing. According to these authors, to achieve perfect printing conditions, values of adhesiveness should be fairly high (15 N) and firmness somewhat low (<10 N). However, in this study, the opposite is observed with doughs presenting lower adhesiveness (control), as these were the ones that were more easily printed, whereas the doughs presenting the highest firmness could not be printed.

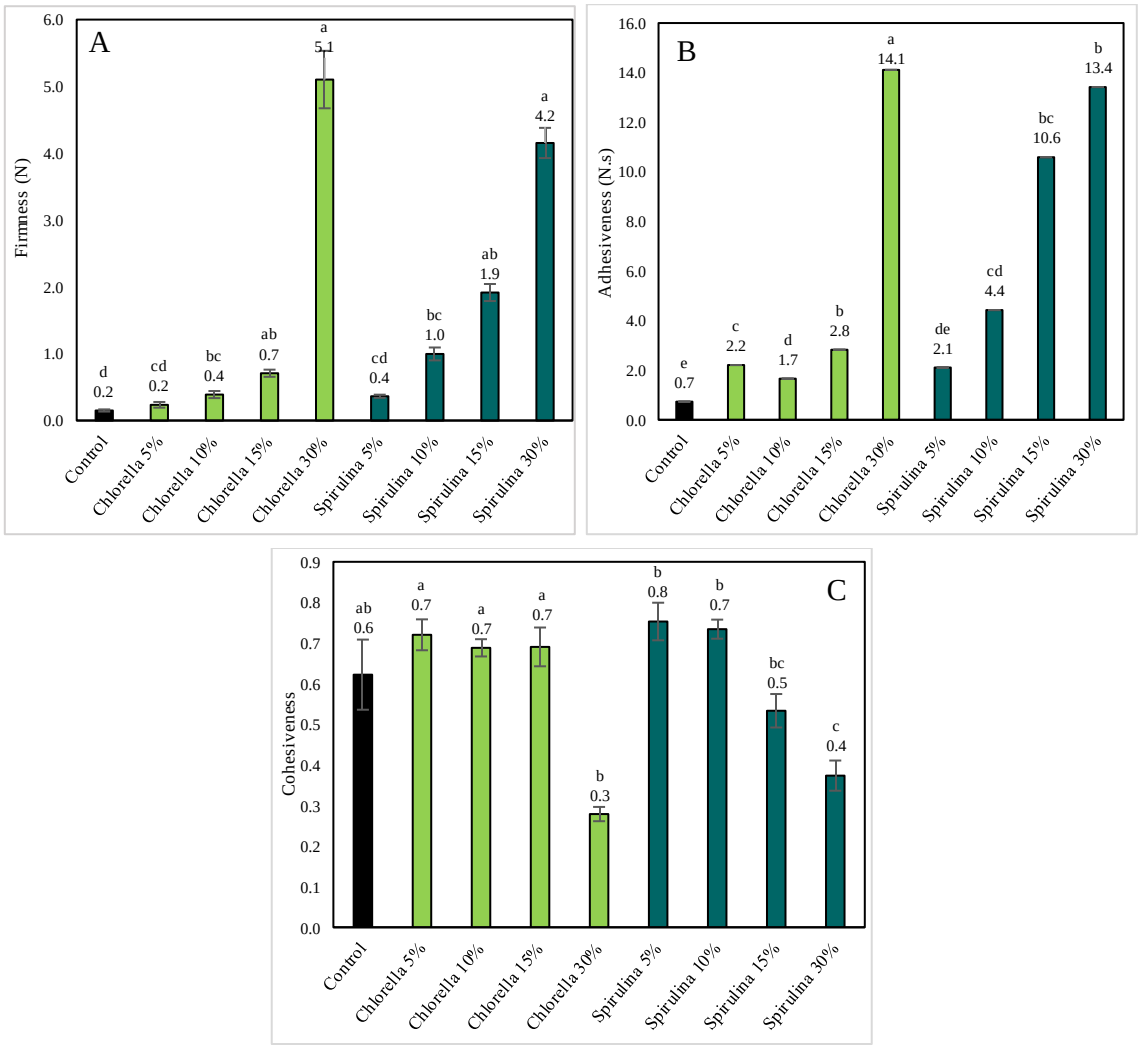

Figure 3.16 – A-C. *Firmness (N) (**A**), Adhesiveness (N.s) (**B**) and Cohesiveness (**C**) of doughs without (control) or with different concentrations of* Chlorella *and Spirulina (5%, 10%, 15% and 30%). Standard deviation is expressed as graphic error bars. Statistical tests were performed relatively to control but independently on Chlorella and Spirulina samples. Different letters represent statistically significant differences between groups ($p < 0.05$) and independently compare* Chlorella *and Spirulina with control doughs.*

3.8.2. Snack texture

In terms of snack texture, the hardness verified in control snacks (32.621 ± 3.442 N.s) is significantly higher ($p < 0.05$) than the hardness found for Spirulina 5% snacks (12.442 ± 4.019 N.s) (**Figure 3.17 – A**). *Chlorella* snacks containing only 5% of algal biomass (34.759 ± 2.078 N.s) presented much higher values in comparison to the latter but were not significantly different from control snacks ($p < 0.05$) (**Figure 3.17 – A**). These results might be supported by previous studies, which revealed that the addition of microalgae had not sufficiently promoted changes in the structure of the snacks enough to alter their resistance to probe penetration (Batista *et al.*, 2017). It is also possible to see a significant ($p < 0.05$) increase in hardness of 10% *Chlorella* (39.053 ± 2.623 N) comparatively to control snacks (**Figure 3.17 – A**). However, for higher levels of incorporation such as 15% *Chlorella* (23.943 ± 1.834 N), there is a significant ($p < 0.05$) drop in hardness of the snacks (**Figure 3.17 – A**). The decrease in *Chlorella* 15% hardness is supported by conclusions reported by Batista *et al.