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Enhancing Food Quality through Probiotic Potential of *Lactobacillus helveticus* C7303: Functional and Safety Attributes from Traditional Iranian Khiki Cheese

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ABSTRACT

Lactobacillus strains, particularly *Lactobacillus helveticus*, serve as excellent candidates for dairy starter cultures and functional probiotics. This suitability stems primarily from their robust capacity for carbohydrate fermentation, efficient lactic acid production, and well-documented health-promoting properties. The present study was designed to characterize the probiotic, functional, and safety attributes of *L. helveticus* C7303, an indigenous strain isolated from traditional Iranian Khiki cheese. A comprehensive suite of evaluations was performed, encompassing carbohydrate fermentation profiles, enzymatic activities, and morph physiological properties. Furthermore, the strain's resilience was tested under various stressors, including NaCl concentrations, thermal fluctuations, acidic conditions, and simulated gastrointestinal environments. Functional indicators, such as cell surface hydrophobicity, auto-aggregation, co-aggregation with clinical pathogens, and cholesterol assimilation, were also rigorously assessed. The results demonstrated that *L. helveticus* C7303 effectively ferments essential dairy sugars including lactose, galactose, glucose, and maltose while exhibiting potent β-galactosidase activity. The strain maintained favorable viability under acidic stress. In the simulated gastrointestinal transit assays, survival rates reached 70.3 ± 10.5% in simulated gastric juice (SGJ) and 85.2 ± 2.8% in simulated intestinal fluid (SIF). Moreover, the isolate displayed high surface hydrophobicity across multiple solvents, time-dependent auto-aggregation, and robust co-aggregation with pathogens. Notably, cholesterol assimilation reached 80.4% in the presence of 0.2% bile. Given these promising functional and safety profiles, *L. helveticus* C7303 represents a high-potential candidate for use as a specialized dairy starter culture and a therapeutic probiotic.

1-Introduction

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host." They have garnered significant attention from global researchers and the modern food industry [1]. According to the FAO/WHO guidelines, probiotic evaluation requires a systematic framework. This includes accurate strain identification, rigorous safety and efficacy testing, and determining suitability for functional foods [2].

Dairy products, especially cheese, are recognized as excellent matrices for probiotic survival during human digestion. Their effectiveness stems from inherent characteristics such as pH buffering capacity, a protective semi-solid structure, and the presence of fat globules [3, 4]. Consequently, traditional cheeses are considered rich sources for isolating novel Lactic Acid Bacteria (LAB) strains due to their natural fermentation and diverse microflora. Recent studies have identified various *Lactiplantibacillus* and *Lacticaseibacillus* species from traditional cheeses in Iran, Mexico, and Ethiopia. Many of these indigenous strains exhibit remarkable acid and bile tolerance, potent antibacterial activity, and notable safety features [5, 6, 7].

The major taxonomic reclassification of the *Lactobacillus* genus in 2020 redefined many common probiotic species into new genera. This shift highlights that accurate molecular identification is now an undeniable necessity for scientific rigor and industrial standards [8]. Such precision ensures that specific health benefits are documented with scientific certainty.

The primary criteria for selecting a potential probiotic include tolerance to gastric acid and bile salts, adhesion to intestinal epithelial cells, and broad-spectrum antibacterial activity [2, 3, 4]. Recent reviews indicate that semi-hard and hard cheeses provide a stable environment for long-term survival due to their lower moisture content [4]. However, safety remains the foremost priority. The European Food Safety Authority (EFSA) emphasizes the need for meticulous strain-level scrutiny via the Qualified Presumption of Safety (QPS) framework [9]. This often involves advanced whole-genome sequencing to confirm the absence of pathogenic traits. [10, 11].

L. helveticus is an industrially significant LAB, used for decades as a primary starter for high-value hard cheeses. This species can produce bioactive peptides through the intensive hydrolysis of milk proteins like casein. These peptides offer physiological effects, including antihypertensive activity, immune regulation, and improved gut health [3, 8].

Identifying new *L. helveticus* strains from underexplored traditional sources adds to the functional diversity of the species. This provides an opportunity to introduce indigenous strains with unique technological and therapeutic traits. Therefore, the present study aims to isolate and molecularly identify the *L. helveticus* C7303 strain from traditional Khiki cheese. We comprehensively evaluated its probiotic traits, including acid/bile resistance, cholesterol assimilation, and adhesion potential. Additionally, its carbohydrate fermentation profile and physiological properties were investigated to determine its suitability for industrial and clinical applications.

2-Materials and Methods

2-1- Isolation and Molecular Identification of the Bacterial Strain

The present experimental research was conducted in 2025 at the Food Microbiology Laboratory within the Faculty of Animal Science and Food Industries at Khuzestan Agricultural Sciences and Natural Resources University. The systematic isolation of the target bacterium was carried out using the method reported by Saboktakin et al. [12], with minor technical modifications. A representative Khiki cheese sample was randomly collected from a local market and immediately transferred to the laboratory

under strictly maintained cold chain conditions to preserve the microbial integrity. A five-gram portion of the sample was aseptically homogenized in 45 mL of a sterile 0.1% peptone water solution, followed by the preparation of a series of decimal dilutions. These dilutions were subsequently plated onto the selective MRS Agar medium to encourage the growth of lactic acid bacteria. After the primary isolation, the resulting colonies were subjected to preliminary phenotypic tests, including Gram staining and the catalase test. For precise molecular identification, the selected strain was first incubated overnight in liquid MRS medium to provide sufficient biomass for downstream analysis. Cells were harvested by high-speed centrifugation, and their genomic DNA was extracted using a specialized commercial kit according to the manufacturer's instructions. Subsequently, a Polymerase Chain Reaction (PCR) was performed using universal primers designed based on the conserved region of the 16S rRNA gene in a final volume of 25.15 µL in a thermal cycler. The optimized thermal program included: an initial denaturation step for 5 min at 95 °C; followed by 35 cycles of denaturation (30 s at 94 °C), primer annealing (30 s at 55 °C), and extension (2 min at 72 °C); and a final extension step for 10 min at 72 °C. The resulting PCR product was then separated via agarose gel electrophoresis (95 V for 45 min) and visualized using a high-resolution gel documentation system. The results confirmed that the selected isolate, which exhibited Gram-positive and catalase-negative characteristics, belongs to the species *L. helveticus* C7303, showing over 99% similarity at the 16S rRNA gene sequence level (Accession code: EU124655)

