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An Assessment of the Functional Properties of Black Amaranth Flour During Fermentation with Probiotic Lactic Acid Bacteria

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Abstract

This study aimed to ferment protein-rich amaranth flour with different strains of lactic acid bacteria (LAB) and to analyse the fermented dough's functional properties. The fermented dough analysis was conducted using titrimetric, spectrophotometric, and chromatographic methods. The antioxidant activity of the fermented doughs was evaluated using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) methods, finding ABTS radical scavenging values ranging from 26.00 ± 1.05% to 58.92 ± 6.05%, while the DPPH values ranged from 21.29 ± 0.83% to 28.24 ± 5.48%. By RP-HPLC (Reversed Phase-High Performance Liquid Chromatography) characterisation, several phenolic acids and flavonoids were identified and quantified. Among these compounds, epigallocatechin was the most abundant, with the highest concentration recorded at 7789.88 ± 17.0 ng/µL in the control sample. This was followed by a 6942.47 ± 5.632 ng/µL concentration in the dough fermented with *Lacticaseibacillus rhamnosus* MIUG BL38 strain and 4983.16 ± 7.29 ng/µL in the dough fermented with *Lactiplantibacillus pentosus* MIUG BL24 strain. These two LAB strains (*Lc. rhamnosus* MIUG BL38 and *Lp. pentosus* MIUG BL24), with probiotic properties previously demonstrated, were selected based on their acidification potential, antioxidant activity, and bioactivity for future optimisation studies. Lactic acid fermentation significantly enhances bioactive characteristics of the amaranth flour, enabling the design of diverse gluten-free products with increased functional properties based on the attributes induced by the prebiotic, probiotic and postbiotic contents (tribiotics).

Keywords: black amaranth; fermentation; *Lactiplantibacillus* spp.; *Lacticaseibacillus* spp.; tribiotics (pre-, pro-, postbiotics); gluten-free

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1. Introduction

Changing lifestyles and global population increases are straining our ability to maintain a sustainable food system. The global demand for meat, dairy, and fish products and their environmental implications are increasing [1]. Thus, it is necessary to discover alternative sources of food protein that consume less energy and water. One critical challenge

for food researchers is to improve the use of sustainable plant proteins with acceptable nutritional and functional qualities [2].

Pseudocereals are structurally similar to cereal grains, with the endosperm, aleurone, testa, and hull covering the majority of the seed components [3]. Generally, pseudocereals possess higher protein and lipid levels and lower carbohydrate levels compared to traditional cereals (maize, wheat, and rice). As opposed to cereal grains, pseudocereals are abundant in bioactive compounds, which include dietary fibre, unsaturated fatty acids, lignans, antioxidants, flavonoids, polyphenols, phytosterols, minerals, vitamins, high-quality proteins with a balanced amino acid profile that offers great digestibility and bioavailability, as well as essential micronutrients [4]. Due to their nutrient-rich profile, pseudocereals are linked with various health benefits [5], including hypolipidemic, anti-inflammatory, anti-hypertensive, anticancer [6], and hepatoprotective effects, along with advantages in preventing obesity and diabetes [3,7].

Nowadays, pseudocereals like amaranth, chia, quinoa, and buckwheat are becoming increasingly popular as gluten-free grains, making them suitable for celiac disease patients [8,9]. Amaranth (*Amaranthus* spp.) is known as one of the New World's oldest crops in Mesoamerica [7]. Among all the species, *Amaranthus caudatus*, *Amaranthus hypochondriacus* and *Amaranthus cruentus* [10] are mainly grown for human consumption [11], being considered a dicotyledonous pseudocereal that belongs to the family of Amaranthaceae, order Caryophyllales [12]. The amaranth industry in Africa is a rapidly emerging sector with significant growth potential. Its cultivation is spreading across various regions, particularly in Nigeria, Tanzania, Uganda, Ghana, and Burkina Faso. Nigeria is currently the largest producer of amaranth on the continent, accounting for over 60% of Africa's total output [13]. In Africa, amaranth is primarily utilised as a food; the leaves and seeds are used to make a variety of foods, including porridges, stews, and soups. Additionally, the crop is used for health purposes, including the treatment of diabetes, hypertension, and anaemia. Amaranth is considered an interesting, unconventional crop, owing to its nutritional and nutraceutical value [14,15], since a few decades ago, amaranth was thought to be an invasive weed [4]. It is high in fats, fibre, vitamins, lipids (including important fatty acids like linoleic (ω6) and α-linolenic (ω3) acids) [7], minerals, polysaccharides [16], antioxidants [17], and amino acids, which are essential for human nutrition (lysine and methionine) [18]. Its protein content (13–19%) is higher than other grains [6,11]. Because of its good composition, it has huge potential as a source of bioactive molecules, such as protein hydrolysates and bioactive peptides [7].

The number of persons who have celiac disease has increased in recent years, resulting in an increasing market demand for gluten-free products [19]. Amaranth flour's lack of gluten makes it perfect for making healthy gluten-free products such as bread, pasta [9], and cookies [20] for people who are gluten hypersensitive [21].

Microbial fermentation, especially by probiotic lactic acid bacteria (LAB), is one of the most popular methods for enhancing the functional characteristics, safety and sensorial properties of pseudocereals like amaranth. Additionally, it improves the nutritional value of food by enhancing the content of vitamins K and B, as well as amino acids like lysine. Chemical profiles and bioactivities of polyphenols of plant-based substrates can be changed by bioprocessing. During lactic acid fermentation, generally, phenolics of different classes are converted into compounds that are often more bioactive. It depends on the substrate composition and the starter cultures' biochemical properties. In addition, during bioprocessing, the vegetal cell walls can suffer alteration with an impact on the release of the intracellular antioxidants, making flavonoids (e.g., catechins) more extractable and detectable [22]. Currently, it is considered a safe food preservation technique,

as antimicrobials are produced with antagonistic activity against spoilage and pathogen microorganisms [23].

