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Characterization and Measurement of Probiotic Properties in Indigenous *Lactobacillus helveticus* C7303: Antimicrobial, Anti-Biofilm, and Antioxidant Activities for Dairy Food Applications

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ABSTRACT

Today, indigenous strains of Lactic Acid Bacteria (LAB) have gained significant importance in the dairy industry. In addition to their inherent adaptability to local environmental conditions, these strains possess unique capabilities in producing desirable flavors and aromas essential for the preparation of various fermented products. This research aimed to investigate the probiotic, functional, safety, and biological characteristics of the indigenous strain *Lactobacillus helveticus* C7303, isolated from Khiki cheese. The evaluation included assessing its antioxidant properties, gene expression profile, anti-biofilm features, resistance to common therapeutic antibiotics, and antimicrobial characteristics against bacterial pathogens. The strain demonstrated high levels of anti-adhesion activity, showing 61.5% inhibition in the competition assay, 53.2% in inhibition, and 30.5% in the displacement assay. *L. helveticus* C7303 exhibited strong antibacterial activity against several Gram-positive and Gram-negative pathogens, including *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* enterica serovar Typhimurium and *Shigella dysenteriae*. In the modified double-layer culture method, the largest zone of inhibition was recorded for *S. aureus* and *L. monocytogenes*, while the least effect was observed against *E. coli* and *S. Typhimurium*. The MTT assay results confirmed that the strain's Cell-Free Supernatant (CFS) caused a significant reduction in the viability of cancer cells. The strain also showed high sensitivity to most tested antibiotics; the largest zone of inhibition was observed for Erythromycin (27.5 mm), and the lowest sensitivity was related to Ampicillin (16.2 mm). Given the positive outcomes from the functional and safety assays, *L. helveticus* C7303 shows promising potential for utilization as a dairy starter and a functional probiotic with both nutritional and therapeutic effects.

1-Introduction

Characterized as Gram-positive, non-spore-forming, and catalase-negative rods or cocci, Lactic Acid Bacteria (LAB) primarily synthesize lactic acid as their major metabolic end-product through the homofermentation or heterofermentation of carbohydrates. Historically associated with dairy niches, these microorganisms serve as pivotal industrial agents in the fermentation of dairy, meat, and vegetable matrices [1]. In food systems, LAB function either as primary starter cultures responsible for initial biochemical transformations or as autochthonous non-starter microbiota that refine the organoleptic and sensory profiles during the secondary stages of maturation [2,3].

Probiotics are diverse microorganisms primarily dominated by the Lactobacillaceae familyrecently reclassified into 25 genera including *Lactobacillus* and *Lactiplantibacillus* and *Bifidobacterium*. Other functional genera include *Enterococcus*, *Lactococcus*, *Pediococcus*, and *Streptococcus thermophilus*. Their health-promoting effects remain strictly strain-dependent, requiring verified gastrointestinal persistence [4].

It is a common taxonomic oversimplification to equate all LAB with probiotics. While the majority of commercial probiotics are members of the Lactobacillaceae family, probiotic designation strictly necessitates the verified capacity to withstand gastrointestinal transit and achieve intestinal persistence. Furthermore, the therapeutic and biochemical attributes of LAB are exclusively strain-specific, meaning that functional diversity is frequently observed even among phylogenetically identical strains [5]. Recently, indigenous LAB isolates have garnered significant industrial attention due to their superior adaptation to local environmental stressors and their unique metabolic repertoire for generating distinctive aromas. Additionally, these native strains often possess robust ecological advantages, including intrinsic resistance to virulent bacteriophages and potent antagonistic activity against competitors [6].

The bio-preservative efficacy of non-starter LAB has been extensively documented, stemming from the synergistic secretion of organic acids, hydrogen peroxide, and diverse bacteriocins [7, 8]. Within the *Lactobacillus* genus, numerous species have been identified for their ability to suppress food spoilage indicators. This inhibitory action is largely mediated by the accumulation of organic acids, notably lactic and phenyllactic acids, which lower the local pH [9]. Moreover, the synthesis of ribosomally-produced antimicrobial peptides (bacteriocins) and bioactive metabolites provides a sustainable strategy for biological food preservation and shelf-life extension [10].

Given that probiotic functionality is inherently genus- and strain-dependent, the systemic characterization of novel isolates remains critical. The present study was designed to rigorously evaluate the probiotic candidacy of a novel *L. helveticus* C7303 strain isolated from traditional Khiki cheese. Our investigation encompasses a comprehensive safety assessment (screening for biogenic amines, DNase, and hemolytic activity), functional profiling (antioxidant and cellular adhesion assays), and an analysis of its antimicrobial spectrum and virulence-related gene expression against key pathogens, including *E. coli*, *S. aureus*, *B. subtilis*, *L. monocytogenes*, *S. Typhimurium*, and *Sh. dysenteriae*.

2-Materials and Methods

2-1- Strain Anti-Adhesion Potential

In this study, the anti-adhesion effect of the probiotic strain against *S. aureus* was assessed using three conventional assays: Competition, Inhibition, and Displacement. Following the method established by Fallah et al. (2018) [11], the Competition assay was performed by simultaneously co-inoculating Caco-2 cells with the probiotic strain and *S. aureus* at an equal ratio (1:1).

The mixture was then incubated for 1 hour at 37 °C under 5% CO_2. After washing with PBS and lysing the cells with 0.05% Triton X-100, the number of adhered *S. aureus* cells was calculated relative to the control. In the Inhibition assay, Caco-2 cells were first incubated with the probiotic strain for 1 h. Following washing with PBS, the cells were then inoculated with *S. aureus*, and the percentage of adhesion inhibition was calculated based on Equation 1. For the Displacement assay, *S. aureus* was first added to the Caco-2 cells; after 1 h of incubation, non-adherent bacteria were removed. The probiotic strain was then added to the wells and incubated again for 1 h. The reduction in the adhesion of *S. aureus* in the presence of the tested strain was recorded as the percentage of displacement [11].

$$\text{Inhibition}=\left(1-\frac{S.aureus \text{ Adhesion in the presence of } L.helveticus}{S.aureus \text{ Adhesion in the abcence of } L.helveticus}\right)\times 100 \qquad \text{Equation (1)}$$

2-2- Antimicrobial Activity

To determine the antimicrobial efficacy, the experimental framework reported in our prior study [12] was employed. Briefly, *L. helveticus* C7303 was inoculated into MRS broth and incubated for 28 h at 37 °C. To obtain the cell-free supernatant (CFS), the biomass was removed via centrifugation (5000 rpm, 20 min, 4 °C), followed by filtration through a 0.22 µm membrane. The resulting CFS was lyophilized for preservation and reconstituted in sterile deionized water prior to experimental use. The inhibitory spectrum was tested against several clinical and foodborne pathogens: *E. coli*, *Sh. dysenteriae*, *S. Typhimurium*, *B. cereus*, *L. monocytogenes*, and *S. aureus*. In the Disc Diffusion Assay (DDA), sterile paper discs were impregnated with a standardized volume of CFS and positioned onto the surface of agar plates previously seeded with the target pathogens. Conversely, for the Well Diffusion Assay (WDA), circular reservoirs were excised in the agar and filled with specific aliquots of the CFS. Then, the diameter of the clear zones of inhibition was measured in millimeters. Furthermore, the Modified Double-Layer approach provided a deeper insight into the inhibitory activity by layering pathogenic suspensions in soft agar over established probiotic colonies. To quantify the potency, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined through the microplate broth dilution technique, identifying the lowest concentrations required to inhibit visible growth and achieve complete bacterial eradication, respectively.

