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A novel probiotic strain of *Lactobacillus fermentum* TIU19 isolated from Haria beer showing both *in vitro* antibacterial and antibiofilm properties upon two multi resistant uro-pathogen strains

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ABSTRACT

Probiotics with antimicrobial activity are gaining interest as a topic in the research field. Urinary tract infections (UTIs), acquired in the hospital or the community, are among the most prevalent infections. The emergence of multidrug resistance (MDR) uro-pathogens has made the current situation more critical in terms of global public health. To face this situation, in this study, *Lactobacillus fermentum* TIU19 (L. *fermentum* TIU19) was isolated and characterized as a new probiotic strain of the rice-based fermented beverage Haria. Subsequently, we also investigated its application as a biological agent that inhibits the growth of multidrug-resistant uro-pathogens, *Escherichia coli,* and *Enterococcus faecalis*. The results showed that, the isolated strain L. *fermentum* TIU19 was sensitive to all antibiotics tested except vancomycin and was devoid of virulence factors, such as haemolytic and gelatinase activities. Therefore, it may be considered safe for public health. It has many probiotic properties, such as survival in simulated gastrointestinal fluid, antioxidant activity, β-galactosidase producing ability, high cell surface hydrophobicity, adhesion ability to epithelial cells, and strong biofilm producer. The growth inhibitory and antibiofilm activities were shown against two uro-pathogens. All these results suggest that L. *fermentum* TIU19 can be explored as a potential probiotic with antagonistic activity against MDR uro-pathogenic *E. coli* and *E. faecalis*.

1. Introduction

Urinary tract infections (UTIs) affect people of all ages around the world, especially young women, children, and the elderly (Magill et al., 2014). According to previous studies, almost 40% of women have suffered from UTIs at some point in their lives (Foxman, 2014). Earlier studies have reported that Gram-positive *Enterococcus faecalis* (Kline and Lewis, 2016) and Gram-negative *Escherichia coli* (Flores-Mireles et al., 2015) are the most prevalent organisms among the UTIs pathogens. Moreover, uro-pathogens are known to form a biofilm that protects the bacteria from host immune response and antimicrobial therapy. As a result, uro-pathogens that are biofilm producers, create obstacles to the treatment of UTIs. Bacteria are attached to the biofilm, helping to share antimicrobial resistance genetic material (Schroeder et al., 2017). Both *E. coli* and *E. faecalis* have also been known to form biofilms in the urogenital tract (Anderson et al., 2003). Furthermore, antibiotic therapy disrupts the normal microbial flora of the urinary tract, which can lead to more severe conditions (Flores-Mireles et al., 2015).

Conventional antibiotic treatment for the management of infections associated with multiple antibiotic-resistant *E. coli* and *E. faecalis* are difficult. In recent days, the continuous rise in multidrug resistance in both *E. coli* and *E. faecalis* has also created a challenge to effective treatment. Antibacterial compounds from natural sources may be an alternative to the treatment of multidrug-resistant bacteria (Vaseeharan et al., 2011). However, they still have some restrictions, such as natural antibacterial agents being less effective than antibiotic therapy (Gavarić et al., 2015). Since both *E. coli* and *E. faecalis* are normal microflora of the urinary tract, they cannot be easily eliminated with antimicrobial compounds. Therefore, more clinical studies need to be carried out to determine their role in UTI prevention (Al-Badr and Al-Shaikh, 2013).

Functional and safety properties should be considered during probiotic selection. Lack of virulence factors, multiple antibiotic resistance, survival under gastrointestinal tract (GI) conditions, biofilm-forming ability, and antimicrobial activity against pathogens are all important

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criteria for the selection of probiotic cultures (Silva et al., 2020). However, there are many commercial probiotics with a narrow spectrum of activity. Therefore, it is important to isolate and characterize novel strains that could have a broad spectrum of antibacterial properties. Among probiotic bacteria, *Lactobacillus* spp. has been shown to have beneficial effects on host health through a variety of mechanisms. (Didari et al., 2014).

In central and eastern India, Haria is the most popular traditional rice beer consumed by the ethnic people of the tribal community of that region. Previously, it has been reported that Haria has many ethnomedicinal properties (Ray et al., 2016), which have not been properly explored. According to a previous study, lactic acid bacteria (LAB) were found to be the dominant microbial community in Haria. (Ghosh et al., 2015a). The presence of probiotic bacteria in Haria, along with their therapeutic potential against pathogenic bacteria, is still limited. To our knowledge, only one study reported the probiotic potential of the L. *fermentum* KKL1 strain from Haria without revealing antimicrobial work using pathogenic bacteria (Ghosh et al., 2015). Probiotics such as *lactobacilli* are the dominant bacteria of the vaginal flora and possess antimicrobial properties that regulate other urogenital microbiota (Barrons and Tassone, 2008). *Lactobacillus* spp can involve acidification of the mucosal surface, inhibition of adhesion of pathogens, production of substances such as vitamins and immunomodulators, and synergistic activity with the host's immune system (Kaur et al., 2002). Clinical studies have established that oral administration of *Lactobacillus* can confirm its effects after reaching the vagina (Reid et al., 2001a). Previous studies stated that probiotic capsules containing *Lactobacillus* spp. were administered orally at a dose to prevent UTIs (Kontiokari et al., 2003; Reid et al., 2001b).

As a result, the study was designed and carried out in the following ways: (i) to isolate a probiotic strain from Haria, showing antibacterial activity upon uro-pathongens; (ii) to assess the characterization of probiotic properties and antibiofilm activity of the isolated strain; and (iii) to investigate the antagonistic efficiency of the isolated probiotic strain against two uro-pathogen strains.

2. Materials and methods

2.1. Isolation and identification of the L. fermentum strain from Haria

To isolate a single probiotic strain, 100 µl of Haria was placed in De Man, Rogosa, and Sharpe (MRS) broth (Himedia, India) and incubated (CO_2 incubator) at 37 °C for 12 h in the presence of 5% CO_2. After incubation, a 100 µl MRS broth culture was transferred to fresh MRS broth (pH 3, acidified with 5 N HCl) and incubated for

3 h more prior to be spread on MRS agar and further incubated for both verifying its viability and isolate their anti-uropathogenic activity *in vitro*.

The antagonistic activity of the isolated probiotic strains was determined by an agar overlaid assay, using *E. coli* MTCC41 as indicator strain. Briefly, a loop full of pure lactobacilli broth culture was inoculated in spot (5 mm in diameter) on MRS agar and incubated at 37 °C for 24 h as described further. (Halder et al., 2017).

