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Original Article

Assessing Protection Mechanisms against *Escherichia coli* by Analyzing Auto-and Co-Aggregation, Adhesion Ability, Antagonistic Activity and Safety Characteristics of Potentially Probiotic *Lactobacillus acidophilus* B103

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ABSTRACT

Background and Objectives: The aim of this study was to assess protection mechanisms against *Escherichia coli* by analyzing aggregation, adherence, antagonistic activity and safety characteristics of potentially probiotic *Lactobacillus acidophilus* B103.

Materials and Methods: Potentially probiotic *L. acidophilus* B103 has been investigated in various aspects. Studied key characteristics included its pH and bile resistance, cell surface hydrophobicity and aggregation, cholesterol removal ability, hydroxyl radical scavenging activity and adhesion ability to Caco-2 cell monolayers. Antagonistic activity of the strain was assessed by adhesion competition on *E. coli* via competition, inhibition and replacement assays. Moreover, safety characteristics were investigated through DNase, hemolytic activity, biogenic amine production and antibiotic susceptibility assays.

Results: *L. acidophilus* B103 included high stability to acidic pH (2.5 and 4.5), simulated gastric and intestinal juices and bile salt concentrations (up to 5% w/v). Moreover, *L. acidophilus* B103 showed relatively high hydrophobicity (51.79%), auto-aggregation (42.38%), co-aggregation (34.48%), cholesterol removal (46.27%) and hydroxyl radical scavenging activities (51.36%). Competition, replacement and inhibition anti-adhesion assays of the strain against *E. coli* were 52.13, 25.20 and 46.40%, respectively. Furthermore, *L. acidophilus* B103 adhered to simulated epithelial cells with a capacity of 11.85%. Neither DNase nor hemolytic activity was observed in the bacterial strain and the strain was highly sensitive to ciprofloxacin.

Conclusions: Based on the results, safety assessments and its food origin, *L. acidophilus* B103 demonstrates potential for use in the food industry. It can serve as starter culture, co-culture and bio-protective agent, effectively enhancing safety and quality of the food products.

Keywords: *Lactobacillus acidophilus* B103, Hemolytic activity, Cholesterol removal, Biogenic amine

1. Introduction

Nowadays, nutrition sciences and associated fields are focusing on using foods to promote well-being, improve health and decrease the risks of diseases (1). As health care costs increase, life expectancy increases and elderly people desire better quality of life, this concept is particularly relevant. Furthermore, the focus has shifted from medication to prevention. A key support of the health care system should therefore include development and contribution of functional foods (e.g., probiotics, prebiotics and synbiotics) (2–5). In addition to providing traditional nutrients, functional foods include further benefits such as promoting health statuses, improving physical and mental health and inhibiting and/or lowering nutrition-associated diseases (6).

Probiotics are living microorganisms, which when administered in enough amounts, promote health functions in animal or human hosts. Of *Lactobacillus* strains, *L. acidophilus, L. fermentum, L. rhamnosus*, *L. plantarum* and *L. delbrueckii* are the most generally known probiotics (7–10). Probiotics exhibit progressive natures in inhibiting growth of pathogenic microorganisms through various mechanisms. These include their ability to adhere to intestinal cells, preventing adhesion of pathogenic

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microorganisms. Probiotics release diverse antimicrobial compounds such as organic acids and bacteriocins, which help fight against growth of the pathogens. In addition, probiotics contribute to improvement of the immune system, further enhancing body defense mechanisms against harmful microorganisms (11–13). Probiotics include potentials to treat various diseases such as obesity, insulin resistance syndrome, type 2 diabetes and non-alcoholic fatty liver disease. They can enhance body immune response, increasing resistance to diseases. Additionally, studies have suggested that specific probiotic bacteria may play significant roles in treatment of certain cancers. It is noteworthy that the benefits of probiotics depend on factors such as the specific strain, dosage and components used to produce a particular probiotic product (7, 14).

The *L. acidophilus*, a well-known species within the *Lactobacillus* genus of lactic acid bacteria (LAB), can be detected in the gastrointestinal tract (GIT) and vagina of humans and animals. These environments are often characterized by their acidity. Morphological structure of *L. acidophilus* consists of Gram-positive non-spore-forming rods with rounded ends. They can be observed either as individual cells, pairs or short chains, typically measuring 0.6–0.9 × 1.5–6 µm. The *L. acidophilus* is addressed as an obligate homofermentative LAB, thriving in anaerobic conditions; although a few strains are facultative heterofermenters. The presence of acidophilus contributes to maintaining an acidic environment within the body, which is critical for inhibiting growth of harmful bacteria. The balance between the good and bad bacteria is essential for overall health and certain medical conditions can disrupt this balance. Use acidophilus microorganisms as probiotics may help restore this equilibrium. Acidophilus microorganisms are naturally detected in various food sources and available as dietary supplements as *L. acidophilus* (15, 16).

The objective of this study was to assess probiotic capabilities of *L. acidophilus* B103 isolated from yogurt. The assessment involved analyzing various factors, including pH and bile resistance, physicochemical characteristics of the strain such as hydrophobicity, auto and co-aggregations, cholesterol removal, hydroxyl radical scavenging activity, adhesion ability to Caco-2 cell monolayers and adhesion competition with *E. coli* through competition, inhibition and replacement assays. Additionally, the current study investigated DNase activity, haemolytic activity, production of biogenic amines and antibiotic susceptibility of the strain.

