Original Article
Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.5327/fst.129522

Characterization of in vitro antagonistic activity of *Lactobacillus helveticus* DLBSA201 and DLBSA202 against *Escherichia coli* 0157:H7

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Abstract

Lactobacillus helveticus DLBSA201 and DLBSA202 were investigated for their potential probiotic traits and protective effects against *Escherichia coli* O157:H7. The survival rate of DLBSA201 and DLBSA202 after being exposed to acid and bile salt was relatively high, although no bile salt hydrolase activity was detected. Both strains also demonstrated an outstanding ability to adhere to intestinal epithelial cells. A combination of DLBSA201 and DLBSA202 could interfere with the growth of *E. coli* O157:H7. Furthermore, DLBSA201 and DLBSA202 also exhibited the ability to remove pre-adhered *E. coli* O157:H7 on intestinal cells. Those strains were able to lower pro-inflammatory genes [TLR-4, tumor necrosis factor-α (TNF-α), and interleukin-8 (IL-8)] in lipopolysaccharides (LPS)-treated intestinal epithelial cells. In addition, the upregulation of occludin and ZO-1 genes by DLBSA201 and DLBSA202 also ameliorates the disruption of the intestinal barrier caused by LPS. The above results suggest that DLBSA201 and DLBSA202 association appear as promising probiotic candidates with the potential to prevent and treat intestinal disease caused by *E. coli* O157:H7.

Keywords: lactic acid bacteria; *Escherichia coli* O157:H7; probiotic; inflammation; intestinal barrier.

Practical Application: DLBSA201 and DLBSA202 are potential probiotic strains to counterattack the harmful effects caused by *E. coli* O157:H7.

1. Introduction

Numerous studies have demonstrated the advantages of probiotic consumption for a wide range of disorders, including inflammatory bowel syndrome (Ishaque et al., 2018), pathogen infection (Raheem et al., 2021), diarrhea (Mekonnen et al., 2020), diabetes (Madempudi et al., 2019), and cholesterol (Tjandrawinata et al., 2022), among other effects. The uniqueness of each probiotic to generate bioactive compounds and to reduce or prevent the colonization of pathogens also contributes to their health benefit effects (Fonseca et al., 2021). The use of probiotics is also proposed as an alternative to antibiotic consumption (Wan et al., 2019). Indeed, the exploration of probiotics to support human health has been increasing over the decades. However, it should be noted that potential probiotic traits and health benefits conferred from every single probiotic cannot be generalized and thus strain-specific.

The most widely used probiotic comes from the *Lactobacillus* genus (Hai et al., 2021). Most strains from *Lactobacillus* are generally safe and proven to resist acid, bile, and enzymes from the human gastrointestinal tract (GIT) (Ozkan et al., 2021). The tolerance in the harsh environment caused by low pH and bile is identified as one of the main traits of *Lactobacillus* because these traits allow the strain to remain viable in the digestion tract (Ozkan et al., 2021). Another favorable trait of *Lactobacillus* is the ability of the strains against pathogens, including secretion of antimicrobial substances, competition of nutritional sources, enhancement of intestinal barrier protein, and immunomodulation (Wan et al., 2019). Additionally, they usually have the ability to adhere to and colonize on intestine epithelium cells, thereby obtaining great advantages against harmful pathogens (Hai et al., 2021).

Based on the Indonesia Social Security Administrative Body (Badan Penyelenggara Jaminan Sosial), it was reported that diarrhea occurrence among all ages in Indonesia was 243,983 cases in 2018 (Ira, 2019), and several studies from different cities in Indonesia revealed that *E. coli* was the main cause of the diarrheal disease (Setyarini et al., 2020; Syahrul et al., 2020; Waturangi et al., 2019). Among several pathogenic strains of *E.* coli, the O157:H7 strain appeared as a major source of food-borne outbreaks (Ibrahim et al., 2011). *E. coli* O157:H7 has also been reported as the causative agent causing diarrhea in children in Indonesia (Syahrul et al., 2020). To date, antibiotics have been identified as the most common method to treat the infection (Li et al., 2021), but the treatment of pathogenic bacteria with antibiotics can also increase the possibility of the development of resistant strains, which will bring a new problem for human

Received Dec. 22, 2022.
Accepted May 5, 2023.
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health (Browne et al., 2020; European Food Safety Authority & European Centre for Disease, Prevention and Control, 2020). Therefore, probiotic, which is a safe and natural biological agent that poses lower side effects, is a perfect candidate to reduce or even replace the use of antibiotics (King et al., 2018).

In this study, the probiotic potentials of two strains of *Lactobacillus helveticus* (DLBSA201 and DLBSA202) were characterized. Besides the tolerance to acid and bile salt, bile salt hydrolase (BSH) activity, and adhesion ability to intestinal cells, we also evaluated the antagonistic activity of both isolates against *E. coli* O157:H7 in vitro.

2. Materials and methods

2.1. Bacterial isolates and growth condition

Two *L. helveticus* strains, namely, DLBSA201 and DLBSA202, used in this study were provided by the Metabolic Engineering Section of Dexa Laboratories of Biomolecular Sciences (Indonesia), while an *Escherichia coli* (O157:H7) derived from ATCC 43888 was obtained from Remel Thermo Fisher Scientific (USA). DLBSA201 and DLBSA202 were previously sequenced with Illumina Hi-Seq PE150 and identified by 16s rRNA. Draft genomes from both isolates had been deposited in GenBank with accession numbers NZ_WWEJ00000000 and NZ_WWEK00000000 for DLBSA201 and DLBSA202, respectively. All isolates were sub-cultured twice at 37°C for 18–22 h in *Lactobacillus* De Man, Rogosa, and Sharpe (MRS) HiVeg Broth (HiMedia, India) for DLBSA201 and DLBSA202 isolates or Tryptic Soy Agar (Merck, Germany) for *E. coli* O157:H7 prior to the experiments. If necessary, a multi-strain probiotic mixture that contains DLBSA201 and DLBSA202 was produced at a ratio of 1:1 after each isolate was standardized using McFarland 3 (~9.0×10^8 cells/mL).