Selected colonies were subjected to Gram staining and catalase test, and further identified *as Lactobacillus* spp. by analytical profile index (API) analysis using the HiLacto Identification Kit (Himedia).

The Gram-positive and catalase-negative pure culture of *Lactobacillus* sp. with the highest antagonistic activity against *E. coli* MTCC41 was identified by 16 s rRNA gene sequencing. Initially, genomic DNA was isolated using a QIAamp DNA Kit (Qiagen) and electrophoresis on a 1.2% agarose gel. 16 s rDNA gene was amplified by PCR (Thermo Fisher SCIENTIFIC) (initial denaturation at 95 °C for 5 min; Thirty-five cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min; final extension at 72 °C for 7 min) from isolated DNA using the universal primers (forward primer 27F 5′-AGAGTTTGATCCTGGCTCAG-3′, and reverse primer 1492R 5′-GGTTACCTTGTTACGACTT-3′) (Srinivasan et al., 2015). The PCR products were analyzed using 1.2% (w/v) agarose gel electrophoresis, and contaminants were removed using a Qiagen Mini Elute Gel extraction kit (Qiagen). The forward and reverse DNA sequencing reactions of the pure PCR amplicon were performed by an ABI genetic analyzer. Nucleotide sequences were obtained and homology was analyzed using the Basic Local Alignment Search Tool (BLAST) (Pearson, 2013). The reference-type bacterial sequences were retrieved from the NCBI database and aligned with the sequences isolated by Clustal W. The CLC sequence viewer has constructed a phylogenetic tree.

2.2. Assessment of safety and virulence factors of isolated L. fermentum

2.2.1. Multiple antibiotic resistance index (MAR)

The Multiple Antibiotic Resistance Index (MAR) was determined using the standard protocol developed by the Clinical and Laboratory Standards Institute (CLSI), USA. The isolated bacteria were assayed for resistance or sensitivity (S-Sensitive and R-Resistant) against multiple antibiotics. The antibiotic sensitivity pattern of the selected strain was examined by using standard antibiotic discs (Ampicillin 10 µg, Vancomycin 30 µg, Gentamycin 10 µg, Kanamycin 30 µg, Streptomycin 10 µg, Erythromycin 15 µg, Chloramphenicol 30 µg, Tetracycline 30 µg) (Himedia, India) placed aseptically over the homogenous bacterial lawn on MRS plates. The plates were incubated at 37 °C for 48 h. After incubation, the diameter of the zone of inhibition (ZOI) was measured (Ayandele et al., 2020). All tests were performed in triplicate. The MAR index was calculated as follows

$$\text{MARIndex} = \frac{\text{Number of antibiotics ineffective}}{\text{Number of antibiotics used}}$$

2.2.2. Haemolytic activity and gelatinase activity

Haemolytic activity was investigated as described by Kaktcham et al. (2018). The overnight grown cells in MRS broth were streaked on a blood agar plate (Himedia, India) and incubated at 37 °C for 72 h. After incubation, a clear zone around the bacterial growth (Red blood cells were haemolyzed completely) demonstrated positive hemolysin activity, whereas no change around the growth (Red blood cells were not haemolyzed) indicated negative results. Experiments were performed in triplicate.

For gelatinase activity, 10 µl of a 6-hour culture was spotted on the nutrient gelatine agar (Himedia, India). The plates were incubated at 37 °C for 48 h. Positive results were indicated by clear zones around the colonies, while negative results were indicated by no zones around the colonies. Experiments were performed in triplicate.

2.3. Assessment of the probiotic potential of isolated L. fermentum

2.3.1. Survival under conditions that simulated the human gastrointestinal tract

The log culture of bacterial cells (10^8 CFU ml^{-1}) was centrifuged at 5000 rpm for 15 min, and the pellet was washed twice with cold and sterile PBS (phosphate-buffered saline). Then the pellet was resuspended in PBS, and the obtained bacterial suspension was used to evaluate the survival of bacteria under human GI tract conditions. For the test of resistance to low pH conditions, cells were incubated in low pH solutions (pH: 2, 3, and 4) adjusted with HCl (1 N) for 0, 1, 2, 3 h at 37 °C. To assess resistance to bile salts, cells were incubated in PBS at pH 7.4, supplemented with 0.1, 0.2, 0.3 and 0.4% (w/v) oxgall (Sigma Aldrich, USA) for 0, 1, 2, 3 h at 37 °C. Simulated gastric fluid (SGF) was prepared according to Lian et al. (2003). At first, pepsin (Sigma Aldrich, USA) (3 g/lit) was suspended in sterile PBS. The solution was adjusted to pH 2, 3, 4 with 12 M HCl, and then filter sterilized through a 0.22 µm filter membrane (Tarson, India). Bacterial cells were re-suspended in simulated gastric fluid (SGF) solution and incubated for 0, 1, 2, 3 h at 37 °C. The simulation of intestinal fluid (SIF) was prepared according to

Amakiri et al. (Amakiri and Thantsha, 2016). Briefly, pancreatin (Sigma Aldrich, USA) (10 g/lit) was suspended in sterile PBS. The solution was adjusted to pH 6.8 and 8 with 0.2 M NaOH or 0.2 M HCl and then sterilized by filter through a 0.22 μm filter membrane (Tarson, India). Bacterial cells were resuspended in a simulated intestinal fluid (SIF) solution and incubated for 0, 1, 2, 3 h at 37 °C.

The resistance of the selected strain in these conditions was assessed in terms of viable colony count on MRS agar after the treatment (Son et al., 2018). Experiments were performed in triplicate. Survival rates were calculated according to the following equation.

$$\%\ \text{of survival} = (N_1 / N_0) \times 100$$

N_1: The total viable count of bacterial cells after treatment.
N_0: The total viable count of bacterial cells before treatment.

2.3.2. Cell surface hydrophobicity

The cell surface hydrophobicity of the selected strain was assessed according to Kaktcham et al. (2017). The bacterial culture was centrifuged at 5000 rpm for 15 mins. The pellet was washed twice with cold and sterile PBS and resuspended in the same buffer to obtain an OD of 600 nm of approximately 1.0 ($OD_{Initial}$). Then, 3 ml of the bacterial cell suspension was mixed with 1 ml of xylene (Sigma-Aldrich, USA) and vortexed for 2 mins. The mixture was then incubated at room temperature for 60 min to separate the two phases (water and xylene). The aqueous phase was collected and its absorbance at 600 nm (OD_{Time}) was measured. Experiments were performed in triplicate.

