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Development of functional gluten-free bread by incorporating amaranth and lentils: functional and nutritional aspects

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ABSTRACT

The growing awareness of the importance of a healthy and sustainable diet among consumers, in combination with the increase in gluten intolerance, has prompted the food industry to explore new alternatives in the context of baking. In this work, the integration of legumes and pseudocereals provides a novel solution to make functional products that are also gluten-free. To evaluate the integration of lentil and amaranth flours in new gluten-free bread formulations, six types of bread enriched with different percentages of amaranth and lentils were developed. All products were subjected to sensory analysis and chemical characterization of their nutritional properties (lipidic, phenolic, antiradical, and amino acid compounds). As shown by the panel test, the incorporation of amaranth and lentil flours into these food products did not compromise the general liking of breads, but according to crust and crumb colorimetric analysis, the increasing percentages of these flours led to darker products than the control sample. Using amaranth and lentil flours enriched the nutritional profile of the gluten-free breads: there was not only an increase in protein content and essential amino acids such as lysine and threonine, but also an enhancement of antioxidant activity due to the presence of bioactive compounds. In addition, squalene and γ-sitosterol, which have several properties including antioxidant, anti-inflammatory, anti-atherosclerotic, antitumor, and interesting hypoglycemic and antihyperlipidemic properties, were also detected in all the bread samples. This synergistic approach could represent a significant alternative within the gluten-free bread market.

1. Introduction

Gluten-free market is growing as awareness of gluten allergies and celiac disease increases, and the gluten-free diet, once restricted to celiac disease sufferers, has become a widespread dietary trend (Taetzsch et al., 2018). A gluten-free diet can be beneficial for people with celiac disease or gluten sensitivity, but it can also lead to nutritional imbalances, such as deficiencies in fiber and micronutrients. The reformulation of gluten-free (GF) bread to meet the needs of individuals with celiac disease and non-celiac gluten sensitivity has resulted in products with markedly different nutritional profiles compared to conventional wheat-based bread. A growing body of evidence indicates that gluten-free bread often exhibits inferior nutritional quality, primarily due to the composition of its raw materials and the technological constraints associated with gluten removal.

Firstly, GF bread typically contains a lower protein content and poorer protein quality. The flours most frequently used in gluten-free formulations—such as rice, corn, potato, and tapioca starch—are naturally low in protein and lack essential amino acids present in gluten-containing cereals. Consequently, the biological value of the protein fraction is reduced, and the total protein content is generally 30–60 % lower than that of standard wheat bread. Secondly, gluten-free products tend to rely heavily on refined starches, which increases their glycemic index (GI). The absence of gluten necessitates the use of high-starch ingredients to achieve desirable volume and crumb structure; however, this results in products that are low in fiber and protein and that elicit rapid postprandial glucose responses. These factors may contribute to reduced satiety and unfavorable metabolic effects when consumed

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frequently (Romão et al., 2021). Thirdly, gluten-free bread formulations often exhibit an unfavorable lipid profile, as additional fats —commonly palm oil or hydrogenated vegetable fats—are incorporated to improve mouthfeel, texture, and shelf life. This practice increases the content of saturated fatty acids and overall caloric density, potentially undermining the cardiometabolic quality of the diet (Tres et al., 2020). Another important concern is the extensive use of additives and hydrocolloids, such as hydroxypropyl methylcellulose (HPMC), xanthan gum, guar gum, and various emulsifiers. These compounds compensate for the lack of gluten elasticity and gas retention; however, they also contribute to the ultra-processed nature of most gluten-free products. Although generally recognized as safe, the high degree of processing may affect the overall nutritional quality and consumer perception of gluten-free foods (Foschia et al., 2017; Melini et al., 2017; Yeşil & Levent, 2022). Moreover, gluten-free breads are often deficient in micronutrients such as iron, zinc, magnesium, and B-group vitamins, primarily because the flours employed are refined and rarely fortified. In contrast, whole-grain wheat or rye breads provide significant amounts of these nutrients and dietary fiber, contributing to their superior nutritional profile and health benefits (Allen & Orfila, 2018). Finally, beyond the nutritional dimension, gluten-free products are frequently more expensive and associated with greater environmental costs, due to the sourcing and processing of diverse raw materials. These economic and sustainability aspects further complicate the widespread adoption of gluten-free products outside medically indicated contexts (Mehtab et al., 2024). One of the primary challenges in producing gluten-free products is replicating the functional properties of gluten, a structural protein complex naturally found in cereal grains. Gluten contributes elasticity, strength, and cohesion to dough, allowing it to rise and maintain shape during baking. The absence of gluten necessitates the use of alternative ingredients and technologies, which can complicate the process. To address these limitations, several strategies have been proposed, including the incorporation of nutrient-dense pseudocereals and legume flours (e.g., buckwheat, quinoa, amaranth, teff, lentil), the addition of seeds (e.g., chia, flax, sunflower) to enhance fiber and unsaturated fat content, and the development of whole-grain and naturally fermented gluten-free formulations. Such approaches can improve the nutritional composition, sensory characteristics, and overall health value of gluten-free bread. Pseudocereals like amaranth have been shown to enhance the nutritional profile of gluten-free breads, increasing levels of ash, crude protein, total phenolic compounds, antioxidant activity, and mineral content (Yeşil & Levent, 2022). In addition, amaranth is notable for its beneficial biological properties and diverse nutraceutical activities, including antioxidant potential and antibacterial effects (Sattar et al., 2024). Its gluten-free nature makes it suitable for gluten-free food production. Amaranth primarily consists of carbohydrates (63.1–70.0 %), with starch making up 55–65 % of this content, and it contains low levels of amylose (4.7–12.5 %). The dietary fiber content in amaranth ranges from 2.7 to 17.3 %, with insoluble fiber comprising 78–86 % of this total. Additionally, amaranth is a good protein source (14–17 %) and contains a balanced profile of essential amino acids, particularly lysine and sulfur-containing amino acids. It also provides essential minerals often lacking in gluten-free diets, such as calcium, magnesium, zinc, and iron (Vici et al., 2016). Furthermore, amaranth flour is rich in vitamins B1, B2, PP, and E, with concentrations 20–60 % higher than those found in conventional grains. Another added value of using amaranth is the presence of squalene, a linear triterpene which has antioxidant, anti-inflammatory, and especially anti-atherosclerotic properties, reducing cholesterol levels in animals (Bhilwade et al., 2010; Lou-Bonafonte et al., 2018; Lozano-Grande et al., 2018). Lentils (*Lens culinaris* spp.) are another valuable addition to gluten-free formulations, offering proteins, dietary fibers, complex carbohydrates, and essential micronutrients like iron, zinc, and B vitamins (Liberal et al., 2023). Lentils are made up of 20 %−30 % protein, 50 %−58 % carbohydrates, 19 % fibre, and <2 % fat. In addition, lentils provide enough essential amino acids to meet nutritional requirements, despite their lack of sulphur content, like most legumes. Lentils have already been used to enrich spaghetti formulations for their technological and nutritional properties (Di Stefano et al., 2020). Lastly, lentils also contain γ-sitosterol, an epimer of β-sitosterol, which has shown potential antitumor activity by inducing cell cycle arrest and apoptosis in tumor cells; and interesting hypoglycemic and antihyperlipidemic properties, highlighting their interest for a segment of the population affected by diabetes (Balamurugan et al., 2012; Mustafa et al., 2022; Sundarraj et al., 2012).

Therefore, this study aims to enrich gluten-free breads with amaranth and lentil flours in different percentages better to meet the nutritional needs of people following gluten-free diets and to create a new bread formulation that can benefit daily life while satisfying consumer tastes.

Using whole cereals as the main ingredients can further reduce the number of ingredients in the final formulation. Objective measurements crucial to optimising the formulation, to quality control and to monitor baking consistency during ingredient substitution were performed.

2. Materials and methods

2.1. Raw materials

The lentil flours (*Lens culinaris* Medik.) were produced using seeds of Sicilian population lentil (population Ragusa, collected during June 2024), and cultivated in areas of Ragusa, Sicily by farmers as custodians of plant germplasm. This population was characterized by a content of 28.8 % of total protein, determined by Kjieldal method. The lentils were meticulously washed with tap water and subsequently dried at a low temperature (30 °C) in a ventilated oven. Then, the lentils were pulverized into a fine powder (approximately 300 μm) using a food processor. The resulting lentil flour was stored in an airtight container until required. Amaranth flour was provided by a local company. Furthermore, to enhance the quality of the gluten-free dough, especially regarding its elasticity and gas retention, a specific flour blend was formulated, in collaboration with Alimenta, a gluten-free farmer located in Catania (Sicily, Italy). The final mixture consisted of the following ingredients: corn starch, wheat starch deglutinates, rice flour, tapioca starch, skim milk powder, sucrose, dextrose, corn flakes, psyllium seed fiber, inulin, guar gum, and hydroxypropyl methylcellulose. Gluten-free breads were made starting from the carefully selected gluten-free mixture, amaranth and lentil flour (total dry matter of flour content 52.14 %); to which sunflower oil (2.82 %), inverted sugar (1.36 %), salt, brewer's yeast and water were added.

