Lacticaseibacillus paracasei 12B0-2: Study of the Functional Properties of a Buffalo Whey Isolate

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Article

Lacticaseibacillus paracasei 12B0-2: Study of the Functional Properties of a Buffalo Whey Isolate

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Abstract: The buffalo whey resulting from cheese production contains lactic acid bacteria (LAB) that may have probiotic potential. The isolate *Lacticaseibacillus paracasei* 12B0-2 from buffalo whey was investigated to identify its potential as a probiotic candidate. Its harmlessness was evaluated through hemolytic activity, presence of the gelatinase enzyme and its susceptibility to antimicrobials. The production of exopolysaccharides (EPS), biofilm formation and antimicrobial activity were considered. Gastrointestinal tract (GIT) tolerance, adhesion capacity using Caco-2 cells, and aggregation and hydrophobicity capabilities were determined. Microencapsulation of the isolate was evaluated in sodium alginate. *L. paracasei* 12B0-2 did not show hemolytic activity or gelatinase production and was sensitive to most of the antimicrobials tested. It produced EPS, showed moderate biofilm formation and promising antimicrobial activity. Its cells were tolerant to the GIT, presenting 99.46 % survival. It showed 75.82 % adhesion to Caco-2 cells, 38.29 % auto-aggregation, 38.36 % co-aggregation with *Escherichia coli* ATCC 10536 and 35.83 % with *Listeria monocytogenes* ATCC 7644. It had 15.9 % hydrophobicity with n-hexadecane and 14.41 % with xylene. Finally, its microencapsulated cells had 93.89 % GIT survival. Concluding that *L. paracasei* 12B0-2 has potential as a probiotic bacterium.

Keywords: buffalo whey; *Lacticaseibacillus*; microbiology; probiotic; safety

1. Introduction

Whey is the liquid fraction resulting from milk protein coagulation during cheese production [1]. It can be a by-product with high nutritional value [2] and a co-product widely used in the food industry [3]. However, it remains the most polluting cheese by-product due to its high organic load [4]. To reduce waste, whey has been employed in industrial bioproduction processes, leading to high-value-added products [5]. Furthermore, it serves as an excellent substrate for the fermentation of lactic acid bacteria (LAB), particularly probiotic strains [6].

Probiotics are "live microorganisms that, when administered in adequate quantities, confer a health benefit on the host" [7]. They must be safe, functional, and suitable for use in both culinary and therapeutic settings, including for immunocompromised individuals [8]. Their survival during food production, storage, and passage through the gastrointestinal tract (GIT) is essential [9]. Probiotics are commonly used in functional dairy foods to control spoilage and pathogenic microorganisms [10]. Interest in, and studies on, the microbiota of dairy products continue to grow [11]. Among these, LAB, especially *Lactobacilli*, play a key role, with various applications and well-documented benefits [12]. Within the genus, *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*) has shown promise, with many studies demonstrating its potential [13–15].

L. paracasei has shown potential in managing diabetes [16,17], hypertension [18,19], cholesterol and triglycerides [20,21], and mental health issues like anxiety and depression [22,23]. It may also alleviate asthma symptoms [24,25], modulate immunity in conditions like celiac disease [26,27], and

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contribute to cancer prevention [28,29]. Furthermore, it demonstrates antimicrobial activity against *Yersinia enterocolitica* subsp. *enterocolitica* [30], *Staphylococcus aureus* [31], *Salmonella* Typhimurium [32], *Escherichia coli* [33], and *Candida albicans* [34]. Therefore, the prospecting of a new probiotic candidate was proposed.

2. Material and Methods

2.1. Microorganism and Cultivation Conditions

Lacticaseibacillus paracasei 12B0-2 was isolated from raw buffalo milk whey collected at the Agricultural Experimental Station of the Federal University of Rio Grande do Sul (Eldorado do Sul-RS), in previous work (data not yet published). The isolate was preserved in 10% glycerol at -20 ºC and reactivated in Man Rogosa and Sharpe broth (MRS; Merck, Darmstadt, Germany) at 37 ºC for 48 hours.

