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Development and optimization of high-protein and low-saturated fat bread formulations enriched with lupin and microalgae

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ABSTRACT

Increased consumer awareness of healthier foods is driving the growth in the functional bread market. In view of this potential, three bread formulations were developed using various types of flour enriched with microalgae. The multigrain breads were composed of lupin and rye (F–R), lupin and spelt (F–S), and lupin, oats, and carob (F-OC) enriched with a mixture of *Chlorella vulgaris* (*C. vulgaris* White and *C. vulgaris* Smooth (4:1)). All breads were high in protein and low in saturated fat. Response surface methodology (RSM) following a central composite design (CCD) was used to evaluate the effect of selected technological parameters, namely water content (64.6–94.6% (w/w of flours)) and microalgae concentration (1.9–3.9% (w/w of flours)) on color, aroma, taste, texture, and overall sensory acceptance of the products. Only water content was found to affect the bread's sensory scores, especially texture, with higher water content increasing bread acceptance. This study allowed the development and optimization of three novel multigrain bread formulations enriched with microalgae that met the requirements of "rich in proteins" and "low in saturated fats" claims.

1. Introduction

Bread is one of the most important foods in the human nutrition and a staple of the diet and cultural identity of communities worldwide. In Europe, bread consumption varies from country to country, nevertheless the average consumption is of 57 kg/person per year (AHFES (Atlantic Area Healthy Food Eco-System), 2021). However, food sensitivities and changing diets are making traditional wheat bread unpopular with many people. A trend that is confirmed by the growth of the functional and fortified bread market, which is expected to reach $260,930.3 Mn by 2027 (The Insight Partners, 2021). Additionally, the consumption of whole-grain breads has been associated with health benefits, such as a reduced risk of developing type 2 diabetes mellitus and other chronic diseases (Sanders, Zhu, Wilcox, Koecher, & Maki, 2023; Schadow et al., 2023).

Taking into account the new food and health trends, some attention has been given to the development of novel multigrain breads with an improved nutritional profile, with the use of alternative flours such as legumes being evaluated for this purpose. Lupin is a high-protein and high dietary fiber legume, that presents a slightly beany flavor and yellow coloration (Hall & Johnson, 2004; Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2016). It has been used as a wheat replacement in various products such as bread (Correia, Gonzaga, Batista, Beirão-costa, & Guiné, 2015; Lee et al., 2006; Pleming, Farahnaky, & Majzoobi, 2021; Villarino et al., 2016), chips (Çoban et al., 2021), and pasta (Jayasena, Leung, & Nasar-Abbas, 2008; Jayasena & Nasar-Abbas, 2012). The low elasticity of the lupin proteins and the high water-holding capacity of its dietary fibre can be a disadvantage when incorporating it into breads, as higher levels of addition will alter the bread's properties (Turnbull, Baxter, & Johnson, 2005; Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2014). Lupin in bread has been shown to improve the mineral content and bioactivities of bread and to increase satiety, which helps to reduce energy intake (Lee et al., 2006; Plustea et al., 2022). Moreover, a lower glycemic index has been found in lupin chips, with the lowest index found in chips with higher lupin content (Çoban et al., 2021).

Algae have also been used in several food products to enhance their nutritional value (Caporgno & Mathys, 2018; Gohara-Beirigo, Matsudo,

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Cezare-Gomes, Carvalho, & Danesi, 2022; Lafarga, 2019). In bread, microalgae such as *Chlorella sorokiniana*, *Nannochloropsis* sp., *Chlamydomonas* sp., and *Tetraselmis* sp. have been applied to enhance the nutritional quality by increasing the protein content, improving the fatty acid and mineral profile and the amount of carotenoid and polyphenol compounds (Diprat, Silveira Thys, Rodrigues, & Rech, 2020; Khemiri et al., 2020; Lafarga et al., 2019; Nunes, Fernandes, Vasco, Sousa, & Raymundo, 2020). Furthermore, environmental concerns and social norms influence positively the willingness to pay more for microalgae-based products, including bread (Maehle & Skjeret, 2022).

Chlorella vulgaris is a green freshwater microalga that has been approved for use in food by the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) (European Commission, 2023; Machado et al., 2020; Molino et al., 2018). However, although it has great theoretical potential for use in food fortification, one of its main drawbacks is its taste, which prevents its use in significant quantities. This has led to the research and development of new strains of C. vulgaris with reduced chlorophyll (the major contributor to the characteristic grassy flavor) (Schüler et al., 2020). These strains have an improved taste that facilitates their application in larger quantities and have reached the market with denominations such as *C. vulgaris* Smooth, *C. vulgaris* White, and *C. vulgaris* Honey (Allma, 2023).

In EU countries, for a food product to bear a nutrition claim, it must meet certain conditions that are explicitly set out in Regulation N° 1924/2020 (European Parliament and of the Council, 2006). The claim “rich in protein” can only be applied to products where the protein provides at least 20% of the energy value, while the claim “low in saturated fat” can only be applied to products where the sum of the saturated fat is less than 1.5 g/100 g and does not provide more than 10% of the energy value (European Parliament and of the Council, 2006).

Considering recent dietary trends and food intolerances, the main objectives of this study were the development and optimization of novel formulations of functional multigrain breads enriched with microalgae, high in protein and low in saturated fat. These novel breads aim to expand the range of fortified bakery products for consumers seeking high-protein and low-saturated fat diets.

2. Materials and methods

2.1. Microalgae

The microalgae, *C. vulgaris* Smooth and *C. vulgaris* White, were generously provided by Allmicroalgae – Natural Products, S.A (Pataias, Portugal). The nutritional values of the microalgae, provided by the manufacturer, are presented in Supplementary material S1.

2.2. Ingredients and reagents

The ingredients used in the preparation of the breads such as no yeast wheat flour T55 (PorSi, Intermarché, Bondoufle, France), salt (Vatel, Alverca, Portugal), fresh yeast (Primo, Senhora da Hora, Portugal), lupin flour (Próvida, Mem Martins, Portugal), wholemeal rye, spelt and oats flours (Próvida, Mem Martins, Portugal), and carob flour (Próvida, Mem Martins, Portugal), sugar (PorSi, Intermarché, Bondoufle, France) were purchased from local supermarkets.

Chloroform (99.2%) was purchased from VWR (Fontenay sous Bois, France), methanol (99.9%) and n-hexane (95.0%) from Carlo Erba (Val-de-Reuil, France), sodium sulfate anhydrous (≥99.0%) and sulfuric acid (95.0–97.0%) from Honeywell (Seelze, Germany), and sodium chloride (99.9%) from Biochem (Cosne-Cours-sur-Loire, France).

2.3. Bread formulations and baking process

Theoretical bread formulations with different flour and microalgae mixtures were analyzed using the nutritional information found on the nutritional labels of the ingredients and from a Portuguese food composition database (INSA, 2022). It has been reported that lupin flour can provide a bitter taste to food products with a potential negative effect on the sensory responses, especially in the taste evaluation (Hall & Johnson, 2004; Villacrés, Cueva, Díaz, & Rosell, 2020). Considering this, and as a way to attenuate the lupin flavor while maintaining the nutritional requirements, various grain flours were considered. The formulations that performed best in terms of nutritional requirements were tested in the laboratory and their sensory properties evaluated. The sensory evaluation at this stage was carried out informally by 3 members of the panel. Then, the formulations that better performed at the sensory level were analyzed to confirm experimentally that they met the nutritional requirements. The three multigrain bread formulations resulting from the selection process were wheat/lupin/rye flours (F–R), wheat/-lupin/spelt flours (F–S), and wheat/lupin/oats/carob flours (F-OC), enriched with a mixture of 1.9% (w/w of flour) of *C. vulgaris* White and *C. vulgaris* Smooth (4:1). The general formulations of the three multigrain breads enriched with microalgae (F–R, F–S and F-OC) and a control wheat bread (O) are presented in Table 1.

The breads were produced following a typical bread production methodology. The water was divided and used to dissolve the salt and the fresh yeast separately. The flours, sugar, and microalgae were weighted, combined in a food processor (Bimby, Vorwerk, Carnaxide, Portugal) and mixed for 5 min (speed 2) with gradual addition of the water with salt. Then, the remaining water with the yeast was slowly added to the dough while it was kneaded for 10 min using the kneading function. Once prepared, the dough was placed on a tray covered with baking paper, shaped, and left to leaven for 60 min at 35 °C in an oven (Memmert, Schwabach, Germany). Additional wheat flour was used in negligible amounts (<2 g per bread) to help shape the breads. The weight of the bread doughs was on average 379.96 ± 5.73 g.

