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Enhancing the Protein, Mineral Content, and Bioactivity of Wheat Bread through the Utilisation of Microalgal Biomass: A Comparative Study of *Chlorella vulgaris*, *Phaeodactylum tricornutum*, and *Tetraselmis chuii*

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Featured Application: While both *Tetraselmis chuii* and *Phaedactylum tricornutum* demonstrate remarkable functional properties, their utilisation in food products remains restricted. Understanding the impact of their addition in terms of dough rheology is crucial for assessing the quality of the resulting bread. Moreover, it is crucial to investigate the volatile composition of microalgae biomass, as it plays a significant role in determining aroma and influencing consumer acceptance.

Abstract: At present, the incorporation of microalgae into bread and related cereal products has attracted attention due to their potential for enhancing nutritional profiles and their impact on health. In this study, 4% of *Chlorella vulgaris*, *Phaeodactylum tricornutum*, and *Tetraselmis chuii* were added into wheat flour to produce bread and assesses their impact on the dough rheology behaviour, quality performance, nutritive value, and bioactive profile of bread. The results showed that *T. chuii* strengthened the dough network, whereas *P. tricornutum* exerted minimal influence. Notably, the incorporation of *C. vulgaris* induced a pronounced weakening of the protein network within the dough matrix, leading to disruptions in dough structure and subsequent alterations in starch gelatinisation and retrogradation. These changes lead to a reduction in the bread volume (22.7%) and a corresponding increase in its firmness when *C. vulgaris* was added. In contrast, *T. chuii* and *P. tricornutum* had no significant effect on bread volume. All microalgae species caused the dark green colour of the bread and enhanced the bread nutritional composition, namely in terms of protein content (14.7% increase in *C. vulgaris* bread) and mineral profile. The breads containing *T. chuii* exhibited a noticeable increase in both total phenolic content (from 7.22 in the control to 38.52 (µg GAE/g)) and antioxidant capacity (from 117.29 to 591.96 (µg TEAC/g) measured by FRAP).

Keywords: microalgae; wheat bread; Mixolab; rheology; texture; bioactivity; volatile organic compounds

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1. Introduction

Bread, a dietary staple consumed worldwide, serves as a vital source of essential nutrients. In recent years, people have become more aware of products that include functional ingredients. Wheat bread has garnered attention among researchers as a medium for delivering essential nutrients and bioactive compounds [1]. Incorporating microalgae into bread can enhance its nutritional profile and provide diverse bioactive constituents [2,3].

Microalgae are known as a sustainable and excellent source of numerous phytochemicals that feature remarkable functional activities and health impacts, including improving gut health, boosting immunity, and reducing the risk of chronic diseases such as cancer, diabetes, and cardiovascular disease [4,5]. According to a report by Market, the global microalgae market size is projected to grow from USD 3.4 billion in 2020 to USD 4.6 billion by 2027 at a compound annual growth rate (CAGR) of 4.3% [6].

In previous studies, microalgae have been incorporated into various food products such as cheese, vegetable soups, pasta, and snacks like cookies and energy bars, consistently demonstrating substantial improvements in nutrient content, bioactive compounds, and antioxidant capacity [7,8]. There has been limited research conducted regarding the inclusion of microalgae in bread products, although the limited findings indicate that their inclusion results in an improvement in the nutritional balance and antioxidant capacity of bread. These studies primarily focused on the most popular and cultivated strains of microalgae and cyanobacteria approved by the European Food Safety Authority (EFSA): *Arthrospira platensis* (Spirulina) sp. and *Chlorella* sp. as ingredients [4,9–11]. *Chlorella vulgaris* is a freshwater green microalga and represents a valuable source of nutrients, including high-quality proteins like those found in eggs and soybeans (its amino acid profile is even more complete than that proposed by FAO/WHO as a standard for human nutrition), polysaccharides (starch and glucans), pigments like carotenoids and chlorophylls, and vitamins [9,12] (which vary according to the growing conditions). If grown under favourable conditions, the resulting lipid profile is more concentrated in polyunsaturated fatty acids (PUFAs), being more suitable for nutritional uses [13]. *C. vulgaris* has already demonstrated immunomodulatory and anticancer properties and displayed protective activities against cardiovascular disease, hypertension, and cataracts [14,15]. It also has the potential to inhibit the growth of pathogenic microorganisms in food, namely in bread [16]. Due to its historical use, *C. vulgaris* is considered a food ingredient and is well established in the market, being exempt from approval by the EFSA as a novel ingredient. It has also already been generally accepted as safe (GRAS) by the Food and Drug Administration (FDA) in the United States of America. *C. vulgaris* is one of the most studied and cultivated microalgae due to its regulatory status. *Tetraselmis chuii*, a marine phytoplankton belonging to the *Prasinophyceae* class, is distinguished for its abundant bioactive compounds and antioxidant enzymes. All essential amino acids are present, making *T. chuii* a complete protein source. Also recognised for its richness in PUFAs, this species contains ω-3 fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). It shows promise in reducing oxidative stress, aiding exercise recovery, and enhancing immune function, as indicated by studies conducted by Toro et al. [17], Sharp et al. [18], and García et al. [19]. Notably, *T. chuii* received approval for use under Regulation (EU) 2017/2470, being limited to sauces, salts, and condiments, with a maximum incorporation level of 250 mg/serving/d. *Phaeodactylum tricornutum,* a marine diatom species, has been extensively investigated, with its genome fully sequenced, since the late 2000s. In Europe, it is cultivated by eight companies, yielding an annual dry biomass production of around four tonnes. *P. tricornutum* is rich in PUFAs, specifically EPA, and pigments such as fucoxanthin. Currently, it is used as a source of fucoxanthin and omega-3-rich oil extracts [20]. However, *P. tricornutum* is not approved, as yet, by the EFSA as a novel food and is still in the authorisation process.

Although both *T. chuii* and *P. tricornutum* exhibit exceptional functional properties, their application in the food matrix is still limited. In the context of bakery products, there is a need for more knowledge on how the baking process affects the microalgae's bioactive qualities [2,21,22]. As far as we know, to date, there is no available data on the incorporation of *P. tricornutum* into bread products.

In fact, microalgae can modify the structure and rheological properties of dough. Previous studies suggest that the presence of microalgae affects starch granules' hydration and protein structure within the gluten network [23], resulting in lower loaf volume and subsequently affecting other quality attributes [2,10,24]. Information about dough properties is important for predicting the potential application of the microalgae and the quality of the produced bread [25].

The aim of the present study was to assess changes in the rheological properties of wheat dough resulting from the addition of *C. vulgaris*, *T. chuii*, and *P. tricornutum*, evaluating their respective impacts on the structure, nutritional value, and bioactive compounds of the resulting wheat bread. This study is part of the YUMAlgae project, which aims to incorporate sustainable and nutritive microalgae ingredients into staple food products, namely

wheat bread. An incorporation level of 4% of freeze-dried microalgae biomass, related to wheat flour, was considered to obtain an interesting nutritional value and health impact without comprising the mechanical behaviour [12] and sensory profile to a large extent.

2. Materials and Methods

2.1. Materials

Freeze-dried microalgae samples (*Chlorella vulgaris*, *Tetraselmis chuii*, and *Phaeodactylum tricornutum*) were provided by the NORCE Norwegian Research Centre (Bergen, Norway). The biochemical composition of these raw microalgae was obtained by Gracio et al. [26] and is detailed in Table 1. Wheat flour (Nacional T65) was obtained from Cerealis Produtos Alimentares (Maia, Portugal). The wheat flour had the following composition per 100.0 g: 10.0 g protein, 73.0 g carbohydrates, 3.3 g fibre, and 1.5 g lipids. Additional raw materials, including yeast (Fermipan, Lallemand Iberia, Setúbal, Portugal), white crystalline saccharose (Sidul, Santa Iria de Azoia, Portugal), and sea salt were purchased from the local market.

Table 1. Biochemical composition of microalgae biomasses (%, Dry weight), from Gracio et al. [26]. *Chlorella vulgaris* (Cv), *Phaeodactylum tricornutum* (Pt) and *Tetraselmis chuii* (Tc). Different letters in the same parameter show significant differences ($p < 0.05$, Tukey test).

Sample	Moisture %	Carbohydrate %	Protein %	Fat %	Ash %
C. vulgaris	4.9 ± 0.14^{a}	48.0 ± 0.01^{a}	43.6 ± 0.00^{a}	0.2 ± 0.09^{b}	16.5 ± 1.93^{a}
P. tricornutum	4.7 ± 0.00^{a}	38.7 ± 0.00^{b}	43.5 ± 0.05^{a}	0.7 ± 0.05^{b}	8.9 ± 0.47^{b}
T. chuii	4.6 ± 0.00^{a}	47.9 ± 0.55^{a}	31.8 ± 0.20^{b}	3.1 ± 0.08^{a}	15.4 ± 2.23^{a}

2.2. Volatile Organic Compounds Microalgae Profile

Static headspace sampling was performed with the headspace autosampler, the TriPlus RSH System (Thermo Finnigan, San Francisco, CA, USA). A 2.5 mL headspace syringe for the PAL System was used for the injection of 2 mL from the 20 mL headspace vials with 1 g of a measured dry sample. The autosampler conditions were set as follows: incubation temperature, 80 °C; incubation time, 10 min; syringe temperature, 100 °C; agitator speed, 500 rpm; fill speed, 100 µL/s; pullup delay, 1 s; injection speed, 500 µL/s; pre- and post-injection delay, 500 ms; flush time, 10 s. After each injection, carryover in the syringe was eliminated by an automatic flush of the syringe with carrier gas. Chromatographic separation was achieved by a Thermo Scientific TRACE 1300 gas chromatograph coupled to a Thermo ISQ mass selective detector. A DB-1 (30 m × 0.25 mm i.d.) (Thermo Fisher Scientific, Austin, TX, USA) fused silica capillary column with a 0.25 µm film thickness was used with helium as the carrier gas (purity > 99.9997 vol % and flow rate = 1.0 mL min^{-1}). The oven temperature program was started at 60 °C (not held) and a linear temperature gradient was applied at a rate of 3 °C/min to a final temperature of 260 °C, then held for 5 min (total run time: 65 min). The ion source temperature was kept at 230 °C, the transfer line was at 150 °C, and the mass spectra were obtained in the 50−500 m/z range at an electron energy of 70 eV. The peak areas in the TIC were determined and expressed as normalised relative percentages. The calculated composition was semi-quantitative/qualitative since no standards for each chemical family were co-injected, nor were their response factors determined. Each aliquot was injected in triplicate.

