Title: Interaction of dough acidity and microalga level on bread quality and antioxidant properties

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Abstract

Microalgae nutritional and healthy dietary pattern might be affected by processes like breadmaking when used as ingredient. This study aims to determine the role of dough acidification on the nutritional pattern of *Chlorella vulgaris* enriched breads. Different levels of microalga (1%, 2% and 3%) were incorporated in the recipe in the presence of either 10% sourdough or chemically acidified doughs. Dough and bread characteristics were evaluated. Addition of microalga reduced the slice area and increased the crumb hardness, but it could be counteracted by increasing dough hydration and adapting proofing time. Doughs and breads enriched with microalga had green color. Dough acidification led to softer breads and enhanced the antioxidant activity of enriched breads. Microalgae incorporation increased the protein and ash content of the breads. Microalgae enriched breads made with chemically acidified doughs or sourdoughs had higher Total Phenolic Content and antioxidant activity as assessed by FRAP and ABTS methods.

Key words: bread; *Chlorella vulgaris*; microalgae; sourdough; bread quality; antioxidant activity

1. Introduction

Bread, as one of the most consumed bakery products worldwide (with average per capita consumption 24.5 kg in 2020 and an expected market growth annually by 2.8% (CAGR (Compound Annual Growth Rate) 2020-2025)) has gained the researchers' interest to be used as a vehicle for delivering bioactive compounds to the consumers (Statista, 2020). To this end, a great number of plant materials rich in bioactive compounds have been incorporated in a bread recipe such as aromatic plants (Skendi, Irakli, Chatzopoulou, & Papageorgiou, 2019), fruits and vegetables (Betoret & Rosell, 2020a), microalgae (Graça, Fradinho, Sousa, & Raymundo, 2018) etc., returning breads with an enhanced nutritional profile. Microalgae have received increased consideration due to the high content of pigments, favourable protein and polyunsaturated fatty acid profiles, phenolics, vitamins, and minerals (Ferreira, et al., 2020; Gopal, Meivelu, Arumugam, Matharasi, Dineshkumar, & Pitchai, 2019; Yun, Kim, & Yoon, 2020). The microalgae *Chlorella luteoviridis*, *Chlorella pyrenoidosa*, and *Chlorella vulgaris*, used prior to May 1997 in Europe, are authorized as food (European Union, Novel Food catalogue). Among the genus *Chlorella*, in the USA, only *Chlorella protothecoides* is included in the GRAS list (FDA, GRAS Notices) and in Australia, all *Chlorella* species (including *Chlorella sorokiniana*) and derivatives for which a novel food application was submitted have been so far considered as traditional food (FSANZ, 2016). *Chlorella* sp. is a rich source of proteins (51-58%), lipids (14-22%) and carbohydrates (2-17%) (Niccolai, Chini Zittelli, Rodolfi, Biondi, & Tredici, 2019; Batista, et al., 2011). Those facts have prompted the research of its incorporation in different bakery products. Incorporation of 2% *C. vulgaris* resulted in "high in selenium" cookies (Uribe-Wandurraga, Igual, García-Segovia, & Martínez-Monzó, 2020) whereas the addition level of both 2% and 6% resulted in higher protein and total phenolic contents as well as

in higher *in vitro* antioxidant capacity while maintaining texture stability and sensory quality of cookies (Batista, et al., 2017). According to Batista, et al. (2019) crackers with 6% *C. vulgaris* could be claimed to be a "source of protein". Fradique, Batista, Nunes, Gouveia, Bandarra, & Raymundo (2010) added *C. vulgaris* (up to 2% w/w) in a pasta recipe rendering pasta with high levels of protein and minerals, without interfering with sensorial quality, cooking and textural properties of the final product. Graça et al. (2018) studied the effect of adding *C. vulgaris* (up to 5% in wheat flour base) on dough and bread quality. Up to 3% addition level they observed a strengthening of the gluten network and thus a positive impact on dough rheology and viscoelastic characteristics. Nevertheless, to fully exploit their potential derived from the pigments and phenolic compounds, the impact of process conditions might be considered, since pH and temperature affect antioxidant activity (Betoret and Rosell, 2020b).

It is already stated the importance of food processing on the bioavailability of functional compounds owing to the impact of process conditions on their physicochemical structure (Dima, Assadpour, Dima, & Jafari, 2020). In the particular case of phenolic compounds and their antioxidant capacity, the processing conditions might play a crucial role modulating bioavailability through chemical modifications or cleavage of phenolic compounds-matrixes bonds (Ribas-Agustí, Martín-Belloso, Soliva-Fortuny, & Elez-Martínez, 2018). Breadmaking is a very complicated process where mechanical constraints are entwined with the microbial action during fermentation encompassing dough acidification and activation of endogenous enzymes (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014). Both endogenous and bacterial enzymes are responsible for the solubilisation of dough components thus increasing their bioavailability and digestibility and/or produce through specific synthetic pathways new bioactive compounds thus improving antioxidative activity (Hur, Lee, Kim, Choi, & Kim, 2014).

Actually, an increase in the amount of free and bound polyphenols was obtained by fermenting KAMUT® khorasan and durum wheat flours with sourdoughs (Saa, Di Silvestro, Dinelli, & Gianotti, 2017). The same trend was observed by Ferri, Serrazanetti, Tassoni, Baldissarri, & Gianotti, (2016) for fermented doughs with sourdoughs containing different lactic acid bacteria strains. Those authors confirmed the positive correlation between short fatty acids with the polyphenol content and antioxidant activity in wheat breads.