* (2017), which suggest that the increase in alga biomass and consequent flour reduction, might cause texture properties on higher percentage snacks to be similar to those found on snacks without algal biomass incorporation (**Figure 3.17 – A**). This sudden drop in hardness may also be attributed to a structural collapse as a result. All Spirulina snacks, on the other hand, displayed significantly ($p < 0.05$) lower hardness values when compared with the control. Among Spirulina snacks, however, it is visible an increase in hardness as more biomass is incorporated, with Spirulina 10% snacks (19.459 ± 3.438 N.s) presenting significantly higher ($p < 0.05$) hardness than 5% Spirulina snacks (**Figure 3.17 – A**). The higher hardness of snacks containing 10 to 15% Spirulina biomass relatively to those only containing 5%, may lie on the fact that the addition of more microalgal biomass causes the reinforcement of the dough structure, resulting in higher snack hardness, for this particular case (Batista *et al.*, 2017; Batista *et al.*, 2019).

Although several studies conducted in the past upon these algae biomass incorporation in cereal snacks, Batista *et al.* (2017, 2019) concluded that there is a sustained increase in hardness proportional to the biomass incorporation level, this was not always true in this case. Furthermore, it seems that this reinforcement is not relevant when comparing 10% and 15% Spirulina. In the case of Spirulina, lower hardness values compared to control, and *Chlorella* 5% and 10% snacks, may be attributed to the lower ability of these biomasses to provide a structural reinforcement compared to that

of *Chlorella* biomass. It is also important to consider the lack of structural basis provided by gluten (absent from our formulation), attributes the responsibility of structural strengthening much to the protein, polysaccharides, hydrocolloids and starch function (Batista *et al.*, 2019). Since all of these molecules can interact with each other and change their conformation during baking as result of high temperatures, it is possible that their final conformations do not favour Spirulina dough hardness (Mota *et al.*, 2020). Batista *et al.* (2019) refers to the fact that a weaker gluten network may lead to the collapse of small gas cells into larger cavities, affecting gas and water retention during baking. In our case, these conclusions might explain why the hardness of Spirulina snacks is lower compared to those of control and *Chlorella* snacks, since Spirulina biomass has been proved to impair starch gelatinization by augmenting gelatinization temperature (Batista *et al.*, 2019).