The cell surface hydrophobicity was calculated using the following formula:

$$\text{Cellsurfacehydrophobicity}(\%) = \left[1 - \frac{\text{ODTime}}{\text{ODInitial}}\right] \times 100$$

Cell surface hydrophobicity (%) was classified as high (51–100%), medium (30–50%), and low (0–29%).

2.3.3. Auto-aggregation

The overnight bacteria culture was centrifuged at 5000 rpm for 15 mins. The pellet was washed twice with cold and sterile PBS and resuspended in the same buffer to obtain an OD of 660 nm of approximately 1.0 (OD_{0min}). After that, this cell suspension was mixed by vortexing for 2 mins, incubated at room temperature for 60 mins, and the optical density (OD_{60min}) of the supernatant was determined at 660 nm. All experiments were performed in triplicate. Auto-aggregation was determined using the following equation:

$$\text{Auto} - \text{aggregation}(\%) = \left[\frac{(\text{OD0min} - \text{OD60min})}{\text{OD0min}}\right] \times 100$$

2.3.4. Adhesion ability

HeLa cells were purchased from the National centre for Cell Science (NCCS) in Pune, India. HeLa cells were grown and maintained in a 24-well tissue culture plate (Tarson, India) in 1 ml of Dulbecco's modified Eagle medium (DMEM) containing 0.11 g/l sodium pyruvate, 2 mM L-glutamine, 4.5 g/l glucose, 10% foetal bovine serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37° C in an atmosphere of 5% CO_2 at constant humidity. For the adhesion ability of the selected strain, HeLa cells were seeded at a concentration of 1×10^5 cells/ml in a 24-well tissue culture plate (Tarson, India). After incubation for 24 h at 37 °C, 10 μl of L. *fermentum* strain was diluted in sterile PBS at a concentration of 1×10^8 cells/ml and added to each well. After incubation for 2 h at 37 °C, the monolayers were washed three times with 500 μl of PBS and lysed with 0.1% (v/v) Triton X-100 (Sigma-Aldrich, USA). Cell lysates were serially diluted, spread on MRS agar, and incubated at 37 °C for 24 h. The adhesion ability (%) of the L. *fermentum* strains to HeLa cells was calculated as the percentage of adhered bacterial cells after 2 h (N_{2h}) divided by the initial number of bacterial cells at 0 h (N_{0h}) (Son et al., 2018). Experiments were performed in triplicate.

$$\text{Adhesionability}(\%) = \left[\frac{(\text{N2h})}{(\text{N0h})}\right] \times 100$$

2.3.5. Biofilm forming ability

With some modifications, quantification of biofilm production was performed as previously described by Borges et al. (2011) with minor modifications. The 2 ml of MRS broth and bacterial cells of the selected strain (10^7–10^8 CFU/ml) were added to each well of a sterile 12-well polystyrene microtiter plate (Tarson, India). The cells were then aerobically incubated at 37 °C for 0, 24, 48, and 72 h. Time 0 hour was used as control. As tests, time intervals of 24, 48, and 72 h are used. After incubation, planktonic cells were removed, and bacterial sessile cells were fixed with 2 ml of methanol (Finar Limited, India) for 15 mins. Subsequently, 2 ml of 2% crystal violet solution (Sigma-Aldrich, USA) was used for bacterial sessile cell staining and kept at room temperature for 10 min, and the excess amount of crystal violet was rinsed off using distilled water. After the dissolution with 2 ml of 30% glacial acetic acid (v / v), biofilm biomass was determined by measuring OD595.

The percentage of biomass formation was determined using the following equation:

$$\text{BiofilmBiomass}(\%) = \frac{\text{OD595}(24/48/72\text{hr})}{\text{OD595}(0\text{hr})} \times 100$$

2.3.6. Study of β-galactosidase production

For determination of β-galactosidase production, a colony of the selected strain was removed from MRS agar plates and thrown into tubes containing Ortho-Nitrophenyl-β-galactoside (ONPG) discs (Sigma-Aldrich, USA) and 100 μl of sterile 0.85% saline solution. Tubes were incubated at 37 °C and observed at an interval of 1 hour to 6 h. The color transition to yellow indicates a positive result in the production of β-galactosidase by releasing the chromogenic compound, o-nitrophenol (Vinderola et al., 2011).

2.3.7. Antioxidant assay

Quantification of the antioxidant assay was performed as previously described by Talib et al. (Talib et al., 2019). 1 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.05 mM) was added to the different numbers of freshly prepared cells from 1.0 to 6.0-log CFU/ml in PBS. The reaction mixture was kept in the dark for 30 mins at room temperature. The DPPH solution in PBS and the different numbers of cells 1.0 to 6.0 log CFU/ml in PBS were used as control and blank, respectively, during this assay. The spectrophotometer (UV–visible Spectrophotometer 2206, SYSTRONICS) was used to measure the optical density (OD) at 517 nm to assess the DPPH scavenging capability. All tests were performed in triplicate.

The percentage of scavenging potential was estimated as follows:

$$\text{DPPHscavengingcapacity}(\%) = \frac{(\text{OD sample} - \text{OD blank})}{\text{OD control}} \times 100$$

2.4. In vitro tests to determine the antagonistic activity of L. fermentum against uro-pathogens

2.4.1. Antagonistic activity of probiotic bacteria against uro-pathogens

Multidrug-resistant uropathogenic *E. coli* (amp^R, tet^R, str^R) and *E. faecalis* (amp^R, tet^R, str^R, chl^R) were used as marker strains to check the antagonistic activity of the isolated strain. The antagonistic activity of an isolated strain was determined by an agar overlay assay (Halder et al., 2017). For this assay, multidrug-resistant pathogenic strains, such as *E. coli* and *E. faecalis*, were used. An aliquot of the selected L. *fermentum* strain (1×10^8 CFU/ml) was spotted on MRS agar and incubated at 37 °C for 24 h. The MRS agar plate was then overlayed with 9 ml of Tryptone soya agar (0.8% agar) mixed with 1 ml of each pathogenic strain (1×10^6 CFU/ml) and incubated at 37 °C for 24 h. The diameter

(mm) of clear zones on the agar plate was measured. All tests were performed in triplicate.

