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Fermentation Unlocks the Functional Role of Amaranth in Modulating Wheat/Amaranth Sourdough Microbiota and Inhibiting Yeast Growth of Refrigerated Doughs

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Abstract

This study focuses on the development of refrigerated doughs without chemical preservatives to obtain a clean-label product. Sourdough-based strategies were applied to replace conventional preservatives, using both spontaneous flour fermentation and a defined starter culture of *Lactiplantibacillus plantarum* CIDCA 8327. In parallel, a partial substitution of wheat flour with 7% amaranth flour was evaluated. To monitor fermentation, pH, titratable acidity, and viable microorganism counts were determined in the sourdoughs, along with culture-independent analyses of microbial communities in two independent spontaneously fermented trials. Dough discs prepared from these sourdoughs were analyzed for pH, titratable acidity, and viable microorganisms, and shelf life was determined based on the appearance of visible mould during refrigerated storage. No substantial differences were observed in the physicochemical parameters of the sourdoughs; however, significant differences in microbial communities were detected, influenced by both amaranth addition and wheat flour batch variability. Dough discs prepared with amaranth flour and spontaneous fermentation showed an extended shelf life and lower mould and yeast counts during refrigerated storage. The use of the starter increased shelf life compared to non-fermented doughs but was less effective than spontaneous sourdough with amaranth. Overall, these results highlight the potential of sourdough technology and amaranth flour for developing clean-label refrigerated products.

Keywords: sourdough; wheat; amaranth; *Lactiplantibacillus plantarum*; shelf life

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1. Introduction

With the advances in the knowledge of the relationship between diet and health, consumers have become increasingly attentive to "clean-label" products, which are characterized by the use of natural ingredients, minimal processing through traditional techniques, and the absence of artificial or synthetic chemicals and additives [1–3]. Market studies conducted in recent years indicate that a substantial proportion of consumers perceive ultra-processed foods negatively, demand greater transparency in product labelling, and are progressively shifting their purchasing preferences toward clean-label products [4,5]. This shift in consumer preferences has spurred renewed interest in research and development in the field of food biopreservation, aiming to meet the demand for these types

of products. Biopreservation involves extending the shelf life and enhancing the safety of foods through the use of natural or controlled microbiota and/or antimicrobial compounds [6–9]. Among the traditional techniques recognized for extending the shelf life of flour-based products, the use of sourdough is one of the oldest methods. Essentially, sourdough consists of a mixture of flour and water in various proportions that ferment spontaneously on account of the microorganisms present in the ingredients and the surrounding environment or by the addition of a defined starter culture [8,10]. In addition to serving as a barrier against bread spoilage, sourdough enhances the organoleptic qualities of the final product, including flavour and texture [8,9,11–14]. Moreover, it improves the nutritional profile by reducing non-digestible oligosaccharides, phytic acid, and gluten content while providing prebiotics and probiotics that may offer health benefits for the consumer's gastrointestinal system [6,15]. Given these numerous advantages, the popularity of sourdough has expanded beyond bread to encompass a range of products, including pasta, tortillas, muffins, and more [15–19]. In pasta, sourdough also enhances nutritional properties by reducing phytic acid content, increasing antioxidant activity, and improving protein digestibility [17,20]. This interest has also stimulated scientific exploration into the selection of starter cultures with novel and enhanced properties to be used as starters for sourdough preparation [21–24]. For instance, the incorporation of sourdough using a selected starter culture with antifungal properties inhibited mould growth more effectively than conventional preservatives during the storage of fresh pasta [25].

Type II sourdough production is characterized by the fermentation of a flour and water mixture without the process of backslopping [10,26]. This fermentation is driven by the presence of microorganisms in the ingredients, particularly from the flour, the surrounding environment, and the addition of starter cultures. Spontaneous fermentations often emerge from the competitive interactions of various native and contaminating microorganisms with lactic acid bacteria (LABs) [27]. LABs play a crucial role in the acidification of the dough matrix through the production of organic acids, primarily lactic acid, which inhibits the growth of non-acid-tolerant species. Additionally, some LABs can produce bacteriocins and antifungal peptides, which help prevent spoilage or contamination of the final product [24,28,29].

Lactiplantibacillus plantarum is a lactic acid bacterium (LAB) that can be isolated from a variety of hosts and environments. These include the human intestinal tract, the oral cavity, dairy products, meat, and spontaneous fermentations of vegetables. It is one of the most frequently isolated species in sourdough due to its wide metabolic diversity [30–35]. *L. plantarum* CIDCA 8327 is a facultative heterofermentative strain isolated from kefir grains. This strain can produce lactic and acetic acid and can survive in extremely low pH conditions [36,37]. Additionally, this strain has exhibited high inhibitory activity against *Salmonella typhimurium*, *S. enterica*, *S. gallinarum*, *S. sonnei*, and *Escherichia coli* [36]. Furthermore, this strain can produce alpha-glucan or heteropolysaccharides depending on growth conditions, which could improve dough characteristics [38].

Empanadas are a traditional Argentine meal consisting of a wheat dough thin disc filled with a mixture of ingredients. The empanada discs are circular doughs (non-fermented product) obtained by the mechanical mixing and kneading of flour and potable water, with or without the addition of salt or edible fats [39,40]. These discs are sealed into a crescent or semi-circle shape using a technique called "repulgue" and can be either baked or fried. Similar culinary creations are found in other parts of the world, such as the Cornish pastry (United Kingdom), panzerotto and calzone (Italy), pierogi (Poland), pirozhki (Russia), fatayer (Arabia), empanada gallega (Spain), brik (Tunisia), börek (Turkey), and sfiha (Middle East).

The dough used for empanada discs is primarily made from refined wheat flour and contains proteins with a low content of lysine, an essential amino acid. To enhance the nutritional value of cereal products, partial replacement with alternative non-cereal protein sources has become a viable strategy [41,42]. Amaranth is a pseudocereal known for the good nutritional value of its protein, which comprises a well-balanced amino acid profile and serves as a precursor of bioactive peptides generated by microbial or enzymatic hydrolysis [41,43–46]. In addition, amaranth is a source of B vitamins, β-carotenes, tocopherols, and squalene and contains essential minerals and dietary fibre [41,47–51]. Notably, during the digestion of amaranth proteins, peptides are released that can serve as physiological modulators, exhibiting various beneficial activities such as antihypertensive, antioxidant, antiproliferative, antithrombotic, anti-inflammatory, and antimicrobial effects [44,45,52–54]. Therefore, amaranth can be used not only to add nutritional value to wheat flour but also for the development of products with added functional and health-promoting properties.

The aim of this study was to evaluate the microbiological characteristics of type II sourdough made with either wheat flour or a blend of wheat flour and amaranth flour for the preparation of empanada discs. To our knowledge, no prior studies have investigated the role of amaranth in sourdough microbiota or its impact on the shelf life of refrigerated doughs. The sourdough was prepared using two methods: spontaneously or by adding *L. plantarum* CIDCA 8327 as a starter culture. The sourdough was explored for producing clean-label dough discs without additives and with improved shelf life under refrigeration.

2. Materials and Methods

2.1. Dough Ingredients and Formulations

Two independent batches of wheat flour 0000, according to Argentinian regulations, were provided by Molino Campodónico (S.A. Miguel Campodónico Ltda., La Plata, Argentina). Amaranth flour was provided by Nutraceutica STURLA S.R.L. (Buenos Aires, Argentina) and high-stearic high-oleic sunflower oil by Advanta/Nutrisun. Table salt (Celusal) was acquired from a hypermarket in La Plata, Buenos Aires, Argentina. These two batches of flour were used to prepare empanada discs in a traditional way. Furthermore, empanada discs prepared with a fermentation step, carried out by the indigenous microorganisms (natural fermentation) or with the addition of *L. plantarum* CIDCA 8327 as a starter, were prepared. The same procedure was applied using wheat–amaranth flour blends (93:7).

