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PROBIOTIC LACTIC ACID BACTERIA FROM ADUWA KO JAANR, A LESSER-KNOWN FERMENTED GINGER BEVERAGE FROM EASTERN HIMALAYAN REGION

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ABSTRACT

The Eastern Himalayan natives enjoy drinking *Aduwa ko jaanr*, a traditionally fermented mild-alcoholic ginger brew. Studies on manufacturing process and microbiological dynamics are infrequent for *Aduwa ko jaanr*. Current study analyzed the probiotic properties of Lactic Acid Bacteria (LAB) associated with *Aduwa ko jaanr*, using in-vitro method, simulating the conditions of intestinal tract. Three LAB isolates were screened and selected for preliminary characterization (morphological and biochemical). Isolates were identified as *Lactiplantibacillus argentoratensis* AKJ(W), *Lactiplantibacillus argentoratensis* AKJ(Y), and *Levilactobacillus brevis* AKJ(P) by 16S rRNA sequencing. Studies on growth kinetics of stationary phase and formation times of secondary metabolites with probiotic effects were conducted. Probiotic tests, namely acid-bile tolerance, phenol tolerance, cell surface hydrophobicity, auto-aggregation property, adherence to mammalian cell line, antibiotic susceptibility, antimicrobial activity and radical scavenging activity were performed. Results indicated the isolates to have health-benefiting properties with antibiotic resistance and inhibiting human pathogens. Isolates are predicted to survive in gut environment as they tolerated high bile salt concentration (0.5%), low pH (pH2) and high phenol concentration (0.6%). Results of cell surface hydrophobicity, auto-aggregation, and mammalian cell line (HEK 293) adherence tests suggested that isolates could adhere and possibly colonize intestine's inner wall. High radical scavenging activity of isolates suggested overall antioxidant property of the fermented beverage. MAR index showed isolates' resistance to different antibiotics, indicating that their action is unaffected even when administered during illness. The present study suggests that LAB in Aduwa ko jaanr has potential probiotic properties which makes it an appropriate traditional beverage conferring health benefits.

Keywords: Aduwa ko Jaanr, fermented ginger beverage, lactic acid bacteria, probiotics

INTRODUCTION

Fermenting food using traditional methods is a good and wise way to preserve food in locations where cultivation is not supported throughout the year. The primary purpose of fermentation is to increase the nutritional value of food by converting complex large molecules into simpler subunits through enzymatic action of fermenting microbes (**Kumar and Kumar, 2015**). Also, fermentation helps to preserve food by maintaining a low pH and preventing spoilage. "Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits on the host" (**Guarnera and Schaafsma, 1998**). Probiotics benefit the body through several processes, including the competitive exclusion of pathogens, synthesis of antimicrobial compounds, immune system regulation, and fermentation of food components into bioactive molecules. Maintaining gut health and general well-being depends significantly on the gut microflora. The composition and functioning of the gut microbiota can be positively influenced by probiotics, potentially improving health. Probiotics' effectiveness in treating gastrointestinal illnesses such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and antibiotic-associated diarrhoea has been thoroughly researched. By increasing the activity of immune cells, supporting anti-inflammatory cytokines, and decreasing pro-inflammatory cytokines, probiotics can influence the immunological response. In addition to gastrointestinal health, probiotics have also been studied for their possible role in treating allergies, urogenital infections, metabolic diseases, and mental health issues (**Joshi, 2007**). Most bacteria used as probiotics belong to Lactic Acid Bacteria (LAB) and *Bifidobacterium* families. LAB are abundant in fermented foods and can be included in the regular diet as they are Generally Regarded As Safe (GRAS) (**Thapa, 2016**). LAB is known to inhabit the intestinal tract of humans and animals (**Rezac *et al.*, 2018**), and the probiotic properties of these bacteria enable them to survive inside the gastrointestinal tract.

Aduwa ko jaanr is a traditionally fermented mild-alcoholic beverage made by fermenting ginger (*Zingiber officinale* Rosc.) rhizome. It is common amongst the native community of the Eastern Himalayas of India, specifically Sikkim, Darjeeling, and adjoining areas of Kalimpong, Kurseong, Siliguri, Dooars and Nepal. The fermentation process is a natural, secure, and essential aspect of indigenous cultural history (**Karki *et al.*, 1983**). It is a heritage passed on from generations and is consumed by the native people as a recreational drink and for relaxing purpose after a tedious work schedule. *Aduwa ko jaanr* is believed to have health benefits offered by the native microbial community. The process of fermentation is entirely traditional and organic. Steamed ginger is cut into pieces, smashed to increase surface area, boiled, allowed to cool, and mixed with natural yeast locally called 'marcha' [Figure. 1 (d)], a mixture of herbs, rice, and spices. The combination is blended thoroughly, packed in an airtight container, and left for fermentation, spanning to about 3 to 4 days in summer and 5 to 7 days during winter [Figure 1 (a)]. *Aduwa ko jaanr* is consumed since many decades, but little is known about its probiotic effects due to the lack of comprehensive research on this fermented food. The present study aimed to isolate and identify predominant LAB from *Aduwa ko jaanr* and characterize their probiotic potential as a report is scarce for this fermented beverage from Eastern Himalayas.

MATERIAL AND METHODS

Sample collection

About 40-50 grams of *Aduwa ko jaanr* samples were collected [Figure 1(c)] from different locations from Eastern Himalayan range. Sterile containers were used for sampling and were transported to laboratory aseptically for analysis within 24 hours.

Process of fermentation

Ginger rhizome is first washed and steamed

It is then dried, cut into small pieces and mixed thoroughly with natural yeast locally called 'marcha'

Ginger is then packed in an air tight container for fermentation

The jar is left for 3-4 days during summer and 5-7 days during winter, at room temperature (25˚ C)

Aduwa ko jaanr

(a)

(b) (c) (d)

Figure 1 (a) Flowchart of *Aduwa ko jaanr* fermentation, (b) Ginger brew, (c) Collected sample of *Aduwa ko jaanr*, (d) Marcha (starter culture)

Isolation of microorganisms

Selective isolation of lactic acid bacteria (LAB) from aseptically collected samples was performed by serial dilution on MRS (Man, Rogosa and Sharpe) agar plates. Homogenization and blending of samples were done with the help of mortar and pestle with slight modifications **(Biswas *et al.*, 2017)**. 5 grams of sample was taken, homogenized, and blended in 45 mL of sterile 0.85% physiological saline water (w/v NaCl). For viable cell count, serially diluted sample was plated into MRS plates and incubated at 37˚C for 48–72 hours **(Rapsang and Joshi, 2015)** under anaerobic condition in CO_2 incubator (ESCO, India), with an input of 5% CO_2 **(Nair and Surendran, 2004)**. Distinct and different colonies were selected from the culture plates based on morphology and streaked onto MRS agar plates to obtain pure cultures which were preserved using 15% glycerol at –20 ^{0}C for further use. Colony count was done for enumerating Colony Forming Units (CFU) per gram of the sample. CFU was calculated by using the following formula **(Ka-ot and Joshi, 2021)**

$$\text{CFU/g or mL} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Volume plated} \times \text{grams of sample taken(for solid sample)}}$$

Morphological and biochemical characterization of isolates

Isolates were identified based on their morphology like Gram staining, motility and different biochemical tests namely, IMViC (Indole, Methyl Red, Voges Proskauer and Citrate), catalase, oxidase, H_2S production, triple sugar utilization test (TSI), starch agar test (amylase) and skim milk agar test (protease test) as per Bergey's Manual of Systematic Bacteriology **(Holt *et al.*, 2000**).