2.4.2. Co-aggregation assay

The experimental protocol for the study of the co-aggregation assay was similar to the auto-aggregation assay. Probiotic bacteria (L. *fermentum* TIU19) and bacterial pathogens (*E. coli* or *E. faecalis*) were inoculated into the respective growth medium and incubated at 37 °C for 18 h. The cells were centrifuged at 5000 g for 15 min, washed twice, and resuspended in phosphate-buffered saline (PBS). Equal volumes (2 ml) of each cell suspension of single species pathogens (*E. coli* or *E. faecalis*) were mixed individually with L. *fermentum* TIU19 by vortexing for 10 s. Control tubes were set up at the same time, containing 4 ml of each bacterial suspension on its own, and the upper cell suspension was measured at OD_{660} nm at 0 h. After 37 °C for 60 min of incubation, the optical density (OD60min) of the upper cell suspension was determined at 660 nm. Samples were taken in the same way as in the auto-aggregation assay (Kos et al., 2003). All tests were performed in triplicate. The percentage of coaggregation was calculated using the equation:

$$\text{Thepercentageofcoaggregation} = \frac{\left(\frac{\text{ODx}+\text{ODy}}{2}\right) - \text{OD}(\text{x}+\text{y})}{\left(\frac{\text{ODx}+\text{ODy}}{2}\right)} \times 100$$

Where x represents L. *fermentum* TIU19 and y represents (*E. coli/E. faecalis*) each of the two strains in the control tubes and (*x + y)* the mixture.

2.4.3. Anti-adhesion assay

HeLa cells were seeded in a 24-well tissue culture plate (Tarson, India). After a 24-hour incubation at 37 °C, single species pathogens (*E. coli, E. faecalis*) and co-culture [(L. *fermentum* TIU19+*E. coli*), (L. *fermentum* TIU19+*E. faecalis*)] (at a ratio of 1:1 diluted in DMEM medium containing L-glutamine) were added to the 24-well tissue culture plate to a final concentration of 1×10^8 CFU/ml. After incubation for 2 h at 37 °C, the monolayers were washed three times with PBS and lysed with 0.1% (v/v) Triton X-100 (Sigma-Aldrich, USA). Cell lysates were serially diluted and spread in selective media, MacConkey sorbitol agar, and Pfizer selective Enterococcus agar for *E. coli* and *E. faecalis, respectively,* and incubated at 37 °C for 24 h. Each experiment was repeated three times. The ability of a given pathogenic strain to adhere to HeLa cells in the absence (N_P) and presence (N_{Mix}) of the L. *fermentum* strain was compared as follows (Son et al., 2017)

$$\text{Anti} - \text{adhesionability}(\%) = [(N_P - N_{Mix}) / N_P] \times 100$$

N_P = number of bacterial cells, when the wells were seeded with only pathogenic bacteria.

N_{Mix} = number of bacterial cells when wells were seeded with L. *fermentum* -pathogenic bacteria mixture.

2.4.4. Biofilm inhibition assay

The isolated strain of L. *fermentum* TIU19 and uro-pathogens (*E. coli,* and *E. faecalis*) were grown overnight in LB media individually. The co-culture [(L. *fermentum* TIU19+*E. coli*), (L. *fermentum* TIU19+*E. faecalis*)] was prepared by an equal volume of the overnight grown single-species culture in a 96-well microtiter plate (Tarson, India) (8.0-log CFU/ml) at the ratio of 1:100 dilution under aerobic conditions at 30 °C for 72 h. Here, single-species *E. coli, E. faecalis* were used as the control, and coculture [(L. *fermentum* TIU19+*E. coli*), (L. *fermentum* TIU19+*E. faecalis*)] used as a test. After incubation for 72 h, planktonic cells were collected from each well and adherent bacteria sessile cells (biofilm) in each well were resuspended with PBS by vigorous pipetting and vortexing followed by 30-s sonication. For the determination of *E. coli* and *E. faecalis* bacterial counts, cultures were seeded on MacConkey sorbitol agar and Pfizer selective Enterococcus agar medium, respectively, and incubated at 37 °C for 24 h (Kaur et al., 2018).

3. Results and discussion

3.1. Isolation, identification, and biochemical characterization of L. fermentum

There are only a few reports of the isolation of probiotic bacteria from Haria. In this study, from Haria, we are therefore attempting to isolate and establish a novel probiotic L. *fermentum* strain with antibacterial activity. Based on our initial screening, we selected bacteria with Gram-positive, catalase-negative, low pH (pH - 3) tolerance, and antagonistic activity against the *E. coli* MTCC41 strain, based on our initial screening. API analysis allows us to select the strain belonging to the genus *Lactobacillus* (data not shown). Analyses of 16S rRNA gene sequence show that the strain has 99.3% identity with the type strain of *Lactobacillus fermentum* LMEM37. The strain was designated as *Lactobacillus fermentum* TIU19 (GenBank: MK752864.1) (Fig. 1A). L. *fermentum* has been previously reported to be a dominant non-pathogenic fermentable species of Haria with probiotic potential (Ghosh et al., 2015).

3.2. Assessment of safety and virulence factors of selected L. fermentum strain

According to the guidelines of FAO and WHO, before defining a strain as a probiotic, it is important to analyze both the functional properties and its safety for public health. We first determined the safety of L. *fermentum* TIU19 by determining the MAR index and assessing virulence factors such as gelatinase and hemolytic activity.

3.2.1. MAR index

The MAR index is a key parameter for understanding the drug-resistant properties of microorganisms. The L. *fermentum* TIU19 strain was found to be sensitive to all antibiotics used, such as ampicillin, gentamycin, kanamycin, streptomycin, erythromycin, and chloramphenicol, tetracycline, and resistant to vancomycin. The MAR index was 0.125 and is shown in Table 1. If the MAR index value was less than 2, the isolate was not considered to be resistant to multiple antibiotics. Hence, the strain was not considered antibiotic-resistant (Resende et al., 2014).

3.2.2. Assay for haemolytic and gelatinase activity

The absence of virulence factors, for example, haemolytic and gelatinase activity, are crucial safety criteria for a probiotic strain. The L. *fermentum* TIU19 strain was tested for both haemolytic and gelatinase activity. The strain showed a lack of these virulence factors (Fig. 1B and1C). This is very important as previously different Lactobacillus probiotic strains had similar results and were therefore considered safe for applications as probiotics (Kaktcham et al., 2018).