2.2. Physicochemical Characterization of Ingredients and Empanada Discs

Protein content was determined according to the Kjeldahl–Arnold–Gunning method (AOAC 1984, 24.027), using factor 5.7 for conversion of nitrogen to protein content. Fat content was determined by the Soxhlet method (AOAC 1990, 920.39) with ethyl ether. Minerals were estimated as the ash content at 550 °C (AOAC 1984, 24.009). Moisture content was indirectly measured by drying in an oven at 105 °C up to constant weight (AOAC 1984, 24.002). Total carbohydrates were determined by the modified Fehling–Causse–Bonnans method after complete acid hydrolysis of the samples (AOAC 1965, p. 495) and by the Anthrone method.

2.3. Starter

L. plantarum CIDCA 8327, belonging to the culture collection of the Centro de Investigación y Desarrollo en Criotecnología de los Alimentos (CIDCA), La Plata, Argentina, was stored at −20 °C in sterile skim milk (La Serenísima, Buenos Aires, Argentina) as cryoprotectant. For the assays, the culture was reactivated by inoculation in sterile DeMan–Rogosa–Sharpe (MRS) broth containing 1.00% universal peptone, 0.50% meat extract, 0.50% yeast

extract, 2.00% D-(+)-glucose, 0.20% K_2HPO_4, 0.10% Tween 80, 0.20% ammonium citrate, 0.50% ammonium citrate, 0.50% ammonium acetate, 0.01% $MgSO_4$, and 0.005 $MnSO_4$, with a pH of 6.5 ± 0.1. After 24 h at 30 °C of aerobic growth, the culture was centrifuged (5 min at 5000 rpm) at room temperature. The pellet was resuspended in saline–tryptone diluent (containing, per litre, 20.0 g of NaCl and 1.0 g of tryptone, pH 7.0 ± 0.2) and used for sourdough elaboration.

2.4. Sourdough Preparation

Wheat flour (W) or flour blend (7%A) was mixed with tap water in equal proportions (1:1) to obtain a dough shield = 200 (DY = dough × 100/g of flour). The mixtures were placed in closed plastic containers at 30 °C for 24 h without agitation to conduct the natural fermentation. To prepare sourdoughs with starter, 1–3 10^7 UFC of *L. plantarum* CIDCA 8327 was added to the mix and was incubated under the same conditions.

2.5. Disc Preparation

Non-fermented discs (controls) were prepared with 100.0 g of wheat flour or flour blend combined with 10.0 g of high-stearic high-oleic sunflower oil, 2.0 g of salt, and 40.00 mL of tap water. To obtain empanada discs prepared from sourdough, 40 g of sourdough was added to 80 g of flour or flour blend. Water (20 g), oil (10 g), and salt (2 g) were added in order to achieve the ingredient ratio used in the controls.

In all cases, the dough was kneaded manually for 20 min or until soft and smooth dough was obtained. It was stretched with the help of a kneading stick and acrylic guides to give a fixed thickness (2 mm) to the dough. Five-centimetre-diameter discs were cut using a circular shape cutter and stored with plastic separators in bags in the fridge at 10 °C.

The experimental design is presented in Figure 1.

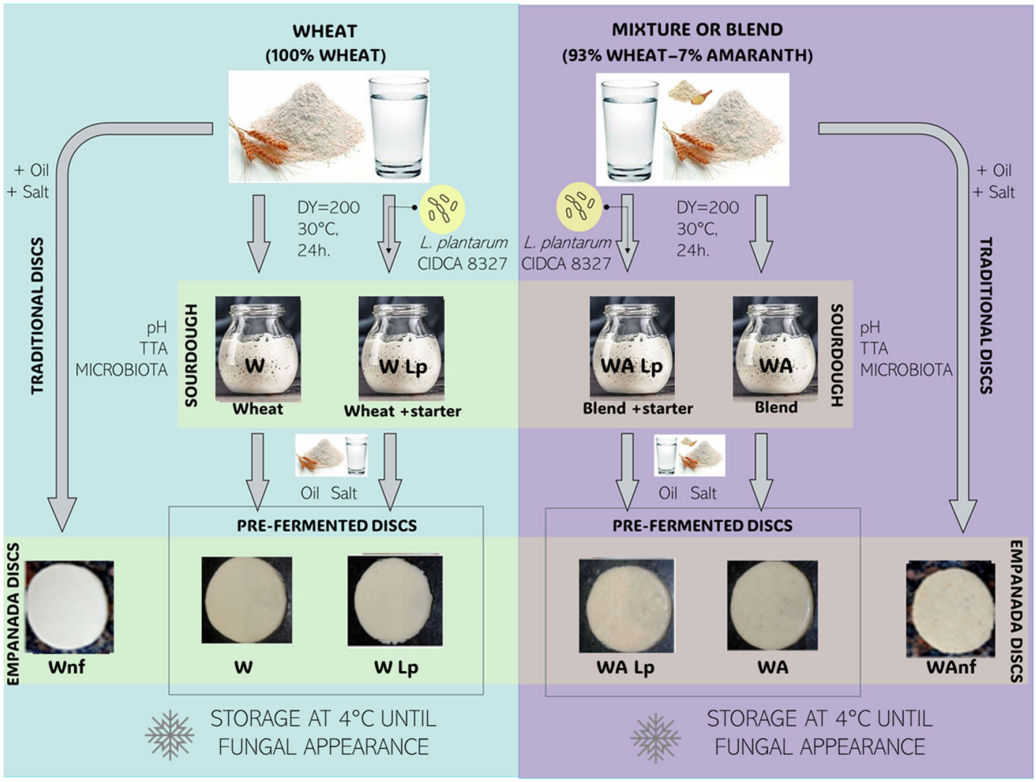


Figure 1. Experimental design for sourdough-based empanada disc preparation. Experimental design for empanada disc preparation with wheat or wheat-amaranth blend flour using sourdough

technology or the traditional method. Spontaneously fermented sourdough or discs prepared with wheat flour (W) or wheat–amaranth blend (WA), as well as sourdough or discs fermented with *L. plantarum* CIDCA 8327 (W Lp and WA Lp), are indicated. Non-fermented discs are denoted as Wnf and WAnf.

2.6. Enumeration of Viable Microorganisms on Sourdoughs and Empanada Discs

Ten grams (10.0 g) of the sample was withdrawn and added aseptically into a sterile bottle with 90.0 mL of sterile tryptone solution and shaken thoroughly. Agar plates were inoculated with appropriate serial decimal dilutions of those suspensions in duplicate. Differential enumeration of colony-forming units was performed on DeMan–Rogosa–Sharpe agar for lactic acid bacteria (LABs). Yeast extract–glucose–chloramphenicol agar (Merck, D64271 Darmstadt, Germany) containing 0.50% yeast extract, 2.00% glucose, 0.01% chloramphenicol, and 1.49% agar, with a pH of 6.6 ± 0.2 was used for yeast enumeration. The Petri plates were incubated at 30 °C for 48 h in aerobiosis. The average counts were expressed as the number of colony-forming units (CFUs)/g of sample.

2.7. Acidification Kinetics

Samples were taken from the sourdough or doughs several times during fermentation or storage to measure pH and total titratable acidity (TTA).

To determine the pH, 1.0 g of the sample was put in a screw-capped tube and 9.00 mL of distilled water previously boiled and cooled was added. The sample was homogenized with vortex equipment and centrifuged at medium speed (Rolco S.R.L. Model 2036, CABA, Argentina) for 10 min. Finally, pH was measured from the supernatant with a pH metre (Altronix Model TPX-1 Girard, OH 44420, USA) that had been previously calibrated.