Indicator cultures were used in this work: *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 10536, *Salmonella* Enteritidis ATCC 13076, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 35984, *Corynebacterium fimi* NCTC 7547, *Lactobacillus rhamnosus* FAGRON™ (Fagron, Brazil), *Citrobacter freundii* F30, *Enterobacter aerogenes* B01, *Listeria innocua* C08, *Staphylococcus sciuri* A0902, *Proteus hauseri* BJX, *Proteus vulgaris* C30, *Listeria seeligeri* BP OXFORD, *Listeria welshmeri* PF OXF, *Listeria seeligeri* PB PALCAM, *Listeria innocua* L07, *Listeria monocytogenes* 4B, *Listeria seeligeri* BQ OXFORD, *Listeria innocua* 6B, *Listeria monocytogenes* 17078/03, *Listeria innocua* L10, *Listeria monocytogenes* 4C and *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella pneumoniae* 376, *Klebsiella pneumoniae* 378, *Klebsiella pneumoniae* 379, *Klebsiella pneumoniae* 380, *Klebsiella pneumoniae* 381, *Klebsiella pneumoniae* 382, *Clostridium difficile* CTI/HU RT 106 (Fiocruz) and *Clostridium difficile* ES11 (Fiocruz).

Lactic acid bacteria were maintained on MRS agar, while pathogenic bacteria were preserved on Tryptic Soy Agar (TSA; Kasvi, São José dos Pinhais, PR, Brazil) cultivated at 37ºC in aerobiosis, except for cultures of *Clostridium difficile* that were grown in Brain Heart Infusion (BHI; Oxoid, Basingstoke, England) at 37ºC in anaerobiosis (Anaerobac).

2.2. Partial Sequencing of the 16S rDNA Gene

The microorganism under study was identified by partial 16S rDNA gene sequencing. Genetic material was extracted by thermal lysis [35], and primers 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 530R (5′-CCGCGGCTGCTGGCACGTA-3′) [36] were used in the polymerase chain reaction (PCR), employing 2.5 U Taq DNA polymerase (Quatro G Biotecnologia), 0.2 mM dNTPs, 50 mM $MgCl_2$, and 10 pmol of each primer. Amplified DNA fragments were analyzed by ACTGene Análises Moleculares Ltd.a. (Biotechnology Center, UFRGS, Porto Alegre, RS) using an AB 3500 Genetic Analyzer sequencer equipped with 50 cm capillaries and POP7 polymer (Applied Biosystems). A phylogenetic tree was constructed from the partial 16S rDNA sequence using Mega 11: Molecular Evolutionary Genetics Analysis software [37], applying the neighbor-joining method [38].

2.3. Safety Assessment

Hemolytic activity was assessed by cultivating the bacterium on Columbia blood agar (CBA; HiMedia, Mumbai, India) [39]. Gelatinase activity was evaluated as previously described [40]. Susceptibility of *L. paracasei* 12B0-2 to ten antimicrobials was tested using the disk diffusion method [41] on Mueller-Hinton agar (MH; BD, Franklin Lakes, USA). The antibiotics were clindamycin (2 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), vancomycin (30 μg), tetracycline (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), erythromycin (15 μg), ampicillin (10 μg), and penicillin G (10 μg). Results were interpreted according to Charteris et al. [42] and the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST) [43]. *Staphylococcus aureus* ATCC 25923 was used as a positive control in both tests.

2.4. Exopolysaccharide Production

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L. paracasei 12B0-2 was cultivated on Congo red agar according to Freeman et al. [44]. A positive result was indicated by the presence of black colonies with a dry crystalline consistency. *S. aureus* ATCC 25923 was used as a positive control.

2.5. Biofilm Formation

Biofilm formation was assessed as described by Stepanović et al. [45], using 96-well plates in octuplicate. *L. paracasei* 12B0-2 (0.5 McFarland) was cultivated in MRS broth, while *Staphylococcus epidermidis* ATCC 35984 in Tryptic Soy Broth (TSB; Kasvi, São José dos Pinhais, PR, Brazil) served as a positive control [46]; TSB alone as a negative control. Optical density (OD) was measured using an ELISA reader. Samples were classified according to Masebe and Thantsha [47] as non-biofilm forming (OD ≤ ODc), weak (ODc ≤ OD ≤ 2x ODc), moderate (2x ODc < OD ≤ 4x ODc), or strong biofilm formers (4x ODc > OD), where ODc is the mean OD of the negative control.