2-2- Evaluation of Carbohydrate Fermentation

The strain's ability to utilize various sugars was investigated using the API 50 CH kit alongside the API 50 CHL medium (bioMérieux, France). The bacterial suspension was prepared in the API 50 CHL medium containing the bromocresol purple indicator and adjusted to a turbidity of 2.0 McFarland. Subsequently, each cupule of the API strip was filled with 100 µL of the inoculum. To ensure anaerobic conditions, a layer of sterile mineral oil was added. The strips were incubated at 37 °C for up to 48 h, and a color change from purple to yellow was recorded as a positive indication of fermentation. Results were read at two distinct time points (24 and 48 h), and the intensity of the reactions was reported as positive, weak, or negative. The final fermentation profile was interpreted based on the data provided by the apiweb™ bioMérieux database and compared with reference patterns [13]

2-3- Investigation of the Strain's Morphological, Physiological, and Biochemical Characteristics

To describe the phenotypic characteristics, the strain was cultured on both solid and liquid media. The key features, including Gram staining, cellular morphology, motility, and sporulation ability, were systematically examined. Furthermore, bacterial growth under different environmental conditions including varying NaCl concentrations (2%, 4%, and 6%) and a range of temperatures (25 °C, 30 °C, 37 °C, and 42 °C) was assessed. The conventional biochemical tests employed included: catalase, urease, oxidase, arginine dihydrolase, gelatin hydrolysis, esculin hydrolysis, and gas production from glucose. These assays were performed according to established standard methods reported for lactic acid bacteria (LAB). These assays were performed according to standard methods reported for Lactic Acid Bacteria and the results were confirmed through three independent replicates for reproducibility [14, 15].

2-4- Measurement of Enzymatic Activity

The enzymatic activities of the strain were assessed semi-quantitatively using the API ZYM kit (bioMérieux, France). For this purpose, a bacterial suspension was

prepared in a sterile saline solution (0.85%) and adjusted to a concentration equivalent to 0.5 McFarland. Each micro-cupule of the strip was then filled with 65 µL of the suspension. The strips were incubated in a humid chamber at 37 °C for four hours. Upon completion of the incubation, ZYM A and ZYM B reagents were added to each cupule, and the resulting color change was recorded within five minutes. The intensity of the color was compared against the API ZYM standard scale and scored from 0 (no activity) to 5 (very strong activity). To enhance the accuracy and reproducibility of the data, results were read independently by two researchers. The obtained enzymatic profile was subsequently matched with the apiweb™ bioMérieux database to confirm the strain's identity [16]

2-5- Evaluation of Bile Resistance

The strain's ability to tolerate bile salts was examined using a method similar to previous reports in the literature. First, the strain was grown in liquid MRS medium for 24 h at 37 °C under strictly anaerobic conditions. Following incubation, the cells were harvested by centrifugation and resuspended in a sterile phosphate-buffered solution. For the tolerance assay, 100 µL of the suspension was plated onto MRS Agar media containing varying concentrations of bile salt (0.3%, 0.5%, and 0.7%). The plates were incubated at 37 °C, and bacterial viability was monitored at specific time intervals of 0, 1, 2, and 3 h. An MRS medium lacking bile salt was used as the positive control to ensure baseline growth. Results were reported as total colony counts and expressed logarithmically in log CFU/mL. This method was adapted with minor modifications from reliable studies focusing on evaluating bile tolerance in *Lactobacillus* species [17].

2-6- Cell Surface Hydrophobicity Assay

The hydrophobicity of the cell surface was evaluated using the method of Shivani et al. [18] with slight modifications. An overnight culture of the strain was prepared in liquid MRS medium (37 °C for 16–18 h). Cells were harvested by centrifugation (6000 rpm for 12 min at 4 °C). The resulting pellet was washed twice and resuspended in sterile PBS, and its initial turbidity was adjusted to approximately 0.6 at 600 nm (A_1). For the hydrophobicity measurement, 4 mL of the bacterial suspension was mixed with 2 mL of each organic solvent including xylene, toluene, and chloroform in separate tubes. The samples were vortexed for 2 min and then allowed to stand undisturbed for one hour at room temperature to facilitate phase separation. After separation, the absorbance of the aqueous phase was measured at 600 nm (A_2). The percentage of surface hydrophobicity was calculated using Equation 1.

$$\text{hydrophobicity\%} = \left[\frac{A_1 - A_2}{A_1}\right] \times 100$$

2-7- Auto-aggregation and Co-aggregation Assay

The strain's ability for auto-aggregation was measured using a spectrophotometric method based on the protocol established by Ruiz-Ramos et al. [19]. For this assay, a fresh bacterial suspension was prepared. After vortexing for 10 s, the initial optical density (OD_1) was recorded at 600 nm. The tubes were then incubated under stationary conditions at 37 °C, with readings taken at 1, 2, 4, and 24 h. At each time point, the final optical density (OD_2) was recorded. The percentage of auto-aggregation was calculated using Equation 2. To measure co-aggregation with pathogens, cultures of the probiotic strain and six indicator pathogenic microorganisms *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Shigella dysenteriae*, and *Bacillus cereus* were prepared under standard conditions. The optical density of all suspensions was adjusted to 0.5 at 600 nm. Each pathogen suspension was then mixed in equal proportions (3 mL of each) with the probiotic strain and incubated at 37 °C for 2 h. Afterward, the optical density of the

mixture (OD_3) was recorded, and the percentage of co-aggregation was calculated using Equation 3:

$$\text{Auto-aggregation } \% = \left[\frac{OD_1 - OD_2}{OD_1}\right] \times 100 \quad \text{Equation (2)}$$

$$\text{Co-aggregation } \% = [(OD_1 + OD_2)/2 - OD_3]/[(OD_1 + OD_2)/2] \times 100 \quad \text{Equation (3)}$$

where OD_1 and OD_2 represent the optical density of the probiotic and the pathogen, respectively, when measured individually, and OD_3 is the optical density of the mixed two-strain suspension.