For titratable acidity determination, ten grams (10 g) of sample was exactly weighed (±0.1 mg), and 90.0 mL of distilled water previously boiled and cooled was added, magnetically agitated, and titrated with 0.1 N NaOH using phenolphthalein as indicator. Results were expressed as acid milliequivalents per gram of sample.

2.8. Lactic Acid and Acetic Acid Quantification

Organic acids were quantified in the supernatant used for pH measurement. To remove sourdough or dough particles, two consecutive centrifugation steps were performed: the first at medium speed using a Rolco S.R.L. Model 2036 (Argentina) for 10 min at room temperature, followed by a second centrifugation at 13,000 rpm (18,000× *g*) for 2 min. The supernatant obtained was filtered with a pore diameter of 0.45 µm (Millipore Corporation, Burlington, MA, USA). Chromatographic analysis was carried out by injecting 20 µL of the sample into HPLC equipment with an Aminex® HPX-87H ionic exclusion column (Bio-Rad Laboratories, Richmond, VA, USA) and an ultraviolet detector (Waters™ 996, Milford, MA, USA) at 214 nm. Chromatographic runs took place for 30 min, with a flow rate of 0.7 mL/min at 60 °C. The mobile phase was prepared by diluting concentrated sulfuric acid to the desired normality (0.009 N) with deionized water. The separation of organic acids was carried out under isocratic conditions throughout the entire analysis.

Organic acids were identified by comparison to standard retention times and quantified with the corresponding peak area using the calibration curve (1–50 mM) and PeakFit® 4.12.00 software.

2.9. DNA Isolation

For DNA extraction, 1.0 g of the sourdough samples was resuspended in 9 mL of sterile phosphate-buffered saline (PBS) solution (containing, per litre, 8.06 g NaCl, 0.22 g KCl, 1.15 g Na_2HPO_4, and 0.20 g KH_2PO_4, with a pH of 7.20 ± 0.20). It was shaken with a Vortex mixer and then disrupted with a homogenizer (Ultra-Turrax T8 IKA LaborTechnik,

Phoenix, AZ, USA) at speed 3 for 5 min. The big particles were decanted by centrifuging for 10 min at low speed (Rolco S.R.L. Model 2036, Argentina). The supernatant was centrifuged for 2 min at 13,000 rpm. Microbial pellets were washed with 1 mL of TE buffer (10 mM Tris-base and 1 mM EDTA; pH 8.0), resuspended with 200 µL of lysis buffer (20 mM Tris-base, 2 mM EDTA, 1.2% *v*/*v* Triton X-100, and 100 mM NaCl; pH 8) containing 20 mg/mL of lysozyme (Sigma Chemical, St. Louis, MO, USA), and incubated an hour at 37 °C. For the next steps of DNA isolation, a commercial kit was used (AccuPrep® Plant Genomic DNA Extraction Kit K-3031, Bioneer Corporation, Daejeon, Republic of Korea) following the manufacturer's protocol. DNA concentration of the obtained extracts was measured in a Nanodrop spectrophotometer (Thermo Scientific Nano Drop 2000 spectrophotometer, Waltham, MA, USA), and the quality of DNA isolation was checked with 260 nm/230 nm and 260 nm/280 nm absorbance ratios.

2.10. PCR Amplification

To analyze the sourdoughs' eubacterial community in a qualitative model, the V3 region of the gene that codifies 16S rRNA [55] was amplified using the following universal synthetic oligonucleotides: 338f-GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGGACTCCTACGGGAGGCAGCAG-3′) with the GC clamp underlined and 518r (5′-ATTACCGCGGCTGCTGG-3′) (Invitrogen, Carlsbad, CA, USA). The yeast community DNA was amplified with the forward primer NL1f-CG (5′-GCGGGCCGCGCGAC CGCCGGGACGCGCGAGCCGGCGGCGGGCCATATCAATAAGCGGAGGAAAAG-3′, with the CG clamp underlined) and the reverse primer LS2 (5′-ATTCCC AAACAACTCGACTC-3′), spanning the D1 region of the 26S rRNA gene [56].

The PCR reaction was performed using Taq polymerase (Inbio Highway, Tandil, Argentina), following the manufacturer's protocol with 0.4 ng/µL of the isolated DNA. The thermal cycling was carried out in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following programme: initial denaturalization step of 5 min at 94 °C, followed by 35 cycles of 30 s at 92 °C, 45 s at 60 °C, and 20 s at 72 °C, with a final 60 s extension step at 72 °C. The obtained amplicons were analyzed by electrophoresis on 1% *w*/*v* agarose gels, stained with ethidium bromide, and visualized under UV light. The amplification mixtures were stored at −20 °C until their utilization.

2.11. Denaturing Gradient Gel Electrophoresis (DGGE)

The PCR products were analyzed by DGGE using a DGGE-2401 analyser (C.B.S. Scientific Co., Del Mar, CA, USA) as described in Bengoa et al., 2020 [57]. An optimal separation of the bands was achieved with a 40–60% urea–formamide denaturing gradient (with 7 M urea and 40% *v*/*v* formamide as 100% denaturing) for eubacteria and 30–70% for yeast PCR products. Electrophoresis was performed at a constant voltage of 120 mV for 16 h at 60 °C. Gels were stained with Sybr Gold (Invitrogen, Eugene, OR, USA) and visualized under UV light.

The PCR products obtained with 338f-GC and 518r primers of the following strains were used for lines of reference in the DGGE gels: *L. plantarum* CIDCA 8327 (48%GC), *Lentilactibacillus kefiri* CIDCA 8348 (51%GC), *Lacticaseibacillus casei* DSMZ 20,011 (55%GC), and *Bifidobacterium adolescentis* CIDCA 5317 (59%GC).

Bands were identified by excising them from the polyacrylamide gel, re-amplifying the DNA using the same primers without the GC clamp and the same PCR cycling conditions, and sequencing the products at Macrogen Inc. (Seoul, Republic of Korea). The sequences were identified using the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 23 April 2024).

2.12. Mass Sequencing Analysis Using Ion Torrent PGM

The mass sequencing analysis was carried out in the MR DNA molecular research laboratory (Shallowater, TX, USA), based on established and validated protocols. The V4 variable region of the 16S RNA gene was achieved using primers 515 and 806, which amplify the gene that codes for 16S rRNA, and the HotStarTaq Plus Master Mix kit (Qiagen, Hilden, Germany). Sequencing was performed using the Ion Torrent Personal Genome Machine (PGM) system (Thermo Fisher Scientific, Waltham, MA, USA). The data obtained were demultiplexed and analyzed using a pipeline developed at the MR DNA Molecular Research Laboratory. Raw sequencing reads were quality trimmed using QIIMEII 2018. Sequencing data were grouped into 3% divergence operating taxonomic units (OTUs) and taxonomically classified using the BLASTn.NET algorithm with the database derived from RDPII3 and NCBI.

2.13. Statistical Analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to identify significant differences among means. Differences were considered statistically significant at $p < 0.05$. All analyses were performed using GraphPad Prism softwareVersion 8.0.2 (GraphPad Software Inc., San Diego, CA, USA). Dendrograms were achieved by the UPGMA algorithm using the Jaccard similarity index in the Gel Compar II program version 1998/2005.

3. Results

3.1. Wheat and Amaranth Flour Composition

Evaluation of wheat and amaranth flour composition revealed that amaranth flour contained lower amounts of carbohydrates and higher levels of proteins, fats, ashes, and fibre (Table 1).

Table 1. Percentual composition of wheat and amaranth flour.