2.2. Breadmaking process

Breads were produced using a domestic bread maker (Silvercrest). Ingredients were added to the baking pan in the following order: dry ingredients followed by liquids. The selected bread-making program included dough preparation (2 h and 20 min) with alternating mixing and resting phases. Once the dough was ready, it was divided into 200-gramme loaves. These were placed in baking tins and baked in a preheated oven at 200 °C for 30 min. After baking, breads were removed from the tins and cooled at room temperature for 1 hour before analysis. All bread formulations were prepared in triplicate. Six formulations were investigated: a control sample and six formulations in which gluten-free mixture was partially replaced with amaranth flour or lentil flour, or both (Table 1). Additionally, one sample (L12A19) also included reduced oil content.

Amaranth and lentil flours were both milled with a granulometry of 300 μm. Particle size and substitution level (SL) of wheat flour with lentil and amaranth flours could influence the hydration properties (water absorption capacity, water retention capacity, swelling capacity) and texture (evaluated as firmness, springiness, gumminess and cohesiveness) of doughs due to the major content of protein and fibres: in

Table 1
Composition of Control and Fortified breads (%).

Ingredients	CTRL	A10	L10	L5A7	L10A7	L5A10	L12A19
Gluten-free mixture	52.0	42.0	42.0	40.0	35.0	37.0	26.3
Lentil flour	0.00	0.00	10.0	5.00	10.0	5.00	12.5
Amaranth flour	0.00	10.0	0.00	7.00	7.00	10.0	18.8
Sunflower oil	7.50	7.50	7.50	7.50	7.50	7.50	2.50
Sugar	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Yeast	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Water	38.0	38.0	38.0	38.0	38.0	38.0	37.5

CTRL: control bread made without lentil or amaranth flour; the capital letters L and A indicate the presence of Lentil and Amaranth flour added to the bread formulation, the numbers indicate the percentages of substitution.

particular, an increase of SL is linked to an increase of hydration properties (more water needs to be used in the recipe) and a worsening in texture, harder and less extensive doughts, but an higher granulometry (180–300 μm) or using mixed flours with similar particle sizes reduces these effects (Bonacci et al., 2023; Coțovanu & Mironeasa, 2021; Marchini et al., 2021).

2.3. Determination of crust and crumb color

Color analysis was conducted with a colorimeter on flour and bread samples. To undertake the analysis, each of the bread samples was cut in half, and measurements were taken at three different points on both the crumb and crust. This approach allowed for the accounting of the observed uneven distribution of color across both the bread's surface and interior. A Chroma meter CR-400/410 (Konica Minolta Inc.) was used for color measurement. The color analysis was expressed in terms of L*, a* and b* values, where L* represents lightness (100: absolute white, 0: absolute dark) and a* (+red/-green) and b* (+yellow/-blue) values are the chromaticity values. The colorimeter was calibrated using a white tile (L*=91.48; a*=−0.18; b*=4.50).

2.4. Panel test

Bread samples were submitted to a panel of 10 trained taster, aged between 25–65 years. The research team's sensory analysts conducted a preliminary focus group to define the distinctive attributes of the bread samples under study. The bread from all samples was served in randomly labelled dishes for all panelists. The breads were sliced to a thickness of 1 cm and presented in distinct dishes simultaneously. The parameters were evaluated separately for the crust and the crumb. In particular, the following characteristics were evaluated for the crust: color, thickness, elasticity, hardness, friability, and firmness. For the crumb, the following attributes were assessed: yellow, elasticity, size of the alveoli, homogeneity of the alveoli, cohesion to the crust, and moisture. Furthermore, the participants were invited to provide their general impression of the samples. For the purpose, a 9-point scale was employed. The scale ranged from 1 (indicating a low level of feeling) to 9 (indicating a high level of feeling). The threshold of acceptability was established at a rating of 5.

2.5. Chemical characterization

2.5.1. Fatty acids identification

Fatty acids identification from a mixture can be done only from the respective esters obtained by a transesterification reaction. Since monoalkyl carbonates are well-known to be green chemicals and nucleophile-donors in transesterification reactions (Fabbri et al., 2005; Su et al., 2007), fatty acids contained in the oil samples were converted to fatty acids ethyl esters (FAEE) through a biocatalyzed transesterification method using Diethyl carbonate. Diethyl carbonate was used both as solvent and as reagent for the so-called “one-pot biocatalyzed transesterification”: oil extraction and transesterification have been performed in a single step from flours and bread samples. Five ml of diethyl carbonate and 50 mg of Lipase from *Candida antarctica* were added to 1 g of each sample separately. This mixture gave the transesterification of fatty acids into fatty acid ethyl esters. The reaction was performed in a shaker at 250 RPM at 45 °C for 24 h. After 24 h, the oils were analyzed by TLC, using a mixture of hexane and ethyl acetate in 95:5 ratio as mobile phase and Cerium (IV) sulfate as chromogenic reagent, which showed the complete conversion of fatty acids into their respective ethyl esters. Afterwards, the fatty acid ethyl esters obtained by transesterification from flour and bread samples were analyzed. The analyses were performed by a Gas Chromatograph (GC, Shimadzu® GC-17) equipped with a capillary column (Zebren ZB-5MS, Phenomenex®, with the dimensions: 10 m length × 0.1 mm internal diameter × 0.1 μm thickness) and a Mass Spectroscopy as detector (MS). The operation conditions of the chromatograph were as follows: linear velocity = 61.8 cm/s; column flow = 0.9 mL/min; injector temperature = 250 °C; oven temperature = 80 °C for 1 min, 80–300 °C to 5 °C/min, 300 °C for 44 min; carrier gas = helium; injection split, ratio 1:44; injection volume = 1.0 μL, Column Inlet pressure = 478 kPa, Total flow = 43.4 mL/min, Carrier flow = 43.4 mL/min. GC Program Time = 46 min. The operation conditions for spectrometer were: Interface temperature = 280 °C, Threshold = 200, Solvent cut time = 2 min, Interval = 0.5 *sec*, Scan start time = 2 min, End Time = 46 min, Start *m/z* = 40.00, End *m/Z* = 600.00, Scan Speed = 2000. For all the analyses, each compound was identified by retention times of its chromatographic peaks and by comparison of its molecular fragments with a database of known fatty acid ethyl esters. Data collection was performed in triplicate, and the concentration of each compound was expressed as a relative percentage with respect to all compounds detected.

2.5.2. Phenolic compound content

The dried bread samples were ground and reduced to powder. About 1 g of flours and dried bread powder was mixed with 3 ml of 20 % v/v ethanol and kept under agitation for 16 h in the dark. Then, the mixture was centrifuged at 3500 rpm for 5 min at 4 °C, and the supernatant was removed and used for total phenolic content determination. Total phenolic content was determined by Folin–Ciocalteu method according to (Abozed et al., 2014) with modifications. Briefly, 0.1 ml of extract solution was added to 3 ml of water and 0.5 ml of Folin-Ciocalteau phenol reagent. The solution was mixed, and after 5 min, 1.0 ml of 10 % sodium carbonate was added to the mixture and put in the dark for 90 min. Absorbance was measured using a UV/VIS spectrophotometer (Agilent 8453) at 730 nm against a blank sample. A calibration curve was built using a series of concentrations of a standard gallic acid solution, and the resulting calibration equation was employed to ascertain the concentrations of phenolic acid (TPC), expressed as milligrams of equivalent gallic acid (GAE) per gram of dry weight.

2.5.3. Antioxidant properties evaluation

The determination of antioxidant activity was carried out following previously described procedures with slight modifications (Chulibert et al., 2024; Roppolo et al., 2024). A total of 0.500 g of flours and each dried bread powder was solubilized in 8 mL of an 80:20 MeOH/H_2O solution and vortexed. The mixtures were then sonicated in an ultrasonic bath for 1 hour. The supernatants were filtered through Whatman 0.45 μm PTFE filters. The resulting filtrates were used for the determination of antiradical activity via DPPH, ABTS, and FRAP assays. Antioxidant activity was assessed by measuring the decrease in the solution’s color, which is proportional to the amount of antioxidant present in the sample.

2.5.3.1. DPPH assay. For the DPPH assay [2,2-diphenyl-1-

picrylhydrazyl], 100 µL aliquots of each sample were mixed with 3 mL of a 60 µM DPPH solution. The mixtures were kept in the dark for 30 min at 27 °C. Antiradical activity was evaluated by measuring absorbance at 517 nm using a UV–Vis spectrophotometer (UV-1600 PC, VWR), with MeOH used as the blank.