3.3. Probiotic potential of selected L. fermentum TIU19 strain

3.3.1. Survival under conditions simulating the human GI tract

In the gastrointestinal tract, the entry of bacteria is restricted due to the acidic pH of the stomach and the antibacterial action of pepsin. These are important criteria for the selection of probiotic bacteria. At the time point of 2 h, there were almost no viable cells left at pH 2, but there was a slight change and no change in numbers at pH 3 and pH 4, respectively. At the time point of 3 h, the percentage of cell survival decreased to 97.2 ± 1.3% at pH 3, again without change at pH 4 (Fig. 2A).

A probiotic bacterium in the small intestine is expected to survive in the presence of bile salt and pancreatin. A thick defensive layer of extracellular polysaccharides and secretion of bile salt hydrolase protect *Lactobacillus* from bile salts (Nawaz et al., 2017). In our study, the bile

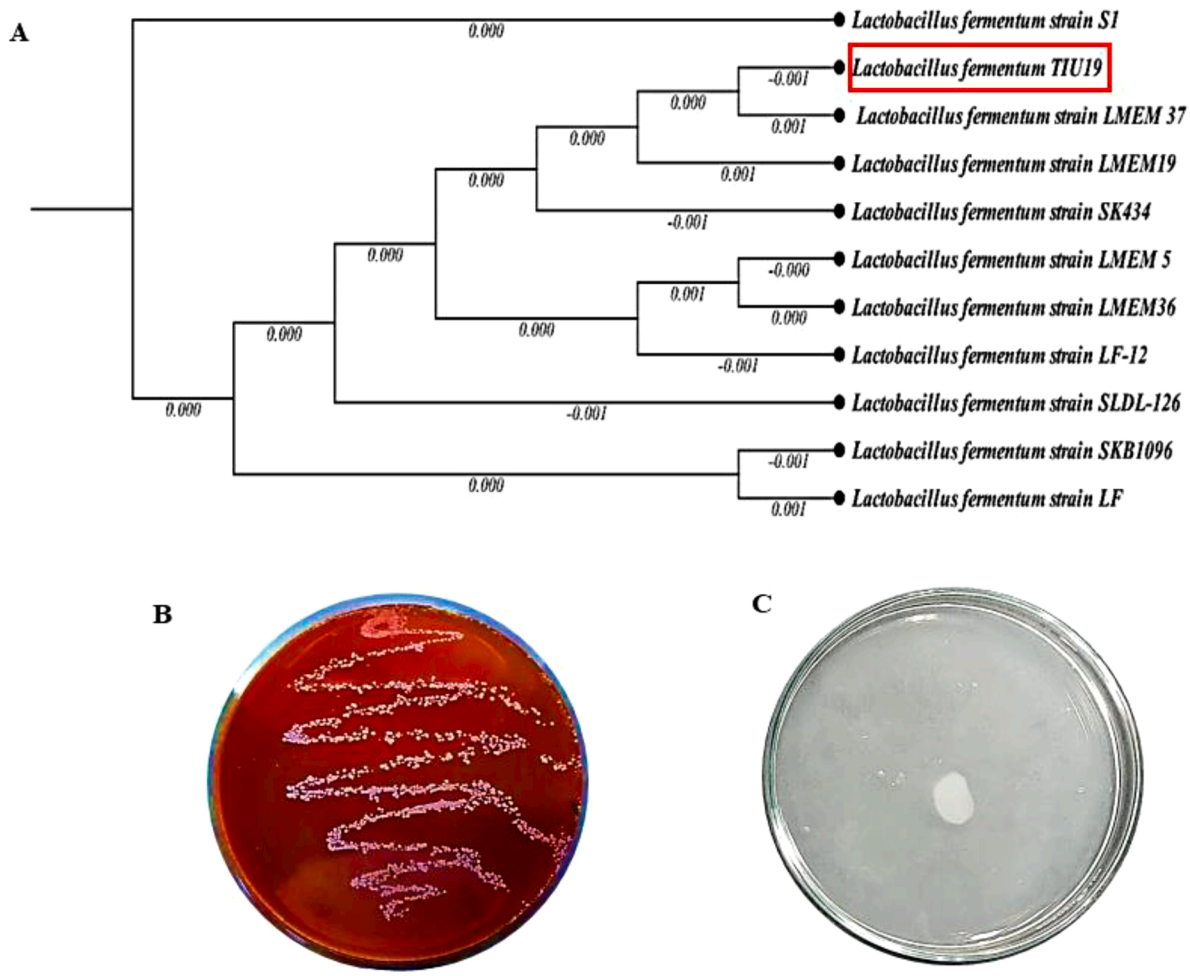

Fig. 1. (A) Cladogram representation of bacteria isolated from Haria. (B) Haemolytic activity of L. *fermentum* TIU19. (C) Gelatinase activity of L. *fermentum* TIU19.

Table 1
MAR Index determination of L. *fermentum* TIU19.

Antibiotics	Type	Amount	Effect	MAR Index
Ampicillin	β-lactams	10 μg	S	0.125
Vancomycin	glycopeptides	30 μg	R	
Gentamycin	aminoglycosides	10 μg	S	
Kanamycin	aminoglycosides	30 μg	S	
Streptomycin	aminoglycosides	10 μg	S	
Erythromycin	macrolides	15 μg	S	
Chloramphenicol		30 μg	S	
Tetracycline		30 μg	S	

salt tolerance profile of the L. *fermentum* TIU19 strain showed that nearly 10% of viable cells were detected at 0.3% and 0.4% oxgall after 3 h of exposure. At the time point of 4 h of treatment with 0.2% and 0.1% oxgall, the number of cells of L. *fermentum* TIU19 decreased by approximately 25% and 3%, respectively (Fig. 2B).

Both the pepsin and pancreatin resistance of the L. *fermentum* TIU19 strain have been shown in Fig. 2C and 2D. At pH 3 and pH 4, when L. *fermentum* TIU19 was treated with pepsin for 3 h, 99% of the bacterial cells survived, but that decreased to 75 ± 3.1% at pH 2. Not only that, viable cells were not completely diminished in the presence of pepsin. Exposure to pancreatin (SIF) at pH 6.8 or pH 8 resulted in nearly 99% of the bacterial cells surviving, indicating that pancreatin had almost no effect on the number of viable cells. The results of the survivability of L. *fermentum* TIU19 under simulated gastric-intestinal juice and bile salt tolerance are quite different from the previously reported probiotic L. *fermentum* KKL1 strain isolated from Haria (Ghosh et al., 2015, p. 1). The L. *fermentum* KKL1 was able to survive in the simulated gastric juice at pH 2 (12.19%) and pH 3 (57.56%) and had strong resistance to bile salt and could tolerate 2% of oxgall for 4 h of incubation periods.