2-3- In vitro Cytotoxicity (MTT Assay)

The anti-proliferative effect of the CFS against HeLa, MCF-7, and HT-29 cancer cell lines was analyzed using the MTT colorimetric technique [13, 14]. Cells were seeded in 96-well plates (3×103 cells/well) and cultured in DMEM supplemented with 10% FBS. Post-incubation (48 h, 37 °C, 5% CO2), cells were exposed to a gradient of CFS concentrations (0–500 mg/mL). After 24 h of treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to facilitate formazan crystal formation over 4 h. These crystals were subsequently solubilized in DMSO, and the absorbance was measured at 570 nm. The results were expressed as the percentage of viable cells relative to the untreated control, and the IC50 values were calculated.

2-4- Strain Antioxidant Activity

The antioxidant capacity of the CFS was quantified using the DPPH and ABTS radical scavenging protocols, along with a linoleic acid peroxidation inhibition test. For the DPPH assay [15], 1 mL of CFS was reacted with 2 mL of methanolic DPPH solution and maintained in total darkness for 30 min. After centrifugation, the absorbance was monitored at 517 nm. Similarly, the ABTS radical was pre-generated by reacting 7 mM potassium

persulfate with 14 mM ABTS in a phosphate buffer (pH 7.4(pH meter; Model 827, Metrohm AG, Herisau, Switzerland)) for 16 h [16]. This solution was adjusted to an optical density (OD) of 0.70 ± 0.01 at 734 nm before being mixed with the CFS. After 30 min, the scavenging activity was calculated based on the reduction in absorbance compared to the control (Equation 2):

$$\text{Scavenging Activity \%} = \left(1 - \frac{A_{control}}{A_{sample}}\right) \times 100$$

Equation (2)

2-5- Inhibition of Linoleic Acid Peroxidation

The ability to inhibit linoleic acid peroxidation was assessed using the Thiobarbituric Acid (TBA) method, with slight modifications [17]. The reaction mixture was prepared containing 1 mL of linoleic acid emulsion, 0.5 mL of PBS buffer, 0.2 mL of 0.01% ferrous sulfate, 0.2 mL of 0.01% ascorbic acid, and 0.5 mL of the CFS or the isolate culture. The mixture was incubated for 12 hours at 37 °C. Following incubation, 2 mL of 0.8% TBA solution, 0.2 mL of 4% trichloroacetic acid (TCA), and 0.2 mL of butylated hydroxytoluene (BHT) were added to the mixture. Samples were then heated at 100 °C for 30 min. After cooling and extraction with 2 mL of chloroform, the OD of the upper phase was measured at a wavelength of 532 nm. The percentage of peroxidation inhibition was calculated relative to the control.

2-6- Antibiotic Susceptibility Test

The antibiotic susceptibility of the strain was evaluated using the Disc Diffusion Assay according to the Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. A fresh culture of the strain was prepared in liquid MRS medium, and after 18–24 h of incubation at 37 °C, the cell density was adjusted to approximately 0.5 McFarland (equivalent to 10^8 CFU/mL). Subsequently, 100 µL of the bacterial suspension was spread uniformly over the surface of sterile MRS agar plates. After the surface dried, antibiotic discs including Imipenem (10 µg), Ampicillin (10 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), Nalidixic acid (30 µg), Nitrofurantoin (300 µg), and Chloramphenicol (30 µg) were placed at appropriate distances on the plate surface. The plates were incubated for 24 h at 37 °C, and the diameter of the growth inhibition zones was measured with a digital caliper. Results were interpreted as resistant, sensitive, or intermediately susceptible based on the CLSI standards.

2-7- Evaluation of Anti-Biofilm Activity

The transcriptional impact of the CFS on *S. aureus* virulence and biofilm-associated genes was analyzed via quantitative Real-Time PCR (qRT-PCR), following the protocol established by Rouhi et al. (2024) with minor adjustments [13]. *S. aureus* cultures were initially grown in Tryptic Soy Broth (TSB) for 24 h at 37 °C, with the inoculum density standardized to 105 CFU/mL. This suspension was subsequently challenged with a sub-inhibitory concentration (1/2 MIC) of the CFS for an additional 24 h, while an untreated control group was maintained in parallel. Total RNA was isolated using the TRIzol™ reagent (Thermo Fisher Scientific, USA), and its concentration and quality were verified using a NanoDrop spectrophotometer. For reverse transcription, the SensiFAST™ cDNA Synthesis Kit (Bioline, UK) was utilized to convert purified RNA into cDNA. The qPCR amplification was conducted on a PikoReal 96 system (Thermo Fisher Scientific, USA) using SYBR™ Green Master Mix. The amplification program involved an initial denaturation step (95 °C, 5 min), followed by 45 cycles of 95 °C for 5 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 30 s (extension). Relative transcript abundance was determined using the $2^{-\Delta\Delta CT}$ method, with normalization against specific primers as detailed in Table 1. All assays were

executed in triplicate to ensure reproducibility.

2-8- Gene Expression Analysis

The effect of the CFS on the expression of genes related to biofilm formation and virulence in *S. aureus* was investigated using the qRT-PCR technique, with slight modifications to the method reported by Rouhi et al. (2024) [13]. Initially, *S. aureus* was cultured in Tryptic Soy Broth (TSB) for 24 h at 37 °C, and the cell density was adjusted to approximately 10^5 CFU/mL. The bacterial suspension was then incubated for another 24 h in the presence of half the Minimum Inhibitory Concentration (1/2 MIC) of the CFS, while the control group was treated only with the culture medium. Total RNA extraction was performed using the TRIzol™ kit (Thermo Fisher Scientific, USA), and the RNA concentration and purity were assessed using a NanoDrop spectrophotometer. cDNA synthesis was carried out from the purified RNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, UK). qPCR reactions were run on a PikoReal 96 instrument (Thermo Fisher Scientific, USA) using the SYBR™ Green Master Mix reagent. The thermal cycling program included initial denaturation at 95 °C for 5 min, followed by 45 cycles, each consisting of: denaturation at 95 °C for 5 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. All reactions were performed in triplicates, and Relative gene expression changes were calculated using the $2^{-\Delta\Delta CT}$ method, and expression levels were ultimately quantified via qRT-PCR using specific primers (Table 1).