Molecular characterization of the bacterial isolates

Genomic DNA was isolated using a bacterial DNA isolation kit (HiMedia, India). 16S rRNA gene amplification was done by polymerase chain reaction (PCR) in Mastercycler Nexus gradient PCR (Eppendorf, AG 22331, Hamburg, Germany.) **(Thokchom and Joshi, 2013; Rapsang and Joshi, 2015)** with slight modifications. Conditions for PCR were denaturation of template DNA at 95˚C for 3 min followed by 30 cycles at 95˚C for 30 seconds, annealing at 51˚C for 30 seconds, extension at 72˚C for 2 min, and final step at 72˚C for 10 mins. 50 µl of PCR mixture contained Taq polymerase (5U/ µl), isolated DNA template (2 µl), 2 µl of Primers (27F and 1492R), 2.0 mM $MgCl_2$ (10X buffer) (HiMedia, India), deoxynucleoside triphosphates (DNTPs: 200µM of dATP, dCTP, dGTP, and dTTP each). For negative control, distilled water was used as a template in lane 3 **(Ka-ot and Joshi, 2021)**. Amplification of 16S rRNA gene was done using universal primers, forward primer 27F (5′-AGA GTT TGA TCA TGG CTC AG-3′) and reverse primer 1492R (5′-TAC GGY TAC CTT GTT ACG ACTT-3′) (HiMedia, India). The amplified product (Figure 2) was eluted and sequenced, giving about 1500 bases.
Phylogenetic neighbours were determined for each of the selected isolates using the Basic Local Alignment Search Tool (BLAST) program against a database of type strains with validly published prokaryotic names available at http://www.eztaxon.org/15 **(Kalita and Joshi, 2017)**. Phylogenetic analysis and evolutionary trees were constructed using the Neighbor-joining method in MEGA software (version 11.0.10)

Assessment of growth kinetics and pH change

Growth curve study along with pH change analysis was done to observe the time required to reach stationary phase and synthesis of organic acids following standard methods (Calumba *et al.*, 2021; Somashekaraiah *et al.*, 2021; Murugan and Mishra, 2022) with minor modifications. MRS broth was inoculated overnight with the LAB isolates at 37˚C. 0.1% of this actively growing culture was inoculated into 100 mL of freshly prepared sterile MRS broth in a conical flask. OD_{600} was measured at each 2 hrs time interval from 0 to 16 hrs till stationary phase was reached. Change in pH was also recorded during the growth period.

Analysis of probiotic properties of LAB:

Survivability, acid and bile salt tolerance of the isolated bacteria

Acid and bile salt tolerance

The acid and bile tolerance test were conducted following the method of Succi *et al.* (2005) with slight modifications. Briefly, the LAB isolates were subjected to pH 2, 3 and 0.1%, 0.3% and 0.5% w/v Oxgall containing bile salt (HiMedia, India) concentration for 0, 2 and 4 hr. Isolates were cultured overnight in MRS broth at 37˚C under anaerobic condition (5% CO_2 input) and was used as inoculum at 1% (v/v). In case of acid tolerance, pH of the media was adjusted with 1N HCl and 1N NaOH. Inoculant without changing the pH was taken as control. For bile salt tolerance, cells grown on MRS broth with bile salt concentrations. Medium without bile salt was taken as control. The absorbance was measured as OD (optical density) at 600nm wavelength using a spectrophotometer (Cecil, CE 7200, England). Percentage of resistance was calculated using the following formula **(Kumar and Kumar, 2015)**:

$$\% \text{ Resistance} = \frac{\text{Increase in OD in MRS broth having bile salt/ pH 2 and 3}}{\text{Incease of OD of MRS broth without bile salt/at pH 7}} \times 100$$

Free radical scavenging activity:

Free radical scavenging potential of the bacterial cultures were assessed using DPPH assay. DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a compound composed of stable free radical molecules which releases hydrogen ions. When dissolved in methanol, DPPH gives purple colouration, which decolourizes and develops different shades of yellow in the presence of antioxidants. This property of DPPH was used to measure the free radical scavenging activity indicating the antioxidant property of the LAB **(Marinova and Batchvarov, 2011)**.
The LAB isolates were cultured in MRS broth and incubated overnight in a shaking incubator at 37°C. The cultures were harvested by centrifugation at 10,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed thrice in PBS (pH 7) and resuspended in the same. 1mL of cell suspension was taken and an equal volume of freshly prepared DPPH (HiMedia, India) in methanol (0.2 mM) solution was mixed by vortexing for five seconds. The setup was incubated for 30 minutes in the dark as DPPH is light-sensitive. After incubation, the intensity of the colour change of DPPH was determined by measuring OD at 517 nm.
Free radical scavenging activity percentage was calculated using the following formula:

$$\% \text{ RS (Radical scavenging)} = \frac{\text{Control} - \text{sample}}{\text{Control}} \times 100$$

Where, Control = distilled water + DPPH; Blank = Cell + PBS (50mM phosphate buffer saline); Sample = DPPH + Cell

Cell Surface properties

Determination of cell surface hydrophobicity

Cell surface hydrophobicity was determined following the method of **Rosenberg and Gutnick (1980)** with minor modifications. *In vitro* analysis was done to see the adherence property of LAB isolates with liquid hydrocarbon, n-hexadecane (HiMedia, India). For comparison, *Lactobacillus plantarum* was taken as control **(Rosenberg and Gutnick, 1980; Rapsang and Joshi, 2015)**. The cells were cultured overnight in MRS broth in a shaking incubator at 150 rpm and 37˚C. The overnight grown, post log and early stationary phase culture was taken for the experiment. Cells were harvested by centrifugation at 5000 rpm for 15 mins at 4˚C. The supernatant was discarded, and the cells were washed twice with phosphate buffer saline (pH 7.0) and resuspended in the same buffer, making a final volume of 1 mL. OD of the resuspended solution was taken at 600 nm wavelength. An equal volume (1 mL) of hydrocarbon (n-hexadecane) was added to the suspension, mixed vigorously in a vortex for 2 mins, and incubated at room temperature for 30 mins. The experimental setup was left undisturbed to separate the organic (hydrocarbon) and the aqueous phases. After incubation period was over, 1 mL of

the aqueous phase was carefully removed and absorbance was measured at 600 nm. The decrease in absorbance of the aqueous phase was considered as % hydrophobicity and was calculated by using the following formula **(Guan *et al.*, 2020)**:

$$\text{Hydrophobicity (\%)} = \frac{\text{OD of aqueous phase at 600 nm before mixing} - \text{OD of aqueous phase at 600 nm after mixing}}{\text{OD of aqueous phase at 600 nm before mixing}} \times 100$$

Autoaggregation property

The autoaggregation property of the cells were examined following the method of **Divisekera *et al.* (2019**) with slight modifications. Cultures were inoculated and grown overnight. The cells were then harvested by centrifugation at 8000 rpm at 4˚C for 10 min. Subsequently, the supernatant was drained and the pellet washed twice with phosphate buffer saline (pH 7.0) and resuspended in the same. The suspension was incubated at 37˚C, keeping it stand still. Upper suspension was taken to measure the absorbance (OD_{600}) at 1,2 and 3 hrs. time intervals. Autoaggregation was calculated in terms of percentage by the following formula (**Meena *et al.*, 2022**)

$$\text{Percentage autoaggregation (\%)} = \frac{x - y}{x} \times 100\ \%$$

Where, x is the initial optical density (at 0 hours) of cell culture suspension and y is the final od of the upper layer of cell suspension after time 't'.

