



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 such 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 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* (“Spirulina”) biomass using 3D printing technology. Doughs without (control) or with microalgal biomass with different incorporation percentages (from 5 to 30 %) were tested and those showing the most adequate rheology (low viscoelastic moduli and apparent viscosity) and texture (low firmness, adhesiveness and high cohesiveness) properties for a correct printing process were selected and baked (control and 5 % microalgae incorporation). Nutritional characterization of the control and 5 % snacks was performed, in terms of total protein and lipid contents, ash, 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 (Control: $a^* -0.953$, $b^* 21.3$; *Chlorella* 5 %: $a^* -2.20$, $b^* 11.8$; *Spirulina* 5 %: $a^* -1.05$, $b^* 3.55$). 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 [1,2]. 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* (“Spirulina”) biomass, which can be incorporated into foods such as pasta [3,4], cheese [5], bread [6,7] and cookies [8], have been explored due to their exceptionally good nutritional characterization, including highly available bioactive molecules (e.g., pigments, polyunsaturated fatty acids, high protein levels and minerals) [8–10]. However, challenges have come to the fore since cultural background, sensorial perception and consumer routines play a major part in the food

industry, and overall food acceptance by customers [1,2]. 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 [1,11–14]. Though, incorporation of microalgae (*C. vulgaris* and *A. platensis*, particularly) in foods 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 [9,15]. 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 of food sensorial experiences by introducing innovative shapes and textures, 3D

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printing has recently been growing in popularity among several stakeholders of the food industry [1]. Besides introducing the possibility of creating complex and personalized designs without any expertise with different types of materials (liquids, powders, or cell cultures), it has the potential to establish new ground for non-traditional food sources as microalgal biomass and insects through the use of different printing techniques such as extrusion-based printing, selective sintering printing, binder jetting, and inkjet printing [1,11–13]. As food shortage is a growing issue due to the exponential global population growth, 3D food printing has the potential to benefit the environment using underexplored food sources as microalgae, with very low greenhouse gas emissions through appealing presentations that help to overcome cultural background barriers to food consumption, compared to other globally established protein sources like meat [1,2]. Additionally, we will delve deeper 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 [16]. Moreover, it is still common to find commercialized gluten-free snacks with poor nutritional value, due to their high sugar and lipid contents [16,17]. Although few studies have explored the incorporation of microalgae into snacks, their scrutiny as an ingredient in gluten-free snacks is still scarce.

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 (*C. vulgaris* and *A. 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, ash, 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; and 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 [58] 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* Smooth (Allmicroalgae – Natural Products, L201950311) (Protein: 26.3; Fat: 7.00; CH: 58.1; Ash: 4.00 g/100 g) and *Spirulina* (Allmicroalgae – Natural Products, PSS0720) (Protein: 64.0; Fat: 7.00; CH: 2.60 g/100 g) 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. The doughs were covered and stabilized for 15 min at room temperature (based on previously performed time sweep tests, in which most of the doughs required 900 s to structure). Afterwards, these doughs were printed in a built-in CAD duck foot shape design (thickness 0.522 ± 0.364 , width 1.935 ± 0.342 , length 2.215 ± 0.655 cm and weight of 0.895 ± 0.114 g, for control snacks), using a commercially available 3D food printer (Foodini, Natural Machines, Spain) with a 1.5

mm nozzle at 20 ± 1 °C, at 1.12 mL/min. This shape has already been used in previous works [18] 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 dough. In each batch, five and snacks were printed in a layer-by-layer deposition technique, forming snacks with four 1.4 mm thick layers (Supplementary material, Fig. A-D), totalizing a total time of six min. Snacks were then baked in a forced-air convection oven (Ariana, Italy), for 6 min and 30 s at 180 ± 5 °C. 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 °C. These were preserved at -25 ± 5 °C until biochemical analysis.

To assess the effect of altering pre-defined built-in designs settings of the 3D food printer, a standard 5 % *C. vulgaris* dough was prepared and printed with an originally designed 4 layered Christmas tree shaped snacks (Supplementary material, Fig. 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. Rheology and texture

Small amplitude oscillatory shear (SAOS) measurements, using a controlled stress rheometer Haake MARS III (Thermo Fisher Scientific, Waltham, MA, USA) were performed to evaluate the dough linear viscoelastic behaviour. Measurements were carried out under controlled temperature $20^\circ \pm 0.5$ °C (controlled by a UTC Peltier), using a 20 mm diameter serrated plate-plate sensor and gap was adjusted 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 in the rheometer device for 5 min (previously determined by time sweep tests at 1 Hz) before performing frequency sweep tests, increasing from 0.01 to 20 Hz, within the linear viscoelastic region. This region was previously determined, through stress sweep tests at 1 Hz, and a constant shear stress of 7 Pa was applied for all samples. All the measurements were performed at least in triplicate.

The time at which maximum torque is reached (s) was also assessed through time sweep tests, performed on doughs immediately after mixing, at 5 °C and 1 Hz, during 1 h, to obtain an equilibrium of the viscoelastic functions.

Steady-state flow measurements were also performed on each dough sample by using the same apparatus, using a logarithmic ramp of shear rates increasing in 10 min from 10^{-6} to 500 s^{-1} . Measurements were performed in triplicate for each formulation (after the 15 min doughs were allowed to rest). Furthermore, flow parameters were estimated through data adjustment of a Williamson-Cross model. The Williamson model (1) is a derivation of the Cross model, which can be used to describe both high and low shear rate regions of shear thinning fluids, considering that $\eta_\infty = 0$ [18]:

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

where η_0 , is the zero-shear viscosity, $\dot{\gamma}$ the shear rate ($d\gamma/dt$), k is the consistency coefficient (s) and m is the flow index (dimensionless) [19].

Texture profile analysis (TPA) tests were performed 9 times on each formulation dough, allowing the determination of texture parameters, 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 0.5 mm/s, 7 mm

penetration distance and 5 s between cycles, at room temperature (20 ± 1 °C) [20]. Snacks were also evaluated through penetration tests, that allowed determination of hardness (N), using the same apparatus to evaluate doughs, but using a 2 mm cylindrical stainless-steel probe, at 1 mm distance and 1 mm/s. Hardness was determined as the force peak (N) in the force *versus* time texturogram, being the required force to penetrate the snack [21].

2.3. Snacks dimensions

Snacks characteristics: 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.

2.4. Nutritional characterization

For biochemical composition determination, snacks were ground to powdered samples, and analysed in triplicate. From each ground formulation, triplicates of 2 g were weighed (Denver Instrument Company, TC-403) and placed in a stove (BINDER, ED56, Germany) at 105 ± 1 °C. Samples were weighed until there were no weight variations in order to determine moisture according to the standard method AACC 44-15.02 [22]. Total ash content was measured by incineration according to the standard method AACC 08-01 [22] using a muffle (SNOL, Lithuania) for 4 h at 550 ± 1 °C. Ash was 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 (Rotronic, Hygropalm, Switzerland), containing a sensor at controlled room temperature of 20 ± 1 °C, 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 \times 6.25 (conversion factor).

Total fat content of each formulation was determined by hydrolysis as described by Doan et al. [23]. Triplicates of 100 mg of each formulation were added to a mixture of methanol, chloroform, and hydrochloric acid was added in a ratio of 10:1:1.5, respectively. The mixture was extracted with *n*-hexane/chloroform (4:1 v/v), taken to a vortex for 2 min and centrifuged (HERMLE, Z383 K, Germany), 10 min at 20 °C 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 °C, 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 *C. vulgaris* and *A. 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. [24]. It was initiated by performing an acid digestion through the addition of 12 mL of hydrochloric acid and 4 mL of nitric acid (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.

2.5. Total phenolic compounds and antioxidant activity

The methods to evaluate total phenolic compounds and antioxidant activity (FRAP - ferric reducing ability of plasma and DPPH - 2,2-diphenyl-1-picryl-hydrazyl-hydrate) 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 μ m 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), obtaining stock solutions at a concentration of 20 mg/mL that were stored at 4 °C to be used in the determination of total phenolic content, antioxidant activity and pigment characterization.

2.5.1. Total phenolic compounds

Total phenolic content of samples was assessed through Mohankumar et al. [25] procedure, based on the extracted obtained as described in 2.5. 150 μ L of snack extract to which 150 μ L of a Folin-Ciocalteu solution (12 %) and 2.4 mL of distilled water were added and then mixed with 300 μ L of sodium carbonate solution (10 %) after 5 min. All tubes were incubated in a dark environment at room temperature for a 2 h 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 equivalents (mg GAE) per g of dry extract.

2.5.2. DPPH method

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 μ g/mL). Triplicates of each standard solution were prepared by adding 3.9 mL of DPPH (60 μ mol/L) to 0.1 mL of each dilution, incubating for 1 h in a dark environment. Upon incubation, methanol was used as blank on the spectrophotometer (Agilent Technologies, Cary 60 UV-Vis, USA), reading the absorbance of the calibration curve at 515 nm. A negative control was performed by replacing the extract with water [26,27].