2.6. Assessment of Antimicrobial Activity

The antimicrobial activity of *L. paracasei* 12B0-2 was evaluated using two methods. First, the modified "Spot-on-the-lawn" technique [48] tested its effectiveness against the indicator strains. An inoculum (1 % v/v) of *L. paracasei* 12B0-2 was prepared in MRS broth and incubated under microaerophilic conditions. The culture was centrifuged at 7000 x g for 15 min to obtain the cell-free supernatant (CFS), which was heated to 80 ºC for 10 min. After cooling, 20 µL aliquots were pipetted onto MH agar plates seeded with indicator strains (0.5 McFarland). Second, *L. paracasei* 12B0-2 was tested against *Clostridium difficile* CTI/HU RT 106 (human clinical sample) and *Clostridium difficile* ES11 (canine clinical sample), using the adapted "Agar spot test" method [49]. *L. paracasei* 12B0-2 was cultured (1 % v/v) in MRS broth under microaerophilic conditions, and aliquots were pipetted onto MRS agar and incubated under the same conditions. *C. difficile* isolates were cultured anaerobically (Anaerobac) on TSA agar. MRS plates were overlaid with thioglycolate agar seeded with *C. difficile* (1.0 McFarland) and incubated anaerobically. Inhibition halos were measured in millimeters (mm).

2.7. Assessment of Probiotic Potential

2.7.1. Gastrointestinal Tract Tolerance

To evaluate tolerance to GIT conditions, cell counts of *L. paracasei* 12B0-2 were performed using the method of Miles, Misra and Irwin [50] on Plate Count Agar (PCA; Kasvi, São José dos Pinhais, PR, Brazil) in three conditions: before exposure to GIT, after exposure to simulated gastric juice (SGJ). and after exposure to simulated intestinal juice (SIJ) [51,52].

2.7.2. Ability to Adhere to Caco-2 Cells

A 96-well plate containing 2 x 10^5 Caco-2 cells was inoculated with a suspension of *L. paracasei* 12B0-2 (10^8 CFU/mL). Aliquots were collected for PCA counting and the culture was incubated at 37 ºC for 3 h. After incubation, the supernatant was discarded and the wells washed with PBS. Then, 0.5 % TRITON-X 100 was added and incubated at room temperature for 10 min. New aliquots were collected for counting. Bacterial adhesion to Caco-2 cells was calculated using the formula (R0/Rt) × 100, where R represents the Ratio = (bacterial cell concentration/adhered CaCo-2 cell concentration). R0 and Rt are the initial ratio and the ratio after the incubation period, respectively [53].

2.7.3. Auto-Aggregation, Co-Aggregation and Hydrophobicity

Auto-aggregation was assessed as described by Kumari et al. [54], with minor modifications. *L. paracasei* 12B0-2, cultured in MRS broth, was centrifuged at 5000 x g for 15 min, washed with PBS (pH 7.2) to an OD (600 nm) of 0.5.

To assess coaggregation between *L. paracasei* 12B0-2 and *E. coli* ATCC 10536 or *L. monocytogenes* ATCC 7644, the method of Handley et al. [55] was applied with modifications. *L. paracasei* 12B0-2 was grown in MRS broth, while the indicator strains were cultured in TSB. All cultures were centrifuged, washed with PBS (pH 7.2) and adjusted to an OD (600 nm) of 0.5. Equal volumes of *L. paracasei* 12B0-

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2 suspension were mixed with each indicator strain. Coaggregation was calculated as (((Ax+Ay) - 2(Axy)) / (Ax+Ay)) * 100, where Ax and Ay are the initial absorbance values of each strain alone, and Axy is the absorbance of the mixture. For both assays, auto-aggregation and co-aggregation, suspensions were incubated at 37 ºC for 24 h. OD measurements were taken at 0 h, 3 h, and 24 h.