Table. 1. PCR Primers for *S. aureus* Virulence Gene Analysis

Gene	**Primer (sequence of primers (5′–3′))**
icaA	Forward CTGGCGCAGTCAATACTATTTCGGGTGTCT Reverse GACCTCCCAATGTTTCTGGAACCAACATCC
agr	Forward TGATAATCCTTATGAGGT GCTT Reverse CACTGTGACTCGTAACGAAAA
hla	Forward GGTTTAGCCTGGCCTTCAGC Reverse ACCAGTAACATTACCGTTGAATCCA
mecA	Forward ACTGCTATCCACCCTCAAAC Reverse CTGGTGAAGTTGTAATCTGG
spa	Forward TAAAGACGATCCTTCGGTGAGC Reverse CAGCAGTAGTGCCGTTTGCTT

2-9- Statistical Analysis

All experiments were performed in three independent biological replicates and three technical replicates. Statistical analysis was conducted using the SPSS software version 22. The Duncan's Multiple Range Test was used to compare means at a 95% confidence level ($P<0.05$), and graphs were plotted using Excel 2016 software.

3-Results and Discussion

3-1- Strain Anti-Adhesion Potential

One of the most important criteria for selecting probiotic strains is their ability to compete with and inhibit the adhesion of pathogens to intestinal epithelial cells. This capability is typically achieved through three main pathways: inhibition, competition, and displacement. These processes act as a biological barrier, playing a crucial role in reducing the colonization of pathogenic agents and preventing gastrointestinal infections [5, 19]. In the current study, the tested strain demonstrated high levels of competitive inhibition (Figure 1). These values indicate that the strain effectively prevented pathogen adhesion by rapidly occupying surface receptors and forming stable microcolonies. In comparison to similar studies, Collado et al. (2008) [5] reported that most *Lactobacillus* strains only achieved inhibition levels above 50% in the competition mode. Generally, the positive correlation between auto-aggregation and co-aggregation indices suggests that the ability to form stable aggregates is an important prerequisite for the competitive

exclusion of pathogens [19]. Therefore, the current results solidify the position of this strain as a strong candidate for probiotic applications.

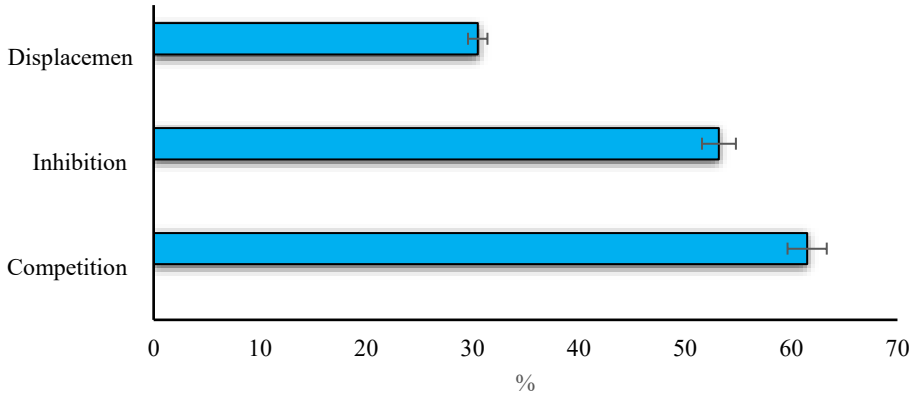

Figure 1. Anti-adhesive potential of *L. helveticus* C7303 against *S. aureus* on Caco-2 epithelial cells. The anti-infection activity was assessed using three distinct mechanistic approaches: competition, inhibition, and displacement assays. Data are presented as mean percentages ± standard deviation (SD) from three independent experiments.

3-2- Antimicrobial Activity

The antimicrobial efficacy of *L. helveticus* C7303 was demonstrated by its robust inhibition of several Gram-positive and Gram-negative pathogens, primarily attributed to the production of organic acids, hydrogen peroxide, and bacteriocins [19, 20]. Evaluation through multiple methodologies including WDA, DDA, and the Modified Double-Layer assay revealed a consistent inhibitory pattern against *S. aureus*, *L. monocytogenes*, *E. coli*, and *Sh. dysenteriae* (Figures 2 and 3). Notably, Gram-positive pathogens exhibited the highest sensitivity, while the weakest inhibition (least effect) was observed against *E. coli* and *S. Typhimurium*. This trend aligns with the findings of Kishilova et al. (2024) and Alizadeh-Behbahani et al. (2025), who reported that lactic acid bacteria (LAB) supernatants significantly reduce the microbial load of *S. aureus* and *L. monocytogenes* compared to Gram-negative bacteria [12].

The significantly higher activity of active CFS (aCFS) compared to neutralized CFS (nCFS) emphasizes the decisive role of acidic metabolites and active growth-phase secretions in pathogen suppression. The observed efficacy is significantly linked to bacteriocinogeny, a key 'colonization resistance' trait. To accurately detect these proteinaceous compounds, CFS was neutralized and catalase-treated, confirming that inhibition persists beyond organic acids. Bacteriocin production provides a competitive advantage by targeting pathogens like *S. aureus* without disrupting the broader microbial ecology [12]. Furthermore, the determined MIC (16–128 mg/mL) and MBC values reinforced the strain's potent killing effect (Figure 4). These results are comparable to, and in some cases exceed, the inhibitory power reported for other indigenous Iranian strains isolated from traditional cheeses [21, 22]. Overall, these findings signify that *L. helveticus* C7303 can effectively inhibit foodborne and intestinal pathogens through the production of antimicrobial compounds acting in parallel with competitive mechanisms. Such strong antimicrobial traits, coupled with high survival in diverse media, strengthen its position as a promising candidate for industrial and therapeutic probiotic applications.

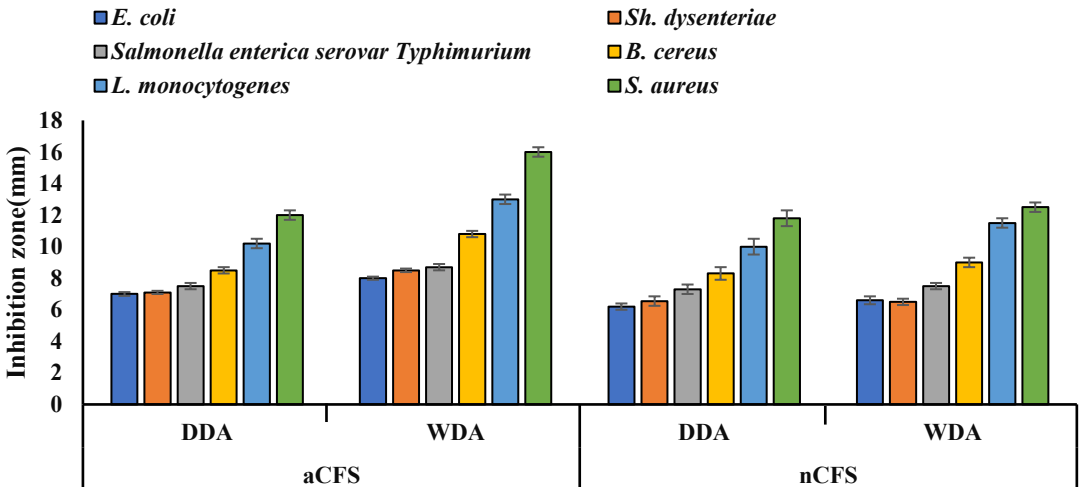

Figure 2. Antibacterial activity of *L. helveticus* C7303 cell-free supernatants (CFS) assessed by agar well diffusion assay (DDA) and disc diffusion assay (WDA) against foodborne pathogens. Both active (aCFS) and neutralized (nCFS) supernatants were tested. Data are shown as mean inhibition zone diameters (mm) ± SD.