In relation to the brittleness of the snacks, it is possible to conclude (**Figure 3.17 – B**) that the incorporation of *Chlorella* did not significantly ($p > 0.05$) affect the brittleness of snacks for any of the incorporation levels (5%-15%). Moreover, brittleness of Spirulina snacks revealed not to be significantly ($p > 0.05$) altered, except in the case of Spirulina 15% snacks (1.412 $\pm$ 0.140 mm), which had significantly ($p < 0.05$) higher brittleness than control snacks (0.978 $\pm$ 0.174 mm) (**Figure 3.17 – B**). This can be explained by the fact that the incorporation of microalgae biomass was not significant enough to affect this parameter.

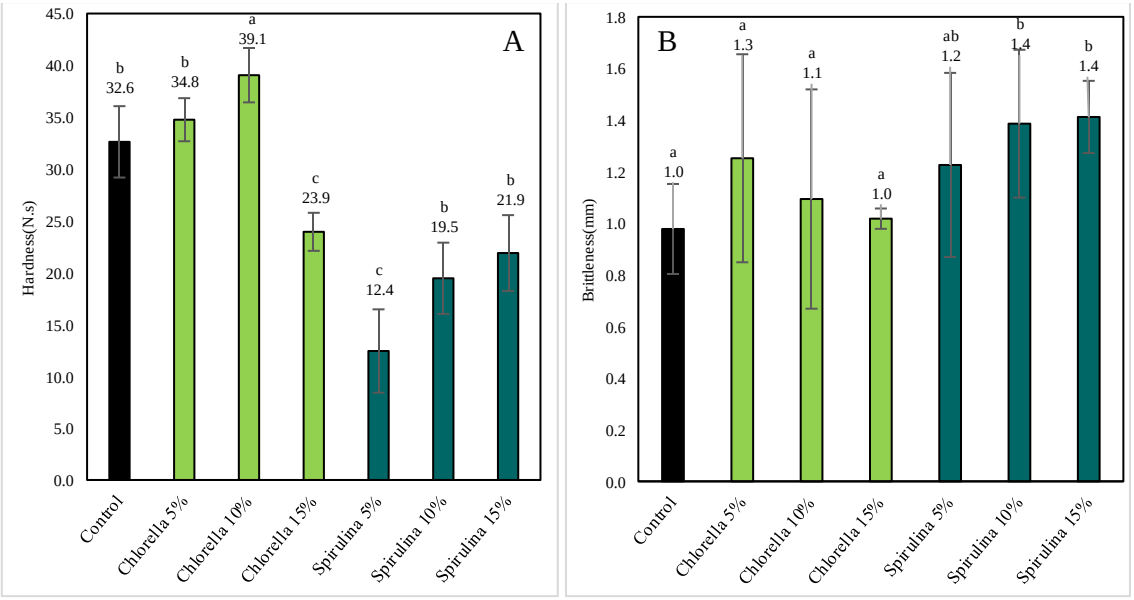

Figure 3.17 – A-B. *Hardness (N) (**A**) and brittleness (mm) (**B**) of control and different concentration of Chlorella and Spirulina (5%, 10% and 15%) snacks. Standard deviation is expressed as graphic error bars. Statistical tests were performed relatively to control but independently on* Chlorella *and Spirulina samples. Different letters represent statistically significant differences between groups ($p < 0.05$) and independently compare* Chlorella *and Spirulina with control doughs.*

