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Physicochemical, Biochemical, Microbiological, and Sensory Characterization of a Fermented Lima Bean (*Phaseolus lunatus*) Probiotic Beverage During Refrigerated Storage

Hassina Bilek [1] ✉ Ana Lúcia Oliveira [2] Manuela Amorim [2] Ana Raquel Madureira [2] Farid Zaid [1] Maria Manuela Pintado [2]

[1] Département des Sciences Alimentaires, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Route Targa Ouzemour, Bejaia 06000, Algeria hassina.bilek@univ-bejaia.dz idiazdiraf@yahoo.fr

[2] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, 172, 4200-374 Porto, Portugal asoliveira@ucp.pt / mamorim@porto.ucp.pt / rmadureira@ucp.pt / mpintado@ucp.pt

ABSTRACT

Background: Growing public health concerns regarding the consumption of conventional dairy products, coupled with the increasing prevalence of plant-based and vegan dietary preferences, have stimulated considerable scientific interest in the development of innovative fermented legume-based beverages. The lima bean (*Phaseolus lunatus*) represents a particularly promising raw material in this context, due to its high protein content, the presence of prebiotic α-galactosides, and its notable endogenous antioxidant compounds.

Aims: This study aimed to evaluate the probiotic viability, physicochemical and biochemical properties, sensory characteristics, and antioxidant potential of fermented lima bean-based beverages subjected to refrigerated storage at 4°C for 28 days.

Material and Methods: Four lima bean-based beverages (Lbs) were formulated by incorporating a 1% inoculum of either the single-strain probiotic *Lactobacillus acidophilus* La5 (LbLa5), *Bifidobacterium animalis* subsp. *lactis* Bb12 (LbBb12), a co-culture of both strains (Lb coculture), or no bacterial inoculum, serving as a non-fermented control beverage.

Results: The probiotic Lbs exhibited a high crude protein content (3.15% – 3.34%) comparable to that of soy-based and bovine milk, and were characterized by a substantial carbohydrate fraction (2.08% – 2.32%). Viable counts of *L. acidophilus* La5 and *B. lactis* Bb12 were maintained at 6 – 7 log CFU/mL throughout the shelf-life period, with *B. lactis* Bb12 demonstrating enhanced survival under co-culture conditions. High-performance liquid chromatography (HPLC) analysis revealed significant reduction in the prebiotic oligosaccharide raffinose throughout storage with final concentrations ranging from 2.17 to 3.35 g/L. Probiotic metabolic activity induced a progressive decline in pH (4.00 – 4.30) accompanied by an increase in titratable acidity (0.70 – 0.89%). Chromatographic profiles demonstrated that both selected strains were proteolytically active during cold storage, resulting in a reduction of free amino acid content and the generation of bioactive low-molecular-weight peptides (≤ 10 kDa). Fermentation promoted an increase in total phenolic compound (TPC) content over the first seven days, whilst DPPH radical scavenging activity increased over the initial three weeks of storage. The co-culture treatment yielded the highest TPC content (1.71 – 2.03 mg GAE/mL) and DPPH radical scavenging activity (0.86 – 1.52 mg TE/mL) throughout the entire storage period. Sensory flavor attributes and overall acceptability were enhanced across all fermented beverage formulations relative to the non-fermented control.

Conclusions: The incorporation of *L. acidophilus* La5 and *B. lactis* Bb12 into lima bean-based beverages yields a nutritionally dense, functionally enriched, and sensorially acceptable plant-based alternative well-suited to vegan and health-conscious dietary regimens. These findings support the valorization of *Phaseolus lunatus* as a viable substrate for the development of probiotic fermented beverages.

Keywords: Lima Bean-Based Beverage; Probiotics; Raffinose; Organic Acids; Free Amino Acids; Bioactive Peptides; Antioxidant Capacity.

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✉ **Corresponding authors:** Hassina Bilek
E-mail : hassina.bilek@univ-bejaia.dz
Tel. +213 (554 341 621)

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1 INTRODUCTION

Over recent years, the demand for plant-based functional foods has increased steadily , driven by rising incidences of lactose intolerance and milk protein allergies, the increasing prevalence of vegetarian diets, and the scarcity of milk supply in developing nations (Demarinis *et al.*, 2022). This expanding market primarily comprises food formulations designed to promote gut health, including probiotics, prebiotics, and symbiotic (Cichońska & Ziarno, 2022).

Legumes represent an ideal matrix for the development of vegan food products due to their high protein content, which satisfies rigorous functional and nutritional standards while maintaining a low glycemic index (Erem & Kilic-Akyilmaz, 2024). However, consumer acceptance of legume-based

products is often hindered by undesirable flavor profiles (beany off-flavors) and the gastrointestinal discomfort induced by indigestible oligosaccharides, such as raffinose (Demarinis *et al.*, 2022). Therefore, probiotic fermentation has emerged as a viable strategy to overcome these limitations. Fermentation processes can significantly enhance the functional properties, nutritional profile, and sensory attributes of the final products (Deziderio *et al.*, 2023).

Novel vegan food formulations frequently incorporate microbial strains belonging to the genera *Lactobacillus* and *Bifidobacterium*, exploiting the innate bifidogenic properties of plant-derived oligosaccharides (Angélica Andrade Lopes *et al.*, 2020). Furthermore, these strains confer well-documented health benefits, including pathogen inhibition, gut microbiota modulation, immune system modulation, and enhanced antioxidant activity (Angélica Andrade Lopes *et al.*, 2020; Cichońska & Ziarno, 2022; Erem & Kilic-Akyilmaz, 2024).

The lima bean (*Phaseolus lunatus*) is a highly promising source for the development of fermented non-dairy beverages owing to its rich nutritional profile and abundant bioactive compounds. Lima beans offer a substantial yield of high-quality protein (14 – 36%) and serve as a rich source of essential amino acids, minerals, B vitamins, dietary fiber, antioxidants, and diverse biologically active constituents (Adebo, 2023). The seeds contain significant concentrations of α-galactosides, which function as prebiotics to stimulate the proliferation of *lactobacilli* and *bifidobacteria* (Chukwu & Onuh, 2020). Previous studies have indicated that lima bean flour and milk represent appropriate substrates for probiotic cultivation (Chukwu & Onuh, 2020). However, to the best of our knowledge, no prior studies have evaluated the specific incorporation of *L. acidophilus* La5 and *B. lactis* Bb12 probiotic cultures into a lima bean milk matrix.

Therefore, the objective of the present study was to develop a probiotic, lima bean-based beverage and to evaluate the changes in cell viability, carbohydrate metabolism, organic acid production, amino acid composition, peptide molecular weight profiles, DPPH radical scavenging capacity, total phenolic content, and sensory acceptability of the final products during a 28-day refrigerated storage period at 4 °C.

2 METHODS

2.1 Materials

Mature *Phaseolus lunatus* seeds were harvested in Souk-El Thenine in the Bejaia region of Algeria. The commercial starter cultures utilized in this research, specifically *L. acidophilus* strain La5 and *B. animalis* ssp. *lactis* strain Bb12 were produced from Chr. Hansen (HØrsholm, Denmark).

2.2 Preparation of Lima Bean Milk

The seeds were manually cleaned to remove impurities, milled employing a SAYONA Ratissier SZJ-12 grinder (China), and electrically sieved (Relesch-AS200) to achieve a particle size of ≤ 0.5 mm. The resulting flour was dispersed in deionized water at a concentration of 10% (m/v) and homogenized using an ULTRA Turrax (T18 Basic; IKA Works, Inc, USA) for 3 min at 18,000 rpm. The resulting slurry was boiled for five minutes to inactivate heat-labile antinutritional factors, particularly lectins and protease inhibitors. Following thermal treatment, the mixture was filtered through a sterile, fine muslin cloth to remove insoluble debris. The collected filtrate (lima bean milk) was immediately utilized for beverage preparation.