2-8- Tolerance Assays

To assess tolerance to acidic conditions, a bacterial suspension was prepared at an approximate concentration of 10^8 CFU/mL in a phosphate buffer adjusted to pH 2.5, 3.5, and 4.5. The samples were incubated at 37 °C for 0, 1, 2, and 3 h. The number of viable bacteria was subsequently calculated and reported as log CFU/mL [22]. To investigate tolerance to simulated digestive conditions, the studied strain was first incubated in SGJ (pH adjusted to 2.5, containing pepsin) for 2 h at 37 °C. Following this, the cells were harvested, washed, and resuspended SIF (pH 7.5, containing 0.3% bile salt and pancreatin). This suspension was further incubated for 4 h at 37 °C. Bacterial survival and viability were determined at the end of the incubation periods by counting colonies on the appropriate culture medium and reported as log CFU/mL [16, 20]

2-9- Cholesterol Assimilation

The capacity of the strain to reduce cholesterol was evaluated according to the method reported by Alizadeh-Behbahani et al. [21], with slight modifications. For this purpose, an MRS liquid medium was prepared to contain polyoxyethylene-cholesteryl sebacate and Oxgall (bile salt), resulting in a final cholesterol concentration of 100 µg/mL in the medium. To examine the effect of the bile salt, the experiment was conducted under two distinct conditions: a medium containing 0.2% (w/v) Oxgall and a medium without Oxgall. Each medium was inoculated with 1% (v/v) of the active bacterial culture and incubated for 48 h at 37 °C under strictly anaerobic conditions. Following incubation, the cultures were centrifuged at 5000 × g for 20 min at 4 °C. The concentration of the residual cholesterol in the supernatant was measured using the CHOD-PAP enzymatic cholesterol kit. The percentage of cholesterol reduction was subsequently determined using the standard calculation method

2-10- Statistical Analysis

All experiments in this study were performed in three independent biological replicates, with three technical replicates for each assay, to ensure maximum reproducibility. Statistical analysis was conducted using the SPSS software version 22. Duncan's multiple range test was employed for post-hoc analysis, and a 95% confidence level ($p < 0.05$) was used to determine significant differences. Graphs were plotted using Microsoft Excel 2016 software, and data are presented as mean ± standard deviation (SD).

3-Results and Discussion

3-1- Evaluation of Carbohydrate Fermentation

The results of the sugar fermentation assay demonstrated that *L. helveticus* C7303 was capable of fermenting a variety of sugars, including D-ribose, D-galactose, D-glucose, D-fructose, maltose, lactose, and sucrose. Furthermore, it exhibited a weakly positive reaction toward trehalose, melibiose, cellobiose, gentiobiose, and starch; however, no metabolic activity was

observed for the other tested carbohydrates (Table 1). This relatively limited yet specific fermentation pattern aligns with characteristics previously reported for specialized probiotic *L. helveticus* strains.

From an industrial perspective, the sugar fermentation profile serves as a vital screening indicator for selecting efficient probiotic strains. Strains capable of fermenting the major milk sugars, such as lactose and maltose, typically exhibit superior performance in dairy fermentation processes, which can lead to reduced ripening times, increased acidity, and improved sensory attributes in fermented products. A study by Fontana et al. [14] on 12 *L. helveticus* strains isolated from dairy products showed that most strains were able to ferment glucose, fructose, galactose, and lactose, while no fermentation activity was observed for mannitol and rhamnose—findings that are consistent with our observations. Similarly, Kido et al. [22] reported that the ability to ferment sucrose and maltose is a common differentiating feature among probiotic *L. helveticus* strains of dairy origin. These collective results indicate that the current strain exhibits a carbohydrate metabolism pattern similar to other strains isolated from dairy products, a finding that is likely linked to its evolutionary adaptation to lactose-rich environments like milk. Conversely, the strain's lack of ability to ferment sugars such as mannose, mannitol, rhamnose, and esculin is in agreement with the report by Tropof et al. [23], who suggested that some *L. helveticus* strains lack the necessary genes for the metabolic pathways of unconventional hexose sugars. This specific feature may suggest an evolutionary selection bias toward the utilization of dominant milk sugar sources (primarily glucose and lactose). Overall, the pattern observed in this study not only confirms the validity of the strain's identification as *L. helveticus* but also suggests that its ability to ferment a restricted range of sugars is likely associated with its ecological fitness for dairy environments and its ultimate probiotic performance.

Table 1. Carbohydrate fermentation profile of *Lactobacillus helveticus* C7303

No.	Carbohydrate	Result	No.	Carbohydrate	Result	No.	Carbohydrate	Result
0	Control	−	17	Inositol	−	34	Gentiobiose	±
1	Glycerol	−	18	D-Mannitol	−	35	Turanose	−
2	Erythritol	−	19	D-Sorbitol	−	36	L-Fucose	−
3	D-Arabinose	−	20	Methyl-α-D-Mannoside	−	37	D-Fucose	−
4	L-Arabinose	−	21	Methyl-α-D-Glucoside	−	38	L-Arabitol	−
5	D-Ribose	+	22	N-Acetyl Glucosamine	−	39	D-Arabitol	−
6	D-Xylose	−	23	Amygdalin	−	40	Gluconate	−
7	L-Xylose	−	24	Arabinitol	−	41	2-Ketogluconate	−
8	D-Adonitol	−	25	Esculin	−	42	5-Ketogluconate	−
9	Methyl-β-D-Xyloside	−	26	Maltose	+	43	Amylopectin	−
10	D-Galactose	+	27	Lactose	+	44	Starch	±
11	D-Glucose	+	28	Melezitose	−	45	Glycogen	−
12	D-Fructose	+	29	Sucrose	+	46	Chitin	−
13	D-Mannose	−	30	Trehalose	±	47	Cellulose	−
14	L-Sorbose	−	31	Inulin	−	48	Pectin	−
15	Rhamnose	−	32	Melibiose	±	49	Xylan	−
16	Dulcitol	−	33	Cellobiose	±	50	Xylanase	−

(+: positive, ±: weak positive, −: negative)