Extracts analysis first involved the preparation of a DPPH solution by dissolving 4.8 mg of DPPH in 200 mL of methanol. Triplicates were made, each containing 3.9 mL of DPPH solution, 0.1 mL of each extract and 0.1 mL of distilled water [26,27]. These were incubated in a dark environment for 1 h and the absorbances were read again at 515 nm, using methanol as blank. 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 [26,27].

2.5.3. FRAP method

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), ferric chloride, 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. Several dilutions (0, 10, 25, 50 and 75 μ g/mL) of an ascorbic acid stock solution (1 mg/mL), using distilled water, were made to obtain a calibration curve; 90 μ L of each ascorbic acid dilution were pipetted, to which 270 μ L 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 °C during 30 min. Simultaneously, 90 μ L triplicates of each snack extract were prepared by adding 270 μ L of distilled water and 2.7 mL of FRAP solution, homogenized using a vortex and incubated in a water bath under the same conditions. Upon time completion, absorbances of these solutions were read at 595 nm with distilled water used as blank. 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 [28].

2.6. Pigments

Pigment characterization was performed by adding 3.8 mL of ethanol (96 %) to 200 μ L 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 Eqs. (2), (3) and (4) for control snack and snacks containing 5 % *C. vulgaris* biomass, whereas Eqs. (5) and (6) were used to better characterize snacks containing *A. platensis* [29,30].

$$\text{Chla } (\mu\text{g/mL}) = 13.36 \times A_{664} - 5.19 \times A_{648}, \quad (2)$$

$$\text{Chlb } (\mu\text{g/mL}) = 27.43 \times A_{648} - 8.12 \times A_{664}, \quad (3)$$

$$\text{Carotenoids } (\mu\text{g/mL}) = (1000 \times A_{470} - 2.13 \times \text{Chla} - 97.64 \times \text{Chlb})/209, \quad (4)$$

$$\text{Chla}_{\text{Art}} (\mu\text{g/mL}) = 12.6 \times A_{665.2}, \quad (5)$$

$$\text{Carotenoids}_{\text{Art}} (\mu\text{g/mL}) = (1000 \times A_{470} - 1.63 \times \text{Chla}_{\text{art}})/221. \quad (6)$$

2.7. Colour

Snack colour was measured using a Minolta CR-400 (Japan) colorimeter with standard illuminant D65 at a visual angle of 2°. Results were expressed according to CIE Lab system colour space defined by the International Commission of Illumination, 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. Nine 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:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (7)$$

The latter Eq. (7) was also used to determine differences between the colour of the dough and the baked snack.

2.8. 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 *C. vulgaris* and *A. 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" (1), except for buying intent, which was also assessed in 7-point hedonic scale but from "would certainly buy" (7) to "would never buy" (1).

2.9. Statistical analysis

Several assumption tests (Bartlett's test, Levene's tests, Kolmogorov-Smirnov 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. Doughs

3.1.1. 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 microalgal biomass [1,12]. 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 [10,11,31]. 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 [10,11,31].

A decrease in water content 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 (Unpublished results). In terms of the actual printing process and in concordance with the rheology results obtained, all doughs including control (without microalgal 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 (Supplementary material, Fig. A-D). An incorporation of 10 % microalgal biomass into doughs led to occasionally faulty printed snacks containing errors, including deficient shapes and misplacement of layers. Moreover, although 15 % algal doughs were printable, these presented a more viscous behaviour. As a result, the first snack was often printed with excess dough, which was originally destined to be left on the waste deposit. Even so, it was observed that control and 5 to 15 % microalgae-containing 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). The printability of the doughs containing higher levels of microalgal biomass incorporation was negatively affected, not only due to the increasing protein and polysaccharide content but also due to their consequently lower flour content [18,32]. A possible solution could be the addition of a plasticizer in order to decrease the viscosity of such doughs. Additionally, dough without xanthan gum was not printable due to its excessive elastic behaviour and consequently fell through the nozzle tip as it lacked structure (Supplementary material, Fig. H) [12,18].

Additionally, in order to assess how printing settings affected the final structure and visual appearance of the product, an original Christmas tree design was printed with different printing options. In fact, the slightest change in parameters such as nozzle height of the first layer, printing speed, layer thickness or even the nozzle size could greatly affect the final aspect of the food (Supplementary material, Fig. A-D). Indeed, smaller or higher nozzle sizes might not be adequate for the pre-defined food design settings, as these nozzles are usually

destined for other food materials (Supplementary material, Fig. A-B). In addition, printing speeds may be adjusted to obtain a better time efficiency in the food production; however, there are limits in terms of detail achievement at higher speeds. First layer nozzle height greatly affects the structure of the food design (Supplementary material, Fig. C). These results suggest that when working with gluten-free cereal doughs, enriched with microalgal biomass, changing these parameters can result in variations on the printing time, design accuracy, food structure, and quantity of dough used (Supplementary material, Fig. 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.

It is thus possible to state that this technology requires further development to become more user friendly as prior knowledge is needed in terms of design settings to print customizable designs with finer details. Other aspects that need to be considered include the ingredients used in the formulations and the post-processing procedures, since these affect the detail, structure and the final design quality of the food.

3.1.2. 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 Texture Profile Analysis (TPA) [10]. In relation to firmness, it was observed that significantly ($p < 0.05$) higher values were recorded on doughs with higher concentrations of microalgal biomass (5.10 ± 0.430 N, for *Chlorella* 30 %) compared to the less concentrated doughs (0.235 ± 0.0430 N, for *Chlorella* 5 %) (Fig. 1A). This increase was also seen in the case of *Chlorella* (0.389 ± 0.0530 N and 0.708 ± 0.0540 N, for *Chlorella* 10 % and 15 %, respectively) and *Spirulina* (0.996 ± 0.0970 and 1.92 ± 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 ± 0.0160 N). These results are backed by former conclusions on this matter, which were attributed to the structuring effect that *C. vulgaris* biomass had on doughs due to its elevated protein and carbohydrate content, causing a higher water absorption, structural reinforcement and, consequently, dough firmness [8,15,33–36]. Firmness increase can also be due to the interaction of the main macromolecules, including proteins as well as polysaccharides, such as starch, from flours and the algal biomass, namely their biochemical properties [36]. These macromolecular interactions occurring in the doughs can be correlated to the linear viscoelastic behaviour doughs presented in frequency sweeps (Figs. 2, 4 and 6) and flow curves (Figs. 3, 5 and 7) [36].