The beneficial effect of *C. vulgaris* addition has been related with its nutritional content and antioxidant properties, but when used as ingredient, those could be affected during the process. The aim of this research was to evaluate the impact of breadmaking (dough acidification) on microalga enriched dough and bread. To this end, three different levels of *C. vulgaris* were used (1%, 2% and 3%) aiming to establish the maximum acceptable content of *C. vulgaris* that could be used in industrial applications while maintaining acceptable bread quality. To unravel the effect of simultaneous addition of microalgae and dough acidification on dough properties and bread quality, two sets of the same experiment were performed. The first set contained 10% sourdough and in the second set chemically acidified dough was prepared by adjusting the pH with organic acids so as to reach the pH of the sourdough.

2. Materials and Methods

2.1. Materials

Wheat flour for breadmaking (12.07% of protein ($N \times 5.7$), 12.9% moisture) was purchased from Harinera la Meta S.A. (Valencia, Spain). Commercial *Chlorella vulgaris* microalga flour was purchased from Allmicroalgae (Lisbon, Portugal). The DPPH (2,2-diphenyl-1-picrylhydrazyl) was from Sigma Aldrich, USA, whereas TPTZ

(2,4,6-tripyridyl-s-triazine) was obtained from Alfa Aesar, GmbH & Go KG, Karlsruhe, Germany. ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), Trolox ((S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and gallic acid were from J&K Scientific GmbH, Pforzheim, Germany. Folin-Ciocalteu reagent, and methanol (HPLC grade) were obtained from Chem-Lab NV, Zedelgem, Belgium. Iron (III) chloride hexahydrate was from Merk, KGaA, Darmstadt, Germany. All the other chemicals used were of analytical grade.

2.2. Chemical composition

The proximate composition of raw materials and breads was determined using the standard methods of the International Organization for Standardization (ISO): moisture (712:2009), protein (16634-2:2016), ash (2171:2007) and total fat (11085:2015). Carbohydrates content was calculated by difference.

2.3. Sourdough preparation and cell count

Sourdough of type IV (Papadimitriou, et al., 2019) was prepared without the use of baker's yeast. Wheat flours (200 g) were mixed with tap water (126 mL) to obtain a final dough yield (DY) [dough weight $\times$ 100 / flour weight] of 163. Fermentation and daily back-slopping (refreshments) were conducted according to Barber et al (1991). To initiate fermentation, 60% of water was substituted with previously macerated wheat bran (that contains endogenous microorganisms) with tap water at a ratio of 20:80 for at least 20 min. Daily refreshments were carried out for the 3 successive days by mixing 50% of the previously fermented dough with flour and water (final dough yield of 163). Dough was incubated at 30 °C and RH 80% under anaerobic conditions. The resulting sourdoughs were freeze-dried, pulverized, packed in plastic bags and stored at 4 °C for further analysis.

Microbial plate counts were determined by plating successive dilutions of sourdough and dough samples onto selective media. Man, Rogosa and Sharpe agar (MRS) and Potato Dextrose Agar (PDA) and standard medium glucose, yeast extract and calcium carbonate (GYC) were used to determined lactic acid bacteria (LAB) and yeasts and acetic bacteria, respectively. MRS plates were anaerobically incubated at 32 °C for 48-72hours, while PDA plates were incubated at 37 °C for 24-48 h and GYC at 30 °C for 72 h, both under aerobic conditions.

2.4. Breadmaking procedure

Reference bread doughs (W) conventionally prepared (by using compressed baker's yeast), bread doughs containing sourdough (SW) and bread doughs chemically acidified (AW) containing different levels of microalgae powder (0, 1, 2 or 3%) were prepared. A basic recipe, based on white wheat flour (f.b.) was used: 2% compressed baker's yeast, 1.5% salt (corrected based on the salt present in the microalgae powder added), and the amount of water that dough needs to reach optimum consistency (1.1 Nm) in Mixolab (Chopin, Paris, France). Sourdough containing doughs were prepared using the basic recipe but replacing 10% wheat flour by freeze-dried sourdough. After preliminary assays for checking the final pHs of the sourdough containing doughs, chemically acidified doughs with pH value of 4.90 ± 0.05 were prepared with the basic recipe and adding 0.15% of a mixture 1:4 of acetic acid (100% w/w) and lactic acid (85% w/v) (Xu, Tang, Hu, Xu, & Gänzle, 2018). Ingredients were mixed in a Farinograph 300 g-bowl (Brabender, Germany) at 30 °C for 10 minutes. The dough was allowed to rest for 10 minutes after which, it was divided in portions of 40 g, shaped manually, placed into silicon pans (100 mm length $\times$ 50 mm width $\times$ 50 mm height) and allowed to ferment at 30 °C till the volume doubles itself. Finally, fermented doughs were baked at 180 °C for

15 minutes and then cooled down at room temperature for 45 minutes. Samples codes indicated the type of bread followed by a number that described the level of microalgae, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3). Two batches in different days were made for each sample.

2.5. Dough characterization during breadmaking

The water absorption (WA) or the amount of necessary water to reach dough optimum consistency (1.1. Nm) for each formulation (without added yeast) was calculated using a Mixolab (Chopin, Paris, France) equipment following the method S defined by the supplier. Consistency of doughs was registered during 30 minutes at 30 °C in the Mixolab. Proofing performance of the doughs was monitored by recording the increment of dough volume (ΔV) every 10 min during 100 minutes. To evaluate the fermentation kinetics, the slope (mL/min) of the volume increase, the maximum volume (mL) and the time to duplicate initial dough volume ($2V$ (min)) were calculated. Samples were analyzed in duplicate.