Probiotics are living, non-pathogenic microorganisms that provide many health benefits to the human body when taken in sufficient concentrations [23]. LAB have a highly specialised proteolytic system, making them one of the most relevant microorganisms for the fermentation-based production of some metabolites (postbiotics) like organic acids, bioactive peptides [17], polyphenol derivatives, volatile compounds, and oligosaccharides with prebiotic activity [16]. Thus, the fermented products combine the tribiotic functional characteristics of pre-, pro- and postbiotics with increased bioactive properties compared to the fermented substrates [24].

Therefore, this work aims to ferment protein-rich amaranth flour with different probiotic strains and to select the best strains in terms of acidification capacity and bioactive properties for the production of nutrient-rich gluten-free bread and bakery products.

2. Materials and Methods

2.1. Plant Material and Amaranth Flour

The black amaranth (BA) (*A. hypochondriacus*) seeds were harvested in the rural commune of Ditinn, Dalaba prefecture (Guinea), in 2024. The samples were divided into batches of 500 g, packed under a vacuum, and stored in the dark, at room temperature [25].

All the experiments were carried out in the Integrated Center for Research, Expertise and Technological Transfer in Food Industry (BioAliment-TehnIA), Dunărea de Jos University of Galati, Romania.

Before obtaining the flour, the seeds were washed with distilled water, under agitation, for 30 min, by using a magnetic stirrer (Ika, model RH digital, Staufen, Germany). Then, the seeds were placed on filter paper and dried in an incubator at 42 °C for 16 h (Stericell Typ 111, München, Germany). The dried seeds were ground using an electric grinder (Heiner HCG150SS, Bucharest, Romania) [26]. The amaranth flour recorded a moisture content of 7.86% ± 0.09%, measured on a moisture analyser (KERN, DAB 100-3, Balingen, Germany). The flour was stored in a glass jar at room temperature for further analysis.

2.2. Chemicals and Reagents

DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), aluminium chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), potassium chloride (KCl), sodium acetate (CH_3COONa), calcium carbonate ($CaCO_3$) and Folin–Ciocalteau reagent were purchased from Sigma-Aldrich (Hamburg, Germany). Culture media such as De Man, Rogosa, and Sharpe (MRS) broth and agar were supplied from Merck Millipore (Darmstadt, Germany). The solvents that were HPLC-grade (ethanol and methanol) were purchased from Scharlau (Barcelona, Spain). The protein assay was performed with the Bio-Rad Protein Assay Kit II (Dubai, United Arab Emirates).

2.3. Physico-Chemical Analysis of Amaranth Seeds

A physico-chemical analysis of the BA seeds was performed for the following parameters, dry matter content, mineral matter, fat content, saturated fatty acids, protein content, and carbohydrates, according to Oncică et al. [27]. The moisture of the samples was determined by drying at 105 °C. Protein content was determined by the Kjeldahl method with the quantification of total nitrogen. The soluble sugars, after extraction, were determined by the difference iodometric method. Fat content was determined by the direct extraction calculation. The ash content was determined by calcination. The fibre amount was obtained by difference.

2.4. Starter Culture of Lactic Acid Bacteria Reactivation and Fermentation

Four probiotic strains, belonging to the Microorganisms Collection of Dunărea de Jos University of Galati (acronym MIUG), presented in Table 1, were used in this study, including *Lacticaseibacillus paracasei* MIUG BL4, *Lacticaseibacillus paracasei* MIUG BL13, *Lactiplantibacillus pentosus* MIUG BL24, and *Lacticaseibacillus rhamnosus* MIUG BL38 [28].

Table 1. Sources of isolation of the studied LAB probiotic strains.

Probiotic Strains	Isolation Source	Accession No.
Lc. paracasei MIUG BL4	Borsch	MT626061.1
Lc. paracasei MIUG BL13	Corn flour	MT611827.1
Lp. pentosus MIUG BL24	Whey	OK325938.1
Lc. rhamnosus MIUG BL38	Chickpea	MT463821.1

To reactivate the stock cultures, which were preserved in 40% (*w*/*w*) glycerol solution at a temperature of −80 °C, 2 mL of stock culture was added to 9 mL of MRS broth (Merck, Darmstadt, Germany) and cultivated for 48 h at 37 °C in an incubator (Binder BF4000, Tuttlingen, Germany).

To obtain single colonies, the reactivated culture (10 µL) was streaked on MRS agar medium (Merck, Darmstadt, Germany) enriched with 30 g/L $CaCO_3$ using a sterile loop [29].

A single colony was transferred to 50 mL of MRS broth and incubated stationary for 48 h at 37 °C to obtain the LAB inoculum. Following that, the optical density at 600 nm (OD600) was measured and adjusted to approximately 2.0 (with fresh MRS broth), indicating a cell concentration of 1×10^8 CFU/mL solution, using a spectrophotometer (Biochrom, Libra 22, Holliston, MA, USA) [30].

The fermentation media consisting of 20% (*w*/*w*) black amaranth flour in distilled water was autoclaved, cooled, inoculated with 2% (*v*/*v*) LAB inoculum, and incubated for 48 h at 37 °C and 100 rpm in an orbital shaker (Lab Companion SI-300, GMI, Minneapolis, MN, USA) [24]. Subsequently, the fermented doughs were freeze-dried at −42 °C and 0.10 mbar using a freeze-drier (Christ Alpha 1–4 LD plus, Osterode am Harz, Germany).

2.5. pH and Total Titratable Acidity (TTA) Measurement

The pH values of the fermented samples were measured with a pH meter (FiveEasy Plus FP20, Mettler Toledo, Greifensee, Switzerland). The total titratable acidity assay was performed with an automatic titrator (TitroLine Easy, Schott Instruments, Mainz, Germany). In brief, 10 g of fermented samples was mixed with 90 mL of distilled water and titrated to an endpoint of 8.50 [31]. The TTA was expressed in mL of 0.1 N NaOH [28].