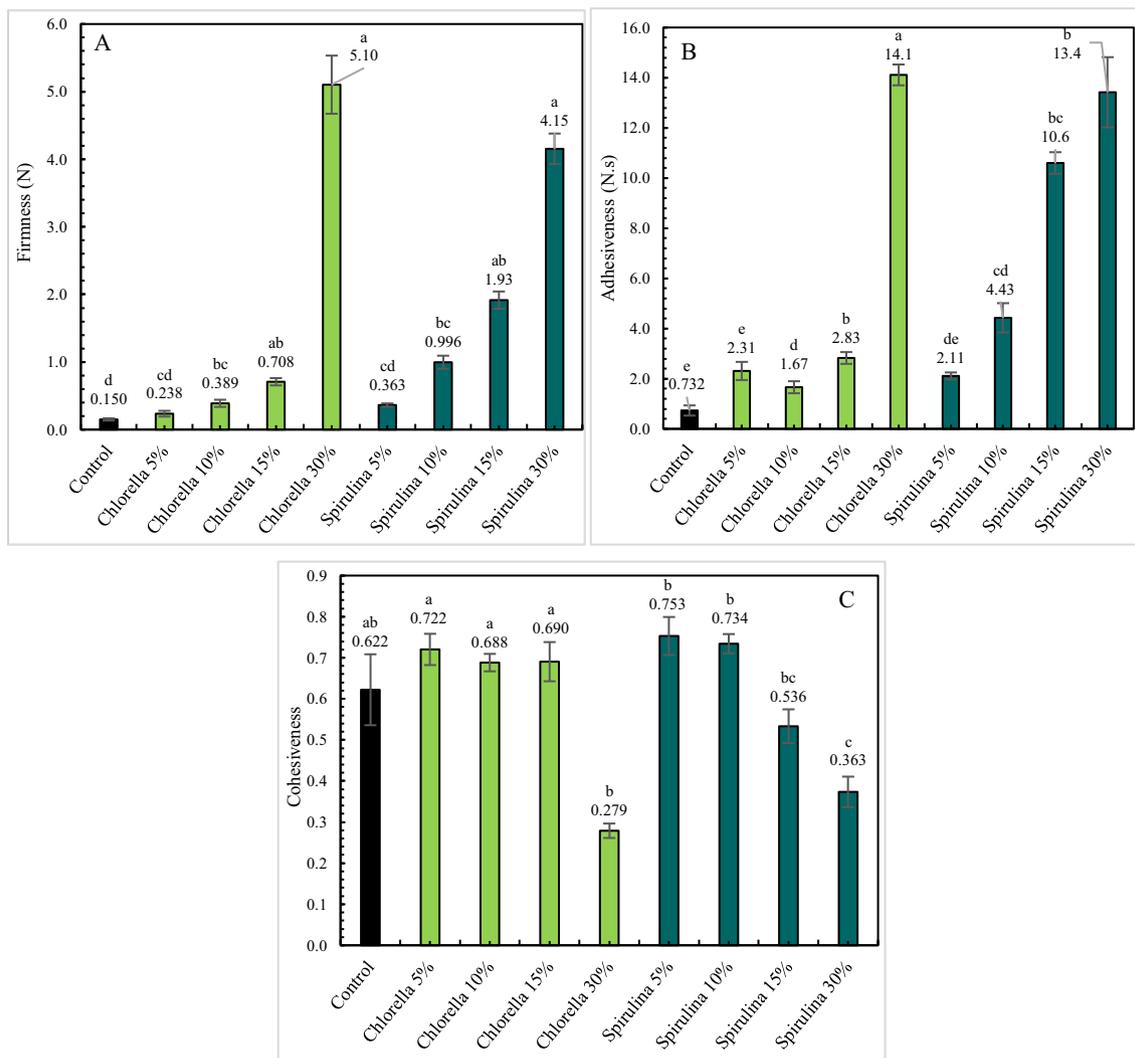


Fig. 1. A-C. Firmness (N) (A), Adhesiveness (N.s) (B) and Cohesiveness (C) of doughs without (control) or with different concentrations of *Chlorella* and *Spirulina* (5 %, 10 %, 15 % and 30 %). Standard deviation is expressed as graphic error bars. Statistical tests were performed relatively to control but independently on *Chlorella* and *Spirulina* samples. Different letters represent statistically significant differences between samples ($p < 0.05$) and independently compare *Chlorella* and *Spirulina* with control doughs.

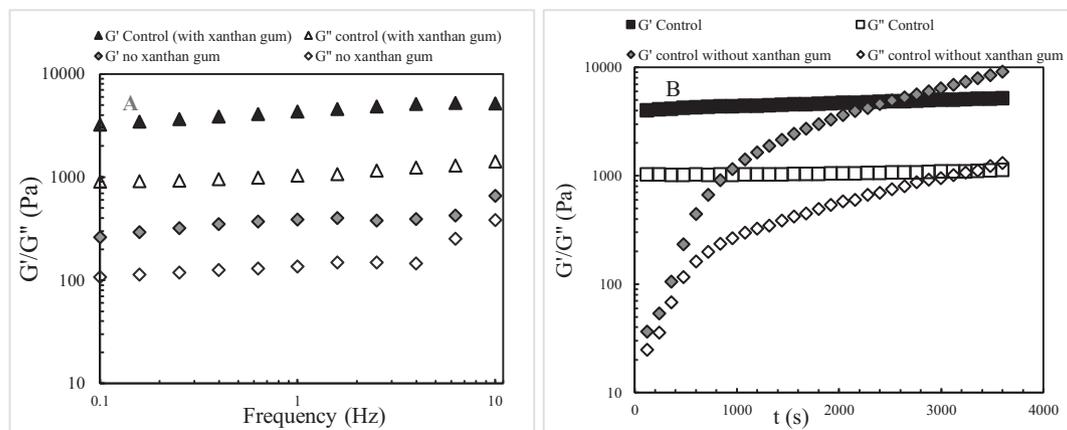


Fig. 2. (A) Storage (G') and loss moduli (G'') (Pa) acquired through frequency sweep tests of control and control without xanthan gum doughs. (B) Storage (G') and loss moduli (G'') (Pa) acquired through time sweep tests at 1 Hz of control and control without xanthan gum doughs. In both A and B, different symbols refer to different formulations, whereas filled and hollow symbols refer to G' and G'' of each formulation, respectively.

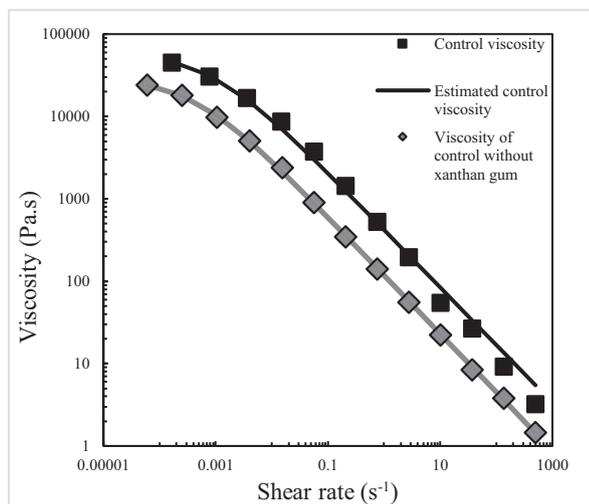


Fig. 3. Flow curves acquired of control and control without xanthan gum doughs. Different symbols refer to different formulations. Values were adjusted to a Cross-Williamson model to obtain the estimated viscosity.

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. [10], who mentioned that extreme high levels of firmness have led to inadequate products and doughs not suitable for 3D printing (Fig. 8).

Regarding adhesiveness, higher values are recorded with increasing incorporation of both *C. vulgaris* and *A. platensis* (Fig. 1B). With even the lowest incorporation of *Chlorella* (2.21 ± 0.190 N.s for *Chlorella* 5 %) it is possible to observe 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 a sustained increase on the dough adhesiveness with increasing microalgae incorporation, since both 15 % (2.29 ± 0.235 N.s) and 30 % (14.1 ± 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, it is possible to conclude that the increase in adhesiveness is only significant when adding >5 % biomass (2.11 ± 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 that doughs incorporating very high levels of microalgal biomass (30 % Spirulina) are significantly ($p <$

0.05) more adhesive (13.4 ± 1.42 N.s, for 30 % Spirulina) than doughs with lower percentages of incorporation such as 10 % (4.43 ± 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 [35].

Dough cohesiveness characterizes the extent to which the product recovers the deformation before it ruptures [35]. This parameter was not significantly altered ($p > 0.05$) by the addition of *C. vulgaris* in comparison to control formulations (Fig. 1C). 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 ± 0.0200) than the remaining *Chlorella* doughs (0.720 ± 0.0380 , 0.688 ± 0.0210 and 0.690 ± 0.0480 , for 5 %, 10 % and 15 % *Chlorella* doughs, respectively). 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 ± 0.0400) comparatively to the control. This abrupt decrease in cohesiveness of 30 % *Chlorella* and Spirulina doughs 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 [36].

There were also doughs which were not printable, namely 30 % doughs, fact which was inherently related to texture parameters as adhesiveness and firmness (Fig. 1). In these doughs we witnessed high values of firmness (Fig. 1A) and adhesiveness (Fig. 1B) comparatively to control and less percentage doughs, which contradicts Álvarez-Castillo et al. [18] 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.

3.1.3. 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 [32]. In this sense, G' (elastic modulus) and G'' (viscous modulus) [10,37] can be consider

important indicators of printability. Elastic modulus has its importance since it indicates mechanical strength and shape retention capability of foods, whereas viscous modulus affects extrusion of dough [32,37]. 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 [14,32,37]. Frequency sweep tests allowed obtaining relevant information (mechanical spectra) 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 linear viscoelastic region, frequency sweep assays were performed on the doughs.

3.1.3.1. Impact of xanthan gum on the dough rheology performance. Xanthan gum is a widely used hydrocolloid in the gluten-free food industry, functioning as a texture improver by increasing rheological properties, hydration, and retarding starch retrogradation [16]. The effect of the addition of hydrocolloid on the viscoelastic behaviour of the dough was investigated by comparing the control formulation (no addition of microalgae, 0.2 % xanthan gum) to a control formula not containing xanthan gum. From Fig. 2A, 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 partially 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 dough for snacks [18], in biscuits dough [36] and in bread dough [6,7,38]. 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 this hydrocolloid displayed excess elasticity, becoming, thus, unprintable.

The importance of xanthan gelling agent incorporation was confirmed through time sweep tests obtained immediately after mixing the doughs (Fig. 2B), where it is perceptible that control doughs (containing xanthan gum) developed structure over time, reaching a plateau after 3.36×10^3 s of both G' (5.10×10^3 Pa) and G'' (1.10×10^3 Pa). In control doughs lacking xanthan gum (7.92×10^3 and 1.11×10^3 Pa, respectively), there is a clear G' growth over time, never reaching a plateau state, a behaviour characteristic of an unstable dough. These results indicate that, in the absence of a gluten matrix, the solely structuring role performed by combining proteins and polysaccharides (mainly starch) present in corn and rice flours as well and on corn starch is insufficient to provide a stable structure to doughs; hence, the addition of hydrocolloids is crucial for this purpose.