Components	Wheat (W)	Amaranth (A)
Carbohydrates, FCB	67.1 ± 1	49 ± 1
Carbohydrates, Anthrone	64.4 ± 1	48.8 ± 0.7
Proteins	8.8 ± 0.2	14.1 ± 0.7
Fats	1.07 ± 0.06	5.6 ± 0.1
Ashes	0.43 ± 0.01	3.05 ± 0.01
Fibre	3.13 ± 0.06	12 ± 1

Values are expressed as mean ± standard deviation, calculated and reported as a percentage (%) per 100 g of flour. W: wheat flour; A: amaranth flour. Carbohydrates were determined by both the Anthrone and Fehling–Causse–Bonnans–methods.

No differences in composition were observed between the two batches of wheat flour, nor were any differences found in breadmaking parameters. The wheat-amaranth blend composition (93% of wheat flour and 7% of amaranth flour) was calculated by considering the individual values of wheat and amaranth flour, as well as their proportion in the blend. The carbohydrate composition of this mixture did not vary significantly. However, a slight increase in the content of proteins, fats, ash, and fibre was observed in the blended flour (Supplementary Table S1).

3.2. Characterization of Sourdough Prepared with Wheat and Wheat–Amaranth Blend

3.2.1. pH and Titratable Acidity of Sourdough Prepared with Wheat and Wheat–Amaranth Blend

The physicochemical and microbiological changes in sourdough obtained by natural fermentation or including *L. plantarum* as starter were studied after 24 h of fermentation at 30 °C using two wheat flour batches in independent assays.

The evolution of pH and total titratable acidity (TTA) of sourdough was measured at the beginning and the end of 24 h spontaneous fermentation of wheat (W) and wheat–amaranth (WA) flour and for sourdough prepared from wheat and wheat-amaranth blend with *L. plantarum* (W Lp and WA Lp). The results obtained with both batches of flour did not show statistical differences (Supplementary Figure S1), so the mean and SD considering both independent assays are presented in Figure 2. Spontaneous fermentation did not show a significant pH decrease in W or WA flour. However, sourdough enriched with *L. plantarum* culture showed a pH decrease after 24 h of fermentation, reaching values of 3.61 ± 0.63 and 3.19 ± 0.19 for W Lp and WA Lp, respectively. Total titratable acidity significantly (p = 0.01) rose after 24 h of fermentation in all samples, and this effect was even greater in the sourdoughs containing amaranth flour. Furthermore, the addition of *L. plantarum* CIDCA 8327 led to higher values of titratable acidity compared with the corresponding spontaneous sourdough.

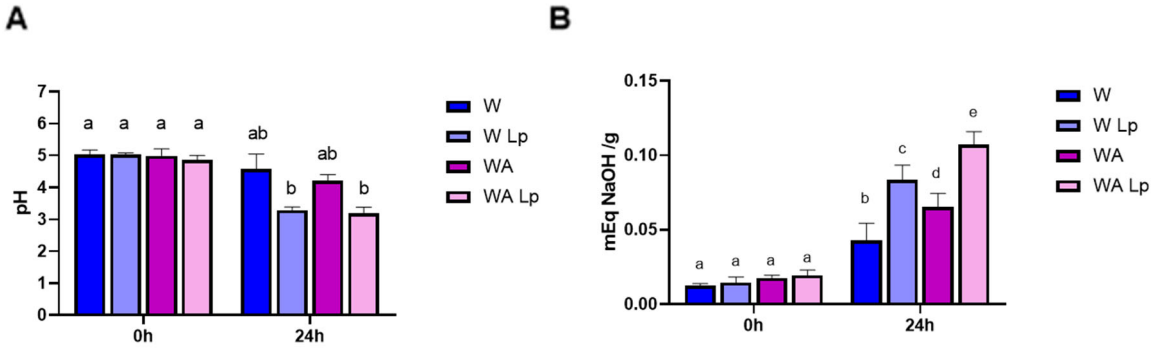


Figure 2. Evolution of pH and titratable acidity in sourdoughs. pH (**A**) and titratable acidity (**B**) of sourdough obtained by natural fermentation of wheat (W) or wheat/amaranth blend (WA) and inoculated with *L. plantarum* (W Lp and WA Lp) at the start and after 24 h spontaneous fermentation at 30 °C. Different letters indicate significant differences using ANOVA with $p \leq 0.05$ for pH and $p \leq 0.01$ for titratable acidity.

When analyzing the concentration of organic acids during fermentation, only trace amounts of acetic acid were detected in all samples at the end of the process. However, lactic acid production was observed in all samples after 24 h of fermentation, reaching values of 4.25 ± 0.29 in W, 2.76 ± 0.44 in WA, and close to 10 mM (9.46 ± 1.31 for W Lp and 10.69 ± 4.56 for WA Lp) in sourdoughs inoculated with *L. plantarum* CIDCA 8327.

3.2.2. Sourdough Microbiota Evolution During Fermentation

Differential enumerations of viable microorganisms were performed on yeast extract–glucose–chloramphenicol agar (YGC) and DeMan–Rogosa–Sharpe agar (MRS) after 24 h of fermentation for the four sourdoughs. The total count of microorganisms on naturally fermented sourdough in MRS agar was not significantly different between all samples (Supplementary Table S2). All the conditions showed low total viable mould and yeast counts, with the statistically lowest being in W Lp compared with the other samples.

DGGE experiments were conducted at the initial, mid, and final stages of sourdough fermentation to analyze changes in yeast and eubacterial populations (Figures 3 and 4).

The yeast diversity observed by the number of bands in the DGGE gels was very low in the two biological replicates, and the yeast composition was clearly different and influenced by the type of flour used in each experiment (Figure 3). In trial 1, the band corresponding to the *Candida*/*Yamadazyma* genus was present at all stages of fermentation in all four sourdough samples. In contrast, yeast belonging to the *Aureobasidium* genus was detected in only two samples (WA and W Lp) at 12 h of fermentation, disappearing at the 24 h sampling. In trial 2, the *Fusarium* genus was present in all sourdoughs but was no longer detected in WA and WA Lp after 24 h of fermentation. Despite differences in band patterns between the two trials, WA from trial 2 showed a change in yeast population after 24 h, with *Candida* becoming the predominant genus by the end of the fermentation process. Therefore, the *Candida* genus was the dominant yeast in WA sourdough across both trials.

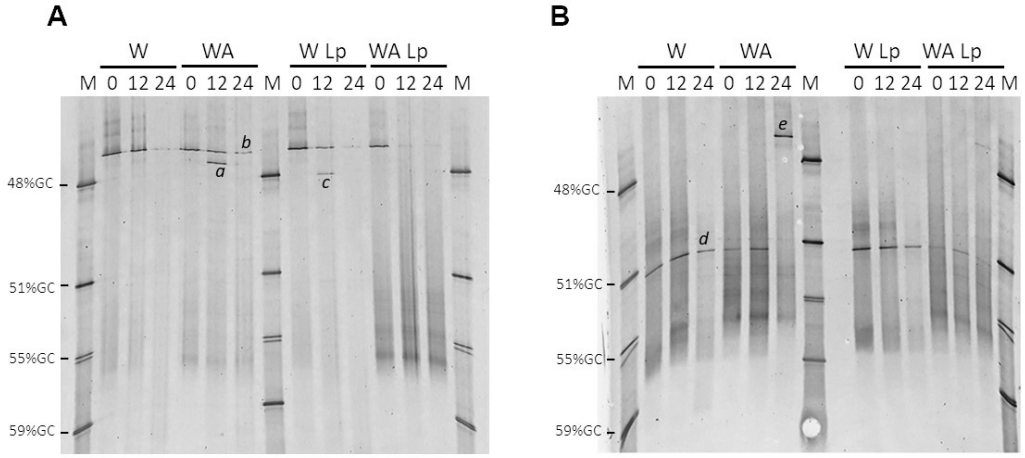


Figure 3. Yeast community profile during sourdough fermentation. PCR-DGGE fingerprints of yeast obtained by amplification of total DNA extracted from sourdough prepared with wheat (W) or wheat/amaranth blend (WA) at different times (0, 12, or 24 h) of spontaneous fermentation or with the starter *L. plantarum* CIDCA 8327 (W Lp and WA Lp) at 37 °C in trial 1 (**A**) and trial 2 (**B**). M: reference marker. Letters indicate the bands that were identified by sequencing. a: *Aureobasidium melanogenum* (98.58% of identity), b: *Candida atlántica*/*Yamadazyma mexicana* (97.62% of identity), c: *Aureobasidium pullulans* (98.04% of identity), d: *Fusarium culmorum* (99.50%), and e: *Candida atlántica*/*Candida spencermartinsiae*/*Yamadazyma mexicana* (93.92%).

