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Transforming chamomile (*Matricaria chamomilla*) infusion into a fermented beverage using sucrose and probiotic lactic acid bacteria

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Abstract
Aromatic and medicinal herbs have long been used worldwide as remedies for a variety of ailments. Among these, chamomile (*Matricaria chamomilla*) is recognized for its analgesic, anti-inflammatory, antimicrobial, antioxidant and antiseptic properties and is commonly consumed as an herbal infusion. Lactic acid fermentation with probiotic lactic acid bacteria (LAB) offers a practical approach to developing functional beverages with improved sensory and physicochemical properties. In this study, we aimed to evaluate whether chamomile infusion, supplemented with sucrose, could serve as a suitable substrate for fermentation by two well-established probiotic strains: *Lacticaseibacillus casei* Shirota (DN-114 001) and *Limosilactobacillus johnsonii* NCC533. We focused on fermentation performance (growth kinetics, acidity, polyphenol content and viable counts) and product stability rather than the detailed nutritional or probiotic metabolite profiles of the strains. Beverages were prepared with 10% and 15% (w/v) sucrose syrups and fermented for 24 h. Statistical analysis revealed significant differences ($p < 0.05$) between treatments in viable counts (CFU/mL), growth rate (μ), and acidity percentage, with the 10% treatment selected based on sensory evaluation. The viable count averaged 7.43 ± 0.11 log CFU/mL, meeting probiotic criteria (>6 log CFU/mL). The average growth rate was 0.18 ± 0.006, pH 4.18 ± 0.042, and acidity 0.285 ± 0.0025%. Sanitary quality was acceptable, with no fungi, yeasts, or coliforms detected. Fermentation also significantly increased the total phenolic content. After four weeks at 4 °C, beverages remained viable, with counts between 6.0 and 6.15 log CFU/mL. These results demonstrate the feasibility of producing a refreshing probiotic beverage based on chamomile infusion, with potential as both a functional and shelf-stable product.

Keywords Matricaria chamomilla, Probiotic beverage, Lacticaseibacillus casei, Lactic acid fermentation, Functional beverage, Phenolic compounds

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1 Introduction

Microorganisms play a crucial role in nature, particularly in biogeochemical cycles. Remarkably, any food consumed by humans—whether plant-based, animal-based, or a combination such as textured proteins—contains microbial loads that interact with its components. These microorganisms use foods as nutrient substrates for growth, leading to changes in the sensory characteristics and appearance of many market products [1]. Modern food science integrates biotechnology, molecular biology, food microbiology and nutrition to develop products that enhance physiological processes and provide essential nutrients. Functional foods, including probiotics, are scientifically proven to confer health benefits. However, most probiotic products are dairy-based. In response, low-calorie beverages using medicinal plants, flowers and fruits have been developed in Mexico for populations dealing with overweight and obesity [2]. Similarly, a fermented beverage from borojó fruit was created in Colombia, incorporating yogurt and honey to provide energizing nutrients in a single matrix [3]. The success of plant matrices as substrates for probiotic beverages highlights a growing consumer segment that prefers plant-based foods or has dairy restrictions [4, 5]. Research indicates a trend toward developing non-dairy probiotic beverages, including fermented vegetable juices and fruit-based formulations. The use of medicinal plants in probiotic beverages also shows promise, contributing to the diversification of functional foods [6, 7]. A major challenge in formulating these beverages is the limited number of adapted microbial strains with probiotic phenotypes that also promote desirable sensory characteristics [8]. Lactic acid bacteria (LAB) are the most widely used microorganisms for producing fermented foods, including dairy, meat, and vegetables. LAB ferment the food matrix, improving sensory properties and nutritional value, while also contributing to food safety [1]. Some *Lactobacillus* strains are well-established probiotics, defined as living microorganisms that, when ingested in sufficient amounts, provide health benefits to the host [9]. The antioxidative and cytotoxic effects of chamomile fermented with *Lactobacillus plantarum* (recently reclassified as *Lactiplantibacillus plantarum*) were investigated to enhance biofunctional activities, suggesting that chamomile fermentation could be applied to develop natural antioxidative and anticancer products [10, 11]. During probiotic powder production, cells are exposed to damaging stresses such as temperature extremes, osmotic pressure, and mechanical forces. To evaluate heat resistance in fatty acid–enriched cultures, *L. johnsonii* (LJ) was exposed to 58–62.5 °C for 5 min [12]. The significant survival rates observed demonstrate this strain's exceptional thermotolerance, highlighting its industrial potential for probiotic processing. Similarly, strains of *Lacticaseibacillus* grow within a variable temperature range but never below 10 °C or above 45 °C [10]. While most *Lactobacillus* species prefer 30–37 °C, some strains (including LJ) exhibit thermotolerant behavior, surviving and even growing at higher temperatures. This is not unusual, as certain LAB, such as *L. delbrueckii* subsp. *bulgaricus* (used in yogurt), thrive at 40–45 °C [13]. These conditions selectively inhibit mesophilic contaminants (e.g., *Enterobacteriaceae* and spoilage microbes), reducing contamination risk without antibiotics or anaerobic agents, a common strategy in probiotic fermentations. The present study aimed to create a non-dairy probiotic beverage from a standardized chamomile infusion fermented with probiotics. The goal was to produce a sensorially pleasing beverage in taste and texture that promotes health and prevents disease, targeting lactose-intolerant individuals, vegetarians, and those avoiding dairy. Chamomile

(*Matricaria chamomilla*), chosen for this purpose, is an aromatic herb with inflorescences that contain over 120 medicinal compounds, including flavonoids, terpenoids, and organic acids. Its volatile oils—such as quercetin, rhamnose, xylose, cineol and limonene—are known for therapeutic properties including anti-inflammatory, anti-ulcer, anti-allergic, bactericidal, antifungal, sedative, analgesic, antispasmodic, anti-diarrheal, and carminative effects. A comprehensive review of its traditional uses, chemical constituents, pharmacological activities, and quality control studies has been reported by Dai et al. (2022) [14]. These properties make chamomile a viable base for producing fermented probiotic beverages. This study hypothesized that chamomile could be used as a base infusion for creating fermented beverages with probiotic bacteria. The objective was to develop an unconventional fermented beverage using a standardized chamomile infusion and simple syrup as a substrate for *Lacticaseibacillus casei* Shirota (DN-114 001) and *Limosilactobacillus johnsonii* NCC533, previously classified as *Lactobacillus casei* Shirota and *Lactobacillus johnsonii*, respectively.

2 Materials and methods

2.1 Strains of LAB used

The *Limosilactobacillus johnsonii* NCC533 (LJ) strain was obtained from the National School of Biological Sciences at National Polytechnic Institute (IPN). The *Lacticaseibacillus casei Shirota* DN-114 001 (LC) strain was obtained from the Technological Institute of Mérida, Microbiology Substation. Both strains were stored in cryopreservation using 20% glycerol as a cryoprotectant.