The previous analysis is reinforced by the values of G' at 1 Hz for formulations with (4.29×10^3 Pa) and without xanthan gum (5.26×10^2 Pa) (Table 1). Therefore, the incorporation of this hydrocolloid contributed to a significant ($p < 0.05$) degree of structuring of the dough, thus enhancing its printing process and allowing the production of more stable doughs.

Gums as xanthan gum are known to increase dough viscosity but also to improve their sensory parameters, viscoelastic properties, and texture [16,39–41]. Results of other viscoelastic parameters are further reinforced by the results obtained from the flow curves (Fig. 3), where it is possible to perceive that control doughs containing xanthan gum had higher apparent viscosity values than formulations lacking such ingredient, for all the range of the selected shear-rates. The following table (Table 2) expresses the parameters of the Cross-Williamson model

Table 1
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 samples ($p < 0.05$).

	G' (1 Hz) Pa
Dough with xanthan gum (0.2 %)	$4.29 \times 10^3 \pm 3.60 \times 10^{01}$ a
Dough without xanthan gum	$5.26 \times 10^2 \pm 3.60 \times 10^{02}$ b

Table 2
Cross-Williamson model parameters of control with and without xanthan gum used for determination and adjustment of estimated viscosity. R^2 is also shown. For each parameter, different letters represent statistically significant differences between samples ($p < 0.05$).

	Samples	
	Control	Control without xanthan gum
η_0 (Pa·s)	6.14×10^{04} a	3.02×10^{04} b
K (s)	1.22×10^{03} b	2.43×10^{03} a
m	7.00×10^{-01} a	7.00×10^{-01} a
R^2	0.999	0.999

adjusted to the flow curves obtained (Fig. 3), where the zero-shear viscosity (η_0) increases ($p < 0.05$) 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 ($p < 0.05$), enhancing the higher viscosity of doughs containing the hydrocolloid xanthan.

3.1.3.2. Impact of *Chlorella vulgaris* on the dough rheology. In terms of *C. vulgaris* incorporation, values of both G' and G'' increased with the level of incorporation of microalgae in the dough (Fig. 4). All the samples presented a similar behaviour in terms of frequency dependency, with G' values being higher than G'' in the whole range of frequency studied. It is also possible to see that doughs containing *Chlorella* have higher viscoelastic moduli 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 [18,32,38]. This is explained by the increase in protein chain interactions, which restricts the dough's mobility, as well as the lack of plasticizing effect due to a minor incorporation of corn starch [18]. In Table 3 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 biomass concentrations. These values suggest a weak gel-like rheology behaviour that is

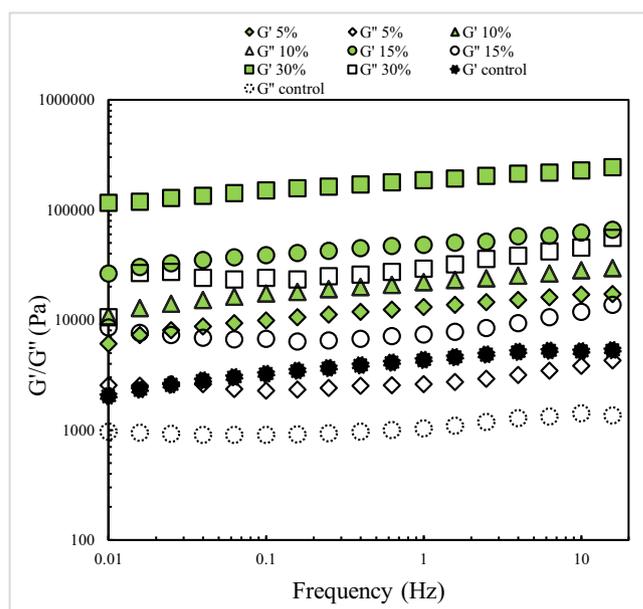


Fig. 4. Storage (G') and loss moduli (G'') (Pa) acquired through frequency sweep tests of different concentration (5 %, 10 %, 15 % and 30 %) of *Chlorella* doughs compared to control. Different symbols refer to different formulations, whereas filled and hollow symbols refer to G' and G'' of each formulation, respectively.

Table 3

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 samples ($p < 0.05$).

	G' (1 Hz) Pa
Control	$4.29 \times 10^3 \pm 3.60 \times 10^{01}$ d
<i>Chlorella</i> 5 %	$1.27 \times 10^4 \pm 5.53 \times 10^{02}$ d
<i>Chlorella</i> 10 %	$2.63 \times 10^4 \pm 3.96 \times 10^{03}$ c
<i>Chlorella</i> 15 %	$4.55 \times 10^4 \pm 2.29 \times 10^{03}$ b
<i>Chlorella</i> 30 %	$1.87 \times 10^5 \pm 5.45 \times 10^{03}$ a

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 [36].

In terms of flow curves, *Chlorella* formulations revealed that apparent viscosity and η_0 increases with the addition of algal biomass, the 30 % formulation showing the highest viscosity and η_0 values (Fig. 5). Such proves the higher strength at rest of doughs containing higher incorporation levels of this microalgal biomass.

Table 4 reflects the viscosity behaviour of doughs containing *Chlorella*, while expressing the Cross-Williamson model parameters. This confirms the higher η_0 and strength of the doughs containing higher concentration of algal biomass (Fig. 5).

3.1.3.3. Impact of *Spirulina* on the dough rheology. Regarding doughs containing *A. platensis*, a similar behaviour was observed; the values of G' and G'' progressively increased with biomass incorporation (Fig. 6). 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. In addition, from Table 5, it can be observed that the G' values (at 1 Hz) drastically increase with the increase in *Spirulina* biomass content, resulting in the increase of the dough structuring. These values have orders of magnitude similar to those found for doughs 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* (Protein: 64.0 g/100 g) comparatively to

Table 4

Control and *Chlorella* (5 %, 10 %, 15 % and 30 %) Cross-Williamson model parameters used for determination and adjustment of estimated viscosity. R^2 is also shown. For each parameter, different letters represent statistically significant differences between samples ($p < 0.05$), results are solely relative to *Chlorella* formulation comparisons.

Samples	η_0 (Pa·s)	K (s)	m	R^2
Control	6.14×10^{04} a	1.22×10^{03} b	7.00×10^{-01} a	0.999
<i>Chlorella</i> 5 %	1.56×10^{05} b	1.93×10^{03} a	9.00×10^{-01} a	0.998
<i>Chlorella</i> 10 %	2.48×10^{05} ab	1.10×10^{03} a	9.00×10^{-01} a	0.994
<i>Chlorella</i> 15 %	6.43×10^{05} ab	1.62×10^{03} a	9.00×10^{-01} a	0.995
<i>Chlorella</i> 30 %	2.83×10^{06} a	4.92×10^{02} a	11×10^{-01} a	0.999

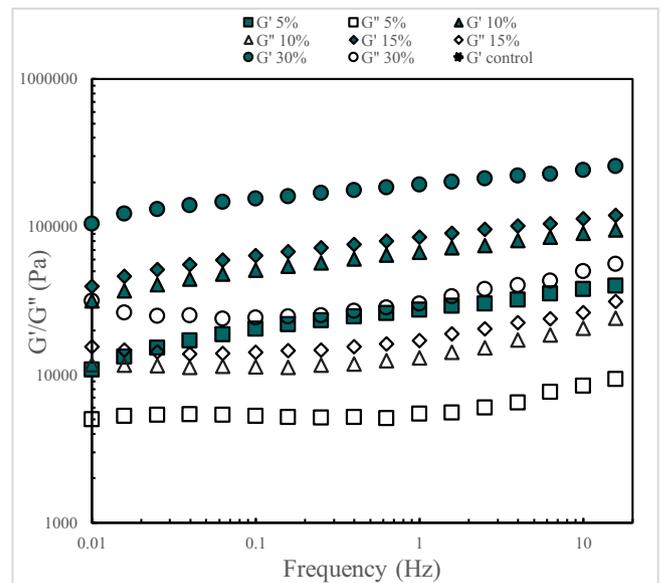


Fig. 6. Storage (G') and loss moduli (G'') (Pa) acquired through frequency sweep tests of different concentration (5 %, 10 %, 15 % and 30 %) of *Spirulina* doughs compared to control. Different symbols refer to different formulations, whereas filled and hollow symbols refer to G' and G'' of each formulation, respectively.

Table 5

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 samples ($p < 0.05$).

	G' (1 Hz) Pa
Control	$4.29 \times 10^3 \pm 3.60 \times 10^{01}$ e
<i>Spirulina</i> 5 %	$2.84 \times 10^4 \pm 3.45 \times 10^{03}$ d
<i>Spirulina</i> 10 %	$6.09 \times 10^4 \pm 6.78 \times 10^{03}$ c
<i>Spirulina</i> 15 %	$8.44 \times 10^4 \pm 9.78 \times 10^{03}$ b
<i>Spirulina</i> 30 %	$1.63 \times 10^5 \pm 8.74 \times 10^{03}$ a

Chlorella (Protein: 26.3 g/100 g).