2.6. Antioxidant Activity (AA)

The antioxidant activity of the freeze-dried samples was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay [32,33].

Firstly, the freeze-dried samples were solubilised in ultrapure water in a ratio of 1:10 (*w*/*v*). The mixture was vortexed for 5 sec and placed in an ultrasonic water bath (DU-32; ARGOLAB, Capri, Italy) for 15 min at 40 °C. After centrifugation (Hettich Universal 320R, Tuttlingen, Germany) at 7000 rpm for 10 min at 4 °C, the supernatant was collected and used for further analysis.

Briefly, for the DPPH assessment, 100 µL of the sample was mixed with 3900 µL of 0.004% (*w*/*v*) DPPH (in methanol) and incubated in the dark for 90 min. The absorbance was measured at 515 nm.

For the ABTS assay, a volume of 1980 µL of ABTS solution (with 0.7 absorbance at OD_{734}) was mixed with 20 µL of sample and homogenised. The mixtures were kept in the dark for 15 min, after which the absorbance was read at 734 nm.

The antioxidant activity was expressed as the radical scavenging activity (RSA, %), based on Equation (1) [33]:

$$RSA\ (\%) = A_{control}/A_{sample} \times 100 \quad (1)$$

where

$A_{control}$—the absorbance of the control sample;

A_{sample}—the absorbance of the analysed sample.

2.7. Total Flavonoid Content (TFC)

To assess the total flavonoid content, the aluminium chloride method was used. Concisely, a volume of 250 µL of samples was mixed with 1250 µL of distilled water and 75 µL of 5% (*w*/*v*) sodium nitrite solution and allowed to stand at room temperature for 5 min. Further, the samples were mixed with 150 µL of 10% (*w*/*v*) aluminium chloride solution, and after 6 min of incubation, 500 µL of 1 M sodium hydroxide and distilled water were added up to a total volume of 3000 µL [34]. The absorbance of the mixtures was immediately measured at 510 nm on a spectrophotometer (Libra S22 UV-VIS, Biochrom, Cambridge, UK).

TFC was expressed in mg catechin equivalents per gram of dry weight (CE)/g DW, based on a calibration curve ($y = 1.856x - 0.0201$, $R^2 = 0.9951$).

2.8. Total Polyphenolic Content (TPC)

The modified Folin–Ciocalteu method was used to measure the total polyphenolic content in the samples. In brief, 200 µL of the samples was diluted in distilled water (7900 µL), to which 500 µL of Folin–Ciocalteu reagent was added. After 5 min, 500 µL of 20% (*w*/*v*) sodium carbonate solution was added, and the samples were allowed to stand for 60 min in the dark. The absorbance was determined at a wavelength of 765 nm [34].

TPC was expressed as mg gallic acid equivalents per gram dry weight (GAE)/g DW, using a gallic acid standard curve ($y = 1.3612x - 0.0205$, $R^2 = 0.9838$).

2.9. Reverse-Phase High-Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds

The identification of phenolic compounds was performed according to the method described by Mërtiri et al. [32] using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA), composed of a degasser, quaternary pumping system, autosampler, column and multi-wavelength detector. The separation of the compounds was performed on a BDS Hypersil C18 column (150 × 4.6 mm, 5 µm), using the following parameters: temperature of 30 °C, injection volume of 10 µL and a flow rate of 1 mL/min. The mobile phases were composed of 100% methanol (solvent A) and 10% formic acid (solvent B). The progression of the gradient is outlined below: 0–20 min. 9% A–91% B, 20–30 min. 35% A–65% B, 30–40 min. 50% A–50% B, and 40–45 min. 9% A–91% B. The results were reported as average values for duplicate measurements ± STDEV in ng/µL based on the peak area and calibration curves with the reference standard.

2.10. Protein Content

The protein content in control and fermented samples was determined by using a Bio-Rad kit (Bio-Rad Laboratories, Dubai, United Arab Emirates [35]. In brief, volumes of 100 µL of samples were added over 5000 µL of diluted dye reagent (1:4, with distillate water),

previously tempered to room temperature. Samples were incubated at room temperature for 5 min in the dark, and then the absorbance at 595 nm was measured.

The protein content was calculated based on a calibration curve, using bovine serum albumin as a standard protein (y = 1.0714x + 0.1679, R^2 = 0.9920) and reported as mg per gram dry weight, mg/g DW.

2.11. Statistical Analysis

The data were analysed using Minitab V19.1 (Minitab LLL, State College, PA, USA). All the experiments were carried out in triplicate. The results were expressed as mean ± standard deviation and analysed using the one-way ANOVA method. The data were analysed for normal distribution (the Ryan–Joiner test) and the homogeneity of variances (Bartlett's test), followed by either the Tukey test ($p < 0.01$) or the Games–Howell test ($p < 0.01$) at a 99% confidence level. Pearson correlation and linear multiple regression were used to assess the data.

3. Results and Discussion

3.1. Proximal Composition of Black Amaranth Seeds

Pseudocereals are edible seeds that belong to dicotyledonous species. Due to their physical similarities with true cereals (Poaceae family), they were classified as pseudocereals [9].

An evaluation of the composition of black amaranth seeds was conducted to explore the potential of this overlooked tropical pseudocereal for food applications. Table 2 provides a summary of the proximate composition of BA seeds.

Table 2. The proximal composition of the black amaranth seeds.