2.3 Chemical Composition of Lima Milk Determination

The proximate composition (protein, fat, ash, and moisture) of the lima milk was determined in accordance with standard AOAC protocols (AOAC, 1990). Briefly, crude protein was quantified utilizing the Kjeldahl method, applying a nitrogen-to-protein conversion factor of 6.25. Crude fat was extracted via a Soxhlet apparatus employing petroleum ether as the solvent. Moisture content was determined gravimetrically by drying a specified sample mass in a hot-air oven at 105 °C until a constant weight was achieved. Ash content was measured by incinerating the samples in a muffle furnace at 550 °C to a constant mass. Total carbohydrate content was calculated by difference, subtracting the cumulative percentages of moisture, crude protein, crude fat, and ash from 100. All analyses were conducted in triplicate.

2.4 Preparation of Lima Beverage (Lbs)

Fresh Lima bean milk was pasteurized at 90°C for 15 minutes (Chukwu & Onuh, 2020) to ensure microbiological safety and inactivate endogenous enzymes. The milk was subsequently cooled and aseptically partitioned into equal volumes within sterile flasks for the preparation of probiotic beverages. Four distinct beverage formulations (Lbs) were established employing the pasteurized milk as a base: (1) fermentation with *L. acidophilus* La5(LbLa5), (2) fermentation with *B. lactis* Bb12 (LbBb12), (3) fermentation with a coculture of both strains (Lbcoculture), and (4) an unfermented control sample.

Fermentation was conducted in duplicate (two independent biological lots per treatment) utilizing 40 mL aliquots. Samples were incubated in a water bath at 37°C for 10 – 18 hours until reaching a target pH value of approximately 4.5. Following fermentation, the beverages were immediately cooled and stored at 4 ± 1°C for an observation period of 28 days. Changes in microbial viability,

pH, TAA%, proximate composition, organic acids, residual carbohydrates, total phenolic compounds (TPC), free amino acids (FAA) contents, peptides molecular weight profiles, DPPH radical scavenging activity, and sensory attributes were systematically monitored at the end of fermentation (Day 0) and at weekly intervals during cold storage (Days 7, 14, 21, and 28). To ensure data consistency, precision, and reproducibility, all analytical determinations were performed in triplicate.

2.5 pH and Titratable Acidity (TAA %) Measurement

The pH was measured at ambient temperature employing a digital pH meter (Crison Micro pH 2002, Spain) equipped with a standard glass electrode. Titratable acidity was determined by titrating 5 mL against a 0.01 N standardized sodium hydroxide (NaOH) solution, with results expressed as grams of lactic acid equivalents per 100 mL of sample.

2.6 Determination of Viable Cell Counts

The viable cell counts of La-5 and BB-12 strains in the fermented beverages during storage were enumerated in accordance with the method proposed by Miles & Misra, (1931). Decimal serial dilutions of each fermented drink were plated on MRS-agar incubated at 24 h at 37 °C in aerobic (*L. acidophilus*) or on MRS-agar with 5% Cysteine-HCl at 48h in anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) (for B. lactis or the mixture), using a Whitley DG250 anaerobic chamber (West Yorkshire, UK). The first dilution used 10g in 100ml of citrate solution to guarantee a representative sample and subject to stomacher (Model 400 Circulator, Seward, Norfolk, England) homogenization during 3 min at 260 rpm. The count results were expressed in log CFU. mL^{-1} of each fermented sample.

To achieve differential enumeration of *Lactobacillus* and *Bifidobacterium* within the co-cultured beverages, MRS agar supplemented with bromophenol blue (MRS-BPB) was employed as a differential medium (Lee & Lee, 2008). Under anaerobic conditions on this medium, *Lactobacillus* colonies exhibit a light blue coloration, whereas *Bifidobacterium* species grow as distinct, smaller colonies with a dark blue morphology.

2.7 Organic Acids and Carbohydrate Analysis

The quantification of carbohydrate and organic acids was carried out according to the method described by Zeppa *et al.* (2001). Each supernatant extract was filtered through a 0.45 µm membrane filter and, immediately prior to injection, through a 0.22 µm filter (Orange Scientific, Belgium). Separation of organic acids and carbohydrates was performed utilizing an Aminex HPX-87H column (BioRad, Hercules, CA, USA). The mobile phase consisted of 0.003 M H_2SO_4 at a flow rate of 0.6 $mL.min^{-1}$, with column temperatures maintained at 65 °C and 40 °C for organic acids and carbohydrates, respectively. Individual compounds were quantified by comparing their retention times with those of authentic standards (Sigma Aldrich®), including succinic, acetic, and lactic acids, as well as glucose, fructose, sucrose, and raffinose, over a concentration range of 0.1 to 5 $g.L^{-1}$.

2.8 Determination of Peptide Molecular Weights by FPLC

The molecular weight (MW) of peptides was determined employing fast protein liquid chromatography–gel filtration (FPLC) on an AKTA Pure 25 system. The analysis utilized two columns in series (Superdex 200 Increase 10/300 GL and Superdex Peptide 10/300 GL) and a buffer solution consisting of 25 mM phosphate buffer (pH 7.0), 150 mM NaCl, and 0.2 g/L sodium azide. The flow rate was set to 0.5 $ml.min^{-1}$, and UV detection was performed at 280 nm. A calibration curve was constructed using standard polypeptide markers, plotting log MW against elution volume.

2.9 Determination of Free Amino Acids Composition

The free amino acid (FAA) profile of each hydrolyzed sample (10 mg) was determined following the procedure of Pripis-nicolau *et al.* (2000). Analyses were performed employing a high-performance liquid chromatography system (Waters Series 2695. Milford, MA, USA) equipped with a Merck column (4.6 x 100 mm, Chromolith® Performance RP18). Fluorescence detection was carried out at excitation and emission wavelengths of 356 nm and 445 nm, respectively utilizing a Jasco-821-FP detector. The mobile phase flow rate was 0.80 $mL.min^{-1}$, and the column temperature was maintained at 60°C for 125 min. Homoserine and norvaline served as internal standards. Amino acid composition was calculated as a percentage based on the peak areas of standard amino acids.

2.10 Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) was measured using the Folin–Ciocalteu method, with gallic acid as the reference standard (Singleton & Rossi, 1965). Absorbance was recorded at 750 nm employing a spectrophotometer (UV mini-1240, Shimadzu, Tokyo, Japan). Results were expressed as milligrams of gallic acid equivalents per milliliter of beverage sample (mg $GAE.mL^{-1}$).

2.11 Determination of Radical Scavenging Activity (DPPH-assay)

The DPPH free radical scavenging activity of the prepared beverages was evaluated according to the method of Alexandre *et al.* (2018), with minor modifications. Briefly, an aliquot of 0.025 mL of diluted beverage (1:1, v/v) was mixed

with 0.975 mL of freshly prepared DPPH• solution (60 µM) in methanol. The mixture was incubated in the dark at 20 ◦C for 20 min, after which absorbance was measured at 515 nm utilizing a spectrophotometer (UV mini-1240, Shimadzu, Tokyo, Japan). Results were expressed as micromoles of Trolox equivalents per milliliter of beverage (µmol TE. mL^{-1}), based on a Trolox calibration curve.

2.12 Sensorial Analysis

Sensory evaluation of the probiotic drinks was conducted after 14 days of refrigerated storage, a time frame considered representative of typical consumption conditions. A panel of sixty randomly selected tasters comprising students and staff from the Escola Superior de Biotecnologia (ESB) – Universidade Católica Portuguesa (UCP), participated in the assessment. Overall liking was rated employing a nine-point hedonic scale ranging from 1 ("dislike very much") to 5 ("neither like nor dislike") and 9 ("like very much")(Peryam & Pilgrim, 1957). Additionally, panelists evaluated sweetness, acidity, and flavor intensity utilizing a five-point Just-About-Right (JAR) scale, where 1 corresponded to "much too weak," 3 to "just about right," and 5 to "much too strong" (Li *et al.*, 2014).