The bread was baked for 30 min at 200 °C and left to cool on a wire rack. The oven temperature was controlled by placing a thermometer right next to the bread during the baking process. Once at room temperature (18 ± 1 °C), the samples were sliced and analyzed.

Triplicates of each formulation were produced on the same day to ensure the reproducibility of the formulations.

Color, pH, a_W, texture, and moisture analyses were performed on the day of production. The remaining samples were freeze-dried, ground to approximately 500 μm, and stored in zipped plastic bags until further analysis.

2.4. Physicochemical analysis

All physicochemical analysis were performed in triplicate on three distinct batches of each formulation.

2.4.1. Weight loss, color, texture, pH, and a_W analyses

The weight loss, color, pH and a_W were analyzed following similar methodologies to those described by Khemiri et al. (2020).

Weight loss was determined by the difference in weight between the dough and the baked bread in three different batches.

pH was evaluated in samples from the dough and bread using a perforation probe on a pH meter (Inolab, WTW, Weilheim, Germany) while a_W of the crumb was performed with a water activity analyzer (HP23-AW-A, Rotronic, Bassersdorf, Switzerland). All readings were done in triplicate.

Color analysis was performed using a Konica Minolta colorimeter (Chroma Meter CR-400, Japan) with a 2° standard observer and a D65 illuminant. The colorimeter was calibrated using a calibration plate ($L^* = 88.50$, $a^* = 0.32$, $b^* = 0.33$) and the results were presented as CIELab coordinates with L* (lightness, black - white, 0–100), a* (green - red, -60 - 60) and b* (blue - yellow, -60 - 60) parameters. Measurements were taken at four different points in the dough, five different points in the crust and three in the crumb. Three slices (with 4.00 ± 0.26 cm of height, 15.50 ± 0.80 cm of length, and 1.50 cm of thickness) from the middle were used and all readings were done five times.

Table 1
General formulations of the breads developed (F–R, F–S, F-OC) and the control used (O) in this study.

Formulations	O		F–R		F–S		F-OC	
Ingredients	%	% (flours)[a]	%	% (flours)[a]	%	% (flours)[a]	%	% (flours)[a]
Wheat flour T55	55.5	100.0	21.4	39.8	21.4	39.8	21.4	39.8
Salt	1.0	1.8	0.6	1.1	0.6	1.1	0.6	1.1
Fresh yeast	1.0	1.8	0.7	1.3	0.7	1.3	0.7	1.3
Water	42.5	76.6	42.7	79.6	42.7	79.6	42.7	79.6
Lupin flour	–	–	20.9	38.9	20.9	38.9	20.9	38.9
Wholemeal Rye/Spelt/Oat flours	–	–	11.4	21.3	11.4	21.3	9.4	17.6
Carob flour	–	–	–	–	–	–	2.0	3.7
White *C. vulgaris*	–	–	0.8	1.5	0.8	1.5	0.8	1.5
Smooth *C. vulgaris*	–	–	0.2	0.4	0.2	0.4	0.2	0.4
Sugar	–	–	1.3	2.4	1.3	2.4	1.3	2.4
Total	100.0	180.2	100.0	186.3	100.0	186.3	100.0	186.3

[a] % of flour was done by adding the values of all flours used for each formulation (i.e. wheat + lupin + rye flours = 100 %).

Texture profile analysis (TPA) of the bread samples was obtained with a TA-XTplus texturometer (Stable MicroSystems, Surrey, UK) adapting a methodology described by Correia et al. (2015) and Nunes, Graça, et al. (2020). The values were obtained at room temperature (18 ± 1 °C) on slices with 1.5 cm thickness using a cylindrical probe P/10 with a 6 mm penetration (corresponding to 40% of the original height). For the analysis, four readings in distinct places were done in each slice using 30 kg trigger load, 5 g trigger force, 1 mm/s test speed and 5 s waiting time. Three slices from the middle of the bread were used. The results of hardness, resilience, cohesion, and springiness were extracted from the software Exponent Connect (Stable Micro Systems, Surrey, UK) using the "Simplified TPA macro".

2.4.2. *Proximate chemical composition*

The proximate analyses were performed following the procedures described below.

Crude protein: the crude protein content was evaluated in an external laboratory using the Dumas method and a nitrogen-to-protein conversion factor of 6.25 (AOAC 992.23-1992, 1998; ISO 16634, 2005).

Total fat: The total fat was evaluated using the protocol adapted from Folch, Lees, and Sloane Stanley (1957). 0.8 mL of water and 5 mL of Folch reagent (chloroform and methanol at 2:1) were added to 1 g of freeze-dried sample and homogenized for 1 min. Then, 5 mL of Folch were added, mixed for 5 min, followed by an addition of 1.2 mL of sodium chloride (0.8%) and mixed for a further 2 min. After centrifugation in an Eppendorf Centrifuge 5810R (Enfield, CT, USA) (6000 rpm, 10 min, 4 °C), the bottom phase was passed through a filtration column (made up of hydrophobic cotton and sodium sulfate anhydrous) and collected to a pear-shaped evaporation flask. 5 mL of chloroform were added to the top phase, and the centrifugation (6000 rpm, 10 min, 4 °C) and filtration steps were repeated. The solvent was evaporated with a rotary evaporator (Laborota 4000, Heidolph, Scwabach, Germany) and the pear-shaped flask incubated in an oven (Memmert, Scwabach, Germany) overnight to ensure total solvent removal. Once at room temperature, the flasks were weighted, and the total fat calculated.

Moisture: 5 g of fresh sample were weighted on a crucible and incubated overnight at 105 °C. Once at room temperature, the crucible was weighted (NP 2282, 2009).

Ash: The crucibles used for the moisture analysis were placed on a furnace (B170, Nabertherm, Lilienthal, Germany) and heated at 535 °C for 5 h. Then, they were left to cool in an exicator until a constant weight was reached (NP 2032, 2009). The moisture and ash were calculated using the following equations:

$$Moisture\ (\%) = \frac{m2 - m3}{m2 - m1} x\ 100$$

$$Ash\ (\%) = \frac{m3 - m1}{m2} x\ 100$$

Where *m1* is the weight of the empty crucible, *m2* is the weight of the fresh sample, *m3* is the weight of the dried sample or the ash.

Carbohydrates and fibers were determined by subtracting the proteins, fats, water, and ash from the total weight (100 g).

2.4.3. *Fatty acid profile*

Fatty acid (FA) profile was obtained following an adaptation of the acid-catalyzed direct transesterification methodology described by Fernández et al. (2015).

2 mL of a methanol/sulfuric acid (98:2) solution were added to 50 mg of freeze-dried bread and placed for 2 h in an 80 °C water bath. After cooling to room temperature, the solution was mixed for 1 min with 1 mL of miliQ water and 2 mL of n-hexane. After centrifugation (1000 rpm, 5 min), 1 mL of the upper organic phase was stored in gas chromatography (GC) vials.

FA analysis was performed using a Finnigan trace GC Ultra chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) equipped with a flame ionization detector (FID), an autosampler (AS 3000, Thermo Electron Corporation), and a TR-FAME capillary column (Thermo TR-FAME, 60 m × 0.25 mm ID × 0.25 μm film thickness). Helium was used as carrier gas (1.5 mL/min flow rate), and air and hydrogen were supplied to the detector at 350 mL/min and 35 mL/min flow rate, respectively.

The temperatures were set at 250 °C for the injector (operating in splitless mode) and at 280 °C for the detector. The temperature of the column was initially set at 75 °C for 1 min, then raised to 170 °C (5 °C/min) and held for 10 min, followed by an increase to 190 °C (5 °C/min) and maintained for 10 min, and then raised to 240 °C (2 °C/min) and held for 10 min.

The FA profile was determined by comparing the resulting retention times with a standard (Supelco 37 component FAME Mix, Sigma Aldrich, Darmstadt, Germany) and the results were expressed as % of total fat. All analyses were done in triplicate.