Cell surface hydrophobicity was evaluated following Lee et al. [56], with adaptations. *L. paracasei* 12B0-2 was inoculated into MRS broth, centrifuged, washed with PBS (pH 7.2) and adjusted to an OD (600 nm) of 0.6-0.8. Equal volumes of cell suspension were homogenized with either n-hexadecane or xylene, vortexed for 5 min, and incubated at room temperature for 30 min for phase separation. OD of the aqueous phase was measured. Hydrophobicity (%) was expressed using the formula (1−A1/A0) × 100, where A1 is the final absorbance and A0 the initial absorbance of the suspension.

2.8. Microencapsulation

Microencapsulation was performed according to Hugues-Ayala et al. [57], with adaptations. *L. paracasei* 12B0-2 was inoculated into 20 mL MRS broth, centrifuged at 5000 x g for 10 min, washed, and resuspended in 5 mL 0.1 % peptone water. The suspension was vortexed with 25 mL 2% sodium alginate and transferred to a syringe with a needle. This mixture was dropped into 0.05 M $CaCl_2$ supplemented with 0.1 % Tween 80. After formation, the microcapsules were stabilized at room temperature for 30 min and stored in peptone water at 4 ºC. Aliquots were counted before and after microencapsulation over sixty days. To estimate its encapsulation efficiency, 1 g of microcapsules was dispersed in 9 mL 1% sodium citrate, vortexed for 5 min., and aliquots were plated on PCA by microdilution. Efficiency (%) was calculated as: Efficiency = post count/initial count * 100. Microencapsulated *L. paracasei* 12B0-2 under simulated GIT conditions was studied according to Bevilacqua et al. [58]. The results were expressed as Log10 CFU/mL.

3. Results

3.1. Partial Sequencing of the 16S rDNA gene

The isolate's identification was confirmed through partial sequencing of the 16S rDNA gene. The sequence was analyzed by the BLAST system (NCBI), finding 99.82 % similarity with others *L. paracasei* sequences available in GenBank (Figure 1).

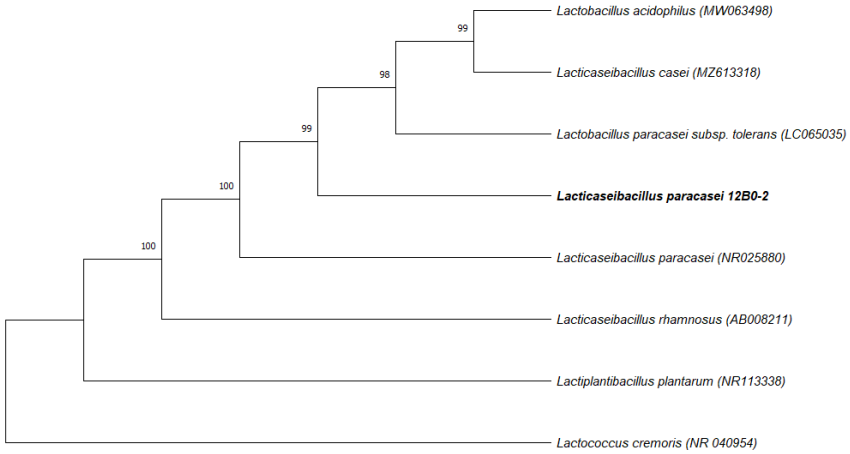

Figure 1. Phylogenetic Tree constructed based on the analysis of partial 16S rDNA using the neighbor-joining methodology. Correlation between the isolate *L. paracasei* 12B0-2 and Genbank models, the accession number in parentheses.

3.2. Safety Assessment

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The *L. paracasei* 12B0-2 isolate showed no hemolytic activity (γ hemolysis) and no gelatinase enzyme activity. *L. paracasei* 12B0-2 was sensitive to 8 of the antimicrobials tested and showed partial susceptibility in the presence of ceftriaxone and resistance to vancomycin.

3.3. Exopolysaccharide Production

EPS production from *L. paracasei* 12B0-2 was observed by the presence of black colonies with dry crystalline consistency on Congo red agar plates.

3.4. Biofilm Formation

L. paracasei 12B0-2 had an OD of 0.363 ± 0.09 and an OD of 0.144 ± 0.01 was obtained for the negative control. As a result, *L. paracasei* 12B0-2 showed moderate biofilm formation.