2. Materials and Methods

2.1. Materials

Chemicals and reagents used in this study included PCR kit (Parstous Biotech, Mashhad, Iran), Genomic DNA Isolation VI kit (Asian Dena-Zist, Iran), Muller-Hinton agar, de Man-Rogosa-Sharpe (MRS) agar and broth, peptone water (Merck, Darmstadt, Germany), DNase media (HiMedia, Mumbai, India), bile salt, pepsin, trypsin, fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), Triton X-100, penicillin-streptomycin (10,000 U/mL), trypsin-EDTA, erythromycin, imipenem, ciprofloxacin, ampicillin, chloramphenicol, nalidixic acid and nitrofurantoin (Sigma-Aldrich, USA).

2.2. Isolation and identification of the probiotic strain

Six samples of yogurt were collected randomly from a local market (Tashan, Khuzestan, Iran) and stored under refrigeration until use. After homogenizing samples (5 g each) in peptone water (0.1%; 45 mL), serial dilutions (10^{-1}–10^{-6}) were prepared and cultured on MRS agar. Following isolation from the culture media, strain was investigated for Gram-staining and catalase activity. Using Genomic DNA Isolation VI kit, genomic DNA was extracted from the strain and the strain was cultured overnight in MRS broth. Moreover, 1492R (5′-GGTTACCTTGTTACGACTT-3′) and 27FYM (5′-AGAGTTTGATYMTGGCTCAG-3′) were used as universal primers for 16S rRNA gene amplification. A 25.15-µl reaction volume, including 16.5 µl of water, 2 µl of dNTP, 2.5 µl of 10× buffer, 1.25 µl of each primer, 1.2 µl of $MgCl_2$, 1.5 µl of the DNA template and 0.2 µl of Taq polymerase, was used to amplify DNA with the aim of master PCR kit. The PCR conditions included (i) one cycle of initial denaturation (95 °C, 5 min), (ii) 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 2 min and (iii) one cycle of final elongation (72 °C, 10 min). In the following steps, PCR products were electrophoresed on agarose gels for 45 min at 95 V to assess if the segments were amplified. To assess homology of the sequences from the amplified genes, nucleotides were sequenced. It was assessed that the isolate, with catalase-negative characteristics and Gram-positive characteristics, belonged to *L. acidophilus* B103 with 99% similarity (17).

2. 3. pH stability

A modified method by Topcu et al. (2020) was used to assess the strain's stability at acidic pH levels (18). Isolate was cultivated in 5 mL MRS broth for 18–24 h at 37 °C under anaerobic conditions. The bacteria were centrifuged at 4 °C for 10 min at 6,000× g. Then, pellets were rinsed twice and resuspended in PBS buffer with pH (HI 221 pH Meter, Hanna Instruments, Woonsocket, RI, USA) adjusted to 2.5 and 4.5. These were then incubated at 37 °C for 0, 1, 2 and 3 h. A serial dilution method was used to count the number of bacteria that survived on MRS agar plates. The count of living *L. acidophilus* B103 was carried out as log colony-forming units (CFU) per mL.

2.4. Bile stability

First, MRS broth was inoculated with the strain and incubated at 37 °C for 24 h. Centrifuge was used to separate

the precipitated cells from the suspension (9,000× g, 4 °C, 5 min) and the cells were recentrifuged after being washed with sterile phosphate buffer. The MRS agar containing bile salts (0.1, 0.3, 0.5 and 0.7% w/v) was used to culture the microbial suspension (100 µl). After anaerobically incubating at 37 °C for 24 h, results were visually observed (19).

2. 5. Resistance to simulated gastrointestinal tract juices

The MRS broth containing 30 mL of the strain (overnight culture) was centrifuged at 8,000× g for 5 min at 4 °C. After removing the supernatant and collecting the cells, 10 mL of 50 mM PBS (pH 6.5) were used twice to wash the cells and resuspension in 3 mL of PBS buffer was carried out. Nine milliliters of the simulated gastric fluid (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$ and 3 g/l pepsin; pH 2.5) were added to 1.0 mL of the strain (9 log CFU/mL). Then, suspension was incubated at 37 ℃ for 3 h. Suspension was centrifuged at 3,800 rpm for 10 min and the supernatant was removed, followed by washing the pellet with PBS. Pellet was resuspended in 1.0 mL of the simulated intestinal fluid (pH 8), containing 0.15% bile salt and 0.1% pancreatin and then suspension was incubated at 37 °C for 3 h. Number of the survival bacteria was counted after incubation and expressed as log CFU/mL (20).

2.6. Hydrophobicity

The probiotic strain was harvested by centrifugation (5000× g, 15 min) and washed with and resuspended in phosphate buffer to reach an absorbance of nearly 1.0 at 600 nm (H_1). Then, an aliquot of n-hexadecane (0.6 mL) was added to the bacterial suspension (3.0 mL) and vortexed for 2 min. The lower phase absorbance was measured at 600 nm (H_2) following incubation (25 °C, 1 h) and surface hydrophobicity of the microbial cells was calculated as follows (8):

$$\text{Surface hydrophobicity (\%)} = \left(\frac{H_1 - H_2}{H_1}\right) \times 100$$