2.2 Pre-inoculum Preparation

Stock culture of the LAB used were thawed at room temperature and activated in 5 mL culture medium prepared using 5 g/L of Difco™ Yeast Extract (YE) and 30 g/L commercial glucose. The culture medium was then incubated in a static condition at 37 °C for 24 h (Ecoshell incubator model 9052 L, Mexico), without atmosphere control.

2.3 Inoculum Preparation

The activated pre-inoculum culture of the LAB after 24 h incubation was inoculated into 400 mL of culture medium containing 5 g/L YE and 30 g/L glucose in 1000 mL Erlenmeyer flasks. The culture medium was cultivated in a Ecoshell incubator model 9052 L, Mexico., at static condition at 42 °C for 24 h, without atmosphere control. The culture was then centrifuged at 2860×g, 37 °C for 15 min (Velab centrifuge model VE4000 Mexico) in order to harvest the cells. The cells pellet was resuspended in volume of 100 mL of saline solution (0.7%) and added to the main medium. This inoculum contained 1.5-2.0×10^6 CFU/mL and standardized for all the fermentations performed.

2.4 Preparation of infusions

Sachets of chamomile tea (Brand: "Bon appétit"), each containing approximately 1.5 g of dry plant matter, were used as the substrate for the probiotic beverage. All sachets were purchased from a local retailer, verified to be from a single production batch to ensure consistency and inspected for quality (intact packaging, appropriate expiration date, no signs of moisture or damage). The preparation of the chamomile infusion was adapted from standard methods for the extraction of bioactive compounds from herbal teas

[15–18]. The solid-to-liquid ratio was standardized based on the manufacturer's recommendation and common consumer practice, using one tea sachet per 250 mL of water, equivalent to 6 g of dry matter per liter of infusion. To achieve the required volume for fermentation, a concentrated infusion was prepared by steeping 16 tea sachets (24 g of dry matter) in 6 L of distilled water. To ensure microbiological safety and prevent contamination during the fermentation process, the entire volume of water along with the tea sachets was subjected to sterilization at 121 °C for 5 min in an autoclave. This sterilization step is critical in food microbiology to eliminate endogenous microbiota that could compete with the probiotic strains [4]. Following sterilization, the concentrated infusion was aseptically divided into three equal portions of 2 L each. Each portion was then transferred into a sterile fermenter containing 2 L of a separately sterilized sucrose solution, resulting in a final infusion with the target concentration (6 g/L) ready for inoculation.

2.5 Fermentations

The fermentations were carried out in 5 L fermentation jars (FAM 5000, ESEVE. Mexico) with an operating volume of 4 L. To prepare the fermentation medium, commercial sucrose was used at concentrations of 100 and 150 g/L. For instance, 1200 g of sucrose were dissolved in a 6 L volume. Subsequently, it was divided into 3 portions of 2 L each and poured into each of the 3 fermenters, which were then individually sterilized at 121 °C for 15 min. The fermenters were kept closed until use, when 2 L chamomile infusion was added. The fermentation medium contained sucrose at a final concentration of 10% and 15%, supplemented with a standardized Chamomile (*Matricaria chamomilla*) infusion (4 sachets per liter, as indicated in Sect. 2.4. The fermentation was conducted in a 5 L fermenter with an operating volume of 4 L, mechanically stirred at 250 rpm, at a temperature of 45 ± 2 °C, and an initial pH of approximately 6.5, for 24 h. All experiments were performed in triplicate.

2.6 Analytical methods

The evaluated parameters were viable counts (CFU/mL), growth rate (μ) [19], pH and percentage of titratable acidity. The ten-fold serial dilution of homogenized samples (0.1 mL) was prepared in sterile solution of 0.8% NaCl and plated on an appropriate agar. The percentage of titratable acidity of each beverage was analyzed as reported elsewhere [20] and calculated with the Eq. 1. medium.

$$AT\,(\%) = \frac{G \times \ N \times \ M_{eq}}{V} \times \ 100 \tag{1}$$

Where: G = NaOH used in the titration (mL); N = Normality of the NaOH used (0.1 N); M_{eq}= Milliequivalent of lactic acid (0.090); V = Volume of the sample (10mL); 100 = Percentage factor.

Each of the analysis was assessed during the fermentation for at least 24 h with readings taken every 4 h. For viable counts, plate dilution using agar medium and the standard method were employed.

2.7 Shelf-life analysis

Upon completion of the fermentations at 24 h at 45 °C, samples of the fermented beverages were stored at a temperature of 4 °C for 4 weeks. Weekly assessments were conducted to determine the viability of the probiotic culture. Viable counts were performed in accordance with the international standards [21]. Briefly, samples were serially diluted in a phosphate buffer solution, plated on MRS agar, and incubated anaerobically at 37 °C for 72 h. The results were reported as colony-forming units per milliliter (CFU/mL) on a logarithmic scale (Log CFU/mL).

2.8 Physicochemical, microbiological, and sensory characterization of fermented beverages

2.8.1 Aerobic mesophiles

For the respective microbiological analyses, each final product was appropriately diluted with a phosphate buffer solution or peptone water (NOM 109-SSA1-1994 and NOM-110-SSA1-1994). The pour plate method with agar was used according to the standard procedure (BIOXON Cat. 134) (NOM-092-SSA1-1994). The plates were incubated at 31 ± 1 °C for 48 ± 2 h. Only plates containing 25 to 250 CFU units were counted. The results are the average (CFU/mL) of three experiment replicates [22].

2.8.2 Fecal coliforms

For this analysis, the methodology described by the NOM-112-SSA1-1994 was followed, using the most probable number (MPN) technique for total coliforms. One loopful from each tube was inoculated into tubes containing lauryl tryptone broth. The tubes showing gas formation were inoculated into tubes containing *Escherichia coli* broth and incubated at 45 ± 1 °C for 48 h. Gas formation confirmed the presence of fecal coliforms. The same procedure was followed to count the total coliform bacteria.

2.8.3 Molds and yeasts

Dextrose and potato agar (BIOXON Cat. 119) were used to determine molds and yeasts, acidifying the medium with 10% w/v tartaric acid, and incubated at approximately 25 ± 1 for 5 days CFU/mL (NOM-111-SSA1-1994; AACC, 1982). Plates containing 10 to 150 colonies were counted and the count was averaged from three experiment replicates.