2.3. Bread Preparation

The following ingredients were used to prepare a control dough: 100 g of flour, 4 g of dried yeast, 1 g of salt, 1 g of sugar, and 59 g of distilled water. Water absorption (14% moisture basis) was adjusted through Mixolab2 (Chopin Technologies, France), while a previously study was used for the control formulation (without algae biomass). The dough containing microalgae was prepared at a level of 4% (w/w, in relation to flour) of each microalgae biomass. In a thermomixer (Bimby Vorwerk, Cloyes-sur-le-Loir, France), the

components were combined. Initially, the yeast and sugar dissolved in water at 37 °C in Position 3 for 30 s. Subsequently, the other ingredients were added and mixed at the ear position (special mode for bakery) for 150 s. The dough was placed in a rectangular mould (18.0 × 9.1 cm for length and width dimensions, respectively) and fermented in the electric camera (Arianna XLT133, Cadoneghe, Italy) for 60 min at 35 °C.

The bread was baked at 180 °C in a Johnson A60 oven (Johnson & Johnson, New Brunswick, NJ, USA) for 20 min. After a two-hour cooling to room temperature, the physicochemical analysis of the bread samples was conducted. Three doughs/loaves of each formulation were prepared, and all the analysis were performed in triplicate at minimum.

2.4. Dough Rheology

2.4.1. Mixolab—Mixing and Pasting Curves

The impact of microalgae on the dough during mixing and pasting was assessed using the Mixolab2 instrument (Chopin Technologies, Paris, France) following the Chopin+ protocol. The moisture of the flour and flour in mixture with microalgae biomass was determined through an automatic moisture analyser PMB 202 (Adam Equipment, Oxford, MS, USA).

The optimal water absorption (WA%) of each formulation was determined by achieving a target consistency during mixing at 30 °C (C1) of 1.1 ± 0.07 Nm torque, according to the AACC International Method 54-60.01 [27]. The Mixolab parameters included: Water absorption (WA% at 14% moisture basis): the amount of water required for achieving a dough of appropriate consistency (target); Dough development time (DDT): the time it takes for dough to develop during mixing to reach C1; Dough stability (DS): the duration during which the dough maintains its structural integrity around C1—C1*11%.; C2 (Nm): minimum torque value when the Mixolab starts heating the dough, reflecting the gluten quality; C3 (Nm): peak torque obtained after C2, expressing starch gelatinisation; C4 (Nm): decrease after C3, representing the cooking stability; C5 (Nm): the torque value obtained by the end of the test, representing starch gelification during the cooling stage. The analyses were performed with three replications for each sample.

2.4.2. Viscoelastic Behaviour

The small amplitude oscillatory shear (SAOS) rheology measurements were conducted using a rheometer (Haake Mars III—Thermo Scientific, Karlsruhe, Germany) with a UTC—Peltier system to determine the viscoelastic properties of the unfermented dough, with and without microalgae, at 20 °C. The frequency sweep test allowed the acquisition of the storage (G') and loss (G'') moduli at frequencies ranging from 0.01 Hz to 100 Hz while maintaining a constant shear stress within the linear viscoelastic region of each sample, previously determined for each sample.

The stress sweep test at 1 Hz was performed before the frequency sweep for the determination of the linear viscoelastic region to select the critical stress to be applied during the SAOS measurements. A serrated parallel-plate sensor system (PP20) with a 2 mm gap was employed. The dough, after kneading, was shaped into small portions and placed in the rheometer device while a thin layer of paraffin oil was applied to prevent the water evaporation. At least three repetitions were conducted for each dough sample after 5 min of resting time for temperature stabilisation and structure recovery, predetermined by using a time sweep test at 1 Hz.

2.4.3. Extensional Evaluation

The extensibility of the dough was determined using the Kieffer Dough and Gluten Extensibility Rig for the TA XTplus Texture Analyser (Stable Micro Systems, Surrey, UK) with a 5 kg load cell. Dough samples were prepared without yeast, as previously described, and dough was shaped into uniform rolls and placed onto the Teflon mould and cut into pieces (5.0 × 0.4 × 0.4 cm for width, hight and depth dimensions, respectively), at

40 °C. The experimental conditions for dough bread testing were standardised with a constant speed of 1.0 mm·s^{-1}, a test distance of 80 mm, and a trigger force of 0.049 N. The parameters assessed included the following: peak force (R_{max}), representing the resistance to extension; corresponding extension distance (E_{max}), indicating the distance stretched without fracturing; ratio between peak force and extension distance (R/E) expressed in N·mm^{-1} [28]. To ensure the accuracy of the results, each dough sample was tested at least six times.

2.5. Bread Quality

2.5.1. Texture, Volume, and Colour Measurements

The texture profile of the crumb bread was determined using a TA XTplus Texture Analyser (Stable Micro Systems, Surrey, UK) with a 5 kg load cell. Texture profile analysis was conducted at 20 °C, as previously described by Khemiri et al. [29], with the following settings: a cylindrical probe with a 10 mm diameter piercing 5 mm of the sample at a speed of 1 mm·s^{-1}. Slices of bread of a 2 cm height were taken from the centre. At least three repetitions were conducted for each bread sample.

The volume of loaf bread was determined using the rapeseed displacement method (AACC 10-05.01). The volume was expressed as cubic centimetres (cm^3). Each sample was subjected to a minimum of four repetitions of the test.

The colour of both the crust and crumb was assessed using a Minolta CR-400 colorimeter (Japan), equipped with a standard illuminant D65 and a visual angle of 2°. The CIELAB system with values (L*, a*, b*) was employed for analysis. In this system, L* represents lightness on a scale from 0 to 100, a* indicates greenness to redness in a range between −60 and +60, and b* varies from blueness to yellowness in a range between −60 and +60, respectively. Three repetitions were conducted, with six readings being taken each time.

2.5.2. Nutritional Composition

The chemical composition of the bread samples was determined using AACC International Methodologies [30]. The water activity of the breads was measured in a LabMaster-aw Neo (Novasina AG, Lachen, Switzerland) tester at 25 °C. Moisture was determined according to AOAC 935.29. Ash was determined by incineration at 550 °C in a muffle, as described in AACC 08-01.01. The total protein was assessed according to ISO 16634-2:2016 [31] by the Dumas Nitrogen Analyser NDA 702 (Velp Scientifica, Usmate, Italy) with a conversion factor of 5.70 (wheat). Fat content was determined by using the Soxhlet method with hexane at reflux for 6 h according to AACC 30-25.01. The carbohydrate content was calculated by difference from 100%, considering the total amount of moisture, ash, protein, and total fat contents.

The mineral content (Na, K, Ca, Mg, P, S, Fe, Cu, Zn, and Mn), expressed in mg (element) per 100 g, was evaluated by inductively coupled plasma optical emission spectrometry (ICP-AES: Thermo System, ICAP-7000 series), according to Beltrão Martins et al. [32]. All measurements were repeated at least three times.

2.5.3. Determination of Total Phenolic Compounds and Antioxidant Capacity

The bread samples were subjected to an extraction following the method described by Khemiri et al. [33] with slight adjustments. A 1:10 ratio of the sample was added to 98% analytical-grade methanol (10 mL). Subsequently, the mixture was vortexed for 2 min. The mixtures were then shaken at 100 rpm (Thermo-Scientific-Model: 2871, Waltham, MA, USA) for 24 h at room temperature. After centrifugation at 10,000 rpm for 10 min, the supernatants were filtered through a 0.45 µm.

The total phenolic content in the extracts was determined using the 96-well microplates Folin–Ciocalteu (FC) method given by Zhang et al. [34] and adjusted by Khemiri et al. [33]. An amount of 20 µL of the extract were mixed with 100 µL of FC reagent (1:4). After 5 min, 80 µL of 7.5% sodium carbonate solution were added to the mixture and, after 2 h in the dark at room temperature, the absorbance was measured at 760 nm (auto mix for 30 s before

reading) in the microplate reader of a Thermo Scientific Multiskan GO spectrophotometer (ThermoFisher Scientific). The results were determined as μg of the gallic acid equivalent (GAE)/g of dry extract.

The extract previously described was used to evaluate the antioxidant capacity of the bread samples. The DPPH assay was performed using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and the method described by Fukumoto and Mazza [35] (and adjusted by Khemiri et al. [33]). Samples (20 μL) at different concentrations were mixed with 180 μL of methanolic DPPH solution (60 μM) in 96-well microplates and incubated in the dark for 30 min. The absorbance was measured at 517 nm. All results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC). The ferric reducing capacity was evaluated using the Ferric Reducing Ability Power (FRAP) [36] adjusted by Khemiri et al. [33]. A sample of 25 μL was mixed with 175 μL of pre-warmed FRAP solution at 37 °C and incubated in the dark for 30 min. The absorbance was measured at 595 nm. All data were represented as TEAC.

2.6. Statistical Analysis

The analysis of variance (one-way ANOVA) of the experimental data was performed using IBM SPSS statistic software (version 24, NY, USA), followed by Tukey's test, based on a significance level of 95% ($p < 0.05$).