Adhesion onto HEK 293 cell line

Adhesion assay was conducted on the HEK 293 cell line (human embryonic kidney cells) obtained from the National Centre For Cell Science (NCCS), Pune, India. Cells were cultured in DMEM (Dulbecco's modified Eagle's medium), with 10% FBS (Foetal Bovine Serum), 1% Pen-strap at a constant temperature of 37˚C and a supply of 5% CO_2. Cells were cultured in T25 culture flasks and left for incubation until 90% cell confluence was observed under the microscope. The assay was done following the method as escribed by **Gonzalez *et al.* (2018)** and **Somashekaraiah *et al.* (2019)** with slight modifications. DMEM and FBS were changed after two days. After the cells reached 90% confluency, they were harvested by trypsinization (200µL) for 2 mins at 37˚C. The cells were centrifuged at 12,000 rpm for 5 mins. The supernatant was discarded and the cell resuspended in DMEM supplemented with 10 % FBS to stop trypsin activity. Cell counting was done using a hemocytometer after which a dilution of 105 cells was made for the adhesion assay. Diluted cells were then seeded into 96 well plates in triplicates and incubated until 90% confluency was observed. Overnight-grown bacterial cells were centrifuged at 9000 rpm for 15 mins to obtain cell pellets. Supernatant was discarded and cell pellet was resuspended in PBS. OD of the suspension was measured and a dilution of 10^7 cells was made to make a 100:1 ratio of bacterial cell to mammalian cell for adhesion. For adhesion assay, media was discarded from 96 well plates and cells were washed twice with PBS, after which 100 µL of 10^7 bacterial cells and 100 µL of DMEM (without FBS) were added into the wells. The setup was incubated for 2 hrs at 37˚C and 5% CO_2 input. After 2 hrs, media was discarded to remove non-adherent bacterial cells and washed twice with PBS. Trypsin (0.25%)/EDTA solution was added to dislodge mammalian cells along with the adherent bacteria. Enumeration of adherent bacterial cells was done by serial dilution of 100 µL content of wells and plating onto MRS agar followed by incubation at 37˚C for 24 hrs.

Phenol tolerance

After evaluating the survival of isolates in simulated gastric conditions, response to phenol concentrations (0.4% and 0.6 % phenol) was observed following the method of **Somasekharah *et al.* (2019)**. Briefly, bacterial cells were grown overnight in MRS broth which was supplemented with 0.4% and 0.6% (v/v) of liquid phenol. The cultures were then spread onto MRS agar plates by 10-fold dilution method for 0 hr and after 24 hrs to check their viability and hence their survival. Viable cells were enumerated through the plate count method.

Antimicrobial activity against the pathogens

The antimicrobial activity of the isolated LAB was assayed against the pathogens, namely, *Escherichia coli* MTCC 730, *Bacillus cereus* MTCC 430, *B. subtilis* MTCC 441, *Staphylococcus aureus* MTCC 2940, *Klebsiella pneumoniae* MTCC 109 and *Candida albicans* MTCC 183.

Spot agar test: With the help of the agar spot test, the antimicrobial activity of probiotic isolates was tested against six strains of pathogens following the method as described by Biswas *et al.* (2017) with minor modifications. Each of three LAB isolates was grown overnight in MRS broth for 12 hrs at 37˚C in a shaking incubator at 150 rpm. Grown cultures were adjusted to 1.5×10^8 CFU/mL and 5 µl of the adjusted culture was spotted on MRS agar surface. The inoculated setup was incubated overnight at 37˚C. The grown colonies were then overlayered with ten millilitres of Brain Heart Infusion soft agar (HiMedia, India), which was pre-inoculated with overnight pathogenic culture adjusted to 0.5 McFarland standard with A_{600} between 0.08 to 0.1 giving approximately 1.5×10^8 CFU/mL and left for incubation for 24 hrs at 37˚C. A clear zone of inhibition was recorded after overnight incubation.

Effect of crude extract of bacteriocin-like proteins against pathogens

To observe the production of bacteriocin-like proteins and their antimicrobial effect on pathogens, crude extract of cell-free supernatant (CFS) was made. CFS was examined following the method of **Imade *et al.* (2021)**. Overnight grown cultures of LAB isolates (10-14 hrs.) at 37˚C in MRS broth was centrifuged at 10,000 rpm for 15 mins. CFS were collected and adjusted to different pH (2,3,4,5 and 6) using 1N HCl and 1N NaOH. The pH of the pre-pH-adjusted CFS was 4.1-4.6. The pH-adjusted extract was then filter sterilized using a 0.2µm filter disc and used against pathogens (*E. coli* and *S. aureus*). To observe their antimicrobial properties, agar well diffusion assay was carried out. Mueller Hinton agar (MHA) (HiMedia, India) plates were swabbed with overnight grown pathogenic bacterial cell suspension (adjusted to 0.5 McFarland standard). The wells with 5 mm diameter were made on MHA plates using a sterile cork borer. 100µL aliquot of CFS with different pH were then inoculated into the wells and incubated at 37˚C for 24 hrs. Clear zone of inhibition was measured after the incubation period.

***In vitro* food safety assessment of the isolates.**

The isolate was assessed for safety by checking DNase activity, Haemolytic activity and Antibiotic susceptibility test.

DNase activity

DNase activity was checked by a method followed by **Somashekaraiah *et al.* (2019)**. A circular smear of overnight grown test isolates was made onto DNase agar plates which was prepared by following manufacturer instructions. *Serratia marcescens* was taken as positive control and *L. plantarum* was taken as negative control. Plates were incubated at 37˚C for 24 hrs. A clear zone around the colony was considered as positive DNase activity.