2.2. Cell culture

Human colorectal adenocarcinoma (Caco-2) HTB-37 was purchased from ATCC and was grown in Minimum Essential Media (MEM) (Gibco, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco, USA) and 1% (v/v) Penicillin-Streptomycin (Gibco, USA). Caco-2 cells were seeded in a six-well plate at a density of 3×10^5 cells/well for assays and were maintained at 5% CO_2 at 37°C throughout the experiment. The media were replaced every 2–3 days for 18 days after cell confluence to produce a Caco-2 monolayer or as indicated. Two hours before treatment, the media were changed into MEM containing 0.5% (v/v) FBS without penicillin-streptomycin.

2.3. Acid resistance test

The acid resistance test was conducted as previously described (Pavli et al., 2016) with some modifications. DLBSA201 and DLBSA202 were inoculated into MRS HiVeg Broth and were incubated at 37°C for 24 h in aerobic conditions. After incubation, 1% (v/v) of each culture was inoculated into 10 mL of 1× phosphate buffer saline (PBS) as a control or into 1× PBS pH 3 that was previously adjusted using 37% HCl. The suspension was incubated at 37°C for 0, 2, and 4 h, and at each specific time, the viable lactic acid bacteria (LAB) was enumerated on MRS HiVeg Agar (HiMedia, India) using the pour plate method. Incubation of viable LAB was held at 37°C for 48 h in aerobic conditions. The experiment was done in triplicate.

2.4. Bile salt resistance test

The bile salt resistance test was carried out following the previous study (Pavli et al., 2016) with modification. Bile salt resistance was investigated by observing the ability of DLBSA201 or DLBSA202 isolates to grow in the presence of 0.5% bile salt with pH 8. A volume of 1 mL of sub-cultured DLBSA201 or DLBSA202 was transferred into 10 mL of 1× PBS containing 0.5% bile salt (Merck, Germany) with pH 8. PBS without the addition of 0.5% bile salt was used as a control. The cultures were then incubated at 37°C for 0, 2, and 4 h. At each specific time, the LAB was enumerated on MRS HiVeg Agar. Incubation of LAB on MRS HiVeg Agar was carried out aerobically at 37°C for 48 h.

2.5. Bile salt hydrolase activity

BSH activity was determined as described previously (Tsai et al., 2014). Briefly, DLBSA201 and DLBSA202 isolates were grown on MRS HiVeg Agar supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA; Sigma, USA), 0.5% (w/v) glycolic acid sodium salt (Sigma, USA), or 0.5% (w/v) bile salt. All media were also supplemented with 0.037% calcium chloride (Sigma, USA). The plates were incubated under anaerobic conditions at 37°C for 72 h and observed for the precipitation zone surrounding colonies that indicated bile salt hydrolase activity of bacteria.

2.6. Adhesion assay on Caco-2 cells

Caco-2 cells were plated into a six-well culture plate and cultivated at 37°C with 5% CO_2. Before the adhesion assay, the cells were washed two times with 1× PBS. DLBSA201 and DLBSA202 were prepared by growing the cells in MRS broth (Merck, Germany) for 24 h at 37°C and were harvested by centrifugation at 8,000 rpm for 10 min at room temperature, washed two times with 0.85% saline, and then were resuspended in MEM (without antibiotic) with a final concentration of 1×10^8 colony-forming units (CFU)/mL. The initial number of DLBSA201 and DLBSA202 was enumerated in this suspension as was recorded as B_0. Adherence assay was performed by adding 1 mL of *Lactobacillus* suspension onto a monolayer of Caco-2 cells and incubating for 2 h at 37°C. Next, MEM was removed, and the Caco-2 monolayer was washed three times with sterile 1× PBS to remove non-adherent bacterial cells. Subsequently, epithelial cells were delicately dissociated by trypsinization with trypsin-EDTA (Gibco, USA), collected by centrifugation at 5500 rpm for 5 min at room temperature, and lysed by incubation with 1% Triton X-100 (Merck, Germany) in PBS for 5 min. Adherent bacteria (B_1) were counted by placing the suspension at appropriate dilution on MRS Agar (Merck, Germany) and incubated at 37°C for 48 h. This assay was carried out in triplicate, and the adhesion percentage was calculated as Equation 1:

$$\%\ adhesion = \frac{B_1}{B_0} \times 100\% \quad (1)$$

2.7. Antibacterial activity

The antimicrobial activity of DLBSA201, DLBSA202, and the mixture of both isolates (1:1) against enterohemorrhagic *E. coli* O157:H7 was tested using agar-well diffusion assay. *E. coli* O157:H7 was grown overnight at 37°C in 10 mL of Nutrient Broth (Oxoid, UK) and then spread on top of Nutrient Agar (Oxoid, UK) with concentration 1×10^7 CFU. A 6-mm diameter hole was made on the agar, and 50 µL of LAB supernatant that was previously grown in MRS HiVeg Broth for 24 h was put into the hole. The agar plates were then incubated at 37°C for 24 h, and nalidixic acid (Oxoid, UK) was used as a positive control. At the end of the experiment, growth inhibition zones were measured in millimeters. All inhibition assays and controls were carried out in triplicate.

2.8. Escherichia coli *O157:H7 growth in cell-free supernatant of DLBSA201 and DLBSA202 isolates*

This assay was conducted as previously described by Delley et al. (2015) with slight modification. One percent of sub-cultured DLBSA201 and DLBSA202 isolates were grown overnight at 37°C in MRS Broth (Merck, Germany). Then, 1×10^8 CFU of the DLBSA201 and DLBSA202 isolates were grown in LAPT media (1.5% Bacto Peptone, 1% Bacto Tryptone, 1% yeast extract, 1% glucose, and 0.1% Tween 80) (Delley et al., 2015), and the supernatant was collected by centrifugation at 13,000 rpm at 4°C for 15 min. Two batches of supernatant [cell-free supernatants (CFS)] were collected. CFS was filter-sterilized using a membrane filter with a size pore of 0.22 µm (Sartorius, Germany) before further application. A volume of 100 µL of CFS and 100 µL of $1\times10^{6-8}$ CFU of overnight *E. coli* O157:H7 in new LAPT media was added to a 96-well plate to obtain a final volume of 200 µL. After 20 h incubation at 37°C, the absorbance was measured at 600 nm, and *E. coli* O157:H7 growth without the addition of CFS was used as a control.