2.6. pH, Total Titratable Acidity (TTA) and acid content in doughs

The pH of fermented doughs was potentiometrically determined using a penetration electrode. The pH of breads was measured after vortexing one gram of bread diluted with 9 mL deionized water. Total Titratable Acidity (TTA) was measured in 10 g of doughs or breads blended with 100 mL distilled water that were titrated with 0.01N NaOH and expressed as mL NaOH/10 g. Concentrations of D-lactic acid and acetic acid were analyzed using a specific kit Acetic Acid Assay Kit (Acetate Kinase) and D-Lactic Acid Assay Kit (D-Lactate) (Megazyme

International, Ireland), respectively, according to the manufacturer's instructions. The results were expressed as g of D-lactic or acetic acid per kilogram of sourdough, fermented dough or bread. Three replicates were averaged for each sample.

2.7. Technological characterization of breads

Breads were automatically cut it in 15 mm slice thickness and then characterized. Color of bread crumb and crust was measured using a chroma meter CR-400 (Konica Minolta, Japan). The color was recorded using CIE- L^* a^* b^* scale, where L^* indicates lightness, a^* indicates hue on a green (-) to red (+) axis, and b^* indicated hue on a blue (-) to yellow (+) axis. Total color difference (ΔE^*) was calculated with the following equation:

$$\Delta E^* = ((L_r^* - L_i^*)^2 + (a_r^* - a_i^*)^2 + (b_r^* - b_i^*)^2)^{1/2}$$

Where L_r^* , a_r^* , b_r^* are color parameters of reference bread without microalgae, and L_i^* , a_i^* , b_i^* are color parameters of breads containing microalgae and/or acidified doughs.

Crumb hardness (N) was determined using a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) applying a TPA test with a double compression. A piece of crumb (20 mm diameter) was taken from the center of each slice and was compressed up to 50% using a 36 mm aluminum probe. The following parameters were calculated from the compression curve: hardness, springiness, cohesiveness, chewiness, and resilience.

The slice 2D area and crumb structure were evaluated by digital image analysis using the ImageJ software (ImageJ 1.52p, National Institutes of Health, USA) following the method described by Espinosa-Ramírez, Garzon, Serna-Saldivar, & Rosell (2018). Briefly, images of bread slices were captured using a flatbed scanner (Epson V550, Japan) at 600 dpi of resolution. Then images were improved by splitting channels,

taking the green channel for the analysis and enhancing the contrast; for segmentation method a predefined algorithm (“Otsu”) was applied. Finally, the 2D slice area (cm²) was measured in order to analyze the crumb cell distribution, mean cell area (mm²) and porosity (%). At least four bread loaves for each recipe were used for the above measurements.

2.8. Extraction of bioactive compounds

Bioactive compounds from the freeze-dried breads were extracted in 70% aqueous methanol as reported in Skendi et al., (2019) with some modifications. Freeze-dried bread (1.5 g) was first homogenized in 4 mL of 70% methanol on a vortex for 30 seconds and then the extraction was performed in an ultrasonic bath for 15 min. Following, the sample was centrifuged at 2264 x g for 15 min and the supernatant was collected. The precipitate was re-dissolved into 3 mL of 70% methanol and extracted for a second time as reported previously. The supernatants were pooled together and centrifuged once more at 8400 x g for 10 min and finally frozen stored until analysis. Extraction of the bioactive compounds from the bread samples was carried out at least in triplicate.

2.9. Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method reported by Singleton, Orthofer, & Lamuela-Raventós (1998). For the determination the extract (200 mL) was first oxidized with 800 mL of the Folin-Ciocalteu reagent diluted with water (1:10 v/v) for 2 min. Following, 2mL sodium carbonate 7.5% w/v was added to the mixture and allowed to react for 2 minutes before adding 8 mL of distilled water. Finally, the mixture was incubated in the dark for one hour at room temperature. The

absorption of the mixtures was measured at 725 nm and the results were expressed as milligrams of gallic acid equivalents per gram of dry weight basis (mg GAE/gdw).

2.10. Determination of antioxidant activity

Three assays were utilized to determine the antioxidant activity of breads based on antiradical activities: a) radical scavenging capacity activity (DPPH), b) radical cation scavenging activity (ABTS) and c) ferric reducing antioxidant power (FRAP). Radical scavenging activity (DPPH) was measured according to Yen & Chen (1995). The extract (150 μ l) was mixed with 2.85 mL of 100 μ M 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent and allowed to rest for 5 minutes before reading the absorbance at 516 nm.

The radical cation scavenging activity (ABTS) was determined by mixing 100 μ L extract with 3.9 mL ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) reagent with an absorbance of 0.70 prepared according to Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans (1999). The absorbance of the mixture was measured at 734 nm after reacting for 4 min at room temperature.

The ferric reducing antioxidant power (FRAP) assay was carried out by mixing 100 μ l of extract with 3 ml of FRAP reagent for 4 min at 37°C under dark conditions as reported from Benzie & Strain (1999). The FRAP assay consisted of a mixture of three solutions: 20 mM ferric chloride solution, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 0.3 mM (pH 3.6) acetate buffer in a proportion of 1:1:10, respectively. The absorbance of the mixture was measured at 593 nm against a blank.

For all the three assays the results were expressed as mg Trolox ((S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents per gram of sample on a dry

weight basis (mg TE/g dw). All the measurements for determination of TPC and antioxidant activity were performed at least in duplicate.