The initial population of eubacteria was similar in W and WA sourdough in each experiment. After 24 h of fermentation, the eubacteria population of W sourdough was completely different in the two biological replicates (Figure 4). In trial 1, the wheat sourdough clustered with those prepared using the *L. plantarum* starter.

As expected, a prominent band corresponding to the standard of *L. plantarum* (band indicated as 48%GC in the marker) was visible in the DGGE gels at 24 h of fermentation in both the W and WA sourdoughs that were supplemented with this starter bacterium. This finding demonstrates that *L. plantarum* effectively controlled and guided the fermentation process. Throughout the fermentative process, *L. plantarum*-enriched sourdoughs consistently grouped within the same clade in the dendrogram for both biological replicates.

In order to characterize the relative abundance of the bacterial community and identify the genera that were present after 24 h of fermentation at 30 °C of spontaneous fermentation (W and WA), massive sequencing of a region of the 16S rRNA gene was performed. Analysis of the Shannon index showed that the samples of experiment 1 presented more bacterial diversity than the samples of experiment 2, and both experiments showed that sourdough samples with amaranth flour presented a higher index than wheat flour sourdough, consistent with an increment of microbial diversity due to the addition of amaranth flour (Supplementary Figure S2).

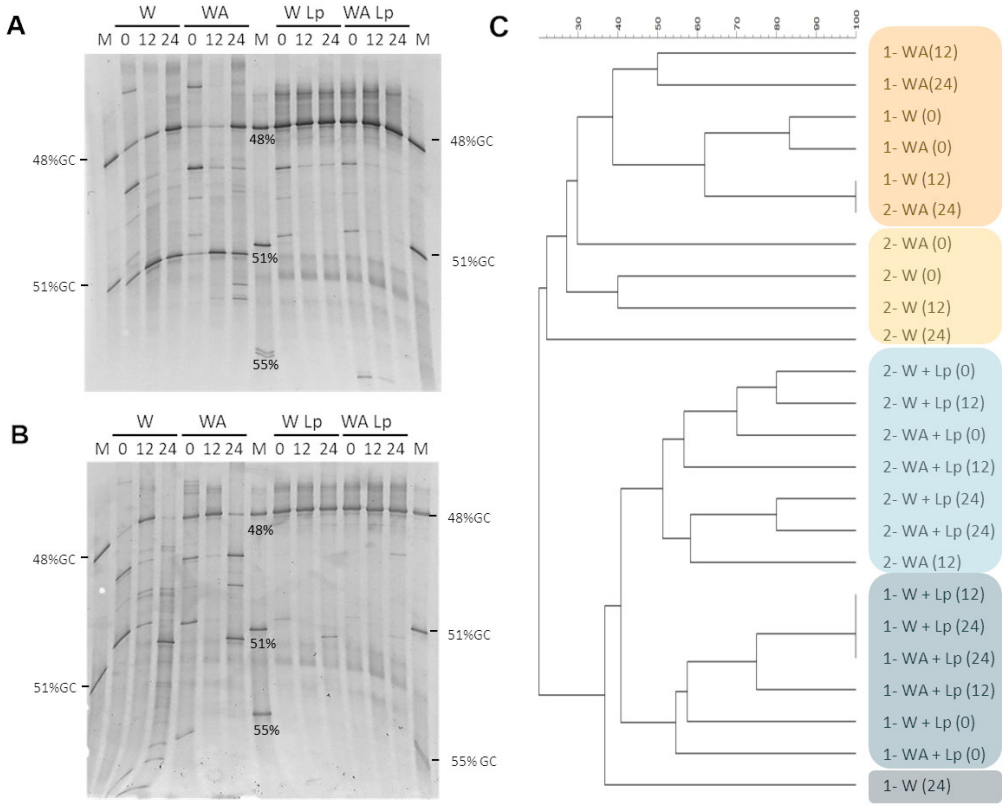


Figure 4. Bacterial community dynamics during fermentation. PCR-DGGE fingerprints of eubacteria obtained by amplification of total DNA extracted from sourdough prepared with wheat or wheat-amaranth blend at different times (0, 12, and 24 h) of spontaneous fermentation (W and WA) or with *L. plantarum* (W Lp and WA Lp) at 37 °C in trial 1 (**A**) and trial 2 (**B**). UPGMA tree of the DGGE fingerprint of trials 1 and 2 (**C**). Data were compared using the Jaccard correlation coefficient.

The principal differences in relative abundances between trials 1 and 2 were analyzed at the genus level, revealing distinct variations in both wheat and wheat–amaranth sourdough. The most representative genera (at least 1% of the total population in one sample) are graphed in Figure 5A. Almost half of the total bacterial population was represented by the former genera *Lactobacillus* and *Bacillus* in all samples. However, the proportion of *Lactobacillus* was higher in trial 1, whereas *Bacillus* was more prevalent in trial 2. This variation may be attributed to differences in the initial composition of the wheat flour. The relative abundance of *Pantoea* in trial 1 was greater when amaranth flour was incorporated, whereas *Pseudomonas* predominated in sourdough made with amaranth flour in trial 2 (Figure 5B).

For a better comprehension of the microbiota variations due to the addition of amaranth flour, the ratio of the relative abundance at the genus level, including all the genera detected, was plotted for each trial (Figure 6). The incorporation of amaranth flour modified the final microbial composition of the sourdough, with trial-dependent variations that resulted in markedly different column patterns. Despite the differences observed between trials, *Pseudomonas*, *Serratia*, *Enterobacter*, *Janthinobacterium*, *Comamonas*, *Clostridium*, and *Acinetobacter* were more abundant in sourdough prepared with the addition of amaranth flour (WA) compared to wheat flour (W), independently of the batch of wheat flour used (Figure 6). Instead, *Cronobacter* and *Fusobacterium* were less represented in sourdough prepared with the addition of amaranth flour (WA) after 24 h of fermentation in both trials.

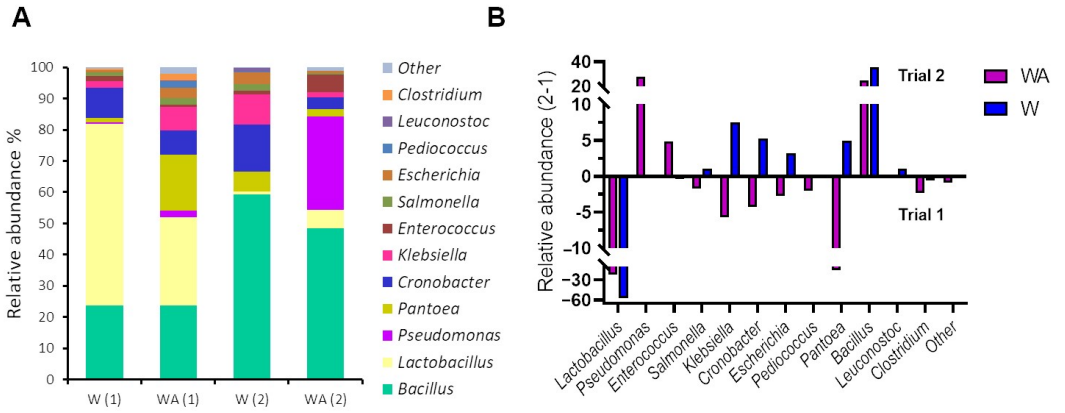


Figure 5. Identification of bacterial genera by high-throughput sequencing in spontaneous sourdough after 24 h of fermentation. (**A**) Relative abundance of the dominant bacterial genera in sourdough prepared by natural fermentation of wheat (W) or wheat-amaranth blend (WA) flour in trial 1 and trial 2. Only the genera higher than 1% in at least one sample were included. (**B**) Differences in bacterial relative abundance at the genus level in samples of sourdough prepared in trial 1 in comparison with the corresponding sourdough obtained in trial 2.