3.5. Assessment of Antimicrobial Activity

The CFS (pH between 3 and 4) showed antimicrobial activity against 51.72 % of the indicator bacteria. The greatest inhibitory activity was identified in the presence of the *K. pneumoniae*, presenting a distance between the indicator bacteria and the isolate of 10.67 mm ± 1.53, followed by the isolates *C. fimi* NCTC 7547, *P. vulgaris* C30, *P. hauseri* BJX and *E. aerogenes* B01 with inhibition halos of: 10.33 mm ± 1.03; 10.0 mm ± 1; 9.67 mm ± 0.58 and 9.5 mm ± 0.71, respectively. A lower inhibitory activity was presented in the presence of *S. sciuri* A0902 with a halo of 3.33 mm ± 0.58. The antimicrobial activity against the remaining isolates: *C. freundii* F30, *E. cloacae*, *K. pneumoniae* (376, 378, 379, 381, 382), *L. innocua* C08, *S.* Enteritidis ATCC 13076, presented halos with an average value of 6.78 mm in diameter. Furthermore, *L. paracasei* 12B0-2 showed antimicrobial activity against both *C. difficile* isolates tested, presenting an inhibitory activity of 6.67 mm ± 1.53 against the human clinical sample *C. difficile* CTI/HU RT 106 (FIOCRUZ) and an activity inhibitory rate of 10.33 mm ± 0.58 against the canine clinical sample *C. difficile* ES11 (FIOCRUZ) (Table 1).

Table 1. Antimicrobial activity of *Lacticaseibacillus paracasei* 12B0-2 against different pathogens, results were expressed in millimeters (mm).

Indicators	Results (mm)
Citrobacter freundii F30	8.0 ± 1
Clostridium difficile CTI/HU RT 106	6.67 ± 1.53
Clostridium difficile ES11	10.33 ± 0.58
Corynebacterium fimi NCTC 7547	10.33 ± 1.03
Enterobacter aerogenes B01	9.5 ± 0.71
Enterobacter cloacae	6.0 ± 1
Klebsiella pneumoniae	10.67 ± 1.53
Klebsiella pneumoniae 376	6.33 ± 0.58
Klebsiella pneumoniae 378	6.0 ± 0
Klebsiella pneumoniae 379	6.67 ± 0.58
Klebsiella pneumoniae 381	5.67 ± 0.58
Klebsiella pneumoniae 382	6.0 ± 1
Listeria innocua C08	8.33 ± 0.58
Proteus hauseri BJX	9.67 ± 0.58
Proteus vulgaris C30	10.0 ± 1
Salmonella Enteritidis ATCC 13076	8.0 ± 1
Staphylococcus sciuri A0902	3.33 ± 0.58

3.6. Assessment of Probiotic Potential

3.6.1. Gastrointestinal Tract Tolerance

L. paracasei 12B0-2 previously the GIT simulation presented 9.87 ± 0.05 Log10 CFU/mL and the control *L. rhamnosus* FAGRON™ 9.59 ± 0.01 Log10 CFU/mL. After SGJ, *L. paracasei* 12B0-2 (9.57 ± 0.35

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Log10 CFU/mL) showed a survival rate of 97.10 % and *L. rhamnosus* FAGRON™ (9, 34 ± 0.09 Log10 CFU/mL) presented a survival rate of 97.43 %. After SIJ *L. paracasei* 12B0-2 (9.82 ± 0.03 Log10 CFU/mL) and *L. rhamnosus* FAGRON™ (9.29 ± 0.06 Log10 CFU/mL) showed a survival percentage of 99.46 % and 96.88 %, respectively (Figure 2).

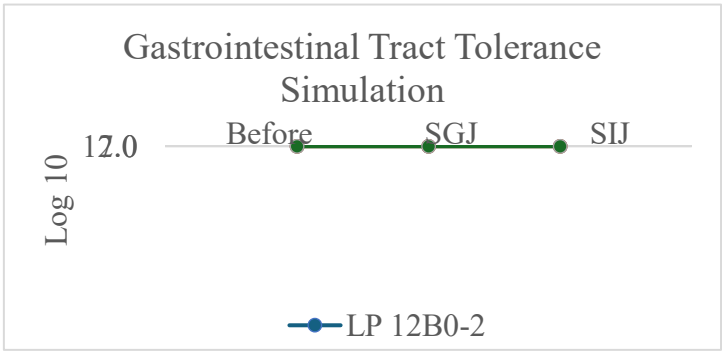

Figure 2. Cellular concentrations of isolates on a logarithmic scale, before exposure, after simulated gastric juice and after simulated intestinal juice. In blue *L. paracasei* 12B0-2, in orange *L. rhamnosus* FAGRON and in green microencapsulated *L. paracasei* 12B0-2.