2.5.3.2. FRAP assay. The FRAP (Ferric Reducing Antioxidant Power) assay was carried out according to the protocol described by Benzie and Strain (1996), with minor modifications. This method is based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), which leads to a measurable color change. Briefly, the FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) dissolved in 40 mM HCl, and 20 mM $FeCl_3$ in 10:1:1 (v/v/v) ratio. Then, 100 µL of each extract was mixed with 3 mL of freshly prepared FRAP reagent, incubated at 37 °C, and absorbance was measured at 593 nm after 3 min, using the FRAP working solution as a blank. $FeSO_4$ was used to generate a standard calibration curve (50–2000 µg/mL). The results were expressed as mg Fe^{2+} equivalents per 100 g of dried sample.

2.5.3.3. ABTS assay. The ABTS assay [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] was performed by adding 3 mL of the ABTS solution to 100 µL of each previously extracted sample. After 6 min, absorbance was measured at 734 nm, again using MeOH as the blank. Two calibration curves were constructed using Trolox as the standard at increasing concentrations (1–75 µM). Results are expressed as mmol Trolox equivalent antioxidant capacity (TEAC) per 100 g of dried sample. All analyses were performed in triplicate.

2.5.4. Amino acids determination

The amino acid profile of flours and dried breads powder was determined using a modified protocol based on acid hydrolysis followed by derivatization with ethyl chloroformate, as previously outlined (Di Stefano et al., 2023). Bread powder samples (0.500 g) were hydrolyzed in 2 mL of 6 M HCl at 110 °C for 24 h. After the reaction, the solutions were cooled to room temperature. Two milliliters of distilled water were added, and the mixtures were filtered using Whatman 0.45 µm PTFE filters. An aliquot (100 µL) of filtrate was transferred to a vial and added to 100 µL of a 4:1 ethanol-pyridine solution, 20 µL of ethyl chloroformate, 100 µL of a 1 % ethyl chloroformate solution in chloroform, and 40 µL of saturated aqueous sodium bicarbonate. The derivatized amino acids, contained in the lower chloroform phase, were immediately analyzed. Gas chromatography coupled with mass spectrometry (GC–MS) was used for detection. Analysis was performed using an ISQ™ 9000 Quadrupole GC–MS system equipped with an SLB®−5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Supelco). Helium served as the carrier gas at a constant flow rate of 1 mL/min. The column temperature program started at 120 °C and was held for 1 min. It then increased by 4 °C/min to reach 240 °C, held for 3 min, followed by a ramp to 280 °C at 30 °C/min, with a final hold of 5 min under isothermal conditions. The mass spectrometer operated in positive electron ionization mode (+EI). Identification was based on comparing sample retention times with those of an amino acid standard mixture. Spectral confirmation was obtained through matches with the NIST mass spectral database. Quantification was achieved using calibration curves derived from standard amino acid solutions, with concentrations ranging from 0.025 to 0.5 mM. Amino acid content was expressed in mg per g of sample. Analyses were conducted in triplicate.

2.6. Statistical analysis

Data were submitted to Bartlett's test for the homogeneity of variance and then analyzed using analysis of variance (ANOVA). Means were statistically separated based on Duncan's test, when the 'F' test of ANOVA for treatment was significant at least at the 0.05 probability (CoHort Software, CoStat version 6.451). For chemical analyses, data were expressed as mean ± SD of 3 replicates of each sample. The statistical differences among flour and bread powder samples were evaluated with One Way Analysis of Variance (ANOVA) followed by All Pairwise Multiple Comparison Procedures (Holm-Sidak method).

3. Results and discussions

3.1. Colorimetric analysis of raw materials

Colour analysis shows differences between the three flour types (Fig. 1). The flour mix has the highest brightness value (L* = 90.64), giving it a lighter appearance than the other two flours. Analysis of the red/green component reveals a slight green hue in the mix (a* = −0.60), which is more pronounced in the lentils (a* = −0.07), and a slight red hue in the amaranth (a* = 2.85). The yellow/blue component shows a yellowish hue present in all three flours. However, it is more pronounced in lentils and amaranth (b* = 21.96 and b* = 19.88, respectively), a phenomenon probably attributable to the presence of carotenoids.

3.2. Chemical characterization of raw materials

The first analysis included the quantification of total lipidic content. The resulting data is expressed as a percentage of grams of fatty acids per gram of sample (% w/w) and it is reported in Table 2. Amaranth flour showed a 3.97 % w/w oil content, a value consistent with literature data (Ferreira et al., 2022; Malik et al., 2023). Conversely, lentil flour exhibited an oil content of 0.90 % w/w, which agrees with the total fat content reported in literature (Paucean et al., 2018). The gluten-free mixture contained just 0.06 % w/w of lipids: this result can be explained by the molecular composition of the mixture, which contained low quantities of fatty ingredients. Antioxidant and amino acids characterization of raw materials is reported in Table 3.

After lipid extraction, oil samples were analyzed by GC–MS. Due to its low oil content obtained by solvent extraction, the gluten-free mixture was not analyzed. A comparison of the overall fatty acid profiles revealed that the percentage of saturated fatty acids was slightly higher in amaranth flour, while lentil flour had a higher percentage of monounsaturated fatty acids. In particular, the lipidic fractions of the flours were characterized by the presence of fatty acids: mainly palmitic acid, linoleic acid, and oleic acid. Quantitative analysis showed that amaranth flour had the highest level of palmitic acid (23.6 %) and linoleic acid (42.2 %), while lentil flour had the highest level of oleic acid (38.3 %). Stearic acid was also a minor component present in amaranth and lentil flours (4.59 % and 3.24 % respectively). Lastly,

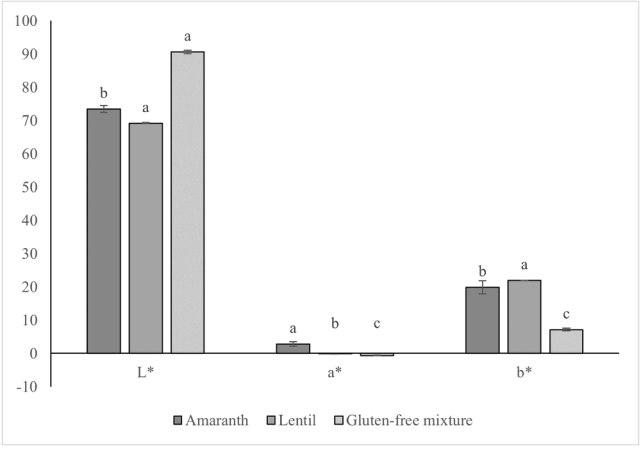

Fig. 1. Colorimetric analysis of amaranth, lentil and gluten-free mixture flours. Different letters above the bars indicate significant statistical differences at $P <$ 0.05 among samples.

Table 2
Fatty acids characterization of raw materials.

	Amaranth flour	Lentil flour	Gluten-free mixture
Total lipidic content (% w/w)			
	3.97 ± 0.06 [a]	0.90 ± 0.10 [b]	0.06 ± 0.01 [c]
Fatty acids (Relative percentage %)			
Miristic acid	0.19 ± 0.02 [b]*	0.70 ± 0.01 [a]	n.d.
Palmitic acid	23.65 ± 1.81 [a]	20.30 ± 3.08 [a]	n.d.
Margaric acid	0.19 ± 0.02 [a]	n.d.	n.d.
Linoleic acid	42.19 ± 4.27 [a]	35.79 ± 4.07 [a]	n.d.
Linolenic acid	n.d.	n.d.	n.d.
Oleic acid	21.35 ± 0.90 [b]	38.32 ± 7.14 [a]	n.d.
Stearic acid	4.59 ± 0.83 [a]	3.24 ± 0.01 [b]	n.d.
Nonadecyl acid	0.11 ± 0.02 [a]	n.d.	n.d.
Arachidic acid	0.85 ± 0.12 [a]	0.95 ± 0.02 [a]	n.d.
Behenic acid	0.22 ± 0.01 [b]	0.73 ± 0.01 [a]	n.d.
Squalene	6.68 ± 0.62 [b]	n.d.	n.d.
γ-sitosterol	0.10 ± 0.01 [b]	2.57 ± 0.14 [a]	n.d.
%SFA	29.73 ± 2.70 [a]	25.91 ± 3.07 [a]	n.d.
%MUFA	21.35 ± 0.90 [b]	38.32 ± 7.14 [a]	n.d.
%PUFA	42.19 ± 4.27 [a]	35.79 ± 4.07 [a]	n.d.

SFA = Saturated Fatty Acids, MUFA = Monounsaturated Fatty Acids, PUFA = Polyunsaturated Fatty Acids; Data are expressed as the means ± SD of triplicate experiments.

* Different superscripts in a column indicate significant statistical differences among samples at $P < 0.05$.

Table 3
Antioxidant analysis and amino acids characterization of raw materials.