The Modified Double-Layer Culture method also confirmed the presence of potent inhibitory activity (Figure 3). In this assay, the largest zone of inhibition was obtained for *S. aureus* and *L. monocytogenes*, while the weakest inhibition (least effect) *E. coli* and *S. Typhimurium*. These results demonstrate the high capability of the *L. helveticus* C7303 strain to secrete antimicrobial compounds that are diffusible within a solid medium. Strong inhibitory characteristics in solid media compared to liquid media have also been affirmed in previous reports [23].

Furthermore, the determined MIC and MBC values indicated the high potency of the strain's killing effect (Figure 4). The MIC values for Gram-positive bacteria (16 mg/mL) were substantially lower than those for Gram-negative bacteria (128 mg/mL), and the MBC values followed the same pattern. These findings align with our previous observations [12], where Gram-positive pathogens exhibited significantly higher susceptibility to probiotic secretions compared to Gram-negative bacteria. The reason for this is likely the higher permeability of the cell wall in Gram-positive bacteria, which facilitates the quicker action of acidic and bacteriocin-like compounds [5, 24].

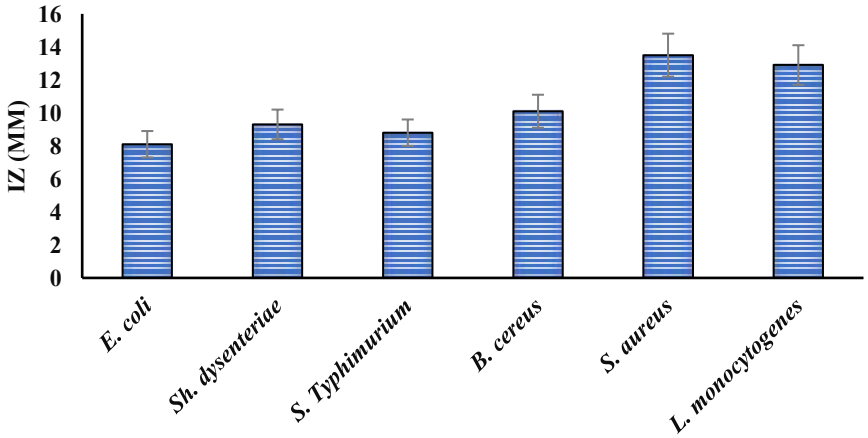

Figure 3. The antibacterial activity of *L. helveticus* C7303 was assessed using the modified double layer method against different pathogenic bacteria. Inhibition zone diameters (mm) are shown as mean ± SD.

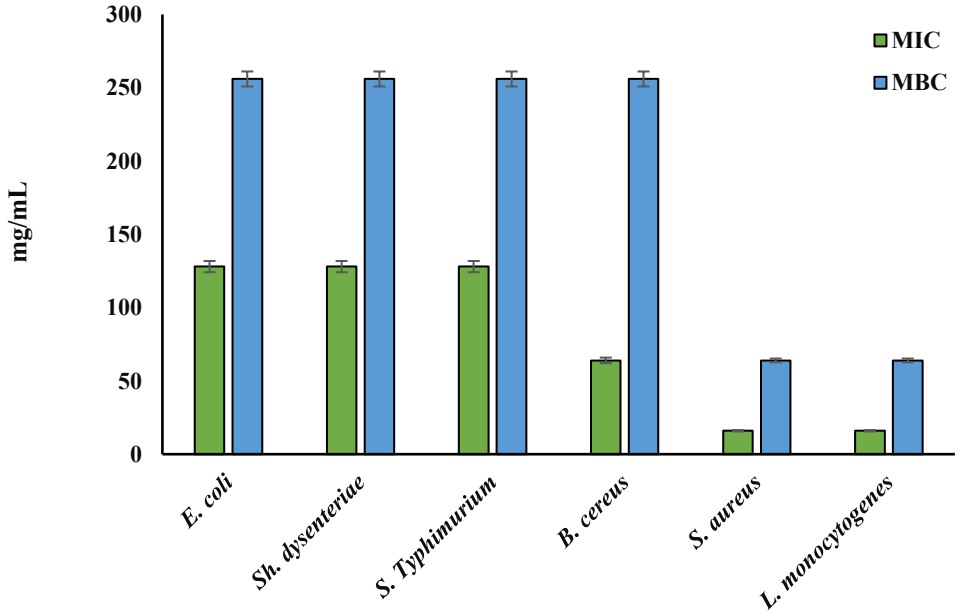

Figure 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *L. helveticus* C7303 cell-free supernatant against the tested pathogenic strains, expressed as mg/mL.

3-3- Cytotoxicity

From a safety perspective, the innocuousness of a probiotic strain must be based on standard frameworks, which include the evaluation of virulence and antibiotic resistance genes, hemolysis assays, and clinical post-market evidence [25]. In Europe, evaluations are often conducted using the Qualified Presumption of Safety framework, which is periodically updated and assigns safety status to many *Lactobacillales* [26, 27]. Beyond policy documents, several recent laboratory studies have demonstrated that combining genomic/phenotypic evaluation with *in vivo* and *in vitro* toxicity assays is a valid approach for confirming the safety of dairy strains [28, 29]. Our results align with these frameworks, indicating that, in addition to its efficacy, the studied strain meets the expected safety indices for food-grade probiotics (Table 2). In this study, the MTT assay results revealed that the strain's CFS caused a significant reduction in the viability of cancer cells. Abedi and Tafvizi (2024) [30] showed that probiotic species' supernatants decrease the viability of the HT-29 and AGS cell lines while activating apoptotic pathways. In another study, *Lactiplantibacillus plantarum* caused a dose-dependent reduction in HT-29 cell proliferation in an *in vitro* model [31]. Furthermore, metabolites from indigenous dairy strains isolated from local Behbahan buttermilk showed no toxicity to human cells, while simultaneously being able to reduce the viability of cancer cells by 40–60% [21].

Table 2. Cytotoxic effect of *L. helveticus* C7303 cell-free supernatant (CFS) against different cancer cell lines expressed as IC_{50} values (mg/mL).

IC_{50}	HT-29 (mg/mL)	Hela (mg/mL)	MCF-7 (mg/mL)
	46.00 ± 1.3^{c}	52.5 ± 1.6^{b}	55.8 ± 1.7^{a}

Values are expressed as mean ± SD. Different superscript letters in the same column indicate significant differences ($p<0.05$).