2.7. Auto-aggregation capacity

The *L. acidophilus* B103 was assessed for auto-aggregation capacity using Fadda et al. (2017) method with modifications (21). This was achieved by centrifuging an overnight culture (10 min at 6,000 rpm) using cold phosphate buffer to wash and dissolve the pellet to achieve an absorbance of 0.60 at 600 nm (A_0). Incubation of the sample was carried out at 25 °C for 30 min and optical density (A_1) of the bacterial suspension was measured. To calculate auto-aggregation of the strain, the following equation was used:

$$\text{Auto-aggregation (\%)} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

2. 8. Co-aggregation

The co-aggregation assay was used by measuring the absorbance of *L. acidophilus* B103 (A_L) and *Staphylococcus aureus* (A_S) suspensions at 600 nm separately. After mixing the suspension for 30 s, the mixture was incubated at 25 °C for 30 min and the absorbance was measured at 600 nm (A_M) for co-aggregation calculation as follows (22):

$$\text{Co-aggregation (\%)} = \left(1 - \frac{A_M}{\frac{A_L + A_S}{2}}\right) \times 100$$

2.9. Adhesion capacity

Strain was assessed for adhesion capacity using Caco-2 cell lines (23). Briefly, DMEM (containing 1% of penicillin-streptomycin and 10% of heat-inactivated FBS) was used to culture the cells at 37 °C under a humidified atmosphere of 5% carbon dioxide (CO_2). When Caco-2 cells had reached 80% confluency, they were trypsinized using 1% trypsin-EDTA and transferred to 6-well tissue plates at density of 30,000 cells/cm^2. It was necessary to incubate the cells and change the media periodically until a differentiated cell monolayer was formed. Then, sterile phosphate buffer was used to wash the plates to remove the old media, particularly penicillin-streptomycin. The overnight bacterial culture was centrifuged (6,000 rpm, 10 min) and the pellet washed with cold phosphate buffer two times before resuspending in DMEM at a concentration of 10^8 bacteria/mL and using to confluent Caco-2 monolayers. Wells were emptied and washed with phosphate buffer and the unbounded bacteria were removed from the plates after incubation (37 °C, 1 h, 95% air/5% CO_2). After incubating for 10 min, Triton X-100 (0.1% v/v) was used to lyse Caco-2 cells and count of bacteria was carried out on MRS agar. This was followed by the calculation of adhesion capacity:

$$\text{Adhesion capacity (\%)} = \left(\frac{\text{Adhered bacteria}}{\text{Total number of bacteria in the wells}}\right) \times 100$$

2.10. Anti-adhesion activity

The competition, inhibition and replacement assessments were carried out to investigate anti-adhesion potential of *L. acidophilus* B103 against *E. coli* (8, 17). Equal quantities of the bacteria were added to the wells and incubated at 37 °C for 1.0 h under 5% CO_2 atmosphere. Sterile phosphate buffer was used to wash free (unbounded) bacteria and 0.05% Triton X-100 was used to detach the adhered bacteria (probiotic and pathogenic species). For the detection of pathogens, selective media were used and competition between the two species for Caco-2 cell adhesion was calculated using the following equation:

$$\text{Competition (\%)} = \left(\frac{\text{Adhered } \textit{E. coli} \text{ combination with } \textit{L. acidophilus} \text{ B103}}{\text{Bounded } \textit{E. coli} \text{ in the absence of } \textit{L. acidophilus} \text{ B103}}\right) \times 100$$

Inhibition assay was used to assess the probiotics ability to inhibit adhesion of *E. coli* to the intestinal cells. The *L. acidophilus* was first transferred to a well containing Caco-2 cells, where it was incubated at 37 °C for 1.0 h under CO_2

pressure. Phosphate buffer wash was used to remove free bacteria and *E. coli* was then added to the well. Incubation for 1.0 h and removal of unbounded *E. coli* were followed by detaching Caco-2 cells and bacteria species with Triton X-100. Bacteria were counted and inhibitory effect was calculated as follows:

$$\text{Inhibition (\%)} = \left(1 - \frac{\textit{E. coli} \text{ adhesion in the presence of } \textit{L. acidophilus} \text{ B103}}{\textit{E. coli} \text{ adhesion in the absence of } \textit{L. acidophilus} \text{ B103}}\right) \times 100$$

The replacement assay was carried out to assess if *L. acidophilus* B103 could replace the pathogenic strain in the intestinal cells. Steps of the assay were repeated, except *E. coli* was added first and then *L. acidophilus* B103 after incubation (1.0 h). Percentage of displacement was calculated by comparing the adhered *E. coli* with those without *L. acidophilus*.

2. 11. Antibacterial activity

2. 11.1. Well diffusion method

In this study, *L. acidophilus* B103 was assessed for its antibacterial characteristics against *S. aureus*, *E. coli*, *S. epidermidis*, *Salmonella enterica* serovar Typhi, *Bacillus cereus*, *Shigella dysenteriae* and *Listeria innocua*), based on the method described by Georgieva et al. (2015) (24). The MRS broth was used to culture the strain (28 h, 37 °C) and centrifuged at 5,000 rpm for 20 min at 4 °C. The pH of half of the cell-free supernatant (CFS) was not changed, while pH adjustment of 5.5 was used to the other half of the solution to neutralize the acidic effect created by organic acids. To achieve the acid and neutralized CFS (aCFSs and nCFSs), mixtures were filtered (0.22 µm) and freeze-dried. The CFS fractions were hydrated with sterile distilled water (DW) (2 mL) and with a quantity of 100 µl were loaded with pathogenic bacteria (0.5 McFarland concentration) in wells of Muller-Hinton agar. After 48 h of incubation at 37 °C, measurements were carried out on the inhibition zone diameters.