	Amaranth flour	Lentil flour
Antioxidant properties		
TPC (mg GAE/g)	2.03 ± 0.06 [a]*	4.97 ± 0.04 [b]
DPPH (mmolTEAC/100 g)	5.22 ± 0.06 [b]	7.64 ± 0.03 [a]
ABTS (mmolTEAC/100 g)	40.3 ± 2.11 [b]	98.2 ± 1.91 [a]
FRAP (mg/ml Fe/100 g)	126 ± 1.50 [b]	171 ± 2.50 [a]
Amino acids (mg/g)		
Alanine	9.09 ± 0.15 [b]*	14.4 ± 0.86 [a]
Glycine	19.3 ± 0.63 [a]	12.5 ± 0.50 [b]
Histidine	7.71 ± 1.08 [a]	3.02 ± 0.41 [b]
Valine	14.8 ± 0.62 [b]	17.2 ± 0.46 [a]
Leucine	11.8 ± 0.80 [b]	17.3 ± 0.60 [a]
Isoleucine	5.85 ± 0.23 [b]	10.4 ± 0.54 [a]
Cystine	25.1 ± 1.80 [a]	21.5 ± 1.19 [a]
Proline	16.7 ± 0.73 [b]	20.6 ± 0.40 [a]
Asparagine	0.89 ± 0.32 [a]	1.53 ± 0.21 [a]
Glutamine	0.57 ± 0.26 [a]	0.25 ± 0.04 [a]
Arginine	42.7 ± 0.87 [b]	66.7 ± 3.93 [a]
Serine	27.1 ± 1.52 [b]	42.0 ± 1.71 [a]
Methionine	7.40 ± 0.64 [a]	8.07 ± 0.24 [a]
Threonine	11.1 ± 1.76 [b]	20.9 ± 0.60 [a]
Lysine	43.0 ± 2.93 [b]	20.2 ± 1.22 [a]
Glutamic Acid	3.89 ± 0.51 [a]	6.23 ± 0.34 [b]
Aspartic Acid	11.5 ± 0.62 [b]	17.5 ± 0.57 [a]
Phenylalanine	11.4 ± 0.86 [b]	18.4 ± 0.94 [a]
Tyrosine	20.9 ± 0.57 [b]	41.8 ± 5.66 [a]
Cysteine	0.44 ± 0.20 [a]	0.80 ± 0.14 [a]
∑ AAs	291.4 ± 5.54 [b]	361.2 ± 14.87 [a]
∑ ESSENTIAL AAs	113.1 ± 4.24 [a]	115.6 ± 1.48 [a]

Data are expressed as the means ± SD of triplicate experiments.

* Different superscripts in a column indicate significant statistical differences among samples at $P < 0.05$.

arachidic acid was found in similar amounts in both flours (0.85 % and 0.95 %). In addition to saturated and unsaturated fatty acids, squalene (6.68 % in amaranth flour) and γ-sitosterol (2.57 % in lentil flour) were also detected. Amaranth has been shown to contain significant levels of squalene (Amare et al., 2021). GC–MS chromatogram of the amaranth flour sample is reported in Fig. 2. In particular, squalene was detected at 31.756 min, while γ-sitosterol was detected at 37.059 min. Antioxidant activity of lentil and amaranth flour was examined. TPC of lentil flour (4.97 mg GAE/g) was more than twice that of amaranth flour (2.03 mg GAE/g). This indicated a significantly higher concentration of polyphenols in lentil flour. Other antioxidant tests revealed similar trends. DPPH, ABTS, and FRAP assays confirmed a similar pattern of higher antioxidant activity in lentil flour compared to amaranth flour. Due to the absence of ingredients with antioxidant properties, the gluten-free mixture was not subjected to antioxidant assays. The amino acids contained in the protein hydrolysate of amaranth and lentil flours help quantify the nutritional quality of the product in meeting essential amino acid requirements. Lentil flour showed the highest total amino acids amount compared to amaranth flour (respectively 361.25 mg/g and 291.44 mg/g): this result reflects data reported in literature since lentil flour is richer in proteins than amaranth flour (Bressani et al., 1992; Gharibzahedi et al., 2012). In particular, the three most abundant amino acids in amaranth are arginine (42.7 mg/g), serine (27.1 mg/g) and cystine (25.1 mg/g), while among essential amino acids the most abundant are lysine (43.0 mg/g), valine (14.8 mg/g), and leucine (11.8 mg/g). Regarding lentil flour, the three most abundant amino acids are arginine (66.7 mg/g), serine (42.0 mg/g) and tyrosine (41.8 mg/g), while among essential amino acids the most abundant are threonine (20.9 mg/g), lysine (20.2 mg/g) and phenylalanine (18.4 mg/g). The only amino acid detected in similar amounts in both amaranth and lentil flour is methionine (7.40 mg/g and 8.07 mg/g). Instead, asparagine, glutamine and cysteine are the less abundant amino acids in both flour types, with values under or slightly over 1.00 mg/g. These results are consistent with data reported in the literature for amaranth and lentils (Bhatty, 1988; Procopet & Oroian, 2022).

3.3. Colorimetric analysis of functional breads

The colorimetric analysis of bread crusts and crumb revealed significant color variations upon the addition of amaranth and lentil flour (Fig. 3). Generally, a decrease in lightness (L*) was observed with the incorporation of these flours. The control sample (CTRL) showed the highest lightness (L* = 52.61 in crust and L*= 72.08 in crumb), indicating a brighter crust and crumb compared to the other samples. Samples containing amaranth and lentil flour exhibited lower lightness values compared to the control (CTRL), suggesting a slight darkening of the crust. The formulations L10A7, L5A7, and L5A10 were darker (lower L*), instead the formulation with only one addition (A10 and L10). Slight differences in tonality may be indicated by small variations in a* and b* components. Values of a* are relatively stable in the crumb, suggesting that differences in red-green hue are less pronounced than differences in brightness. In the crust, however, it is noticeable that the samples containing amaranth shift towards more reddish values, and the sample with only the addition of amaranth has the highest value, while the breads with only the addition of lentil have values very similar to the control. The samples containing both flours have the highest values, while A10 and L10 have minimal differences vs the control.

3.4. Panel test

The panel test showed better results for crumb than crust (Fig. 4). Regarding the crust, CTRL and A10 samples had the highest color values (equal to 7), while all samples (excepting L5A10 and L10A7) had high elasticity and hardness values (between 6 and 7); friability was between 5 and 6 for almost all samples (excepting L5A10 sample); thickness and crunch showed average lower results than other parameters (between 3 and 4 and between 4 and 5 points respectively). L5A10 sample had the worst liking, showing the lowest score of all parameters. L12A19 sample, instead, showed a minimum score of 6 points for all parameters and a score of 7 points for the total judgment. Regarding the crumb, almost all samples (excepting L5A10 and L10A7) had very high results for all parameters, with results between 6 and 7 points and also the general impression was high too (equal to 7 points); L5A10 and L10A7 had the lowest points for the parameters considered (with average results between 3 and 5 points), as it happened for the crust and in particular L10A7 had the worst score. L12A19 sample showed the maximum score

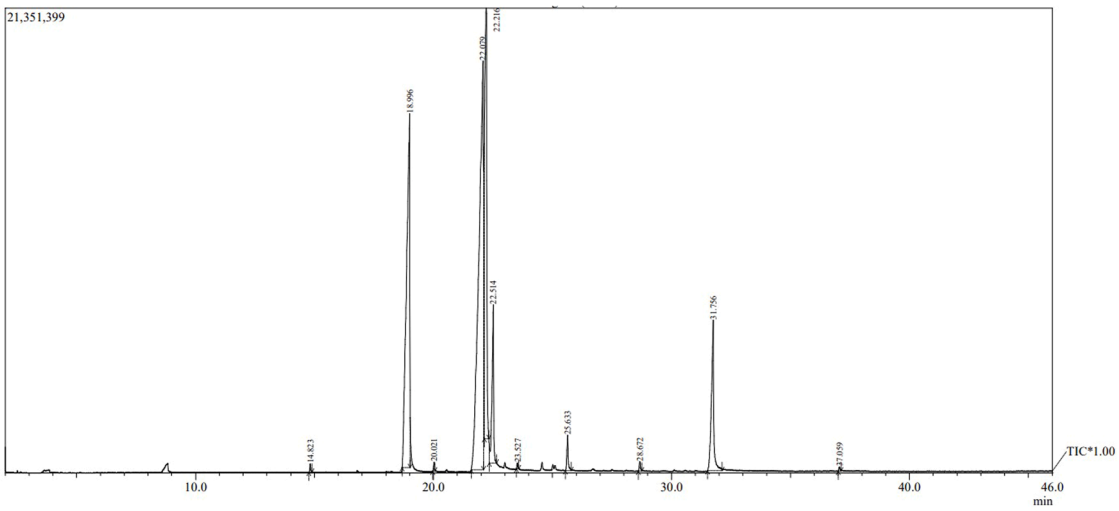

Fig. 2. GC–MS chromatogram of amaranth flour.