3. Results

3.1. Volatile Organic Compounds Profile

The sensory experience of eating is significantly influenced by volatile compounds in food, contributing to flavour, texture, and overall appeal [37]. The composition of volatile organic compounds obtained through GC–MS analysis is outlined in Table 2. Notably, aldehydes constitute a significant portion (17.2% in *C. vulgaris*), as do alcohols (6.7% and 31.4% in *T. chuii* and *P. tricornutum*, respectively), ketones (22.9% in *T. chuii*), alkanes (16.8% in *C.vulgaris*), alkenes (ranging from 0.2% in *T. chuii* to 22.1% in *C. vulgaris*), and alkynes (from 0.1% in *T. chuii* to 12.0% in *C. vulgaris*). Additionally, *N*-based compounds were identified across different microalgae strains, with around 48% featuring in *T. chuii*. Terpenoids represented 13% of all compounds in *P. tricornutum*. The presence of 2-pentylfuran was also noted in smaller amounts (1–4%).

Table 2. The volatile compounds composition of microalgae biomasses: *Chlorella vulgaris*, *Phaeodactylum tricornutum* and *Tetraselmis chuii*. *Chlorella vulgaris* (Cv), *Phaeodactylum tricornutum* (Pt) and *Tetraselmis chuii* (Tc).

Compound	Cv	Pt	Tc
Aldehydes	17.2 ± 1.28	8.6 ±2.03	10.4 ± 1.03
Alcohols	11.5 ± 1.66	31.4 ± 3.50	6.7 ± 0.80
Ketones	2.2 ± 0.56	4.1 ± 0.48	22.9 ± 9.48
Alkanes	16.8 ± 4.00	5.6 ± 1.34	1.2 ± 0.22
Alkenes	22.1 ± 3.46	0.7 ± 0.00	0.2 ± 0.03
Alkynes	12.0 ± 0.90	-	0.1 ± 0.00
S-based compounds	4.2 ± 0.31	-	3.0 ± 0.01
N-based compounds	9.4 ± 1.60	32.4 ± 4.75	47.5 ± 6.58
Terpenoids	2.1 ± 0.36	12.9 ± 2.12	6.9 ± 0.89
Other	0.4 ± 0.13	0.6 ± 0.40	0.6 ± 0.44
Total identified compounds	97.8 ± 7.44	96.2 ± 12.46	99.4 ± 14.93
Non-identified compounds	2.2	3.8	0.5
Total	100.0	100.0	100.0

Examining each microalgae strain individually, *T. chuii* stood out for its richness in N-based compounds (48% of all compounds). Ketones represented around 23% of all compounds. Aldehydes, terpenoids, alcohols, and *S*-based compounds constituted 10%, 7%,

7%, and 3%, respectively. Alkanes, alkenes, and alkynes collectively represented under 2% of all compounds identified in *T. chuii*. The volatile composition exhibited significant variation within the same microalgae strain, influenced by growth conditions, harvesting, and storage [37]. For *C. vulgaris*, alkenes constituted 22% of all compounds. Aldehydes ranked as the second most abundant volatile class. Alkanes, alkynes, and alcohols represented approximately 17%, 12%, and 12% of all identified compounds, respectively. N-based compounds were identified in the *C. vulgaris* biomass, accounting for 9% of all compounds. Some authors highlighted aldehydes and *S*-based compounds as major identified volatiles in *C. vulgaris* [38]. *P. tricornutum* showcased richness in both alcohols and *N*-based compounds, representing around 63% of all identified compounds. Terpenoids accounted for 13% of all compounds. Aldehydes were identified in considerable amounts (around 9%), while alkanes and ketones were identified in smaller amounts (6% and 4%, respectively).

As shown in a previous publication about gluten-free breads and the addition of different microalgae species [39], alcohols and terpenes/terpenoids are the main compounds contributing to the overall unpleasant aroma in microalgae-containing breads.

3.2. Dough Rheology

3.2.1. Mixing and Pasting Properties

It is important to investigate the influence of microalgae incorporation on dough rheology during mixing and pasting. Mixolab2 was employed to assess the doughs' behaviours and determine the optimum water absorption % (WA) for optimal consistency (Table 3).

Table 3. Parameters extracted from the pasting curves of control (without algae) and 4% (*w*/*w*) microalgae dough. *Chlorella vulgaris* (Cv), *Phaeodactylum tricornutum* (Pt) and *Tetraselmis chuii* (Tc). Different letters in the same parameter show significant differences ($p < 0.05$, Tukey test).

Sample	WA %	DDT (s)	DS (s)	C2 (N.m)	C3			C4 (N.m)	C5 (N.m)
					Time (s)	Torque (N.m)	T (°C)		
Control	59.0	86 ± 19 ^{c}	574 ± 4 ^{a}	0.44 ± 0.01 ^{a}	1376 ± 6 ^{b}	2.58 ± 0.01 ^{b}	71.50 ± 0.76 ^{c}	2.00 ± 0.00 ^{c}	4.09 ± 0.02 ^{ab}
Cv	60.5	221 ± 23 ^{b}	496 ± 0 ^{b}	0.23 ± 0.00 ^{d}	1465 ± 10 ^{a}	2.12 ± 0.03 ^{c}	72.80 ± 2.42 ^{bc}	2.11 ± 0.02 ^{bc}	3.06 ± 0.04 ^{c}
Pt	58.5	299 ± 5 ^{a}	592 ± 15 ^{a}	0.34 ±0.03 ^{b}	1452 ± 40 ^{a}	2.58 ± 0.07 ^{b}	76.20 ± 0.66 ^{a}	2.24 ± 0.91 ^{b}	4.27 ± 0.12 ^{a}
Tc	57.0	286 ± 23 ^{a}	568 ± 32 ^{a}	0.28 ± 0.00 ^{c}	1478 ± 21 ^{a}	2.74 ± 0.02 ^{a}	76.80 ± 0.14 ^{a}	2.41 ± 0.45 ^{a}	3.98 ± 0.04 ^{b}

The control dough's optimal consistency was set at a water absorption rate of 59.0%. The addition of microalgae resulted in noticeable differences in the dough's water absorption capacity. *C. vulgaris* increased water absorption to 60.5%, while *T. chuii* and *P. tricornutum* decreased WA to 57.0% and 58.5%, respectively (Table 3). The reduction in the water absorption may be due to the high fat content in *T. chuii* compared with the other algae [40]. Additionally,, the increased water absorption in the *C. vulgaris* sample could be attributed to its high cell wall polysaccharides and protein content, which can entrap more water. The available free water is distributed among the hydrophilic components in the microalgae and wheat flour, potentially hindering the formation of the gluten network [41]. Like our findings, Garzon et al. [1] observed significant water absorption when incorporating *C. vulgaris* into sourdough. In contrast to our results, Qazi et al. [42] reported higher water absorption with the incorporation of 12% *T. chuii.* This may be attributed to the high levels of algae, which led to increased protein and polysaccharide content, as explained by Lazo-Velez et al. [43].

Dough development time (DDT) and stability (DS) usually describe the dough's status, with high values indicating strong dough, as documented by Amjid et al. [44]. In this study, it was observed that the microalgae-enriched dough required more time for development compared to the control dough ($p < 0.05$) (Table 3). Specifically, *C. vulgaris* exhibited the shortest development time when compared to *T. chuii* and *P. tricornutum*, although it was

higher than the control dough (221 s vs. 86 s). Interestingly, the presence of *T. chuii* and *P. tricornutum* did not significantly affect the dough's stability ($p > 0.05$) when compared to control. However, the addition of *C. vulgaris* led to a slight but statistically significant ($p < 0.05$) reduction in stability from 574 s to 496 s. The lower stability observed in the *C. vulgaris* dough may be attributed to its increased water absorption. This last result is aligned with the study conducted by Gracia et al. [12]; however, they reported a more pronounced reduction in stability when *C. vulgaris* was added at the same level to wheat flour.

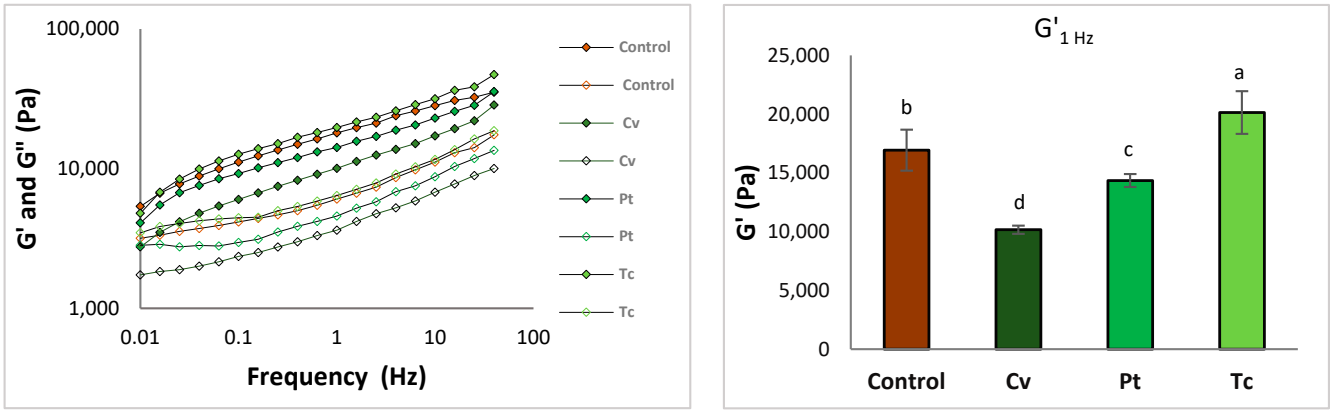
The pasting curve, specifically characterised by the C2 and C3 parameters, revealed distinct patterns (Table 3). In comparison, samples containing microalgae exhibited a significant ($p < 0.05$) decrease in C2 values, which was in contrast to the control (0.44 N.m), with the C. *vulgaris* sample having the lowest C2 value (0.23 N.m) and the *P. tricornutum* having the highest C2 value (0.34 N.m) (Table 3). The decrease in C2 suggests a potential destabilisation of protein structures, as the proteins of microalgae formed a discontinuous matrix with the gluten network [45]. The weaker gluten network significantly impacts gas retention during fermentation, leading to reduced bread volume. This result is supported by research from Dhaka and Khatkar [46], which links lower values of C2, dough stability (DS), and development time (DDT) to reduced dough strength and decreased bread volume. The addition of microalgae significantly ($p < 0.05$) increased the temperature and time required for starch gelatinisation, as indicated by the C3 parameter values. This phenomenon may be attributed to fibre interference and the microalgae's role in hindering water accessibility within starch granules, thereby rendering them more stable at higher temperatures [23,47]. The *T. chuii* sample recorded a torque value of 2.74 N.m, which was higher than that of the control (2.58 N.m), while the *C. vulgaris* sample yielded a lower value of 2.11 N.m. This indicates that the *C. vulgaris* biomass could promote impaired starch gelatinisation, possibly due to the ability of *C. vulgaris* to hold more water, which competes with starch for available water. In a similar trend, *A. platensis* (Spirulina) increased the C3 temperature and impaired starch gelatinisation in the model gel system [41]. In the case of *P. tricornutum*, there was no significant difference in C3 torque compared to the control.