3.3.2. Cell surface hydrophobicity

For any good probiotic strain, the nonspecific hydrophobic surface interaction between the microorganism and the host cell acts as a key adhesion mechanism (Ramos et al., 2013). In our study, the cell surface hydrophobicity of L. *fermentum* TIU19 was 61.53%. This indicates a significantly high surface hydrophobicity property. As previously isolated L. *fermentum* KKL1 from Haria was reported to exhibit only moderate hydrophobicity (31.03%), it can be hypothesized that newly isolated L. *fermentum* TIU19 significantly improves the probiotic potential, at least based on hydrophobicity.

3.3.3. Auto-aggregation and adhesion ability

The probiotic strain should have the capacity to adhere to the mucosal surface (Muryany et al., 2018). In general, the higher hydrophobicity of the probiotic strain shows its auto-aggregation and high adhesion capability. In our study, L. *fermentum* TIU19 demonstrated a good autoaggregation ability of approximately 70.23 ± 4.2% at 37 °C, which is very similar to the previously reported probiotic strains *Lactobacillus pentosus* ST712BZ1 and *Lactobacillus plantarum* ST284BZ2, isolated from traditional Bulgarian milk products. The auto-aggregation ability of *Lactobacillus pentosus* ST712BZ1 and *Lactobacillus plantarum* ST284BZ2 was found to be approximately 67% and 99% (Todorov et al., 2007). Aggregation capacity is an important property for probiotic

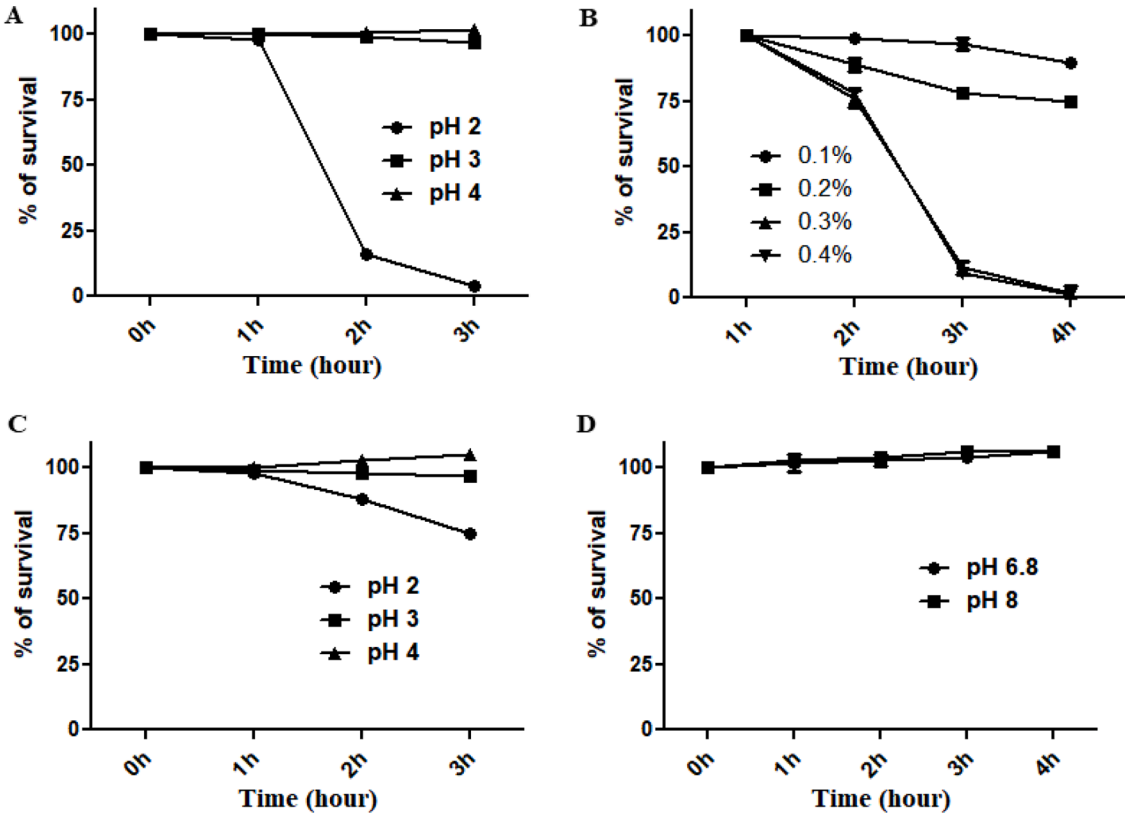

Fig. 2. Survival of L. *fermentum* TIU19 in (A) the presence of acid (B) bile-containing buffer (C) simulated gastric fluid (D) simulated intestinal fluid.

strains; it leads to the formation of biofilms to protect the host from pathogen invasion (Grigoryan et al., 2018). This property of lactobacilli gives them the ability to adhere to the surface of intestinal cells.

The adhesion ability of the selected strain was determined by using the HeLa cell line. For the adherence study of various pathogens, including uro-pathogens, the use of Hela cells has already been reported (Leccese Terraf et al., 2017). In addition to this, the morphology and characteristics of urethral epithelial cells are very similar to Hela cells (Zuiverloon et al., 2018).

The adhesion ability of the isolated strain to HeLa cells was 77.42 ± 1.7%. It is very similar to the previous report where four LAB strains (*Leu. mesenteroides* H40, L. *plantarum* FI10604, L. *brevis* FI10700, and L. *perolens* FI10842) showed high adhesion ability (Son et al., 2018). Therefore, it can be hypothesized that; the adhesion ability of the isolated strain of L. *fermentum* could play a role in its function as probiotics and may allow bacteria to grow on the mucosal surface.

3.3.4. *Biofilm forming ability*

Biofilm formation is another important factor in the selection of probiotic strains. The biofilm biomass of the selected L. *fermentum* TIU19 strain was increased in a time-dependent manner, as illustrated in Figs. 3A and 3B. After 72 h of incubation, the biofilm biomass of the isolated strain was found to be 84.29 ± 1.7%. It is very similar to the previous report, where *Lactobacillus* strains were found to be good biofilm producers (Terraf et al., 2012).