2.11. Statistical analysis

The results are reported as the average and standard deviation of measurements. Analysis of variance (ANOVA) was applied using Statgraphics Centurion XVII (Statistical Graphics Corporation, UK) and SPSS Statistics 25.0 software (SPSS Inc., Chicago, USA). The Duncan test was used to assess significant differences ($P < 0.05$) among samples. The statistical analysis of TPC and antioxidant activity includes the application of general linear model for multiple variables as well as correlation analysis among the variables. Additionally, a principal component analysis (PCA) was carried out to detect possible discrimination among samples.

3. Results and Discussion

Different levels of *C. vulgaris* powder (0, 1, 2 or 3%) were incorporated into breads. Based on the optimum amount reported by Graça et al. (2018), 3% was selected as the maximum incorporation level. To evaluate the potential impact of acidification, doughs fermented with a spontaneous sourdough and doughs chemically acidified were carried out and the resulting breads evaluated. The final yeast, LAB and acetic acids counts (CFU/g) in the lyophilized sourdough were 2×10^3 , 3×10^8 ; and 1×10^5 , respectively.

3.1. Dough characteristics

Dough rheological properties during mixing were determined using the Mixolab, which also allowed defining the water absorption to be used in the breadmaking for obtaining doughs with constant consistency. The addition of microalgae powder resulted in a steady increase of the water absorption required to reach constant consistency of 1.1 Nm

(Table 1). This increase might be expected considering that microalgae are a source of hydrocolloids, which are generally recognized for their ability to enhance water absorption in breadmaking (Rosell, Rojas, & Benedito de Barber, 2001). Specifically, in *C. vulgaris*-based crackers, Batista et al. (2019) reported that the addition of high concentrations of microalgae biomass could lead to a weaker gluten network, unable to efficiently trap gas bubbles and water molecules. Batista et al. (2011) also reported that *A. platensis* biomass (commercially known as spirulina) impairs starch gelatinization in model gel systems, namely by increasing the gelatinization temperature, likely due to competition for water binding zones during the hydration of starch granules. In fact, during dough mixing, the available free water is partitioned between microalgae and wheat flour hydrophilic sites, which might also impair gluten protein network development. It is possible that the formation of a weaker gluten network might result in the collapse of small gas cells into larger cavities, which might influence gas and water trapping during baking (Batista et al., 2019). Likewise, sourdough containing doughs required significantly higher water absorption compared to their counterparts (reference and chemically acidified dough). The presence of microalgae at different levels did not significantly modify the doughs' stability during mixing (Figure S1). All doughs showed a good performance during mixing for 30 min, although those containing sourdough showed some decay after 20 min of mixing. The effect was more pronounced in samples with added microalgae powder, showing that the interaction between microalgae and sourdough resulted in a slight weakening of the dough when overmixed. *C. vulgaris* effect on dough mixing agrees with results reported by Graça et al. (2018) within the level range used in the present study, showing the increase in water absorption but without affecting dough stability, effects that these authors ascribed to the supplemented proteins from the microalga. Same absorption trend has been reported

with red and brown seaweeds powder when added up to 8% to wheat flour (Arufe, Della Valle, Chiron, Chenlo, Sineiro, & Moreira, 2018; Mamat, Akanda, Zainol, & Ling, 2018), due to their high water retention capacity.

The fermentation of the doughs was monitored to compare breads obtained with the same level of fermentation. Doughs were baked when dough volume doubled itself and to reach that level, doughs containing sourdough (SW0) showed the faster fermentation (50 min) compared to reference dough (W0) (60 min) and chemically acidified dough (AW0) (60 min) (Figure S2 and Table S1). Addition of the microalga in the recipe accelerated the fermentation of the chemically acidified doughs (40 min) but not of the reference dough and sourdough. Therefore, *C. vulgaris* addition up to 3% did not affect the fermentation kinetics by bakers' yeast as reported by Graça et al. (2018), but in the presence of acids an underlying more complex mechanism than the obvious "lowering of pH" might affect fermentation, thus speeding the process. The compounds comprising *C. vulgaris* could represent an attractive media for yeast growth. It is recognized that it is rich in amino acids, fatty acids and minerals (Niccolai, Chini Zittelli, Rodolfi, Biondi, & Tredici, 2019). The presence of these compounds in certain amounts could represent the right combination that possibly boosts the yeast metabolism and increase the fermentation rate. Nevertheless, that effect was not observed in the presence of sourdough due to the LAB competition with yeast for nutrients.

As expected, the presence of the sourdough significantly ($P<0.05$) decreased the dough pH and increased the TTA, which was similar to that of chemically acidified doughs (Table 1). Addition of the microalga decreased only the pH of the reference dough without affecting significantly that of other types of dough that already had lower pH due to the presence of the sourdough or acidification process. The TTA values in the

reference dough were affected by the presence of the microalga but not by the addition level, whereas a progressive increase with the fortification level was observed for the other two types of dough.

The type of dough significantly ($P<0.05$) affected the content of lactic and acetic acids in fermented doughs (Table 1). Sourdough containing doughs showed higher content of lactic acid than their counterparts without sourdough, whereas, chemically acidified dough exhibited the highest concentration of acetic acid (Table 1). The ANOVA statistical analysis indicated that microalga addition did not affect significantly the amount of lactic and acetic acids of the doughs. Lactic and acetic acids are the main metabolites of the sourdough processes; lactic acid bacteria being a part of the stable biota of the mature sourdoughs (Gobbetti et al., 2014). In consequence, compared to conventionally produced breads with baker's yeast, sourdough fermentation increased lactic and acetic acid values of doughs, increasing acidity and reducing pH, whereas the presence of microalga, no related to the level of incorporation, increased the acidity and reduced the pH in minor extent possibly due to the presence of short fatty acids diverse than lactic and acetic acids.