3.9. Colour evaluation

3.9.1. Dough colour

The final visual traits of a product are crucial for its public acceptance and commercialization, being colour part of these traits (Uribe-Wandurraga *et al.*, 2020b). Some authors consider this to be the most important sensory trait for consumer's acceptance due to expectations created around the taste a food could have based on its colour (Uribe-Wandurraga *et al.*, 2020b). Results from this study (**Figure 3.1**; **Table 3.11; Table 3.12**) show that control doughs (81.933 ± 0.241) are the brightest of all, and there is a significant ($p < 0.05$) darkening effect as lightness (L*) values lower with increasing incorporation of either *Chlorella vulgaris* (37.223 ± 0.428, at 10% concentration) or *Arthrospira platensis* (19.997 ± 0.559, at 10% concentration) (**Table 3.11; Table 3.12**). The darkening of doughs due to increasing microalgae concentration can be attributed to the higher pigments content in microalgal biomass (Gouveia *et al.*, 2006; Gouveia *et al.*, 2007; Batista *et al.*, 2017; Batista *et al.*, 2019; Khemiri *et al.*, 2020). Another possible explanation for the darkening of snacks when alga biomass is added is the Maillard reaction, which consists in a series of complex cascade reactions involving proteins that cause a darkening effect during heating (Mota *et al.*, 2020). This darkening is seen as positive since the majority of gluten-free doughs generally lack attractive colouration (Khemiri *et al.*, 2020).

Other key colour parameters of a sample are a*, which ranges from green to red, and b*, that ranges from yellow to blue; both varying from -60 to 60 (Uribe-Wandurraga *et al.*, 2020b). It is possible to verify that 5% doughs of both *Chlorella* (-13.056 ± 0.052) and Spirulina (-6.64 ± 0.336) present significantly ($p < 0.05$) smaller (more negative) a* values than doughs not containing either biomass (-2.289 ± 0.146, for control doughs) (**Table 3.11; Table 3.12**). It is also possible to see progressively more positive b* values as microalgae incorporation increases in doughs, indicating a higher green to blue chromaticity than control doughs. This is associated with the pigment characterization of each dough, being that those containing algae biomass have higher content of pigments (**Figure** 3.**11**) that justify these colours. In terms of yellowness (b*), there is a clear progressive and significant ($p < 0.05$) decrease in values from the incorporation of more than 5% *Chlorella* biomass (17.865 ± 0.252 and 5.586 ± 0.378 for 10% and 30%, respectively) comparatively to control dough (19.724 ± 0.236) (**Table 3.11; Table 3.12**). In Spirulina doughs, b* values are significantly ($p <$

0.05) more negative (0.253 ± 0.047 and -0.563 ± 0.067 for 10% and 30%, respectively) comparatively to control. These results enhance the fact that Spirulina doughs have a much lower yellow chromaticity, as b* values are much closer to zero or negative in higher concentrated doughs, reflecting their darker blue colour. On both *Chlorella* and Spirulina formulations there is an evident decrease in green and yellow chromaticity as the level of biomass incorporation increases, with values reaching zero that are accompanied by much lower values of luminosity, indicating the darkening of doughs (Batista *et al.*, 2019). The higher yellow chromaticity (b* values) and lower greener chromaticity (a* values) of control dough, confirms the absence of algae biomass and consequent pigments which are responsible for attributing a greener and darker colour to doughs. Furthermore, these higher values of yellowness reflect the typical yellow colour of gluten-free doughs incorporating corn flour (Preichardt *et al.*, 2011).

As it is expected, in terms of colour parameter values (**Tables 3.11; Table 3.12**), it is possible to observe significant ($p < 0.05$) and visually perceptible increases ($\Delta E^* > 5$) in colour differences (ΔE^*) as a result of microalgae incorporation (Khemiri *et al.*, 2020). In comparison to control dough, the highest differences are found on 30% doughs (61.224 and 69.522 for *Chlorella* and Spirulina, respectively) and the lowest on 5% incorporation (36.061 and 59.337 for *Chlorella* and Spirulina, respectively). These higher differences between control and higher concentrated doughs are related to the concentration and saturation of pigments present on each of the algae biomass. This results in colour improvement of the doughs, which are inevitably related with the presence and type of pigments in the microalgae biomass used (Khemiri *et al.*, 2020). This is particularly evident in terms of chlorophyll, as this is the predominant pigment found in green microalgae such as *Chlorella vulgaris* (Khemiri *et al.*, 2020).