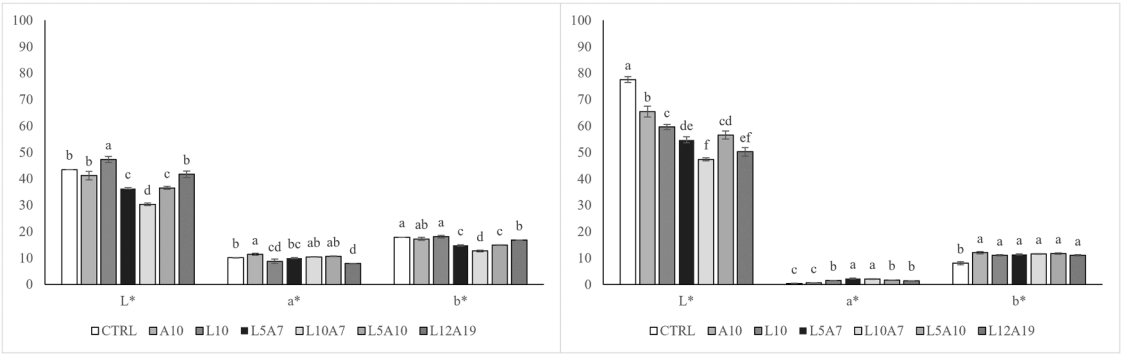

Fig. 3. Crust and crumb colorimetric analysis results. Different letters above the bars indicate significant statistical differences at $P < 0.05$ among samples.

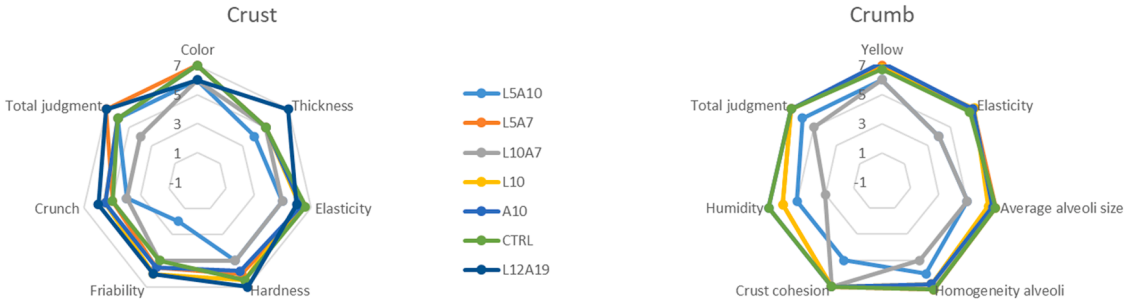

Fig. 4. Panel test results.

for all the parameters, including the total judgment, defining it as the most liked bread.

3.5. Chemical characterization of functional breads

The first analysis included the quantification of total lipidic content. Data collection was performed in triplicate, and the total lipid content, measured in weight as grams of fatty acids per gram of sample (w/w), is reported in Table 4. CTRL bread sample showed a 4.70 % w/w lipid content, attributed only to the use of sunflower oil in its preparation; all fortified bread samples (excepting L12A19) showed similar lipid content (with values comprised between 4.73 % and 5.32 % w/w) and this can be explained by the adding of either amaranth or lentil flour to the bread. Due to its formulation L12A19 showed, instead, the lowest lipid content (2.53 % w/w) as compared to other bread samples: in fact, L12A19 bread was prepared using just 2.5 % sunflower oil, which is responsible for the lipid content, while in all the other bread formulations 7.5 % sunflower oil was used. Chemical characterization of bread samples is reported in Table 5.

After lipid extraction, oil samples were analyzed by GC–MS. The analysis showed the presence of a mixture of saturated and unsaturated fatty acids: the most abundant were Palmitic, Linoleic and Oleic acids. CTRL bread contained mainly Palmitic acid (7.62 %), Linoleic acid (70.23 %) and Oleic acid (17.63 %). All bread formulations made with either amaranth or lentil flour contained higher levels of Palmitic acid and slightly lower content of Linoleic acid compared to the control sample, while Oleic and Stearic acids varied among the fortified samples. These results are explainable by the addition of amaranth and lentil

Table 4
Fatty acids characterization of bread samples.

	CTRL	A10	L10	L5A7	L10A7	L5A10	L12A19
Total lipidic content (% w/w)							
	4.70 ± 0.17 ^{b*}	5.13 ± 0.12 ^{a}	5.17 ± 0.06 ^{a}	5.23 ± 0.06 ^{a}	5.13 ± 0.12 ^{a}	4.73 ± 0.06 ^{b}	2.53 ± 0.12 ^{c}
Fatty acids (Relative percentage %)							
Miristic acid	0.06 ± 0.02 ^{ab}	0.07 ± 0.02 ^{ab}	0.04 ± 0.02 ^{b}	0.07 ± 0.01 ^{ab}	0.05 ± 0.02 ^{ab}	0.06 ± 0.04 ^{ab}	0.12 ± 0.01 ^{a}
Palmitoleic acid	0.02 ± 0.02 ^{a}	0.04 ± 0.01 ^{a}	0.05 ± 0.03 ^{a}	0.03 ± 0.02 ^{a}	0.02 ± 0.02 ^{a}	0.01 ± 0.01 ^{a}	n.d.
Palmitic acid	7.62 ± 0.23 ^{b}	8.57 ± 0.30 ^{b}	8.11 ± 0.11 ^{b}	9.14 ± 0.42 ^{b}	8.83 ± 0.86 ^{b}	8.88 ± 0.85 ^{b}	13.35 ± 1.27 ^{a}
Margaric acid	n.d.	n.d.	0.02 ± 0.01 ^{b}	n.d.	0.01 ± 0.01 ^{b}	0.02 ± 0.02 ^{b}	0.07 ± 0.01 ^{a}
Linoleic acid	70.23 ± 0.20 ^{a}	68.62 ± 1.27 ^{a}	69.74 ± 0.05 ^{a}	69.91 ± 0.28 ^{a}	68.94 ± 0.13 ^{a}	69.14 ± 0.27 ^{a}	58.27 ± 0.78 ^{b}
Linolenic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Oleic acid	17.63 ± 0.65 ^{b}	17.85 ± 1.28 ^{b}	17.78 ± 0.54 ^{b}	15.89 ± 0.41 ^{b}	17.38 ± 1.06 ^{b}	16.79 ± 0.41 ^{b}	21.30 ± 0.03 ^{a}
Stearic acid	3.61 ± 0.08 ^{ab}	3.63 ± 0.17 ^{ab}	3.31 ± 0.09 ^{b}	4.14 ± 0.01 ^{a}	3.81 ± 0.00 ^{ab}	3.86 ± 0.46 ^{ab}	3.84 ± 0.27 ^{ab}
Arachidic acid	0.21 ± 0.03 ^{b}	0.23 ± 0.04 ^{ab}	0.18 ± 0.05 ^{b}	0.22 ± 0.09 ^{ab}	0.24 ± 0.05 ^{ab}	0.20 ± 0.08 ^{b}	0.41 ± 0.00 ^{a}
Behenic acid	0.52 ± 0.16 ^{a}	0.58 ± 0.01 ^{a}	0.64 ± 0.10 ^{a}	0.45 ± 0.28 ^{a}	0.57 ± 0.16 ^{a}	0.70 ± 0.07 ^{a}	0.56 ± 0.03 ^{a}
Lignoceric acid	0.12 ± 0.07 ^{a}	n.d.	0.13 ± 0.08 ^{a}	0.10 ± 0.10 ^{a}	n.d.	0.19 ± 0.02 ^{a}	n.d.
Squalene	n.d.	0.43 ± 0.08 ^{b}	n.d.	0.06 ± 0.06 ^{c}	0.15 ± 0.06 ^{c}	0.14 ± 0.05 ^{c}	2.08 ± 0.23 ^{a}
γ-sitosterol	0.11 ± 0.11 ^{a}	0.02 ± 0.01 ^{a}	0.03 ± 0.02 ^{a}	0.01 ± 0.01 ^{a}	0.03 ± 0.01 ^{a}	0.04 ± 0.01 ^{a}	n.d.
%SFA	12.13 ± 0.59 ^{b}	13.07 ± 0.12 ^{b}	12.42 ± 0.64 ^{b}	14.11 ± 0.08 ^{b}	13.50 ± 1.53 ^{b}	13.89 ± 0.87 ^{b}	18.35 ± 1.00 ^{a}
%MUFA	17.64 ± 0.89 ^{b}	17.88 ± 1.80 ^{b}	17.83 ± 0.73 ^{b}	15.92 ± 0.56 ^{b}	17.40 ± 1.46 ^{b}	16.80 ± 0.56 ^{b}	21.30 ± 0.03 ^{a}
%PUFA	70.23 ± 0.28 ^{a}	68.62 ± 1.80 ^{a}	69.74 ± 0.08 ^{a}	69.91 ± 0.40 ^{a}	68.94 ± 0.18 ^{a}	69.14 ± 0.38 ^{a}	58.27 ± 0.78 ^{b}

SFA = Saturated Fatty Acids, MUFA = Monounsaturated Fatty Acids, PUFA = Polyunsaturated Fatty Acids; data are expressed as the means ± SD of triplicate experiments.