2.8.4 Total phenol content

The total phenol content was determined using the colorimetric method of Singleton & Rossi et al.(1965) [23] with some modifications [24]. Considering a 40 mg/mL sample ratio, the control solution was prepared in test tubes by adding 1 mL of gallic acid (Sigma-Aldrich®), 2.5 mL of Folin reagent (1:2), and 2 mL of 7.5% Na_2CO_3. The mixture was agitated and heated to 50 °C for 10 min to measure the absorbance at 760 nm in a spectrophotometer (Perkin Elmer Lambda 25, USA). Then, in a reaction tube, 5 µL of each sample, 500 µL of water, and 1350 µL of Folin-Ciocalteu reagent (Sigma-Aldrich®), (700 mL of distilled water + 100 g of sodium tungstate, + 25 g of sodium molybdate + 50 mL of 85% phosphoric acid + 100 mL of concentrated hydrochloric acid) were added. Subsequently, 1100 µL of 7.5% Na_2CO_3 were added and the tubes were subjected to a water bath for 15 min due to the endothermic reaction. The mixture was then cooled and aliquots of the reactions were taken in photometer cells for reading at 760 nm in a

spectrophotometer (Perkin Elmer Lambda 25, USA) to determine absorbance [7]. Gallic acid (GA) solutions between 0 and 40 μg/mL were used to construct the calibration curve. The results were expressed as μg of GA per mL of sample obtained from the slope equation of the standard curve; values are presented as the mean of triplicate analyses ± standard deviation (SD).

2.8.5 Sensory acceptance test

The sensory analysis was conducted using a panel of thirty-five ($n = 35$) evaluators recruited from the Food Engineering and Biotechnology program at the University of Papaloapan. The panelists, who had academic background in food engineering were instructed to evaluate the products under controlled conditions. Each participant was presented with four coded samples: LC 10%, LC 15%, LJ 10%, and LJ 15%. The samples were served in identical 15 mL aliquots at room temperature. Panelists were instructed to rinse their palates with water between samples to neutralize residual flavors. The evaluation was performed using a structured survey divided into two sections (one for each bacterial strain). For each sample, panelists were asked to rate their degree of liking for the attributes of odor, color, taste and texture on a 5-point hedonic scale with the following categories:

Strongly dislike.

Slightly dislike.

Neither like nor dislike.

Slightly like.

Strongly like.

In addition to the scaled ratings, panelists were provided with open-ended sections to offer free comments on each product and to indicate whether they would consider purchasing it in the future. After all data were collected, a sensory analysis session was held to discuss and interpret the findings. Data were analyzed using non-parametric statistical methods appropriate for ordinal data, given the nature of the hedonic scale. To assess the association between categorical variables, a Chi-square test was used. (Supplemental materials, Tables S1,2,3).

2.9 Experimental design and statistical analysis

The experimental design was conducted using MINITAB 19 and consisted of a 2×2 full factorial design, where the evaluated treatments were the levels of sucrose syrup concentration in the beverage: 10% w/v and 15% w/v, respectively. Each block represented a different strain, although no randomization of runs existed. The response variables considered were the growth parameters at 24 h of fermentation: viable counts (CFU/mL), growth rate (μ). Each treatment was performed in three replicates. Furthermore, the collected data from the variables were separately analyzed using one-way ANOVA to test for differences or significant effects at a significance level of 0.05. Similarly, the phenolic content was statistically analyzed, considering the resulting absorbance as the response factor of the samples and using a significance level of 0.05.

3 Results and discussion

3.1 Viable counts

The incorporation of sucrose syrup (10% and 15% w/v) into standardized chamomile (*Matricaria chamomilla*) infusion beverages significantly influenced the growth kinetics of the probiotic lactic acid bacteria (LAB) strains tested ($p < 0.05$). Both LC and LJ exhibited a typical microbial growth pattern: an initial lag phase (0–4 h) for metabolic adaptation, a pronounced exponential growth phase (8–20 h), followed by a notable decline in viable counts after 20 h. Although a similar study used the same strains, the growth was not as remarkable, probably due to the nature of the infusion used was also the same [25]. (Fig. 1). This decline is particularly noteworthy as sucrose, the primary energy source, was still present in the medium, pointing to limitations beyond carbon availability. For LC, viable counts increased from 6.20 to 7.35 Log CFU/mL in the 10% sucrose treatment and from 6.30 to 7.61 Log CFU/mL in the 15% sucrose treatment. LJ reached slightly higher values, from 6.20 to 7.51 Log CFU/mL at 10% sucrose and from 6.31 to 7.70 Log CFU/mL at 15% sucrose after 20 h. The modestly superior performance of LJ, especially at the higher sucrose concentration, suggests a slightly greater efficiency in utilizing this carbohydrate under the specific constraints of the chamomile matrix. The observed growth kinetics and the subsequent population decline are a direct consequence of the chamomile infusion's suboptimal nutritional profile for supporting sustained LAB proliferation. LAB are fastidious microorganisms with complex nutritional requirements; they are auxotrophic for multiple amino acids, vitamins and nucleic acid precursors, and thus depend entirely on the fermentation substrate to supply these essential nutrients [26]. Although sucrose provided an adequate energy source, the chamomile infusion was critically deficient in nitrogenous compounds, despite the fact that its amino acid profile has been characterized [27]. Our preparation, using four tea bags, resulted in a medium

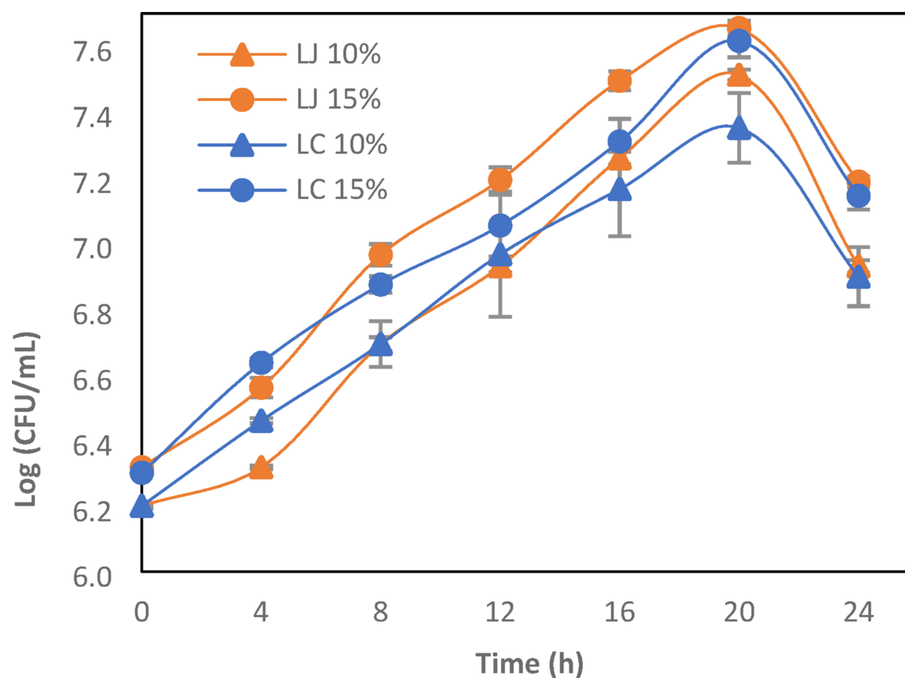

Fig. 1 Kinetics of LAB grown in a medium based on sucrose and chamomile infusion. (Triangles) 10% sucrose; (Circle) 15% sucrose. *LJ Limosilactobacillus johnsonii NCC533*, *LC Lacticaseibacillus casei*. Log (CFU/mL), Log transformation of colony forming units per milliliter

with a protein availability of only ~ 0.3 g/L. This value is orders of magnitude lower than the nutrient-rich environment of MRS broth and is a key factor limiting growth. This nutritional inadequacy is further compounded by the presence of inherent antimicrobial compounds in chamomile, such as flavonoids (e.g., apigenin) and phenolic derivatives, which can exert bacteriostatic effects and inhibit microbial growth [28]. The combination of nutrient scarcity and mild antimicrobial pressure creates a challenging environment that prevents the cultures from maintaining stationary phase populations, leading to the observed decline after 20 h.