Table 3.11. *Colour parameters (L*, a*, b*) and colour differences (ΔE*) doughs containing* Chlorella *biomass incorporated at different concentrations (5%, 10%, 15% and 30%) compared to the control where algal biomass was omitted. Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups (p < 0.05).*

	Dough			
	L*	**a***	**b***	**ΔE***
Control	81.933 ± 0.241^{a}	-2.289 ± 0.146^{d}	19.724 ± 0.236^{ab}	-
***Chlorella* 5%**	47.516 ± 0.183^{ab}	-13.056 ± 0.052^{a}	21.677 ± 0.063^{a}	36.115
***Chlorella* 10%**	37.223 ± 0.428^{bc}	-11.074 ± 0.186^{ab}	17.865 ± 0.252^{bc}	45.603
***Chlorella* 15%**	32.042 ± 0.594^{cd}	-9.589 ± 0.182^{bc}	14.887 ± 0.321^{cd}	50.654
***Chlorella* 30%**	22.375 ± 1.056^{d}	-3.47 ± 0.323^{cd}	5.586 ± 0.378^{d}	61.224

Table 3.12. *Colour parameters (L*, a*, b*) and colour differences (ΔE*) doughs containing Spirulina biomass incorporated at different concentrations (5%, 10%, 15% and 30%) compared to the control where algal biomass was omitted. Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups (p < 0.05).*

	Dough			
	L*	**a***	**b***	**ΔE***
Control	81.933 ± 0.241^{a}	-2.289 ± 0.146^{bc}	19.724 ± 0.236^{a}	-
Spirulina 5%	25.656 ± 0.396^{ab}	-6.64 ± 0.336^{a}	1.427 ± 0.059^{ab}	59.336
Spirulina 10%	19.997 ± 0.559^{bc}	-3.197 ± 0.117^{ab}	0.253 ± 0.047^{bc}	64.931
Spirulina 15%	18.565 ± 1.208^{cd}	-1.091 ± 0.089^{cd}	-0.271 ± 0.051^{cd}	66.459
Spirulina 30%	15.513 ± 0.590^{d}	0.891 ± 0.118^{d}	-0.563 ± 0.067^{d}	69.522

3.9.2. Snacks colour

In snack colour measurements (**Table 3.13; Table 3.14**), L* values of control snacks (62.908 ± 2.437) were significantly lighter ($p < 0.05$) than those incorporating biomass from *Chlorella* (28.431 ± 1.483, 10% incorporation) and Spirulina (21.406 ± 1.274, 10% incorporation). Luminosity decrease is related with higher incorporations of alga biomass, suggesting that the increasing incorporation leads to pigment saturation of snacks, causing a darkening of colour (Batista *et al.*, 2019; Khemiri *et al.*, 2020).

In relation to a* (**Table 3.13; Table 3.14**), control snacks (-0.953 ± 1.036) displayed significantly higher values than those found on *Chlorella* (-2.203 ± 0.342, 5% *Chlorella*) and Spirulina snacks (-1.053 ± 0.079, 5% Spirulina). The higher greener tonality for *Chlorella* and Spirulina snacks may be related to the high chlorophyll

concentration of *Chlorella vulgaris* and *Arthrospira platensis* (Batista *et al.*, 2017). When it comes to yellow chromaticity, the incorporation of even the lowest percentage (5%), of either *Chlorella* (11.806 ± 0.789) or Spirulina (3.549 ± 0.378), resulted in snacks with significantly ($p < 0.05$) lower values comparatively to control snacks (21.333 ± 0.670), which were more yellow. The reduction in a* and b* values with the increase in algal biomass incorporation may be related with higher pigment degradation, pigment saturation effect or could even be attributed to the kinetics of pigment degradation (chlorophylls in particular) (Batista *et al.*, 2017; Batista *et al.*, 2019).

An increase in ΔE* is denoted as the incorporation of both *Chlorella* and Spirulina increases, with the latter presenting highest differences, compared to those found on *Chlorella* snacks incorporating the same amount of biomass. These differences, much like in the case of doughs, can be attributed to the pigment properties of microalgae incorporated in the snacks.