3-4- Strain Antioxidant Activity

Lactobacillus species can protect human cells against oxidative stress by scavenging free radicals [32]. In this research, the CFS of the *L. helveticus* C7303 strain exhibited significant antioxidant activity (Figure 5). This level of activity suggests that the studied strain possesses a considerable ability to neutralize free radicals, an effect likely stemming from the production of antioxidant metabolites, including bioactive peptides, organic acids, and antioxidant enzymes like superoxide dismutase (SOD) [33]. Zhao et al. (2024) [34] demonstrated that the metabolites of *L. rhamnosus* and *L. plantarum* strains, isolated from fermented dairy products, achieved 70% and 75% inhibition of the ABTS radical and approximately 60% inhibition of the DPPH radical, respectively, which aligns with the activity level observed in the current strain. They reported that these effects resulted from the production of antioxidant enzymes (such as SOD) and bioactive peptides generated during fermentation [34]. Given the known role of oxidative stress in the pathogenesis of many inflammatory and metabolic diseases, these results indicate that *L. helveticus* C7303 can be considered not only for its classic probiotic traits but also as a strain with high functional antioxidant potential.

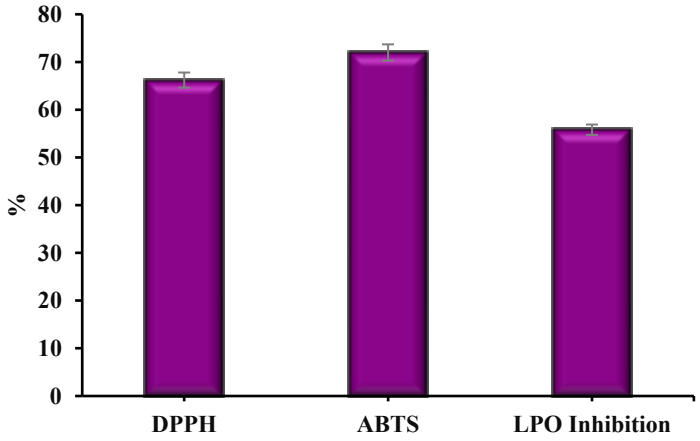

Figure 5. Antioxidant activity of the cell-free supernatant of *L. helveticus* C7303, including the percentage of DPPH and ABTS radical scavenging activity and inhibition of lipid peroxidation

3-5- Antibiotic Resistance

The assessment of antibiotic susceptibility is a fundamental requirement for evaluating the safety of probiotic strains, as the presence of transferable resistance genes can pose a risk of horizontal resistance transfer to pathogens [25]. In this study, the investigated strain demonstrated high sensitivity to most of the tested antibiotics (Figure 6). Specifically, the largest zone of inhibition was recorded for Erythromycin (27.5 mm), while the lowest degree of sensitivity was observed for Ampicillin (16.2 mm), although it remained within the sensitive range. In a similar study, most *Lactobacillus* strains isolated from dairy products were reported to be sensitive to Imipenem and Erythromycin but showed relative resistance to Ampicillin. That study also emphasized that the observed resistance genes were primarily chromosomal and non-transferable, posing no threat to safety. This pattern indicates that intrinsic resistance in some *Lactobacilli* is a recognized characteristic that is typically not horizontally transmitted [35]. The study by Floris et al. (2025) [36] showed that *Lactobacillus* strains isolated from traditional raw milk dairy products exhibited resistance to certain antibiotics;

however, the predominant resistances were associated with Vancomycin and Tetracycline, while other antibiotics showed desirable sensitivity. Similarly, the report by Dach et al. (2023) [37] also noted some resistances in food-grade and human-derived *Lactobacilli* from Nigeria, but the majority of strains maintained a sensitive phenotype, and transferable resistance genes were rarely detected. Based on these findings and established biosafety criteria, the resistance patterns of *L. helveticus* C7303 appear to be intrinsic (chromosomal) rather than acquired. While resistance mediated by mobile genetic elements poses a risk of horizontal transfer, intrinsic resistance is a stable, non-transferable trait. This characteristic offers a functional advantage, as it allows the probiotic to be co-administered with specific antibiotic therapies to maintain gut microbiota balance without the risk of disseminating resistance genes.

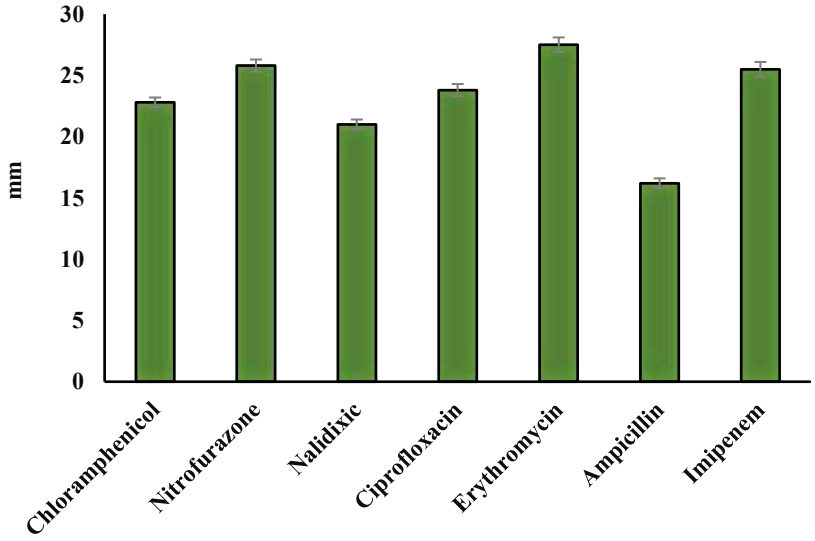

Figure 6. Effect of common antibiotics on the growth of *L. helveticus* C7303, expressed as mean inhibition zone diameter (mm).

3-6- Gene Expression Analysis

The results from the analysis of virulence gene expression demonstrated that treatment with the strain's CFS caused a significant reduction in the expression of key pathogenicity genes in *S. aureus* compared to the control sample (Figure 7). These findings indicate that the metabolites secreted by *L. helveticus* C7303 are capable of inhibiting the virulence activities of *S. aureus* by interfering with the transcriptional regulation of these genes. Recent studies have reported similar effects. Kash et al. (2023) [38] showed that the supernatant of *Lacticaseibacillus rhamnosus* and *Lpb. plantarum* strains led to a significant decrease in the expression of the *mecA, icaA*, and *hla* genes in Methicillin-Resistant *S. aureus* (MRSA), which was accompanied by reduced biofilm formation and hemolytic toxicity. Furthermore, other studies suggest that peptidic metabolites derived from *Lactobacilli* can downregulate the expression of *agr* and *spa* by inhibiting the signaling pathway [33].