Physico-Chemical Characteristics	Results
Ash, %	3.50 ± 0.14
Moisture, %	11.05 ± 0.21
Fats, %,	5.25 ± 0.13
Saturated fatty acids, %	0.80 ± 0.14
Proteins, %	18.85 ± 0.35
Carbohydrates, %	61.09 ± 0.90
Sugars, %	3.50 ± 0.35

The proximal composition of the Guinea BA seeds revealed carbohydrates and protein content of 61.09 ± 0.90% and 18.85 ± 0.35%, respectively. The contents of fats and sugars were evaluated at 5.25 ± 0.13%, respectively, 3.50 ± 0.35%. The protein content in Guinea BA seeds (18.85 ± 0.35) exceeds the 12–18% value described by Różewicz [36]. A higher protein content is directly associated with an improved binding capacity [32]. Therefore, amaranth flour can be used as a binder in baking many goods due to its higher protein content. Pseudocereals, such as quinoa, amaranth, and buckwheat, have a high protein content of 10–18 g/100 g. Compared to rice and maize, which have 7.23 g/100 g and 7.40 g/100 g, respectively, amaranth protein content is significantly higher [9]. This emphasises the significance of amaranth seeds as a plant-based protein source, particularly for vegan and vegetarian diets. Amaranth proteins are also renowned for having a balanced profile of important amino acids, especially lysine, which is often limited in most cereals. Amaranth seeds are also characterised by a relatively high fat content (about 7–9%) [37,38]. Instead, the Guinea BA seeds exhibited a lower fat content, measuring 5.25 ± 0.13%, indicating a favourable lipid profile with possible cardioprotective benefits. The black amaranth seeds' ash level (3.50 ± 0.14%) indicates a high nutritional density and a significant content of

mineral salts. From a nutritional perspective, this is significant since it may help meet daily requirements for vital minerals. The moisture level of 11.05 ± 0.21% indicates good physico-chemical stability and a minimal risk of oxidative or microbiological spoiling. However, the composition of amaranth seeds is determined by both varietal factors and meteorological conditions, as well as the level of nitrogen fertilisation [36].

3.2. pH and Total Titratable Acidity (TTA)

The probiotic strains were selected based on a combination of technological, functional, and safety-related criteria. All strains were chosen for their proven ability to survive in gastrointestinal-like conditions (low pH and bile salt tolerance) and their capacity to adhere to intestinal epithelial cells (assessed previously in vitro). Furthermore, the selected strains demonstrated antimicrobial activity against common foodborne pathogens, lacked haemolytic activity, and showed no acquired antibiotic resistance, supporting their safety for use in food or nutraceutical applications. Their origin from traditional or plant-based fermented matrices also indicates their adaptability to various food substrates and their relevance for functional food development [28].

As shown in Figure 1, the dough fermented with the *Lp. pentosus* MIUG BL24 strain exhibited the lowest pH value (3.90 ± 0.06), followed by *Lc. paracasei* MIUG BL13 and *Lc. paracasei* MIUG BL4, which listed pH values of 4.01 ± 0.01 and 4.08 ± 0.10, respectively. However, no significant differences ($p < 0.01$) were found among the doughs fermented with the strains *Lp. pentosus* MIUG BL24, *Lc. paracasei* MIUG BL13 and *Lc. paracasei* MIUG BL4 in terms of pH. In a study, Calabrò et al., 2022 [14], reported that for three accessions of two amaranth species, *Amaranthus cruentus* (origin: Mexico, Montana, Illinois) and *A. hypochondriacus* (origin: India, Nebraska, Pennsylvania), the pH obtained during *in vitro* fermentation was 6.46–6.59, at 39 °C, under anaerobic conditions.

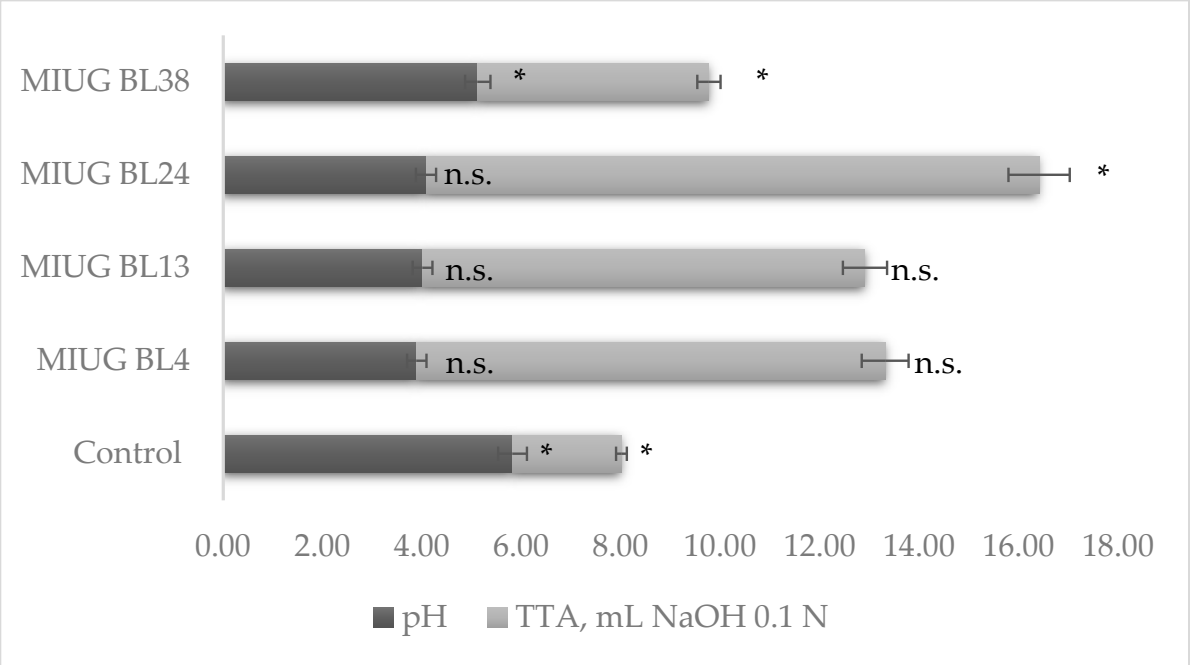

Figure 1. Dough's pH (■) and TTA (■) after 48 h of lactic acid fermentation. n.s.—not significantly different, *—significantly different ($p < 0.01$), based on the Tukey test.