C4 significantly increased ($p < 0.05$) for *P. tricornutum* and *T. chuii* samples, indicating that these microalgae exhibited high amylase activity. There was a decrease in C5 torque when *C. vulgaris* was incorporated, whereas *P. tricornutum*, and *T. chuii* samples did not significantly differ from the control. This suggests that *C. vulgaris* affects starch gelification, possibly due to interactions between *C. vulgaris* components and gluten and starch in the dough system. A similar result was found when *Saccharina latissima* was added to a wheat bread formulation [48].

3.2.2. Dough Viscoelastic Behaviour

Evaluation of the viscoelastic properties of unfermented dough was conducted using oscillatory tests where the results are expressed by the storage (G′) and loss (G″) moduli. As shown in Figure 1, the results revealed that G′ was greater than G″, indicating the viscoelastic nature of the dough with elastic-like behaviour. The presented data demonstrated an increase in both viscoelastic functions with increasing frequencies, for the control and microalgae dough samples. This suggests that the slow recovery of the stressed dough network resulted from its lack of complete elasticity. A similar trend has been reported in other dough formulations, such as gluten-free bread enriched with microalgae, investigated by Nunes et al. [2].

The addition of microalgae resulted in changes in the magnitudes of G′ and G″. In the *T. chuii* dough, higher G′ and G″ values were recorded compared to the control, indicating an improved degree of dough structure (Figure 1a). Conversely, both *P. tricornutum* and *C. vulgaris* recorded lower G′ and G″ values than the control. In terms of the elastic moduli G′ at 1 Hz frequency, *C. vulgaris* had the lowest G′ value, whereas *T.* chuii had the highest (Figure 1b). This suggests that *C. vulgaris* and *P. tricornutum* reduced dough elasticity ($p < 0.005$). The findings obtained from the mechanical spectra are in agreement with the Mixolab results.



(**a**) (**b**)

Figure 1. Mechanical spectra at 20 °C (G′ storage modulus—filled symbol, G″ loss modulus—open symbol) (**a**), and G′ at 1 Hz extracted from each mechanical spectrum (**b**). Control: dough without microalgae; Cv: dough with *Chlorella vulgaris*; Pt: dough with *Phaeodactylum tricornutum*; Tc: dough with *Tetraselmis chuii*. Different letters show significant differences ($p < 0.05$, Tukey test).

The results of this study highlight the variable influence of microalgae on the dough's viscoelasticity and structure, suggesting that it may depend on factors such as their composition, including protein, fat, carbohydrates, and fibre, as well as physical structure factors like cell wall nature and particle size [2,49]. Enzyme activity may also play a role in this variability [50–52].

3.2.3. Dough Extensibility Properties

Considering the impact of microalgae on dough extension, the Kieffer Dough and Gluten Extensibility Rig was used to assess extensibility properties. It is noteworthy that these properties greatly depend on gluten protein quality, and they are important for determining bread volume [53]. A specific level of dough extensibility is essential for achieving optimal baking performance. Excessive dough strength can hinder the formation of sufficient CO_2 gas bubbles during fermentation, resulting in a small loaf. Thus, the dough should have enough extensibility to trap an adequate amount of gas while retaining the strength to prevent gas-cell collapse [54]. Figure 2 presents the relationship between R_{max} and E_{max}, with the low value of the ratio indicating a dough with low R_{max} compared to its E_{max}.

Compared to the control dough, both *P. tricornutum* and *C. vulgaris* doughs exhibited significantly reduced R_{max} ($p < 0.05$), indicating a decrease in dough strength. In contrast, *T. chuii* dough displayed no significant difference from the control, suggesting that it did not impact dough strength. The extensibility parameter did not significantly differ between the microalgae-enriched doughs and the control dough, indicating that microalgae only had a slight effect on the dough's stretching capacity. The R/E ratio provided further insights, with *C. vulgaris* enriched dough demonstrating a notably lower ratio compared to the control and other microalgae-enriched doughs, indicative of an altered balance between strength and extensibility. Nunes et al. [2] found a similar trend, noting that adding freshwater *C. vulgaris* biomass to wheat bread decreased both the R_{max} and (R/E) ratio.

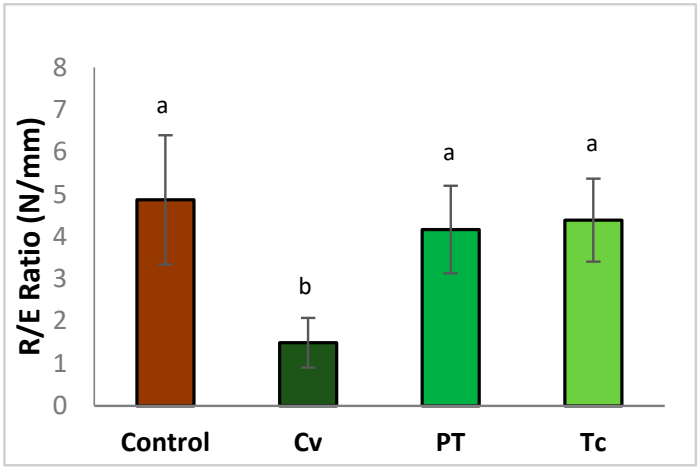


Figure 2. Relationship between the resistance to extension and the extensibility of the dough (R/E) determined using the Kieffer Dough and Gluten Extensibility Rig. Control: dough without microalgae; Cv: dough with *Chlorella vulgaris*; Pt: dough with *Phaeodactylum tricornutum*; Tc: dough with *Tetraselmis chuii.* Different letters in the same parameter show significant differences ($p < 0.05$, Tukey test).

3.3. Technological and Chemical Properties of Bread

The technological quality of bread, including colour, texture, volume, moisture, and water activity, were evaluated and are presented in Tables 4 and 5.

Table 4. Colour parameters (CIELAB system) of bread crumb and crust. Control: without microalgae; *Chlorella vulgaris* (Cv), *Phaeodactylum tricornutum* (Pt), and *Tetraselmis chuii* (Tc). Different letters in the same parameter show significant differences ($p < 0.05$, Tukey test).

Sample	Crust			Crumb		
	L*	a*	b*	L*	a*	b*
Control	63.26 ± 2.31^{a}	7.90 ± 1.88^{a}	32.23 ± 2.52^{a}	63.76 ± 3.28^{a}	-1.05 ± 0.26^{b}	17.70 ± 0.84^{c}
Cv	32.79 ± 1.78^{c}	0.24 ± 0.19^{b}	18.21 ± 1.45^{c}	31.01 ± 2.32^{b}	-4.32 ± 0.23^{c}	25.77 ± 1.04^{b}
Pt	39.63 ± 1.30^{b}	-1.51 ± 0.72^{c}	22.95 ± 1.97^{b}	29.63 ± 1.65^{b}	0.67 ± 0.15^{a}	27.50 ± 1.43^{a}
Tc	38.70 ± 2.40^{b}	0.40 ± 0.70^{b}	22.98 ± 2.00^{b}	31.87 ± 1.88^{b}	-6.46 ± 0.92^{d}	28.83 ± 1.88^{a}

Table 5. Effects of microalgae on the texture properties, volume, moisture, and water activity of bread samples. Control: without microalgae; *Chlorella vulgaris* (Cv), *Phaeodactylum tricornutum* (Pt), and *Tetraselmis chuii* (Tc). Different letters in the same parameter show significant differences ($p < 0.05$, Tukey test).

Sample	Firmness (N)	Cohesiveness	Elasticity	Volume (cm^3)	Moisture (%)	a_w
Control	1.80 ± 0.36^{b}	0.76 ± 0.03^{a}	0.99 ± 0.02^{a}	437.5 ± 46.30^{a}	43 ± 0.13^{a}	0.97 ± 0.00^{a}
Cv	2.42 ± 0.72^{a}	0.74 ± 0.02^{a}	1.04 ± 0.10^{a}	338.0 ± 22.80^{b}	42.8 ± 0.21^{a}	0.97 ± 0.00^{a}
Pt	2.28 ± 0.23^{a}	0.76 ± 0.02^{a}	1.02 ± 0.05^{a}	372.5 ± 45.00^{ab}	42.9 ± 0.50^{a}	0.96 ± 0.00^{b}
Tc	1.69 ± 0.14^{b}	0.77 ± 0.03^{a}	0.99 ± 0.02^{a}	390.0 ± 42.81^{ab}	41.5 ± 0.11^{b}	0.95 ± 0.00^{c}

3.3.1. Bread Colour and Volume

Figure 3 illustrates that bread containing microalgae had a distinctive dark green colour in both the crumbs and crust.