2.13 Statistical Analysis

Data analyses were performed employing Statistical software version 5.5 (StatSoft, Inc., Tulsa, USA) and XLSTAT version 2013.2.05 (Addinsoft, New York, NY, USA). All assays were conducted in triplicate (n = 3 analytical replicates per lot). Data were analyzed through one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to identify significant differences at a threshold of $p < 0.05$. Results are expressed as means ± standard deviation (SD).

3 Results & Discussion

3.1 Chemical Composition of the Lima Bean-Based Beverages (Lbs)

Table 1 outlines the chemical composition of the formulated Lbs samples. The nutritional profiles obtained were consistent with previous literature reports concerning lima bean, soy, and alternative plant-based matrices (Chukwu & Onuh, 2020). Fermentation induced a significant increase in crude protein content, which may be attributed to the proliferation of microbial biomass or to specific anabolic pathways facilitating the synthesis of proteinaceous components (Emkani *et al.*, 2022). The resulting macronutrient profile characterizes these beverages as having a well-balanced composition.

Conversely, the observed reduction in other nutrients (fat, fiber, ash, and total carbohydrate) during the fermentation process may attributed to their leaching into the aqueous medium or their catabolism by the starter cultures via hydrolytic enzyme activities. Comparable dynamics have previously been reported in fermented beverages formulated from other legumes, namely common bean, soy, lupin, and cowpea (Chukwu & Onuh, 2020; Cichońska & Ziarno, 2022). Conclusively, lima bean milk represents a robust source of vegetable protein and digestible carbohydrates that can be strategically exploited in functional food design.

3.2 Acidification During Refrigerated Storage

Figures 1c and 1d illustrate the temporal variations in pH and titratable acidity (TAA%) within the Lbs drinks over the storage period. The mean pH values of all fermented Lbs decreased significantly ($p < 0.05$) during the 28-day storage period under refrigeration conditions (4 °C), whereas the control beverage maintained stable values. As anticipated, the TAA profiles exhibited an inverse trend to the pH measurements. Figure 1c highlights significant differences ($p < 0.05$) in acidity profiles among the probiotic strains utilized for fermentation. Furthermore, the elevated TAA% values observed in the coculture indicate intensive growth and pronounced metabolic activity of probiotic bacteria.

The concomitant drop in pH and elevation of TAA during storage are primarily driven by the post-acidification activity of *L. acidophilus* La5 and *B. lactis* Bb12. This metabolic activity leads to a progressive accumulation of organic acids within the matrix, even under low-temperature conditions (4 °C) (Cui *et al.*, 2021). Consistent with the present findings, higher acidity levels have been reported when utilizing mixed cultures of lactic acid bacteria and *bifidobacteria* compared to monocultures fermented with *L. acidophilus* alone (Bujna *et al.*, 2018; Cui *et al.*, 2021).

Table 1. Chemical Composition of Lima beverage samples (g.100 mL^{-1})

	Beverage samples			
Parameters	**Lbcontrol**	**LbLa5**	**LbBb12**	**Lbcoculture**
Moisture	92.61 ± 0.10^{c}	93.42 ± 0.08^{b}	93.17 ± 0.09^{a}	93.31 ± 0.11^{ab}
Ash	0.45 ± 0.03^{b}	0.35 ± 0.01^{a}	0.36 ± 0.04^{a}	0.33 ± 0.02^{a}
Fat	0.65 ± 0.03^{c}	0.50 ± 0.01^{a}	0.57 ± 0.04^{b}	0.49 ± 0.03^{a}
Protein	2.95 ± 0.02^{b}	3.19 ± 0.01^{a}	3.15 ± 0.02^{a}	3.34 ± 0.02^{c}
Fiber	0.82 ± 0.01^{b}	0.45 ± 0.01^{a}	0.43 ± 0.00^{a}	0.40 ± 0.01^{a}
Carbohydrates	2.52 ± 0.04^{b}	2.08 ± 0.02^{a}	2.32 ± 0.03^{b}	2.13 ± 0.00^{a}

Note: Values are means ± standard deviation of three analytical replicates from two independent experimental lots per treatment. Different superscript letters (a, b, c) within the same row indicate significant differences at $p \leq 0.05$; means sharing the same letter are not significantly different (one-way ANOVA followed by Tukey's test).

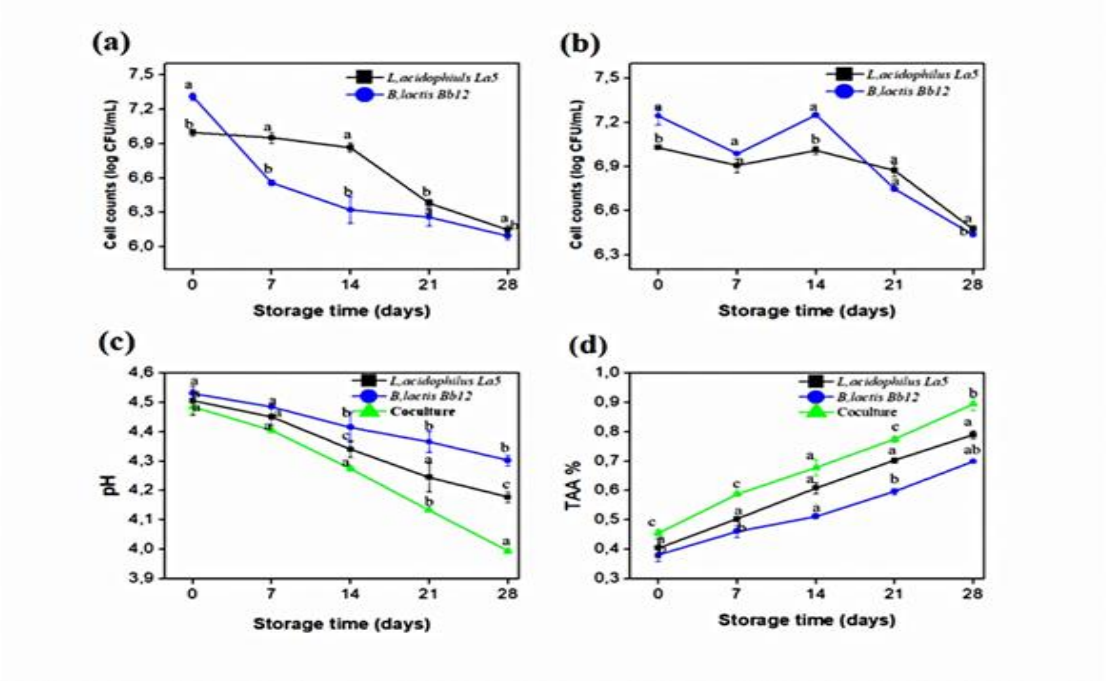

Figure 1. Changes in the Viable Cell Counts (log CFU.mL^{-1})
in Monocultures (a) and in Combined Cultures (b), in Titratable Acidity (c) and pH Values (d) of the Fermented Lima Beverages During Refrigeration Period of 28 Days

Notably, excessive over-acidification did not occur during cold storage, thereby preserving both the viability of probiotic strains and the organoleptic attributes of the fermented beverages (Demarinis *et al.*, 2022). Overall, the acidity profiles adheres to the standard criteria established for traditional fermented milks and commercial fermented plant-based counterparts (Cichońska & Ziarno, 2022; Deziderio *et al.*, 2023), confirming the efficacy of the fermentation process within these legume-based matrices.