Haemolytic activity

Test for haemolytic activity was done by following the method of **Kowsalya *et al.* (2022)**. Positive control was taken as *S. marscenses* and negative test organism as *L. plantarum.* Blood agar plates were prepared using tryptic soy agar supplemented with 5% defibrinated sheep blood. Smear was made using test organisms. Plates were incubated for 24hrs. at 37˚C. α (characterized by a green halo zone), β (clear zone of hemolysis) and γ haemolysis (having no zone) were used to characterize the type of haemolysis (**Ayyash *et al*., 2018).**

Antibiotic sensitivity assay

The antibiotic sensitivity test was conducted by following the disc diffusion method (Bauer *et al.*;1966) against 20 known commercially available antibiotics (HiMedia, India). Out of twenty, 19 antibiotics viz. Ampicillin (25µg), Cefotaxime (10µg), Ceftazidime (30µg), Chloramphenicol (30µg), Ciprofloxacin (1µg), Co-trimoxazole (25µg), Erythromycin (10µg), Gentamycin (10µg), Meropenem (10µg), Methicillin (5µg), Miconazole (30µg), Nalidixic acid (30µg), Neomycin (30µg), Penicillin G (10µg), Polymyxin B (300µg), Rifampicin (2µg), Streptomycin (300µg), Tetracycline (10µg) and Vancomycin (30µg) were anti-bacterial while one i.e. Fluconazole (25µg) was antifungal. The overnight cultures of the LAB isolates were adjusted to 0.5 McFarland standard (~1.5×10^8 CFU/mL). 0.1 mL volume of the adjusted overnight culture was spread plated onto Muller Hinton agar (MHA) plates. The plates were allowed to dry for some time. Sterile antibiotic discs were
placed aseptically onto the surface of the dried inoculated MHA plates in an aseptic condition. Zone of growth inhibition was measured after 24 h, excluding the disc diameter. Isolates showing zone of resistance ≥20 mm were considered as sensitive (S) and between 15-19 mm as intermediate (I) and ≤14 mm diameter were considered as resistant (R) [**Vlkova *et al*., 2006; CLSI, 2012**]. Multiple Antibiotic Resistance (MAR) index for the isolates was calculated by the formula *a/b* where *a* denotes the number of antibiotics to which the isolates showed resistance and *b* denotes the total number of antibiotics taken for assay (**Chettri and Joshi, 2022**). Isolates with MAR index >0.2 were considered multiple antibiotic resistant, and ≤ 0.2 was considered negligible or less resistant.

Cumulative probiotic score of isolates

The cumulative probiotic potential score is calculated by using a standard scorecard **(Gautam and Sharma, 2015)**. The score is obtained by adding all the probiotic test scores such as test for acid and bile tolerance, phenol tolerance, antioxidant property, antibiotic susceptibility test, cell surface hydrophobicity test, autoaggregation test, adhesion to HEK 293 cell line, antagonistic activity against pathogens, DNase test, Haemolysis test, antimicrobial activity of crude extract of

bacteriocin against pathogens. The following equation is used to calculate probiotic potential,

$$\text{Probiotic potential} = \frac{\text{observed score}}{\text{maximum score}} \times 100$$

Statistical Analysis

Data were analyzed using different statistical tools. Standard deviation and standard error of mean were used for data that was taken from triplicate readings. Evaluation of data was done statistically using Graph Pad Prism V.9 and graphs were plotted using Origin software 2023b (v.10.0.5.157). Statistical significance determination was done by two-way ANOVA followed by Tukey's test for Post Hoc analysis taking $p<0.05$.

RESULTS AND DISCUSSION

Isolation of microorganisms

Isolates were chosen and characterized based on morphological features of the colony such as colour, colony size, opacity, elevation, and margin. Three physiologically distinct colonies obtained were chosen for further study. The average CFU was found to be 3.4×10^8 cells^{-g}.

Morphological (Gram positive or negative and motility test) and biochemical characterization

Morphological, motility and biochemical test of the isolates, AKJ (W), AKJ(Y) and AKJ(P) showed positive for Lactic acid bacteria (Table 1). The isolates were Gram-positive and non-motile, indicating to be Lactic acid bacteria.

Biochemical test of the isolates

The observations for biochemical tests namely IMViC (Indole, Methyl Red, Voges Proskauer and Citrate), catalase, oxidase, H_2S production, triple sugar utilization test (TSI), starch agar test (amylase) and skim milk agar test (protease test) are presented in Table 1. The isolates could utilize a diverse range of carbon sources namely, Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose and L-Arabinose along with the production of organic acids.

Table 1 Morphological and biochemical characteristics of the isolates AKJ (W), AKJ (Y) and AKJ (P)

Characteristics	AKJ(W)	AKJ(Y)	AKJ(P)
Morphological Characteristics			
Colony morphology	Circular white colonies	Yellow circular colonies	Slightly pinkish circular
Gram staining (+/-)	+	+	+
Cell morphology	Rod shaped bacilli	Rod shaped bacilli	Rod shaped bacilli
Biochemical Characteristics			
Oxidase	-	-	-
Catalase	-	-	-
MR (Methyl Red test)	+	+	+
VP (Voges Proskauer test)	-	-	-
Citrate	-	-	-
Skim Milk Agar (protease test)	-	-	-
Urease test	-	-	-
SIM test for motility	-	-	-
H_2S test	-	-	-
Indole test	-	-	-
Starch agar test (amylase test)	-	-	+
Carbohydrate fermentation			
Lactose	+	+	+
Xylose	+	+	+
Maltose	+	+	+
Fructose	+	+	+
Dextrose	+	+	+
Galactose	+	+	+
Raffinose	+	+	+
Trehalose	+	+	+
Melibiose	+	+	+
Sucrose	+	+	+
L-Arabinose	+	+	+
TSI (Triple sugar metabolism test)			
(Stab/Bud)	K/K	A/A	K/K
Gas	-	-	-

Legend: K/K = red/yellow (indicates only glucose fermentation); A/A = yellow/yellow (indicates lactose and glucose and/or sucrose is fermented)

Molecular identification of the LAB isolates

PCR-amplified DNA was observed to be around 1500kb marked in agarose gel with a 100bp DNA marker (HiMedia, India) (Figure 2)

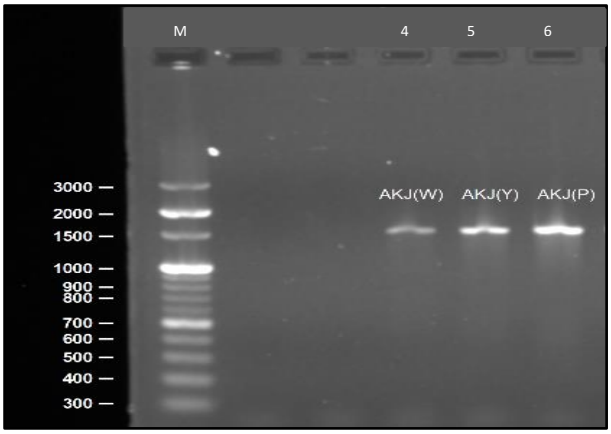


Figure 2 PCR amplification of 16S rDNA region of three LAB isolates with 100bp DNA ladder (M).

Phylogenetic analysis: Phylogenetic characterization showed that all three isolates belonged to *Lactobacillus*. BLAST results showed AKJ(P) with 98.7% similarity to *Lactobacillus brevis*, AKJ(Y) showed 98.88% similarity with *Lactiplantibacillus argentoratensis* and AKJ(W) showed 97.49% similarity with *Lactiplantibacillus argentoratensis* (Table 3).