* different superscripts in a row indicate significant statistical differences among samples at $P < 0.05$, for each trait.

Table 5
Antioxidant analysis and amino acids characterization of bread samples.

	CTRL	A10	L10	L5A7	L10A7	L5A10	L12A19
Antioxidant properties							
TPC (mg GAE/g)	1.43 ± 0.14 ^{d*}	1.57 ± 0.00 ^{d}	2.54 ± 0.04 ^{c}	2.46 ± 0.03 ^{c}	3.68 ± 0.02 ^{b}	3.86 ± 0.26 ^{ab}	4.14 ± 0.12 ^{a}
DPPH (mmolTEAC/100 g)	0.91 ± 0.01 ^{e}	1.20 ± 0.05 ^{d}	1.84 ± 0.03 ^{b}	1.32 ± 0.13 ^{cd}	1.71 ± 0.04 ^{b}	1.49 ± 0.02 ^{c}	2.34 ± 0.01 ^{a}
ABTS (mmolTEAC/100 g)	4.50 ± 0.35 ^{f}	14.49 ± 0.02 ^{d}	18.27 ± 0.02 ^{b}	11.64 ± 0.07 ^{e}	16.31 ± 0.19 ^{c}	12.57 ± 0.24 ^{e}	25.06 ± 1.33 ^{a}
FRAP (mg/ml Fe/100 g)	10.83 ± 1.45 ^{e}	36.58 ± 2.60 ^{d}	54.33 ± 1.05 ^{c}	32.78 ± 4.00 ^{d}	74.13 ± 3.45 ^{b}	54.93 ± 1.05 ^{c}	86.40 ± 0.50 ^{a}
Amino acids (mg/g)							
Alanine	0.07 ± 0.00 ^{g}	0.50 ± 0.03 ^{f}	1.31 ± 0.03 ^{e}	2.03 ± 0.08 ^{d}	9.81 ± 0.18 ^{a}	5.04 ± 0.33 ^{b}	2.75 ± 0.15 ^{c}
Glycine	n.d.	1.20 ± 0.04 ^{d}	1.43 ± 0.07 ^{c}	1.69 ± 0.05 ^{b}	1.08 ± 0.07 ^{e}	0.60 ± 0.04 ^{f}	3.33 ± 0.05 ^{a}
Histidine	0.91 ± 0.03 ^{d}	0.56 ± 0.06 ^{e}	7.97 ± 0.06 ^{a}	4.25 ± 0.06 ^{b}	1.11 ± 0.34 ^{d}	0.48 ± 0.03 ^{e}	3.23 ± 0.12 ^{c}
Valine	0.18 ± 0.02 ^{e}	1.05 ± 0.05 ^{e}	2.90 ± 0.05 ^{d}	5.90 ± 0.09 ^{b}	7.86 ± 1.24 ^{a}	4.80 ± 0.09 ^{c}	4.79 ± 0.10 ^{c}
Leucine	0.66 ± 0.04 ^{e}	0.22 ± 0.03 ^{f}	1.21 ± 0.03 ^{d}	1.92 ± 0.06 ^{c}	9.22 ± 0.06 ^{a}	4.70 ± 0.08 ^{b}	1.86 ± 0.09 ^{c}
Isoleucine	0.61 ± 0.02 ^{c}	0.35 ± 0.03 ^{d}	0.18 ± 0.02 ^{e}	0.02 ± 0.01 ^{f}	3.33 ± 0.05 ^{a}	1.85 ± 0.04 ^{b}	0.41 ± 0.06 ^{d}
Cystine	0.45 ± 0.03 ^{e}	0.44 ± 0.03 ^{e}	2.54 ± 0.06 ^{d}	5.30 ± 0.07 ^{c}	8.82 ± 0.56 ^{a}	7.46 ± 0.11 ^{b}	2.42 ± 0.07 ^{d}
Proline	0.80 ± 0.03 ^{e}	0.30 ± 0.02 ^{f}	2.50 ± 0.05 ^{c}	0.88 ± 0.03 ^{e}	6.42 ± 0.11 ^{b}	8.12 ± 0.10 ^{a}	1.13 ± 0.08 ^{d}
Asparagine	n.d.	0.02 ± 0.00 ^{e}	0.12 ± 0.01 ^{d}	0.21 ± 0.01 ^{c}	0.50 ± 0.03 ^{a}	0.32 ± 0.04 ^{b}	0.10 ± 0.01 ^{d}
Glutamine	0.02 ± 0.01 ^{c}	0.02 ± 0.00 ^{c}	0.03 ± 0.00 ^{c}	0.04 ± 0.01 ^{c}	0.17 ± 0.01 ^{a}	0.12 ± 0.02 ^{b}	0.04 ± 0.01 ^{c}
Arginine	5.31 ± 0.07 ^{e}	9.30 ± 0.08 ^{c}	4.58 ± 0.07 ^{f}	4.48 ± 0.07 ^{f}	28.11 ± 0.06 ^{a}	23.16 ± 0.15 ^{b}	8.71 ± 0.08 ^{d}
Serine	n.d.	0.09 ± 0.01 ^{c}	0.21 ± 0.01 ^{b}	n.d.	0.04 ± 0.01 ^{cd}	0.01 ± 0.00 ^{d}	3.06 ± 0.08 ^{a}
Methionine	n.d.	n.d.	0.19 ± 0.01 ^{d}	0.80 ± 0.01 ^{b}	1.87 ± 0.03 ^{a}	1.82 ± 0.06 ^{a}	0.59 ± 0.03 ^{c}
Threonine	n.d.	0.22 ± 0.01 ^{d}	0.07 ± 0.01 ^{e}	0.23 ± 0.01 ^{d}	13.05 ± 0.07 ^{a}	11.71 ± 0.08 ^{b}	0.74 ± 0.06 ^{c}
Lysine	n.d.	0.29 ± 0.01 ^{ef}	0.50 ± 0.02 ^{e}	7.22 ± 0.08 ^{c}	11.28 ± 0.41 ^{a}	9.57 ± 0.10 ^{b}	3.15 ± 0.04 ^{d}
Glutamic Acid	n.d.	n.d.	0.27 ± 0.02 ^{c}	n.d.	3.05 ± 0.04 ^{a}	1.19 ± 0.03 ^{b}	n.d.
Aspartic Acid	n.d.	n.d.	0.73 ± 0.02 ^{d}	2.63 ± 0.06 ^{c}	6.07 ± 0.02 ^{b}	7.70 ± 0.11 ^{a}	n.d.
Phenylalanine	n.d.	0.95 ± 0.04 ^{c}	0.82 ± 0.03 ^{d}	0.32 ± 0.02 ^{e}	8.23 ± 0.10 ^{a}	1.63 ± 0.05 ^{b}	0.10 ± 0.01 ^{f}
Tyrosine	n.d.	1.12 ± 0.03 ^{b}	0.33 ± 0.01 ^{c}	0.10 ± 0.01 ^{e}	7.43 ± 0.06 ^{a}	7.49 ± 0.06 ^{a}	0.24 ± 0.02 ^{d}
Cysteine	n.d.	n.d.	0.04 ± 0.00 ^{c}	0.05 ± 0.00 ^{c}	0.46 ± 0.01 ^{a}	0.20 ± 0.01 ^{b}	n.d.
∑ AAs	9.01 ± 0.05 ^{g}	16.62 ± 0.32 ^{f}	27.91 ± 0.27 ^{e}	38.05 ± 0.14 ^{c}	127.91 ± 0.25 ^{a}	97.97 ± 0.46 ^{b}	36.66 ± 0.16 ^{d}
∑ EAAs	2.35 ± 0.05 ^{g}	3.62 ± 0.16 ^{f}	13.83 ± 0.07 ^{e}	20.66 ± 0.04 ^{c}	55.95 ± 0.90 ^{a}	36.56 ± 0.23 ^{b}	14.87 ± 0.14 ^{d}

Data are expressed as the means ± SD of triplicate experiments.