When contextualized with other plant-based substrates (Table 1), the nutritional limitations of chamomile become starkly evident. For instance, beetroot juice, while relatively low in protein (~ 1.35%), supported growth up to 9.22 Log CFU/mL for LC [29]. , likely due to a richer profile of minerals and other growth factors. Similarly, *Aloe vera* gel, with a higher crude protein content (2.2% in leaf powder), supported populations as high as 10.91 Log CFU/mL. Similarly, González et al. (2007) used *Aloe vera* as the plant-based medium to obtain high concentrations of viable cells from two probiotic bacteria, *Lacticaseibacillus casei* Shirota (NRRL-1445) and Lb. plantarum (NCIMB 11718) [30]. They reported growths of 6.6×10^{10} and 5.7×10^{9} CFU/mL for LC Shirota and *L. plantarum*, respectively, after 48 h of fermentation at a temperature of 37 °C. Elsewhere, *L. plantarum* and LC strains were used to ferment sterile Roselle juice (*Hibiscus sabdariffa*) at a temperature of 30 °C for 72 h [31]. Roselle calyces boast a high crude protein content ranging from 17.4% to 17.9% on a dry basis [32]. The high nitrogen content of

Table 1 Comparison of fermentation parameters between current study (Manzanilla, et al.) and literature data

Used substrate Concentration (%)	Strain	Viable count (Log CFU/mL)	μ (h^{-1})	Process Time (h)	Temp. (℃)	pH	Titratable Acidity (%)
Matricaria chamomilla (Current study)							
10	LC	7.35	0.301	24	45	4.21	0.283
15		6.65	0.389			4.02	0.302
10	LJ	7.51	0.312	24	45	4.15	0.286
15		7.7	0.393			3.96	0.315
Beta vulgaris (Beetroot) [29]							
5.78	LC	9.22	1.75	24	30	4	0.251
	L. plantarum	9.06	0.1			4.1	0.520
Aloe vera [30]							
25	LC	7.14	0.32	24	30	4.62	0.701
50		7.54	0.24				
75		9.32	0.15				
100		10.81	0.02				
25	L. Plantarum	8.2	0.31	24	30	5.6	0.200
50		8.43	0.22				
75		9.39	0.19				
100		9.75	0.16				
Hibiscus sabdariffa (Roselle) [31]							
7	LC	9.67	0.165	72	30	3.3	1.130
14		9.80	0.181			3.3	1.240
7	*L. Plantarum*	8.55	0.049			3.26	1.190
14		8.78	0.35			3.19	1.400

(Log CFU/mL), Log 10 transformation of colony forming units per milliliter. *Lactobacillus plantarum* has been reclassified as *Lactiplantibacillus plantarum* [10]

Roselle calyces contributes to sustaining elevated viable cell counts (up to 9.80 Log CFU/mL) throughout extended fermentation periods of up to 72 h. This comparative analysis underscores that the substrate's composition, particularly its protein and essential micronutrient content, is a decisive factor in fermentation performance and probiotic viability. The maximum specific growth rates (μ) observed for our strains (0.301–0.393 h^{-1}) are substantially lower than those often reported in rich laboratory media (>1 h^{-1}) but are highly meaningful within the context of a nutrient-challenged plant matrix. These rates indicate that both LC and LJ successfully adapted their metabolism to the chamomile infusion, initiating steady growth despite the constraints. The moderated growth is not an indicator of poor strain viability but rather a reflection of a nutrient-limited system where cellular machinery operates at a reduced capacity. The energy is likely diverted towards stress response mechanisms and the synthesis of essential compounds that cannot be scavenged from the environment. This is a well-established principle, as the nutritional composition of the substrate is a primary determinant of microbial growth dynamics and metabolic activity in fermented plant-based beverages as reported by Tangyu et al., (2019), who highlight that the nutritional composition of the substrate is a primary determinant of microbial growth dynamics and metabolic activity in fermented beverages [33]. In conclusion, while chamomile infusion can support the initial growth of probiotic LAB, its inherent nutritional deficiencies, specifically, its extremely low protein content and the presence of mild antimicrobials limit the maximum achievable biomass and prevent the sustained maintenance of viable populations. This highlights the necessity of nutritional fortification (e.g., with protein sources, yeast extract, or peptides) to develop a chamomile-based beverage that can effectively deliver high doses of probiotics to the consumer.

3.2 Growth rate of LAB

The specific growth rate (μ) of the LAB strains showed significant differences ($p < 0.05$) depending on sucrose concentration (Fig. 2). For LJ, the growth rates were 0.313 h^{-1} at 10% sucrose and 0.394 h^{-1} at 15% sucrose, while LC exhibited slightly lower values of 0.301 h^{-1} and 0.389 h^{-1}, respectively. Although the differences between strains under the same treatment were not statistically significant, LJ consistently showed a modest

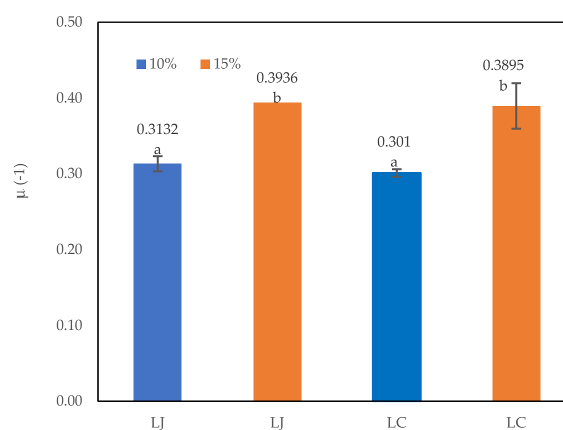

Fig. 2 Specific grow rate (μ) of the LAB growing in sucrose based medium and chamomile infusion at the end of 24 h of fermentation. Different letters showed significant difference ($p < 0.05$)

advantage over LC, suggesting a higher metabolic efficiency in utilizing sucrose under the tested conditions. Both strains demonstrated steady and reproducible growth across treatments, and while the values are lower than the growth rates (> 1 h^{-1}) reported in sugar-rich media by other authors, the performance observed here is notable given the nitrogen-limiting nature of the chamomile infusion. The results indicate that LAB can adapt to the plant-based substrate and maintain stable metabolic activity, albeit at moderated growth rates, which reflects the nutritional constraints of chamomile compared with protein-rich substrates such as milk. Thus, the lower μ values do not reflect poor viability but rather the expected performance in a nutrient-limited system.