3.3 Viability of Probiotic Bacteria During Beverage Storage

Figure 1a and 1b depict the variations in viable cell counts within the fermented Lbs over the 28-day refrigerated storage interval. The data indicate that the probiotic cell counts for all fermented variants remained well within the therapeutic threshold (≥ 10^6 CFU.mL^{-1}) throughout the entire storage duration (Cichońska *et al.*, 2023). This demonstrates that lima bean milk, even in the absence of exogenous nutrient supplementation, serves as an effective vehicle for probiotic delivery. This high suitability is likely mediated by the intrinsic presence of oligosaccharides and accessible nitrogen sources required to sustain probiotic proliferation. Furthermore, endogenous polyphenols may stimulate ùicrobial growth due to their prebiotic-like properties (Piekarska-Radzik & Klewicka, 2021). Statistical analysis further revealed that survivability of *B. lactis* Bb12 was significantly enhanced ($p < 0.05$) upon co-inoculation with *L. acidophilus* La5, validating the stimulatory effects of the latter strain during the initial 14 days of storage.

However, a progressive decline in total viable counts was observed over extended storage, which is likely attributable to the depletion of carbohydrates in the medium, advanced proteolysis disrupting the matrix equilibrium, elevated acidity, and the accumulation of growth-inhibitory metabolic byproducts (Arab *et al.*, 2022; Li *et al.*, 2019). Similar survival trend for *L. acidophilus* and *B. lactis* strains have been documented in various plant-based milk and cheese alternatives (Chaves & Gigante, 2016; Deziderio *et al.*, 2023).

Throughout the storage period, *L. acidophilus* La5 maintained a higher viable count than *B. lactis* Bb12 in the monoculture treatments, indicating that psychrotolerance and growth kinetics within plant-based milk are strain-dependent. In agreement with these observations, Ozturkoglu-Budak *et al.* (2019) reported superior viability of the La5 relative to Bb12 during prolonged storage. The enhanced persistence of Bb12 in the coculture system may be attributed to the strain's competitive behavior for carbon sources and its metabolic interaction with La5, which generates bifidogenic amino acids and low-molecular-weight peptides (Arab *et al.*, 2022; Demarinis *et al.*, 2022).

Comparable synergistic growth improvements have been reported in fermented fruit juices and soy-based yogurt alternative formulated with both genera (Cichońska *et al.*, 2023; Cui *et al.*, 2021), suggesting a robust mutualistic symbiosis between the two strains.

Incorporating *L. acidophilus* La5 and *B. lactis* Bb12 into a lima bean milk matrix directly supports the sustainable utilization and valorization of this agricultural resource. Consequently, processing parameters should be optimized to in future production scales to maximize microbial viability and metabolic functionality.

3.4 Carbohydrate and Organic Acid Dynamics

Table 2 details the chronological shifts in the concentrations of free carbohydrates (raffinose, glucose, and galactose) and organic acids (lactic and acetic acids) during storage. High-performance liquid chromatography (HPLC) analysis revealed that raffinose content in the fermented Lbs decreased significantly ($p < 0.05$) following two weeks of storage. Meanwhile, monosaccharide levels (glucose and galactose) expanded during the first week—a phenomenon driven by the enzymatic hydrolysis of raffinose—before progressively declining thereafter. Conversely, the unfermented control sample exhibited a stable carbohydrate profile throughout the evaluation period. Fructose remained undetected post-fermentation, indicating its preferential and rapid catabolism by the starter cultures as a primary carbon source.

The residual carbohydrate content confirms the enzymatic activity of alpha-galactosidase (raffinose galactohydrolase) within the La5 and Bb12 strains evaluated (Schöpping *et al.*, 2021). The catabolism of raffinose varied significantly depending on the inoculated culture, with the co-culture matrix demonstrating a superior capacity for oligosaccharide consumption. These observations concur with previous reports detailing the metabolic degradation of raffinose during the storage of plant-based milk alternatives (Cichońska *et al.*, 2023). In contrast, Ziarno *et al.*(2019) reported that oligosaccharide breakdown was inhibited during the refrigerated storage of kidney bean-based beverages. Such discrepancies can be attributed to strain-specific probiotic behaior, distinct carbohydrate consumption kinetics under cold stress, and the structural complexity of the food matrix (Ozturkoglu-Budak *et al.*, 2019). The reduction in raffinose content reduces a primary flatulence-inducing factor, thereby enhancing the overall consumer acceptability and functionality of the legume-based beverage.

Table 2. Changes in Viable Cell Counts (log CFU. mL^{-1}) of Individual *L. acidophilus* La5 and *B. animalis* Bb12 and of their Combination pH, titratable acidity (% lactic acid), acetic acid, lactic acid, raffinose family oligosaccharides (RFOs), galactose and glucose (g.L^{-1}) content compared to control during 28 days of refrigerated storage at 4°C

Storage time(days)	LbLa5	LbBb12	Lb coculture	Lb control
Acetic Acid (g.L^{-1})				
0	ND	2.13 ± 0.02^{A,a}	3.09 ± 0.07^{A,b}	ND
7	ND	2.45 ± 0.04^{A,a}	3.30 ± 0.03^{A,b}	ND
14	ND	3.34 ± 0.01^{B,a}	3.60 ± 0.03^{B,a}	ND
21	ND	3.80 ± 0.01^{B,b}	4.13 ± 0.01^{C,c}	ND
28	ND	4.04 ± 0.02^{C,b}	4.62 ± 0.08^{C,c}	ND
Lactic Acid (g.L^{-1})				
0	3.85 ± 0.18^{A,b}	1.64 ± 0.04^{A,a}	4.05 ± 0.02^{A,b}	ND
7	3.90 ± 0.06^{A,b}	1.78 ± 0.01^{A,a}	4.46 ± 0.15^{AB,b}	ND
14	5.04 ± 0.28^{B,b}	2.08 ± 0.04^{B,a}	4.99 ± 0.36^{B,b}	ND
21	5.57 ± 0.01^{C,b}	2.60 ± 0.00^{C,a}	8.39 ± 0.68^{C,c}	ND
28	6.48 ± 0.05^{C,b}	3.23 ± 0.02^{D,a}	11.83 ± 0.72^{D,c}	ND
Raffinose (g.L^{-1})				
0	13.42 ± 0.39^{D,a}	15.77 ± 0.04^{C,b}	11.43 ± 0.18^{B,a}	30.83 ± 1.09^{A,c}
7	12.61 ± 0.17^{D,a}	16.93 ± 0.56^{C,b}	11.95 ± 0.24^{B,a}	31.26 ± 1.33^{A,c}
14	4.97 ± 0.01^{B,a}	5.65 ± 0.00^{B,a}	4.28 ± 0.02^{B,a}	25.95 ± 1.57^{B,b}
21	2.81 ± 0.05^{A,a}	5.60 ± 0.06^{B,b}	2.47 ± 0.01^{A,a}	28.12 ± 0.49^{B,c}
28	2.41 ± 0.02^{A,a}	3.35 ± 0.04^{A,b}	2.17 ± 0.02^{A,a}	30.67 ± 1.23^{A,c}
Glucose + Galactose (g.L^{-1})				
0	1.14 ± 0.01^{D,a}	3.10 ± 0.04^{C,b}	1.33 ± 0.01^{D,a}	0.29 ± 0.00^{A,c}
7	1.24 ± 0.04^{D,a}	3.40 ± 0.05^{C,b}	1.42 ± 0.02^{D,a}	0.31 ± 0.04^{A,c}
14	0.42 ± 0.00^{B,a}	1.96 ± 0.02^{B,b}	0.45 ± 0.04^{B,a}	0.22 ± 0.00^{A,c}
21	0.70 ± 0.02^{C,a}	0.83 ± 0.00^{B,a}	0.55 ± 0.00^{C,a}	0.27 ± 0.02^{A,a}
28	0.03 ± 0.00^{A,a}	0.61 ± 0.01^{A,b}	0.22 ± 0.01^{A,ab}	0.29 ± 0.00^{A,b}

Note: Values are expressed as mean ± standard deviation (n = 3 analytical replicates per lot). Different lowercase letters (a–c) within the same row indicate statistically significant differences ($p < 0.05$) among treatments (LbLa5, LbBb12, Lb coculture, and Lb control) on the same storage day. Different uppercase letters (A–D) within the same column indicate statistically significant differences ($p < 0.05$) among storage times within the same treatment.