3.2. Bread technological characteristics and composition

The presence of microalga to doughs and respective breads was readily visible from the change in their color (Figure 1), showing a greenish color that was intensified with the amount of *C. vulgaris* powder. Nevertheless, a nice crumb was obtained from all the recipes. At first glance it was observed that acidified breads and sourdough breads had lighter crumb than their reference counterparts. As it was visually detected, microalga powder significantly ($P<0.05$) altered the color of both the crust and crumb of the breads (Table S2). The crust showed a steady reduction of the luminosity when increasing the amount of microalga, independently of the type of dough. Likewise, b^*

parameter decreased with the amount of microalga in all the breads, thus reducing the yellowish tone of the crust. The total color difference (ΔE^*) confirmed the drastic color change, since values were higher than 3.0 (Arufe et al., 2018). Similarly, the luminosity of the crumb was reduced when augmenting the level of microalga powder. Conversely to the trend observed in the crust, b^* values of the crumbs increased in the presence of microalga, with a steady decrease when augmenting the level of microalga powder, although in sourdough breads, no trend was detected. Again, the total color difference (ΔE^*) of the crumb confirmed the substantial change in the color of the crumbs containing the microalga but also the very different color induced by the type of dough (AW0, SW0).

Breads prepared with sourdough had significantly ($P < 0.05$) lower pH followed by breads obtained from chemically acidified doughs. Breads containing *C. vulgaris* had significantly higher pH than the respective controls, with exception of the reference recipe, in which no trend was observed when increasing the level of the microalga. In regard to concentration of organic acids in breads, *D*-lactic acid enhanced its concentration compared to its dough counterparts, either by type of dough and microalgae levels, with exception of the reference recipe (Table 1). Meanwhile, acetic acid was significantly lower in breads than in doughs (Table 1). In a similar way, Brandt (2019), noticed that, during baking or drying, acetic acid is volatilized, while lactic acid is more stable, due to the non-volatility of this compound.

When the bread technological characteristics were statistically analyzed considering the type of dough and the microalgae level, it was observed that the type of dough significantly affected the bread hardness and the mean cells area of the crumb, whereas the level of microalgae significantly affected the bread 2D slice area (related to bread

volume) and the porosity of the crumb, as well (Table 2). The textural properties of the breads are included in Table S1. The 2D crumb area decreased when increasing microalgae level, with exception of sourdough-bread, where no significant direct trend was observed. In spite of reference and acidified doughs were fermented to the same level, the oven rise during baking seems to be affected by microalgae incorporation, leading to a reduction of the bread expansion and in consequence the bread volume. Arufe et al. (2018) described a decrease in the dough porosity during fermentation, in the presence of brown seaweeds, which was associated to the high elongational viscosity provided by the seaweeds, but that effect was more ascribed to proofing operation than baking. Similar bread volume reduction was reported by Graça et al. (2018), who described noticeable changes in the crumb structure, with bigger gas cells due to dough weakening when adding *C. vulgaris*. The same trend was observed in the present study, microalgae addition led to an increase in porosity and the size of gas cells showed a steady increase, but the effect was only significant ($P<0.05$) at the higher microalgae level tested. Probably, the adapted fermentation time limited the bubbles coalescence during fermentation, minimizing the microalgae impact on crumb structure, and only the highest level tested impacted on the dough viscoelasticity.

Breads containing sourdough showed softer crumbs, although slight increase was observed when increasing the microalgae level. Taking into account the crumb image analysis, in general those breads displayed higher porosity and bigger gas cells that suggests a more open crumb structure due to dough bubble coalescence, likely derived from the weakening of gluten matrix due to both microalgae addition and lactic acid bacteria metabolites.

The chemical composition of the breads was significantly affected by the microalgae level, with exception of the fat content, and no significant differences were induced with

the type of dough (Table 3). Despite the different water absorption of the diverse doughs, no pattern was identified among the samples. As expected, the protein as well as the ash contents were significantly increased with the microalga's addition due to the higher presence of these two components in *C. vulgaris*. This is also reported in the literature for other bakery products (Batista et al., 2019). On the other hand, addition of *Chlorella vulgaris* did not significantly affected the fat content of the resulting breads.

3.3. Variation of total phenolics and antioxidant activity among bread samples

The TPC and antioxidant activity of breads were significantly increased with increasing level of the microalgae (Figure 2). The application of General Linear Model revealed that only microalgae levels equal or greater than 2% were able to increase significantly the TPC. Furthermore, it was found that the type of dough plays an important role in the amount of TPC. The TPC increases in the following order: reference dough < sourdough < acidified dough. It is recognized that low pH values facilitate the extraction of phenolic compounds. Thus, sourdough and acidified breads theoretically should have more extracted phenolics in the free form compared to the control dough that has higher pH among the doughs. Since in the case of acidified dough the dough matrix is exposed for longer time to lower pH than in the sourdough, it is probable that this could explain the greater TPC content of acidifies dough.

FRAP antioxidant activity was also significantly increased with increasing of the microalgae level compared to the reference (bread without *C. vulgaris*) regardless the type of dough used (Figure 2b). It seems that type of dough used for bread preparation played also a significant role in the amount of increase in the FRAP values (reference dough $\leq$ sourdough $\leq$ acidified dough).