2.9. Displacement of Escherichia coli *O157:H7 by DLBSA201 and DLBSA202 isolates*

The displacement of *E. coli* O157:H7 by DLBSA201 and DLBSA202 isolates was conducted according to the previous study (Jankowska et al., 2008) with some modifications. Overnight DLBSA201 and DLBSA202 isolates and *E. coli* O157:H7 were harvested by centrifugation at 8000 rpm for 10 min at room temperature. The cell pellets were washed two times with 0.85% sterile saline and were adjusted to 1×10^8 CFU before resuspending in antibiotic-free MEM media. Before co-incubation with bacteria, Caco-2 cells were washed three times with warm PBS to remove any antibiotics. Next, Caco-2 cells were co-incubated with *E. coli* O157:H7 at 37°C for 2 h. At the end of incubation time, Caco-2 cells were carefully washed three times with warm sterile 1× PBS to remove any non-adherent *E. coli* O157:H7. Afterward, 1×10^8 CFU of single- or mixed-strain DLBSA201 and DLBSA202 were added to Caco-2 cells for another 2 h. At the end of the experiments, Caco-2 cells were washed three times with warm sterile 1× PBS to remove non-adherent bacteria. The cells were then detached by 1% trypsin and harvested by centrifugation at 6,000 rpm at room temperature for 5 min. One percent (1%) of Triton X-100 in PBS was added to detach the bacteria from Caco-2 cells. After being detached, viable detached bacteria were placed on MRS Agar and TSA after serial dilution. The growth of *E. coli* O157:H7 or *Lactobacillus* isolates was used as a control.

2.10. Detection of inflammation-related gene expression in Caco-2 cells treated with LPS and DLBSA201 and DLBSA202 isolates

For this assay, DLBSA201 and DLBSA202 isolates were prepared by centrifuging the overnight cultures at 8,000 rpm for 10 min at room temperature. After being washed two times with 1× sterile PBS, DLBSA201 and DLBSA202 concentrations were adjusted to 1×10^7 CFU. The media from confluence Caco-2 cells were replaced with serum-free media and incubated for 2 h before the addition of 1 ng/mL lipopolysaccharides (LPS) from *E. coli* O55:B5 (Sigma Aldrich, USA) to induce inflammatory condition. Then, DLBSA201 and DLBSA202 or their mixture was added to Caco-2 cells and incubated at 37°C for 20 h with 5% CO_2. The mRNA levels of genes related to inflammation were measured by quantitative real-time polymerase chain reaction (qRT-PCR).

2.11. Effects of DLBSA201 and DLBSA202 isolates in tight-junction-related gene expression after being treated with LPS

LPS from *E. coli* O55:B5 with a concentration of 1 µg/mL was added for 4 h before viable DLBSA201 and DLBSA202 were added to the Caco-2 cell monolayer. Single- or mixed-strain DLBSA201 and DLBSA202 isolates with a concentration of 1×10^9 CFU were used for this assay. After 20 h of DLBSA201 and/or DLBSA202 exposure, the media was removed, and Caco-2 cells were rinsed three times with 1× PBS, followed by total RNA extraction and mRNA level quantification by qRT-PCR.

2.12. RNA extraction, cDNA synthesis, and Real-Time Quantitative polymerase chain reaction

For gene expression analysis, RNA from Caco-2 cells after treatment was isolated using TRIzol® reagent (Thermo Fisher Scientific, USA), reversed into cDNA using Revertra Ace® (Toyobo, Osaka), and amplified by RT-PCR using KAPA SYBR® FAST qPCR kit master mix (Kapa Biosystem, USA). The primers for housekeeping and target genes are shown in Table 1, and the β-actin encoded gene was used as the internal control.

2.13. Statistical analysis

Data obtained from all experiments were expressed as mean±standard deviation (SD) and were statistically analyzed using SPSS version 25 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, United States). One-way analysis for variance (ANOVA) followed by Duncan's multiple comparison tests was used to determine significant differences between the groups with a significance level at α=0.05.

3. Results

3.1. Acid and bile tolerance

No significant reduction was found when DLBSA201 and DLBSA202 were incubated in 1× PBS with pH 3 for 2 h

(Figure 1A). However, after being further incubated for 4 h, the number of viable DLBSA201 and DLBSA202 was significantly decreased (Figure 1A). Log reduction of DLBSA201 and DLBSA202 after 4 h incubation was 3.83 and 3.71 log CFU/mL, respectively. In other words, the survival rate of DLBSA201 and DLBSA202 toward low pH was 56.02% and 58.05%. In the case of bile resistance, log reduction of DLBSA201 and DLBSA202 that were incubated into 1× PBS supplemented with 0.5% bile salt for 4 h was not significant (Figure 1B). Both of those isolates showed a high survival rate (97.06% for DLBSA201 and 90.23% for DLBSA202) in the presence of bile salt.

3.2. Bile salt hydrolase activity

The ability of DLBSA201 and DLBSA202 to hydrolyze BSH was tested by growing each isolate in MRS Agar supplemented with 0.5% bile salt, 0.5% taurodeoxycholic acid sodium salt, or 0.5% glycolic acid sodium salt. At the end of incubation time, no precipitation was found around the colonies (Figure 2). This result indicated that DLBSA201 and DLBSA202 do not have any BSH activity.

Table 1. List of primers used for RT-qPCR for quantification of lowering pro-inflammatory genes and upregulating intestinal gut barrier genes.