Interestingly, the accumulation of reducing carbohydrates recorded after seven days of refrigeration indicates a transient metabolic equilibrium between monosaccharide generation via oligosaccharide cleavage and its subsequent utilization as an energy substrate during early storage. These results align with biochemical trends documented for alternative fermented legume-based beverages and functional fruit juices (Bujna *et al.*, 2018; Tindjau *et al.*, 2023).

A fundamental attribute of LAB and *bifidobacteria* fermentation is the production of various organic acids such as lactic acid and acetic acid as the principal end-products of carbohydrate metabolism (Ozturkoglu-Budak *et al.*, 2019). As summarized in Table 2, statistically significant variations ($p < 0.05$) in organic acid accumulation were observed for each strain. The La5 synthesized substantial quantities of lactic acid as its primary end-product, whereas Bb12 generated a mixed profile of both acetic and lactic acids during monoculture and co-culture fermentations. Conversely, organic acids remained completely undetected in the unfermented control beverage, validating the efficacy of the thermal processing step and the maintenance of a sterile matrix environment. These distinct acid profiles originate from divergent be resulted from metabolic pathways and varying substrate assimilation rates of the respective strains.

As an obligate homofermentative microorganism, *L. acidophilus* utilizes the Embden-Meyerhof-Parnas (EMP) pathway to produce lactic acid isomer mixtures (Erem & Kilic-Akyilmaz, 2024), which justifies the high titratable acidity observed in La5-containing matrices. Acetic acid , by contrast, represents an intrinsic metabolite of the *Bifidobacteria* genus synthesized via the fructose-6-phosphate phosphoketolase pathway ("bifid shunt") (Ozturkoglu-Budak *et al.*, 2019); hence, its concentration was significantly higher in co-cultivation systems. This mirrors prior research detailing elevated acetic acid yields during the co-culture of *B. lactis* Bb12 with Lactobacilli (Bujna *et al.*, 2018). Eventually, these organic acid profiles confirm the successful implementation of probiotic fermentation employing La5 and Bb12 probiotic strains, contributing to the extension of the microbiological shelf life of the lima bean beverages.

3.5 Peptide Molecular Weight (MW) Distribution via FPLC

The FPLC chromatograms exhibiting peptide resolution within the fermented LBs across the storage period are displayed in Figure 2a, 2b, and 2c. The FPLC profiles revealed that all fermented Lbs maintained broadly comparable molecular weight (MW) distributions, characterized by the progressive evolution, disappearance, or emergence of specific peptide peaks relative to the unfermented control. Low-molecular-weight fractions (MW < 1 kDa) were liberated at elution volumes of 73, 60, and 65 mL across both mono- and co-cultures. By day 14 of storage, the total area under the curve for peaks eluting at 73 mL and 60 mL expanded or materialized within the La5 and Bb12 monocultures, respectively, as well as within the co-culture

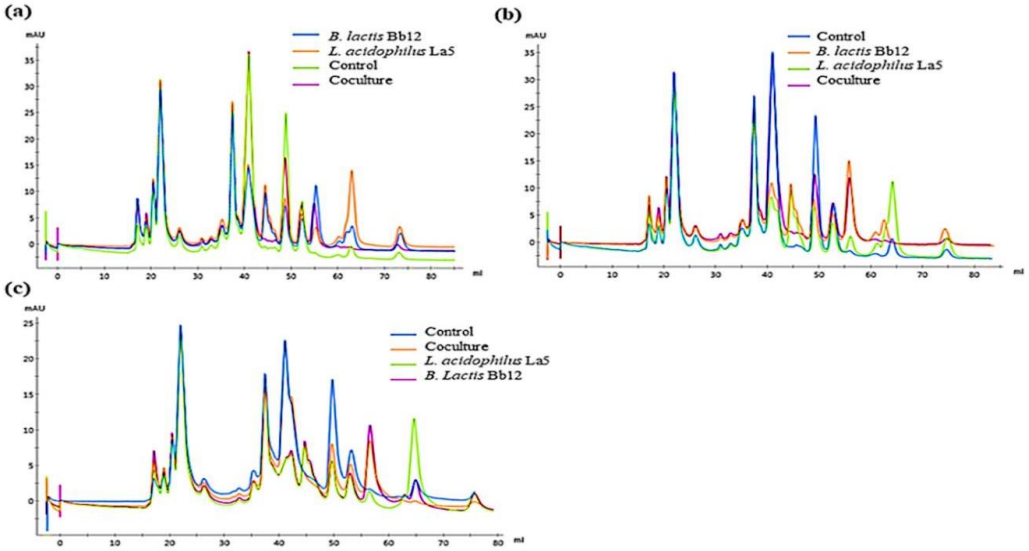

Figure 2. FPLC-Chromatograms of Protein Fraction and Peptides of Fermented Lima Bean Beverages

(a): Production Day, (b): Following 14-day Cold Storage (c): Following 28-day Cold Storage

matrix. Conversely, the peaks at 60 mL and 63 mL resolved completely in the co-culture. By the termination of the 28-day storage trial, a reduction in specific peak areas occurred alongside the manifestation of novel peaks, especially in the monocultures. For instance, a distinctive peak emerged at an elution volume of 75 mL for both individual strains. The disappearance of peaks at 73 mL and 60 mL was observed across all fermented matrices, while fraction peaks at 30 mL and 44 mL were depleted universally.

These chronological modifications in the chromatogram topology demonstrate that *L. acidophilus* and *B. lactis* maintain active proteolytic systems during cold storage at 4 °C. The reduction or complete elimination of discrete peptide fractions within the probiotic LBs may be attributed to their assimilation as a nitrogen source by fermenting bacteria during cold storage to sustain survival (Schöpping *et al.*, 2021). Conversely, the amplification of alternative peak areas indicates progressive extracellular peptide release due to ongoing protein hydrolysis (Chen *et al.*, 2019). Consequently, this structural degradation of native lima bean storage proteins yields low-MW bioactive peptides (< 10kDa) known to possess putative antioxidant functionalities and human health benefits (Hidalgo-fuentes *et al.*, 2024), which should be studied further *in vitro* and *in vivo*.

3.6 FAA Composition During Storage

Table 3 documents the variations in the FAA profiles of the LbLa5a and LbBb12 formulations at days 0, 14, and 28 of storage. Statistically significant discrepancies ($p < 0.05$) were observed across distinct treatments and storage intervals, as differentiated by Tukey's post-hoc stratification. At day 0, a notable accumulation of specific FAAs was recorded in the fermented variants compared to the control; this included significant enrichments in Cys, Asp, Asn, and Met in the La5-fermented beverage, as well as Thr in LbBb12. This This initial elevation in FAA content can be attributed to the active biosynthetic and metabolic secretomes of the probiotic strains (Tindjau *et al.*, 2023).

Over the full course of refrigerated storage, the majority of individual amino acid fractions exhibited a downward trajectory, a trend particularly pronounced within the fermented matrices, Notably, branched-chain amino acids (Ileu, Leu, and Val) along with sulfur-containing species (Met and Cys) decreased significantly from day 0 to day 28, especially in *L. acidophilus* La5 and co-culture treatments. This systematic depletion implies active microbial assimilation for cellular maintenance, conversion into volatile flavor compounds, or catalytic enzyme synthesis. Alternatively, it may reflect an adaptive response of the strains to nutritional deficits within the matrix over extended periods (Bezerra *et al.*, 2016; Tindjau *et al.*, 2023). Polyphenols may modulate the transcriptional expression of genes governing microbial proteolysis and amino acid metabolism. (Piekarska-Radzik & Klewicka, 2021).