Regarding *Spirulina*, dough flow curves presented a similar trend to that found on *Chlorella* doughs: the dough containing 30 % displayed the highest apparent viscosity values, whereas the dough with only 5 % microalgal biomass had the lowest viscosity (Fig. 7). Like in the case of *Chlorella*, there is a noticeable higher strength at rest of doughs containing higher levels of algal biomass incorporation.

Table 6 reveals the parameter values of the Cross-Williamson model adjusted to the viscosity of doughs containing *Spirulina* (Fig. 7), 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

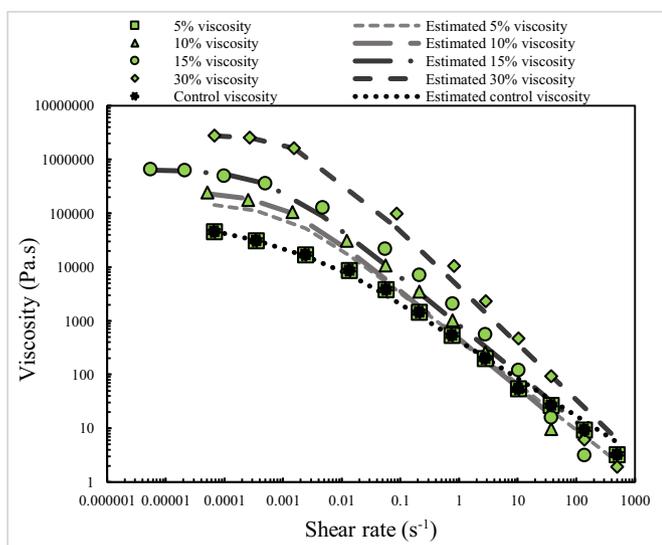


Fig. 5. Flow curves of different concentration *Chlorella* doughs (5 %, 10 %, 15 % and 30 %) compared to control. Different symbols refer to different formulations. Values were adjusted to a Cross-Williamson model to obtain the estimated viscosity.

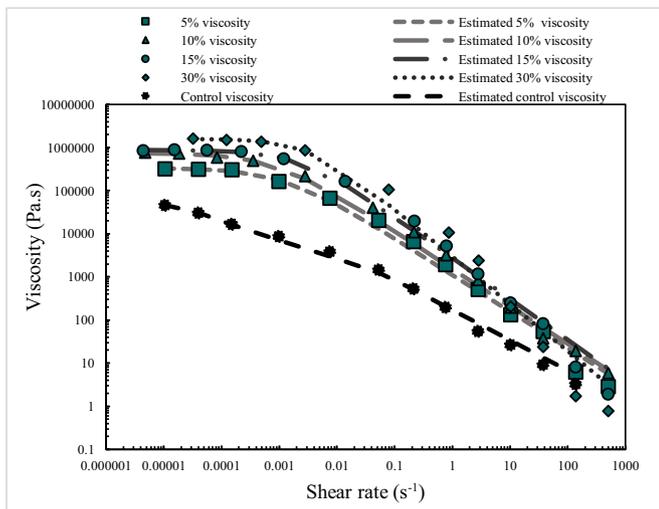


Fig. 7. Flow curves acquired of different concentration Spirulina doughs (5 %, 10 %, 15 % and 30 %) compared to control. Different symbols refer to different formulations. Values were adjusted to a Cross-Williamson model to obtain the estimated viscosity.

Table 6

Control and Spirulina (5 %, 10 %, 15 % and 30 %) Cross-Williamson model parameters used for determination and adjustment of estimated viscosity. R² is also shown. For each parameter, different letters represent statistically significant differences between samples (*p* < 0.05), results are solely relative to Spirulina formulation comparisons.

Samples	η_0 (Pa·s)	<i>k</i> (s)	<i>m</i>	R ²
Control	6.14×10^{04} a	1.22×10^{03} b	7.00×10^{-01} a	0.999
Spirulina 5 %	3.35×10^{05} b	8.45×10^{02} a	8.50×10^{-01} b	0.996
Spirulina 10 %	7.41×10^{05} b	1.21×10^{03} a	8.70×10^{-01} b	0.994
Spirulina 15 %	8.79×10^{05} ab	4.31×10^{03} ab	9.50×10^{-01} ab	0.998
Spirulina 30 %	1.58×10^{06} a	3.01×10^{03} a	11×10^{-01} a	0.998

and strength of the doughs containing algae biomass, that was equally observed from the flow curves. Lastly, there is an increase of *m* values with increasing Spirulina incorporation, reflecting the same difficulty associated with the handling of higher algae percentage doughs as in the case of *Chlorella*, due to their higher shear-thinning behaviour.

It was observed an increase in η_0 and *m* proportional to the incorporation of *Chlorella* and Spirulina algal biomass, translating into doughs that were also progressively harder to extrude out of the cartridge nozzle and faultier printing outcomes for higher percentage doughs. This can be due to higher protein and polysaccharide content from microalgal biomass [42]. Past studies revealed similar results where an increase in viscosity values was correlated with higher levels of microalgal biomass incorporation, which was also attributed to the protein, polysaccharide, and fiber contents of the samples, since these molecules have a high-water retention capacity, consequently increasing viscosity [32].

3.2. Snacks

3.2.1. Snack texture

In terms of snack texture, the hardness verified in control snacks (32.6 ± 3.44 N.s) is significantly higher (*p* < 0.05) than the hardness found for Spirulina 5 % snacks (12.4 ± 4.02 N.s) (Fig. 8). *Chlorella* snacks containing only 5 % of algal biomass (34.8 ± 2.08 N.s) presented much higher values in comparison to the latter but were not significantly different from control snacks (*p* < 0.05). 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

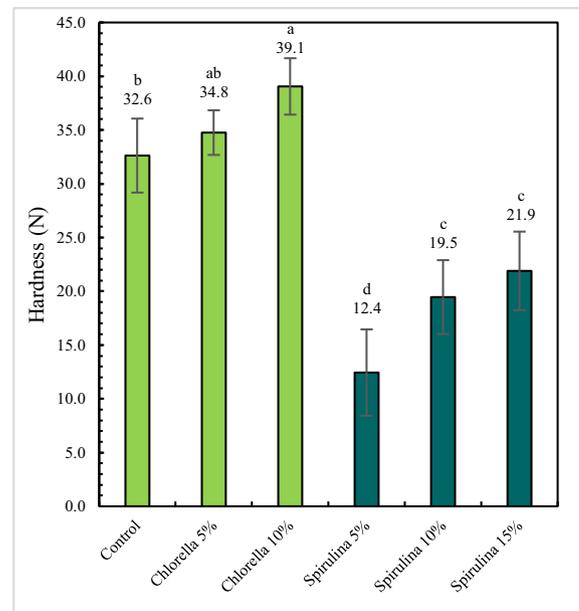


Fig. 8. Hardness (N) of control and different concentration of *Chlorella* and Spirulina (5 %, 10 % and 15 %) snacks. Standard deviation is expressed as graphic error bars. Statistical tests were performed relatively to control but independently on *Chlorella* and Spirulina samples. Different letters represent statistically significant differences between samples (*p* < 0.05) and independently compare *Chlorella* and Spirulina with control doughs.

snacks enough to alter their resistance to probe penetration [8]. It is also possible to see a significant (*p* < 0.05) increase in hardness of 10 % *Chlorella* (39.1 ± 2.62 N) comparatively to control snacks. 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 (19.5 ± 3.44 N.s) presenting significantly higher (*p* < 0.05) hardness than 5 % Spirulina snacks. 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 [8,21].

As several studies [8,21] concluded, there is a sustained increase in hardness proportional to the biomass incorporation level, similar to what is observed in this study (Fig. 8). However, 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 [21]. 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 [36]. Batista et al. [21] 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 [21].

3.2.2. Thickness of samples

Regarding the thickness 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 7). 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. In relation to width, length, and weight of the snacks, these were not significantly ($p > 0.05$) affected as result of any percentage of either *Chlorella* or *Spirulina* biomass incorporation (5 %–15 %) (results not shown).

3.2.3. Nutritional characterization

The baking process leads to changes in the chemical characteristics of the final product, in relation to the initial printed design [1,10]. 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, ash, protein, carbohydrates content (calculated by difference) and its energy value (kcal) (Table 8). 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 nutritional characterization.

Snacks containing microalgal biomass revealed an improved nutritional characterization when compared to the control formulations (Table 8). The incorporation of microalgae *C. vulgaris* and *A. platensis* resulted in snacks with significantly ($p < 0.05$) lower humidity values (11.2 and 10.5 %, for *Chlorella* and *Spirulina*, respectively) than when compared to control snacks (12.5 %). 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 [8,38,43]. This is supported by the significantly ($p < 0.05$) higher protein content in the 5 % *Spirulina* (9.87 %) snacks compared to both 5 % *Chlorella* (7.86 %) and control (5.66 %) snacks. 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 [8,21]. These results are in agreement with other baked-products studies incorporating these microalgae, which indicate a similarly consequent protein increase [8,32,38,44,45]. *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 [8,46,47]. 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.