3.3 Fermented broth pH

During fermentation, both strains exhibited a marked decline in pH across treatments, with a significant effect of sucrose concentration ($p < 0.05$) but no significant differences between strains within the same treatment (Fig. 3A). These results showed increased production of lactic acid. At 10% sucrose, LJ and LC reached final pH values of 4.15 and 4.21, respectively, while at 15% sucrose the values were lower, at 3.96 and 4.02 (Fig. 3A). This enhanced acidification at higher sucrose levels is a direct result of increased glycolytic flux and subsequent LA production. The antimicrobial effect of this acidification is not solely due to the low pH but is profoundly influenced by the dissociation state of the LA produced. LA (pKa ≈ 3.86) exists in a dynamic equilibrium between its undissociated (non-polar) and dissociated (anionic) forms. In the acidic environment achieved here (pH ~ 4.0), a significant fraction of the acid remains undissociated. This uncharged molecule can freely diffuse across the microbial cell membrane. Once inside the more neutral cytoplasm of competing microorganisms, it dissociates, releasing hydrogen ions (H^+) and acid anions. This intracellular acidification overwhelms the cell's pH homeostasis mechanisms, collapsing the proton motive force and inhibiting crucial enzymatic activity, thereby providing a strong barrier against the cell growing [34–36]. Interestingly, the producing LAB strains themselves are highly adapted to this acidic environment. They possess efficient proton extrusion systems, such as the F_0F_1-ATPase, which consumes ATP to pump excess H^+ out of the cell, maintaining a viable intracellular pH [37]. This explains why both strains maintained acidification without experiencing strong product inhibition themselves. The observed gradual stabilization of pH beyond 24 h, therefore, is not primarily due to LA toxicity to the producers but rather indicates a metabolic slowdown. This shift is likely associated with the exhaustion of limited nitrogen sources, combined with the cumulative bacteriostatic effect of intrinsic chamomile compounds such as α-bisabolol and flavonoids, which are known to act synergistically

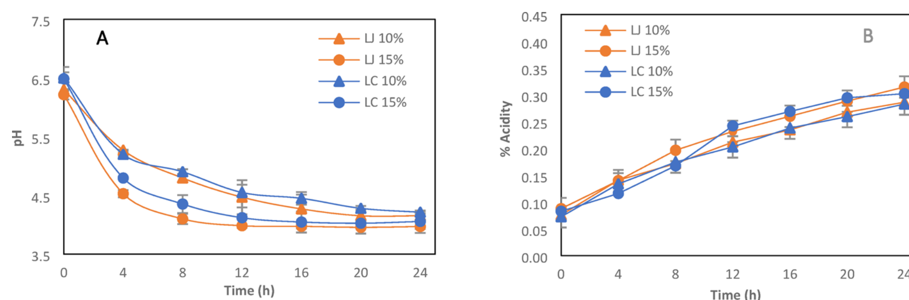

Fig. 3 Kinetics of pH (**A**) and acidity (**B**) during chamomilla infusion fermentation by LAB. There was a significant effect of sucrose concentration ($p < 0.05$) but no significant differences between strains within the same treatment

to inhibit microbial growth [38–40], forcing the cultures into dormancy-like survival states. LA production reflected these patterns, with significant differences ($p < 0.05$) between sucrose concentrations but not between strains within the same treatment (Fig. 3B). At 10% sucrose, LC and LJ produced 0.282% and 0.286% LA (± 0.04), while at 15% sucrose the values rose to 0.302% and 0.314% (± 0.06) (Fig. 3B). Once again, LJ exhibited a slightly higher acidification capacity. Although these concentrations are lower than those reported in nutrient-rich substrates, the achieved levels are sufficient to act as an effective preservative due to the mechanism described above. The reduced titratable acidity likely stems from the absence of sufficient nutrients (nitrogen, phosphorus, magnesium) essential for sustained high-yield metabolism, limiting the total biomass and thus the volumetric acid production [41, 42]. Overall, these results confirm that sucrose supplementation enables both strains to effectively acidify the chamomile infusion. The achieved pH values below 4.2, coupled with the presence of undissociated LA, create a synergistic hurdle that not only ensures probiotic viability but also guarantees microbial safety by severely inhibiting the growth of pathogenic bacteria and fungi.

3.4 Microbiological analysis of fermented beverages

Microbiological safety is a critical parameter in the development of functional beverages, as it ensures both consumer protection and regulatory compliance. In this study, microbiological analyses were performed on the final fermented products to evaluate the effectiveness of sterilization and hygienic measures, as well as to confirm the absence of potentially harmful microorganisms. The parameters tested included mesophilic aerobic bacteria (MAB), fungi and yeasts (F-Y), total coliforms (TC), and fecal coliforms (FC). All results were within the permissible limits established by international and national regulations, including the Mexican Official Standard NOM-093-SSA1-1994 and the standards governing fermented dairy products such as live yogurt cultures (Codex A-11[A]/1975; RD 179/2003; BOE 18/02/03). Importantly, the observed microbial counts for undesirable microorganisms were far below the viable counts of the probiotic strains themselves ($\geq 1 \times 10^6$ CFU/mL), confirming that the beneficial LAB dominated the fermentation process (Table 2). This dominance is essential, as it reduces ecological niches for spoilage or pathogenic organisms, thereby enhancing the microbiological stability of the beverage. A notable finding was that fermentations supplemented with 15% sucrose supported higher LAB counts compared to the 10% treatments. This observation suggests that sucrose concentration not only promoted probiotic growth but also indirectly contributed to microbial safety by accelerating acidification and lowering pH,

Table 2 Microbiological counts in the product

Strain	Substrate (%)	Microbiological counts				
		LAB	**MAB**	**F-Y**	**TC**	**FC**
		***Allowed Limit**				
		≥ 6	**≤ 3.7**	**1.0**	**< 2**	**< 3**
			****Log [CFU/mL]**		**MPN /mL**	
LC	10	7.35 ± 0.09	3.42 ± 0.12	ND	ND	ND
	15	7.61 ± 0.05	3.58 ± 0.12	ND	ND	ND
LJ	10	7.51 ± 0.09	3.46 ± 0.07	ND	ND	ND
	15	7.7 ± 0.03	3.62 ± 0.01	ND	ND	ND

ND no detected, *MAB* Mesophilic Aerobic Bacteria, *F-Y* Fungus-Yeast, *TC* Total coliforms, and *FC* fecal coliforms, *MPN* Most Probable Number. **(Log CFU/mL), Log_{10} transformation of colony forming units per milliliter. *The allowed limits were stablished by the Mexican Official Standard NOM-093-SSA1-1994

conditions under which spoilage bacteria and fungi are less likely to proliferate. This dual effect improved probiotic viability and enhanced microbiological stability underscores the role of sugar supplementation as both a metabolic substrate and a protective factor in plant-based probiotic fermentations. In addition, the turbidity observed in the final products can be attributed to dense LAB growth, which is often considered a positive indicator of fermentation success. In probiotic beverage development, such turbidity has been associated with viable microbial presence and is generally accepted by consumers as a natural characteristic of "living" fermented products. However, further optimization of sensory quality (e.g., flavor, texture, appearance) may be necessary for broader consumer acceptance. Overall, the microbiological profile obtained demonstrates that chamomile-based probiotic beverages, when produced under controlled fermentation conditions, are both safe for consumption and effective in delivering viable probiotic populations. This outcome is particularly relevant for positioning chamomile infusion as a potential non-dairy alternative substrate for probiotic beverages.