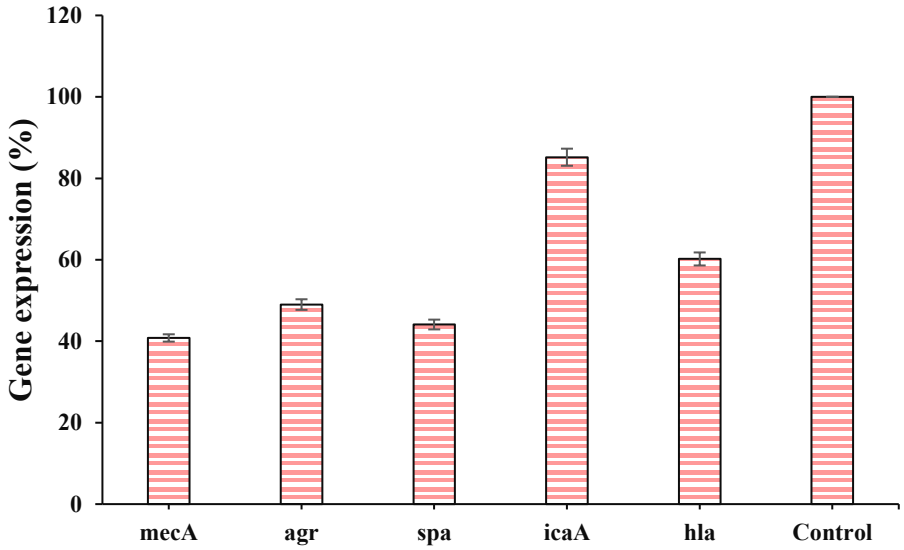

Figure 7. Relative expression levels of *S. aureus* virulence genes in the untreated control and in the presence of *L. helveticus* C7303.

3-7- Anti-Biofilm Activity Assessment

A significant reduction was observed in the amount of biofilm formed by *S. aureus* in both the initial and mature stages when exposed to varying concentrations of the treatment. In the initial biofilm stage, a sharp decrease was recorded at concentrations near the MIC. Biofilm formation was reduced from 100% in the control to 53% at MIC, and further dropped to 18.5% at 4MIC (Figure 8). A similar trend was observed in the mature biofilm stage; the residual biofilm amount decreased from 100% in the control to 48% at MIC and sharply declined to 16.5% at 4MIC (Figure 8). These results indicate that the anti-biofilm effect of *L. helveticus* C7303 is dose-dependent, with the efficacy of biofilm elimination increasing as the concentration rises. In comparable studies, it has been reported that the supernatant of *Lpb. plantarum* caused a 70–80% reduction in *S. aureus* biofilm formation at MIC and achieved complete elimination at 4MIC. Even sub-MIC concentrations are capable of weakening the biofilm structure by inhibiting the expression of biofilm-related genes (icaA, sarA) [39, 40].

Beyond its probiotic potential, *L. helveticus* C7303 shows significant promise as a protective starter culture for the dairy industry. Incorporating this strain into fermented products at a target concentration of 10^6 to 10^8 CFU/g could leverage its potent antimicrobial and antioxidant activities to enhance food safety and offer functional health benefits to consumers.

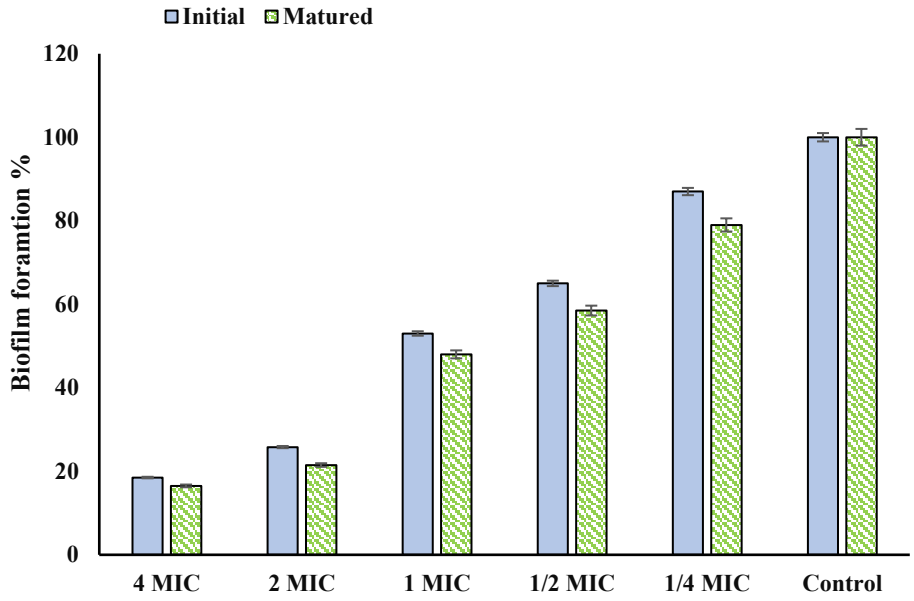

Figure 8. Antibiofilm activity of cell-free supernatant (CFS) of *L. helveticus* C7303 against the biofilm formation ability of S. aureus at initial and mature biofilm formation statuses.

4-Conclusion

The indigenous strain *L. helveticus* C7303, isolated from Khiki cheese, presents an integrated profile of valuable probiotic and industrial characteristics. The suppression of virulence gene expression highlights the strain's ability to compete with harmful microorganisms and support the intestinal mucosal barrier. Results from the Modified Double-Layer Culture method also confirmed potent inhibitory activity. Furthermore, the anti-biofilm effect of the strain was found to be dose-dependent, with the efficiency of biofilm elimination increasing with higher concentrations. The MTT assay results demonstrated that the strain's CFS caused a significant reduction in the viability of cancer cells. On the safety and functionality fronts, evidence regarding its antioxidant activity and the desirable antibiotic sensitivity pattern indicates that this strain is reliable, not only in terms of performance but also from a safety perspective. Although *L. helveticus* C7303 showed robust *in vitro* functional traits, future *in vivo* trials are necessary to validate its gastrointestinal survival, colonization, and health-promoting effects under complex biological conditions.

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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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.

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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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5-References

[1] Shemshad, N., Roozbeh Nasiraie, L., & Majidzadeh Heravi, R. (2021). Isolation of probiotic lactobacilli bacteria from traditional Naein dairy product (Koome). *Iranian Journal of Medical Microbiology*, 15(1), 85-106.

[2] Rezaei, Z., Nickfar, F., Salari, A., Yousefi, M., Khodaparast, M. H. H., & Shamloo, E. (2023). Feasibility of biofilm production capacity by *Levilactobacillus brevis* isolated from motal cheese and evaluation of biofilm resistance produced in vitro and in yogurt. *Arabian Journal of Chemistry*, 16(5), 104702.

[3] Rezaei, Z., Khanzadi, S., & Salari, A. (2021). Biofilm formation and antagonistic activity of Lacticaseibacillus rhamnosus (PTCC1712) and Lactiplantibacillus plantarum (PTCC1745). *Amb Express*, 11(1), 156.