2.5. *Optimization of the production process*

The production process was optimized using a response surface methodology (RSM) with a central composite design (CCD). The effect of two independent variables was studied and its effect on the sensory analysis (color, aroma, taste, texture, and overall appreciation) was evaluated. The chosen variables were the percentage of water (64.6–94.6% (w/w of flours), corresponding to a 15% reduction and a 15% increase in water compared to the starting bread recipe (with 76.6% water)), and the percentage of microalgae (1.9–3.9% (w/w of flours)). The percentages of water and microalgae were established as function of the weight of the combined flours (w/w of flours). The water upper and lower limits were selected based on preliminary experiments that established the extreme levels of water that still allowed the manufacturing of the breads (data not shown). The levels of microalgae were chosen through preliminary sensory analysis (informal tastings with 3 panellists), selecting the upper end as the limit of microalgae that could be added to the breads without causing product rejection (data not shown).

Table 2 represents the coded and decoded matrix used in the optimization.

Ten runs with two replications of the center points were used for the model's construction.

2.6. Sensory analysis

To optimize the production process, sensory evaluation was performed using a semi-trained panel. The matrix was divided, randomized, and served on two distinct days for each bread formulation (F–S, F–R and F-OC), so as not to overwhelm the panelists. The panel was composed by 13 panelists for the F–S bread (10 female and 3 male), 14 panelists for the F–R bread (12 female and 2 male) and 15 panelists for the F-OC bread (13 female and 2 male). The ages ranged from 18 to 64.

Portions of the breads (without crust) were served in plates labelled with 3-random number codes and evaluated in terms of color, aroma, taste, texture, and overall appreciation following a five-point hedonic scale (1-dislike extremely, 2-dislike, 3-neither like nor dislike, 4-like, and 5-like extremely). The RSM results were composed by the average of the sensory attributes.

Water was provided to cleanse the palate between samples.

The sensory validation of the optimized breads was carried out using the same methodology. Two samples of each optimized bread were provided to a panel composed by 14 females and 3 males.

2.7. Bread shelf-life

Seeing as all breads presented similar general formulations (with minor changes in terms of water and grains used), the storage behavior should be similar between all of them. One of the breads, rye bread (F–R), was therefore chosen to assess shelf-life. The shelf-life was evaluated during a four-day period, from Monday (day of production (day 0)) to Friday (day 4)), using five distinct samples. The samples were stored in polyethylene zipped bags and stored at room temperature (18 ± 1 °C) protected from light. The parameters pH, a_W, moisture, and texture were monitored as previously described.

The microbiological analyses were performed in six breads and evaluated from day 1 to day 5 after production. Twenty-five grams of each sample were added to 225 mL of sterile buffered Ringer's solution (Biokar Diagnostics, Beauvais, France) and homogenized in a stomacher (Interscience, Saint Nom la Brèteche, France) for 2 min. Appropriate decimal dilutions were prepared in Ringer's solution for microbial enumeration along the mentioned time period: Total viable counts at 30 °C (ISO 4833-1, 2013), yeast and molds at 25 °C (NP 3277-1, 1987), *Bacillus* spp. (Health Protection Agency, 2004) and *B. cereus* (ISO 7932, 2004).

Table 2
Matrix with the coded and decoded values of the percentage of water and percentage of microalgae.

Runs	Coded		Decoded	
	Water (%)	Microalgae (%)	Water (%)	Microalgae (%)
1	−1.00	−1.00	69.00	2.10
2	−1.00	1.00	69.00	3.60
3	1.00	−1.00	90.20	2.10
4	1.00	1.00	90.20	3.60
5	−1.41	0.00	64.60	2.90
6	1.41	0.00	94.60	2.90
7	0.00	−1.41	79.60	1.90
8	0.00	1.41	79.60	3.90
C	0.00	0.00	79.60	2.90
C	0.00	0.00	79.60	2.90

2.8. Statistical analysis

Analysis of variance (ANOVA) was used to generate the regression coefficients of the effects (linear, quadratic and interactions) involved in the model resulting from the RSM. Coefficient of determination (R^2) was used to evaluate the suitability of the model. In addition, ANOVA one-way was used to compare the nutritional (moisture, ash, protein, fats, fatty acids, carbohydrates, and energy) and physicochemical (weight loss, pH, a_W, texture, and color) characteristics of the different breads (O, F–R, F–S, F-OC). Differences in pH, a_W, texture, and moisture of F–R were also analyzed using an ANOVA one-way. All assumptions inherent to the performance of the ANOVA (normality of data and homogeneity of variances) were validated. Whenever the requirements were not met, the Kruskal-Wallis non-parametric test was used. In order to carry out the multiple comparisons, the Tukey HSD test (whenever the ANOVA requirements were met) and the Games-Howell test (for the remaining cases) were used. The comparison of the control (wheat bread) with the remaining breads was done using a Dunnett's test. Results are presented as mean and standard deviation (SD). In all analyses, the results were considered statistically significant at the 5% level (that is, whenever p-value <0.05). RSM analysis was performed on Statistica 10 (StatSoft, Inc., Minneapolis, USA) while the remaining statistical analyses were done with the software IBM SPSS Statistics 28 (Copyright IBM Corp. ©1989–2023, Armonk, NY 10504-1722, USA).

3. Results and discussion

3.1. Development of the bread formulations

Three multigrain bread formulations were developed using wheat/lupin/rye flours (F–R), wheat/lupin/spelt flours (F–S), and wheat/lupin/oats/carob flours (F-OC). A mixture of *C. vulgaris* White and *C. vulgaris* Smooth (4:1) was incorporated into each formulation at a concentration of 1.9% (w/w of flour). A visual representation of the breads can be found in Fig. 1.

Table 3 presents the results of the proximate analysis of the developed breads and a control wheat bread. All of the developed formulations presented higher protein, total fat, fatty acids, ash, and energy values when compared with a wheat bread (Table 3). On the other hand, the multigrain breads had lower average values for carbohydrate and fibre. These results were consistent with those reported on a multigrain bread comprising oat/rye/buckwheat/wheat (Angioloni & Collar, 2011). Previously, lupin flour originated breads with higher protein, fat, ash, and energy values and lower carbohydrate values (Plustea et al., 2022). Oats have been shown to increase the ash, fat and protein content and lower the carbohydrate and energy value (Chauhan, Kumar, Kumar, & Kumar, 2018) while rye increases the protein, fat, and ash content (Pourafshar, Rosentrater, & Krishnan, 2015).

Earlier studies with lupin as a wheat substitute reported a decrease in bread's moisture with increasing substitution (Plustea et al., 2022). This could be due to the high water-binding capacity of the lupin's fibre (Turnbull et al., 2005). In the present study, all bread types had similar moisture content of approximately 48%, with the exception of F-OC bread, which had a lower moisture content (p-value <0.05; Table 3). This reduction in moisture could be the result of the combination of carob and lupin due to the cumulative water-binding capacity of the two legumes (Tsatsaragkou, Gounaropoulos, & Mandala, 2014; Turnbull et al., 2005).

All breads showed significant improvements in protein content (p-value <0.05; Table 3) with increases of 82, 87, and 91% for the F–R, F-OC, and F–S breads, respectively. The substantial amounts of lupin used in the formulations contributed significantly to this increase since it has a remarkably high protein content (Table S2, supplementary material). Significant differences were found between the multigrain breads due to the distinct protein profile of the grains used (p-value <0.05; Table 3). Rye presents lower protein (Table S2, supplementary material) resulting