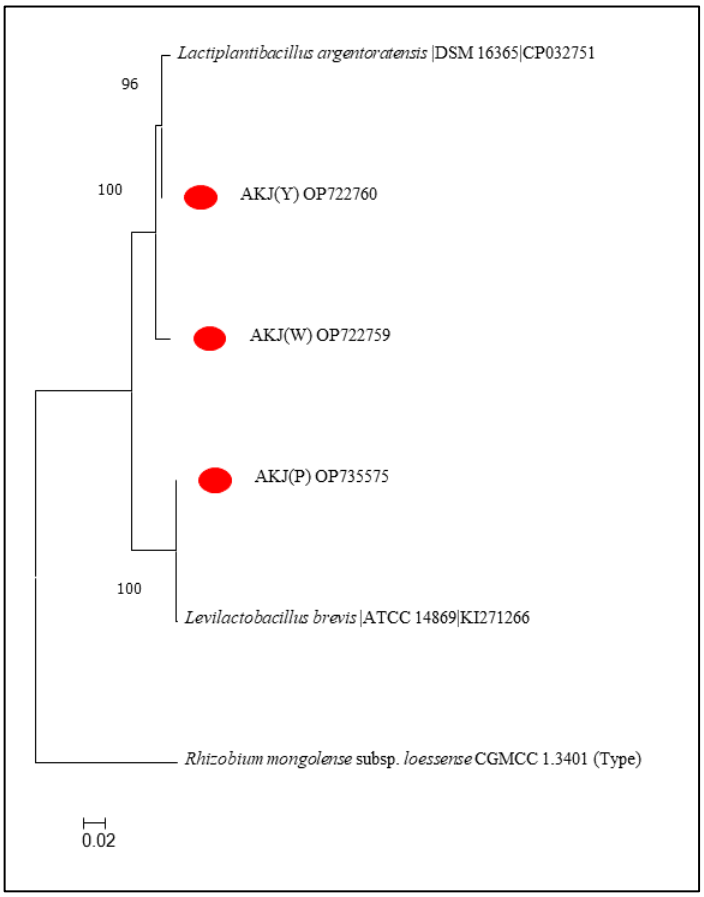


Figure 3 Phylogenetic tree of characterized LAB isolates AKJ(W), AKJ(Y), and AKJ(P) constructed using the Neighbour-joining method with a bootstrap of 1000 replicates and using *Rhizobium mongolense* subsp. *loessense* as an outgroup organism in MEGA (V11.0.10) software.

The growth nature of the isolates

Figure 4 shows the growth patterns of the LAB isolates, as well as the alteration in media pH caused by generation of organic acids**.** Growth kinetic study was done from the growth curve, and growth parameters were derived (Table 2). From the logistic growth curve slow growth was observed till 2 hrs. as the cells were in the lag phase (Figure 4). This is due to the cells adapting physiologically to the environment (**Murugan and Mishra, 2022**). This phase was followed by the log phase, where rapid increase in cell growth was observed. *L. argentorantensis* AKJ(W), *L. argentorantensis* AKJ(Y) and *L. brevis* AKJ(P) all reached the stationary phase after 14 hours, 12 hours, and 14 hours, respectively, after they reached maximum growth and the absorbance remained stable. Doubling time for *Lactiplantibacillus argentoratensis* AKJ(W), *Lactiplantibacillus argentoratensis* AKJ(Y), and *Levilactobacillus brevis* AKJ(P) was calculated from the growth curve using the formula: **$Y=Y_0*exp(k*X)$,** and was found to be 2.77 hrs, 2.85 hrs and 2.90 hrs respectively. Thus, inoculant was taken between 10-14 hrs of incubation for other probiotic property tests. Change in pH was also observed from pH 6.2 to lower pH 4.2 which is in accordance with the results of **Ghosh *et al.* (2014)** and **Somashekaraiah *et al.* (2021**), indicating the production and accumulation of organic acids.

Assessment of probiotic properties

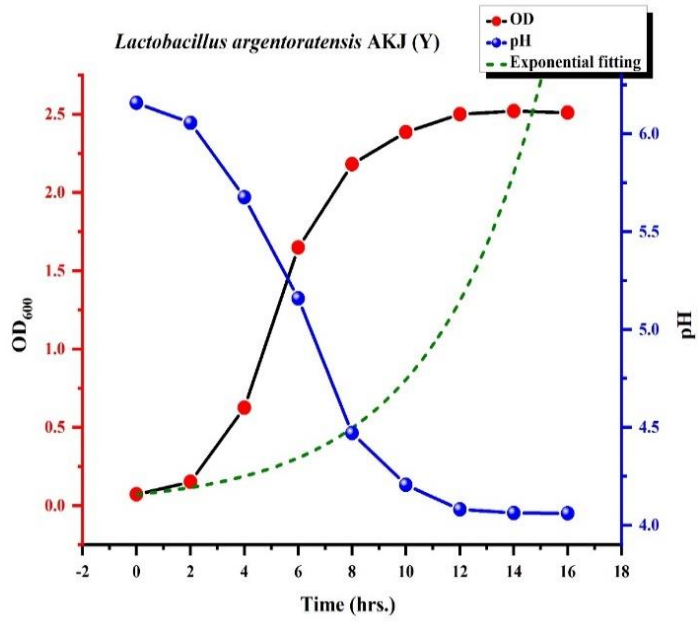
Tolerance to acid and bile salts and taxonomic position of the isolates

Probiotics must pass through human gastric transit and survive against harsh acidic and bile salt conditions in the intestine to remain alive, colonize and exert their probiotic properties (**Nithya and Halami, 2013**). Test for acid and bile tolerance was to check the survival of LAB isolates in simulated acid and bile conditions of the intestine. The pH of the stomach generally ranges from 2.5 to 3.5 and can go as low as pH 1.5, which may alter immediately after having food or prolonged fasting and depending upon the kind of food, the usual time for food passage in the stomach is between 2 to 4 hrs (**Huang and Adams, 2004**). The intestinal tract is basic in nature due to the secretion of bile salts, whose concentration is around 0.3% (w/v) (**Yang *et al.*, 2020**). *Levilactobacillus brevis* AKJ(P) demonstrated the highest percentage (73%) of acid tolerance, whereas *Lactiplantibacillus argentoratensis* AKJ(W) showed the highest (79%) tolerance to (0.5 %) bile salt after 4 hrs. The results show that all LAB isolates are more than 50% tolerant to pH 2, and 0.5% (w/v) bile salt for 4 hours, indicating that the isolates can survive in the human intestinal system extremely effectively (Table 3; Figure 5,6). Survivability of the isolates was significantly dependent on pH and time of exposure (with $p < 0.05$), as shown by previous workers (**Nithya and Halami, 2013; Kumar and Kumar, 2015**).
Tolerance of LAB isolates towards low pH are associated with pH profile of H^+-ATPase, also the composition of cytoplasmic membrane (**Pieniz *et al.*, 2014**). But pH resistance majorly depends upon the bacterial isolate, media composition and other growth factors. Whereas tolerance of isolates towards high bile salt concentration is reported by earlier due to the presence of bile salt hydrolase (BSH) enzyme, which reduces the toxic effect of conjugating bile (**Toit *et al.*, 1998**).