In respect to ash and total fatty acids content, the incorporation of

Table 7

Control, *Chlorella* and *Spirulina* snacks height (cm). Standard deviation is displayed with each value. Different letters represent significantly different statistical samples ($p < 0.05$) between control and snacks containing different microalgal concentrations (0 %, 5 %, 10 % and 15 %).

		0 %	5 %	10 %	15 %
Height (cm)	Control	0.522 ± 0.364 ^{bc}	/	/	/
	<i>Chlorella</i>	/	0.502 ± 0.190 ^c	0.574 ± 0.165 ^{ab}	0.607 ± 0.122 ^a
	<i>Spirulina</i>	/	0.598 ± 0.103 ^{bc}	0.621 ± 0.210 ^{ab}	0.646 ± 0.306 ^a

Table 8

Nutritional characterization including humidity (%), ash (%), total fatty acids (%), protein (%), carbohydrates (%) and energy value (kcal/100 g) of control, *Chlorella*, and *Spirulina* snacks at 5 % concentration. Standard deviation is displayed with each value. Different letters represent statistically significant differences between samples ($p < 0.05$). *Calculated by difference.

Samples	Control	<i>Chlorella</i> 5 %	<i>Spirulina</i> 5 %
Humidity (%)	12.5 ± 0.503 ^a	11.2 ± 0.215 ^b	10.5 ± 0.0980 ^b
Ash (%)	1.75 ± 0.332 ^a	2.06 ± 0.349 ^a	2.15 ± 0.142 ^a
Total fatty acids (%)	9.15 ± 0.666 ^a	7.65 ± 0.492 ^a	8.23 ± 0.799 ^a
Protein (%)	5.66 ± 0.0505 ^c	7.86 ± 0.0341 ^b	9.87 ± 0.184 ^a
Carbohydrates (%)	70.9	71.2	69.2
Energy (kcal/100 g)	315	324	325

both microalgae did not significantly ($p > 0.05$) alter in the snacks tested (Table 8). These ash contents are in concordance with past studies upon using equal levels of biomass incorporation [8,21].

The amount of carbohydrates (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* (69.2 %) compared to 5 % *Chlorella* (71.2 %) or even control snacks (70.9 %) (Table 8). This similar and high percentage of carbohydrates in snacks either 5 % *Spirulina*, 5 % *Chlorella* or control, could be attributed to the high carbohydrate content of corn flour and corn starch [47]. Finally, energy values (kcal) were found to be higher in snacks containing 5 % *Spirulina* (325 kcal) than those containing 5 % *Chlorella* (324 kcal) and control snacks (315 kcal). 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, physical-chemical stability and their sensory perception [10,21]. 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 [10]. a_w of control snacks (0.682 ± 0.00500) had significantly higher values ($p < 0.05$) than those of *Chlorella* 5 % (0.613 ± 0.00300) and *Spirulina* 5 % (0.640 ± 0.0130). Since values under or equal to 0.800 and 0.600 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* [10,35]. As snacks containing 5 % *Chlorella* have significantly lower a_w than 5 % *Spirulina*, 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 [8,21]. Although these values do present improvements to control snacks, they are somewhat insufficient to ensure their crispiness, since they surpass the 0.500 threshold mentioned by several authors [21,36], 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 assays. 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 [21,35].

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 [48]. As can be seen in Table 9, an increase in important minerals that are involved in a balanced nutrition in snacks containing either *C. vulgaris* or *A. platensis*, compared to control snacks, was obtained. Specifically, there were significantly ($p < 0.05$) higher iron values in 5 % *Spirulina* snacks (0.439 mg/100 g) compared to those of the control (0.125 mg/100 g) and 5 % *Chlorella* (0.192 mg/100 g) snacks, which may be crucial for increasing physical performance in

Table 9

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

Sample	Control	<i>Chlorella</i> 5 %	<i>Spirulina</i> 5 %
Na	54.3 ± 1.55 ^a	55.3 ± 1.11 ^a	57.1 ± 1.44 ^a
K	4.90 ± 0.888 ^c	8.87 ± 0.108^b	12.1 ± 0.523^a
Ca	0.382 ± 0.0960 ^c	2.14 ± 0.0730^a	1.60 ± 0.0140^b
Mg	1.57 ± 0.0290 ^c	2.32 ± 0.0660 ^{bc}	4.03 ± 0.0900^a
P	6.21 ± 0.152 ^c	13.7 ± 0.114^a	12.5 ± 0.245^b
S	5.02 ± 0.181 ^c	7.21 ± 0.0680 ^b	9.83 ± 0.167 ^a
Fe	0.125 ± 0.0440 ^b	0.192 ± 0.0360 ^b	0.439 ± 0.0420^a
Cu	0.0390 ± 0.00100 ^{ab}	0.0410 ± 0.00300 ^a	0.0360 ± 0.00300 ^b
Zn	0.0970 ± 0.00300 ^b	0.180 ± 0.00200^a	0.0650 ± 0.00200 ^c
Mn	0.0260 ± 0.00100 ^b	0.0550 ± 0.00100 ^a	0.0400 ± 0.00100 ^{ab}
B	0.00500 ± 0.0 ^b	0.0100 ± 0.00100 ^a	0.00600 ± 0.00100 ^{ab}
Pb	0.00700 ± 0.00300 ^a	0.00500 ± 0.00200 ^a	0.00600 ± 0.00200 ^a
Cr	0.0134 ± 0.00300 ^a	0.0120 ± 0.00200 ^a	0.0170 ± 0.00600 ^a
Ni	0.00700 ± 0.00200 ^a	0.00700 ± 0.00200 ^a	0.00900 ± 0.00200 ^a
Cd	0 ± 0.0 ^a	0 ± 0.0 ^a	0.000 ± 0.00100 ^a

Bold highlights important minerals to human nutrition found in significant quantities.

consumers of all ages [35,49]. Potassium, being associated to intracellular fluid balance, carbohydrates metabolism, protein synthesis and nerve impulses, is an important mineral [50]. Snacks incorporating 5 % *Chlorella* (8.87 mg/100 g) and 5 % *Spirulina* (12.2 mg/100 g) presented significantly ($p < 0.05$) higher potassium values than those found in

control snacks (4.90 mg/100 g), with the latter clearly surpassing those of the remaining snacks. Both *Chlorella* and *Spirulina* (2.14 and 1.60 mg/100 g, respectively) presented significantly higher ($p < 0.05$) calcium content than the control snack (0.380 mg/100 g), which can be important for bone built in youngsters lacking access to other calcium-rich foods [35,51]. Another equally important mineral is zinc, as it participates in a series of metabolic processes, including synthesis of carbohydrates, lipids, and proteins [50]. In 5 % *Chlorella* snacks (0.180 mg/100 g), zinc was found in significantly ($p < 0.05$) higher quantities compared to those of the control snack (0.0970 mg/100 g). Since microalgae can accumulate contaminants in their cells, upon the assessment of their levels, both snacks were considered safe for consumption. For example, neither the levels of lead (0.00700, 0.00500, 0.00600 mg/100 g, for control, *Chlorella* 5 %, and *Spirulina* 5 %, respectively) nor of other possible contaminants such as cadmium (0.000 mg/100 g, for control, *Chlorella* 5 %, and *Spirulina* 5 % snacks) presented any risk to human health. In fact, these low values were within the recommended limits imposed by the European Commission Regulation [56,57]. 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 [6,7,35]. Finally, the mineral content improvement resulting 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 [35,52].