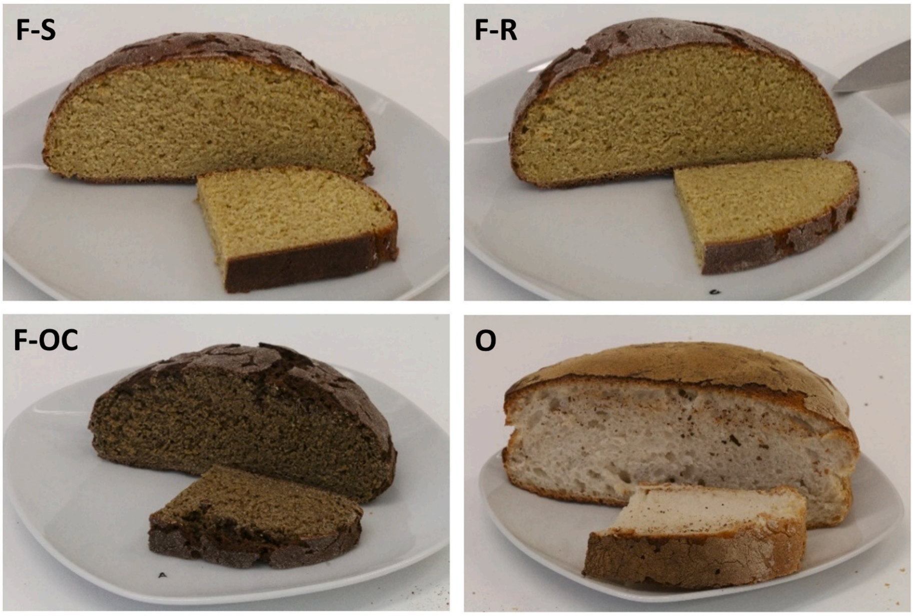

Fig. 1. Representation of the multigrain bread formulations enriched with 1% of *C. vulgaris* developed. Bread with Wheat/Lupin/Rye (F–R), Wheat/Lupin/Spelt (F–S), Wheat/Lupin/Oats/Carob (F-OC), and a Wheat bread (O).

Table 3
Proximate nutritional analysis of the adopted multigrain bread formulations enriched with 1.9 % (w/w of flour) of *Chlorella vulgaris*. O – wheat bread, F–S – spelt and lupin bread, F-R– rye and lupin bread, F-OC – carob, oats, and lupin bread.

	O	F–S	F–R	F-OC
Moisture (%)	47.82 ± 0.10 ^{a}	47.67 ± 0.11 ^{a}	47.70 ± 0.14 ^{a}	46.50 ± 0.25 ^{b}
Ash (%)	1.28 ± 0.01 ^{a}	1.60 ± 0.01 ^{b}	1.55 ± 0.02 ^{c}	1.64 ± 0.01 ^{d}
Protein (g/100 g)	6.18 ± 0.20 ^{a}	11.83 ± 0.05 ^{b}	11.26 ± 0.15 ^{c}	11.56 ± 0.17 ^{d}
Total Fat (g/100 g)	1.06 ± 0.15 ^{a}	2.72 ± 0.07 ^{b}	2.55 ± 0.08 ^{c}	3.24 ± 0.05 ^{d}
Fatty Acids (% of total fat)				
SFA	27 ^{a}	18 ^{b}	18 ^{b}	18 ^{c}
MUFA	16 ^{a}	47 ^{b}	48 ^{b}	48 ^{c}
PUFA	57 ^{a}	35 ^{b}	34 ^{b}	34 ^{c}
Carbohydrates + Fibers (g/100 g)	43.70 ± 0.27	36.23 ± 0.14	37.10 ± 0.22	37.04 ± 0.31
Energy (Kcal/100 g)	209.29 ± 0.36	217.07 ± 0.16	216.31 ± 0.27	224.42 ± 0.34

Different letters on the same line demonstrates differences (p-value < 0.05, ANOVA, Tuckey HSD or p-value <0.05, Kruskal-Wallis, Games-Howell).

in breads with only 11.26 ± 0.15 g/100 g. On the other hand, oats present higher protein content than spelt (Table S2, supplementary material), however since the F-OC bread had a mixture of oats and carob, the protein content was lower (11.56 ± 0.17 g/100 g) than the F–S bread (11.83 ± 0.05 g/100 g). Similar observations were reported in previous studies with breads containing lupin, oats and spelt (Angioloni & Collar, 2011; Chauhan et al., 2018; Plustea et al., 2022; Villarino, Jayasena, Coorey, Chakrabarti-Bell, Foley, et al., 2015). Other rye breads also showed reduced protein (Angioloni & Collar, 2011; Pourafshar et al., 2015).

The proteins contributed 22% to the energy value of F–S bread and 21% to the energy value of F–R and F-OC breads, confirming the compliance with the requirement for the claim 'rich in protein'. However, protein was obtained using the Dumas methodology, which is known to obtain higher values than the conventional Kjeldhal method. To obtain more accurate protein results, the optimization of the methodology for the multigrain breads could have been necessary, as reported by Serrano, Rincón, and García-Olmo (2013).

Higher fat contents were found in all multigrain breads, with values of 2.55 ± 0.08, 2.72 ± 0.07, and 3.24 ± 0.05 g/100 g for the F–S, F–R, and F-OC, respectively, when compared to wheat bread (1.06 ± 0.15 g/100 g) (p-value <0.05; Table 3). This increase was expected due to the higher amount of fat present in the lupin flour (Table S2, supplementary material), which was the major contributor in the novel formulations. The remaining flours and microalgae also present fats, but in lower amounts. Higher fat amounts were found in F-OC due to the combination of the fat present in lupin and oat flours (Table S2, supplementary material). These results are consistent with those reported for the use of lupin, rye, oats, and spelt in breads (Angioloni & Collar, 2011; Plustea et al., 2022; Pourafshar et al., 2015).

The multigrain breads also presented higher SFA compared to the control (p-value <0.05). Nevertheless, the claim 'low in saturated fat' was fulfilled, as the total amounts were less than 1.5 g/100 g. Higher MUFA and PUFA contents were also found in these breads (p-value <0.05; Table 3).

Additionally, the increase in ash suggests an improvement in mineral content. Although the mineral content was not analyzed, previous research has demonstrated an improvement in the macro- and micro-elements in lupin bread (Plustea et al., 2022). Plustea et al. (2022) found significant increases in calcium, potassium, magnesium, iron, zinc, copper, chromium, nickel, and manganese in lupin bread. On the other hand, *C. vulgaris* also contains considerable amounts of iron, potassium, and selenium (Bito, Okumura, Fujishima, & Watanabe, 2020).

3.2. *Optimization of the bread formulations*

The formulations were optimized using RSM and the results of the sensory analysis are shown in Table 4.

RSM allows the evaluation of the effect of the water and microalgae additions on the breads by correlating the individual and combined effects of the variables with sensory responses (Mudgil, Barak, & Khatkar, 2016). By relating the sensory responses to different levels of microalgae and water, a model is constructed that allows the prediction of the conditions to maximize the bread's sensory scores (Mudgil et al., 2016).

As previously mentioned, lupin flour has been known to provide a slightly bitter taste to the products which may adversely affect sensory

Table 4
Results of the response surface methodology of the breads.

Runs	Decoded		F–S					F-OC					F–R				
	Water (%)[a]	Microalgae (%)[a]	Color	Aroma	Taste	Texture	Overall appreciation	Color	Aroma	Taste	Texture	Overall appreciation	Color	Aroma	Taste	Texture	Overall appreciation
1	69.0	2.1	3.9	3.8	3.3	3.8	3.3	3.9	3.7	3.8	3.3	3.8	4.0	3.6	3.3	3.1	3.3
2	69.0	3.6	3.6	3.6	3.1	3.1	2.9	4.0	3.6	3.7	2.9	3.5	3.6	3.9	2.8	3.2	2.9
3	90.2	2.1	4.1	3.9	3.8	4.0	3.7	4.1	3.9	4.2	4.0	3.9	4.1	3.9	3.8	3.9	3.8
4	90.2	3.6	4.2	4.1	3.7	4.4	3.7	3.9	3.4	3.6	3.4	3.6	4.2	3.9	3.3	3.8	3.6
5	64.6	2.9	3.7	3.3	3.4	3.4	3.4	3.8	3.6	3.7	3.1	3.7	3.8	3.7	3.6	3.3	3.5
6	94.6	2.9	4.2	4.0	3.3	4.3	3.5	4.0	3.8	3.7	3.9	3.8	4.2	4.1	3.6	3.7	3.6
7	79.6	1.9	4.3	4.0	3.1	3.8	3.2	3.9	3.7	3.8	3.8	3.8	4.3	4.1	3.6	4.1	3.7
8	79.6	3.9	4.1	3.9	3.3	3.8	3.3	3.9	3.8	3.8	3.9	3.8	4.1	3.7	3.1	3.6	3.4
C	79.6	2.9	**4.3**	**4.0**	**3.6**	**3.9**	**3.7**	**4.0**	**3.7**	**3.8**	**3.7**	**3.8**	**4.0**	**3.8**	**3.7**	**3.9**	**3.6**
C	79.6	2.9	**4.1**	**3.9**	**3.9**	**4.0**	**3.9**	**4.1**	**3.6**	**4.0**	**3.8**	**3.9**	**3.9**	**3.8**	**3.7**	**3.8**	**3.6**

[a] % in terms of w/w of flours.

perception (Hall & Johnson, 2004; Villacrés et al., 2020). In the present study, considerable amounts of lupin flour were used (20–21%), and thus a bitter taste was noticed by the panelist. However, the addition of water seemed to help soften this taste, with the panelists mentioning that the breads with lower water contents presented bitterer taste and aftertaste. Indeed, this tendency was confirmed by the sensory responses, as the breads with the lowest water content (64.6 and 69%) received the lowest scores. As can be seen in Figs. 2 and 3, water addition was the most significant independent variable in all multigrain breads.