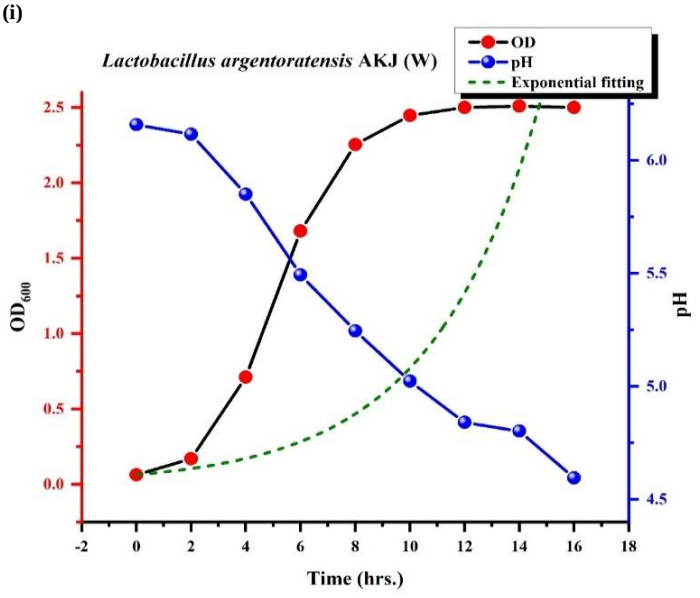
Table 2 Exponential growth parameters of the isolates

Isolates	**Exponential growth equation: $Y=Y_0*exp(k*X)$**			
	Y_0	**k**	**Tau**	**Doubling time (dT) in hrs.**
***Lactobacillus argentoratensis* AKJ (W)**	0.063	0.250	4.0	2.77
***Lactobacillus argentoratensis* AKJ (Y)**	0.071	0.243	4.12	2.85
***Levilactobacillus brevis* AKJ (P)**	0.073	0.239	4.18	2.90

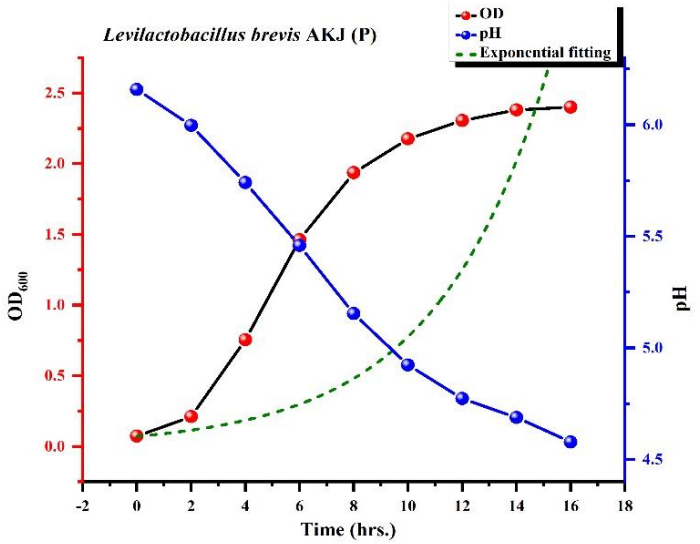
Legend: Y_0 is Y when time (X) is zero; k (rate constant) = 1/X (expressed in hrs.$^{-1}$); Tau (time constant) = 1/k (expressed in hrs.); Doubling time (dT) = ln (2)/k (expressed in hrs.)



(i)



(ii)



(iii)

Figure 4 Growth pattern (red circle), pH change profile (blue circle), exponential fitting (green dashes) for (i) *Lactiplantibacillus argentoratensis* AKJ (W), (ii) *Lactiplantibacillus argentoratensis* AKJ (Y) and (iii) *Levilactobacillus brevis* AKJ (P)

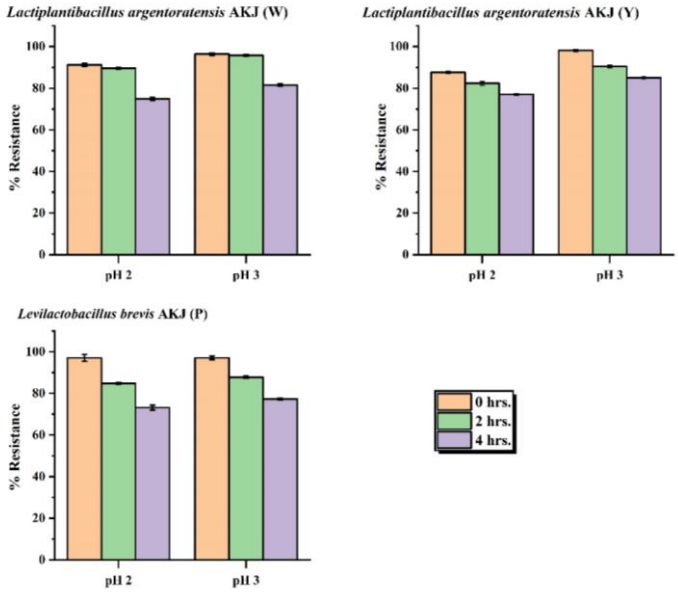


Figure 5 Acid tolerance of LAB isolates: *Lactiplantibacillus argentoratensis* AKJ (W), *Lactiplantibacillus argentoratensis* AKJ (Y) and *Levilactobacillus brevis* AKJ (P). Error bar signifies the standard deviation of mean for results obtained from triplicate readings with $p < 0.05$.

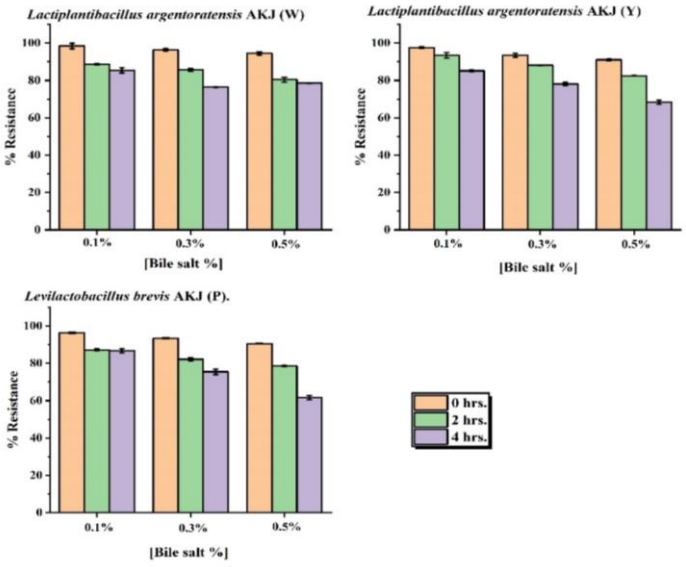


Figure 6 Bile tolerance of LAB isolates: *Lactiplantibacillus argentoratensis* AKJ (W), *Lactiplantibacillus argentoratensis* AKJ (Y) and *Levilactobacillus brevis* AKJ (P). Error bar signifies the standard deviation of mean for results obtained from triplicate readings with $p < 0.05$.