3-2- Enzymatic Activity Profile

The enzymatic activity profile of the *L. helveticus* C7303 strain was determined using the API ZYM system (scored from 0 to 5). The highest activities observed were associated with β-galactosidase, α-glucosidase, and β-glucosidase. This finding underscores the strain's potent ability to degrade milk sugars, particularly lactose and its derivatives (Figure 1). These results are consistent with the findings of Zhao et al. [24], who reported that the presence of strong glycosidase enzymes is a distinguishing feature of dairy *Lactobacillus* strains and plays a crucial role in their adaptation to lactose-rich environments. Furthermore, aryl-amidase activity was found to be low to moderate, while general proteolytic enzymes, such as trypsin and α-chymotrypsin, were notably

absent. This pattern suggests that protein degradation in this strain is primarily mediated by membrane-bound proteinases and specific peptidases, rather than by general serum enzymes. This observation aligns with previous reports by Griffiths et al. [25] regarding the specialized proteolytic system of *L. helveticus*. In addition, moderate activity was observed for both alkaline phosphatase and acid phosphatase, mirroring the profile reported for dairy lactobacilli in a study by Nemska et al. [26]. From a clinical safety perspective, the absolute absence of β-glucuronidase activity is a significant positive indicator. This enzyme has been linked in some bacteria to the production of carcinogenic and potentially harmful metabolites in the gut; thus, its absence is a key regulatory criterion for selecting safe probiotic strains [27]. Collectively, this comprehensive enzymatic profile suggests that the *L. helveticus* C7303 strain possesses the desired capacity for utilizing milk sugars and exhibits a safe enzymatic repertoire, positioning it as a viable candidate for both probiotic applications and the dairy industry.

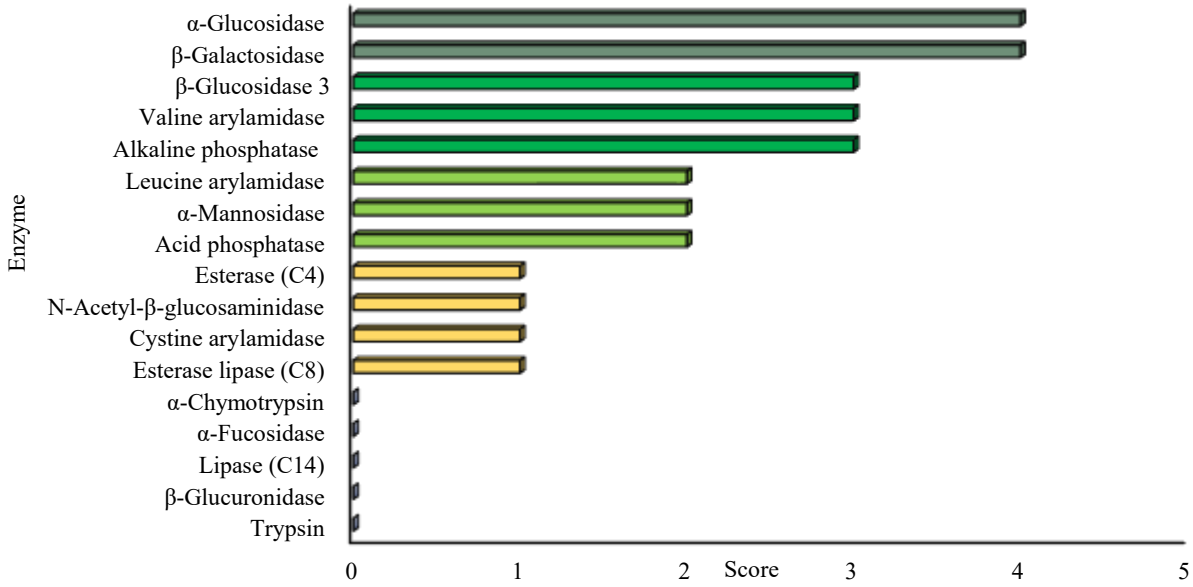

Figure 1. Enzymatic activity profile of *L. helveticus* C7303 determined using the API ZYM system (score 0–5).

3-3- Morphological, Physiological, and Physicochemical Characteristics

Examination of the basic characteristics of the *L. helveticus* C7303 strain revealed that the bacterium is Gram-positive, rod-shaped, non-motile, and non-spore-forming. Notably, it lacked the enzymatic activities of catalase, urease, and oxidase (Table 2). Biochemical tests showed that the strain was unable to hydrolyze gelatin or esculin. Additionally, it did not produce gas from glucose and lacked arginine dihydrolase activity. This phenotypic pattern aligns consistently with the established profile for *L. helveticus*. This species is typically identified as Gram-positive, facultatively anaerobic, and catalase-negative bacilli [28, 29]. These findings provide phenotypic validation for the molecular identification results.

Table 2. Morphological, physiological features of *L. helveticus* C7303

Characteristics	Result
Morphology & physiology	
Gram reaction	Positive (+)
Cell shape	Rod
Motility	Non-motile
Spore formation	Negative (−)
Catalase	Negative (−)
Urease	Negative (−)
Oxidase	Negative (−)
Arginine dihydrolase	Negative (−)
Gelatin hydrolysis	Negative (−)
Esculin hydrolysis	Negative (−)
Gas production from glucose	Negative (−)

Regarding growth under stress, the evaluation of various sodium chloride (NaCl) concentrations showed that the strain exhibited favorable growth at 2% and 4% NaCl. However, a significant reduction in viability occurred at the 6% concentration. This suggests that *L. helveticus* C7303 falls within the typical salt tolerance range for dairy lactobacilli. According to Vidyastovi et al. [30], most *L. helveticus* strains grow well at 2% to 4% salt but decline at higher levels. Furthermore, temperature assessments indicated that the highest growth rate was at 37 °C. Favorable growth was also observed between 30 °C and 42 °C, while growth was significantly inhibited at 25 °C (Table 3). This pattern matches the facultatively thermophilic nature of *L. helveticus*. As reported by Griffa et al. [29], this species achieves optimal growth within the 37 °C to 42 °C range. Such a characteristic is significant for industrial milk fermentation, as most processes occur within this temperature window. These combined results demonstrate that *L. helveticus* C7303 aligns with its species' standard profile. Moreover, it exhibits desirable technological traits, including salt tolerance and an optimal growth range, ensuring its effective utilization in fermented dairy products.