DPPH and ABTS activity of breads augmented with increasing of the microalgae level for all the types of dough (Figure 2c, d), indicating higher levels of hydrophilic compounds like ascorbic acid and hydrophobic compounds like pigments, respectively. Again, the type of dough plays an important role in the increase observed. Sourdough seemed to increase ABTS values at a greater extent than acidified dough and reference dough, that showed a similar magnitude of increase. The opposite was observed when comparing the effect of dough type on the increase of DPPH values, with greater DPPH values in the reference dough followed by acidified dough and then sourdough. It was surprising the significant increase observed in DPPH values in breads made with 3% microalga and acidified dough. Likely, the combination of high amount of acetic acid and the low lactic acid content might be responsible of that response. Considering that the ABTS method has higher sensitivity towards hydrophobic compounds, results could be related with such compounds released by lactic acid bacteria, whereas DPPH with higher sensitivity towards hydrophilic compounds were closely related to reference dough.

In the present study, the total phenolic content showed significantly moderate correlation with antioxidant activity of breads determined by FRAP ($r= 0.541$) and ABTS ($r= 0.486$) assays. Nevertheless, no correlation was observed between the antioxidant activity measured by DPPH assay and the TPC. FRAP antioxidant activity correlated very well with ABTS ($r= 0.816$) and moderately with DPPH ($r= 0.520$), while the data obtained from DPPH assay did not correlate with those gained from ABTS assay.

Phenolic compounds must be first released from the bread matrix during extraction in order to be detected for their antioxidant activity. Their chemical structure as well as the food matrix interactions (such as with carbohydrates, proteins and lipids) are factors that

impede bioaccessibility and bioavailability of phenolics (Dima et al., 2020; Ribas-Agustí et al., 2018). This could explain the small increase in the amount of phenolic compounds with increasing of microalgae level as well as the different patterns among the three assays. The literature cited several possible processing conditions (such as mechanical processing, enzymatic and chemical treatments, thermal processing, pasteurization and sterilization, extrusion cooking, and bioprocessing, drying, ultrasounds, high pressure, pulsed electric fields, etc.) that affect the release of phenolics from the food matrix (Ribas-Agustí et al., 2018; Wang, He, & Chen, 2014). It is obvious that the type of fermentation plays an important role not only in the amount of phenolics but also in the antioxidant activity of the bread. Although the amount of free phenolic compounds detected in the acidified dough bread was higher than in sourdough and reference bread only FRAP assay data follow the same trend. However, with reference to DPPH and ABTS, reference dough and sourdough bread showed the highest antioxidant activity in each respective assay. According to Skendi, Irakli, & Chatzopoulou (2017), factors such as mechanisms of action of the applied assays, presence of different compounds with different antioxidant activity, synergistic/antagonistic effects between phenolic compounds have an important effect on the overall antioxidant activity of the extracts. Thus, fermentation type affects not only the amount of bioactive compounds (Ferri et al., 2016) but also their profile since other compounds may be synthesized resulting in an alteration of the antioxidant activity (Hur et al., 2014).

3.4. Principal component analysis

A principal component analysis (PCA) was carried out with all the variables evaluated to identify possible discrimination among samples containing microalgae and the

impact of the dough type (Figure 3). Two components were able to explain 63.3% of the samples' variability. Component 1 explained 37% whereas Component 2 described 26.3% of the variation. The score plot revealed that samples were grouped based on the dough type, with clear discrimination between reference breads and those containing acidified doughs (sourdough or chemically acidified). Component 1 allowed discrimination of the breads obtained from different type of doughs, whereas Component 2 discriminated between reference breads and acidified breads (sourdough or chemically acidified). Breads containing microalgae were located in the positive axis of Component 1, with exception of AW1, being discriminated by the lactic acid content and TTA of doughs and breads, the crumb structure (porosity and gas cells size), the textural properties, breads composition and the total phenolic content and antioxidant activities. However, Component 2 allowed better discrimination of the acidified breads that were grouped in the negative axis of PC2, with exception of AW3, owing their textural properties, acids content, total phenolic content and ABTS activity. Furthermore, reference breads were grouped in the positive axis of PC2, mainly due to their higher pHs, longer fermentation, higher crumb hardness and DDPH activity. Therefore, the PCA confirmed the role of acid doughs for promoting the antioxidant activity and TPC when incorporating *Chlorella vulgaris* into breadmaking process.

4. Conclusions

Incorporation of *C. vulgaris* into wheat breads allowed obtaining breads with innovative technological characteristics but also enriched in bioactive compounds. However, its supplementation to acidified doughs permitted to enhance the antioxidant activity. The incorporation of *C. vulgaris* into bread recipes required the water absorption adjustment but no further impact on dough stability was observed. Breads enriched with *Chlorella*

vulgaris up to 3% were mainly characterized by intense green hue, slightly lower slice 2D area and higher crumb hardness that was associated to the thicker gas cell walls compared to the control bread. Particularly, in microalgae enriched breads, the use of chemically acidified doughs or sourdoughs allows enhancing the TPC and antioxidant activity in the resulting breads. Nevertheless, antioxidant assays suggested differences in the profile and amount of phenolics released since the increase in the antioxidant activity was different among the prepared breads. Thus, to optimize the health benefits of *Chlorella vulgaris* in breads, it would be advisable to use acidic doughs, prepared chemically or by applying sourdough fermentation.

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Conflict of Interest The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

The following are the Supplementary data to this article:

5. References

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Figure captions

Figure 1. Captured images of doughs and bread slices. Reference (W): a - d, Chemically acidified (AW): e - h and Sourdough (SW): i - l, with 0, 1, 2 or 3% of *C. vulgaris* powder from left to right.