Recently, Zhao et al. [39] reported that red-fruited amaranth fermented with *Limosilactobacillus fermentum*, *Latilactobacillus graminis*, and *Lactiplantibacillus plantarum* (a commercial starter) had a positive effect on the silage fermentation characteristics, with a pH of 4.16 to

4.38. In addition, the water amaranth mash fermented by *L. rhamnosus* GG at 37 ± 1 °C for 14 h has the lowest final pH value (4.39) [40]. Fermenting amaranth flour (25% in water) with *Propionibacterium freudenreichii* and *Levilactobacillus brevis* to produce vitamin B12 resulted in a decrease in the pH from 6.3 to 3.7 after 72 h. Additionally, the TTA increased from 3.3 mL to 22.1 mL of 0.1 N NaOH [41].

Alternately, BA dough fermented with *Lp. pentosus* MIUG BL24 exhibited the highest TTA value (12.33 ± 0.35 mL of 0.1N NaOH). No significant differences ($p < 0.01$) were found among the doughs fermented with the strains *Lc. paracasei* MIUG BL13 and *Lc. paracasei* MIUG BL4, in terms of acidifying potential.

The results of this investigation are in agreement with those conducted by Xie et al. [41], who reported a TTA value of 15.5 mL 0.1 mol/L NaOH (and pH 4.2) for amaranth flour fermented by *Propionibacterium freudenreichii* and *Levilactobacillus brevis*, after 24 h of fermentation at 30 °C. Additionally, *L. plantarum* RTa12, *L. sakei* RTa14, and *P. pentosaceus* RTa11, which were isolated from spontaneous amaranth sourdough, were used to inoculate the amaranth flours in a study by Sterr et al. [18]. The sourdough's pH level reached and remained at 4.0 after 48 h of fermentation. Inverse proportional values were displayed by the TTA; however, it remained constant from the second day until the fermentation was complete (25 mL 0.1N NaOH).

Usually, LAB convert carbohydrates into organic acids, such as lactic and acetic acid, during their growth, which lowers the pH and increases the acidity of the medium [42,43].

pH and TTA are important indicators for assessing the microbiological quality of food products since they are linked to the antibacterial and antifungal attributes that come from the organic acid content of fermented doughs.

3.3. Antioxidant Activity (AA) of the BA Doughs

The dynamic changes in the antioxidant activity of the fermented samples were evaluated using the DPPH and ABTS methods, finding ABTS radical scavenging values ranging from 26.00 ± 11.05% to 58.92 ± 6.05%, while the DPPH values ranged from 19.74 ± 0.97% to 28.24 ± 5.48% (Figure 2).

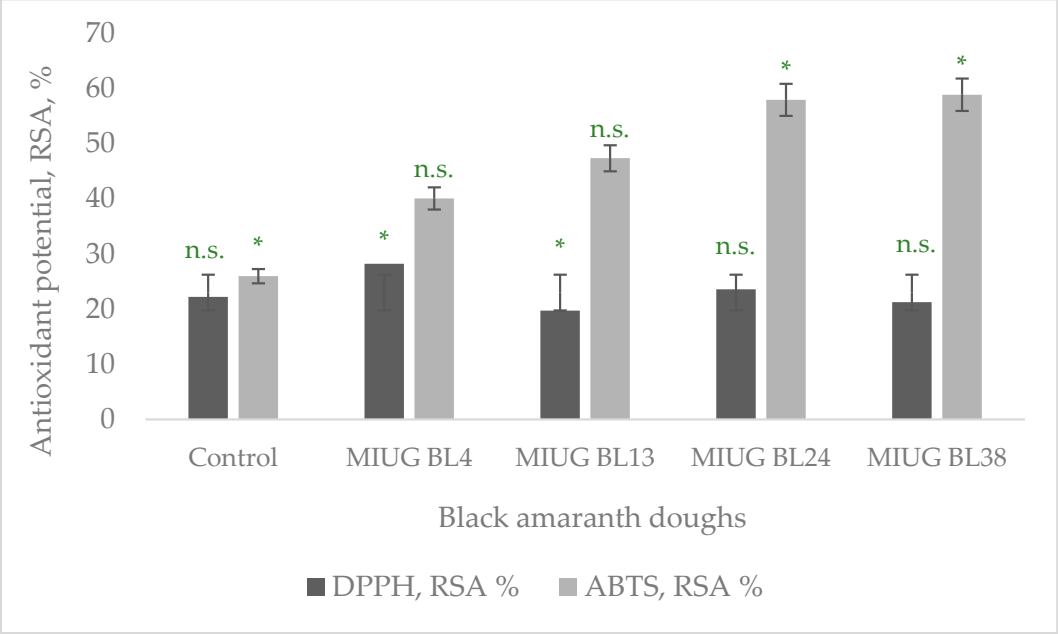

Figure 2. The antioxidant activity of the BA doughs. n.s.—not significantly different, *—significantly different ($p < 0.01$), based on the Tukey test.

A stronger scavenging effect of the DPPH radical was found for dough fermented with the *Lc. paracasei* MIUG BL4 (28.24 ± 5.48%) strain. The highest inhibition percentages of the ABTS radical were shown in doughs fermented with the *Lp. pentosus* MIUG BL24 strain and the *Lc. rhamnosus* MIUG BL38 strain (58.00 ± 1.81% and 58.92 ± 6.05%), which were almost 2.2 times higher than the control.