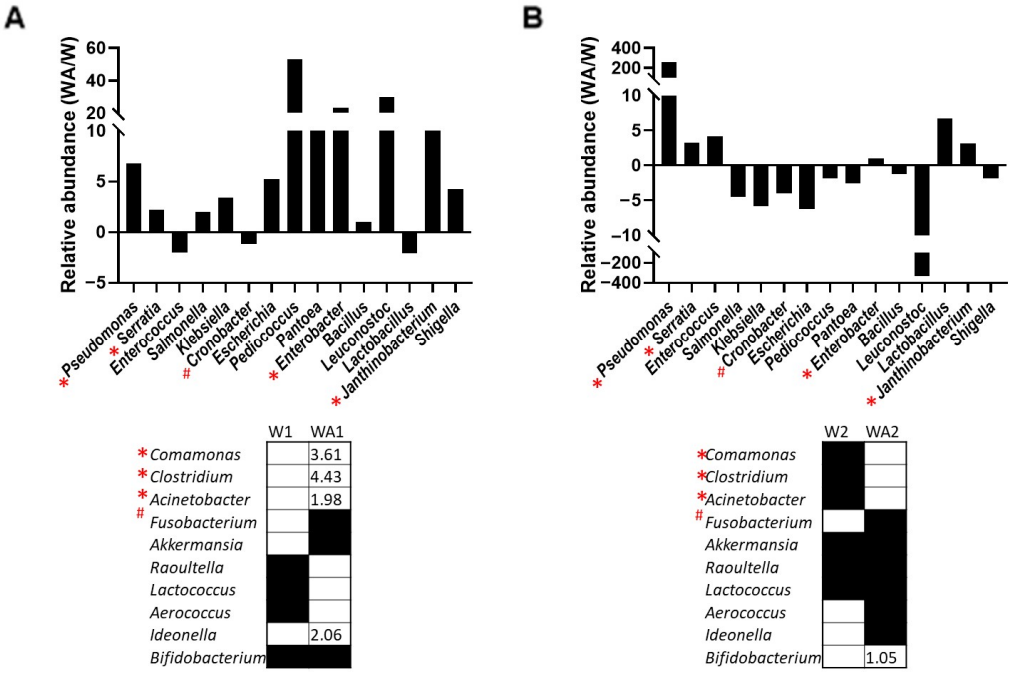


Figure 6. Relative abundance of bacterial genera in wheat–amaranth sourdoughs compared to wheat sourdoughs across two trials. Relative abundance of bacterial genera in sourdoughs prepared with the wheat–amaranth blend (WA) compared to those with wheat (W) from trial 1 (**A**) and trial 2 (**B**). Only genera detected in all four samples are shown in the column chart; additional genera are listed in the table below. Black boxes indicate genera not detected in a given sample, while white boxes indicate their presence. Numbers inside white boxes represent the relative abundance ratio for that sample. * indicates genera that increased in both trials, whereas # indicates genera that decreased in both trials.

3.3. Characterization of Empanada Discs During Refrigerated Storage

Empanada discs prepared with wheat flour or with a wheat–amaranth flour blend, either without fermentation or from sourdough obtained by natural fermentation or inoculated with a defined starter culture, were stored at 10 °C. *Empanada* discs that were prepared without a pre-fermentation process (Wnf and WAnf) exhibited the shortest shelf life during refrigerated storage, lasting almost two weeks before showing visible signs

of fungal growth, and were consistent in both trials (Table 2). These data suggest that sourdough fermentation could be a viable strategy to replace chemical preservatives during refrigerated dough storage. In contrast, the shelf life of discs made from sourdough in trial 2 was nearly twice as long as that of the discs from trial 1 across all four conditions. The addition of the *L. plantarum* starter to the wheat sourdough did not show differences in the shelf life of the empanada discs compared to those produced through spontaneous fermentation in both trials. However, spontaneous fermentation using wheat–amaranth flour increased the shelf life of the empanada discs by 3.77 times in trial 1 and 7.92 times in trial 2 compared to the non-fermented discs. For discs prepared with *L. plantarum* sourdough using wheat–amaranth flour, the shelf life increase was nearly double that of unfermented discs, but it was lower than that for discs prepared with spontaneous sourdough.

Table 2. Shelf life * of the refrigerated discs.

DISCS	Trial 1		Trial 2	
	Days of Storage	Relative Relation (X/Wnf)	Days of Storage	Relative Relation (X/Wnf)
Wnf	13	1	13	1
W	21	1.61	48	3.69
W Lp	22	1.69	40	3.08
WAnf	13	1	18	1.38
WA	49	3.77	103	7.92
WA Lp	25	1.92	53	4.08

* Shelf life was defined as the maximum period without the appearance of mould on the disc surface. X/Wnf: relation between the shelf life of the disc prepared with sourdough and the shelf life of the control disc prepared with sourdough in the same experiment. Non-fermented discs made of wheat flour (Wnf) or wheat–amaranth blend (WAnf). Discs made with wheat and wheat–amaranth sourdough obtained by natural fermentation (W and WA) or by addition of *L. plantarum* (W Lp and WA Lp).

The shelf life of non-fermented discs during refrigerated storage was used as an endpoint to compare the microbiological properties of dough prepared with and without sourdough technology (13 days). Initially, low levels of viable mesophiles and yeast were detected in the non-fermented discs, but these levels increased by 4 log cycles until visible mould was detected (Table 3).

Table 3. Viable microorganism count on discs expressed as log UFC/g and pH values at the beginning and after two weeks of refrigerated storage.

	AN		YGC		pH	
	Initial	Two Weeks	Initial	Two Weeks	Initial	Two Weeks
Wnf	3.68 ± 0.34^{A}	$7.88 \pm 0.43^{A,*}$	3.94 ± 0.69^{A}	$7.5 \pm 0.11^{A,*}$	5.40 ± 0.28^{A}	$4.29 \pm 0.36^{A,C}$
W	7.90 ± 0.09^{B}	$8.56 \pm 0.07^{B,*}$	3.26 ± 0.20^{A}	$5.38 \pm 0.09^{B,*}$	5.12 ± 0.16^{A}	$3.90 \pm 0.27^{A,B,*}$
W Lp	8.88 ± 0.16^{C}	$8.69 \pm 0.07^{B,D}$	3.91 ± 0.74^{A}	$6.19 \pm 0.28^{B,*}$	4.255 ± 0.26^{B}	3.52 ± 0.01^{B}
WAnf	3.49 ± 0.18^{A}	$6.96 \pm 0.20^{C,*}$	3.37 ± 0.18^{A}	$6.58 \pm 0.61^{A,*}$	5.03 ± 0.03^{A}	4.67 ± 0.10^{C}
WA	7.87 ± 0.01^{B}	8.14 ± 0.18^{A}	4.06 ± 0.47^{A}	3.68 ± 0.77^{C}	5.025 ± 0.05^{A}	$3.88 \pm 0.10^{A,B,*}$
WA Lp	8.82 ± 0.07^{C}	9.16 ± 0.02^{D}	3.24 ± 0.34^{A}	3.92 ± 0.04^{C}	3.99 ± 0.21^{B}	$3.72 \pm 0.27^{A,B}$

Different letters indicate statistically significant differences between discs on the same storage day (ANOVA, $p < 0.05$). Asterisks (*) indicate significant differences compared to the initial count for each condition (ANOVA, $p \leq 0.0001$).