Figure 2. Total phenolic content (TPC, expressed as $\mu\text{g GAE/g}$ of bread dw, a), and antioxidant activity (ferrous reducing capacity (FRAP); b), DPPH free radical scavenging activity; c), and ABTS scavenging activity; d), expressed as $\mu\text{g Trolox equivalent (TE)/g}$ of bread dw, for the different breads fortified with *C. vulgaris* at three addition levels. Different numbers above error bars within the same type of bread indicate significant differences ($P \leq 0.05$) among the means, as determined by the Duncan's multiple range test. Different letters above error bars within the same microalgae concentration indicate significant differences ($P \leq 0.05$) among the means, as determined by the Duncan's multiple range test.

Figure 3. Plot (PC 1 $\times$ PC 2) of the set of bread samples and evaluated variables obtained by principal component analysis (PCA). Samples are labelled as in the text. pH, TTA and acids content preceded by D or B are referred to dough or bread, respectively. Dotted lines are grouping the samples obtained from different type of doughs.

Table 1. Impact of the microalgae level and type of dough on water absorption (WA), pH, TTA and acids content in doughs and breads. Samples codes indicated the type of bread followed by a number that described the level of microalgae, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3).

Sample	WA (g/100g)	pH		TTA (ml NaOH/10 g)		D-Lactic acid (g/kg)		Acetic acid (g/kg)	
		Dough	Bread	Dough	Bread	Dough	Bread	Dough	Bread
W0	56.6	5.48 ± 0.00 ^c	5.82 ± 0.02 ^f	4.2 ± 0.1 ^a	0.4 ± 0.0 ^a	0.04 ± 0.01 ^a	0.03 ± 0.01 ^a	1.86 ± 0.17 ^a	0.30 ± 0.07 ^{abcd}
W1	56.8	5.38 ± 0.01 ^d	5.77 ± 0.01 ^e	5.3 ± 0.2 ^{bc}	0.6 ± 0.1 ^b	0.07 ± 0.02 ^a	0.04 ± 0.00 ^a	2.06 ± 0.16 ^{abcd}	0.27 ± 0.03 ^{abc}
W2	58.5	5.39 ± 0.01 ^d	5.81 ± 0.01 ^{ef}	5.6 ± 0.2 ^{cd}	0.6 ± 0 ^c	0.03 ± 0.01 ^a	0.06 ± 0.01 ^{ab}	1.98 ± 0.13 ^{abcd}	0.26 ± 0.07 ^{ab}
W3	60.4	5.40 ± 0.01 ^d	5.83 ± 0.04 ^f	5.3 ± 0.9 ^{bc}	0.7 ± 0 ^d	0.06 ± 0.00 ^a	0.06 ± 0.02 ^{ab}	1.93 ± 0.19 ^{ab}	0.20 ± 0.06 ^a
AW0	56.6	4.88 ± 0.02 ^{ab}	5.38 ± 0.01 ^c	4.8 ± 0 ^{ab}	0.7 ± 0 ^d	0.05 ± 0.00 ^a	0.05 ± 0.01 ^a	2.30 ± 0.09 ^e	0.52 ± 0.07 ^d
AW1	56.8	4.86 ± 0.01 ^a	5.34 ± 0.01 ^b	6.3 ± 0.2 ^{de}	0.8 ± 0 ^f	0.06 ± 0.01 ^a	0.09 ± 0.04 ^{ab}	1.97 ± 0.04 ^{abc}	0.49 ± 0.14 ^{cd}
AW2	58.5	4.88 ± 0.03 ^{abc}	5.44 ± 0.01 ^d	6.9 ± 0.1 ^e	0.8 ± 0 ^{ef}	0.06 ± 0.01 ^a	0.07 ± 0.03 ^{ab}	2.01 ± 0.05 ^{abcd}	0.44 ± 0.13 ^{bcd}
AW3	60.4	4.88 ± 0.01 ^{ab}	5.45 ± 0 ^d	8.2 ± 0.0 ^f	0.9 ± 0 ^g	0.04 ± 0.00 ^a	0.12 ± 0.03 ^b	1.89 ± 0.06 ^a	0.37 ± 0.11 ^{abcd}
SW0	61.5	4.90 ± 0.01 ^{bc}	5.27 ± 0.03 ^a	4.9 ± 0.1 ^{ab}	0.6 ± 0 ^b	0.48 ± 0.06 ^b	0.51 ± 0.04 ^c	2.15 ± 0.04 ^{bcd}	0.27 ± 0.08 ^{abc}
SW1	63.9	4.91 ± 0.01 ^c	5.32 ± 0.02 ^b	6.3 ± 0.4 ^e	0.7 ± 0 ^d	0.49 ± 0.05 ^b	0.54 ± 0.09 ^c	2.17 ± 0.21 ^{cde}	0.51 ± 0.18 ^d
SW2	64.1	4.89 ± 0.01 ^{abc}	5.38 ± 0.01 ^c	7.0 ± 0.4 ^e	0.8 ± 0 ^{de}	0.44 ± 0.06 ^b	0.56 ± 0.03 ^c	2.13 ± 0.01 ^{bcd}	0.30 ± 0.09 ^{abcd}
SW3	64.6	4.88 ± 0.01 ^{ab}	5.35 ± 0.01 ^{bc}	9.0 ± 0.4 ^g	0.7 ± 0 ^{de}	0.49 ± 0.04 ^b	0.68 ± 0.03 ^d	2.20 ± 0.03 ^{de}	0.31 ± 0.02 ^{abcd}
<i>P-value</i>									
Type of dough		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0072	0.0010
<i>Chlorella vulgaris</i> level		0.0562	0.0008	0.0000	0.0000	0.4078	0.0008	0.5900	0.1169

Means with different letters within the same column denote significant differences ($P \leq 0.05$) among the means, as determined by the Duncan test.