In a recent study, Araujo-León et al. [44] reported that some extracts from the leaves and inflorescences of *A. cruentus* neutralised the DPPH radical by 52% and 46%, respectively. [45] Yeşil and Levent [45] reported an AA value of gluten-free bread made from amaranth flour of 6.68 ± 2.45%. In contrast, the raw flour exhibited a higher antioxidant potential of 18.46 ± 0.93%. According to a study conducted by Vento et al. [46], the highest results were observed for amaranth seeds after spontaneous fermentation (18.34 RSA%, 5 times increase) and in germinated seeds after co-fermentation with *Saccharomyces cerevisiae* and *L. plantarum* strains or fermentation with the *L. plantarum* strain (46.31 and 52.89 RSA%, 5.1 and 5.8 times increases, respectively).

These results are directly linked to the fermentation of amaranth flour, which plays a crucial role in enhancing the antioxidant activity of pseudocereals [23,47]. The consumption of fermented amaranth rich in pre-, pro- and postbiotics may enhance the body's ability to combat free radicals. This could help prevent an imbalance of reactive oxygen species, which can lead to certain types of cancer, high blood pressure, and degenerative diseases [21].

The difference in antioxidant mechanisms can occur because ABTS activity involves hydrogen atom transfer, whereas DPPH activity occurs via electron transfer. Additionally, the reduction in ABTS free radical-scavenging activity may be linked to the synergy and redox interactions among various compounds. Furthermore, the specific antioxidant mechanisms can vary among different bacterial strains. Therefore, the reducing power is an important indicator commonly used to assess phenolic reducing ability [48,49].

3.4. Bioactive and Protein Composition Characterisation of Black Amaranth Doughs

3.4.1. Total Polyphenol Content (TPC) of the BA Doughs

The total polyphenol content in doughs fermented with the selected probiotic strains was decisively reduced compared to the control. However, no significant differences ($p < 0.01$) were found among the doughs fermented with the *Lc. paracasei* MIUG BL13 strain, the *Lc. rhamnosus* MIUG BL38 strain and the control sample (Table 3).

Table 3. The bioactive composition characterisation of the fermented samples.

	Samples Fermented with LAB Strains				
	Control	**MIUG BL4**	**MIUG BL13**	**MIUG BL24**	**MIUG BL38**
TPC, mg GAE/g DM	2.62 ± 0.18	2.24 ± 0.22 *	2.02 ± 0.22 **	2.00 ± 0.09 **	2.33 ± 0.20 *
TFC, mg CE/g DM	2.00 ± 0.21	2.20 ± 0.16	2.14 ± 0.02	2.12 ± 0.09	1.97 ± 0.19
PC, mg/g DM	1.23 ± 0.03	0.75 ± 0.07 ****	1.01 ± 0.03 ***	1.05 ± 0.08 **	1.18 ± 0.06 *

Statistical significance was determined using a one-way ANOVA with Tukey's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The results obtained align with those of Calabrò et al. (2022) [14], who found that three accessions of two amaranth species, *Amaranthus cruentus* (from Mexico, Montana, and Illinois) and *A. hypochondriacus* (from India, Nebraska, and Pennsylvania), exhibited total phenolic content ranging from 0.18 to 0.40 mg GAE/g of seeds during in vitro fermentation.

Yeşil and Levent [45] reported a TPC for gluten-free bread made from amaranth flour of 1.64 ± 0.18 mg GAE/g, while raw flour exhibited a higher TPC of 2.55 ± 0.20 mg GAE/g. Recently, Vento et al. [46] stated that the TPC in raw and cooked amaranth seeds was signif-

icantly lower (0.5 mg GAE/g DW), whereas, sprouting the seeds significantly increased the polyphenol content up to 2.5 mg GAE/g DW.

In particular, the content of polyphenols in unfermented seeds of amaranth was 0.54 mg GAE/g DW, and the highest content was reached after co-fermentation with *S. cerevisiae* and *L. plantarum* strains (5.22 mg GAE/g DW, 10 times more). In the germinated seeds of non-fermented amaranth, 2.54 mg GAE/g DW of polyphenols was found, and the highest content was after spontaneous fermentation (9.92 mg GAE/g DW, about four times higher) [46].

Previous studies demonstrated a decline in polyphenol content during fermentation across various fermented foods, including millet fermented with yeast and *Lactobacillus* strains. This finding contrasts with the research conducted by Đorđević et al. [50], which emphasised that the type of fermentation plays a critical role in altering the levels of bioactive compounds in the examined cereals. The observed discrepancy can be linked to pH variations across different fermentation processes, as the optimal pH is essential for releasing cell wall-degrading enzymes derived from cereal grains [51].

Other studies have demonstrated that fermentation positively influences the total phenolic content (TPC) and antioxidant activity of cereals. However, the extent of this impact varies depending on the species of microorganisms involved [52]. To better understand the mechanisms that enhance the nutritional value of fermented cereals, further research is necessary to examine the changes in microbial populations and the activities of relevant enzymes during fermentation. Additionally, it is important to recognise that the Folin–Ciocalteu method, which is used to measure the total amount of phenolic compounds, is nonspecific and has limitations when applied to fermented samples.

3.4.2. Total Flavonoid Content (TFC) of the BA Doughs

Amaranth dough fermented with the *Lc. paracasei* MIUG BL4 strain, *Lc. paracasei* MIUG BL13 strain, and *Lp. pentosus* MIUG BL24 strain showed an increase in total flavonoid content compared to the control dough. However, no significant differences ($p < 0.01$) were found between the doughs fermented with these strains and the control.