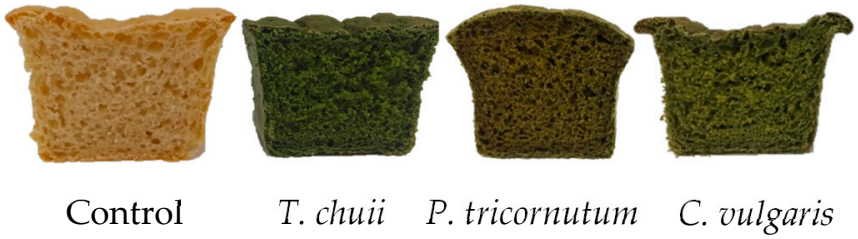


Figure 3. Appearance of breadcrumbs prepared with 4% (*w*/*w*) microalgae in comparison to control bread (without microalgae).

Notably, the crust colour closely corresponds with the crumb. The results in Table 4 revealed significant differences ($p < 0.05$) in the L*, a*, and b* values between bread with microalgae and the control bread. Specifically, *C. vulgaris* had the darkest crust colour and an L* value of 32.79, which was significantly different from *T. chuii* (38.7) and *P. tricornutum* (39.63). Bread containing *P. tricornutum* displayed a more prominent green hue, as indicated by a negative a* value for the crust, while the crumb tended towards redness, characterised by a positive a* value. In all samples, both the crust and crumb were predominantly characterised by a yellow hue, as evidenced by positive b* values. Notably, the crust and crumb of *C. vulgaris* displayed slightly less in degree yellow (b* = 18.2 and b* = 25.8, respectively). The green colour in microalgae-enriched bread is attributed to chlorophylls and carotenoid pigments. Its intensity varies with microalgae levels, aligning with previous research [12].

In this study, the bread volume results (Table 5) showed no significant difference between the control bread and the bread with *P. tricornutum* and *T. chuii*. However, the bread with *C. vulgaris* exhibited a significant reduction ($p < 0.05$) of volume (22.7% decrease). Previous studies have reported variations in the bread volume when different percentages of microalgae were used. Notably, bread enriched with higher levels of up to 8% of *A. platensis*, *C. vulgaris*, *Microchloropsis gaditana,* and *T. chuii* showed a substantial reduction in loaf volume, possibly reaching up to a 40% decrease compared to those with lower additions of 4% [10]. This reduction in volume can be attributed to the interference in the gluten matrix, resulting from the partial replacement of flour with microalgae [55].

3.3.2. Bread Texture

In the investigation of bread crumb texture, no statistically significant differences were observed in cohesiveness and elasticity when compared to the control bread. However, a noticeable increase in bread firmness was evident ($p < 0.05$) in bread formulations containing *P. tricornutum* or *C. vulgaris* compared to the control bread (Table 5). Conversely, *T. chuii* algae did not exhibit significant differences from the control bread, resulting in a softer crumb.

The results obtained from volume evaluation showed consistency with the dough's elastic modulus (G′ at 1 Hz, Figure 1b) and were inversely related to bread firmness (Table 5). The firmness increase has been associated with a volume decrease, resulting in a more compact structure. Similar patterns were noticed in gluten-free dough when incorporating *C. vulgaris* and *T. chuii* (4% *w*/*w*) [22]. However, while the *P. tricornutum* increased the firmness, there was no effect on the bread volume, with is an advantage for the use of this microalgae.

3.3.3. Bread Moisture and Water Activity

Moisture content and water activity (a_w) are relevant factors in assessing both bread quality and its susceptibility to microbial spoilage. Moisture content affects the texture and overall quality of bread, while water activity is important to determining the potential for microbial growth [56]. The results in Table 5 showed no significant differences in moisture content among the control, *C. vulgaris*, and *P. tricornutum* bread samples, whereas the addition of *T. chuii* resulted in a significant reduction ($p < 0.05$) in moisture content.

Likewise, a reduction in moisture content was also observed in wheat bread and crackers containing microalgae [7]. Conversely, another study on gluten-free bread with microalgae indicated no significant change in moisture content [22]. However, the moisture content of enriched bread is aligned with the acceptable range of normal bread, which is between 35 and 45% [57]. No significant changes in water activity were noticed between the control bread and *C. vulgaris* bread samples; lower a_w levels ($p < 0.05$) were obtained in the *T. chuii* and *P. tricornutum* samples (Table 5). Sukhikh et al. [24] observed a reduction in water activity in bread as microalgae levels increased, with the most significant decrease occurring at higher microalgae concentrations. Reduced water activity contributes positively to shelf life. For instance, optimal bread quality is achieved by maintaining a lower water activity level, as higher values increase microbial spoilage. Specifically, the threshold for bacteria and mould growth is around 0.91 a_w [58].

3.4. Nutritional Composition and Bioactivity

3.4.1. Nutritive Value

The incorporation of different microalgae strains into bread resulted in significant changes in their nutritional composition (Table 6) compared to the control sample ($p < 0.05$). The microalgae significantly increased the bread´s protein content, with *C. vulgaris* biomass exhibiting the most pronounced increase (14.7%) in protein content compared to the other algae. In previous research, a significant increase in protein was observed when microalgae were added to baked products, which aligns with our results [1,7]. However, the protein content in the bread samples accounted for at least 12% of the energy value, suggesting that all bread samples, including the control, potentially meet the criteria for nutrition claims according to Regulation (EC) No. 1924/2006 [59]. In terms of lipids and ash, they also increased with the addition of microalgae. However, breads with *C. vulgaris* and *T. chuii* exhibited higher content in regard to both ash and lipid compared to *P. tricornutum*. A similar trend was found when *M. gaditana* and *Chlamydomonas* sp were added to gluten-free bread, resulting in an increase in the lipids and ash [29]. Breads containing *C. vulgaris* and *P. tricornutum* exhibited lower carbohydrate content than the control, while the addition of *T. chuii* resulted in no significant variation in carbohydrate content. In general, it is important to indicate that the protein, lipid, and ash contents in the end products depend on the specific type of algae utilised and the conditions under which they are cultivated.

Table 6. Centesimal composition and energy value of bread samples. Control: Without microalgae; *Chlorella vulgaris* (Cv), *Phaeodactylum tricornutum* (Pt), and *Tetraselmis chuii* (Tc). Different letters in the same parameter show significant differences ($p < 0.05$, Tukey test).

Sample	Moisture g/100 g	Carbohydrates g/100 g	Protein g/100 g	Lipids g/100 g	Ash g/100 g	Energy kJ/100 g
Control	42.97 ± 0.12^{a}	44.75 ± 0.06^{a}	10.62 ± 0.05^{d}	0.03 ± 0.00^{d}	1.63 ± 0.02^{c}	221.62
Cv	42.75 ± 0.15^{a}	42.30 ± 0.21^{c}	12.18 ± 0.02^{a}	0.16 ± 0.00^{a}	2.61 ± 0.06^{a}	219.19
Pt	42.93 ± 0.50^{a}	43.87 ± 0.47^{b}	11.31 ± 0.02^{b}	0.04 ± 0.00^{c}	1.84 ± 0.03^{b}	221.23
Tc	41.47 ± 0.12^{b}	44.75 ± 0.17^{a}	11.08 ± 0.03^{c}	0.14 ± 0.00^{b}	2.56 ± 0.04^{a}	224.46

In general, wheat grains typically contain low concentrations of calcium (Ca), potassium (K), and magnesium (Mg), which can be partially removed with the bran during the milling processes [60]. The addition of microalgae biomass can enhance these mineral contents, which are essential for bone health, nerve function, and maintaining overall well-being. Their presence in a diet is vital for preventing various health issues and supporting vital bodily functions [61].

The mineral profile of wheat bread improved when enriched with microalgae biomass, as shown in Table 7. Bread containing *C. vulgaris* exhibited higher levels of iron (Fe) compared to the control ($p < 0.05$). In contrast, bread with *T. chuii* and *P. tricornutum* did not exhibit a significant difference ($p > 0.05$) (Table 7). It is worth noting that the

incorporation of microalgae increased the levels of potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), sulphur (S), and manganese (Mn). The addition of *T. chuii* resulted in a substantial increase in both Ca and Mg levels, surpassing all other bread formulations. However, *P. tricornutum* increased the K, Mg, S, and Mn in comparison to the other bread samples. According to Regulation (EC) No. 1924/2006 [59], all bread samples, including the control, met the recommended daily values (RDV) for manganese (Mn). At a 4% incorporation level of microalgae, while the remaining studied mineral contents may not currently meet the criteria for nutritional claims, this does offer valuable insights into the fortification potential of microalgae.

Table 7. Minerals content (mg/100 g) of bread samples. Control: Without microalgae; *Chlorella vulgaris* (Cv), *Phaeodactylum tricornutum* (Pt) and *Tetraselmis chuii* (Tc). Different letters in the same parameter show significant differences ($p < 0.05$, Tukey test). Nd—non determined.