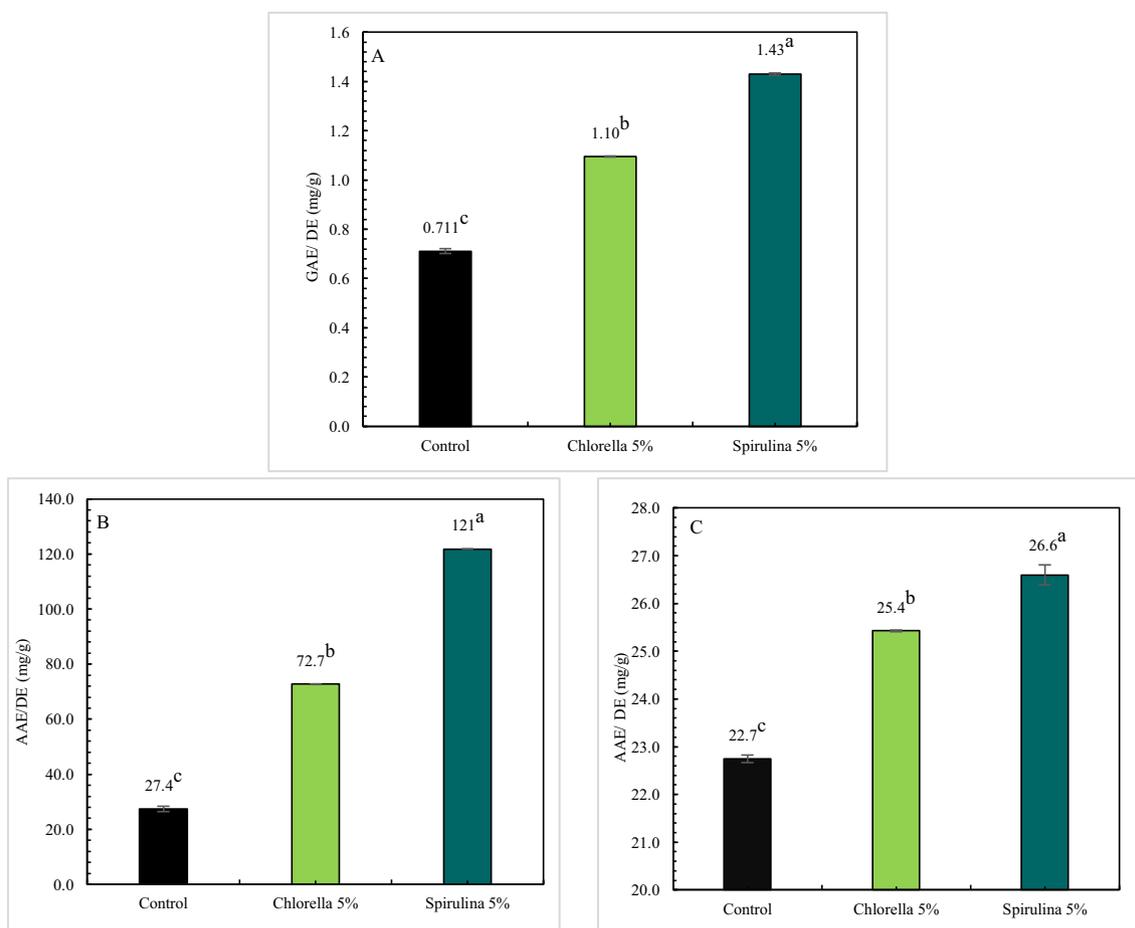


Fig. 9. (A) Total phenolic content (expressed as gallic acid equivalents (GAE) mg/g dry weight) of control, *Chlorella*, and *Spirulina* 5 % concentration snacks. (B) Antioxidant activity (expressed as ascorbic acid equivalents (AAE) mg/g dry weight) determined by FRAP of control, *Chlorella*, and *Spirulina* 5 % concentrations snacks. (C) Antioxidant activity (expressed as ascorbic acid equivalents (AAE) mg/g dry weight) determined by DPPH of control, *Chlorella*, and *Spirulina* 5 % concentrations snacks. Results are expressed as average ± standard deviation. Different letters represent statistically significant differences between samples ($p < 0.05$).

3.2.4. Total phenolic compounds and antioxidant capacity

Phenolic compounds as phenols, tannins, lignins and phenolic acids are microalgae secondary metabolites considered to be a very important class of natural antioxidants [8]. These were evaluated as a whole (total phenolics) in terms of total presence in the selected snacks. Total phenolic content results revealed that control snack had a lower total phenolic content (0.710 mg GAE/g DE) than both *Chlorella* 5 % (1.10 mg GAE/g DE) and *Spirulina* 5 % (1.43 mg GAE/g DE) (Fig. 9A). 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 [8,21,53]. *C. vulgaris*, however, presented lower values compared to those obtained in the latter studies, which may be attributed to the use of different strains of this species, since the one used in this study was grown heterotrophically. Furthermore, as the total phenolic content of *A. platensis* is higher than that of *Chlorella*, this difference could also explain these results, as shown by most previous reports [8,21,52,54]. In this sense, past studies [8,21] 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 (Fig. 9A). 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 [54]. 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*.

The antioxidant activity was assessed by two different methods including FRAP and DPPH assays. Past studies [8] revealed higher antioxidant activity of green microalgae such as *Chlorella vulgaris*, justified by their higher content on chlorophyll *a* and *b*, compared to those of other microalgae. In this study, FRAP assay indicated that control snack (27.4 mg AAE/g DE) presented a lower ($p < 0.05$) antioxidant activity compared to that of *Chlorella* 5 % (72.7 mg AAE/g DE), however, *Spirulina* (121 mg AAE/g DE) snacks showed a significantly (p

> 0.05) higher antioxidant activity than those of *Chlorella* or the control snack (Fig. 9B). 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 [21,44,46]. Finally, the lower levels of antioxidant activity observed in the case of *Chlorella* 5 % snacks can be related to the fact that the *Chlorella vulgaris* biomass incorporated was grown heterotrophically, possessing lower content of phytopigments and thus explaining the lower antioxidant activity found in these snacks.

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 5 % *Spirulina* (26.6 mg AAE/g DE) in comparison to 5 % *Chlorella* snacks (25.4 mg AAE/g DE). Conversely, control snacks displayed the lowest antioxidant activity (22.7 mg AAE/g DE) (Fig. 9C). 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 [21,52]. Ultimately, such elevated antioxidant activity could be attributed to the presence of chlorophyll *a*, and phycobiliproteins, namely phycocyanin [8,21,52,55].

3.2.5. Pigments

Pigment analysis revealed that control snacks contained far less ($p < 0.05$) chlorophyll *a* (0.0578 mg/g) and chlorophyll *b* (0.127 mg/g) compared to *Chlorella* 5 % snacks values (1.12 and 0.175 mg/g, for chlorophyll *a* and *b*, respectively) (Fig. 10). Snacks containing 5 % of *Spirulina* biomass presented significantly ($p < 0.05$) higher values of chlorophyll *a* and carotenoids (0.270 and 0.0692 mg/g, respectively) than the remainder, whereas chlorophyll *b* content was not accounted for in this snack as *Spirulina* biomass lacks this pigment [30]. Despite pigment composition in *Chlorella* 5 % snacks not being higher in chlorophyll *a* compared to *Spirulina* snacks, as it was expected since *C. vulgaris* is a green alga, it additionally presented high chlorophyll *b* levels. Higher carotenoid content was also detected on *Spirulina* snacks.

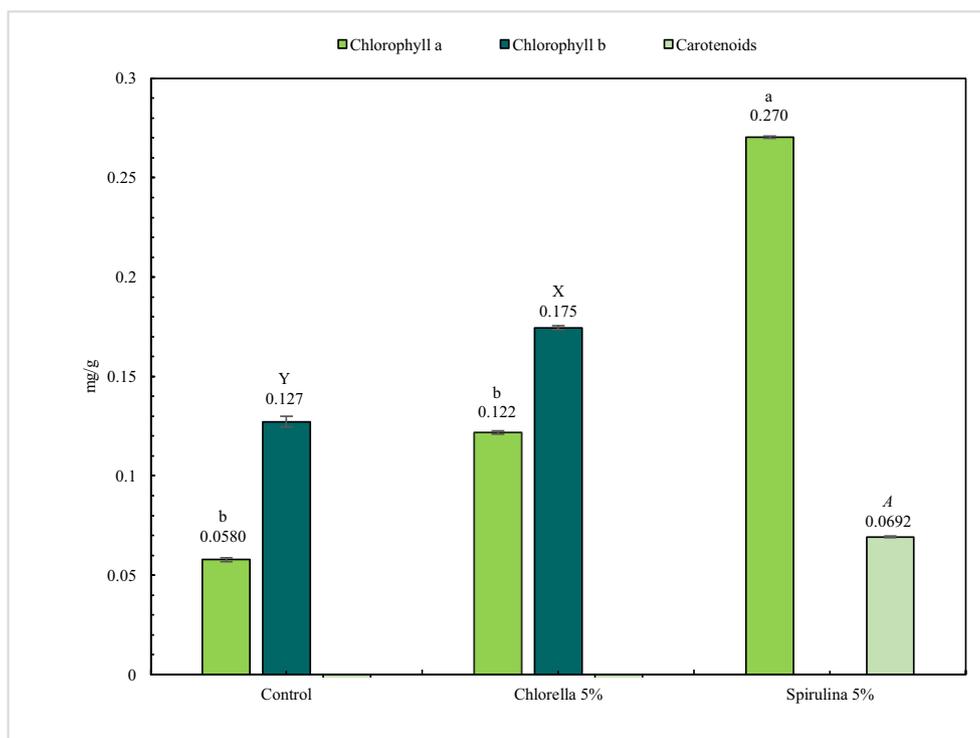


Fig. 10. Pigment characterization (mg/g dry weight) including chlorophyll *a*, *b* and carotenoid content of control, *Chlorella*, and *Spirulina* 5 % concentrations snacks. Results are expressed as average \pm standard deviation. Different letters represent statistically significant differences between samples ($p < 0.05$).