2.11.2. Modified double layer method

Double layer or spot-on-the-lawn method was used to assess antagonistic activity of the isolate (25). The MRS broth was used to culture the probiotic strain (12 h at 37 °C). After spotting onto MRS agar, plates were incubated at 37 °C for 24 h and then overlaid with melted Muller-Hinton agar. The solidified media were inoculated with 100 µl of the pathogenic bacteria and re-incubated for 24 h at 37 °C. To assess pathogen sensitivity to the isolate, clear zones were assessed around the spots at the end of the process.

2.12. Cholesterol assimilation

Method of Ladha and Jeevaratnam (2018) with modifications was used to assess the cholesterol removal ability of *L. acidophilus* B103 (26). The activated *L. acidophilus* B103 was inoculated into the sterilized MRS broth with 0.3% oxgall bile salt and 100 µg/mL of cholesterol stock solution at 37 °C for 28 h. As a control, MRS broth was used without inoculation. Then, fermented media were centrifuged at 6,000 rpm for 8 min at 4 °C. Three milliliters of 95% ethanol and 2 mL of 50% KOH were added to the supernatants (0.5 mL), mixed and heated (60 °C, 10 min). Then, DW (3 mL) and hexane (5 mL) were added to the solution. To separate the phases, mixture was stored (15 min, 25 °C), then 2 mL of hexane were evaporated at 60 °C. The o-phthalaldehyde reagent (2 mL) was added to the mixture, incubated at 25 °C for 10 min and then mixed with 1 mL of concentrated sulfuric acid. For the inoculated and uninoculated samples, absorbance was measured at 570 nm after 10–15 min of incubation at room temperature (RM). A standard curve of cholesterol was established and the cholesterol assimilation (%) was measured as follows:

$$\text{Cholesterol assimilation (\%)} = \left(1 - \frac{T}{C}\right) \times 100$$

Where, T and C were the concentration of cholesterol (mg/mL) in fermentation broth supernatant and uninoculated culture media, respectively.

2. 13. Antioxidant activity

Antioxidant activity of the isolate was assessed according to the report by Somashekaraiah et al. (2019) (27). A mixture of $FeSO_4$ (1.0 mL, 0.75 mM), sodium phosphate buffer (2.0 mL, pH 7.4) and 1,10-phenanthroline (1.0 mL, 0.75 mM) were mixed and vortexed for this assay. Then, *L. acidophilus* B103 (1.0 mL, 10^8 CFU/mL) was added to the mixture. A solution containing hydrogen peroxide (H_2O_2) (1.0 mL, 0.01% v/v) was added to the mixture and incubated (1.5 h at 37 °C) to initiate the reaction. Then, mixture was centrifuged at 8,000 rpm for 10 min at 4 °C and absorbance of the supernatant was measured at 536 nm. To calculate the hydroxyl radical scavenging effect, the following equation was used:

$$\text{Antioxidant activity (\%)} = \left(\frac{As - Ac}{Ab - Ac}\right) \times 100$$

In this equation, As represented absorbance of the test sample, Ac indicated absorbance of the control containing 1,10-phenanthroline, $FeSO_4$ and H_2O_2, while Ab represented absorbance of the blank, containing 1,10-phenanthroline and $FeSO_4$.

2. 14. Safety assessment

2. 14.1. Antibiotic susceptibility

A modified method of Zhou et al. (2005) was used to assess antibiotic susceptibility of *L. acidophilus* B103 to erythromycin, imipenem, ciprofloxacin, ampicillin, chloramphenicol, nalidixic acid and nitrofurantoin (28). First, MRS agar was used to culture the strain (0.5 McFarland), followed by placement of antibiotic discs on the media. After fixing for 10 min at RM, plates were incubated at 37 °C for 48 h and inhibition zones around the antibiotic discs were measured.

2.14.2. DNase and haemolytic activity

The DNase and haemolytic characteristics of the strain were assessed based on the method of Vasiee et al. (2020) (23). The DNase activity of the probiotic strain was assessed using DNase media for 48 h at 37 °C. Presence of a clear pinkish zone around the colonies showed production of DNase. To assess haemolytic activity of *L. acidophilus* B103, the strain was streaked on tryptic soy agar (TSA) supplemented with 7% (v/v) sheep blood. Following incubation at 37 °C for 24 h, plates were assessed for the changes. Presence of blood lysis zones surrounding the colonies on media showed haemolytic activity (β-haemolysis), while presence of green color in the media (α-haemolysis) or no color changes (γ-haemolysis) demonstrated non-haemolytic activity.

2. 14.3. Biogenic amine production

To assess biogenic amine production ability of *L. acidophilus* B103, media containing L-histidine monohydrochloride, L-ornithine monohydrochloride, tyrosine di-sodium salt and L-lysine monohydrochloride were used. The aim was to assess potentials of the strain to catalyze decarboxylation of amino acids, leading to biogenic amine production. First, strain was sub-cultured twice in MRS broth supplemented with 0.005% pyridoxal-5-phosphate and 0.1% precursor amino acids. Then, strain was spotted on MRS agar, containing 0.06% bromocresol purple with or without amino acids. It was reported positive if purple color was observed in surrounding colonies after 2–5 d of incubation (20).