In F–S (Fig. 2a, b, and 2c and Fig. 3a, b, and 3c), water presented a positive linear and negative quadratic effect on color ($R^2 = 0.90$), aroma ($R^2 = 0.96$) and texture ($R^2 = 0.99$) (Tables S3, S4, and S5 supplementary material). It also had a positive linear effect of water, a correlation between water and microalgae, and a negative quadratic effect of the microalgae on texture ($R^2 = 0.99$) (Table S5, supplementary material). The quadratic effects of the water in color (p-value = 0.048) and microalgae in texture (p-value = 0.042) were not very strong since they presented p-values close to the limit of significance (p-value <0.05). The linear effect of the water was the most significant, indicating that an increase in water enhances the bread's color, aroma, and texture. On the other hand, the negative and quadratic effects suggest that water only increases the response to a certain point, beyond which further increases may hinder the sensory responses.

For F–R bread (Fig. 2e, f, 3e, and 3f), water was also the most significant variable for color ($R^2 = 0.87$) and texture ($R^2 = 0.83$), showing a positive and linear effect on the scores (Tables S7 and S8, supplementary material).

The F-OC bread only presented a positive linear effect of water on texture ($R^2 = 0.72$) (Figs. 2d and 3d and Table S6, supplementary material). However, this effect was very weak (p-value = 0.047). During the sensory evaluation, carob showed the potential to reduce the impact of the lupin's bitter taste, even in small amounts. Carob could also have contributed to the lower R^2 values, which accounted for only 72% of the results. The inclusion of an assessment regarding the addition of carob to the breads could have increased the regression coefficient.

Other works have used RSM to optimize the water content in breads. The quadratic effect and interaction of water and lupin flour on the texture and overall acceptability of breads has been previously reported (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2015). In oat breads, water affected the evenness of the crust and the hardness and sensory softness, elasticity, and moistness of the crumb (Flander, Salmenkallio-Marttila, Suortti, & Autio, 2007). In carob gluten-free breads, water also affected the texture, resulting in breads with a softer crumb (Tsatsaragkou et al., 2014).

Texture is one of the most important attributes for consumer acceptability, because lower hardness is associated with more desirable breads (He, Li, Chen, Huang, & Tao, 2023). As seen, texture was the attribute most affected by the water, with higher amounts improving the acceptability. Tsatsaragkou et al. (2014) mentioned that a low water content may cause negative effects on bread properties due to the repartition of the available water with the different dough components. As mentioned, lupin has a high water-binding capacity and low elasticity proteins resulting in harder breads and thus the optimization of the water is necessary to reduce its negative effects (Villarino et al., 2016).

After understanding the effects of the variables on the sensory scores, the levels of addition were predicted to maximize the acceptability of the bread. The optimal predicted conditions are presented in Table 5 and the resulting optimized breads are shown in Fig. 4. The suitability of the statistical model to predict the sensory score was evaluated through a confirmatory sensory analysis with breads produced with the optimal conditions (Fig. 5).

Bread F–S, with the lower water content and higher microalgae addition, obtained the lowest taste (3.18 ± 0.76) and overall appreciation (3.26 ± 0.67) scores. The remaining sensory parameters were evaluated as a 3.97 ± 0.17, 4.06 ± 0.42, and 3.94 ± 0.55 for color,

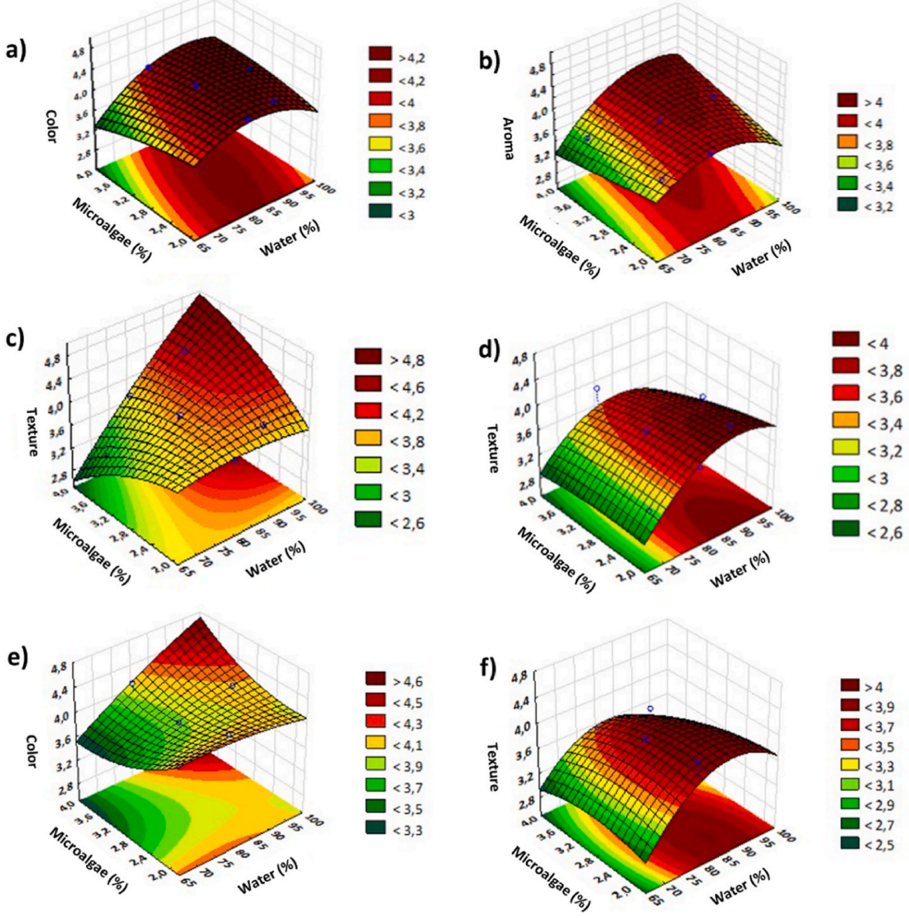

Fig. 2. Response surface graphs of the effect of the parameters studied on the bread's sensory response. Effect of the microalgae and water addition on the a) color of the F–S bread, b) aroma of the F–S bread, c) texture of the F–S bread, d) texture of the F-OC bread, e) color of the F–R bread, and f) texture of the F–R bread. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

aroma, and texture, respectively.

F–R bread obtained the highest scores in all sensory parameters, achieving an average of 4.06 ± 0.34 for color, 4.12 ± 0.41 for aroma, 4.00 ± 0.65 for taste and 4.00 ± 0.55 for texture, and 3.97 ± 0.63 for overall appreciation. Lastly, F-OC bread scored slightly lower F–R with 4.00 ± 0.70, 3.91 ± 0.75, 3.91 ± 0.83, 4.00 ± 0.43, and 3.85 ± 0.70 for color, aroma, taste, texture, and overall appreciation, respectively.

Considering the results, it was possible to confirm the model's suitability to predict the sensory results of multigrain breads, because the sensory scores were within the predicted values of the model.