Table 3. Growth performance of *L. helveticus* C7303 under different NaCl concentrations and incubation temperatures (OD_{600} at 24 h, mean ± SD)

Growth at different NaCl concentrations	(OD_{600} at 24 h)
2% NaCl	1.05 ± 0.07
4% NaCl	0.92 ± 0.06
6% NaCl	0.47 ± 0.08
Growth at different temperatures (°C)	**(OD_{600} at 24 h)**
25 °C	0.78 ± 0.06
30 °C	1.00 ± 0.07
37 °C	1.15 ± 0.08
42 °C	0.85 ± 0.05

3-4- Bile Resistance

The bile tolerance assay showed a time-dependent decrease in viable cells across all concentrations during the 3 h incubation. Notably, the highest survival rate occurred in the 0.5% bile treatment (Figure 2). This pattern indicates a gradual reduction in viability. It reflects the intrinsic sensitivity of the cells to the progressive accumulation of bile acids. Bile stress reduces viability in lactobacilli primarily due to the detergent properties of bile acids. These acids disrupt cell membrane integrity, alter permeability, and cause the leakage of cytoplasmic contents [31, 32]. However, the higher survival at 0.5% concentration, compared to 0.3% and 0.7%, may stem from induced stress responses. Such responses often upregulate protective genes, including bile salt hydrolase (BSH) [33, 34]. The presence of BSH genes and bile-inducible operons in *L. helveticus* is well-documented. These factors play a critical role in conferring bile resistance [14, 35]. Similar studies on *L. helveticus* strains reported a gradual but acceptable decrease in survival during bile assays [36]. These findings align with our results, where a continuous decline in the viable population was observed. Noshad et al. [37] also reported that lactobacilli from traditional doogh showed stability in 0.5% bile, showing relative resistance to bile stress. Collectively, these results suggest that the studied strain can traverse the bile barrier in the small intestine. Survival in bile is a fundamental criterion for probiotic selection. It is a prerequisite for reaching the colon to exert beneficial effects [38].

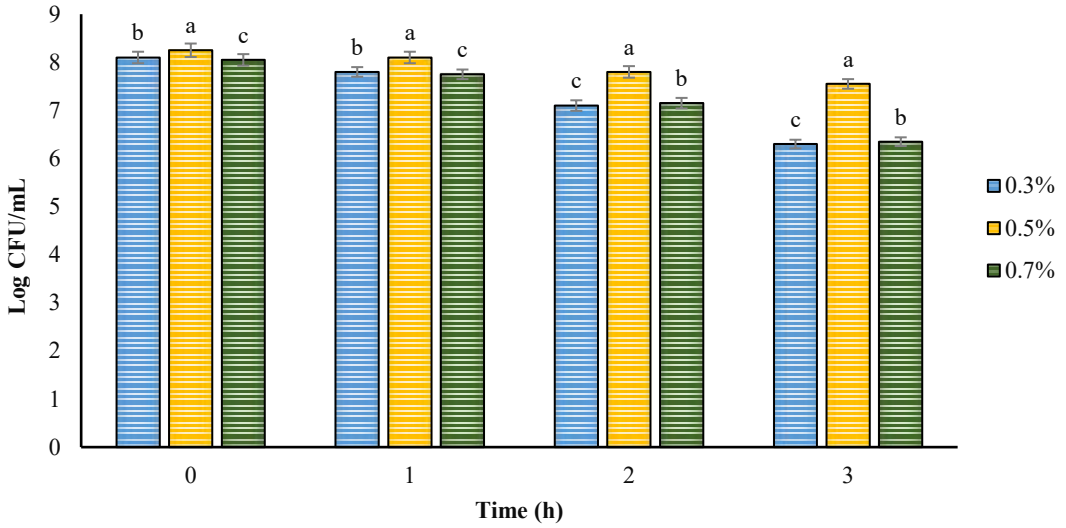

Figure 2. Survival of *L. helveticus* C7303 in the presence of different bile salt concentrations (0.3%, 0.5%, and 0.7%) during 3 h of incubation at 37 °C. Data are expressed as mean ± SD (n=3). Different letters (a–c) indicate significant differences (P<0.05) among samples.

3-5- Cell Surface Hydrophobicity

High hydrophobicity is considered an important characteristic when selecting probiotic strains, as initial adhesion to epithelial cells is the fundamental first step toward colonization and the elicitation of beneficial effects [39, 40]. The results of this study indicated that the *L. helveticus* C7303 strain possessed relatively high levels of cell surface hydrophobicity. Figure 3 illustrates the varying hydrophobicity values across different solvents. These values signify a high tendency of the cells to interact with the non-polar phase, which is generally regarded as an indicator of the potential adhesion ability to the intestinal mucosa and epithelium [41]. In various studies concerning *Lactobacillus* species, values exceeding 50% have typically been classified as high hydrophobicity, correlating with a greater adhesion capacity to Caco-2 and HT-29 cell lines [42]. The values obtained in the current research fall within this optimal range, suggesting that the studied strain has a high potential for adhesion to host cells based on its surface characteristics.

Recent genomic investigations have also indicated the presence of genes related to surface-layer proteins (S-layer) and adhesins in some *L. helveticus* strains, which can play a significant role in enhancing hydrophobicity and interfacial adhesion [14, 43]. Barzegar et al. [44] reported that lactobacilli isolated from traditional Iranian cheese exhibited high hydrophobicity values, and this trait showed a positive correlation with their superior adhesion ability to Caco-2 cells. Overall, these results confirm that the studied strain is highly suitable in terms of its surface properties for potential application as a functional probiotic.

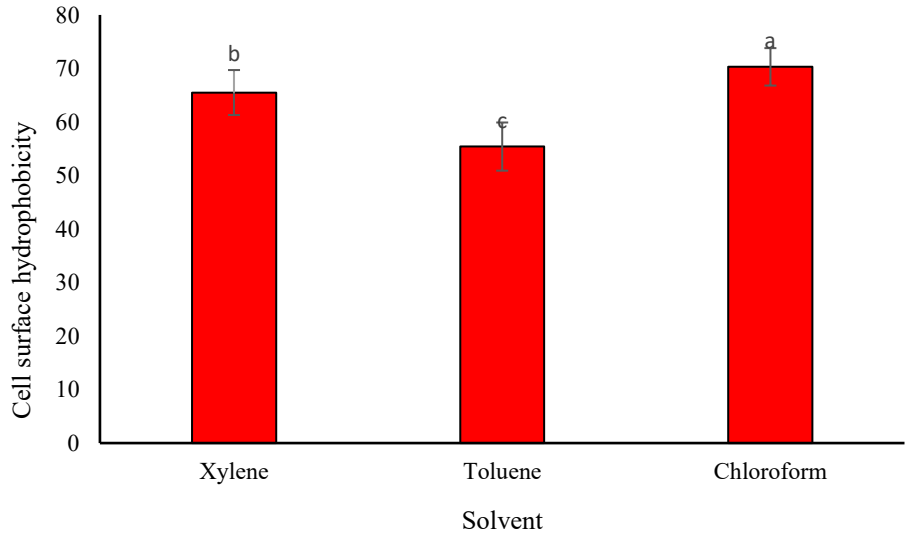

Figure 3. Cell surface hydrophobicity (%) of *L. helveticus* C7303 toward three organic solvents (Xylene, Toluene, and Chloroform). Data are expressed as mean ± SD (n=3). Different letters (a–c) indicate significant differences (P<0.05) among samples.