2.15. Statistical analysis

To analyze data, analysis of variance (ANOVA) was carried out using SPSS software v.26. Means were compared using Duncan test at a 95% confidence level to identify significant differences ($p < 0.05$). All experiments were repeated three times to ensure reliable results.

3. Results

3.1. Tolerance to acid, bile salt and simulated GIT juices

Cell viability at pH 2.5 decreased significantly from 9.12 to 6.10 log CFU/mL as the exposure time increased from 0 to 3 h (Fig. 1). However, viability of the probiotic cells was not affected by pH 4.5 (Fig. 1). Simulated GIT juice led to a 18.20% decrease in the count of viable cells (Fig. 1). Viability of the isolate cells under bile salt treatments was measured (Table 1).

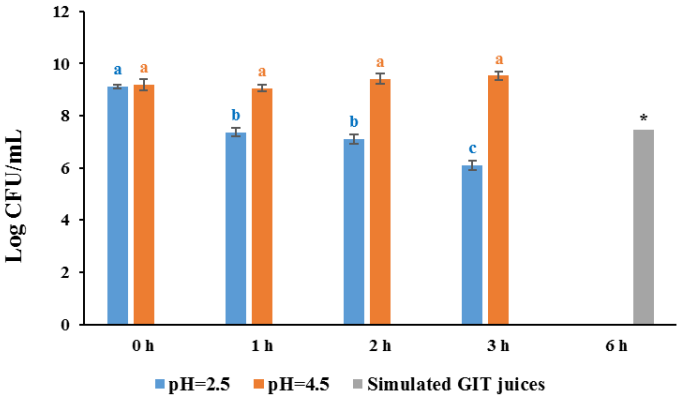


Figure 1. Survivability of *L. acidophilus* B103 under acidic conditions (pH 2.5 and 4.5, 3 h) and simulated GIT juice (6 h). Different letters indicate significant differences between the treatments at $p < 0.05$. * This treatment was not included in statistical analysis.

Table 1. Survivability of *L. acidophilus* B103 under various bile salt concentrations

	0.1%	0.3%	0.5%	0.7%
Survivability	++++	+++	+++	++

++ Moderate survivability
+++ High survivability
++++ Very high survivability

3.2. Cell surface hydrophobicity, auto-aggregation, co-aggregation and adhesion capacity

Surface hydrophobicity assesses ability of the probiotic cells to adhere to the surface of epithelial cells. Isolate showed 51.79% ±0.68 surface hydrophobicity (Fig. 2). Isolate showed 42.38% ±0.44 auto-aggregation and 34.48% ±0.52 co-aggregation (Fig. 2). Adhesion capacity of *L. acidophilus* B103 to Caco-2 cells was 11.85% (Fig. 2).

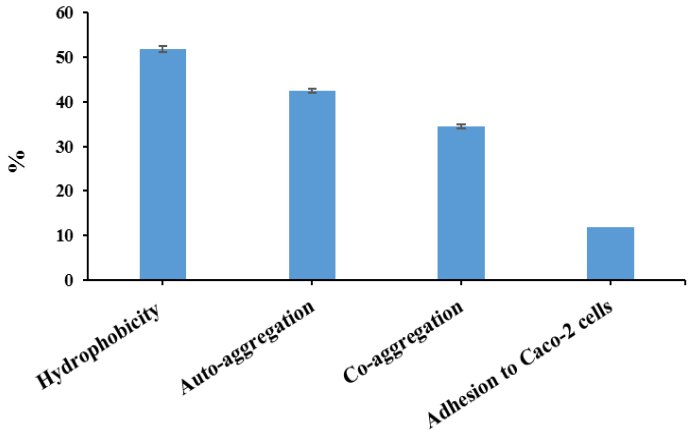


Figure 2. Surface hydrophobicity, auto-aggregation, co-aggregation and adhesion characteristics of *L. acidophilus* B103.

3.3. Anti-adhesion characteristics

Adhesion of *E. coli* to Caco-2 cells decreased by 52.13% ±0.17 when the probiotic strain was added to the culture media simultaneously with *E. coli* (Fig. 3).

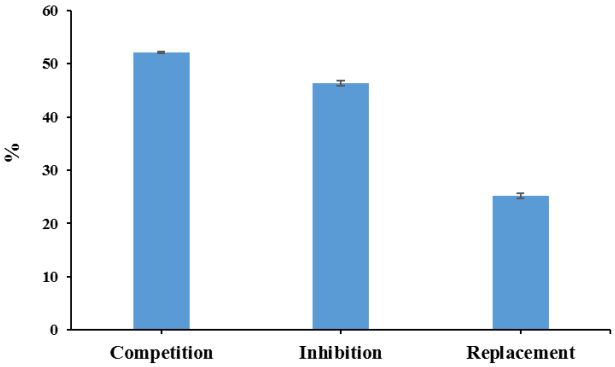


Figure 3. Anti-adhesion effects of *L. acidophilus* B103 against *E. coli* based on the competition, replacement and inhibition assays.

3.4. Antibacterial effects

Antimicrobial effects of aCFS and nCFS fractions of *L. acidophilus* B103 against pathogenic bacteria based on the well diffusion agar method is shown in Table 2. As shown in the table, aCFS and nCFS were able to suppress growth of pathogens; however, the former included generally higher antimicrobial activity than that the latter did. The highest and the lowest antimicrobial effects of nCFS were reported for *S. aureus* and *E. coli*, respectively ($p < 0.05$). Nonetheless, *S. typhi* and *E. coli* were respectively the most sensitive and the most resistant bacterial species to aCFS ($p < 0.05$). Table 2 provides results of antimicrobial effects of the probiotic strain against pathogenic bacteria through the modified double-layer method. The highest and the lowest inhibition zones were observed for *S. aureus* and *S. dysenteriae*, respectively ($p < 0.05$).