The inclusion of alternative ingredients as wheat substitutes has been shown to influence the sensory evaluation of breads. According to Correia et al. (2015) the addition of lupin to wheat bread up to a proportion of 10% does not have adverse effects on the bread's sensory analysis (Correia et al., 2015). However, at higher substitution levels (20 and 30%), a reduction in scores for appearance, taste, texture, and general acceptability were verified (Plustea et al., 2022). The bread's color was the only parameter that improved with the increase of lupin (Kefale & Yetenayet, 2020; Plustea et al., 2022). Compared to a wheat bread, the use of alternative grains such as rye or oats has resulted in lower acceptability (Angioloni & Collar, 2011; Chauhan et al., 2018). On the other hand, carob and spelt has led to enhanced acceptability, yet excessive amounts can result in negative responses (Angioloni & Collar, 2011; Frakolaki, Giannou, & Tzia, 2020; Issaoui, Flamini, & Delgado, 2021). These findings demonstrate the significance of conducting sensory analysis on novel products made with alternative ingredients as wheat substitutes.

3.3. Physical and chemical characterization of the optimized breads

The optimized breads were further characterized, and the results of the physicochemical and nutritional analyses are presented (Table 6).

As expected, the optimized breads demonstrated greater moisture values, as they were produced with higher water contents. They also presented higher a_W, suggesting high perishability of the product due to its high-water availability for the development of spoilage organism (Chirife, del PilarBuera, & Labuza, 1996; Magan, Arroyo, & Aldred, 2003).

Lower ash, carbohydrates, and energy values were obtained for the optimized breads compared to the original recipes. Compared to wheat bread, the multigrain breads also contained on average higher ash and lower carbohydrate (all breads) and energy value (F–R and F-OC bread).

Slightly lower protein values were found in the optimized bread than in the original recipes, nevertheless the values are still over the limit for the protein claim. The F-OC bread presented the highest protein content followed by the F–S and F–R bread. In terms of protein contribution to energy value, the F-OC showed an increase of approximately 22%, higher than the 21% of the original recipe. On the other hand, in the F–S bread, the protein contribution was lowered to 20% of the energy value (*vs.* 22% for the non-optimized recipe) and the F–R bread maintained its protein contribution at 21% of the energy value.

An increase in total fat was also verified (compared to the non-optimized formulations). This increase is due to the increase in microalgae in the optimized breads. Optimized bread F-OC presented the same concentration of microalgae as the original, and thus showed similar fat

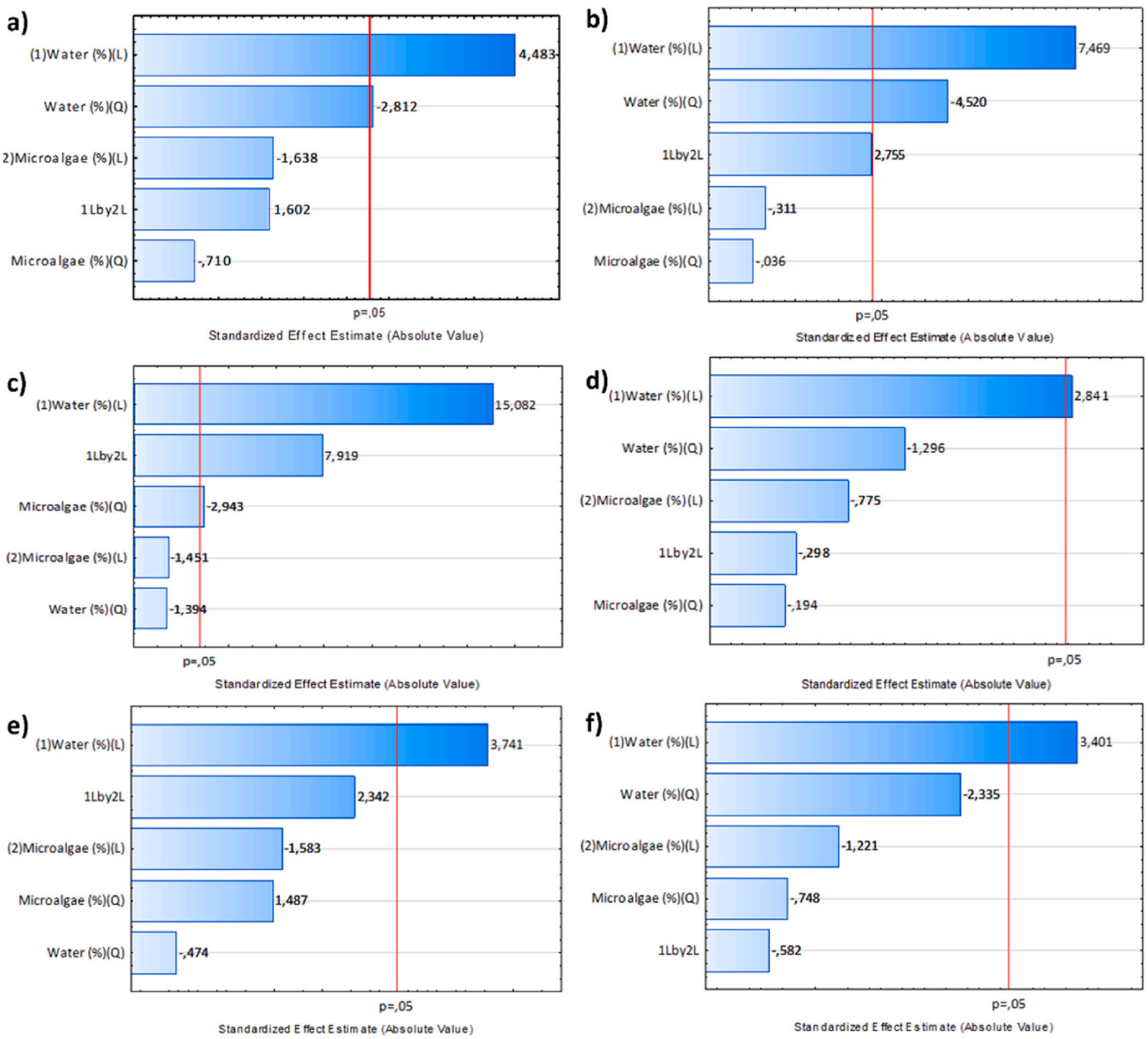

Fig. 3. Pareto charts of the standardized effects of the studied variables on the a) color of the F–S bread, b) aroma of the F–S bread, c) texture of the F–S bread, d) texture of the F-OC bread, e) color of the F–R bread and f) texture of the F–R bread. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Optimal conditions of water and microalgae predicted for the bread formulations.

	Optimal Conditions	
	Water (%)	Microalgae (%)
F–R	94.6	2.4
F–S	87.1	2.9
F-OC	94.6	1.9

values (3.24 ± 0.05 vs 3.38 ± 0.06 g/100 g for the original and the optimized, respectively). A small increase in microalgae (from 1.9 to 2.4%) in F–R bread caused an increase in fat of about 16%, while a larger increase (from 1.9 to 2.9%) in F–S bread saw an increase of 22%. Previously, increasing *C. vulgaris* by 1–2% did not induce any changes in the fat content (Garzon, Skendi, Antonio Lazo-Velez, Papageorgiou, & Rosell, 2021). On the other hand, an increase from 2.5 to 5% of *C. sorokiniana* originated breads with 77% more lipids (Diprat et al., 2020).

Differences were also verified in the fatty acid's quantifications, with a rearrangement of their classes, especially MUFA and PUFA. Optimized F–S bread presented higher MUFA content and lower PUFA content, whilst F–R and F-OC breads presented higher MUFA content and lower SFA and PUFA contents.

The breads had similar hardness values (except F–S bread), but showed some differences in terms of resilience, cohesion, and springiness (p-value <0.05; Table 6). F–R bread presented lower values of resilience, cohesion, and springiness overall. F–S bread, on the other hand, presented higher hardness, resilience, cohesion, and springiness. F-OC bread showed hardness closer to the F–R bread, and values of resilience, cohesion, and springiness between those of the other two breads. In previous studies, adding oat, rye, or spelt to breads resulted in higher hardness and lower cohesiveness, compared to a wheat bread (Angioloni & Collar, 2011). In oats bread, only an increase in the hardness was observed, with the springiness and cohesiveness remaining unchanged (Astiz, Guardianelli, Salinas, Brites, & Puppo, 2023; He et al., 2023). Carob also increased the hardness and cohesiveness of bread, while contrasting results have been reported for the bread's elasticity (springiness) (Issaoui et al., 2021; Salinas, Carbas, Brites, & Puppo, 2015). These different texture profiles are the result of the different gluten contents of the minority flours used (rye, spelt, and oats) and their interaction with the wheat present (Pruska-Kedzior, Kedzior, & Klockiewicz-Kaminska, 2008; Schalk, Lexhaller, Koehler, & Scherf, 2017). Lupin and carob do not contribute to gluten but could interfere in the matrix due to the dilution of the gluten protein structures

Fig. 4. Representation of the optimized breads. Bread with Wheat/Lupin/Rye (F–R), Wheat/Lupin/Spelt (F–S), and Wheat/Lupin/Oats/Carob (F-OC).