	Major Minerals (mg/100 g)					
	Na	**K**	**Ca**	**Mg**	**P**	**S**
Control	166.40 ± 0.45 ^{d}	111.97 ± 0.61 ^{d}	9.56 ± 0.07 ^{d}	12.27 ± 0.24 ^{d}	60.63 ± 0.87 ^{d}	58.27 ± 0.27 ^{d}
Cv	208.36 ± 1.31 ^{c}	132.73 ± 1.91 ^{c}	11.07 ± 0.30 ^{c}	14.05 ± 0.06 ^{c}	74.28 ± 1.24 ^{a}	72.50 ± 1.08 ^{c}
Pt	281.18 ± 2.19 ^{a}	186.62 ± 1.05 ^{a}	18.33 ± 0.65 ^{b}	25.10 ± 0.44 ^{a}	66.82 ± 0.67 ^{b}	112.53 ± 1.41 ^{a}
Tc	260.05 ± 1.28 ^{b}	141.01 ± 1.62 ^{b}	40.61 ± 0.32 ^{a}	24.89 ± 0.11 ^{b}	61.78 ± 0.20 ^{c}	84.82 ± 0.38 ^{b}
15% RDV (mg/100 g)	Nd	300.0	120.0	56.3	105.0	Nd
	Trace Minerals (mg/100 g)					
	Fe	Cu	Zn	Mn		
Control	0.93 ± 0.04 ^{b}	0.06 ± 0.00 ^{b}	0.69 ± 0.00 ^{b}	0.37 ± 0.00 ^{c}		
Cv	1.14 ± 0.09 ^{a}	0.09 ± 0.01 ^{a}	0.71 ± 0.00 ^{a}	0.43 ± 0.00 ^{b}		
Pt	0.93 ± 0.00 ^{b}	0.07 ± 0.00 ^{ab}	0.71 ± 0.02 ^{a}	0.50 ± 0.00 ^{a}		
Tc	0.96 ± 0.00 ^{b}	0.08 ± 0.00 ^{ab}	0.68 ± 0.01 ^{b}	0.41 ± 0.00 ^{b}		
15% RDV (mg/100 g)	2.1	0.2	1.5	0.3		

Considering the sodium content (Na), it was observed in Table 7 that bread samples enriched with *P. tricornutum* and *T. chuii* contained 281.2 and 260.1 mg/100 g, respectively. However, it is imperative to emphasise that addressing this high sodium content can be achieved through the reduction of sodium derived from the salt source.

3.4.2. Total Phenolic Compounds and Antioxidant Activity

In recent years, there has been a growing interest in phenolic compounds derived from algae due to their antioxidant, antimicrobial, and immunomodulatory properties [38,62–64]. Murray et al. [4] demonstrated their potential in addressing cardiovascular disease and diabetes, while Jerez-Martel et al. [65] emphasised their antioxidant potential. Despite these promising results, it is important to assess the biological activity of the phenolic compounds within the food matrix and evaluate their stability during baking.

In this study, the addition of microalgae to bread formulations significantly increased ($p < 0.05$) the total phenolic compounds (TPC) and antioxidant capacity (FRAP and DPPH) (Figure 4). *T. chuii* bread presented the highest phenolic content (38.52 µg GAE/g), followed by *P. tricornutum* (32.38 µg GAE/g) and *C. vulgaris* (17.94 µg GAE/g) (Figure 4a). These results are consistent with the findings of Grácio et al. [26], who characterised the nutritional profile and bioactivity of *C. vulgaris* and *T. chuii* biomasses used in the present study, indicating the exceptionally high phenolic content of the *T. chuii* biomass. In a study conducted by Qazi et al. [22], a comparable trend was identified, indicating that, upon incorporation into gluten-free bread, *T. chuii* exhibited a greater phenolic content than *C. vulgaris*.

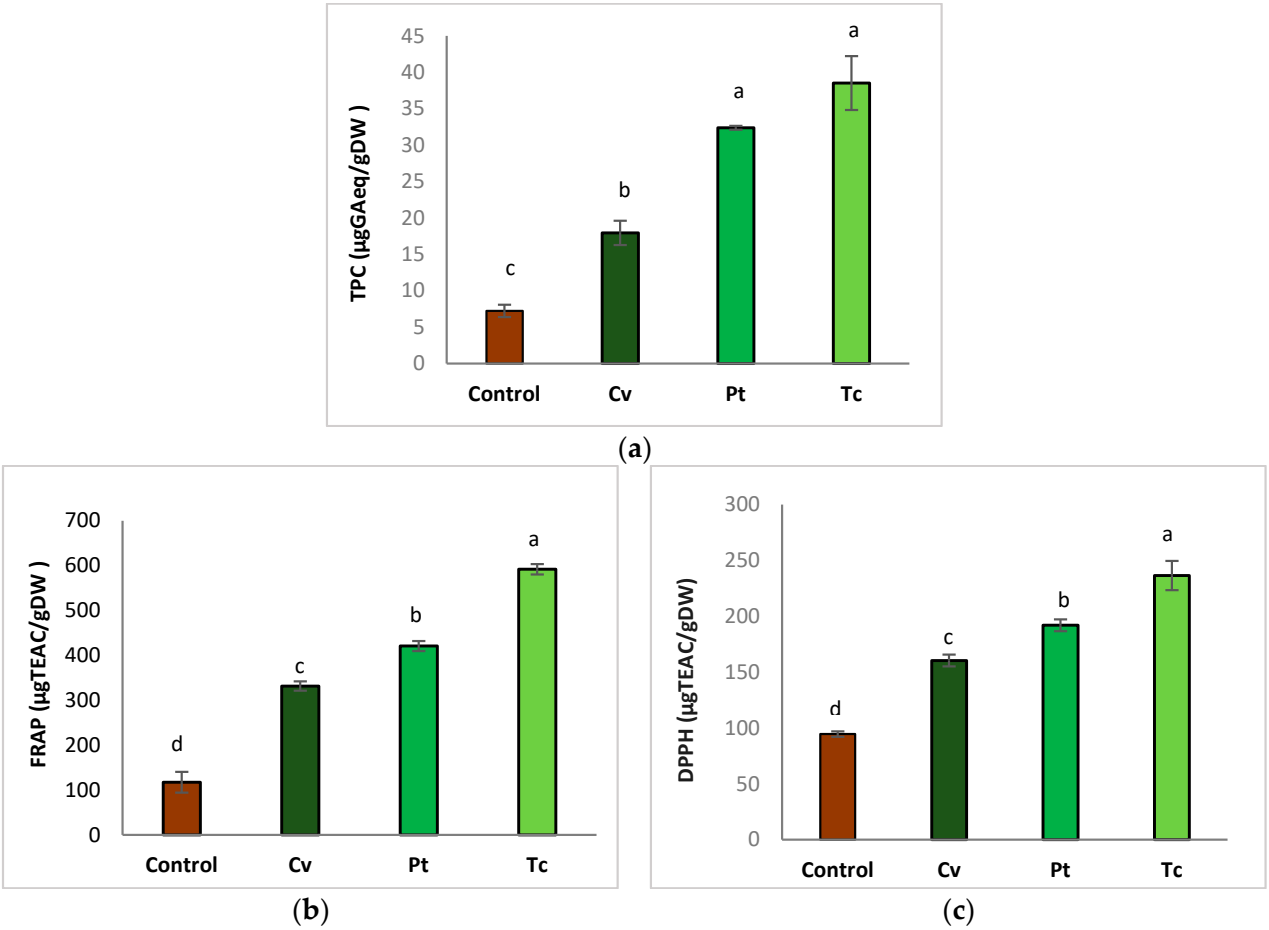


Figure 4. Total phenolic content (**a**), antioxidant capacity by FRAP (ferric ion reducing antioxidant power) (**b**), and DPPH (radical scavenging activity) (**c**). Control: bread without microalgae; Cv: bread with *Chlorella vulgaris*; Pt: bread with *Phaeodactylum tricornutum*; Tc: bread with *Tetraselmis chuii*. Different letters in the same parameter show significant differences ($p < 0.05$, Tukey test).

The antioxidant capacity of the bread samples was assessed using the FRAP and DPPH assays, which revealed a positive relation between the total phenolic content and antioxidant capacity (Figure 4b,c). Results indicate that *T. chuii*-enriched bread displayed the highest antioxidant capacity, with a FRAP value of 592.0 µg TEAC/g, followed by *P. tricornutum* (420.9 µg TEAC/g) and *C. vulgaris* (331.7 µg TEAC/g). Similarly, the DPPH assay showed values of 160.3 µg TEAC/g for bread containing *C. vulgaris*, 236.4 µg TEAC/g for *T. chuii*, and 192.0 µg TEAC/g for *P. tricornutum*. A similar trend was observed in a study conducted by Hernández-López et al. [66] where the addition of Spirulina to bread formulation resulted in high antioxidant capacity. Additionally, an increase in antioxidant capacity was observed when microalgae were added to other staple foods, such as pasta [67] and broccoli soup [7]. In addition to phenolic compounds, microalgae are rich in chlorophyll, carotenoids, and vitamin E, all of which contribute to their antioxidant activity [21,68]. *T. chuii* is notable also for its high content of protein and peptide fractions, which play a key role in its antioxidant activity [69], while *P. tricornutum* is distinguished by its high fucoxanthin content [21].

4. Conclusions

In this study, three different algae species clearly showed their effect on dough rheology, bread quality, and nutritional attributes. Notably, *T. chuii* and *P. tricornutum* performed better than *C. vulgaris* in these attributes. *C. vulgaris* led to increased water absorption, coupled with decreasing dough stability, protein network, starch gelatinisation, starch ret-

rogradation, and elasticity, resulting in lower bread volume and a higher crumb firmness. *P. tricornutum* caused decreased water absorption and a reduction in the protein network but exhibited no significant impact on dough stability, starch gelatinisation, or starch retrogradation. Consequently, it had no significant effect on bread volume. *T. chuii* decreased water absorption and the protein network, with no significant impact on dough stability, starch gelatinisation, or starch retrogradation. However, it notably increased elasticity, which did not impact bread volume or texture. Regarding nutritional attributes, the incorporation of microalgae enhanced the nutritional composition of the final product, resulting in higher levels of protein and some minerals. Additionally, it led to improvements in total phenolics and antioxidant capacity, with *T. chuii* exhibiting the highest increase. Overall, *T. chuii* emerged as the best formulation, exhibiting superior mechanical properties and bioactivity across the bread samples. Within the scope of the YUMAlgae project, sensory enzymes will be employed to enhance the overall aroma and colour of microalgae ingredients. These improved ingredients will undergo testing in bread formulations, aiming to attain elevated levels of microalgae that result in favourable health impacts and high sensory acceptance from consumers.