3.5 Quantification of reducible phenolic antioxidants (Folin-Ciocalteu Assay)

The absorbance of gallic acid solutions was measured to generate a standard curve, which served to quantify the fermented samples that received the highest ratings from the sensory evaluation panel, corresponding to samples LJ10% and LJ15%, and compared against a fraction of the base plant infusion (BPI) before fermentation. All measurements were performed at 760 nm in triplicate, with samples previously centrifuged at 10,000 rpm for 10 min and the cell mass removed to avoid analysis errors. It is known that the Folin reagent contains molybdate and sodium tungstate, which react with any type of phenol to form complexes, a phenomenon in which electron transfer occurs at a basic pH, reducing the complexes into intensely blue chromogenic oxides when heat is applied, proportionate to the number of hydroxyl groups in the molecule in question [23]. The experimental results showed that there is a significant difference between each evaluated sample, and visually, the intensity of the blue coloration pattern increased according to the type of treatment, with a higher intensity observed when the substrate concentration was 15% compared to 10%. This response has been observed in these strains as they increase the content of biologically relevant compounds (including phenols) when they undergo fermentative processes compared to the original compounds present in the base infusion, highlighting their generative potential. Considering that the base infusion had a very low concentration of antioxidants, it was also observed that the blue coloration pattern is proportional to the quantity of phenols, albeit in very small proportions that are typically consumed by humans. Both treatments increased their oxidative capacity by 2.62 and 5.20 times, respectively, compared to the base infusion as reported in Table 3. It has been reported that various factors contribute to the development or increase in oxidative power during fermentative processes, including the concentration of the infusion, the age of the plant base, strain age, nutrient availability, osmotic stress and the

Table 3 Total oxidative capacity content expressed in µg of Gallic acid per mL of sample

Sample	µg GA/mL	Times increase
IPB	0.168 ± 0.011	0.0
LJ10%	0.610 ± 0.010	2.62
LJ15%	1.042 ± 0.014	5.20

µg GA/mL, microgram of Gallic Acid per milliliter of product

The data are the average of three replicates of the experiment

possibility that the gradual increase observed in the treatments might be related to survival strategies of the strains, such as the release of metabolic byproducts (e.g., organic acids, hydrogen peroxide, or exopolysaccharides) into their environment [43]. Studies have found that seemingly identical products of *M. chamomilla* exhibit significant heterogeneity in terms of extraction yield, chemical composition, and antioxidant effects [44]. It has also been demonstrated that the content of polyphenols increases during fermentation. For example, the fermentation with yeast and Lactobacillus has been shown to increase the content of phenolic compounds during millet fermentation [45]. In other studies, lactic acid fermentation was found to increase the concentrations of total phenols, flavonoids, and anthocyanins, which were 5 to 10 times higher than those found in chemically acidified and non-fermented controls. The ability of *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) to degrade polyphenolic compounds has been documented and considered a metabolic strategy to adapt to hostile environmental niches [46]. Furthermore, Curiel et al. found that all phenolic acids increased, but primarily gallic and ellagic acids [47]. Both compounds can be released through tannase activity. Tannase, or tannin acyl hydrolase (EC 3.1.1.20), catalyzes the hydrolysis of ester bonds present in hydrolysable tannins and gallic acid esters. Therefore, the presence of carbon-rich sources and phenolic compounds (such as those found in beverages with the infusion) in a fermentative medium would allow these microorganisms to efficiently degrade gallotannins using this enzyme, undoubtedly leading to high energy expenditure depending on the number of compounds present (assuming this phenomenon occurred in the current study). Consequently, it was determined that there is a significant effect ($p < 0.05$) on the total polyphenol content in the incorporation of both treatments compared to the control Fig. 4.

3.6 Sensory acceptance and product implications

In general, favorable results were obtained in the evaluation of the beverages. Figure 5 Shows the preference for the strain LJ, which resulted in the beverage with the higher acceptance. We fail to reject the null hypothesis since there is no difference in the average ratings between the four attributes (Odor, Color, Taste and Texture). In other words, all attributes are liked or disliked equally overall. There is no statistically significant evidence that the average rating differs between Odor, Color, Taste and Texture. The slight variations in the data (e.g., Color has a higher "neither" rating) are not large enough to be considered different from random chance in this statistical model since the p-value is 1 which is extremely high. It is not even close to being significant. However, for rating categories (Columns) there is a highly significant difference ($p = 0.0033$). Consumers showed a clear pattern in how they rated the product, favoring the neutral-to-positive options. The main insight is that the product's sensory attributes are perceived consistently, and the overall product reception is cautiously positive. Particularly, the panel showed a slight preference for beverages with a higher concentration of sugar (those who prefer sweeter tastes), engaging their senses with treatments LC and LJ with 15% of sucrose. Additional details regarding beverage preference and purchase intention are provided in the Supplemental Material (Figs. S1–S6). These figures present a broader view of the panelists' responses across all treatments, highlighting differences in sensory acceptance and willingness to purchase. This supplemental analysis supports the main findings shown in Fig. 5, reinforcing the observed preference for LC and the