Target genes	Sequences (5'-3')	Tm (°C)	Product size (bp)
β-actin	F: AGAGGGAATTCGTGCGTGAC R: CAATAGTGATGACCTGGCCGT	59	137
TNF-α	F: CTCGAACCCCGAGTGACAAG R: TGAGGTACAGGCCCTCTGAT	59	159
TLR-4	F: TGAGCAGTCGTGCTGGTATC R: CAGGGCTTTTCTGACTCGTC	59	167
IL-8	F: ATGACTTCCAAGCTGGCCGTG R: TGAATTCTCAGCCCTCTTCAAAA ACTTCTC	60	297
CLDN-2	F: ACCTGCTACCGCCACTCTGT R: CTCCCTGGCCTGCATTATCTC	60	91
OCN	F: CCCATCTGACTATGTGGAAAGA R: AAAACCGCTTGTCATTCACTTTG	58	77
ZO-1	F: GGACCAGCTGAAGGACAGCT R: TCCGTTAACCATTGCAACTCG	59	54

3.3. Adhesion assay on Caco-2 cells

Both DLBSA201 and DLBSA202 strains showed adherence ability to human epithelial cells. The number of viable LAB attached to Caco-2 cells is proportional to the number of initial LAB. Our result showed that DLBSA202 (95.08%) had a significantly higher adherence rate than DLBSA201 (89.03%) (Figure 3).

3.4. Antibacterial activity

Figure 4 represents the zone inhibition of DLBSA201, DLBSA202, and their combination toward *E. coli* O157:H7.

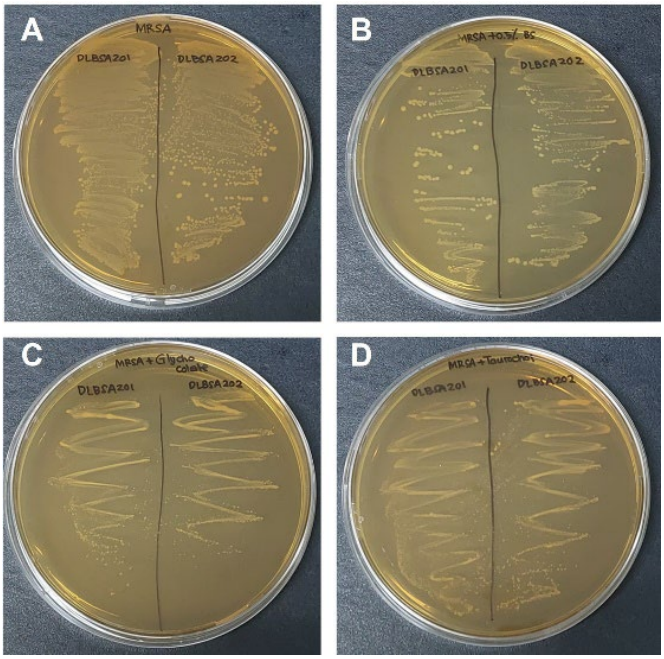


Figure 2. Colonies of DLBSA201 and DLBSA202 isolates on (A) MRS Agar, (B) MRS Agar+0.5% bile salt, (C) MRS Agar+0.5% sodium glycocholate, and (D) MRS Agar+0.5% sodium taurocholate.

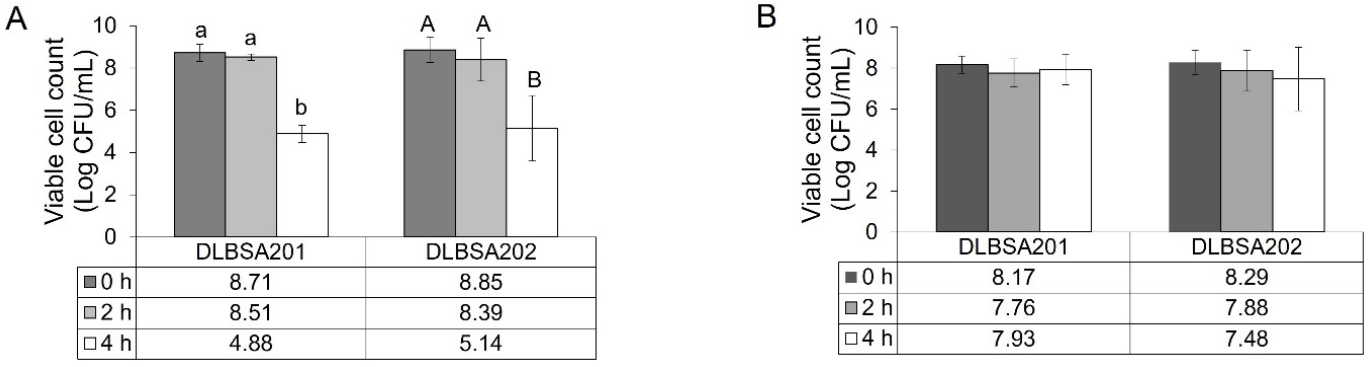


Figure 1. Survival ability from DLBSA201 and DLBSA202 isolates was measured in PBS with (A) pH 3.0 or (B) 0.5% bile salt after incubation at 37°C for 0, 2, and 4 h. The result is expressed as mean±SD of at least three independent experiments. Different letters (a and b, or A and B) represent significant differences ($p<0.05$) for the comparison of every isolate group by one-way ANOVA.

The mean inhibition (mm) of DLBSA201 and DLBSA202 against *E. coli* O157:H7 was 12 and 13 mm, respectively. In addition, the combination of DLBSA201 and DLBSA202 showed slightly higher inhibition, i.e., 15 mm.