3.6.2. Ability to adhere to Caco-2 Cells

L. paracasei 12B0-2 showed cell viability of 98.61 % ± 1, an average of 5708 ± 1074 bacterial cells for each Caco-2 cell and an adhesion percentage of 75.82 % ± 15.64.

3.6.3. Auto-Aggregation, Co-Aggregation and Hydrophobicity

The ability of *L. paracasei* 12B0-2 auto-aggregate after 3 h of incubation was 6.03 % ± 3.2 and at the end of 24 h it was 38.29 % ± 3.6. Its co-aggregation was 7.22 % ± 2 after 3 h and 38.36 % ± 2.3 in 24 h with *E. coli* ATCC 10536, compared to the co-aggregation with *L. monocytogenes* ATCC 7644 which was 11 .99 % ± 3.7 after 3 h and 35.83 % ± 7.2 after 24 h of incubation. The hydrophobicity of *L. paracasei* 12B0-2 in the presence of n-hexadecane was 15.9 % ± 3.51 and 14.41 % ± 2.11 in the presence of xylene (Table 2).

Table 2. Aggregation capacity and hydrophobicity of *Lacticaseibacillus paracasei* 12B0-2.

Hydrophobicity		**Auto-aggregation**		**Co-aggregation**			
				E.coli		***L. monocytogenes***	
				ATCC 10536		**ATCC 7644**	
n-hexadecane	xylene	3h	24h	3h	24h	3h	24h
15.9 ± 3.51	14.41 ± 2.11	6.03 ± 3.2	38.29 ± 3.6	7.22 ± 2	38.36 ± 2.3	11.99 ± 3.7	35.83 ± 7.2

3.7. Microencapsulation

L. paracasei 12B0-2 showed 85.67 % microencapsulation efficiency and remained viable over 60 days. Its initial count was 7.59 ± 0.34 Log10 CFU/mL, increasing to 8.04 ± 0.06 on day 10. On day 20, the count was 7.77 ± 0.18, decreasing to 7.36 ± 0.23 on day 40. At the end of the period, on day 60, the final count was 6.73 ± 0.06 Log10 CFU/mL (Figure 3). Microencapsulated *L. paracasei* 12B0-2 (8.34 ± 0.14 Log10 CFU/mL) was subjected to simulated GIT conditions, with a count of 8.28 ± 0.08 after SGJ and 7.92 ± 0.04 after SIJ (Figure 2), corresponding to 98.17 % and 93.89% survival, respectively.

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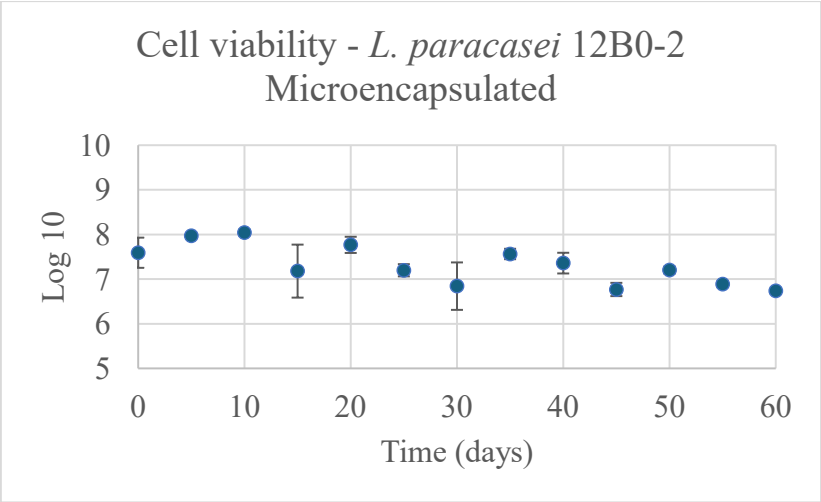

Figure 3. Maintenance of cell viability of microencapsulated *Lacticaseibacillus paracasei* 12B0-2 on a logarithmic scale.