According to a recent study conducted by Vento et al. [46], the TFC was comparable in raw and cooked seeds (1.8 mg QE/g DW), while sprouting significantly increased flavonoid content to 5.0 mg QE/g DW. Additionally, all the conditions of fermentation induced an increase, except for a decrease after fermentation with the *S. cerevisiae* strain and co-fermentation with the *S. cerevisiae* and *L. plantarum* strains in amaranth seeds (from 2.35 to 1.58 and 1.31 mg QE/g DW, respectively). In amaranth seeds, the highest values were obtained after spontaneous fermentation (6.1 mg QE/g DW) (about 2.6 times more than unfermented seeds). In the germinated seeds, the highest flavonoid content was detected after spontaneous fermentation (9.41 mg QE/g DW in amaranth, 1.9 times more than unfermented germinated seeds, respectively), while a slight decrease was only after fermentation with *S. cerevisiae* strain [46].

Flavonoids are powerful antioxidants that act through multiple mechanisms to neutralise free radicals and prevent oxidative damage in the body [53]. Flavonoids are known to lower the incidence of chronic diseases and help stop oxidative damage in cells. Antioxidant, anti-inflammatory, and anticancer characteristics are among the many health advantages linked to flavonoid supplementation. The frequent use of supplements high in flavonoids can help lower the risk of developing chronic conditions like diabetes, heart disease, and some types of cancer [17,53].

The nutritional and anti-nutritional components of foods change in terms of bioactivity and digestibility during the fermentation process. For example, polyphenols are mostly present in bound forms in unfermented cereals, where they are conjugated with sugars,

fatty acids, or proteins. However, during fermentation, these polyphenols are converted into free forms that can be used by the metabolism or enzymatic activity of the fermenting microorganisms. Free phenolic compounds are more bioavailable, which can enhance antioxidant activity as they release free polyphenols and aglycones. Conversely, in some types of fermentation, the content of free phenolic compounds may decrease due to their bonding with other molecules in the food matrix or due to degradation by microbial enzymes through hydrolysis conducted by specific microbial strains [46].

3.4.3. Protein Content (PC) of the BA Doughs

Protein contents ranging from 0.75 ± 0.07 to 1.23 ± 0.03 mg/g DM were obtained from amaranth fermented doughs (Table 3).

The doughs fermented with the *Lp. pentosus* MIUG BL24 strain and *Lc. rhamnosus* MIUG BL38 strain demonstrated comparable effects on protein hydrolysis during the amaranth flour fermentation. The dough fermented with the *Lc. rhamnosus* MIUG BL38 strain had the highest protein content (1.18 ± 0.06 mg/g DM); however, it was lower than the control (1.23 ± 0.03 mg/g DM). These results are in agreement with those of Cruz-Casas et al. [17], who indicated that fermentation produces protein hydrolysates through a strain-dependent process.

During sourdough fermentation, enzymes found in pseudocereals influence the breakdown of native proteins in cereals and pseudocereals. These enzymes first release oligopeptides via primary proteolysis. Following this, the metabolic activity of microbial peptidases and proteinases causes the release of smaller peptides and free amino acids, known as secondary proteolysis [44].

Fermentation is a relatively unexplored method for hydrolysing amaranth protein. This approach uses microorganisms to create protein hydrolysates and bioactive peptides by breaking down parental proteins using peptidases produced during fermentation [17]. Cruz-Casas et al. [17] noticed that the fermentation type, protein source, and microorganisms used have a significant impact on the synthesis of bioactive protein hydrolysates.

Hydrolysing amaranth proteins resulted in peptide-rich fractions with anti-inflammatory, antidiabetic [54], antihypertensive [55], and antioxidant qualities, among other health advantages. These proteins were used in pasta and baked goods, as well as emulsions, food gels, drinks, edible films, and microfibers. Overall, amaranth proteins and peptides have significant potential for diverse food and nutritional applications [6].

3.5. RP-HPLC Characterisation of BA Fermented Dough

The polyphenolic compounds identified through RP-HPLC characterisation are listed in Table 4.

Table 4. RP-HPLC characterisation of BA fermented dough.

Identified Compounds, ng/µL	Control	*Lc. paracasei* MIUG BL4	*Lc. paracasei* MIUG BL13	*Lp. pentosus* MIUG BL24	*Lc. rhamnosus* MIUG BL38
Phenolic acids					
Gallic acid	161.21 ± 0.82	19.15 ± 0.25 ****	27.67 ± 7.98 ****	n.d.	n.d.
Protocatechuic acid	13.34 ± 1.47	12.57 ± 0.70 n.s.	15.33 ± 0.33 n.s.	16.25 ± 0.59 *	19.61 ± 0.54 **
4-Hydroxybenzoic acid	3.73 ± 0.09	n.d.	n.d.	n.d.	2.96 ± 0.02 **
p-Coumaric acid	9.49 ± 0.48	n.d.	n.d.	n.d.	n.d.
Flavonoids					
Epigallocatechin	7789.88 ± 17.07	3941.11 ± 11.61 ****	3566.02 ± 11.24 ****	4983.16 ± 7.29 ****	6942.47 ± 5.63 ***
Epicatechin	880.02 ± 15.33	415.13 ± 13.11 ***	274.92 ± 7.43 ****	337.72 ± 2.53 ****	242.50 ± 9.14 ****
Catechin	n.d.	217.33 ± 4.85	237.20 ± 2.74	226.48 ± 2.78	434.81 ± 5.63 **

n.d.—Not detected, n.s.—not significantly different. Statistical significance was determined using a one-way ANOVA with Tukey's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The black amaranth fermented doughs exhibited a higher concentration of flavonoids compared to the polyphenol content, with epigallocatechin showing the highest concentration, followed by epicatechin and catechin. Among the quantified compounds, epigallocatechin was the most prevalent, with the highest concentration of 7789.88 ± 17.0 ng/µL in the control sample, followed by a concentration of 6942.47 ± 5.632 ng/µL in the dough fermented with the *Lc. rhamnosus* MIUG BL38 strain and 4983.16 ± 7.29 ng/µL in the dough fermented with the *Lp. pentosus* MIUG BL24 strain. In contrast, bioactive compounds such as *p*-coumaric acid and 4-hydroxybenzoic acid were identified in small amounts only in the control sample. The dough fermented with the *Lc. rhamnosus* MIUG BL38 strain demonstrated the highest content of polyphenols, including protocatechuic acid (19.61 ± 0.54 ng/µL), 4-hydroxybenzoic acid (2.96 ± 0.02 ng/µL), epigallocatechin (6942.47 ± 5.63 ng/µL), and epicatechin (242.50 ± 9.14 ng/µL). These results demonstrate that the phenolic content of the fermented product is correlated with the biochemical properties of the inoculum.