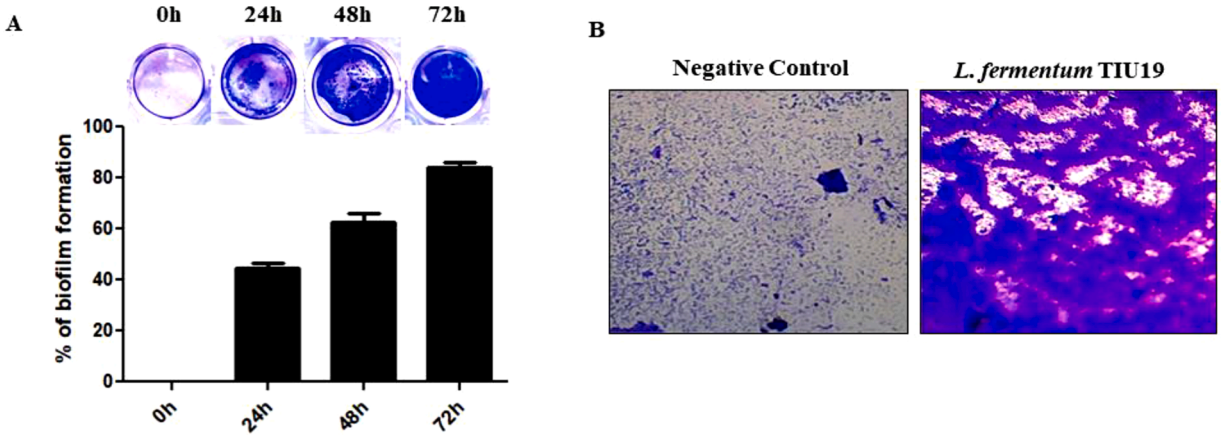

Fig. 3. (A) The bar diagram represents the biofilm-forming potential of L. *fermentum* TIU19 at different time points. Data are represented as the mean ± SEM, where $n = 3$. The pictures above the bar are representing respective well stained with crystal violet. (B) Light microscopic observation of wells stained with crystal violet after 72 h of incubation in the absence and presence of L. *fermentum* TIU19 depicted the good biofilm-forming ability of the isolated L. *fermentum*.

3.3.5. β-galactosidase production

From a technological point of view, the ability of the probiotic strain to produce β-galactosidase enzyme production is an important factor. Bacterial cells were incubated with a reaction buffer and ONPG substrate. β-galactosidase converts the colourless ONPG substrate into galactose and the chromophore o-nitrophenol, producing a bright yellow solution. The L. *fermentum* TIU19 probiotic strain was found to have positive β-galactosidase activity, which should provide an additional benefit, as reported earlier (Zárate and Chaia, 2012).

3.3.6. Antioxidant property

The DPPH radical scavenging activity of L. *fermentum* TIU19 is shown in Fig. 4A. 4-log to 6-log CFU/ml bacterial cells showed free radical scavenging activity ranging from 46.7 ± 2.10% to 86.58 ± 1.06%. In the present study, L. *fermentum* TIU19 showed significant concentration-dependent free radical scavenging activity, which is very similar to the previously reported probiotic strain L. *plantarum* KCC-24, isolated from Italian ryegrass. The scavenging activity of L. *plantarum* KCC-24 was found to be approximately 70%.

3.4. In vitro test to determine the antagonistic activity of L. fermentum TIU19 against uro-pathogens

3.4.1. Antagonistic activity against uro-pathogens

The antagonistic activity of L. *fermentum* TIU19 against uro-pathogens shows that L. *fermentum* TIU19 inhibited the multi resistant *E. coli* and *E. faecalis* strains. The inhibition zones observed were 11 ± 1.2 mm and 19 ± 0.75 mm, respectively. Various probiotic *Lactobacillus* strains have been reported to exhibit antibacterial activity against a variety of pathogenic bacteria, including *E. coli* and *E. faecalis* (Prabhurajeshwar and Chandrakanth, 2017).

3.4.2. Effects of L. fermentum TIU19 on the co-aggregation

Co-aggregation ability may form a barrier that prevents colonization by pathogenic microorganisms (Kos et al., 2003, p. 92). To understand the co-aggregation activity of the probiotic bacteria with both uro-pathogens, single species pathogens were mixed individually with L. *fermentum* TIU19 (*E. coli* + L. *fermentum* TIU19 and *E. faecalis* + L. *fermentum* TIU19) and co-incubated for 60 mins. We found that the co-aggregation activity of L. *fermentum* TIU19 with uro-pathogenic *E. coli* was 61 ± 2.9% and 52 ± 1.8% for *E. faecalis*. These findings are comparable to earlier reports, where a high coaggregation percentage of *lactobacilli* was obtained with *E. coli* ATCC 11,229 (Ekmekci et al., 2009). In this study, L. *fermentum* TIU19 demonstrated strong co-aggregative behavior with both uro-pathogenic strains.

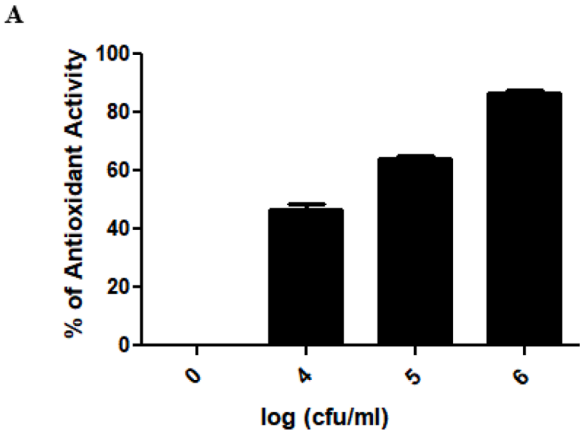

Fig. 4. (A) DPPH radical scavenging property of L. *fermentum* TIU19 in a concentration-dependent manner. Data are represented as the mean ± SEM, where $n = 3$.

3.4.3. Effects of L. fermentum TIU19 on the adhesion of uropathogens to Hela cells

Probiotic bacteria may establish a defensive barrier that prevents pathogenic bacteria from colonizing the epithelial cell lining (Odenwald and Turner, 2017; Takiishi et al., 2017). For the adherence study of various pathogens, including uro-pathogens, Hela cells have already been reported (Leccese Terraf et al., 2017). In addition to this, the morphology and characteristics of urethral epithelial cells are very similar to Hela cells (Zuiverloon et al., 2018). To investigate the effect of L. *fermentum* TIU19 on the adherence capacity of the two uro-pathogenic strains, L. *fermentum* TIU19 was co-inoculated into HeLa cells by each uro-pathogen (Fig. 5A). We found that L. *fermentum* TIU19 was found to be competitive and significantly reduced the adhesion of uro-pathogens *E. coli* and *E. faecalis* to HeLa cells by 69.93 ± 2.2% and 71.62 ± 3.1% respectively. In this study, L. *fermentum* TIU19 demonstrated strong inhibition of bacterial uro-pathogenic adhesion to HeLa cells.