4. Discussion

Identifying species through genotypic and phenotypic methods, following current binomial nomenclature, is required to confirm the use of an internationally recognized culture deposit [60]. Romero-Luna et al. [59] found 99 % similarity between *L. paracasei* CT12 from water kefir, and Cui et al. [61] studied four *L. paracasei* isolates (BY12, BY2, BY20, BY25) from fermented products, all with over 99 % similarity.

Hemolysis is undesirable in probiotics [62]. Gelatinase hydrolyzes gelatin, damaging host tissue and aiding bacterial spread [63], thus, its absence protects against inflammation [64]. Valdiviezo-Marcelo et al. [65] evaluated 11 LAB from artisanal cheese that lacked hemolytic and gelatinase activity. *L. paracasei* 12B0-2 was sensitive to 8 antimicrobials, partially susceptible to ceftriaxone, and resistant to vancomycin. According to Kullar et al. [66], *Lactobacillus* spp. are intrinsically resistant to vancomycin and aminoglycosides. Tang et al. [67] found *L. paracasei* LP10266 resistant to ceftriaxone, vancomycin, cefuroxime, cefazolin, and meropenem.

EPS enhances survival under adverse conditions by protecting against low pH, bile salts, and digestive enzymes, ensuring probiotic survival through the GIT [68]. They also support cell aggregation, biofilm formation, and intestinal adhesion [69]. Bhat and Bajaj [70] screened EPS production in *L. paracasei* M7 with Congo red medium, then purified the EPS to study its biotechnological potential as an antioxidant, antibiofilm agent, cholesterol reducer, and emulsifier.

Biofilms are complex bacterial communities attached to surfaces created by the extracellular matrix, composed of EPS, nucleic acids, and proteins [71]. The biofilm state confers tolerance to stressful GIT conditions, potentially enhancing probiotic activity, immunomodulatory properties, and intestinal permeability [72]. These results align with those of Chen et al. [73], in which *L. paracasei* R3 showed moderate biofilm formation. Although many LAB are strong biofilm formers, this characteristic is variable. Baliyan et al. [74] 43 LAB isolates: three were weak biofilm formers, seven showed moderate formation, and the remaining were strong formers.

LAB support balanced intestinal microbiota, enhance resistance against pathogens, and boost immune function. These benefits may stem from the production of organic acids, bacteriocins, hydrogen peroxide, and compounds such as vitamins and other metabolites with antibacterial activity [48]. Several *L. paracasei* isolates exhibit antimicrobial activity against pathogens, including: *L. monocytogenes* [75,76], *K. pneumoniae* [63], β-hemolytic *Streptococcus* and *S. aureus* [77], including methicillin-resistant *S. aureus* (MRSA) [78], *Salmonella* sp. [59], *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Enterococcus faecalis*, *Bacillus cereus* [79] and also *C. difficile* [49,80]. *C. difficile* infections often develop during antibiotic therapy, which reduces intestinal microbial diversity [81]. Probiotics help regulate the GIT microenvironment disrupted by *C. difficile* overgrowth [82].

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The GIT survival of *L. paracasei* 12B0-2 aligned with findings by Lee et al. [83] for *L. paracasei* MG4693, which showed 98.2 % survival after simulated GIT transit. Viable probiotics are essential for health benefits, requiring delivery to the intestine via oral administration, since this is their target site [84]. Zommara et al. [85] evaluated 15 LAB isolates; all survived, including *L. paracasei* BD3, which endured SGJ with a count reduction from 7.02 to 7.01 log10 CFU/mL, and after SIJ exposure reached 4.67 log10 CFU/mL.

Adhesion promotes colonization by enhancing interaction between LAB and GIT, supporting probiotic function, and enabling binding to intestinal epithelial cells [86,87]. Caco-2 models are widely used to assess probiotic adhesion capacity [88], as these cells exhibit morphological and functional differentiation, mimicking mature enterocytes [89]. Rocha-Mendoza et al. [90] reported over 85 % adhesion for *L. paracasei* OSU-PECh-BA and *L. paracasei* OSU-PECh-3B. Juntarachot et al. [91] observed 67.5 % adhesion for *L. paracasei* CNCM I-1572.