* different superscripts in a row indicate significant statistical differences among samples at $P < 0.05$, for each trait.

flours. Besides, the higher concentrations of palmitic and stearic acid were detected in bread samples made with higher amounts of amaranth flour: A10, L5A10, L12A19 and L5A7. This result is explainable considering the composition of amaranth flour, which is richer in fatty acids compared to lentil flour. The highest concentrations of palmitic acid (13.35 %) and oleic acid (21.30 %) were detected in L12A19 sample. Furthermore, considering the low oil content in gluten-free mixture, we can assume that it did not contribute to fatty acids composition of bread samples. Like flours, also bread samples contained squalene and γ-sitosterol: squalene was more abundant in A10, L10A7, L5A10 and L12A19 samples, γ-sitosterol instead in L10, L10A7 and L5A10 samples. Data reported in literature explain why γ-sitosterol levels resulted higher in bread samples made with more lentil flour (Mustafa et al., 2022). GC–MS chromatogram of L12A19 bread sample is reported in Fig. 5. Squalene was still detectable as in amaranth flour, but in lower concentration; γ-sitosterol was not detectable. Considering also flours analysis results, we can affirm L12A19 fatty acids composition derives mainly from amaranth flour. Out of the percentage of substitution, the average fatty acids values for breads containing both amaranth and lentil flour (LxAx) have been calculated, and a second statistical analysis has been performed as reported in Table 6.

The most abundant fatty acids were palmitic acid (10.05 %), linoleic

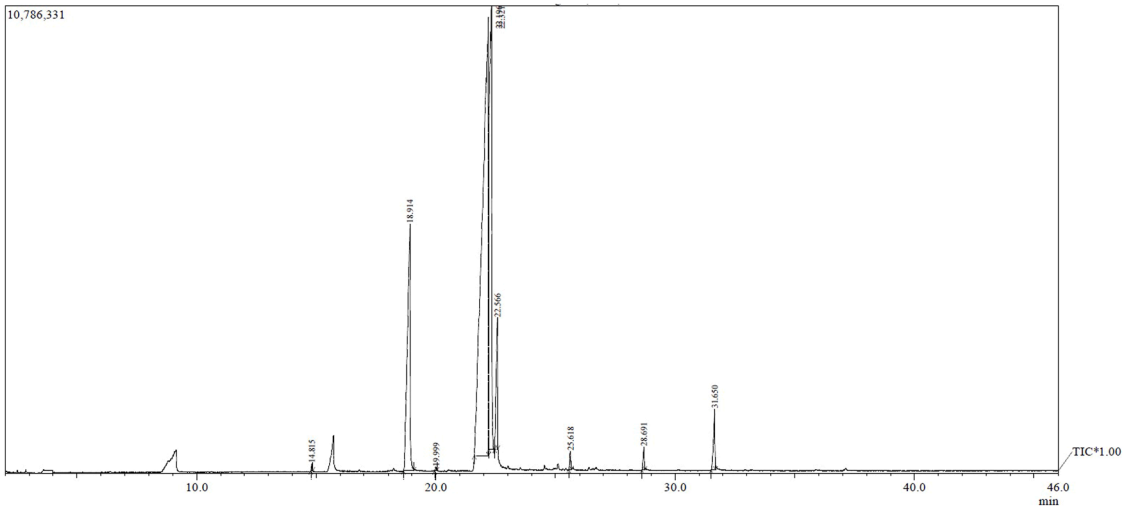

Fig. 5. GC–MS chromatogram of L12A19 bread sample.

Table 6
Fatty acids composition (Relative percentage %) of LxAx bread samples.

	CTRL	A10	L10	LxAx
Myristic acid	0.06 ± 0.02 ^{a}	0.07 ± 0.02 ^{a}	0.04 ± 0.02 ^{a}	0.07 ± 0.02 ^{a}
Palmitoleic acid	0.02 ± 0.02 ^{a}	0.04 ± 0.01 ^{a}	0.05 ± 0.03 ^{a}	0.01 ± 0.01 ^{a}
Palmitic acid	7.62 ± 0.23 ^{a}	8.57 ± 0.30 ^{b}	8.11 ± 0.11 ^{b}	10.1 ± 0.85 ^{a}
Margaric acid	n.d.	n.d.	0.02 ± 0.01 ^{ab}	0.02 ± 0.01 ^{a}
Linoleic acid	70.2 ± 0.20 ^{a}	68.6 ± 1.27 ^{ab}	69.7 ± 0.05 ^{a}	66.6 ± 0.36 ^{b}
Linolenic acid	n.d.	n.d.	n.d.	n.d.
Oleic acid	17.6 ± 0.65 ^{a}	17.9 ± 1.28 ^{a}	17.8 ± 0.54 ^{a}	17.8 ± 0.48 ^{a}
Stearic acid	3.61 ± 0.08 ^{ab}	3.63 ± 0.17 ^{ab}	3.31 ± 0.09 ^{b}	3.91 ± 0.18 ^{a}
Arachidic acid	0.21 ± 0.03 ^{a}	0.23 ± 0.04 ^{a}	0.18 ± 0.05 ^{a}	0.27 ± 0.05 ^{a}
Behenic acid	0.52 ± 0.16 ^{a}	0.58 ± 0.01 ^{a}	0.64 ± 0.10 ^{a}	0.57 ± 0.14 ^{a}
Lignoceric acid	0.12 ± 0.07 ^{a}	n.d.	0.13 ± 0.08 ^{a}	0.07 ± 0.03 ^{a}
Squalene	n.d.	0.43 ± 0.08 ^{b}	n.d.	0.61 ± 0.10 ^{a}
γ-sitosterol	0.11 ± 0.11 ^{a}	0.02 ± 0.01 ^{a}	0.03 ± 0.02 ^{a}	0.02 ± 0.01 ^{a}
%SFA	12.1 ± 0.59 ^{b}	13.1 ± 0.12 ^{b}	12.4 ± 0.64 ^{b}	15.0 ± 0.87 ^{a}
%MUFA	17.6 ± 0.89 ^{a}	17.9 ± 1.80 ^{a}	17.8 ± 0.73 ^{a}	17.9 ± 0.65 ^{a}
%PUFA	70.2 ± 0.28 ^{ab}	68.6 ± 1.80 ^{ab}	69.7 ± 0.08 ^{ab}	66.6 ± 0.44 ^{b}

SFA = Saturated Fatty Acids, MUFA = Monounsaturated Fatty Acids, PUFA = Polyunsaturated Fatty Acids. Data are expressed as the means ± SD of triplicate experiments; *different superscripts in a row indicate significant statistical differences among samples at $P < 0.05$, for each trait.

acid (66.56 %), and oleic acid (17.84 %). Regarding antioxidant properties, all the tests showed the ability of amaranth and lentil flours to increase the antioxidant potential of fortified bread samples in comparison with the CTRL (Table 5). In bread samples, the highest total polyphenol content was found in the sample with the addition of 10 % lentil flour and 7 % amaranth flour, while the lowest was found in bread with the addition of amaranth only (in percentage of 10 %). The results obtained from the analysis of the samples tested reflected the characteristics of the raw materials. Specifically, samples containing only amaranth flour exhibited lower levels of polyphenols. Data indicates that lentil flour is significantly richer in polyphenols compared to amaranth flour. When incorporated into bread, the combination of both flours, especially with a higher percentage of lentil, leads to a substantial increase in the overall polyphenol content. Data from ABTS, DPPH, and FRAP assays consistently demonstrated a significant positive correlation between the incorporation of lentil and amaranth flours and the antioxidant capacity of the bread samples. Compared to the control and the amaranth-only sample (A10), bread samples enriched with lentil flour (L10, L5A7, L10A7, L5A10) generally showed higher antioxidant levels. These findings suggest, again, that lentil flour is a primary contributor to the enhanced antioxidant profile of the bread. The combination of lentil and amaranth flours (L5A7, L10A7, L5A10) often seems to have a synergistic effect on the antioxidant capacity. The values of these samples are generally higher than the sum of the individual contributions of lentil and amaranth flours. The combination of different antioxidants in the mixed flour samples may lead to synergistic effects, enhancing their overall antioxidant capacity. However, absolute values may vary slightly between tests due to differences in the reactivity of the antioxidant compounds. A more detailed analysis of ABTS data revealed that the addition of 10 % lentil flour yielded the most significant increase in terms of antioxidant activity (18.27 mmolTEAC/100 g), confirming the nutritional value of both flours, the addition of 10 % amaranth flour also contributed to an increase in the antioxidant activity of the bread compared to the control sample, even if the interaction between the antioxidant compounds of the two flours may be complex and dependent on the proportions used. The DPPH assay reveals subtle differences in trends among the samples. While the highest antioxidant activity, consistent with the ABTS results, is observed in samples enriched with 10 % lentil flour, a notable synergistic effect is evident when both lentil and amaranth flours are incorporated. This suggests that the combination of these two flours enhances antioxidant capacity beyond the sum of their individual contributions. FRAP assay confirmed the consistent impact of lentil flour on the antioxidant capacity of bread. Additionally, the results highlighted the complex interplay between the antioxidant compounds of lentil and amaranth flours. While a synergistic effect was not consistently observed, the combination of these two flours still contributed to a significant improvement in the overall antioxidant profile of the bread (Table 7).