3.5. Cell-free supernatant of DLBSA201 and DLBSA202 isolates reduces the growth of E. coli *O157:H7*

Escherichia coli O157:H7 were grown in the presence of CFS from DLBSA201 and DLBSA202 to assess the possibility of cell-free factors in CFS that affect the *E. coli* O157:H7 growth. CFS of DLBSA201, DLBSA202, and their mixture significantly reduced the growth of *E. coli* O157:H7 in all concentrations (Figure 5A). Additionally, higher growth reduction of 1×10^9 CFU *E. coli* O157:H7 strain was found when CFS combination (~30%) was added rather than the addition of CFS of single-strain *Lactobacillus* (15.1% for DLBSA201 and 19.7% for DLBSA202).

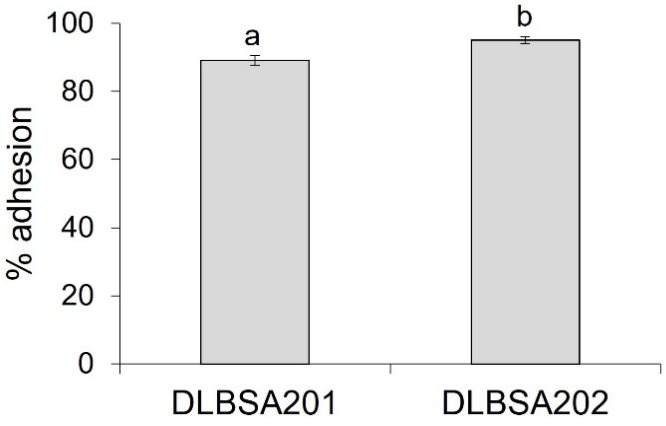


Figure 3. Adhesion ability of DLBSA201 and DLBSA202 isolates on Caco-2 cells. The result is expressed as mean±SD of at least three independent experiments. Different letters (a and b) represent significant differences ($p<0.05$).

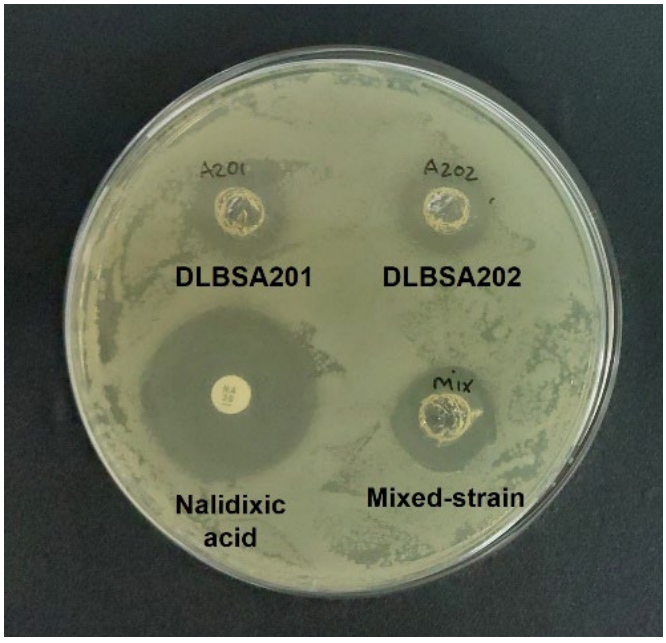


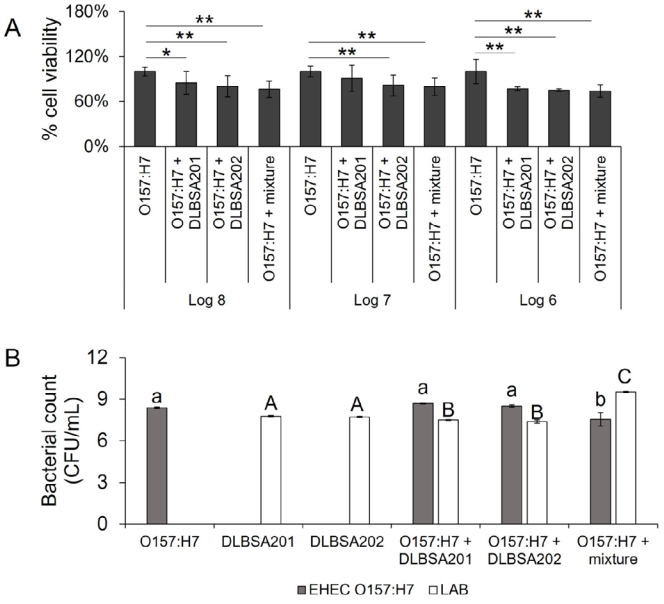
Figure 4. Antibacterial activity from DLBSA201 and DLBSA202 isolates against *E. coli* O157:H7 as demonstrated by the inhibition zones. The assay was performed three times in duplicate, and nalidixic acid was used as the positive control.

3.6. Displacement of Escherichia coli *O157:H7 by DLBSA201 and DLBSA202 isolates*

DLBSA201 and DLBSA202 showed the ability to inhibit the growth of *E. coli* 0157:H7. Next, we assessed the ability of those *Lactobacillus* isolates to replace *E. coli* O157:H7 that was previously adhered to epithelial cells. The addition of a single strain of DLBSA201 or DLBSA202 could not replace the number of attached *E. coli* O157:H7 on Caco-2 cells (Figure 5B). However, when DLBSA201 and DLBSA202 were added as a mixture, they significantly reduced the number of attached *E. coli* O157:H7 cells (Figure 5B). This result indicated that a combination of DLBSA201 and DLBSA202 is preferable to get better inhibition result toward pathogen bacteria.

3.7. Anti-inflammatory activity of DLBSA201 and DLBSA202 isolates in human intestinal cells

The modulation of several pro-inflammatory genes was examined in LPS-stimulated human intestinal epithelial cells (Caco-2). The addition of 1 ng/mL LPS for 2 h induced the expression levels of TLR-4, IL-8, and TNF-α, but the introduction of a single or mixed strain of DLBSA201 and DLBSA202 markedly repressed the expression of TLR-4 and IL-8 (Figure 6).



*p<0.05; **p<0.01.