Table 3.13. *Colour parameters (L*, a*, b*) and colour differences (ΔE*) snacks containing* Chlorella *biomass incorporated at different concentrations (5%, 10% and 15%) compared to the control where algal biomass was omitted. Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups (p < 0.05).*

	Snacks			
	L*	**a***	**b***	**ΔE***
Control	62.908± 2.437^{a}	-0.953± 1.036^{a}	21.333 ± 0.670^{a}	-
***Chlorella* 5%**	32.677 ± 1.922^{ab}	-2.203± 0.342^{b}	11.806 ± 0.789^{b}	30.770
***Chlorella* 10%**	28.431 ± 1.483^{bc}	-2.285 ± 0.477^{b}	9.716± 1.061^{c}	35.535
***Chlorella* 15%**	27.077 ± 0.665^{c}	-2.370± 0.405^{b}	8.736± 0.554^{d}	37.141

Table 3.14. *Colour parameters (L*, a*, b*) and colour differences (ΔE*) snacks containing Spirulina biomass incorporated at different concentrations (5%, 10% and 15%) compared to the control where algal biomass was omitted. Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups (p < 0.05).*

	Snacks			
	L*	**a***	**b***	**ΔE***
Control	62.908± 2.437^{a}	-0.953± 1.036^{a}	21.333 ± 0.670^{a}	-
Spirulina 5%	23.526 ± 0.725^{ab}	-1.053 ± 0.079^{ab}	3.549 ± 0.378^{b}	42.374
Spirulina 10%	21.406 ± 1.274^{bc}	-0.550 ± 0.217^{b}	2.447 ± 0.452^{c}	44.763
Spirulina 15%	20.178 ± 0.902^{c}	-0.577 ± 0.217^{b}	2.084 ± 0.337^{c}	46.030

The differences found on snacks colour relatively to the doughs colouration are clear (**Table 3.15**), indicating a pigment degradation as a result of elevated temperatures during the baking process (Batista *et al.*, 2019; Vieira *et al.*, 2020). Possible factors

affecting the colour space of the snacks include changes in volume, moisture, and the formation of chlorophyll degradation by-products (pheophorbides and pyropheophorbides) (Batista *et al.*, 2019). Such differences can also be attributed to the oxidation of microalgal pigments incorporated in the doughs that took place during the baking process (Fradique *et al.*, 2010).

Table 3.15. *Colour difference (ΔE*) between control, Chlorella and Spirulina doughs and the correspondent snacks at different concentrations (5%, 10% and 15%). Standard deviation is displayed with each value. Different letters represent statistically significant differences between groups ($p < 0.05$).*

	Control	***Chlorella 5%***	***Chlorella 10%***	***Chlorella 15%***	**Spirulina 5%**	**Spirulina 10%**	**Spirulina 15%**
ΔE*	20.134	20.867	14.864	10.705	6.345	3.716	2.9

3.10. Sensory analysis

On a hedonic scale of 1 to 7, with 1 being the worst and 7 the best, sensory analysis indicated that the panellists did not like the texture of snacks. The control snack (PK8) was the most disliked (3.6), followed by the 5% *Chlorella* snack (7C7) (4.1), and the 5% Spirulina snack (9LL) (4.5) (**Figure 3.18**). In terms of taste, it is possible to observe that there was a similar sensory assessment for control snacks (5.1) and those containing Spirulina (5.1), being *Chlorella* the least appreciated (4.8). In terms of appearance, the control snack presented the most pleasant appearance with a score of 5.8, compared to *Chlorella* (5.6) and Spirulina (5.5). Regarding colour, snacks incorporating Spirulina did not impress the panel (5.1) neither did those containing *Chlorella* (5.4), with the control snacks being considered to be the most appealing (5.8). In terms of scent, Spirulina was the most liked snack (4.5), followed by *Chlorella* (4.1) and control (3.6). The global appreciation and buying intent did not reveal great potential for control snacks, as these would probably not be bought by most panellists (3.2). The same could be concluded for those containing 5% *Chlorella* (3.3), whereas 5% Spirulina snacks (4.0) would be occasionally bought. It can be said that the most liked snack was the one containing 5% Spirulina (5.1), followed by 5% *Chlorella* (4.9) and control snacks (4.8). Based on our sensory analysis results (**Figure 3.18**) it can be concluded that the majority of the panellists did not appreciate the texture characteristics of snacks, with Spirulina 5% snacks being the most accepted. This may be attributed to the baking time of the snacks, which was adjusted in an attempt to achieve a crunchy snack, since shorter baking times caused the snacks to acquire a soft texture, whereas