3.5. Cholesterol assimilation and antioxidant activity

Cholesterol assimilation activity of the isolate was 46.27% ±0.66. Ability of *L. acidophilus* B103 in scavenging hydroxyl radicals was 51.36% ±0.85.

3.6. Safety assessment

The *L. acidophilus* B103 did not show haemolytic and DNase activities and was not able to produce biogenic amines. Another important characteristic of the probiotics is their antibiotic resistance to assess their safety status for use in foods. Results showed that *L. acidophilus* B103 was sensitive to ciprofloxacin, chloramphenicol, imipenem and erythromycin, semi-sensitive to nitrofurantoin and resistant to nalidixic acid and ampicillin (Fig. 4).

4. Discussion

The bacterial survival and growth in GIT can be predicted by assessing their resistance to acidic pH and bile salts. This characteristic is necessary for choosing isolates with probiotic characteristics (24). It is possible that the constant gradient between the extracellular and intracellular pH values is responsible for the isolate ability to resist acidic pH. Gram-positive microorganisms can be protected against acidic conditions by F_1F_0-ATPase, which can increase intracellular pH under acidic circumstances and then decrease lethal effects induced by such conditions (25). Similarly, it has been reported that *L. acidophilus* (BG2FO4) show rapid decreases in numbers at pH 2.0, but the viable cell number do not increase significantly at pH 4.0 (29). Moreover, Lankaputhra (1995) reported that *L. acidophilus* strains survived well at pH ≥ 3.0 and the counts of viable cells were > 10^7 CFU/g after 3 h of incubation (30); majorly because of the acidic condition of the juice and antimicrobial activity of pepsin (17). Similarly, Barzegar et al. (2021) reported that *L. acidophilus* (B14) showed the greatest resistance to simulated GIT, compared to other probiotic strains (20). Ashraf and Smith (2016) detected that *L. acidophilus* 388, *L. reuteri*, *L. rhamnosus* G5435, *Bifidobacterium lactis* BB12, *Streptococcus thermophilus* 1,342 and *S. thermophilus* M5 were able to resist gastric and small intestinal transits (31).

Table 2. Antibacterial effects of *L. acidophilus* B103 against the pathogenic bacteria, based on well diffusion agar and modified double-layer methods

Pathogen	Well diffusion agar (mm)			Modified double layer method (mm)
	Control (distilled water)	aCFS	nCFS	
Escherichia coli	-	7.00 ± 0.50 ^{f}	6.20 ± 0.52 ^{d}	14.17 ± 0.24 ^{ef}
Salmonella enterica serovar Typhi	-	23.10 ± 0.32 ^{a}	12.30 ± 0.41 ^{b}	15.20 ± 0.43 ^{e}
Shigella dysenteriae	-	11.20 ± 0.46 ^{e}	8.10 ± 0.30 ^{c}	13.15 ± 0.19 ^{f}
Bacillus cereus	-	13.40 ± 0.40 ^{d}	9.40 ± 0.24 ^{c}	21.70 ± 0.43 ^{b}
Staphylococcus epidermidis	-	13.15 ± 0.26 ^{d}	11.40 ± 0.54 ^{b}	20.30 ± 0.19 ^{c}
Staphylococcus aureus	-	17.50 ± 0.43 ^{c}	17.40 ± 0.67 ^{a}	23.20 ± 0.10 ^{a}
Listeria innocua	-	21.20 ± 0.42 ^{b}	12.10 ± 0.37 ^{b}	18.30 ± 0.15 ^{d}

Different letters in each column indicate significant differences between samples at $p < 0.05$.
CFS; cell-free supernatant
aCFS; acid CFS
nCFS; neutralized CFS