According to Kardooni et al. [92], even low co-aggregation may inhibit pathogen biofilms in the GIT. Bacterial adhesion to non-polar solvents reflects cell surface hydrophobicity [93]. More hydrophobic bacteria adhere better to surfaces, making them promising probiotic candidates [94]. The low hydrophobicity of *L. paracasei* 12B0-2 may be attributed to surface EPS [54].

Reuben et al. [95] reported auto-aggregation of 63 LAB strains ranging from 38.5 % to 54.50 %. Conversely, Baliyan et al. [72] found 36.4 % to 90.7 % among 43 LAB isolates. Fonseca et al. [88] assessed co-aggregation of 5 LAB isolates with *E. coli* (EPEC) CDC 055, observing values from 2.39 % to 65.15 %. In contrast, Reuben et al. [95] reported that *L. paracasei* G10 co-aggregated with *E. coli* ATCC 10536 (35.4 %), *E. coli* O157:H7 ATCC 43894 (34.03 %) and *L. monocytogenes* ATCC 19113 (59.96 %). Additionally, Amini et al. [96] observed 56.74 % co-aggregation between *L. paracasei* AS20 and *L. monocytogenes* ATCC 19114.

Klimko et al. [97] evaluated the hydrophobicity of 33 LAB using n-hexadecane, including three *L. paracasei* isolates (CM MSU 527, CM MSU 531, CM MSU 544), which showed 85 %, 18 % and 4 %, respectively. Furthermore, only five strains exhibited hydrophobicity above 50 %. Fonseca et al. [88] also reported wide variability using xylene, finding values ranging from 6.67 % to 96.06 % among 19 LAB strains, and 13 isolates exhibiting less than 35 %.

Probiotic microencapsulation enhances resistance to GIT stress and increase viability after processing and storage [98]. The encapsulating material is crucial for efficiency, and alginate is widely used due to its pH-responsive release behavior [99,100]. However, alginate alone presents disadvantages: its porosity may result in a faster release of loaded molecules, reducing encapsulation efficiency [101,102].

A similar microencapsulation efficiency to that of *L. paracasei* 12B0-2 was obtained by Han et al. [103], who achieved 84.46 % for a probiotic mix containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. paracasei* subsp. *paracasei*.

The cells adapted to the alginate, making it possible to maintain cell viability over the sixty days, showing a reduction of 0.86 Log10 CFU/mL compared to time zero. Encapsulation improves probiotic viability, supporting the development of dairy and non-dairy probiotic foods without altering sensory or physical properties, which can benefit human health [104].

Devarajan et al. [105] found survival rates of 71.39 % for *L. rhamnosus* MF00960, 89.34 % for *Pediococcus pentosaceus* MF000967, and 87.39 % for *L. paracasei* DSM20258 when microencapsulated in sodium alginate. These values increased to 99.01 %, 97.93 % and 92.62 %, respectively, with the addition of casein derived from camel milk. Sekhavatizadeh, Afrasiabi and Montaseri [106] assessed the GIT survival of *Lactobacillus acidophilus* ATCC 4356, reporting 30.57 % in its free form, compared to 48.43 % when microencapsulated in sodium alginate and galbanum gum.

5. Conclusion

This study investigated an isolate from buffalo milk whey, a potencial source of functional bacteria. *Lacticaseibacillus paracasei* 12B0-2 was selected as a probiotic candidate. The strain was safe, produced exopolysaccharides, formed moderate biofilm, and adhered to Caco-2 cells. Althought auto-

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aggregation, co-aggregation and hydrophobicity were lower than expected, it exhibited notable antimicrobial activity against several indicator bacteria, including *Clostridium difficile.* Importantly, *L. paracasei* 12B0-2 showed high survival rates in SGJ and SIJ, indicating potential for GIT tolerance. When microencapsulated, it maintained high viability over 60 days, despite a slight reduction in survival compared to free cells. In summary, *L. paracasei* 12B0-2 exhibited key traits of a promising probiotic candidate.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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