3-6- Auto-aggregation and Co-aggregation Capacity

In the present study, the results for auto-aggregation demonstrated a time-dependent increasing trend (Figure 4), rising from 20.1% at the first hour to 80.34% at 24 h. This increase in spontaneous aggregation over time is typically attributed to the gradual expression of surface proteins and polysaccharides that facilitate intercellular interactions [45]. This phenomenon is a crucial criterion in the selection of probiotic strains, as increased auto-aggregation can contribute to the formation of microcolonies and achieve more stable colonization within the intestinal mucosa [41]. Abushelebi et al. [42] reported similar results in *Lactobacillus* strains isolated from dairy products. Furthermore, a study by Wang et al. [46] indicated that *Lactobacillus* strains isolated from non-dairy fermented environments exhibit considerable auto-aggregation and co-aggregation abilities, in addition to acid and bile tolerance.

Zheng et al. [8], in a comprehensive screening of 18 *Lactobacillus* strains, reported that only three strains managed to exceed 75% auto-aggregation capacity after 24 h. This observation suggests that the current strain ranks at the level of selected industrial strains in terms of its aggregative potential. Moreover, the study by Grosso et al. [47] demonstrated that surface-layer (S-layer) proteins play a significant role in the auto-aggregation of *Lactobacillus* strains; specifically, the deletion or modification of these proteins led to a drastic reduction in aggregation capacity in dairy strains. These results collectively confirm the importance of surface factors in the aggregative behavior of the bacterium.

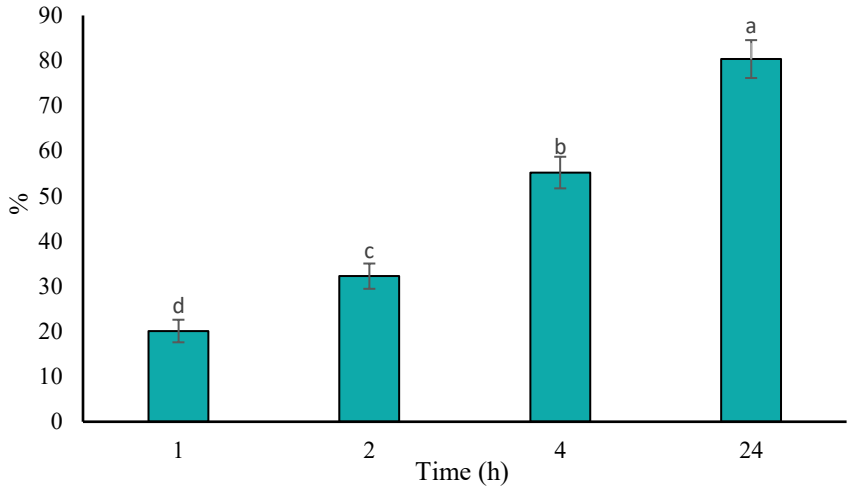

Figure 4. Auto-aggregation (%) of *L. helveticus* C7303 over time (1, 2, 4, and 24 h) at 37 °C. Data are expressed as mean ± SD (n=3). Different letters (a–c) indicate significant differences (P<0.05) among samples.

In the cell co-aggregation assay with pathogenic bacteria, the studied strain demonstrated high aggregation values, particularly against *S. aureus*, *B. cereus*, and *L. monocytogenes* (Figure 5). These values were notably higher than the 30%–45% range reported in the study by Collado et al. [48]. Co-aggregation with pathogens can play a crucial role in preventing their adhesion to host cells, as probiotic cells interfere with the direct attachment of pathogenic bacteria to the mucosa by surrounding or entrapping the pathogenic microorganisms [48]. This characteristic is highly significant in terms of food safety and the principle of competitive exclusion within the intestinal habitat [49]. Cordoni et al. [50] also highlighted the role of aggregative behaviors and adhesion to epithelial cells in the competitive inhibition of pathogens, demonstrating that strains with superior aggregation capacity performed better in inhibiting the adhesion of *E. coli* and *S. aureus* to Caco-2 cells. The present results align with this perspective, as the studied strain achieved co-aggregation values exceeding 50% with several clinically important pathogens. This property is likely mediated by the involvement of specific surface proteins and polysaccharides, leading to the formation of mixed microbial complexes. Such strong co-aggregative potential underscores the strain's ability to create a biological barrier against pathogen colonization.

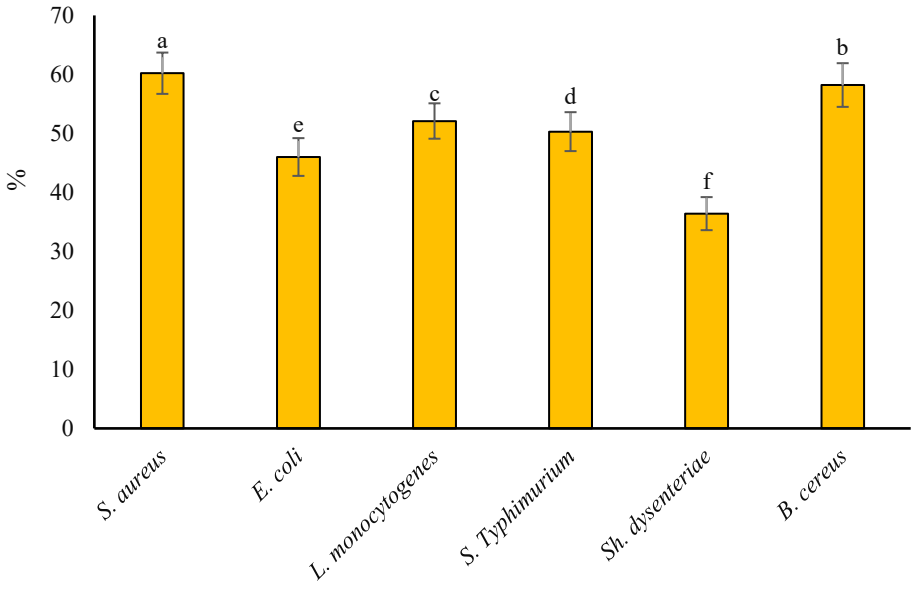

Figure 5. Co-aggregation (%) of *L. helveticus* C7303 with selected pathogenic bacteria. Data are expressed as mean ± SD (n=3). Different letters (a–f) indicate significant differences (P<0.05) among samples.