Table 3 Tolerance of isolates to acid and bile salt and their taxonomic placement

Taxonomic placement	Isolates	Accession number[a] (rDNA type representative isolates)	Highest hit on the similarity basis[b]	Percentage similarity (%)	Acid tolerance (lowest pH 2)	Bile tolerance (highest 0.5% oxgall concentration)
Bacteria, Firmicutes, Lactobacillus	AKJ(W)	OP722759	*Lactiplantibacillus argentoratensis*	97.49	+++	+++
	AKJ(Y)	OP722760	*Lactiplantibacillus argentoratensis*	98.88	++	+
	AKJ(P)	OP735575	*Levilactobacillus brevis*	99.86	+	++

Legend: [a] GenBank NCBI; [b]Species published validly in EzTaxon; +++ = maximum tolerance (more than 70%); ++ = medium tolerance (60-70%) and + = low tolerance (50-60%)

Free radical scavenging property/ DPPH scavenging activity/ antioxidant property

Free radical scavenging activity was significantly high in all three isolates (Figure 7). Antioxidant property ranged from a minimum 75.37 % [*L. argentoratensis* AKJ(Y)], medium [*L. argentoratensis* AKJ(W)] to a maximum 95.27% [*L. brevis* AKJ (P)]. Results indicate the isolates have suitable antioxidant properties and can scavenge free radicals.

During metabolism in a biological system, oxidative reactions occur in abundance. Thus, due to endogenous metabolic processes in a human body or exogenous chemicals in food systems, free radicals and reactive oxygen species (ROS) are produced in the human body (**Pieniz *et al.*, 2015**). Thus, accumulation of these ROS (such as hydroxyl radicals, superoxide anion and hydrogen peroxide) and free radicals in the human body causes disturbance in the prooxidant-antioxidant balance in the cell which results in the hydroxylation of DNA, lipid peroxidation, denaturation proteins and apoptosis which will ultimately compromise the cell's viability (**Wang *et al.*, 2017**). Most biological systems possess innate antioxidant defense systems (enzymatic and non-enzymatic), which protect the cells and organs in synergy from damage caused by free radicals (**Kurutas, 2016**). However, the defense system may not be sufficient to deal with all the damage caused by oxidative stress (**Raman *et al.*, 2015**). Studies have shown that probiotics have suitable antioxidant properties mainly due to the presence of exopolysaccharides on the cell wall and the presence of antioxidant enzymes [such as peroxidase, SOD (superoxide dismutase), catalase, glutathione peroxidase) and antioxidant compounds (such as tocopherol, glutathione and ascorbic acid) (**Yang *et al.*, 2020**). Thus, incorporating probiotics with antioxidative properties in food can support the innate defense system and reduce the damage caused by oxidation more efficiently.

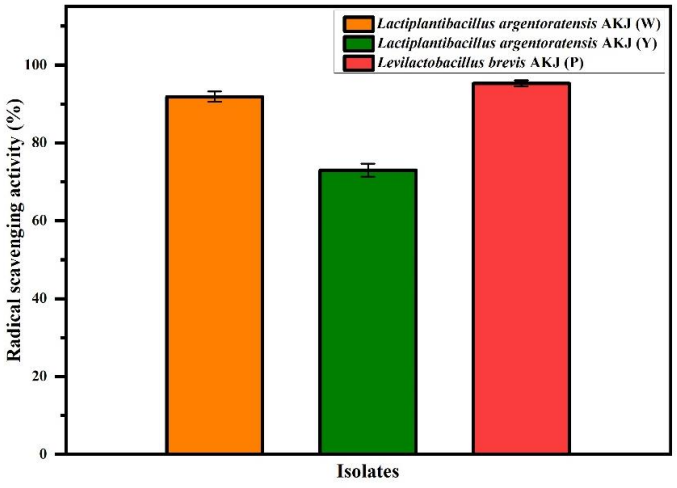


Figure 7 Antioxidant property of the isolates measured in terms of radical scavenging from DPPH. Error bar denotes the standard deviation of mean ($p < 0.05$) obtained from three independent experiments.

Cell surface properties

Assay of cell surface hydrophobicity (CSH)

The isolates *L. brevis* AKJ (P) showed the highest CSH (34.61%), *L. argentoratensis* AKJ(W) showed medium (25.15%) and *L. argentoratensis* AKJ(Y) showed less CSH (3.41%) compared to control *Lactobacillus plantarum* (31.04%) (Figure 8). This indicates that the isolates specifically *L. brevis* AKJ (P) and *L. argentoratensis* AKJ(W), can firmly adhere to the mucus layer and epithelial cells of the intestinal lining. A more hydrophobic cell surface is typically anticipated for probiotic microorganisms so that they are not easily dislodged by polar solvents (like water) and colonize to display their probiotic properties in the gastrointestinal tract (**Krausova *et al.*, 2019**). The hydrophobicity of cell surface depends upon the surface components and the differences in the CSH among the isolates from the same sample might be due to factors such as types of appendages (hydrophilic or hydrophobic), such as hydrophobic amino acids, polysaccharides, differences in expression level of cell surface proteins (such as S- layer proteins), lipoteichoic acid, mucus binding proteins and other constituents (**Guan *et al.*, 2020; Alp and Kuleasan, 2019**). While the CSH experiment is carried out in-vitro utilizing hydrocarbons (n-Hexadecane in our case), it presents us with a qualitative understanding of the hydrophobicity of the cell surface, indicating the extent to which the probiotic isolates can adhere to the intestinal walls. (**Pieniz *et al.*, 2014**).

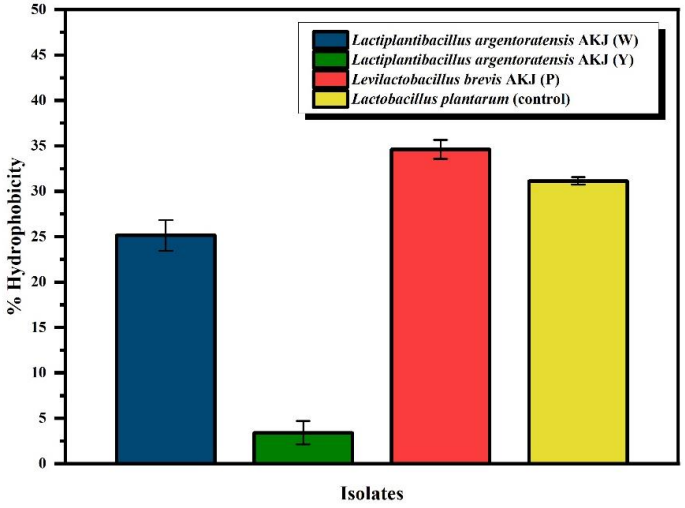


Figure 8 Cell surface hydrophobicity profile of characterized LAB with respect to adhesion with hydrocarbon, n -Hexadecane.