Although there are significant changes, this pigment characterization of both snacks containing either *C. vulgaris* or *A. 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 [8,50]. This *Chlorella* snacks pigment characterization is again due to the heterotrophic mode of growing this microalga, that presented inferior antioxidant activity compared to *Spirulina* snacks.

3.2.6. Colour evaluation

Arguably one of the most important sensory traits, the final visual traits, and most specifically, the colour of a product are crucial for its public acceptance and commercialization [14]. Contrary to the frequent neutral coloured gluten-free products, the darkening of doughs (Table 10) observed in this study is attributed to the pigments of the microalgal biomass incorporated or even the Maillard reaction, and is seen as an attractive and distinct feature [8,21,33–36].

Through snack colour measurements (Table 10), we can see significantly higher ($p < 0.05$) L^* values of control snacks (62.9 ± 2.44) compared to those containing biomass from *Chlorella* (28.4, 10 %) and *Spirulina* (21.4, 10 %). Such 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 [21,35]. In relation to a^* , control snacks (-0.953) displayed significantly higher values than those found on *Chlorella* (-2.20 , 5 %) and *Spirulina* snacks (-1.05 , 5 % *Spirulina*). This higher greener tonality of *Chlorella* and *Spirulina* snacks may be related to the high chlorophyll concentration and overall pigment characterization of *C. vulgaris* and *A. platensis* [8,35]. When it comes to yellow chromaticity (b^*), the incorporation of even the lowest percentage (5 %), of either *Chlorella* (11.8) or *Spirulina* (3.55), resulted in snacks with significantly ($p < 0.05$) lower values comparatively to control snacks (21.3), which were more yellow. The higher yellowness of control doughs reflects the typical yellow colour of gluten-free doughs incorporating corn flour [40]. 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) [8,21].

An increase in ΔE^* (Table 10) 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.

The differences found on snacks colour relatively to the doughs colouration are clear (Table 11), indicating a pigment degradation as a result of elevated temperatures during the baking process [10,21]. Possible factors affecting the colour space of the snacks include changes in volume, moisture, the formation of chlorophyll degradation by-products (pheophorbides and pyropheophorbides) or even due to oxidation of microalgal pigments [3,21].

Table 10

Colour parameters (L^* , a^* , b^*) and total colour differences (ΔE^* , in relation to control) snacks containing *Chlorella* and *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 samples ($p < 0.05$).

Snacks	Snacks			ΔE^*
	L^*	a^*	b^*	
Control	62.9 ± 2.44^a	-0.953 ± 1.04^a	21.3 ± 0.670^a	–
<i>Chlorella</i> 5 %	32.7 ± 1.92^{ab}	-2.20 ± 0.342^b	11.8 ± 0.789^b	30.8
<i>Chlorella</i> 10 %	28.4 ± 1.48^{bc}	-2.29 ± 0.477^b	9.72 ± 1.06^c	35.5
<i>Chlorella</i> 15 %	27.1 ± 0.665^c	-2.37 ± 0.405^b	8.74 ± 0.554^d	37.1
<i>Spirulina</i> 5 %	23.5 ± 0.725^{ab}	-1.05 ± 0.0790^{ab}	3.55 ± 0.378^b	42.4
<i>Spirulina</i> 10 %	21.4 ± 1.27^{bc}	-0.550 ± 0.217^b	2.45 ± 0.452^c	44.8
<i>Spirulina</i> 15 %	20.2 ± 0.902^c	-0.577 ± 0.217^b	2.08 ± 0.337^c	46.0

3.2.7. 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 5 % *Spirulina* snack (9LL) was the most liked (4.5), followed by the 5 % *Chlorella* snack (7C7) (4.1) and lastly the control snack (PK8) (3.6) (Fig. 11). 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 did not reveal great potential for control snacks, as these would most likely not be bought by most panellists (3.2). Although similar conclusions can be done for those containing 5 % *Chlorella* (3.3), 5 % *Spirulina* snacks (4.0) showed potential as these would occasionally be bought. Hence the most liked snack in terms of global appreciation was the one containing 5 % *Spirulina* (5.1), followed by 5 % *Chlorella* (4.9) and control snacks (4.8). Based on the sensory results it can be concluded that the majority of the panellists did not appreciate the texture characteristics of snacks, being related 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 the results on taste, we observed that both *Spirulina* 5 % and control snacks were the favourite ones. 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*.

4. Conclusion

Overall, 5 % *Spirulina* snacks presented the best nutritional profile with higher antioxidant activity, phenolic content, and an interesting pigment characterization. Furthermore, these snacks' mineral and protein content (considered as "a protein source"), not only has the potential to cause a positive impact on consumer nutrition but can also improve perception on gluten-free products incorporating microalgae. Despite 5 % percentage *Spirulina* snacks were manufactured, future studies could venture into exploring the creation of snacks containing higher concentration of algal biomass, since 10 % microalgal doughs in this study revealed good rheological properties, that transpired into a fairly smooth printing process. Nevertheless, 3D food printing is still somewhat limited to the built-in computer assisted design (CAD) software printers provide, as results obtained in this study showed consistent structural failures associated with alterations to the existent and original designs. In this sense, microalgal biomass 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.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2022.102863>.

CRedit authorship contribution statement

As the corresponding author, I declare that the present work has not been published previously (except in the form of an abstract, a published lecture or academic thesis), that is not under consideration for publication elsewhere. I also declare that this work is approved by all authors and explicitly by the responsible authorities where the work was carried out, and that, if accepted, will not be published elsewhere in the same form, in English or in any other language, including electronically

Table 11

Total 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 samples ($p < 0.05$).

	Control	<i>Chlorella</i> 5 %	<i>Chlorella</i> 10 %	<i>Chlorella</i> 15 %	<i>Spirulina</i> 5 %	<i>Spirulina</i> 10 %	<i>Spirulina</i> 15 %
ΔE^*	20.1	20.9	14.9	10.7	6.35	3.72	2.90

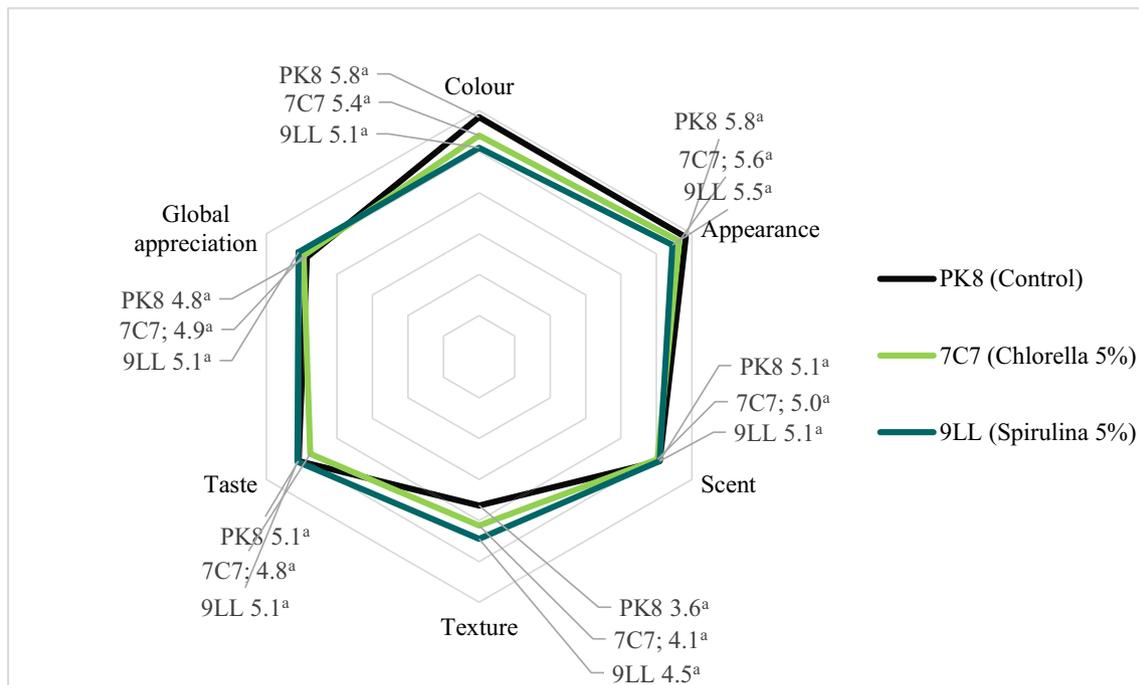


Fig. 11. Sensory analysis results ($n = 30$) of control (PK8), *Chlorella* (7C7) and *Spirulina* (9LL) 5 % concentration snacks. Different letters represent statistically significant differences between ($p < 0.05$).

without the written consent of the copyright-holder.

Declaration of competing interest

As the corresponding author, I disclose that there were no personal or financial relationships with other people or organisations which inappropriately influenced this work. All sources of financial support were disclosed in the manuscript.

Data availability

Data will be made available on request.

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