Certain amino acids—specifically Glu, Gln, Lys, Ser, Ala, Gly, Cys, Ile, Leu, Met, Phe, Thr, Tyr, Val, Trp, and Arg—are metabolized by, or actively participate in the metabolic pathways of, *L. acidophilus* La5 and *B. lactis* Bb12 bacteria (Meng et al., 2021; Tindjau et al., 2023). These metabolic dynamics elucidate the observed depletion of FAAs within the fermented Lbs. Analogous reductions in FAA have been reported in dairy and vegan products fermented with *L. acidophilus* La5 and *B. lactis* Bb12 (Bezerra *et al.*, 2016; S. Li *et al.*, 2019; Meng *et al.*, 2021).

Elsewhere, the decrease of FAA in LbLa5 and co-culture underscores the dominant role of the La5 microorganism in driving proteolytic evolution attributed to its robust proteolytic activity and peptidase action (Chaves & Gigante, 2016). Collectively, both treatment and the duration of storage significantly influenced FAA composition. Depending on the strain, monocultures promoted rapid proteolysis, which was followed by amino acid depletion. Conversely, the co-culture systems exhibited a more stable FAA profile, which could enhance the nutritional quality and sensory profile of the final functional beverage.

3.7 Dynamics of TPC During Storage

Figure 3a displays the TPC variations in Lbs over 28 days across the various Lb formulations. Probiotic fermentation induced a significant elevation in TPC content compared to the control drink during the first week of storage (Figure 3a). followed by a gradual decline. The highest TPC values were recorded for La5 and its coculture throughout the shelf-life evaluation, reaching peak concentration of 1.73 mg GAE.mL^{-1} and 2.03 mg GAE.mL^{-1}, respectively, at day 7. In contrast, the lowest TPC levels were recorded in LbBb 12, which reached the lowest level (1.16 mg GAE.mL^{-1}) by day 28.

Phenolic compounds constitute a major group of antioxidants present in beans (Chen *et al.*, 2019). Predictably, TPC fluctuations during cold storage are modulated by the metabolic activity of the viable microflora, which alters the matrix pH and liberate bacterial hydrolytic enzymes into the bean matrix. The release of bioactive molecules by *L. acidophilus* La5 and *B. lactis* Bb12 intensified over the first seven days of storage. This increase is due to the hydrolysis of complex phytochemical compounds into simplified structures via synthesized hydrolytic enzymes (Hidalgo-fuentes *et al.*, 2024). Thus, the significant increase in TPC observed in La5 and its coculture is consistent with previous literature regarding fermented legume-based products (AL Zahrani & Shori, 2023; Cichońska *et al.*, 2023). This elevated polyphenol retention positions the fermented Lbs as promising functional vehicles, particularly when consumed within an optimized one-week shelf-life window.

Table 3. Content changes of free amino acids (mg.100 mL-1) during the cold storage (4 °C, 0 – 14 – 28 day) in the control and the fermented Lbs with *L. acidophilus* La5, *B. lactis* Bb12, and their co-culture

FAA %	Control sample			*L. acidophilus* La5			*B. lactis* Bb12			Co-culture		
days	0	14	28	0	14	28	0	14	28	0	14	28
Ileu	0.81 ± 0.03^{bA}	0.72 ± 0.02^{aA}	0.68 ± 0.00^{aA}	0.78 ± 0.02^{cB}	0.57 ± 0.00^{bB}	0.30 ± 0.02^{aC}	1.06 ± 0.00^{aA}	0.81 ± 0.03^{bA}	0.64 ± 0.00^{bA}	0.55 ± 0.01^{bC}	0.38 ± 0.01^{cC}	0.23 ± 0.00^{aC}
Leu	2.48 ± 0.06^{bB}	2.39 ± 0.02^{bB}	2.32 ± 0.02^{bB}	2.29 ± 0.00^{aB}	1.26 ± 0.01^{aA}	0.83 ± 0.01^{aA}	2.37 ± 0.02^{bB}	1.22 ± 0.02^{bA}	1.10 ± 0.01^{bA}	1.85 ± 0.03^{aA}	0.99 ± 0.01^{aB}	0.63 ± 0.02^{aB}
Lys	1.61 ± 0.01^{bB}	1.70 ± 0.03^{bA}	2.02 ± 0.02^{bA}	1.03 ± 0.04^{aA}	0.75 ± 0.02^{aB}	0.31 ± 0.01^{aC}	0.94 ± 0.04^{aA}	0.52 ± 0.05^{aB}	0.39 ± 0.00^{aC}	1.67 ± 0.07^{bA}	0.81 ± 0.01^{bB}	0.46 ± 0.00^{bC}
Met	0.37 ± 0.01^{aA}	0.36 ± 0.00^{aA}	0.35 ± 0.00^{aA}	0.54 ± 0.02^{bB}	0.16 ± 0.00^{aB}	0.09 ± 0.00^{aC}	0.25 ± 0.01^{aA}	0.17 ± 0.01^{aA}	0.06 ± 0.00^{aB}	0.44 ± 0.03^{bA}	0.17 ± 0.00^{bB}	0.10 ± 0.00^{bC}
Phe	1.76 ± 0.02^{aA}	1.79 ± 0.01^{aA}	1.75 ± 0.01^{aA}	1.82 ± 0.03^{bB}	1.16 ± 0.01^{bA}	0.85 ± 0.00^{bA}	2.19 ± 0.04^{cA}	1.50 ± 0.04^{cB}	1.25 ± 0.00^{cB}	1.61 ± 0.01^{aA}	1.63 ± 0.01^{aA}	0.98 ± 0.01^{aB}
Thr	1.18 ± 0.03^{bA}	1.15 ± 0.01^{bA}	1.08 ± 0.01^{bA}	0.90 ± 0.04^{aB}	0.74 ± 0.03^{aB}	0.62 ± 0.02^{aB}	1.26 ± 0.03^{cA}	0.93 ± 0.01^{cB}	0.88 ± 0.05^{cC}	0.92 ± 0.05^{bB}	0.82 ± 0.01^{bC}	0.77 ± 0.02^{bC}
Val	1.46 ± 0.01^{bA}	1.40 ± 0.01^{bA}	1.34 ± 0.01^{bA}	1.64 ± 0.04^{aB}	1.31 ± 0.01^{aB}	1.21 ± 0.01^{aB}	1.58 ± 0.05^{aB}	1.45 ± 0.05^{aB}	1.11 ± 0.01^{aC}	1.66 ± 0.06^{bA}	1.38 ± 0.04^{bB}	0.93 ± 0.03^{bC}
Trp	0.99 ± 0.03^{aA}	0.93 ± 0.01^{aA}	0.82 ± 0.01^{aB}	1.01 ± 0.03^{bA}	0.82 ± 0.01^{bB}	0.53 ± 0.02^{bC}	0.91 ± 0.01^{aA}	1.02 ± 0.04^{aA}	0.97 ± 0.01^{aA}	0.91 ± 0.02^{bA}	0.99 ± 0.00^{bA}	0.77 ± 0.02^{bB}
Ala	1.19 ± 0.07^{bB}	1.12 ± 0.01^{abB}	1.10 ± 0.03^{aA}	1.07 ± 0.05^{aB}	1.10 ± 0.01^{abB}	1.07 ± 0.03^{aA}	1.16 ± 0.02^{bB}	0.97 ± 0.02^{bc}	1.12 ± 0.01^{aA}	1.29 ± 0.04^{aA}	1.38 ± 0.03^{aA}	0.97 ± 0.03^{bB}
Arg	1.09 ± 0.05^{bB}	1.00 ± 0.00^{bB}	0.91 ± 0.01^{bB}	1.15 ± 0.05^{aA}	1.08 ± 0.01^{aB}	0.99 ± 0.01^{aB}	0.93 ± 0.02^{aB}	0.94 ± 0.05^{aB}	1.07 ± 0.01^{aA}	1.11 ± 0.05^{aA}	0.89 ± 0.04^{aB}	1.04 ± 0.04^{aA}
Asp+Asn	3.05 ± 0.06^{bB}	3.15 ± 0.02^{bB}	2.97 ± 0.02^{bB}	3.56 ± 0.06^{aA}	3.07 ± 0.02^{bB}	2.62 ± 0.00^{bC}	3.13 ± 0.06^{bB}	3.47 ± 0.02^{aA}	2.81 ± 0.01^{bB}	3.01 ± 0.04^{bB}	2.49 ± 0.02^{ac}	1.98 ± 0.03^{ac}
Cys	0.16 ± 0.01^{aB}	0.16 ± 0.00^{aB}	0.14 ± 0.00^{ac}	0.23 ± 0.01^{aA}	0.14 ± 0.00^{bc}	0.07 ± 0.00^{bc}	0.14 ± 0.02^{bB}	0.03 ± 0.00^{bc}	0.01 ± 0.00^{bc}	0.28 ± 0.01^{cA}	0.10 ± 0.01^{bc}	0.06 ± 0.00^{bc}
Glu+Gln	6.79 ± 0.02^{aB}	6.90 ± 0.05^{aB}	6.41 ± 0.04^{ac}	11.15 ± 0.10^{bA}	4.79 ± 0.03^{bc}	2.31 ± 0.05^{bc}	9.19 ± 0.02^{bA}	4.96 ± 0.05^{bc}	3.84 ± 0.03^{bc}	9.82 ± 0.08^{aA}	9.42 ± 0.01^{aA}	8.65 ± 0.00^{aB}
Gly	1.11 ± 0.06^{bB}	1.26 ± 0.01^{bB}	1.05 ± 0.01^{bB}	0.81 ± 0.03^{aB}	0.55 ± 0.02^{aC}	0.38 ± 0.00^{aC}	1.12 ± 0.06^{bB}	0.70 ± 0.01^{bC}	0.66 ± 0.00^{bC}	0.90 ± 0.04^{aB}	0.50 ± 0.01^{aC}	0.31 ± 0.00^{aC}
Ser	2.42 ± 0.01^{bB}	2.49 ± 0.02^{bB}	2.37 ± 0.03^{bB}	2.70 ± 0.04^{cA}	1.75 ± 0.03^{bc}	1.04 ± 0.01^{ac}	2.43 ± 0.02^{bB}	1.96 ± 0.05^{bB}	1.62 ± 0.01^{bc}	2.77 ± 0.04^{cA}	1.85 ± 0.01^{bc}	1.40 ± 0.01^{bc}
Tyr	0.63 ± 0.00^{bB}	0.66 ± 0.00^{bB}	0.61 ± 0.02^{bB}	0.97 ± 0.05^{aA}	1.08 ± 0.02^{aA}	0.57 ± 0.00^{ab}	1.16 ± 0.03^{aA}	1.08 ± 0.05^{aA}	0.38 ± 0.00^{bc}	1.12 ± 0.01^{aA}	1.13 ± 0.01^{aA}	0.74 ± 0.01^{ab}