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References

1. Garzon, R.; Skendi, A.; Lazo-Velez, M.A.; Papageorgiou, M.; Rosell, C.M. Interaction of dough acidity and microalga level on bread quality and antioxidant properties. *Food Chem.* **2021**, *344*, 128710. [CrossRef] [PubMed]
2. Nunes, M.C.; Graça, C.; Vlaisavljević, S.; Tenreiro, A.; Sousa, I.; Raymundo, A. Microalgal cell disruption: Effect on the bioactivity and rheology of wheat bread. *Algal Res.* **2020**, *45*, 101749. [CrossRef]
3. Demarco, M.; Oliveira de Moraes, J.; Matos, Â.P.; Derner, R.B.; de Farias Neves, F.; Tribuzi, G. Digestibility, bioaccessibility and bioactivity of compounds from algae. *Trends Food Sci. Technol.* **2022**, *121*, 114–128. [CrossRef]
4. Murray, M.; Dordevic, A.L.; Ryan, L.; Bonham, M.P. An emerging trend in functional foods for the prevention of cardiovascular disease and diabetes: Marine algal polyphenols. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 1342–1358. [CrossRef] [PubMed]
5. Verni, M.; Demarinis, C.; Rizzello, C.G.; Pontonio, E. Bioprocessing to preserve and improve microalgae nutritional and functional potential: Novel insight and perspectives. *Foods* **2023**, *12*, 983. [CrossRef] [PubMed]
6. Loke Show, P. Global market and economic analysis of microalgae technology: Status and perspectives. *Bioresour. Technol.* **2022**, *357*, 127329. [CrossRef]
7. Lafarga, T.; Mayre, E.; Echeverria, G.; Vinas, I.; Villaro, S.; Acien-Fernandez, F.G.; Castellari, M.; Aguilo-Aguayo, I. Potential of the microalgae *Nannochloropsis* and *Tetraselmis* for being used as innovative ingredients in baked goods. *LWT-Food Sci. Technol.* **2019**, *115*, 108439. [CrossRef]
8. Ampofo, J.; Abbey, L. Microalgae: Bioactive Composition, Health benefits, safety and prospects as potential high-value ingredients for the functional food industry. *Foods* **2022**, *11*, 1744. [CrossRef]
9. Caporgno, M.P.; Mathys, A. Trends in microalgae incorporation into innovative food products with potential health benefits. *Front. Nutr.* **2018**, *5*, 58. [CrossRef]
10. Qazi, W.M.; Ballance, S.; Kousoulaki, K.; Uhlen, A.K.; Kleinegris, D.M.M.; Skjanes, K.; Rieder, A. Protein enrichment of wheat bread with microalgae: *Microchloropsis gaditana*, *Tetraselmis chuii* and *Chlorella vulgaris*. *Foods* **2021**, *10*, 3078. [CrossRef]
11. Pereira, T.; Costa, S.; Barroso, S.; Teixeira, P.; Mendes, S.; Gil, M.M. Development and optimization of high-protein and low-saturated fat bread formulations enriched with lupin and microalgae. *LWT-Food Sci. Technol.* **2024**, *191*, 115612. [CrossRef]

12. Graça, C.; Fradinho, P.; Sousa, I.; Raymundo, A. Impact of *Chlorella vulgaris* on the rheology of wheat flour dough and bread texture. *LWT-Food Sci. Technol.* **2017**, *89*, 466–474. [CrossRef]
13. Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.Y.; Vaca-Garcia, C. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renew. Sustain. Energy Rev.* **2012**, *35*, 265–278. [CrossRef]
14. Justo, G.Z.; Silva, M.R.; Queiroz, M.L.S. Effects of the green algae *Chlorella vulgaris* on the response of the host hematopoietic system to intraperitoneal Ehrlich ascites tumor transplantation in mice. *Immunopharmacol. Immunotoxicol.* **2001**, *23*, 119–132. [CrossRef]
15. de Souza Queiroz, J.; Barbosa, C.M.V.; da Rocha, M.C.; Bincoletto, C.; Paredes-Gamero, E.J.; de Souza Queiroz, M.L.; Palermo Neto, J. *Chlorella vulgaris* treatment ameliorates the suppressive effects of single and repeated stressors on hematopoiesis. *Brain Behav. Immun.* **2013**, *29*, 39–50. [CrossRef]
16. Pina-Pérez, M.C.; Rivas, A.; Martínez, A.; Rodrigo, D. Antimicrobial potential of macro and microalgae against pathogenic and spoilage microorganisms in food. *Food Chem.* **2017**, *235*, 34–44. [CrossRef] [PubMed]
17. Toro, V.; Siquier-Coll, J.; Bartolomé, I.; Robles-Gil, M.C.; Rodrigo, J.; Maynar-Mariño, M. Effects of *Tetraselmis chuii* microalgae supplementation on ergospirometric, haematological and biochemical parameters in amateur soccer players. *Int. J. Environ. Res. Public Health* **2020**, *17*, 6885. [CrossRef]
18. Sharp, M.; Wilson, J.; Stefan, M.; Gheith, R.; Lowery, R.; Ottinger, C.; Reber, D.; Orhan, C.; Sahin, N.; Tuzcu, M.; et al. Marine phytoplankton improves recovery and sustains immune function in humans and lowers proinflammatory immunoregulatory cytokines in a rat model. *Phys. Act. Nutr.* **2021**, *25*, 42–55. [CrossRef] [PubMed]
19. García, Á.; Toro-Román, V.; Siquier-Coll, J.; Bartolomé, I.; Muñoz, D.; Maynar-Mariño, M. Effects of *Tetraselmis chuii* microalgae supplementation on anthropometric, hormonal and hematological parameters in healthy young men: A double-blind study. *Int. J. Environ. Res. Public Health* **2022**, *19*, 6060. [CrossRef] [PubMed]
20. Celi, C.; Fino, D.; Savorani, F. *Phaeodactylum tricornutum* as a source of value-added products: A review on recent developments in cultivation and extraction technologies. *Bioresour. Technol. Rep.* **2022**, *19*, 101122. [CrossRef]
21. Batista, A.P.; Niccolai, A.; Fradinho, P.; Fragoso, S.; Bursic, I.; Rodolfi, L.; Biondi, N.; Tredici, M.R.; Sousa, I.; Raymundo, A. Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and in vitro digestibility. *Algal Res.* **2017**, *26*, 161–171. [CrossRef]
22. Qazi, M.W.; de Sousa, I.G.; Nunes, M.C.; Raymundo, A. Improving the nutritional, structural, and sensory properties of gluten-free bread with different species of microalgae. *Foods* **2022**, *11*, 397. [CrossRef] [PubMed]
23. Bhatnagar, P.; Gururani, P.; Parveen, A.; Gautam, P.; Kumar, V. Algae: A promising and sustainable protein-rich food ingredient for bakery and dairy products. *Food Chem.* **2023**, *441*, 138322. [CrossRef] [PubMed]
24. Sukhikh, S.; Ivanova, S.; Dolganyuk, V.; Pilevinova, I.; Prosekov, A.; Ulrikh, E.; Noskova, S.; Michaud, P.; Babich, O. Evaluation of the prospects for the use of microalgae in functional bread production. *Appl. Sci.* **2022**, *12*, 12563. [CrossRef]
25. Švec, I.; Hrušková, M. The Mixolab parameters of composite wheat/hemp flour and their relation to quality features. *LWT-Food Sci. Technol.* **2015**, *60*, 623–629. [CrossRef]
26. Grácio, M.; Ferreira, J.; Pagarete, A.; Nunes, C.; Raymundo, A. Potential health benefits of different microalgae strains. In Proceedings of the Young Algaeneers Symposium (YAS), Faro, Portugal, 9–11 May 2023.
27. AACC International Method. *Determination of Rheological Behavior as a Function of Mixing and Temperature Increase in Wheat Flour and Whole Wheat Meal by Mixolab*; Method 54–60; AACC Int: St. Paul, MN, USA, 2010; pp. 1–6.
28. Macedo, C.; Nunes, M.C.; Sousa, I.; Raymundo, A. Rheology methods as a tool to study the impact of whey powder on the dough and breadmaking performance of wheat flour. *Fluids* **2020**, *5*, 50. [CrossRef]
29. Khemiri, S.; Khelifi, N.; Nunes, M.C.; Ferreira, A.; Gouveia, L.; Smaali, I.; Raymundo, A. Microalgae biomass as an additional ingredient of gluten-free bread: Dough rheology, texture quality and nutritional properties. *Algal Res.* **2020**, *50*, 101998. [CrossRef]
30. AACC. Approved Methods of Analysis 2023. 11th ed. Available online: https://www.cerealsgrains.org/resources/Methods/Pages/default.aspx (accessed on 17 September 2023).
31. *ISO 16634-2:2016*; Food Products. Determination of the Total Nitrogen Content by Combustion according to the Dumas Principle and Calculation of the Crude Protein Content. Part 2: Cereals, Pulses And Milled Cereal Products. ISO: Geneva, Switzerland, 2016.
32. Martins, R.B.; Gouvinhas, I.; Nunes, M.C.; Peres, J.A.; Raymundo, A.; Barros, A.I.R.N.A. Acorn flour as a source of bioactive compounds in gluten-free bread. *Molecules* **2020**, *25*, 3568. [CrossRef]
33. Khemiri, S.; Khelifi, N.; Messaoud, C.; Smaali, I. Bioprospecting of microalgae for a potential use as enzyme inhibitors, anti-ageing and prebiotic agents. *Biocatal. Agric. Biotechnol.* **2023**, *51*, 102759. [CrossRef]
34. Zhang, Q.; Zhang, J.; Shen, J.; Silva, A.; Dennis, D.A.; Barrow, C.J. A simple 96-well microplate method for estimation of total polyphenol content in seaweeds. *J. Appl. Phycol.* **2006**, *18*, 445–450. [CrossRef]
35. Fukumoto, L.R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **2002**, *48*, 3597–3604. [CrossRef]
36. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol.* **1995**, *28*, 25–30. [CrossRef]
37. Nunes, M.C.; Ferreira, J.; Raymundo, A. Volatile fingerprint impact on the sensory properties of microalgae and development of mitigation strategies. *Curr. Opin. Food Sci.* **2023**, *51*, 101040. [CrossRef]