Figure 5. The effects of cell-free supernatant (A) or DLBSA201, DLBSA202, or mixture of DLBSA201 and DLBSA202 isolates (B) in *E. coli* O157:H7 cell viability or adhesion in Caco-2 cell culture. The growth of *E. coli* O157:H7 and DLBSA201 or DLBSA202 was used as a control. Student's t-test was used to compare the control with another group,), and different letters (a and b, or A–C) represent significant differences ($p<0.05$) by one-way ANOVA.

In addition, a single strain of DLBSA201 or DLBSA202 was not enough to bring a significant reduction of TNF-α, but the mixture of both isolates was able to significantly bring down the TNF-α expression level (Figure 6).

3.8. Effects of DLBSA201 and DLBSA202 isolates in tight-junction-related gene expression after treated with LPS

In this study, we observed the ability of DLBSA201 and DLBSA202 to change the mRNA levels of tight-junction-related genes in Caco-2 cells under LPS stimulation. The LPS addition decreased the expression of OCN but not ZO-1 mRNA levels, and the addition of mixed DLBSA201 and DLBSA202 (1:1) significantly increased OCN and ZO-1 expression levels (Figure 7). The expression of CLDN-2 was slightly increased by LPS, but the exposure of single as well as mixed DLBSA201 and DLBSA202 isolates did not significantly change the CLDN-2 expression although there was a tendency of CLDN-2 mRNA level back to normal after the cell was treated with mixed DLBSA201 and DLBSA202 (p=0.09).

4. Discussion

In this study, two potential *Lactobacillus* strains, namely, *L. helveticus* DLBSA201 and *L. helveticus* DLBSA202 were characterized for probiotic prerequisites, and their ability to inhibit *E. coli* O157:H7 was assessed. Probiotic ability to resist stress while present in the GIT, such as survival in low pH conditions and the presence of bile salt, is a key functional feature of probiotics (Byakika et al., 2019). This feature is species- and strain-dependent (Missaoui et al., 2019; Sadeghi et al., 2022), although *Lactobacillus* species are commonly known to be able to resist acid and bile salt (Jung et al., 2021; Pino et al., 2019; Xu et al., 2020). LAB could be divided into four groups according to its resistance in low pH conditions, including susceptible (survival rate<10%), moderate (10–60%), good (60–80%), and very good resistance (>80%) (Sadeghi et al., 2022). Based on that classification, DLBSA201 and DLBSA202 showed moderate resistance with a survival rate of about 56–58% after being exposed to an acidic environment (Figure 1A). The ability of those isolates to survive in an acidic environment partly contributed to their ability to maintain constant gradient between extracellular and cytoplasmic pH (Ostadzadeh et al., 2023). The bacterial cell prevents the hydrogen ions from the cytoplasm to enter the interior of the cell and simultaneously releases the equivalent hydrogen ions from the cytoplasm to protect vital components such as DNA and adenosine triphosphate (ATP) in a cell (Ostadzadeh et al., 2023). For instance, teichoic acid – one of the major components of *Lactobacilli* cell walls – was reported to help in creating a pH gradient across the cell wall (Alcantara et al., 2020). The structure of teichoic acid is diverse among Gram-positive bacteria, but they share a linear backbone consisting of phosphate groups linked to different alditol groups (Alcantara et al., 2020). Consequently, teichoic acid is responsible as a reservoir for phosphate and acts as a major contributor to the net negative charge to neutralize acid (Alcantara et al., 2020). The regulation of F_0F_1-ATPase at the transcriptional level is also believed to protect Gram-positive bacteria to survive in an acidic environment (Mendonca et al., 2022).