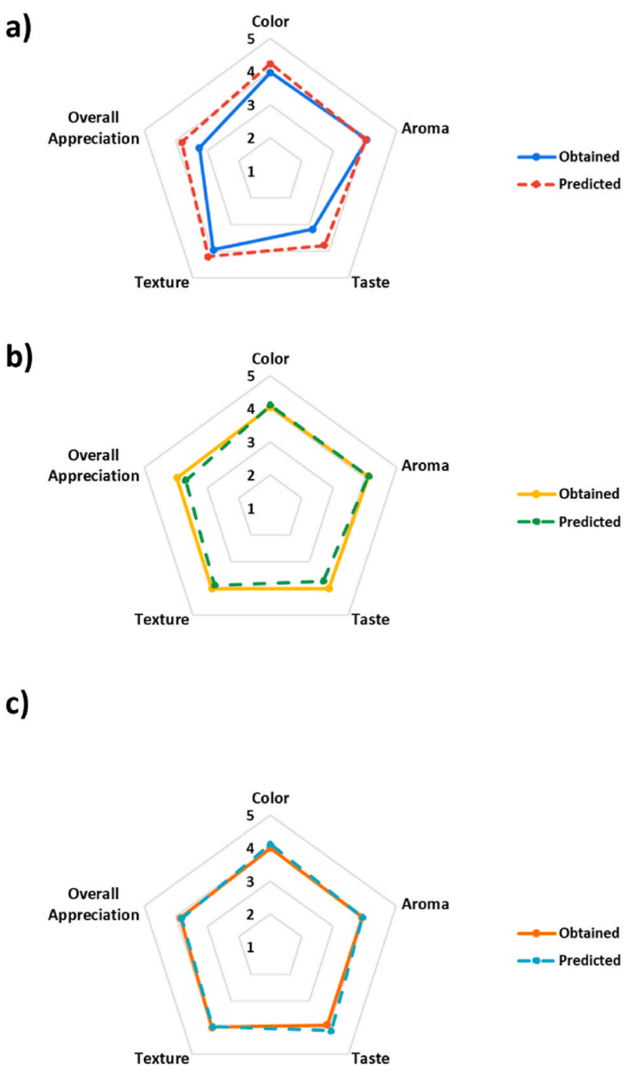

Fig. 5. Predicted and obtained sensory scores of the optimized a) bread F–S, b) bread F–R, and c) bread F-OC.

Table 6
Physicochemical and nutritional characterization of the optimized breads.

Weight loss (%)	F–S	F–R	F-OC
	12.50 ± 0.40 ^{a}	12.47 ± 0.28 ^{a}	12.29 ± 0.26 ^{a}
PH			
Dough	5.34 ± 0.01 ^{a}	5.32 ± 0.02 ^{a}	5.26 ± 0.02 ^{b}
Baked	5.45 ± 0.01 ^{a}	5.47 ± 0.02 ^{a}	5.36 ± 0.02 ^{b}
a_W	0.97 ± 0.01 ^{a}	0.98 ± 0.01 ^{b}	0.97 ± 0.01 ^{ab}
Texture			
Hardness (N)	3.78 ± 0.59 ^{a}	3.23 ± 0.43 ^{b}	3.23 ± 0.54 ^{b}
Resilience (%)	31.79 ± 3.92 ^{a}	20.81 ± 3.08 ^{b}	28.24 ± 2.46 ^{c}
Cohesion (%)	64.49 ± 3.12 ^{a}	52.16 ± 5.63 ^{b}	59.99 ± 2.75 ^{c}
Springiness (%)	82.66 ± 1.92 ^{a}	75.23 ± 3.07 ^{b}	79.42 ± 2.65 ^{c}
Color			
L* (dough)	67.87 ± 2.81 ^{a}	68.06 ± 1.94 ^{a}	51.62 ± 1.73 ^{b}
L* (crust)	40.30 ± 5.83 ^{a}	40.53 ± 4.26 ^{a}	37.04 ± 3.35 ^{b}
L* (crumb)	53.94 ± 2.16 ^{a}	52.37 ± 2.00 ^{b}	35.01 ± 1.33 ^{c}
a* (dough)	−0.48 ± 0.22 ^{a}	1.30 ± 0.25 ^{b}	7.97 ± 0.25 ^{c}
a* (crust)	12.64 ± 3.69 ^{a}	12.66 ± 4.11 ^{a}	11.51 ± 2.77 ^{b}
a* (crumb)	3.03 ± 0.27 ^{a}	3.61 ± 0.18 ^{b}	7.83 ± 0.22 ^{c}
b* (dough)	35.89 ± 0.84 ^{a}	33.65 ± 1.08 ^{b}	24.77 ± 0.78 ^{c}
b* (crust)	14.67 ± 8.06 ^{a}	14.74 ± 8.69 ^{a}	12.34 ± 5.61 ^{b}
b* (crumb)	35.39 ± 1.09 ^{a}	31.74 ± 0.77 ^{b}	21.73 ± 0.46 ^{c}
Moisture (%)	50.36 ± 0.29 ^{a}	52.20 ± 0.20 ^{b}	52.06 ± 0.24 ^{b}
Ash (%)	1.45 ± 0.04 ^{a}	1.38 ± 0.03 ^{b}	1.39 ± 0.08 ^{ab}
Protein (g/100 g)	10.60 ± 0.23 ^{a}	10.54 ± 0.20 ^{a}	10.96 ± 0.26 ^{a}
Total Fat (g/100 g)	3.32 ± 0.15 ^{a}	2.95 ± 0.08 ^{b}	3.38 ± 0.06 ^{c}
Fatty Acids (% of total fat)			
SFA	18 ^{a}	17 ^{a}	17 ^{b}
MUFA	50 ^{a}	51 ^{a}	49 ^{b}
PUFA	33 ^{a}	32 ^{a}	32 ^{b}
Carbohydrates + Fibers (g/100 g)	34.29 ± 0.40 ^{a}	32.93 ± 0.08 ^{b}	32.22 ± 0.23 ^{c}
Energy (Kcal/100 g)	209.41 ± 0.74 ^{a}	200.43 ± 0.27 ^{b}	203.10 ± 0.96 ^{c}

Different letters on the same line demonstrates differences (p-value < 0.05, ANOVA, Tuckey HSD or p-value <0.05, Kruskal-Wallis, Games-Howell).

(Doxastakis, Zafiriadis, Irakli, Marlani, & Tananaki, 2002; Salinas et al., 2015).

3.4. *Shelf-life of the bread with rye, lupin, and microalgae*

The bread with the best overall sensory acceptance, F–R bread, was selected for the shelf-life evaluation. Monitoring of pH, a_W, moisture, microbiological parameters, and texture was performed for five days of storage (Fig. 6 and Table 7).

As expected, differences in moisture, pH, a_W, and texture were verified over time (p-value <0.05; Fig. 6).

Water activity and moisture decreased significantly (p-value <0.05; Fig. 6) during storage, with the greatest differences being observed after four days. Moisture presented a steady decrease of 2.4, 3.2, and 6.5% from the initial 52.30 ± 0.12% after 2, 3 and 4 days, respectively. Similar results were obtained for the a_W, with a decrease of the initial 0.975 to 0.952 on day 4. From day 4 to day 5, a slight increase of 0.5% in moisture and 0.7% in a_W were verified. In previous studies with wheat/lupin breads, the moisture and a_W was stable during a 5-day storage (Correia et al., 2015). This contrast can be the result of the lower concentrations of lupin (10%) being used in the aforementioned study and the potential contribution of the rye.