38. Cichoński, J.; Chrzanowski, G. Microalgae as a source of valuable phenolic compounds and carotenoids. *Molecules* **2022**, *27*, 8852. [CrossRef] [PubMed]
39. Lafarge, C.; Cayot, N. Insight on a comprehensive profile of volatile compounds of *Chlorella vulgaris* extracted by two "green" methods. *Food Sci. Nutr.* **2019**, *7*, 918–929. [CrossRef] [PubMed]
40. Sudha, M.L.; Srivastava, A.K.; Vetrimani, R.; Leelavathi, K. Fat replacement in soft dough biscuits: Its implications on dough rheology and biscuit quality. *J. Food Eng.* **2007**, *80*, 922–930. [CrossRef]
41. Batista, A.P.; Nunes, M.C.; Raymundo, A.; Gouveia, L.; Sousa, I.; Cordobés, F.; Guerrero, A.; Franco, J.M. Microalgae biomass interaction in biopolymer gelled systems. *Food Hydrocoll.* **2011**, *25*, 817–825. [CrossRef]
42. Qazi, W.M.; Ballance, S.; Uhlen, A.K.; Kousoulaki, K.; Haugen, J.E.; Rieder, A. Protein enrichment of wheat bread with the marine green microalgae *Tetraselmis chuii*—Impact on dough rheology and bread quality. *LWT-Food Sci. Technol.* **2021**, *143*, 111115. [CrossRef]
43. Lazo-Velez, M.A.; Mata-Ramírez, D.; Serna-Saldivar, S.O.; Chuck-Hernandez, C. Functional effects of soybean concentrates obtained from sprouted seeds enriched in selenium in wheat breadmaking. *Cereal Chem.* **2017**, *94*, 740–745. [CrossRef]
44. Amjid, M.R.; Shehzad, A.; Hussain, S.; Shabbir, M.A.; Khan, M.R.; Shoaib, M. A comprehensive review on wheat flour dough rheology. *Pak. J. Food Sci.* **2013**, *23*, 105–123.
45. Rosell, C.M.; Collar, C.; Haros, M. Assessment of hydrocolloid effects on the thermo-mechanical properties of wheat using the Mixolab. *Food Hydrocoll.* **2007**, *21*, 452–462. [CrossRef]
46. Dhaka, V.; Khatkar, B.S. Mixolab thermomechanical characteristics of dough and bread making quality of Indian wheat varieties. *Qual. Assur. Saf. Crops Foods* **2013**, *5*, 311–323. [CrossRef]
47. Martínez-Sanz, M.; Fabra, M.J.; Gómez-Mascaraque, L.G.; López-Rubio, A. Structural effects of microalgae additives on the starch gelatinisation process. *Food Hydrocoll.* **2018**, *77*, 257–269. [CrossRef]
48. Oprea, O.B.; Tolstorebrov, I.; Claussen, I.C.; Sannan, S.; Apostol, L.; Moșoiu, C.; Gaceu, L. Potential for *Saccharina latissima* flour as a functional ingredient in the baking sector. *Foods* **2023**, *12*, 4498. [CrossRef] [PubMed]
49. Ahmed, J.; Kumar, V. Effect of high-pressure treatment on oscillatory rheology, particle size distribution and microstructure of microalgae *Chlorella vulgaris* and *Arthrospira platensis*. *Algal Res.* **2022**, *62*, 102617. [CrossRef]
50. dos Brasil, B.S.A.F.; de Siqueira, F.G.; Salum, T.F.C.; Zanette, C.M.; Spier, M.R. Microalgae and cyanobacteria as enzyme biofactories. *Algal Res.* **2017**, *25*, 76–89. [CrossRef]
51. Dura, A.; Rosell, C.M. Enzymes in baking. In *Microbrobial Enzyme Technology in Food Applications*; CRC Press: Boca Raton, FL, USA, 2017; pp. 295–314. [CrossRef]
52. Thapa, S.; Li, H.; OHair, J.; Bhatti, S.; Chen, F.C.; Nasr, K.A.; Johnson, T.; Zhou, S. Biochemical characteristics of microbial enzymes and their significance from industrial perspectives. *Mol. Biotechnol.* **2019**, *61*, 579–601. [CrossRef] [PubMed]
53. Dapcevic, T.; Pojic, M.; Hadnaev, M.; Torbic, A. The Role of Empirical Rheology in Flour Quality Control. In *Wide Spectra of Quality Control*; InTech: London, UK, 2011. [CrossRef]
54. Caffe-Treml, M.; Glover, K.D.; Krishnan, P.G.; Hareland, G.A.; Bondalapati, K.D.; Stein, J. Effect of wheat genotype and environment on relationships between dough extensibility and breadmaking quality. *Cereal Chem.* **2011**, *88*, 201–208. [CrossRef]
55. Jaekel, L.Z.; da Silva, C.B.; Steel, C.J.; Chang, Y.K. Influência da adição de xilanase nas características de pão de forma preparado com farinha de trigo comum ou farinha de trigo de grão inteiro. *Cienc. Tecnol. Aliment.* **2012**, *32*, 844–849. [CrossRef]
56. Ayub, M.; Wahab, S.; Durrani, Y. Effect of water activity (Aw), moisture content and total microbial count on the overall quality of bread. *Int. J. Agric. Biol.* **2003**, *5*, 274–278.
57. Ibrahim, U.K.; Rahman, N.A.A.; Suzihaque, M.U.H.; Hashib, S.A.; Aziz, R.A.A. Effect of baking conditions on the physical properties of bread incorporated with green coffee beans (GCB). *IOP Conf. Ser. Mater. Sci. Eng.* **2020**, *736*, 062019. [CrossRef]
58. Allen, L.V. Quality Control: Water Activity Considerations for Beyond-use Dates. *Int. J. Pharm. Compd.* **2018**, *22*, 288–293.
59. European Parliament & Council. Regulation (EC) 1924/2006 on nutrition and health claims made on foods. *Off. J. Eur. Communities* **2006**, *L404*, 1–15.
60. Cobalchin, F.; Volpato, M.; Modena, A.; Finotti, L.; Manni, F.; Panozzo, A.; Vamerali, T. Biofortification of common wheat grains with combined Ca, Mg, and K through foliar fertilisation. *Agronomy* **2021**, *11*, 1718. [CrossRef]
61. Gharibzahedi, S.M.T.; Jafari, S.M. The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation. *Trends Food Sci. Technol.* **2017**, *62*, 119–132. [CrossRef]
62. Silva, M.; Kamberovic, F.; Uota, S.T.; Kovan, I.M.; Viegas, C.S.B.; Simes, D.C.; Gangadhar, K.N.; Varela, J.; Barreira, L. Microalgae as potential sources of bioactive compounds for functional foods and pharmaceuticals. *Appl. Sci.* **2022**, *12*, 5877. [CrossRef]
63. Jimenez-Lopez, C.; Pereira, A.G.; Lourenço-Lopes, C.; Garcia-Oliveira, P.; Cassani, L.; Fraga-Corral, M.; Prieto, M.; Simal-Gandara, J. Main bioactive phenolic compounds in marine algae and their mechanisms of action supporting potential health benefits. *Food Chem.* **2021**, *341 Pt 2*, 128262. [CrossRef]
64. Machu, L.; Misurcova, L.; Ambrozova, J.V.; Orsavova, J.; Mlcek, J.; Sochor, J.; Jurikova, T. Phenolic content and antioxidant capacity in algal food products. *Molecules* **2015**, *20*, 1118–1133. [CrossRef] [PubMed]
65. Jerez-Martel, I.; García-Poza, S.; Rodríguez-Martel, G.; Rico, M.; Afonso-Olivares, C.; Gómez-Pinchetti, J.L. Phenolic profile and antioxidant activity of crude extracts from microalgae and cyanobacteria strains. *J. Food Qual.* **2017**, *2017*, 2924508. [CrossRef]
66. Hernández-López, I.; Alamprese, C.; Cappa, C.; Prieto-Santiago, V.; Abadias, M.; Aguiló-Aguayo, I. Effect of Spirulina in bread formulated with wheat flours of different alveograph strength. *Foods* **2023**, *12*, 3724. [CrossRef] [PubMed]

67. Rodríguez De Marco, E.; Steffolani, M.E.; Martínez, C.S.; León, A.E. Effects of spirulina biomass on the technological and nutritional quality of bread wheat pasta. *LWT-Food Sci. Technol.* **2014**, *58*, 102–108. [CrossRef]
68. Wang, H.M.; Pan, J.L.; Chen, C.Y.; Chiu, C.C.; Yang, M.H.; Chang, H.W.; Chang, J.S. Identification of anti-lung cancer extract from *Chlorella vulgaris* C-C by antioxidant property using supercritical carbon dioxide extraction. *Process Biochem.* **2010**, *45*, 1865–1872. [CrossRef]
69. Coulombier, N.; Blanchier, P.; Le Dean, L.; Barthelemy, V.; Lebouvier, N.; Jauffrais, T. The effects of CO_2-induced acidification on *Tetraselmis* biomass production, photophysiology and antioxidant activity: A comparison using batch and continuous culture. *J. Biotechnol.* **2021**, *325*, 312–324. [CrossRef] [PubMed]