The amino acid concentration in breads enriched with amaranth, lentils, and a blend of the two pseudocereals has proven useful for assessing the nutritional quality and protein completeness of the product in meeting essential amino acid requirements. Table 5 shows the characterization of amino acids after acid hydrolysis of proteins in dried bread powder. The bread sample with the lowest amount of total amino

Table 7
Antioxidant properties of LxAx bread samples.

	CTRL	A10	L10	LxAx
TPC (mg GAE/g)	1.43 ± 0.14 [c*]	1.57 ± 0.00 [c]	2.54 ± 0.04 [b]	3.54 ± 0.11 [a]
DPPH (mmolTEAC/100 g)	0.91 ± 0.01 [d]	1.20 ± 0.05 [c]	1.84 ± 0.03 [a]	1.71 ± 0.05 [b]
ABTS (mmolTEAC/100 g)	4.50 ± 0.35 [d]	14.5 ± 0.02 [b]	18.3 ± 0.02 [a]	16.4 ± 0.46 [c]
FRAP (mg/ml Fe/100 g)	10.8 ± 1.45 [c]	36.6 ± 2.60 [b]	54.3 ± 2.25 [a]	62.1 ± 2.25 [a]

Data are expressed as the means ± SD of triplicate experiments.
* different superscripts in a row indicate significant statistical differences among samples at $P < 0.05$, for each trait.

acids (9.01 mg/g) is CTRL bread, and this can be explained by the fact that the recipe foresees 52 % of gluten-free mixture, but not amaranth or lentil flour. Following, A10 sample contained 16.62 mg/g of amino acids, while L10 contained 27.91 mg/g of amino acids, and this behavior can be explained by the introduction of 10 % amaranth or 10 % lentil in bread formulations. L5A7 and L10A7 showed very different total amino acid amounts (38.05 mg/g and 127.91 mg/g, respectively), and this can be explained by the 5 % more lentil flour used in the recipe, maintaining the same amount of amaranth flour, since it is well known that it is richer in proteins. L5A10 has 2.5 times more amino acids than L5A7, and this time the difference can be explained by the 3 % more amaranth flour used in the recipe. Lastly, L12A19 showed a total amino acid amount of just 36.7 mg/g, less than L5A7 and L5A10 samples, despite L12A19 contains a double amount of both flours: this behavior can be explained by the smaller quantity of gluten-free mixture used in the recipe (just 26.25 %), which is a blend rich in protein components. The most interesting bread sample, according to its composition, is L10A7, which has 55.95 mg/g of total essential amino acids. The three most abundant essential amino acids in this sample are threonine (13.05 mg/g), lysine (11.28 mg/g), and leucine (9.22 mg/g), which are the same recurrent amino acids detected in amaranth and lentil flours (together with valine and phenylalanine). Instead, asparagine, cysteine, and glutamine are the least present amino acids, with values under 0.50 mg/g. Lastly, the total amino acid amounts in L10A7 sample are 127.91 mg/g. The average amino acid values for breads containing both amaranth and lentil flour (LxAx) have been calculated, and a second statistical analysis has been made as reported in Table 8. The most abundant essential amino acids are lysine (7.81 mg/g), threonine (6.43 mg/g) not present in the control bread sample, and valine (5.84 mg/g), while the total amino acids amount is 75.2 mg/g.

Table 8
Amino acids composition (mg/g) of LxAx bread samples.

	CTRL	A10	L10	LxAx
Alanine	0.07 ± 0.00 [d]	0.50 ± 0.03 [c]	1.31 ± 0.03 [b]	4.91 ± 0.19 [a]
Glycine	n.d.	1.20 ± 0.04 [c]	1.43 ± 0.07 [b]	1.67 ± 0.05 [a]
Histidine	0.91 ± 0.03 [c]	0.56 ± 0.06 [d]	7.97 ± 0.06 [a]	2.27 ± 0.14 [a]
Valine	0.18 ± 0.02 [d]	1.05 ± 0.05 [c]	2.90 ± 0.05 [b]	5.84 ± 0.38 [a]
Leucine	0.66 ± 0.04 [c]	0.22 ± 0.03 [d]	1.21 ± 0.03 [b]	4.43 ± 0.07 [a]
Isoleucine	0.61 ± 0.02 [b]	0.35 ± 0.03 [c]	0.18 ± 0.02 [d]	1.41 ± 0.04 [a]
Cystine	0.45 ± 0.03 [c]	0.44 ± 0.03 [c]	2.54 ± 0.06 [b]	6.00 ± 0.20 [a]
Proline	0.80 ± 0.03 [c]	0.30 ± 0.02 [d]	2.50 ± 0.05 [b]	4.14 ± 0.08 [a]
Asparagine	n.d.	0.02 ± 0.00 [c]	0.12 ± 0.01 [b]	0.28 ± 0.02 [a]
Glutamine	0.02 ± 0.01 [b]	0.02 ± 0.00 [b]	0.03 ± 0.00 [b]	0.09 ± 0.01 [a]
Arginine	5.31 ± 0.07 [c]	9.30 ± 0.08 [b]	4.58 ± 0.07 [d]	16.1 ± 0.09 [a]
Serine	n.d.	0.09 ± 0.01 [c]	0.21 ± 0.01 [b]	0.78 ± 0.02 [a]
Methionine	n.d.	n.d.	0.19 ± 0.01 [b]	1.27 ± 0.03 [a]
Threonine	n.d.	0.22 ± 0.01 [b]	0.07 ± 0.01 [c]	6.43 ± 0.05 [a]
Lysine	n.d.	0.29 ± 0.01 [c]	0.50 ± 0.02 [b]	7.81 ± 0.16 [a]
Glutamic Acid	n.d.	n.d.	0.27 ± 0.02 [b]	1.06 ± 0.02 [a]
Aspartic Acid	n.d.	n.d.	0.73 ± 0.02 [b]	4.10 ± 0.05 [a]
Phenylalanine	n.d.	0.95 ± 0.04 [b]	0.82 ± 0.03 [c]	2.57 ± 0.04 [a]
Tyrosine	n.d.	1.12 ± 0.03 [b]	0.33 ± 0.01 [c]	3.81 ± 0.04 [a]
Cysteine	n.d.	n.d.	0.04 ± 0.00 [b]	0.18 ± 0.01 [a]
∑ AAs	9.01 ± 0.05 [d]	16.62 ± 0.32 [c]	27.9 ± 0.27 [b]	75.2 ± 0.25 [a]
∑ ESSENTIAL AAs	2.35 ± 0.05 [d]	3.62 ± 0.16 [c]	13.8 ± 0.07 [b]	32.0 ± 0.33 [a]

Data are expressed as the means ± SD of triplicate experiments; *different superscripts in a row indicate significant statistical differences among samples at $P < 0.05$, for each trait.

4. Conclusions

The incorporation of lentil and amaranth flours has modified the color, lipid profile, antioxidant activity, and amino acid content of gluten-free breads, generally improving their nutritional value and maintaining or improving sensory properties. In terms of lipid content, the gluten-free mix had a low fat content, suggesting its limited contribution to the lipid profile of the fortified breads. The selection and the proportion of lentil and amaranth flours are crucial in modulating the lipidic profile of breads; the process enables the production of products characterized by specific nutritional properties. In addition, the incorporation of flours has been demonstrated to be a significant source of bioactive compounds, including squalene and γ-sitosterol. The antioxidant activity of the fortified breads was consistently demonstrated at higher levels compared to the control bread. The bread enriched by 10 % of lentil and 7 % of amaranth showed the highest total phenols content. In general, lentil flour appears to be the primary factor contributing to the enhanced antioxidant activity observed in breads, as substantiated by the results of TPC, DPPH, ABTS, and FRAP tests. The combination of lentil flour and amaranth frequently demonstrates a synergistic effect, enhancing antioxidant capacity. From the amino acid profile aspect, the strategic combination of the different amaranth and lentil flours compensated for any deficiencies present in each flour taken individually, providing a broad supply of all essential and nonessential amino acids needed for adequate nutrition. This synergistic approach has the potential to enhance the overall protein value of the final food product. As for total phenols content, breads enriched by 10 % of lentil and 7 % of amaranth demonstrated the highest amino acid content. These new food products have the potential to represent a significant alternative within the gluten-free product market.

Ethical statement

Studies in humans and animals: No animal testing was conducted. Informed consent was obtained from all subjects involved in the study. The blank informed consent we used is enclosed. It is written in Italian language.

CRediT authorship contribution statement

Enrica Pistorio: Writing – original draft, Methodology, Investigation, Formal analysis. **Alessandro Gallina:** Writing – original draft,

Methodology, Investigation, Formal analysis. **Carla Buzzanca:** Methodology, Investigation. **Angela D'Amico:** Methodology, Investigation. **Vita Di Stefano:** Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation. **Maria Grazia Melilli:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

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.

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Data availability

Data will be made available on request.

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