Note: Values are expressed as mean ± standard deviation (n = 3). Different lowercase letters within the same column indicate significant differences among time points (0, 14, and 28 days) within each treatment ($p < 0.05$. Different uppercase letters within the same row indicate significant differences among treatments (Control, L. acidophilus La5, B. lactis Bb12, and co-culture) at each sampling time ($p < 0.05$).

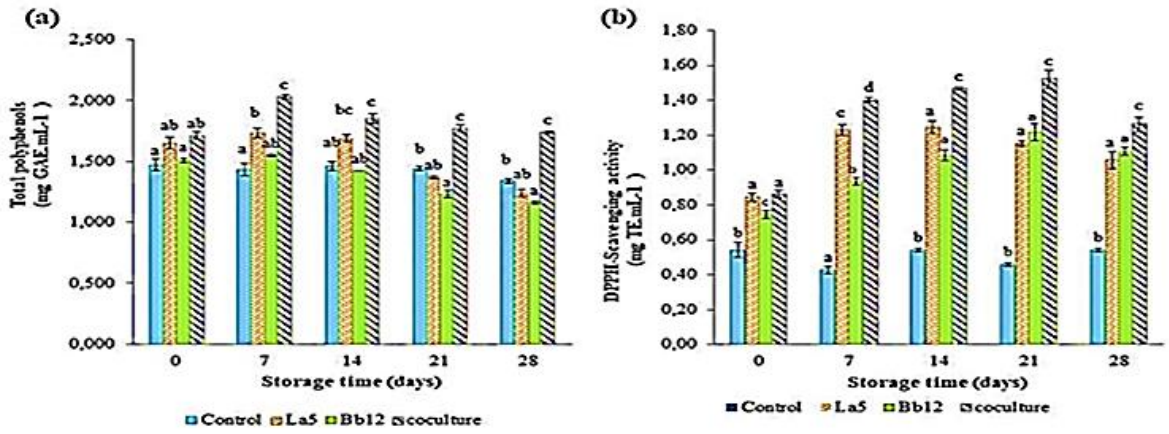

Figure 3. Evolution of Total Phenolic Content (TPC)

(a) and value of antioxidant activities (b) analysed by DPPH assay of the different Lima beverages (Control, La5, Bb12 and Mix) during four weeks under refrigeration. Different letters mean significant differences between samples at each storage period determined by Turkey's test ($p \leq 0.05$)

Conversely, the reduction in polyphenol concentrations during the final three weeks of storage may be attributed to the continuous biotransformation of polymeric phenolics by specific probiotic bacterial enzymes, such as phenolic acid decarboxylases (AL Zahrani & Shori, 2023). These findings align with prior investigations (AL Zahrani & Shori, 2023; Chen *et al.*, 2019) that reported a decrease in TPC with extended refrigerated storage. Paradoxically, the microbial metabolism and enzymatic degradation of polyphenols by probiotic strains may yield localized bioactive derivatives that offer supplementary functional benefits (Piekarska-Radzik & Klewicka, 2021). Notably, it is critical to acknowledge that non-phenolic organic constituents within the matrix, including reducing carbohydrates can cross-react with the Folin-Ciocalteu reagent (Cichońska *et al.*, 2023); this phenomenon may lead to an underestimation of the true phenolic variations due to background matrix interference.

3.8 DPPH Radical Scavenging Activity During Beverage Storage

The temporal changes in DPPH radical scavenging activity across the Lb formulations throughout the storage period are illustrated in Figure 3(b). *L. acidophilus* La5 and its corresponding co-culture system exhibited higher scavenging activity than the *Bifidobacterium lactis* Bb12 monoculture during storage ($p < 0.05$), whereas the non-fermented control beverage maintained a relatively stable antioxidant baseline. The DPPH scavenging capacity intensified, reaching peak values of 1.52 mg TE.mL^{-1} and 1.22 mg TE.mL^{-1} after three weeks of storage in the co-culture system and the B12 monoculture, respectively, and peaking at 1.25 mg TE.mL^{-1} after two weeks in the La5 formulation. However, the antioxidant properties of all fermented Lb underwent a significant decline during the final week of evaluation.

In the present study, the *L. acidophilus* La5 and *B. lactis* Bb12 strains exerted a positive influence on the DPPH inhibitory capacity of the Lbs over the first three weeks of storage, despite the concurrent decrease in TPC. This phenomenon is likely modulated by active microbial metabolism, which generates alternative antioxidant intermediates, such as low-molecular-weight bioactive peptides and FAA (Cichońska *et al.*, 2023; Yang *et al.*, 2024). Additionally, the quantitative reduction of complex polyphenols during cold storage may facilitate the structural breakdown and digestibility of macronutrients, thereby enhancing the antioxidant value of fermented beverages (AL Zahrani & Shori, 2023). Identical improvements in antioxidative capacity driven by *Lactobacillus* spp. and *Bifidobacterium* spp. have been previously documented in alternative fermented plant-based matrices (Bujna *et al.*, 2018; Naprasert *et al.*, 2019).