3-7- Tolerance Assay

The *L. helveticus* C7303, isolated from Khiki cheese, demonstrated a considerable capacity for tolerating acidic stress and simulated digestive environments. In the acid tolerance assay, the viable cell count decreased from 8.70 log CFU/mL at pH 2.5 to 6.95 log CFU/mL over 3 h (Figure 6). This marginal reduction across the pH 2.5–3.5 range indicates a high-level acid tolerance. Such results align with ranges reported in classic screening studies of *Lactobacillus* strains [14]. Furthermore, the strain exhibited acceptable survival in both SGJ and SIF over 4 h (Table 4). These viability ranges are considered reference tolerance levels for probiotic candidates [2]. This profile is consistent with strain-specific reports on *L. helveticus*. For instance, the LH10 strain showed appropriate tolerance to simulated digestive fluids in previous genotypic and phenotypic evaluations [51]. From a mechanistic perspective, the resilience of the current strain may be attributed to canonical acid and bile stress responses. These include F1 F0-ATPase activity for proton extrusion and the regulation of membrane lipid composition. Additionally, the induction of acid shock proteins and bile salt hydrolase (BSH) activity play critical roles. These mechanisms are well-documented in *L. helveticus* and other lactobacilli [14, 43, 52]. Aligned with these responses, factors such as metabolizable sugars can enhance survival in acidic environments. This phenomenon has been systematically demonstrated in various lactobacilli (Corcoran et al., 2005).

The observed tolerance is comparable to the survival ranges of commercial reference strains, such as *L. rhamnosus* GG. In some reports, the current strain presents equivalent or superior performance. However, protocol differences including model, matrix, and duration must be considered in the final interpretation [53, 54]. In summary, the acid data and survival in SGJ and SIF suggest that the studied strain can effectively navigate the upper gastrointestinal tract. This capacity, coupled with its traditional dairy origin, makes it a promising candidate for functional dairy products. It warrants further evaluations, including adhesion, pathogen competition, and genomic safety screening.

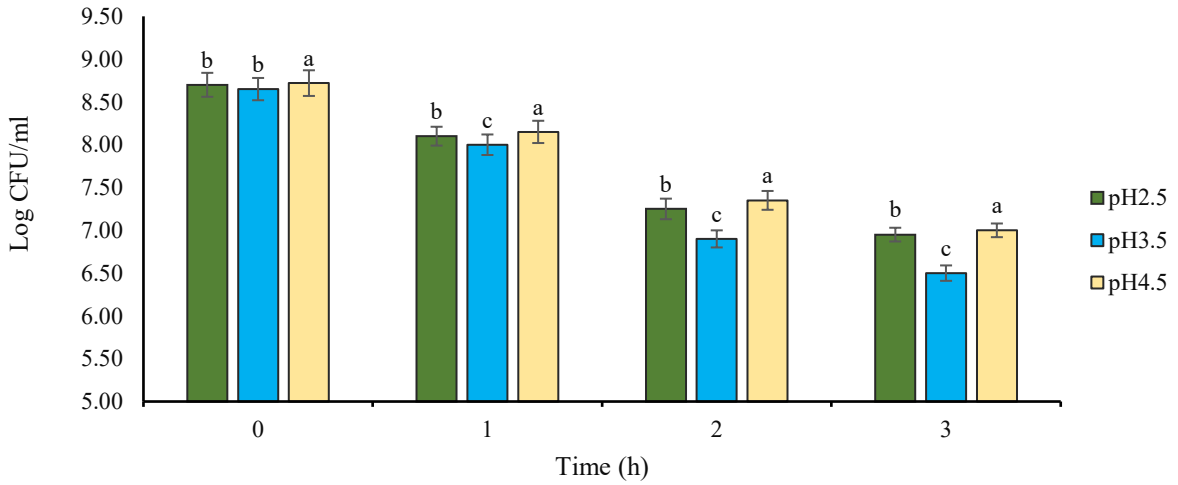

Figure 6. Survival and acid tolerance of *L. helveticus* C7303 at varying pH levels (2.5, 3.5, and 4.5) over a 3 h period at 37 °C. Data are expressed as mean ± SD (n=3). Different letters (a–c) indicate significant differences ($P<0.05$) among samples.

Table 4. Survival of *L. helveticus* C7303 under simulated gastrointestinal conditions

Simulated condition	pH	Duration (h)	Bile (%)	Survival (%) ± SD
Simulated gastric juice (SGJ)	2.5	2	–	70.10 ± 3.5
Simulated intestinal fluid (SIF)	7.5	4	0.3	85.22 ± 2.8

3-8- Cholesterol Assimilation

The present results demonstrated that the indigenous *L. helveticus* C7303 strain possesses a remarkable capacity for reducing cholesterol. This ability is significantly influenced by the presence of bile salts. In the presence of 0.2% bile salt, the strain left only 59.20 µg/mL remaining from an initial 100 µg/mL concentration after incubation (Table 5). This pattern aligns with recognized mechanisms of cholesterol reduction by LAB. A primary mechanism involves the activity of the bile salt hydrolase enzyme. This enzyme facilitates the deconjugation of bile salts, leading to the precipitation of free cholesterol or its integration into the cellular membrane [55]. This process is enhanced by bile salt, explaining the significant increase in cholesterol removal observed under these conditions [56]. Regarding the reduction scope, our findings are consistent with data reported for reference *L. helveticus* strains. For instance, Hassan et al. [36] reported approximately 35% cholesterol removal for the LH10 strain in 0.2% bile, which dropped below 15% without bile. Furthermore, Levy et al. [43] reported a 25%–45% reduction for other indigenous dairy strains. These findings are significant for both public health and food technology. Elevated cholesterol is a definitive risk factor for atherosclerosis and cardiovascular diseases. The use of probiotics to modulate lipid profiles has shown promise in both human and animal studies [57, 58]. Specifically, *L. helveticus* is renowned for producing angiotensin-converting enzyme (ACE)-inhibitory peptides. These can simultaneously modulate blood pressure and cholesterol levels [59]. Therefore, the

coexistence of digestive tolerance and cholesterol reduction makes this strain a multi-functional candidate for developing indigenous functional dairy products.