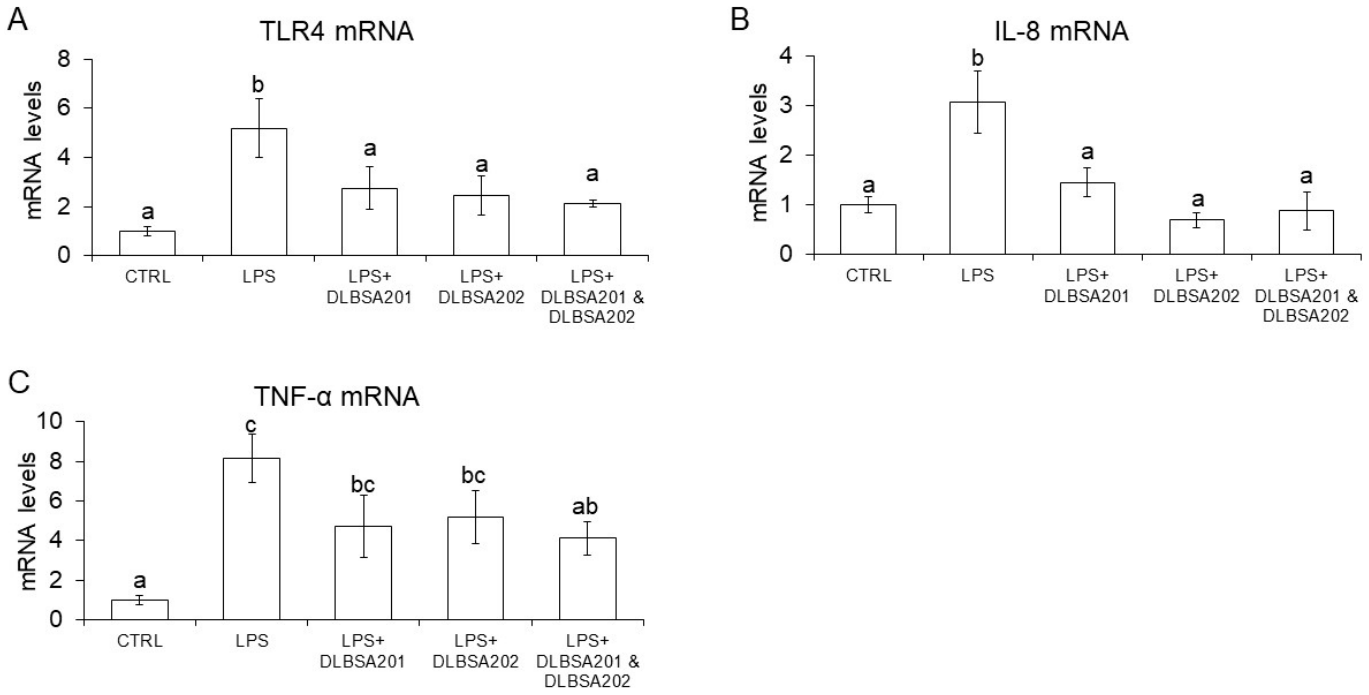


Figure 6. Transcript levels of pro-inflammatory genes (A) TLR-4, (B) IL-8, and (C) TNF-α of Caco-2 cells treated with DLBSA201, DLBSA202, or a combination of DLBSA201 and DLBSA202 isolates (1:1). LPS was used to induce inflammation before Caco-2 cell was treated with probiotics. The result is expressed as mean±SD of at least three independent experiments. Different letters (a–c) represent significant differences ($p<0.05$) by one-way ANOVA.

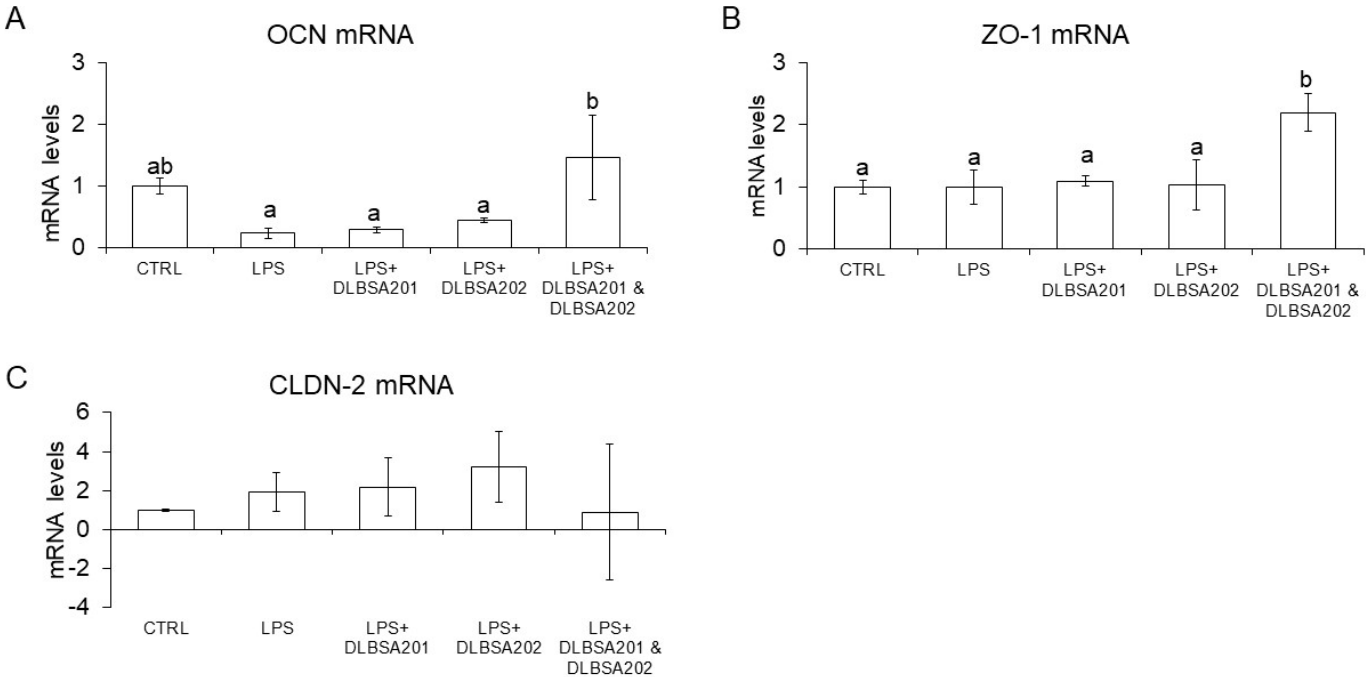


Figure 7. Transcript levels of (A) occluding, (B) ZO-1, and (C) claudin-2 genes treated with 1 µg/mL LPS and DLBSA201, DLBSA202, or a combination of DLBSA201 and DLBSA202 isolates (1:1). The result is expressed as mean±SD of at least three independent experiments. Different letters (a–c) represent significant differences ($p<0.05$) by one-way ANOVA.

Based on the classification by Sadeghi et al. (2022), bacterial strains that have survival rates >60% in the presence of 0.3% bile salt can be categorized as highly resistant strains. Therefore, DLBSA201 and DLBSA202, which exhibited a high survival rate (>90%) in the presence of 0.5% bile salt (Figure 1B), could be categorized as highly resistant strains. A slight reduction in the presence of bile salt may be caused by the production of a harmful and toxic compound due to taurine metabolism (Tjandrawinata et al., 2022). Moreover, although DLBA201 and DLBSA202 have a high resistance toward bile salt, no BSH activity was found (Figure 2). BSH activity has been postulated to offer protection to the bacteria against the harmful effect of bile, but recent findings reported that BSH activity was unrelated to the bile salt resistance ability of probiotics (Tjandrawinata et al., 2022). In addition, *L. helveticus* R0052, which is already used in several commercial probiotic products, did not also show any bile salt deconjugate activity, and its partial bile hydrolases genes are not active (FDA, 2018). The high resistance ability of DLBSA201 and DLBSA202 could be related to other mechanisms such as stress response protein, alteration of the bacterial cell wall, and efflux pumps (Bustos et al., 2018; Horackova et al., 2020). In brief, other mechanisms should be responsible for the ability of DLBSA201 and DLBSA202 to survive in a medium containing a high concentration of bile salt.