A strong negative correlation was observed between epigallocatechin and TFC (r = −0.920, $p < 0.05$), meaning higher epigallocatechin levels are related to lower total flavonoid content. Similarly, protein content also showed a significant negative correlation with TFC (r = −0.916, $p < 0.05$). Other correlations, although sometimes moderately strong, such as those between epigallocatechin and TPC or PC, were not statistically significant (Table 5).

Table 5. Pearson's correlation coefficient of epigallocatechin, TFC, TPC, PC, DPPH, ABTS for the dough.

	Epigallocatechin	TFC	TPC	PC	DPPH	ABTS
Epigallocatechin	1.000					
TFC	−0.920 *	1.000				
TPC	0.822 $^{n.s.}$	−0.638 $^{n.s.}$	1.000			
PC	0.822 $^{n.s.}$	−0.916 *	0.466 $^{n.s.}$	1.000		
DPPH	−0.266 $^{n.s.}$	0.560 $^{n.s.}$	0.037 $^{n.s.}$	−0.741 $^{n.s.}$	1.000	
ABTS	−0.265 $^{n.s.}$	0.006 $^{n.s.}$	−0.692 $^{n.s.}$	−0.020 $^{n.s.}$	−0.195 $^{n.s.}$	1.000

TFC = total flavonoid content, TPC = total polyphenolic content, PC = protein content, ABTS = antioxidant capacity measured in ABTS assay, DPPH = antioxidant capacity measured in DPPH assay, $^{n.s.}$ = not significant, * = significant at $p < 0.05$.

TPC, DPPH, and ABTS had no effect on epigallocatechin content prediction; however, TFC accounts for 84.58% of the variation in epigallocatechin content. This statistically significant linear relationship ($p = 0.027$) suggests that higher total flavonoid content may contribute to reduced levels of epigallocatechin, possibly due to transformation, degradation, or competition during the dough fermentation (Table 6).

Table 6. Regression analysis of epigallocatechin versus TFC, TPC, PC, DPPH, ABTS for dough.

Source	DF	Adj SS	Adj MS	F-Value	*p*-Value
Regression	1	11,626,204.0	11,626,204.0	16.45	0.027
TFC	1	11,626,204.0	11,626,204.0	16.45	0.027
Error	3	2,120,221.0	706,740.0		
Total	4	13,746,425.0			

R^2 = 0.846, DF = degrees of freedom, SS = sums of squares, MS = mean square, F = variance ratio, *p* = probability.

Phenolic compounds, including flavonoids, hydroxycinnamic acids, and hydroxybenzoic acids, are usually available in *Amaranthus* species. Comparative analyses of *A. tricolor* red and *A. lividus* green genotypes show that the red variations are richer in flavonoids

and phenolic acids. Red genotype, in particular, had phenolic contents between 85 and 312 μg/g DW, which was higher than the range of 71 to 220 μg/g DW for the green genotype. The majority of these compounds consist of protocatechin, caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, vanillic acid, salicylic acid, and trans-cinnamic acids [45]. In two types of *A. cruentus* seeds, gallic and protocatechuic acids were shown to prevail after *p*-hydroxybenzoic acid [56]. Protocatechuic and *p*-hydroxybenzoic acids are among the phenolic compounds with antioxidant activity in amaranth [3]. Catechins (epigallocatechin and epicatechin) have strong antioxidant effects and can be controlled directly, such as via metal ion chelation and scavenging ROS, or indirectly, by producing phase II detoxifying enzymes, inhibiting prooxidant enzymes, and stimulating the activation of antioxidant enzymes.

Amaranth contains various phenolic compounds, including gallic acid, vanillic acid, ferulic acid, and their derivatives. It also has flavonoids such as kaempferol, quercetin, rutin, and their glycosides, as well as tannins. These compounds are primarily known for their antioxidant, anti-inflammatory, anti-diabetic, and anticancer properties. Therefore, they may play a significant role in developing functional foods aimed at improving human health [46]. Therefore, it is known that phenolic acids and flavonoids are responsible for amaranth's antioxidant capacity [7].

4. Conclusions

This study focused on the biotication of black amaranth flour by lactic acid fermentation using probiotics LAB to ensure that individuals with celiac disease receive adequate nutrition and to combat malnutrition, particularly in underdeveloped countries. The obtained fermented doughs were characterised regarding their bioactive properties, particularly regarding acidification capacity and potential to produce antioxidant and polyphenolic compounds. As a result, the probiotic strains *Lc. rhamnosus* MIUG BL38 and *Lp. pentosus* MIUG BL24 showed beneficial properties for obtaining gluten-free and plant protein-rich doughs by black amaranth flour fermentation and were chosen for further investigations that targeted fermentation process optimisation. Fermented amaranth products can effectively be used to create various functional foods, including synbiotic foods, gluten-free products, and supplements for other fermented foods with tribiotic characteristics (pre-, pro-, or postbiotic), with beneficial technological and functional characteristics. Although amaranth has high nutritional value, it also contains several anti-nutrient compounds that can diminish the nutrients' bioavailability, especially their protein content. Unwanted flavours or textures may also develop in some fermented amaranth products, which could affect consumer acceptance. Additionally, the variability in composition and the quality of the raw material (black amaranth seeds) might impose certain limitations on the utilisation of the flour and the bioactive properties of the fermented product.

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