Table 5. Cholesterol reduction by *L. helveticus* C7303 in the presence and absence of bile

Condition	Initial cholesterol (µg/mL)	Residual cholesterol (µg/mL ± SD)	Reduction (%) ± SD
With 0.2% bile	100	59.20 ± 3.5	40.80 ± 3.5
Without bile	100	82.10 ± 3.2	17.90 ± 3.2

4-Conclusion

The indigenous strain *L. helveticus* C7303, isolated from traditional Khiki cheese, presents a comprehensive profile of superior probiotic and industrial characteristics. The strain demonstrated significant resistance to acidic and bile stresses and maintained its stability under simulated gastrointestinal conditions, indicating its high potential for traversing the upper digestive tract and effectively colonizing the human gut. Its specific fermentation pattern for key dairy sugars, coupled with the high activity of its glycosidase enzymes, confirm the bacterium's natural adaptation to lactose-rich environments, reinforcing its role in optimizing commercial dairy fermentation processes. Furthermore, surface characteristics including high hydrophobicity, considerable auto-aggregation capacity, and effective co-aggregation with pathogens highlight the strain's ability to competitively exclude harmful microorganisms and fortify the intestinal mucosal barrier. Finally, the remarkable cholesterol assimilation capacity of *L. helveticus* C7303 positions it as a promising multifunctional candidate for developing indigenous functional dairy products with both nutritional and therapeutic benefits. Given its thermophilic nature and NaCl tolerance, this strain is particularly suited for application as an adjunct probiotic culture in industrial-scale cheese production. Moving forward, we plan to conduct in-depth genomic safety evaluations, including the screening for antibiotic resistance genes and virulence factors, to fulfill regulatory safety requirements. Additionally, future in vivo studies are warranted to confirm its health-promoting effects and performance within a complex biological system. Future genomic mapping using Whole Genome Sequencing (WGS) will further elucidate its functional genetic architecture.

Data Availability

All data relevant to the study are included in the article.

Conflict of 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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Not applicable.

Authors Contributions

Zeinab Mousavi: Writing – review & editing, Resources, Methodology, Investigation. **Behrooz Alizadeh Behbahani:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Hossein Jooyandeh & Morteza Taki:** Writing – original draft, Resources, Methodology. **Alireza Vasiee**: Writing – original draft, Methodology.

Ethical approval

This article does not involve any studies with human or animal subjects.

Clinical trial number

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used "Microsoft Copilot" in order to paraphrase and grammatically check the sentences. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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مقاله علمی_پژوهشی

افزایش کیفیت غذا از طریق پتانسیل پروبیوتیکی باکتری *لاکتوباسیلوس هلوتیکوس* C7303: ویژگی‌های عملکردی و ایمنی پنیر خیکی سنتی ایرانی

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چکیده

سویه‌های *Lactobacillus*، به‌ویژه *Lactobacillus helveticus* به دلیل توانایی بالای آن‌ها در تخمیر کربوهیدرات‌ها، تولید اسید لاکتیک و اثرات شناخته‌شده سلامت‌محور، گزینه‌های بسیار مناسبی برای استفاده به‌عنوان کشت‌های آغازگر لبنی و پروبیوتیک‌های عملکردی محسوب می‌شوند. هدف این مطالعه بررسی ویژگی‌های پروبیوتیکی، عملکردی، ایمنی و بیولوژیکی سویه بومی *L. helveticus* C7303 بود که از پنیر خیکی (یکی از پنیرهای سنتی ایرانی) جداسازی شده است. در این پژوهش چندین شاخص مورد ارزیابی قرار گرفت: توانایی تخمیر کربوهیدرات‌ها، فعالیت آنزیمی، ویژگی‌های مورفولوژیک، فیزیولوژیک و فیزیکوشیمیایی، همچنین تحمل به شرایط اسیدی، نمک‌های صفراوی و شیره‌های شبیه‌سازی‌شده دستگاه گوارش. ویژگی‌های رشد نیز در غلظت‌های مختلف NaCl و دماهای متفاوت بررسی شد. علاوه بر این، شاخص‌های عملکردی شامل آب‌گریزی سطحی، خودتجمعی، هم‌تجمعی با پاتوژن‌ها و توانایی جذب کلسترول مورد مطالعه قرار گرفت. این سویه توانست قندهای مهم لبنی مانند لاکتوز، گالاکتوز، گلوکز و مالتوز را به‌خوبی تخمیر کند و فعالیت بالای β-گالاکتوزیداز را نشان داد. بقای سویه در شرایط اسیدی مطلوب بود. در آزمون شیره ی گوارشی شبیه‌سازی‌شده، میزان زنده‌مانی در شیره معده شبیه‌سازی‌شده ۱۰/۵ ± ۷۰٪/۳ و در شیره روده شبیه‌سازی‌شده ۲/۸ ± ۸۵/۲ ٪ گزارش شد. این سویه آب‌گریزی سطحی بالا در برابر حلال‌های مختلف، خودتجمعی وابسته به زمان و هم‌تجمعی قوی با پاتوژن‌ها را نشان داد. میزان جذب کلسترول در حضور ۰/۲ ٪ صفرا به ۸۰/٤٪ رسید. با توجه به نتایج مثبت و پایدار حاصل از آزمون‌های عملکردی و ایمنی، *L. helveticus* C7303 پتانسیل بالایی برای استفاده به‌عنوان یک کشت آغازگر لبنی کارآمد و یک پروبیوتیک عملکردی با اثرات تغذیه‌ای و درمانی قابل توجه دارد.