[4] Moradi, M., Kousheh, S. A., Almasi, H., Alizadeh, A., Guimarães, J. T., Yılmaz, N., & Lotfi, A. (2020). Postbiotics produced by lactic acid bacteria: The next frontier in food safety. *Comprehensive reviews in food science and food safety*, 19(6), 3390-3415.

[5] Collado, M. C., Meriluoto, J., & Salminen, S. (2007). In vitro analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus. *Food Research International*, 40(5), 629-636.

[6] Touret, T., Oliveira, M., & Semedo-Lemsaddek, T. (2018). Putative probiotic lactic acid bacteria isolated from sauerkraut fermentations. *PloS one*, 13(9), e0203501.

[7] Medina-Pradas, E., Pérez-Díaz, I. M., Garrido-Fernández, A., & Arroyo-López, F. N. (2017). Review of vegetable fermentations with particular emphasis on processing modifications, microbial ecology, and spoilage. In *The microbiological quality of food* (pp. 211-236). Woodhead Publishing.

[8] Rokhtabnak, N., Khaleghi, M., & Sasan, H. (2016). Isolation and identification of *Lactobacillus* bacteria with probiotic potential from traditional dairy in Kerman. *Iranian Journal of Medical Microbiology*, 10(1), 24-34.

[9] Rodríguez-Pazo, N., Vázquez-Araújo, L., Pérez-Rodríguez, N., Cortés-Diéguez, S., & Domínguez, J. M. (2013). Cell-free supernatants obtained from fermentation of cheese whey hydrolyzates and phenylpyruvic acid by *Lactobacillus plantarum* as a source of antimicrobial compounds, bacteriocins, and natural aromas. *Applied biochemistry and biotechnology*, *171*(4), 1042-1060.

[10] de Oliveira, C. M. S., Grisi, C. V. B., de Souza Silva, G., Neto, J. H. P. L., de Medeiros, L. L., dos Santos, K. M. O., & Cardarelli, H. R. (2023). Use of *Lactiplantibacillus plantarum* CNPC 003 for the manufacture of functional skimmed fresh cheese. *International Dairy Journal*, 141, 105628.

[11] Fallah, F., Mortazavi, S.A., and Tabatabai Yazdi, F. (2018). Investigating the properties of probiotic bacteria *Lactobacillus brevis* strain PML1 based on its ability to adhere to intestinal epithelial cells. *Applied Microbiology in Food Industry*, 5(1), 41-53. [in Persian]

[12] Behbahani, B. A., Rahmati-Joneidabad, M., & Taki, M. (2025). Examining the impact of probiotic *Lactiplantibacillus pentosus* 6MMI on inhibiting biofilm formation, adhesion, and virulence gene expression in Listeria monocytogenes ATCC 19115. *Biofilm*, 9, 100255.

[13] Rouhi, A., Falah, F., Azghandi, M., Behbahani, B. A., Mortazavi, S. A., Tabatabaei-Yazdi, F., & Vasiee, A. (2024). Investigating the effect of *Lactiplantibacillus plantarum* TW57-4 in preventing biofilm formation and expression of virulence genes in Listeria monocytogenes ATCC 19115. *Lwt*, 191, 115669.

[14] Liu, C., Xu, L., Chen, S., Wang, S., Lv, M., Dong, K., ... & Cao, Z. (2025). Whole-genome sequencing and probiotic properties of *Lactobacillus helveticus* KM7 isolated from the gut of the Chinese honey bee (Apis cerana): A promising exopolysaccharide-producing strain. *BMC microbiology*, 25(1), 530.

[15] Gu, X., Wang, H., Wang, L., Zhang, K., Tian, Y., Wang, X., ... & Wang, X. (2024). The antioxidant activity and metabolomic analysis of the supernatant of Streptococcus alactolyticus strain FGM. *Scientific Reports*, 14(1), 8413.

[16] Behbahani, B. A., Jooyandeh, H., Vasiee, A., & Zeraatpisheh, F. (2023). Evaluation of anti-yeast metabolites produced by Lactobacillus strains and their potential application as bio-preservatives in traditional yogurt drink. *Lwt*, 188, 115428.

[17] Oancea, A. G., Untea, A. E., Dragomir, C., & Radu, G. L. (2022). Determination of optimum TBARS conditions for evaluation of cow and sheep milk oxidative stability. *Applied Sciences*, 12(13), 6508.

[18] Behbahani, B. A., Jooyandeh, H., Taki, M., & Falah, F. (2024). Evaluation of the probiotic, anti-bacterial, anti-biofilm, and safety properties of Lacticaseibacillus paracasei B31-2. *Lwt*, 207, 116676.

[19] Zawistowska-Rojek, A., Kośmider, A., Stępień, K., & Tyski, S. (2022). Adhesion and aggregation properties of Lactobacillaceae strains as protection ways against enteropathogenic bacteria. *Archives of Microbiology*, *204*(5), 285.

[20] Abushelaibi, A., Al-Mahadin, S., El-Tarabily, K., Shah, N. P., & Ayyash, M. (2017). Characterization of potential probiotic lactic acid bacteria isolated from camel milk. *LWT-food Science and Technology*, 79, 316-325.

[21] Noshad, M., Alizadeh Behbahani, B., & Hojjati, M. (2021). Investigation of probiotic and technological characteristics of lactic acid bacteria isolated from native Doogh of Behbahan. *Food Research Journal*, 31(4), 169-186.

[22] Barzegar, H., Alizadeh Behbahani, B., & Falah, F. (2021). Safety, probiotic properties, antimicrobial activity, and technological performance of Lactobacillus strains isolated from Iranian raw milk cheeses. *Food Science & Nutrition*, 9(8), 4094-4107.

[23] Kishilova, S. A., Rozhkova, I. V., Kolokolova, A. Y., Yurova, E. A., Leonova, V. A., & Mitrova, V. A. (2024). Assessment of the Biological Activity of a Probiotic Fermented Milk Product with the Addition of Lactobacillus helveticus Cell-Free Supernatant. *Fermentation*, 10(10), 503.

[24] Piwat, S., Sophatha, B., & Teanpaisan, R. (2015). An assessment of adhesion, aggregation and surface charges of Lactobacillus strains derived from the human oral cavity. *Letters in Applied Microbiology*, 61(1), 98-105.

[25] Sanders, M. E., Akkermans, L. M., Haller, D., Hammerman, C., Heimbach, J. T., Hörmannsperger, G., & Huys, G. (2010). Safety assessment of probiotics for human use. *Gut microbes*, 1(3), 164-185.

[26] EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Bampidis, V., Azimonti, G., Bastos, M. D. L., Christensen, H., Dusemund, B., ... & Radovnikovic, A. (2023). Safety and efficacy of a feed additive consisting of copper (II)-betaine complex for all animal species (Biochem Zusatzstoffe Handels-und Produktionsges. mbH). *EFSA Journal*, 21(2), e07817.

[27] EFSA Panel on Biological Hazards (BIOHAZ), Koutsoumanis, K., Allende, A., Álvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., ... & Herman, L. (2023). Update of the list of qualified presumption of safety (QPS) recommended microorganisms intentionally added to food or feed as notified to EFSA. *EFSA Journal*, *21*(1), e07747.