higher baking times resulted in burnt and even harder snacks. Regarding taste results, we observed that both Spirulina 5% and control snacks were the favourite ones, with *Chlorella* 5% being the least pleasant. It is clear that foods with green colour raise significantly more doubts in potential consumers than more neutrally coloured foods. The snacks which revealed the most potential for commercialization and overall appreciation were those incorporating 5% Spirulina.

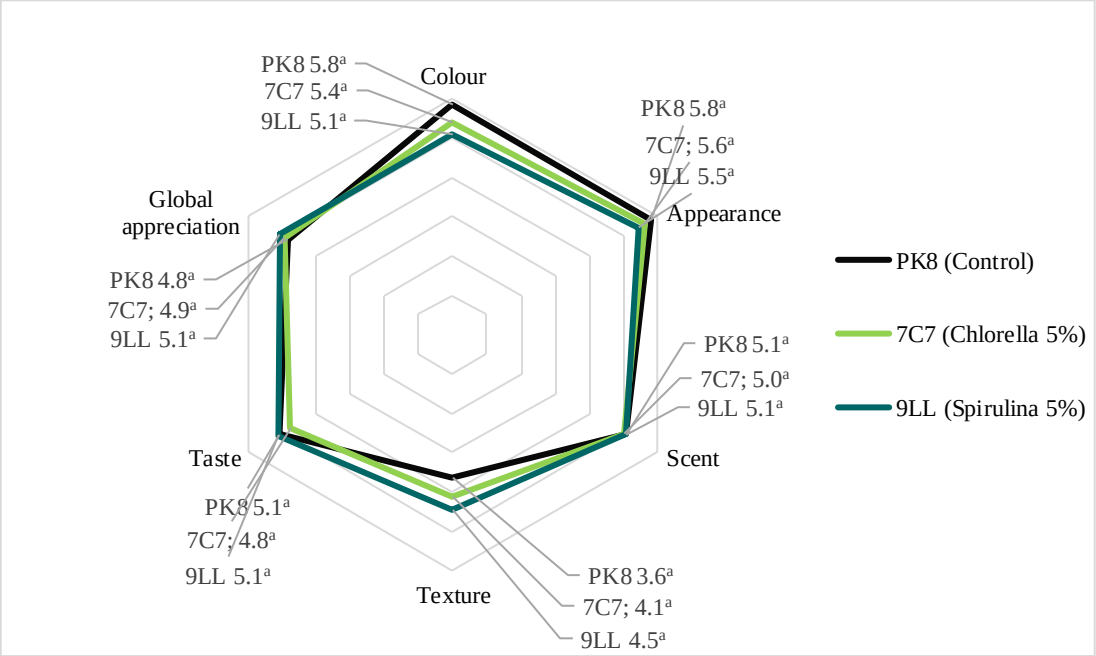

Figure 3.18. *Sensory analysis results (n = 30) of control (PK8),* Chlorella vulgaris *(7C7) and* Arthrospira platensis *(9LL) 5% concentration snacks. Different letters represent statistically significant differences between groups ($p < 0.05$).*

3.11. Conclusion

Overall, 5% Spirulina snacks presented the best nutritional profile with higher antioxidant activity, mineral and protein content, while improving consumer's perception on gluten-free products incorporating microalgae. However, 3D food printing is still somewhat limited to the built-in computer assisted design (CAD) software printers provide. In this sense, microalgae incorporation in gluten-free foods using 3D printing still requires further studying in order to allow food market commercialization to become a reality, while helping to create a more sustainable diet among consumers and to respond to the current resource scarcity.