Table 2. Technological characteristics of breads supplemented with different levels (0, 1, 2, 3%) of *C. vulgaris* powder. Reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3) and sourdough breads (SW0, SW1, SW2, SW3) were evaluated.

Sample	2D Slice Area (cm ²)			Hardness (N)			Mean cell area (mm ²)			Porosity (%)		
W0	15.6	±	0.4 ^{ef}	2.6	±	0.2 ^g	0.61	±	0.12 ^{bc}	26.96	±	4.03 ^{abcd}
W1	14.4	±	0.4 ^{abc}	2.3	±	0.2 ^f	0.61	±	0.08 ^{bc}	31.96	±	3.99 ^{de}
W2	13.8	±	0.1 ^{abc}	2.8	±	0.2 ^h	0.60	±	0.05 ^{bc}	31.41	±	0.72 ^{cde}
W3	13.4	±	0.3 ^a	2.6	±	0.2 ^{gh}	0.88	±	0.04 ^{ef}	36.31	±	2.07 ^{ef}
AW0	16.0	±	0.5 ^f	1.9	±	0.1 ^{cd}	0.43	±	0.05 ^a	22.46	±	1.08 ^{ab}
AW1	15.4	±	0.2 ^{def}	1.7	±	0.1 ^{abc}	0.48	±	0.08 ^{ab}	24.82	±	2.42 ^{ab}
AW2	14.5	±	0.1 ^{bcd}	2.1	±	0.2 ^{def}	0.50	±	0.03 ^{ab}	22.04	±	2.20 ^a
AW3	13.8	±	1.0 ^{abc}	2.2	±	0.2 ^{ef}	0.65	±	0.05 ^c	35.19	±	1.11 ^{ef}
SW0	13.6	±	0.0 ^{ab}	1.6	±	0.3 ^{ab}	0.55	±	0.01 ^{abc}	25.40	±	1.99 ^{abc}
SW1	14.4	±	0.7 ^{abc}	1.4	±	0.2 ^a	0.69	±	0.02 ^{cd}	28.58	±	4.17 ^{bcd}
SW2	14.7	±	0.1 ^{cde}	1.8	±	0.1 ^{bc}	0.80	±	0.09 ^{de}	32.03	±	1.57 ^{de}
SW3	13.9	±	0.1 ^{abc}	1.9	±	0.2 ^{cde}	0.97	±	0.02 ^f	38.80	±	2.52 ^f
<i>P-value</i>												
Type of dough	0.0839			0.0000			0.0000			0.0014		
<i>Chlorella vulgaris</i> level	0.0202			0.0000			0.0000			0.0002		

*Means with different letters within a column were significantly different ($P < 0.05$).

Table 3. Chemical composition in g /100 g (as is) of breads supplemented with seaweed powder. Samples codes indicated the type of bread followed by a number that described the level of seaweed, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3),
sourdough breads (SW0, SW1, SW2, SW3)

Type of dough	Moisture	Protein	Ash	Total Fat	Carbohydrates
W0	35.39 ± 0.51 ^{bc}	12.17 ± 0.02 ^a	0.84 ± 0.05 ^a	0.24 ± 0.06	51.36
W1	35.16 ± 0.13 ^b	12.67 ± 0.03 ^c	0.99 ± 0.03 ^b	0.25 ± 0.05	50.94
W2	35.41 ± 0.29 ^{bc}	13.27 ± 0.00 ^e	1.10 ± 0.05 ^{cde}	0.22 ± 0.06	50.00
W3	35.49 ± 0.96 ^{bcd}	13.75 ± 0.06 ^f	1.12 ± 0.08 ^{def}	0.25 ± 0.07	49.38
AW0	35.30 ± 0.70 ^b	12.25 ± 0.03 ^a	0.86 ± 0.02 ^a	0.21 ± 0.04	51.28
AW1	33.89 ± 0.10 ^a	12.93 ± 0.06 ^d	0.99 ± 0.05 ^{bc}	0.25 ± 0.02	51.94
AW2	34.79 ± 0.32 ^{ab}	13.31 ± 0.11 ^e	1.04 ± 0.08 ^{bcd}	0.25 ± 0.02	50.60
AW3	35.01 ± 0.06 ^b	13.90 ± 0.03 ^g	1.21 ± 0.07 ^f	0.28 ± 0.03	49.61
SW0	35.68 ± 0.55 ^{bcd}	12.18 ± 0.10 ^a	0.83 ± 0.04 ^a	0.26 ± 0.10	51.05
SW1	36.46 ± 0.17 ^{cd}	12.50 ± 0.11 ^b	0.97 ± 0.05 ^b	0.26 ± 0.05	49.81
SW2	35.02 ± 0.70 ^b	13.22 ± 0.02 ^e	1.04 ± 0 ^{bcd}	0.27 ± 0.03	50.45
SW3	36.57 ± 0.06 ^d	13.66 ± 0.04 ^f	1.17 ± 0.02 ^{ef}	0.24 ± 0.09	48.36
<i>P-value</i>					
Type of dough	0.0043	0.3802	0.6067	0.8118	0.1112
Seaweed quantity	0.3172	0.0000	0.0000	0.9610	0.0106

*Means with different letters within a column were significantly different ($P < 0.05$).

Figure 1.

Figure 2.

Figure 3.