[28] Kim, T., Mondal, S. C., Jeong, C. R., Kim, S. R., Ban, O. H., Jung, Y. H., ... & Kim, S. J. (2022). Safety evaluation of *Lactococcus lactis* IDCC 2301 isolated from homemade cheese. *Food Science & Nutrition*, 10(1), 67-74.

[29] Lee, M., Bang, W. Y., Lee, H. B., Yang, S. Y., Lee, K. S., Kang, H. J., ... & Yang, J. (2024). Safety Assessment and Evaluation of Probiotic Potential of *Lactobacillus bulgaricus* IDCC 3601 for Human Use. *Microorganisms*, 12(10), 2063.

[30] Abedi, A., Tafvizi, F., Akbari, N., & Jafari, P. (2024). Cytotoxic activity and apoptosis induction by supernatant of *Lentilactobacillus buchneri* on HT-29 colon cancer cells. *Iranian Journal of Microbiology*, *16*(2), 219.

[31] Begley, M., Gahan, C. G., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS microbiology reviews*, 29(4), 625-651.

[32] Wang, J., Wang, J., Yang, K., Liu, M., Zhang, J., Wei, X., & Fan, M. (2018). Screening for potential probiotic from spontaneously fermented non-dairy foods based on in vitro probiotic and safety properties. *Annals of Microbiology*, 68(12), 803-813.

[33] Zhang, W., Lai, S., Zhou, Z., Yang, J., Liu, H., Zhong, Z., ... & Peng, G. (2022). Screening and evaluation of lactic acid bacteria with probiotic potential from local Holstein raw milk. *Frontiers in microbiology*, 13, 918774.

[34] Zhang, W., Lai, S., Zhou, Z., Yang, J., Liu, H., Zhong, Z., ... & Peng, G. (2022). Screening and evaluation of lactic acid bacteria with probiotic potential from local Holstein raw milk. *Frontiers in microbiology*, 13, 918774.

[35] Zhao, J., Zeng, X., Xi, Y., & Li, J. (2024). Recent advances in the applications of *Lactobacillus helveticus* in the fermentation of plant-based beverages: A review. *Trends in Food Science & Technology*, 147, 104427.

[36] Papadimitriou, K., Alegría, Á., Bron, P. A., De Angelis, M., Gobbetti, M., Kleerebezem, M., ... & Kok, J. (2016). Stress physiology of lactic acid bacteria. *Microbiology and Molecular Biology Reviews*, 80(3), 837-890.

[37] Floris, I., Battistini, R., Tramuta, C., Garcia-Vozmediano, A., Musolino, N., Scardino, G., ... & Bianchi, D. M. (2025). Antibiotic resistance in Lactic Acid Bacteria from dairy products in Northern Italy. *Antibiotics*, 14(4), 375.

[38] Duche, R. T., Singh, A., Wandhare, A. G., Sangwan, V., Sihag, M. K., Nwagu, T. N., ... & Ezeogu, L. I. (2023). Antibiotic resistance in potential probiotic lactic acid bacteria of fermented foods and human origin from Nigeria. *BMC microbiology*, 23(1), 142.

[39] Kos, B. V. Z. E., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., & Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of applied microbiology*, 94(6), 981-987.

[40] García-Cano, I., Rocha-Mendoza, D., Ortega-Anaya, J., Wang, K., Kosmerl, E., & Jiménez-Flores, R. (2019). Lactic acid bacteria isolated from dairy products as potential producers of lipolytic, proteolytic and antibacterial proteins. *Applied microbiology and biotechnology*, 103(13), 5243-5257.

[41] Hasali, N. H. M., Zamri, A. I., Lani, M. N., Matthews, V., & Mubarak, A. (2024). Cheese containing probiotic *Lactobacillus brevis* NJ42 isolated from stingless bee honey reduces weight gain, fat accumulation, and glucose intolerance in mice. *Heliyon*, 10(4).

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مقاله علمی_پژوهشی

شناسایی و اندازه‌گیری ویژگی‌های پروبیوتیکی در سویه بومی *Lactobacillus helveticus* C7303: فعالیت‌های ضدمیکروبی، ضدبیوفیلم و آنتی‌اکسیدانی برای کاربرد در فرآورده‌های لبنی

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چکیده

امروزه، سویه‌های بومی باکتری‌های اسید لاکتیک (LAB) در صنعت لبنیات از اهمیت قابل توجهی برخوردار شده‌اند. این سویه‌ها علاوه بر سازگاری ذاتی با شرایط محیطی محلی، توانایی‌های منحصربه‌فردی در تولید طعم و عطر مطلوب برای تهیه محصولات تخمیری مختلف دارند. این پژوهش با هدف بررسی ویژگی‌های پروبیوتیکی، عملکردی، ایمنی و زیستی سویه بومی *Lactobacillus helveticus* C7303 جداسازی شده از پنیر خیکی انجام شد. ارزیابی شامل بررسی خواص آنتی‌اکسیدانی، پروفایل بیان ژن، ویژگی‌های ضدبیوفیلم، مقاومت به آنتی‌بیوتیک‌های درمانی رایج و ویژگی‌های ضدمیکروبی علیه پاتوژن‌های باکتریایی بود. این سویه سطوح بالایی از فعالیت ضدچسبندگی نشان داد، به طوری که ۶۱/۵ درصد مهار در آزمون رقابتی، ۵۳/۲ درصد در آزمون مهار و ۳۰/۵ درصد در آزمون جابجایی نشان داده شد. *L. helveticus* C7303 فعالیت ضدباکتریایی قوی علیه چندین پاتوژن گرم‌مثبت و گرم‌منفی از جمله *Staphylococcus aureus*، *Bacillus cereus*، *Listeria monocytogenes*، *Escherichia coli*، *Salmonella* enterica serovar Typhimurium و *Shigella dysenteriae* از خود نشان داد. در روش کشت دولایه اصلاح‌شده، بیشترین هاله مهار برای *S. aureus* و *L. monocytogenes* ثبت شد، در حالی که کمترین اثر علیه *E. coli* و *S. Typhimurium* مشاهده گردید. نتایج آزمون MTT تأیید کرد که سوپرناتانت عاری از سلول (CFS) این سویه باعث کاهش معنی‌داری در زنده‌مانی سلول‌های سرطانی شد. این سویه همچنین حساسیت بالایی به بیشتر آنتی‌بیوتیک‌های آزمایش‌شده نشان داد؛ بیشترین هاله مهار مربوط به اریترومایسین (۲۷/۵ میلی‌متر) و کمترین حساسیت مربوط به آمپی‌سیلین (۱۶/۲ میلی‌متر) بود. با توجه به نتایج مثبت حاصل از آزمون‌های عملکردی و ایمنی، *L. helveticus* C7303 پتانسیل امیدوارکننده‌ای برای استفاده به عنوان کشت آغازگر لبنی و یک پروبیوتیک عملکردی با اثرات تغذیه‌ای و درمانی نشان می‌دهد.