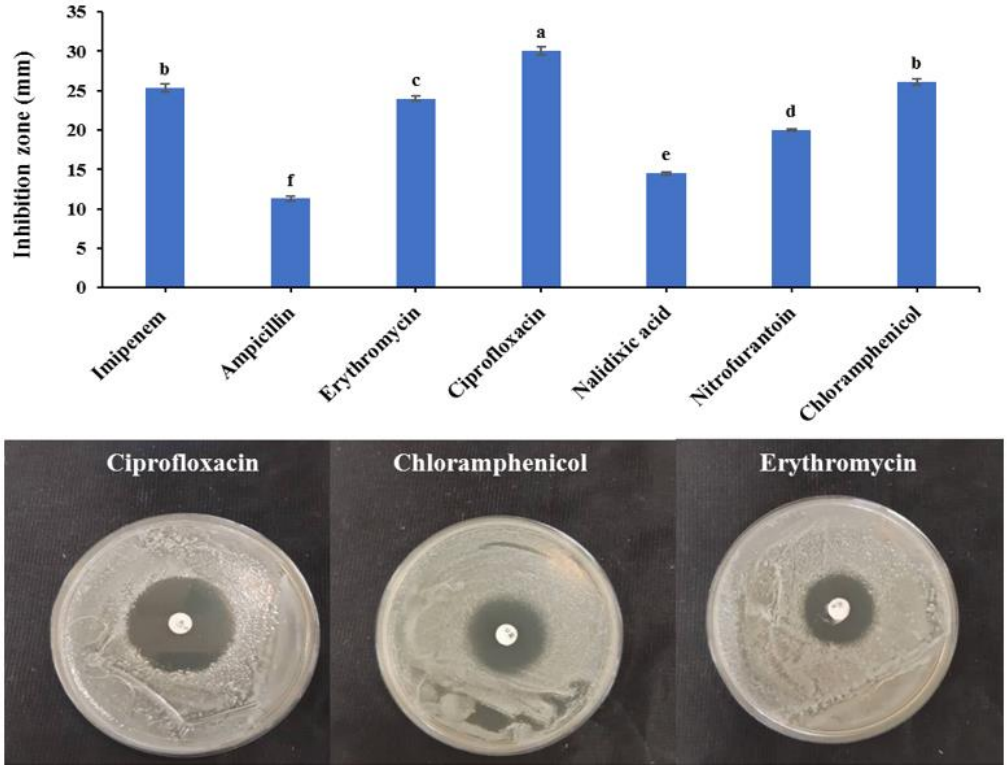


Figure 4. Effects of common antibiotics on the growth of *L. acidophilus* B103. Different letters indicate significant differences between the treatments at $p < 0.05$.

Generally, cell viability decreased significantly as the bile salt concentration increased from 0.1 to 0.7%. Concentration of bile salts in the body do not usually exceed 0.3% (23). At this point, the probiotic strain demonstrated high cell viability in the bile concentration range of 0.1–0.5%. Similar results on the ability of LAB strains to resist high bile salt concentrations have been reported by Pieniz et al. (2014) (32) and Vasiee et al. (2020) (23). Resistant potential of the probiotic strains to bile salts might be linked to their activity in de-conjugating bile salts to cholesterol and amino acids (33).

Cell surface hydrophobicity of LAB and *Lactobacillus* spp. has been reported by studies. Barzegar et al. (2021) showed that the highest and the lowest hydrophobicity values were associated to *L. acidophilus* B14 (65.9%) and *L. casei* B22 (25.6%), respectively, isolated from Iranian raw milk cheeses (20). Similarly, *L. plantarum* strain L15 showed 53% surface hydrophobicity (7). Cell hydrophobicity values of 6.58–73.3, 48.07 and 53.3% have been reported for *L. plantarum* strains (34), *L. brevis* KU15006 from kimchi (35) and *L. brevis* ku200019 (36), respectively. Therefore, the probiotic isolate might be capable of adhering to intestinal cells.

The auto-aggregation characteristic mediated adhesion of the probiotic strain to mucosal surfaces and epithelial cells, while co-aggregation facilitated binding of the strain to pathogenic bacteria (37). Results were similar to those reported by Barzegar et al. (2021), who reported that probiotic strains of *L. acidophilus* B14 and *L. acidophilus* B14 included auto-aggregation levels of 51.3 and 45.0%, respectively (20). The corresponding isolates were able to show nearly 25% of co-aggregation capacity with *S. enterica* serovar Typhimurium (20). In another study by Jena et al. (2013), *Lactobacillus* strains presented 33.2–47.2% of auto-aggregation and 11.89–38.22% of co-aggregation (38). Surface hydrophobicity and auto-aggregation ability of the probiotic bacteria are directly correlated as strains with strong surface hydrophobicity and auto-aggregation potential can attach to the human intestinal cell monolayers further easily (39). Low co-aggregation effects of the probiotic stains can suppress biofilm formation by pathogens in the GIT (17). Auto-aggregating and adhering ability of the probiotic cells are majorly based on the presence of surface-bound proteins and other macromolecules in their structures (20).

Probiotics can be selected by investigating their potential to adhere, at least temporarily, to the epithelial cells (40). Similarly, it was demonstrated that *L. acidophilus* B14, *L. acidophilus* B15 and *L. acidophilus* B17, isolated from Iranian raw milk cheeses included adhesion capacity of approximately 15, 11 and 6%, respectively (20). Moreover, adhesion values of 12.2 and 10.2% were observed in *L. plantarum* L15 and *L. plantarum* Lp91, respectively (7, 41). Direct relationships have been detected between *in-vitro* adhesion abilities of the probiotics and their stability/colonization in the GIT. Relatively, adherence of the probiotics to the intestinal receptor cells can be facilitated by lectin-like protein, S-layer, glyceraldehyde-3-

phosphate dehydrogenase and lipoteichoic acid. Subsequent colonization of the probiotic cells in the intestine may improve the immune system (7, 17, 20)

Infection is caused by pathogenic bacteria, adhering to the host gut cells and releasing toxins (42). Probiotics include potential to decrease attachment of the pathogenic bacteria by blocking receptors and secreting antimicrobial agents (17). Anti-adhesive effects of the probiotics might be linked to the competition between *E. coli* and *L. acidophilus* for nutrients/receptors or the ability of the probiotic strain to produce antimicrobial compounds (e.g., H_2O_2, organic acids, bacteriocins and polysaccharides) (17). Moreover, probiotic strain was able to inhibit adherence of *E. coli* to the intestinal cells (46.40% ±0.50) (Fig. 3). Furthermore, the replacement effect of the strain on *E. coli* was 25.20% ±0.53 (Fig. 3). Similarly, Hojjati et al. (2020) reported that the anti-adhesion capacities of *L. brevis* gp104 against adhesion of *S. aureus* to Caco-2 cells were 52, 47 and 21% for the competition, inhibition and displacement, respectively (8). In another study, it was shown that *Lactobacillus* strains isolated from traditional fermented dairy foods in China were able to inhibit adhesion of *E. coli* to the intestinal epithelial cells (~20–95%) (43). Several factors such as bacterial concentration, growth media, incubation time, normal intestinal microbiota and food matrix digestion can affect adhesion characteristics of the pathogens and probiotic strains to the intestinal cells (44).