In contrast, the fermented discs, whether they included *L. plantarum* as a starter or not, showed high levels of mesophiles at the initiation of storage, with no significant changes during storage, with the exception of wheat discs prepared from spontaneous

sourdough. Regarding yeast and fungal growth, an increase in viable yeast counts over the storage period was observed in the dough disc. However, discs made from sourdough with amaranth flour (WA and WA Lp) maintained low yeast counts during refrigerated storage. For trial 1, the total amount of mesophiles and yeast was determined in storage until the appearance of visible mould for each condition (Supplementary Figure S3). The pH values during refrigerated storage revealed that non-fermented discs made with wheat flour or flour blend started with initial pH values above 5 in both cases, which decreased over the course of refrigeration. Similar results were observed in the discs prepared with spontaneous sourdough made from wheat or wheat–amaranth flour, but those discs reached lower pH values (pH = 4) after two weeks of storage. Instead, discs made with sourdough inoculated with *L. plantarum* CIDCA 8327 started with a pH close to 4, which remained constant after two weeks of storage (Table 2). Acetic acid levels were lower than the detection limit at the beginning and at 14 days of storage. Lactic acid was detected only in discs prepared with sourdough with *L. plantarum* CIDCA 8327 at the beginning of storage, and the concentration was maintained after two weeks (5.42 ± 1.13 mM and 4.89 ± 0.95 mM for W Lp and WA Lp, respectively). Discs prepared with spontaneous sourdough reached similar lactic acid levels (4.53 ± 1.65 mM and 6.55 ± 1.10 mM for W and WA, respectively) after two weeks of storage.

The evolution of pH and total titratable acidity (TTA) was monitored throughout the entire storage period (Figure 7). The drop in the pH of discs prepared with spontaneous sourdough was more pronounced than the non-fermented discs. In contrast, discs prepared with the starter culture maintained their initial pH throughout storage. Titration acidity increases correlated with pH drop, but at the same pH level, the titratable acidity was higher in discs made from sourdough with wheat/amaranth blend flour. This difference may be attributed to the buffering capacity of amaranth proteins.

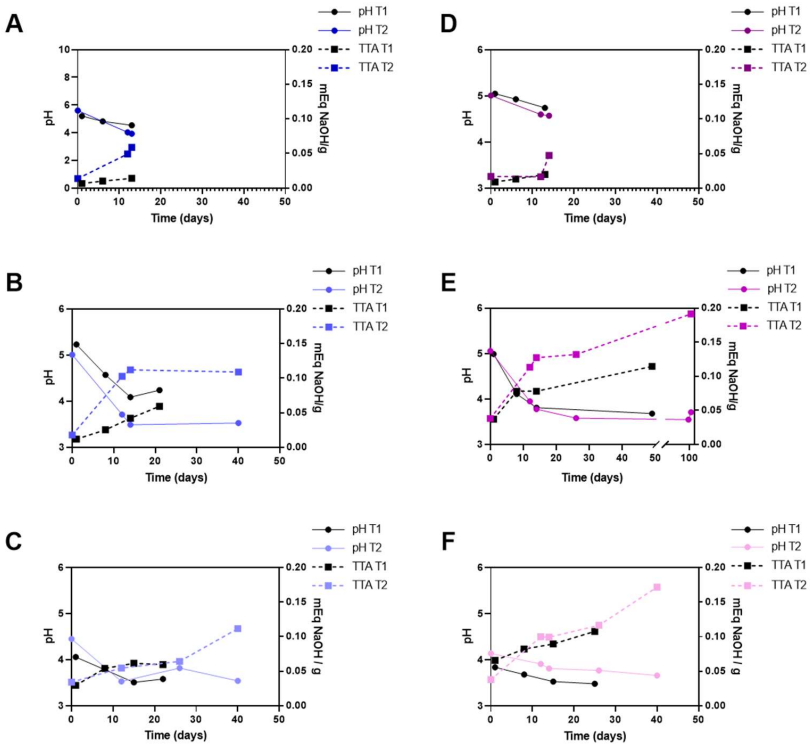


Figure 7. Evolution of pH and titratable acidity in the refrigerated discs. pH and titratable acidity of empanada discs during refrigerated storage prepared using the traditional method (**A**,**D**), with sourdough obtained by natural fermentation (**B**,**E**) or inoculated with a defined starter (**C**,**F**) using wheat or wheat-amaranth blend flour, respectively. T1 and T2 indicate each trial.

When comparing the changes in pH and titratable acidity between the discs made with the two wheat flour batches, a more significant decrease in pH and a greater increase in titratable acidity were observed in trial 2 for both wheat and wheat–amaranth discs subjected to spontaneous fermentation. However, these differences were not observed when *L. plantarum* was used as a starter culture.

4. Discussion

The present study demonstrated the feasibility of producing empanada dough discs using sourdough from wheat flour partially substituted with 7% amaranth flour, a pseudocereal known for its high protein content, balanced amino acid profile, and presence of bioactive compounds [41,43,44,53,54]. This is the first study to demonstrate that amaranth-enriched sourdough improves the shelf life of empanada discs during refrigerated storage through the inhibition of yeast growth.

Empanada dough discs are typically elaborated by mixing the ingredients and preserved using chemical additives such as propionic acid, sorbic acid, and their derivatives, as authorized by the Argentine Food Code https://www.argentina.gob.ar/anmat/codigoalimentario (accessed on 23 April 2024) (Chapter IX, Article 722). The inclusion of a pre-fermentation step (Type II sourdough) in the elaboration of empanada discs has contributed to an improvement in the shelf life of the final product (Table 2). Consequently, the results of the present work indicate that empanada prepared with sourdough is an alternative suitable for the growing consumer demand for preservative-free foods [58]. Even though the sourdough application delayed the visual appearance of mould on dough discs during storage, the effect depended on the sourdough microbiome, which is determined by the batch of flour used, the inclusion of *L. plantarum* as a starter, and the contribution of amaranth microbiota.

The sourdough microbiome is a complex ecosystem that depends on several variables, such as temperature of fermentation, type and composition of flour, and flour microbiota, among others [9,10,59]. For this reason, the reproducibility of spontaneous sourdoughs is low, and consequently, the product that will be generated is not constant, as was evident in the shelf life observed in discs prepared with sourdough, whether spontaneous or inoculated [26,60]. Spoilage occurred earlier in trial 1 compared to trial 2, with the shelf life nearly doubling in the latter, which may be due to variances in the initial microbiota of the batches of flour, changes in the quantities and qualities of the composition of flour, and/or different microbial growth factors or inhibitors that affect microbial consortia evolution during the fermentation process [15]. Sequencing of spontaneous sourdoughs prepared with wheat or wheat–amaranth flour and fermented for 24 h showed differences in bacterial composition between trials (Figure 5). *Bacillus* and formerly *Lactobacillus* genera [32] were the most represented in each trial and are frequently found in sourdough [59]. Among the observed microbial differences, sourdough made with wheat flour from batch 1 exhibited higher alpha diversity (Shannon index), a greater relative abundance of *Lactobacillus*, and a lower proportion of *Bacillus* species compared to batch 2. Some *Bacillus* species can produce diverse antimicrobial and bioactive compounds [8,45,61]. This fact may be ascribed to the extended shelf life of the empanada disc prepared in trial 2. Moreover, sourdough microbiota differs between both trials in other genera, whose presence/absence can induce metabolic changes in the sourdough that may lead to changes in shelf life. These microbiota differences were not reflected in physicochemical parameters such as the final pH or total titratable acidity (TTA) of sourdoughs. However, they were clearly associated with variations in the shelf life of the refrigerated sourdough discs. Yeast counts before 24 h of fermentation were low across all samples, and DGGE profiles revealed limited yeast diversity, with clear differences observed between trials (Supplementary Table S2 and Figure S3). In

trial 1, the genera *Candida*/*Yamadazyma* were detected during all the stages of fermentation. In contrast, trial 2 showed the presence of *Fusarium* in the early stages of fermentation. However, the *Fusarium* band disappeared over time, and a band corresponding to *Candida*/*Yamadazyma* appeared at 24 h—exclusively in the sourdough supplemented with amaranth flour with or without starter culture. These differences may be influenced not only by the flour composition but also by microbial interactions during fermentation. Previous studies have identified specific associations between lactic acid bacteria and yeasts, which could play a key role in shaping the dynamics of the microbial community and may help explain the observed shifts in yeast populations between trials [15,54,62–64].