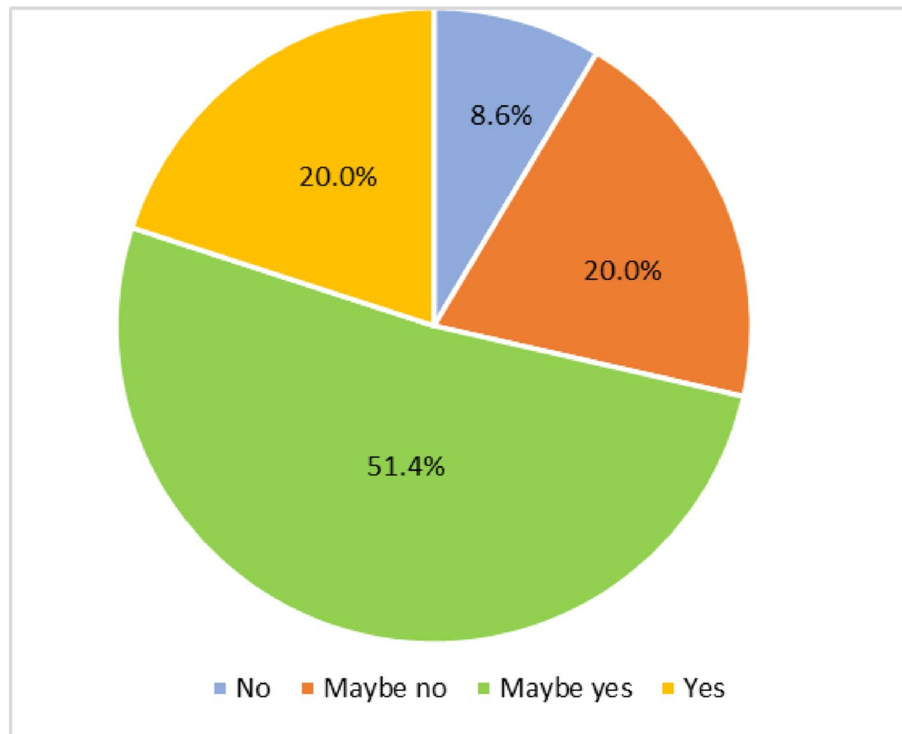

Fig. 4 Preference of the product from LJ after its consumption regarding to buy or not buy it

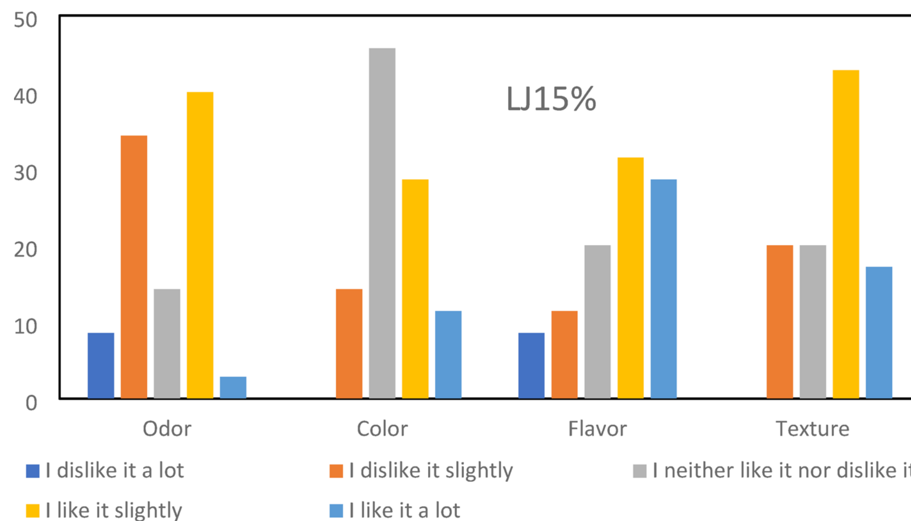

Fig. 5 Product attributes used to evaluate product preference for LJ using 15% of substrate. For rating categories (Columns) there is a highly significant difference ($p = 0.0033$)

enhanced acceptance of formulations with higher sucrose content, particularly those with 15% sucrose in treatments LC15% and LJ15%. These treatments stood out more in terms of taste, aroma, and texture, and received positive feedback and suggestions after consumption. Additionally, 37% and 51% mentioned that they would possibly buy each beverage if it were to be commercialized (Fig. 4). Treatments of 10% for LC and LJ received greater acceptance from the health-conscious audience (those who do not consume too much sugar). They were particularly appreciated for their taste and special

color parameters. They also provided feedback for potential product improvements in terms of presentation and coloration. Furthermore, 31% and 49% expressed interest in purchasing each beverage if it were to be commercialized. Statistically significant results revealed that the beverages with the highest overall acceptance in all evaluated parameters were those of the LJ strain, especially the 10% concentration showed in Fig. 4. The results regarding the preference of the beverage containing the probiotics are showed in Fig. 5. As can be seen, there is a positive result toward the acceptance of the product. According to the comments of the participants, perhaps the product could have a better acceptance if the sugar concentration is reduced, and the acidity is increased. Both cases are possible, because the concentration of sugar is manageable, and the acidity could be increased by adding nitrogen sources to favor microorganism growth. The sensory evaluation revealed a generally favorable reception for the developed probiotic beverages. Consistent with the statistical analysis indicating no significant difference in the average ratings between Odor, Color, Taste and Texture ($p = 1$), our data suggests that the product's sensory profile is perceived as cohesive. This internal consistency is crucial, as dissonance between sensory attributes can lead to consumer rejection [48]. However, a deeper, attribute-level analysis of the most accepted strain, LJ, provides more nuanced insights. While the overall model showed no difference, the distribution of hedonic responses indicates that Color was the leading attribute, achieving the highest net acceptance index (+ 65%), followed by Flavor (+ 40%) and Texture (+ 15%) (Fig. 5). This suggests that the visual appeal of the beverage was a primary driver of initial acceptance, a factor well-documented in food science as critical for first-time consumers [49]. The highly significant difference ($p = 0.0033$) between rating categories confirms that panelists distinctly favored neutral-to-positive options, indicating a cautiously positive overall reception rather than a polarized response. This pattern is common in novel functional foods, where consumers may be hesitant but open to new products [50].

Our findings agree with the well-established principle that sucrose content is a dominant factor in the acceptance of fermented beverages. The clear preference for treatments with 15% sucrose (LC15% and LJ15%) (Supplemental materials, Tables $S_{1,2,3}$) is consistent with studies on other probiotic products, where sweetness effectively masks the sour and bitter notes often associated with lactic acid bacteria (LAB) [51]. For instance, our overall acceptance rates of 37–51% for the 15% sucrose formulations are comparable to those reported for probiotic fruit-based beverages, which often struggle to balance palatability with functional claims [52]. However, a significant segment of health-conscious consumers (31–49%) showed a willingness to purchase the 10% sucrose formulations. This bifurcation in consumer preference highlights a critical market trend: the simultaneous demand for indulgent and "better-for-you" options. Our ability to achieve measurable acceptance even at a lower sugar level is a promising finding, suggesting that with further optimization, a reduced-sugar variant could successfully cater to this growing market segment.

3.6.1 Study limitations

While this study provides valuable insights, certain limitations must be acknowledged. The use of a convenience sample of 30 students, while common in preliminary sensory tests, limits the generalizability of the findings to the broader population. Furthermore, the single-exposure tasting protocol effectively measures initial acceptance but cannot

predict long-term consumer liking, which is essential for a successful commercial product [53, 54]. Finally, the study design confounds the effects of sucrose concentration and potential acidity; the higher sugar content not only adds sweetness but may also be balancing the acidity produced by the LAB, a variable that was not independently measured in this phase.

3.6.2 Future perspectives

Based on these findings, several avenues for future research are proposed.