Antimicrobial effects of the probiotic strains have been attributed to the production of short-chain fatty acids (CSFAs), organic acids and bacteriocins. Bacteriocins are further active against the growth of Gram-positive pathogens, whereas H_2O_2, hydroxyl fatty acids and organic acids provide greater antimicrobial activities against Gram-negative ones (7, 17). Similarly, Barzegar et al. (2021) reported that *L. acidophilus* strains isolated from Iranian raw milk cheeses were able to inhibit growth of *S. aureus*, *E. coli*, *Pseudomonas aeruginosa* and *S. enterica* serovar Typhimurium (20). Moreover, it has been demonstrated that *L. plantarum* AF1 and *L. plantarum* NO1 included great antibacterial characteristics against *S. aureus*, *E. coli*, *L. monocytogenes* and *S. typhi*, majorly due to the formation of inhibitory compounds such as bacteriocins, CO_2, H_2O_2, organic acids and δ-dodecalactone (45). Antibacterial activity of *L. acidophilus* LAP5 has been reported against pathogenic microorganisms (46).

Despite the fact that cholesterol is an essential part of the body tissues, its high levels (hypercholesterolemia) is the major reason for cardiovascular diseases (CVDs), which is the leading causes of death worldwide. Although drug therapy can be effective in lowering cholesterol levels, long-term medication can be hazardous to the body. Hence, consumption of the fermented foods containing probiotic bacteria is widely accepted (47, 48). Potency of the probiotics in decreasing cholesterol levels is entirely strain-dependent and can be explained by various mechanisms such as bile deconjugation via bile salt hydrolase activity, cholesterol coprecipitation with deconjugated bile, cholesterol-to-coprostanol conversion and cholesterol binding to the probiotic cell surfaces and incorporation into their cell membranes (47). Lipid decreasing capacity of *B. animalis* subsp. *lactis* F1-7 and *L. vaginalis* FN3 have been demonstrated by Liang et al. (2020) (49). Similarly, Anila et al. (2016) reported that cholesterol could be assimilated by fermented food-derived *Lactobacillus* strains, especially if bile salts were present (50).

Probiotic strains may scavenge radicals in the gut after entering and colonizing the intestines (23). Bacteria can produce their own antioxidant enzymes, the most important of which is superoxide dismutase that can catalyze superoxide breakdown into water and H_2O_2. Moreover, antioxidant characteristic of the probiotic strains could be attributed to their metabolites such as butyrate, glutathione and folic acid (51). The ABTS radical scavenging activity of *Enterococcus durans* LAB18s has been verified by Pieniz et al. (2014) (32) and it was suggested that the strain could be used to decrease oxidative damages in human and animal foods. Moreover, ability of *S. thermophilus* 821 in chelating ferrous and cupric ions has been reported by Lin and Yen (1999) (52). *Lactobacillus brevis* LAP2 (53) and *L. mucosae* AN1 and *L. fermentum* SNR1 (54) have been potent antioxidant probiotic strains. It could be therefore considered a safe strain for health-promoting uses. Similar findings have been reported in the literature (20, 23, 45).

Antibiotics can present antimicrobial activity via protein synthesis inhibition, mRNA synthesis prevention and bacterial cell destruction. It is noteworthy that probiotics with native antibiotic resistance may be beneficial for restoring gut microbiota after antibiotic therapy. However, probiotics may carry genes that are resistant to therapeutic antibiotics and their transfer to pathogens can cause severe safety problems (17). Similarly, it has been addressed that *L. acidophilus* isolates are sensitive to chloramphenicol and erythromycin (20). Moreover, it has been reported that all the *Lactobacillus* strains isolated from rat fecal microbiota were sensitive to chloramphenicol, tetracycline, clindamycin, ampicillin, erythromycin, kanamycin streptomycin and gentamycin (38).

Conclusion

This study has verified that *L. acidophilus* B103 is a promising probiotic strain. The strain includes high stability to acidic pH (pH 2.5 and 4.5), simulated gastric and intestinal juices and bile salt concentrations (up to 5% w/v). Moreover, *L. acidophilus* B103 has shown relatively high hydrophobicity (51.79%), auto-aggregation (42.38%), co-aggregation (34.48%), cholesterol removal (46.27%) and hydroxyl radical scavenging activity (51.36%).

Competition, replacement and inhibition anti-adhesion assays of the strain against *E. coli* were 52.13, 25.20 and 46.40%, respectively. Furthermore, *L. acidophilus* B103 adheres to simulated epithelial cells with a capacity of 11.85%. Neither DNase nor haemolytic activity is seen in the strain and the strain is highly sensitive to ciprofloxacin. Based on the findings of probiotic assays, safety assessment and food origin of *L. acidophilus* B103, this strain can be used in the food industry as starter and co-culture as well as bio-protective agent to improve safety and quality of food products.

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Ethical approval

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

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