The most used way to improve the stability of sourdough is the addition of single or mixed starter cultures, usually autochthonous or allochthonous lactic acid bacteria [65]. *L. plantarum* is one of the LAB species that is found in sourdoughs and is frequently selected as a starter culture because of its capability to ferment a wide range of carbohydrates [23,31,32,34]. The DGGE dendrogram showed that *L. plantarum* CIDCA 8327 modified the sourdough microbiome, homogenizing the wheat and wheat–amaranth bacterial profile after 24 h of fermentation at 30 °C (Figure 4). A considerable pH drop (Figure 2) was observed along with an increment of lactic acid production, whether in the case of sourdough prepared from wheat flour or a blend. Those are desirable parameters when a culture starter is chosen since the ability to diminish pH, produce organic acids, and antifungal metabolites are important to improve the stability and reproducibility of sourdough [65]. The addition of *L. plantarum* CIDCA 8327 delayed the appearance of visible fungal growth in the discs compared with non-fermented discs, regardless of whether wheat or wheat–amaranth flour was used. Microbial counts and pH values of the discs were comparable to those obtained with spontaneous sourdough (Table 3 and Figure 7). Empanada discs made with sourdough prepared with wheat flour by natural fermentation or by *L. plantarum* addition showed the same shelf life. Nevertheless, visible fungal growth appeared earlier in the starter-fermented wheat–amaranth discs compared to their spontaneously fermented counterparts. It is important to note that, after sourdough fermentation, additional ingredients such as oil, salt, and fresh flour were incorporated during disc preparation. These changes altered the environmental conditions and increased microbial diversity, potentially reintroducing competition within the dough microbiota during refrigerated storage. This suggests that the competition between *L. plantarum* and the native wheat–amaranth microbiota results in earlier fungal spoilage.

Beyond the differences observed in both trials mentioned previously, supplementation with amaranth flour modified the spontaneous sourdough microbiota, and discs prepared with it were the ones with the most an extended shelf life during refrigeration (Table 2 and Figure 6). A recent bibliography described antimicrobial and antiviral effects of some *Amaranthus* species, which were obtained from ethanol/methanol extract, seed oil, and betacyanin fraction [66]. Moreover, Moyer et al. identified a peptide rich in proline with an antimicrobial effect, and Guo et al. elucidated the mechanisms involved in the antibacterial activity of *A. tricolor* against *S. aureus* [67,68]. Lactic acid bacteria during fermentation can produce metabolites such as organic and phenolic acids that inhibit mould growth [24,69], and some *Bacillus subtilis* present antifungal properties against *Fusarium oxysporum* [70] that may contribute to the extended shelf life. In addition, it has been demonstrated that some bacteria belonging to the *Lactobacillus* and *Bacillus* genera are capable of digesting amaranth proteins and producing peptides with biological activity [45]. The addition of amaranth flour to the formulation of empanada discs without prior fermentation did not alter their shelf life during refrigerated storage; however, empanada discs formulated with wheat–amaranth sourdough had an extended shelf life in both assays. This indicates that fermentation plays an important role, either due to the establishment of a different

microbial consortium and its fermentation products, by activation of amaranth's own antimicrobial components, or by both factors together.

Future research, such as a deep study of metabolic profiles as well as the search for a consolidated stable consortium of isolated microorganisms, will shed light on determining the factors responsible for shelf life incrementation and inhibition of yeast growth during refrigerated storage. In this sense, the use of amaranth might represent an alternative to food preservatives used currently, a new property that could be added to the numerous benefits of amaranth in terms of nutritional value and the health benefits already known.

5. Conclusions

This research demonstrates that incorporating a pre-fermentation step, specifically using Type II sourdough, in the preparation of empanada discs can enhance the shelf life of the final product. The dough discs made with Type II sourdough, whether through spontaneous fermentation or by using *L. plantarum* CIDCA 8327 as a starter culture, effectively had a delayed appearance of visible mould during refrigeration. This method presents a promising alternative for "clean-label" products. However, the effectiveness of this approach depends on the sourdough microbiome, which varies based on the specific batch of flour used, the inclusion of *L. plantarum* as a starter, and the contribution of amaranth microbiota. It was observed that the microbiota from spontaneous fermentation differed between flour batches. Although these differences did not lead to significant changes in pH or total titratable acidity (TTA) of the sourdough, they were associated with variations in the shelf life and TTA of the refrigerated discs. Discs prepared with spontaneous sourdough and 7% amaranth flour demonstrated the longest shelf life before spoilage and exhibited lower yeast and mould counts compared to traditionally prepared discs. In these doughs, fermentation was identified as a crucial factor in extending the shelf life of empanada discs made from wheat–amaranth flour, as the addition of amaranth flour alone did not significantly impact shelf life during refrigeration. While the use of *L. plantarum* as a starter culture dominated the sourdough microbiota, the shelf life of wheat dough discs was similar to those prepared with natural sourdough. Interestingly, visible fungal growth appeared earlier in the discs fermented with the starter culture compared to those made through spontaneous fermentation, indicating that the prevalence of the starter limited the growth or activity of microbiota present in wheat–amaranth sourdough obtained through natural fermentation. The addition of a small amount of amaranth flour, combined with sourdough fermentation, effectively delays spoilage and inhibits yeast growth during the refrigerated storage of empanada discs. This finding opens new inquiries for research into the contributions of amaranth microbiota to sourdough. Overall, the findings of this study suggest that empanadas made with sourdough are a viable alternative for consumers seeking additive-free foods.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation12020080/s1, Table S1: Percentual composition of wheat flour (batches 1 and 2), amaranth flour, and amaranth–wheat blend; Table S2: Enumeration of viable microorganisms in sourdoughs after 24 h fermentation at 30 °C; Figure S1: Evolution of pH and titratable acidity in sourdoughs; Figure S2: Alpha diversity of bacterial communities in spontaneous sourdough after 24 h of fermentation; Figure S3: Microbial counts in empanada discs during storage (trial 1).

Author Contributions: Conceptualization, M.C.A. and A.G.A.; methodology, C.D. and E.B.; validation, C.D., E.B., M.C.A. and A.G.A.; investigation, C.D., E.B., M.C.A. and A.G.A.; data curation, C.D. and E.B.; writing—original draft preparation, C.D. and E.B.; writing—review and editing, C.D., M.C.A. and A.G.A.; supervision, M.C.A. and A.G.A.; project administration, A.G.A.; funding acquisition, A.G.A. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

Wnf	non-fermented discs made with wheat flour
WAnf	non-fermented discs made with wheat and amaranth flour
W	sourdough or discs made with wheat flour
WA	sourdough or discs made with wheat flour wheat and amaranth flour
W Lp	sourdough or discs made with wheat flour and the starter culture *L. plantarum* CIDCA 8327
WA Lp	sourdough or discs made with wheat flour, amaranth flour, and the starter culture *L. plantarum* CIDCA 8327

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