The ability to adhere in the intestinal cells is another important prerequisite for probiotics as this ability can increase the possibility of probiotics colonizing without being removed from the intestine by peristalsis (Byakika et al., 2019; Yu et al., 2022). Probiotics that are successfully colonized in the intestine could offer more health benefits to the host, such as control of harmful microbes and modulation of the immune system, among other benefits (Jung et al., 2021; Sadeghi et al., 2022). The result showed that both of our probiotic strains have a good adherence ability (Figure 3). The ability of the LAB strain to adhere to epithelial cells may occur through the specific and/or non-specific adhesion between epithelial cells and bacterial surface components such as exopolysaccharides, pili, glycolytic enzymes, polysaccharide A, collagen-binding protein, mucus-binding protein, mucus adhesion promoting protein, and sortase A (Gorreja & Walker, 2022; Yu et al., 2022). In general, *Lactobacillus* species have surface molecules such as lipoteichoic acid (LTA), surface layer-associated proteins, fibronectin-binding proteins, and mucin-binding proteins to help the bacteria to adhere (Monteagudo-Mera et al., 2019).

Alongside its essential role to maintain a healthy gut microbiome population, probiotic offers a new strategy to prevent and treat foodborne infection and gastrointestinal disorder (Bhat et al., 2020). Following the promising result of our strains exhibiting potential probiotic traits, we examined the potential of *L. helveticus* DLBSA201 and *L. helveticus* DLBSA202 to protect and restore damage caused by *E. coli* O157:H7 strain.

DLBSA201, DLBSA202, and their combination showed antagonistic effects against *E. coli* O157:H7, as shown in Figure 4. Furthermore, CFS of DLBSA201 and DLBSA202 indicated significant growth reduction of *E. coli* O157:H7 (Figure 5A). Besides the ability to create an acidic environment, it is possible that other compounds that may be secreted by *Lactobacillus* sp. Such as bacteriocin may act synergistically to provide less conducive habitat for *E. coli* to grow (Lu et al., 2020; Qiao et al., 2021).

Our result also clearly showed that the combination of DLBSA201 and DLBSA202, but not a single strain, could

remarkably decrease the adhesion of *E. coli* O157:H7 in Caco-2 cells (Figure 5B). Similar to our result, the ability of LAB such as *Lactobacillus fermentum* strain 8711 (Jayashree et al., 2018), *Lactobacillus acidophilus* AD125 (Xing et al., 2023), *Lactobacillus casei* NA-2 (Ma et al., 2022), and *Lactobacillus brevis* DF01 (S. H. Kim et al., 2019) to reduce the adhesion of pathogens was also reported. The displacement of a pre-adhered pathogen by probiotics could be related to the competition for the specific receptor (Zawistowska-Rojek et al., 2022). Furthermore, antimicrobial substances produced by probiotic isolates with specific effects on certain pathogens may play a role in displacement effects (Esteban-Fernandez et al., 2019; Jayashree et al., 2018).

Exposure of Caco-2 cells to LPS increases the expression of TLR-4 and stimulates the activation of pro-inflammatory cytokines such as IL-8 and TNF-α. Our study proved that the combination of DLBSA201 and DLBSA202 impressively reduced the TLR-mediated inflammatory cytokine production (Figure 6). Metabolite production, such as butyrate, by probiotic bacteria may confer anti-inflammatory response (Zhu et al., 2021). Additionally, LTAs produced by many *Lactobacillus* species, such as *L. plantarum* Biocenol™ LP96 (Noviardi et al., 2022), *L. rhamnosus* GG, and *L. brevis* KU15152 (Kim et al., 2022), have been reported to repress IL-8 production.

LPS from bacteria is also known to increase gut permeability and play an essential role in the gut inflammation process. The effects of DLBSA201 and DLBSA202 to enhance the intestinal barrier through the upregulation of two tight junction genes, which are ZO-1 and OCN, were found in this study. The ability of our probiotic strains to increase ZO-1 and OCN genes could be related to their capacity to bring down TLR-4 and TNF-α, as previously seen in *Bifidobacterium dentium* N8 (Zhao et al., 2021). Bacteriocin produced by potential probiotic bacteria was previously reported to repair intestinal barrier dysfunction by assembling the Occludin and Claudin-1 of impaired intestinal cells (Yu et al., 2018) and plantaricin of *L. plantarum* was also able to maintain intestinal barrier in Caco-2 cells by preventing the increase in transcellular permeability and IL-8 level (Bu et al., 2022). Moreover, citrulline-enriched fermented milk with *L. helveticus* ASCC511 has been reported to ameliorate inflammation and disrupted tight junction caused by LPS of *E. coli* O55:B5 through the restoration of the transepithelial electrical resistance (TEER) and regulation of tight junction proteins (ZO-1, occluding, and claudin-1) (Ho et al., 2020). Thus, it appears that the combination of DLBSA201 and DLBSA202 may maintain gut health by improving intestinal barrier functions.

5. Conclusions

We believed that *L. helveticus* DLBSA201 and *L. helveticus* DLBSA202 possess the traits of a potential probiotic. Both isolates demonstrated acid and bile salt tolerance as well as high adhesion capacity. Additionally, the mixture of DLBSA201 and DLBSA202 showed their ability to compete with *E. coli* O157:H7. Protective effects of those isolates against inflammation response by lowering pro-inflammatory genes and upregulating intestinal gut barrier genes were also noticed in LPS-stimulated condition. *In vivo* studies regarding their potential to suppress *E. coli* growth and infection need to be performed in detail in the future.

Data availability

The draft genomes of *L. helveticus* DLBSA201 and *L. helveticus* DLBSA202 were deposited in GenBank under the accession numbers NZ_WWEJ00000000 and NZ_WWEK00000000, respectively, under bioproject number PRJNA224116. The versions used in this study are the first ones.

Acknowledgments

The authors would like to thank Prof. Lilis Nuraida, Destrina Grace, and Jeni Rustan for their assistance in editing the manuscript.

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