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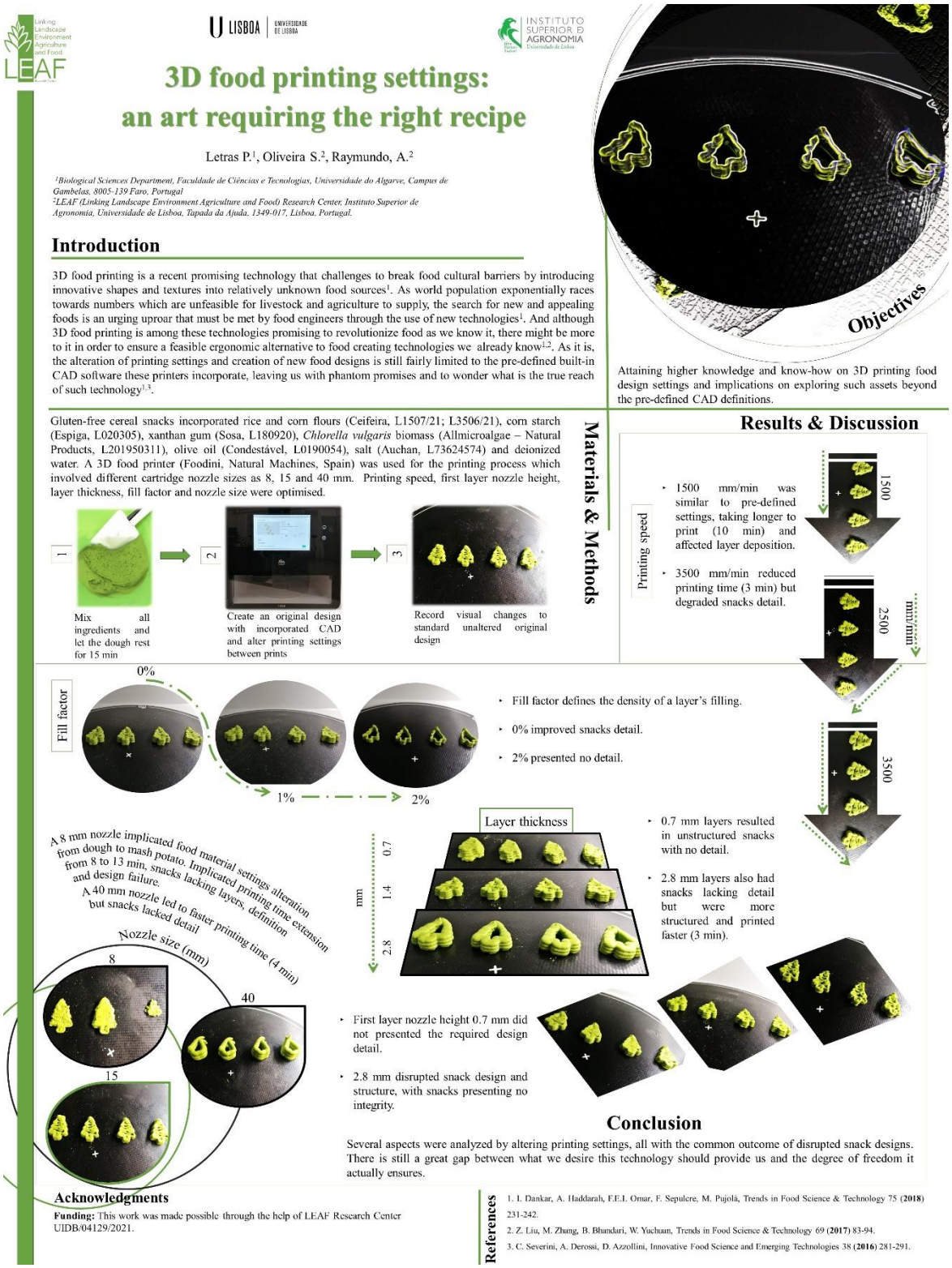
Annexes



Annex I. *3D food printing settings: an art requiring the right recipe Poster. Presented at XV - Encontro de Alimentos of the Portuguese Chemical Society (5-8 September) -* https://xveqa.events.chemistry.pt *in Madeira.*