1. Sugar-Acid Optimization: Future work should focus on systematically varying sucrose concentration and titratable acidity to identify the optimal sensory "sweet spot," potentially allowing for a reduction in sugar without compromising overall liking, as suggested by our panelists.
2. Alternative Sweeteners: Investigating the use of natural non-nutritive sweeteners (e.g., stevia, monk fruit) could help develop a low-calorie version that maintains consumer acceptance for health-conscious segments.
3. Longitudinal Acceptance Studies: Conducting repeated exposure tests (e.g., over 5–7 days) would determine if the initial cautious positivity translates into sustained liking, a key factor for repeat purchases.
4. Probiotic Viability in the Final Formulation: The ultimate success of a probiotic beverage depends on the survival of viable bacteria until consumption. Future studies should correlate sensory acceptance with the stability of the probiotic strains in the optimized formulation throughout its shelf life.

In conclusion, the LJ strain, particularly at 15% sucrose concentration, yielded a probiotic beverage with promising sensory acceptance, primarily driven by its color and flavor. The study successfully identifies both the potential and the current limitations of the product. By addressing the optimization of the texture and sugar-acid balance and by validating these findings with a larger and more diverse consumer group, this research lays a solid foundation for the development of a marketable probiotic beverage that meets the evolving demands of modern consumers.

3.7 Cell viability

A crucial part of the various food tests involves checking their shelf life, and for this reason, this process was carried out under controlled time and temperature conditions (refrigeration at 4 °C for 4 weeks). The results obtained showed a clear decrease in the viable counts of probiotic lactic acid bacteria (BAL) in general for both strains of beverages (LJ & LC) in both treatments (10% & 15% w/v) over time. During fermentation, both strains exhibited differences across treatments, with a significant effect of sucrose concentration ($p < 0.05$) but no significant differences between strains within the same treatment (Fig. 6). Aliquots were taken from each sample at 0, 7, 14, 21, and at the end of the 28 days (4 weeks). Specifically, the lactic acid cultures in both treatments for LC beverages gradually reduced their viability, starting with logarithmic values of 6.84 and 7.06, respectively, and ending at 6.0 and 6.15 after the 4-week period. On the other hand, both treatments for LJ beverages also reduced their culture viability, starting with values of 6.93 and 7.20, respectively and ending at 6.15 and 6.30 by the end of the period (Fig. 6). Although the fermentation conditions were not the optimum for instance, there

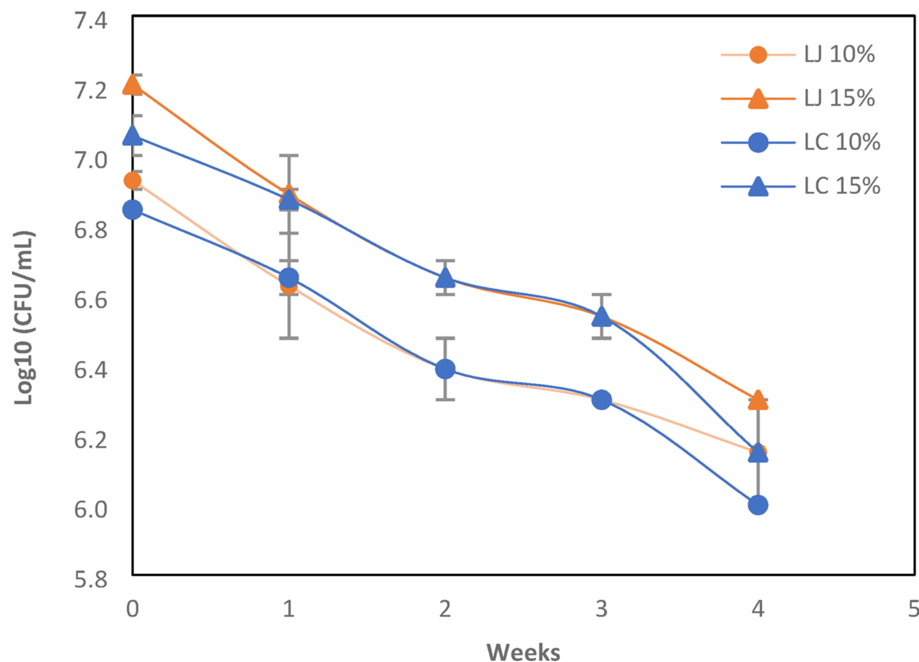

Fig. 6 Microbial shelf-life kinetics of probiotic beverage expressed as the Log (CFU/mL). There was a significant effect of sucrose concentration ($p < 0.05$) but no significant differences between strains within the same treatment. (Log_{10} CFU/mL), Log_{10} transformation of colony forming units per milliliter of product

was a lack of nitrogen source. Even with the presence of the previously mentioned factors, they still retained their sensory characteristics (Fig. 5). To be considered a probiotic food, it generally needs to have counts of >106–108 or 108–1010 CFU/mL for each ingested dose [55, 56]. Following this criterion, each generated beverage proved to be viable under the evaluated conditions for the growth of probiotic BAL. Other factors contributing to the decrease in probiotic organism viability may depend on the level of oxygen in the products, oxygen permeability of the packaging, handling, pH reduction of the medium, and the accumulation of organic acid as a result of growth [57].

4 Conclusion

This study demonstrated that *Lacticaseibacillus casei* Shirota (DN-114 001) and *Limosilactobacillus johnsonii* (NCC533) can successfully ferment chamomile infusion supplemented with sucrose, producing beverages that met probiotic standards (≥ 10^6 CFU/mL) and complied with Mexican sanitary regulations. Both strains responded to substrate concentration, but showed comparable growth performance under the same conditions. The beverage produced with *L. johnsonii* at 10% sucrose achieved the highest sensory acceptance, indicating that both sucrose concentration and strain selection are critical parameters for optimizing consumer appeal.

Chamomile infusion proved to be a versatile fermentation substrate, supporting microbial growth, lactic acid production, and pH reduction, while also enhancing total phenolic content. These results confirm the feasibility of developing a refreshing, shelf-stable probiotic beverage using chamomile infusion. Future studies should focus on elucidating strain-specific metabolic contributions and their interaction with plant-derived bioactive compounds to further improve product quality and functionality.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Author contributions

M.H.H.E.,—writing original draft. M.H.H.E ., N.M.L. G., H.S.F.,—formal analysis. C.T.J., C.Z.O, N.M. A.,—data analysis, review & editing. N.H. C., C.T. J, O.O.A—data curation. N. M. A., M.H.H.E., N.H.C—conceptualization, original draft, review & editing. H.S.F., O.O.A, N. H.C.,—conceptualization, review & supervision. All authors read and approved of the final manuscript.

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Data availability

Please contact authors for data requests.Nolasco-Hipolito CiriloUniversidad del Papalaoapan. Scientific Research Centere-mail: cnolasco@unpa.edu.mx.

Code availability

Not applicable.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Code of Ethics, Rules of Integrity and Code of Conduct of the University of Papaloapan and was approved by the University's Research Ethics Committee. All procedures involving human participants were in accordance with the institutional guidelines and the ethical principles of the Declaration Helsinki.

Consent for publication

The participants have consented to the submission of the data collected in this study to the journal.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare no competing interests.

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