3.4.4. Biofilm inhibition assay

Lactobacillus species have been considered beneficial microflora since they are capable of inhibiting the growth and/or colonization of numerous pathogenic bacteria (Di Cerbo et al., 2016).To evaluate the isolated L. *fermentum*'s ability to prevent biofilm formation of uro-pathogens, single species of uro-pathogens were mixed separately with it and co-incubated to form a dual-species biofilm. After incubation, we determined the number of *E. coli* and *E. faecalis* cells. Planktonic and sessile cells of *E. coli* decreased by 4-log and 2.1-log (Fig. 5B), and *E. faecalis* was reduced by 3.8-log and 4.1-log, respectively, compared to the control (Fig. 5C). From these results, we assumed that the L. *fermentum* TIU19 strain may inhibit the formation of *E. coli* and *E. faecalis* biofilms. Our findings are consistent with previous research, which found that different *Lactobacillus* species decreased biofilm-associated cells of *E. coli* O157:H7, L. *monocytogenes,* and *S. Typhimurium* by 5-log, 4-log, and 7-log, respectively (Gómez et al., 2016).

4. Conclusions

The results presented show that L. *fermentum* TIU19 has safety properties *in vitro* and inhibitory activity upon multi resistant uro-pathogens *E. coli* and *E. faecium*. Thus, this strain harbor promising probiotic traits, but further analyses are needed to verify *in vivo* the probiotic behavior of L. *fermentum* TIU19 too.

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on a reasonable request.

Author's contribution

Conceptualization: Arnab Ganguli **Supervision:** Arnab Ganguli **Data curation:** Shatabdi Das, Arnab Ganguli **Formal Analysis:** Shatabdi Das, Arnab Ganguli **Investigation:** Shatabdi Das, Arnab Ganguli, Kumari Vishakha **Methodology:** Shatabdi Das, Arnab Ganguli **Validation:** Shatabdi Das, Arnab Ganguli, Kumari Vishakha, **Visualization:** Shatabdi Das, Arnab Ganguli **Project administration:** Shatabdi Das, Arnab Ganguli **Writing - Original Draft:** Shatabdi Das, Arnab Ganguli

Ethics approval

Not applicable

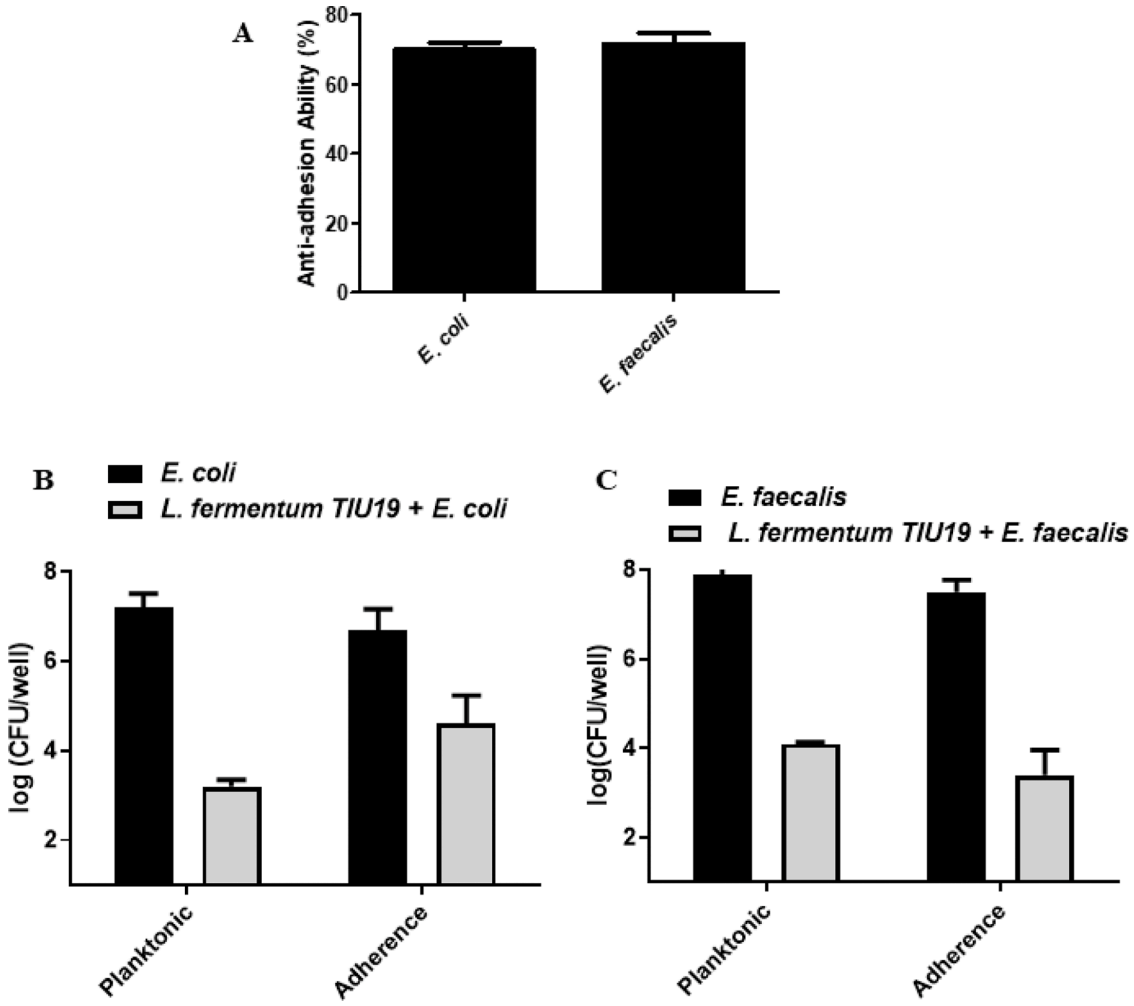

Fig. 5. (A) The bar diagram represents the anti-adhesion ability of L. *fermentum* TIU19 against *E. coli* and *E. faecalis*. (B and C) The bar diagram represents the log (CFU/ml) of planktonic and adherence cells of *E. coli* (B) and *E. faecalis* (C) in the presence and absence of L. *fermentum* TIU19. Data are represented as the mean ± SEM, where $n = 3$.

Declaration of Competing Interest

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

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