Furthermore, the specific enzymatic repertoire of the starter cultures directly governs the availability of antioxidant constituents within fermented Lbs (Cichońska *et al.*, 2023). The DPPH radical scavenging dynamics appeared to be synergistically enhanced in the presence of *B. lactis* Bb12, a strain widely reported to possess superior intrinsic antioxidant mechanisms compared to standard LAB (Cichońska *et al.*, 2023). Correspondingly, elevated antioxidant activities have been observed in plant-based beverages fermented with a combination of bacterial concentrates containing *B. lactis*

Bb12 (AL Zahrani & Shori, 2023; Cichońska *et al.*, 2023). The optimization of these antioxidant properties through targeted fermentation serves as a critical mechanism for mitigating risks associated with cellular oxidative stress (Erem & Kilic-Akyilmaz, 2024).

Nevertheless, the final drop in DPPH radical scavenging activity at the end of the storage is strongly associated with the quantitative loss of key phenolic compounds via advanced probiotic degradation pathways (Arab *et al.*, 2022). Consequently, further characterization of the isolated phenolic, FAA, and peptide fractions is required to isolate their discrete contributions to the overall antioxidant kinetics.

3.9 Sensory and Organoleptic Profiling

The overall acceptability scores and the Just-About-Right (JAR) attribute distributions for the formulated Lbs are presented in Figures 4(a) and 4(b), respectively. Statistically significant variations ($p \leq 0.05$) were observed in the mean overall acceptability scores between the fermented Lbs and the non-fermented control. The co-culture beverage exhibited the highest sensory preference, recording a mean score of 5.54. This was followed by the La5 and Bb12 single-strain formulations, which achieved scores of 5.23 and 5.03, respectively , whereas the control beverage received the lowest consumer preference ratings. Panelists predominantly characterized the control matrix as excessively sweet, lacking sufficient acidity, and presenting a moderate flavor intensity (Figure 4b). In contrast, the fermented variants (LbLa5, co-culture, and LbBb12) were perceived to possess a significantly attenuated sweetness profile (identified by 65%, 66%, and 52% of panelists, respectively). Moreover, the La5 monoculture and the coculture system were characterized as exhibiting a highly pronounced flavor (39% and 49%, respectively) with robust acidity (69% and 64%, respectively).

The hedonic evaluation revealed mean overall acceptability scores below 6.0 on a 9-point scale (Figure 4a), indicating a modest primary consumer acceptance of these beverage formulations. This constrained appreciation may stem from consumer unfamiliarity with this specific beverage

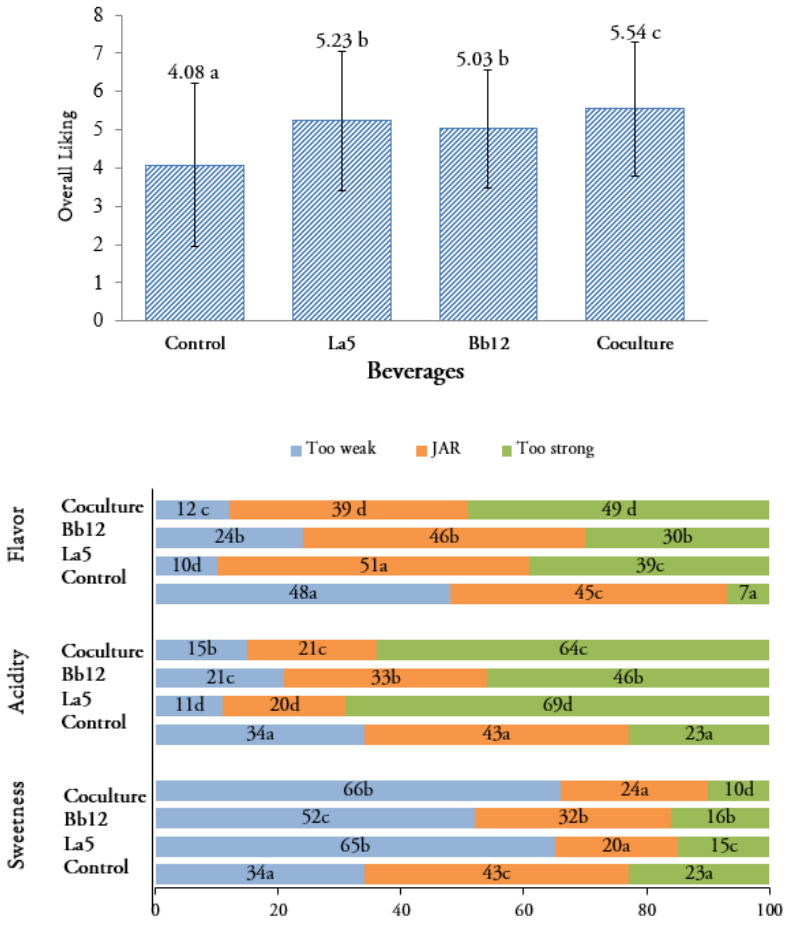

Figure 4. Sensory Assessment of Consumer Acceptability of Lima Unfermented and Fermented Beverages by Overall Preferencing Scores and of Attributes Just-About-Right (JAR) for Sweetness, Acidity and Flavor

archetype within the target geographic market. Previous studies have reported comparable hedonic baselines for lima bean and other legume-based formulations (Chukwu & Onuh, 2020).

The enhanced flavor profiles of the fermented Lb samples are directly attributable to the microbial generation of volatile organic compounds and organic acids during fermentation. These metabolites, such as lactic acid, glutamic acid, and flavor-active amino acids (e.g., Val, Leu, and Ile), impart characteristic aromatic notes and desirable acidity to the matrix (Hidalgo-fuentes *et al.*, 2024; Li *et al.*, 2019), thereby validating the metabolic efficacy of the La5 and Bb12 fermentation processes. Furthermore, a major part of the objectionable "beany" off-flavors typical of raw legume-based products are reduced or eliminated during fermentation (Erem & Kilic-Akyilmaz, 2024). Although probiotic fermentation significantly advanced consumer acceptance of the Lb matrices, these sensory benchmarks underscore the necessity of further organoleptic optimization. Future developments should explore the integration of natural fruit pulps, prebiotic carbohydrates, or masking flavor agents, as well as blending with complementary botanical sources to achieve an optimal sensory profile.

4 Conclusions

In conclusion, the present study demonstrates that lima bean extract serves as an excellent nutritional matrix rich in protein, carbohydrates, and bioactive components, rendering it an ideal medium for probiotic propagation. The probiotic strains *L. acidophilus* La5 and *B. lactis* Bb12 maintained high viability at sufficient levels throughout the 28-day refrigerated storage period, satisfying the therapeutic minimums required for functional foods. The microbial metabolic activity during cold storage was highly strain-dependent, inducing substantial alterations in the profiles of potentially bioactive compounds, such as free phenolics, low-molecular-weight bioactive peptides, and FAAs, which consequently dictated the radical scavenging kinetics of the fermented beverages.

Importantly, probiotic fermentation efficiently eliminated non-digestible alpha-galactosides (raffinose oligosaccharides), which may enhance gastrointestinal tolerance and nutrient bioavailability. Additionally, probiotic fermentation process significantly improved the flavor architecture and consumer acceptability of the Lbs. Nevertheless, future investigations must focus on optimizing microbial kinetics and metabolic yields to refine the final sensory profile. Taken together, our findings indicate that these fermented legume-based beverages represent a promising category of novel, lactose-free functional drinks, ideally suited for vegan demographics and individuals presenting with lactose intolerance and vegans, thereby expanding the non-dairy functional beverage market and promoting the diversification of legume-based food products.

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Conflicts of Interest: The authors declare no conflicts of interest associated with this research study.

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