In terms of texture, hardness increased significantly (p-value <0.05; Fig. 6) by 15, 47, 79 and 92% over the storage period. With increasing hardness, a decrease in the bread's resilience, cohesion, and springiness by 33, 32 and 19% was verified by the end of storage. Similar tendencies were previously verified for lupin breads (Correia et al., 2015) and breads with lupine protein isolates (López & Goldner, 2015) where the increase in hardness was accompanied by the decrease in cohesiveness

Table 7
Microbiological (microbial counts at 30 °C, molds at 30 °C, yeast at 25 °C) results of the control and F–R breads over five days.

	Day 1	Day 2	Day 3	Day 4	Day 5
Microbial count at 30 °C, (CFU/g) – Control	–	$<1.0 \times 10^1$	Present but $<4.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Microbial count at 30 °C, (CFU/g)-F-R	–	$<1.0 \times 10^1$	Present but $<4,0 \times 10^1$	EN = 9.0×10^1	Present but $<4.0 \times 10^1$
Molds at 25 °C, (CFU/g) – Control	–	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Molds at 25 °C, (ufc/g) – F–R	–	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.1×10^2	$<1.0 \times 10^1$
Yeast at 25 °C, (CFU/g)- Control	–	$<1.0 \times 10^1$	Present but $<4.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Yeast at 25 °C, (CFU/g)- F–R	–	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Bacillus spp., (CFU/g)- Control	–	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Bacillus spp., (CFU/g)- F–R	–	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
B. cereus, (CFU/g)- Control	–	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
B. cereus, (CFU/g)- F–R	–	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$

Estimated number, Different letters on the same line demonstrates differences (p-value < 0.05, ANOVA, Tuckey HSD or p-value <0.05, Kruskal-Wallis, Games-Howell).

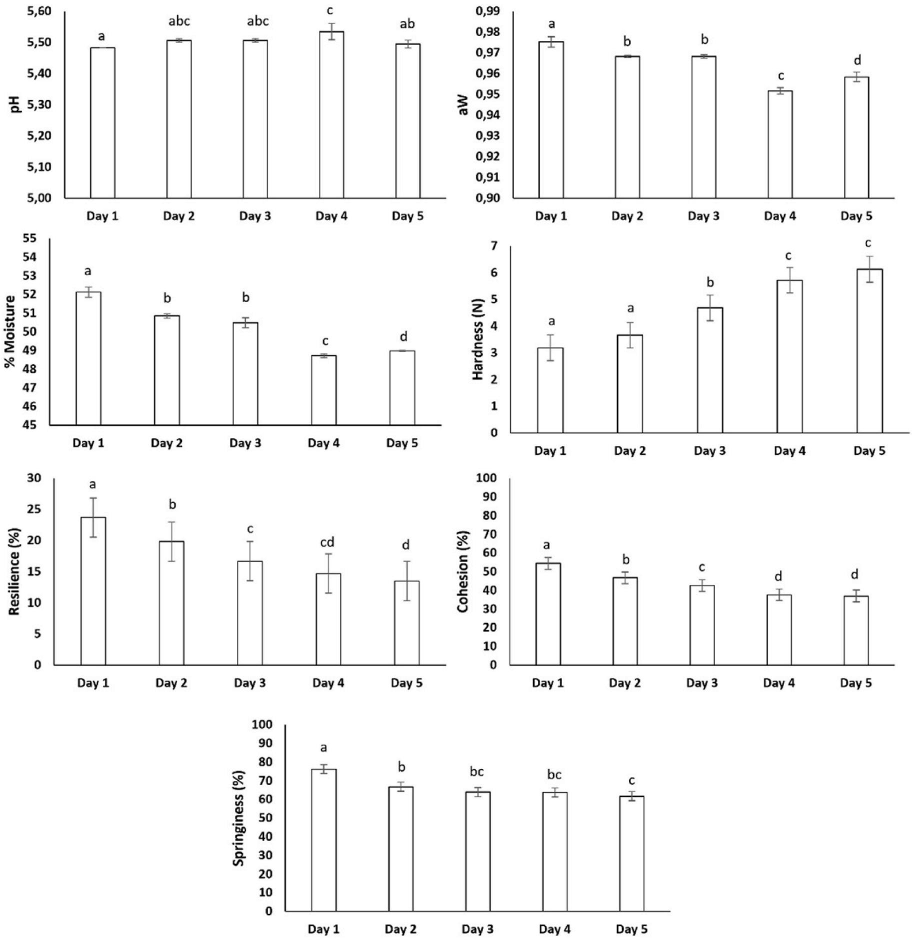

Fig. 6. PH, a_W, moisture, texture (hardness, resilience, cohesion, springiness) results of the F–R bread over five days.

and elasticity (springiness) during storage.

The increase in hardness and the decrease in moisture and water activity were expected. A negative correlation has been reported between moisture and crumb hardness in breads with lupine protein isolates (López & Goldner, 2015). The staling process has been associated mainly with the redistribution of the water in the bread which allows starch crystallization and gluten dehydration causing the firming of the crumb (Villarino et al., 2016). The migration of the moisture from the crumb to the crust also influences bread staling (Baik & Chinachoti, 2000; Villarino et al., 2016). In texture, resilience evaluates how well the bread fights to regain its original height, springiness is the capacity to regain its original structure and the cohesiveness is the capacity to deform before breaking (Szczesniak, 2002; Texture Technologies Corp. & Stable Micro Systems, 2023). The loss of water also seems to influence the bread's resilience, cohesiveness, and springiness, with this loss decreasing the ability to regain the original structure (López & Goldner, 2015).

Villarino et al. (2016) suggested that the lupin flour's high fibre contents, with its water-binding capacity, could contribute to delaying the staling process by preventing gluten dehydration and helping to retain the crumb moisture. Nevertheless, the authors recognize that further studies were necessary to concretely explore the potential of lupin to reduce bread staling (Villarino et al., 2016).

The breads proved to be microbially stable during the storage period (Table 7). A possible mold contamination was detected on the fourth day, however this seemed to be an external contamination, as it was not confirmed in the fifth day's samples. Nevertheless, all microbiological parameters were within the satisfactory reference values for food hygiene and safety (Instituto Nacional de Saúde Doutor Ricardo Jorge, 2019). Therefore, the bread was deemed safe for consumption for at least five days.

4. Conclusion

Multigrain bread formulations with high-protein and low-saturated fat were developed using a combination of lupin with different grains (rye, spelt, oat and carob) and *C. vulgaris*.

The effect of the water and microalgae percentages in bread formulations was evaluated using the response surface methodology. This methodology was successful in optimizing the water and microalgae percentages in the breads and in the predicting the bread's sensory responses. Only water was shown to influence the responses of the breads, with the rye/lupin bread receiving the highest scores for taste and overall appreciation. The spelt/lupin bread had the lowest overall sensory scores.

A study on the shelf-life of the rye/lupin bread saw an increase in hardness and decrease in moisture, a_W, springiness, cohesiveness, and resilience during storage. Nevertheless, the microbial safety was confirmed for at least five days.

Regarding the limitations of this study, there are some aspects that could be enhanced. The protein content was determined by the Dumas method, which should be optimized for the matrix studied. A comparison with the protein content obtained by Kjeldhal method would be desirable. Moreover, the sensory panel was mainly composed of women, who could have influenced the resulting formulations, so a possible gender bias should not be excluded. Further characterization of bread dough and the nutritional profile, such as mineral, dietary fibre and amino acid composition, antioxidant activity, microstructure, specific volume, spread ratio, water holding capacity, and *in vitro* digestibility could enhance the bread's novelty and strengthen their market potential.

Despite of the referred limitations, savoury multigrain bread formulations were successfully developed, and all multigrain breads met the requirements for the nutrition claims 'rich in proteins' and 'low in saturated fats', making them good alternatives for consumers looking for healthy and nutritious bread options.

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Ethical statement

Participants gave their verbal consent for the sensory evaluation and could withdraw from the survey at any time, without the need to indicate the reason. The products tested were safe for consumption.

CRediT authorship contribution statement

Tatiana Pereira: Investigation, Writing – original draft, Visualization. **Sandrina Costa:** Investigation. **Sónia Barroso:** Supervision, Writing – review & editing, Visualization. **Paula Teixeira:** Investigation, Writing – review & editing. **Susana Mendes:** Formal analysis, Writing – review & editing, Validation. **Maria M. Gil:** Conceptualization, Supervision, Funding acquisition, Project administration, Resources, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115612.

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