Autoaggregation

The ability of probiotics to auto-aggregate is regarded as an essential attribute for an optimal probiotic candidate. It helps the bacteria to adhere and colonize the epithelial cells of the intestinal walls, subsequently preventing the colonization of pathogens. This characteristic makes the probiotic microorganisms extremely capable to withstand the peristaltic action of the intestine and wash down by polar solvents like water which is advantageous for their survival in the ecosystem and the manifestation of their probiotic effects. (**Gautam and Sharma, 2015**). In this study, *Lactiplantibacillus argentoratensis* AKJ(Y) showed the highest autoaggregation (19.12 %) and the increase in percentage was highly significant after 3 hrs of incubation. The increase in autoaggregation was significantly different ($p < 0.05$) in all isolates (Figure 9). *Levilactobacillus brevis* AKJ (P) showed less autoaggregation (4.98 %) and the increase in percentage was less significant. Whereas *Lactiplantibacillus argentoratensis* AKJ(W) showed the least autoaggregation (4.59 %) and the percentage increase was less significant. Results indicated that the autoaggregative property of LAB isolates is time-dependent and is directly proportional to time, which can be related to the previous works (**Divisekera *et al.*, 2019; Kowsalya *et al.*, 2022**)

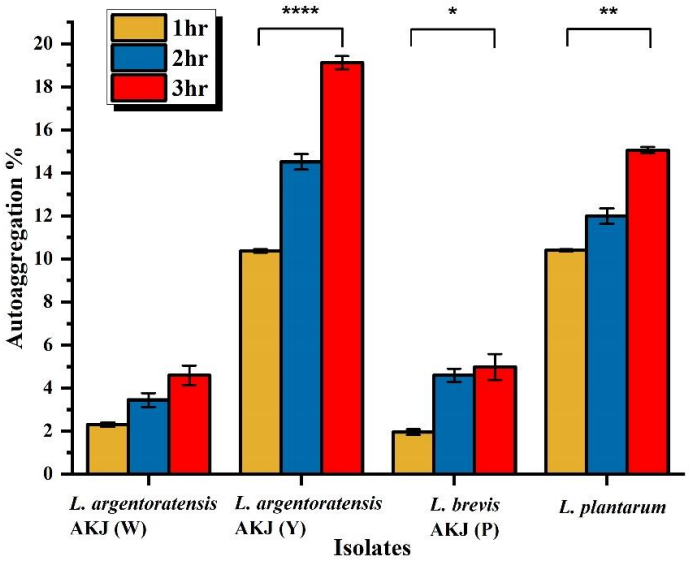


Figure 9 Autoaggregation property of the isolates compared to the reference organism (*L. plantarum*). The error bar denotes the standard deviation of mean. Bar with asterisk represents the degree of significant increase in autoaggregation with $p < 0.05$.

Adhesion assay onto HEK 293 cell line

Lactiplantibacillus argentoratensis AKJ(Y) showed the highest adhesion amongst all three isolates (41%). *Levilactobacillus brevis* AKJ(P) showed 39% and *Lactiplantibacillus argentoratensis* AKJ(W) showed 22% adhesion onto HEK 293 cells.

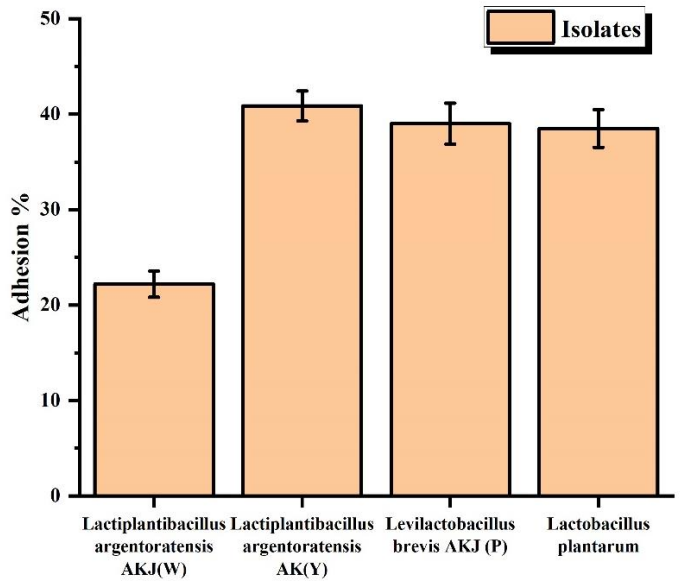


Figure 10 Adhesion assay onto HEK 293 cell line

Phenol tolerance

After 24 hrs. of incubation, colony count was performed to assess the viable cells. *Lactiplantibacillus argentoratensis* AKJ(W) showed the most significant increase in growth, 29% at 0.4% and 22%, at 0.6% phenol concentration after 24 hrs. with $p < 0.05$. *Lactiplantibacillus argentoratensis* AKJ(Y) showed 17% and 20% and *Levilactobacillus brevis* AKJ (P) showed 16% and 19% increase in growth against 0.4% and 0.6 % phenol concentration, respectively (Figure 11). *Lactiplantibacillus argentoratensis* AKJ(W) was the most tolerant isolate towards different concentrations of phenol. All three isolates showed varying degrees of sensitivity towards increasing phenol concentrations. But, their survival at 0.6% phenol indicates they are tolerant to high phenol concentrations and can survive in a gut environment. Tolerance to phenol is an essential characteristic for a probiotic to survive in gastrointestinal conditions since gut microbiota is capable of deaminating aromatic amino acids of food into phenols and phenolic compounds, which might be toxic for probiotic microorganisms (**Yadav *et al.*, 2016; Divisekera *et al*., 2019; Somashekaraiah *et al*., 2019**)

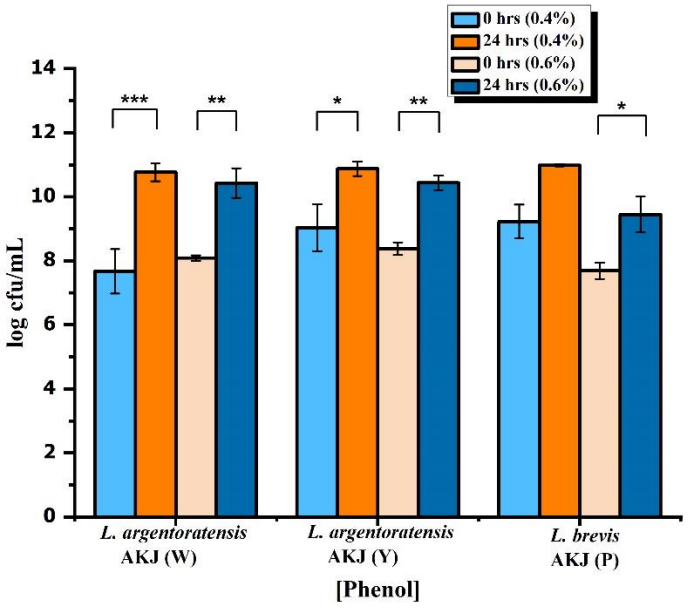


Figure 11 Tolerance of isolates to different phenol concentrations (0.4 % and 0.6 %). The error bar denotes the standard deviation of mean. The bar with an asterisk represents the degree of significant increase ($p < 0.05$) in the bacterial population before (0 hr) and after (24 hrs) treatment with phenol concentrations.

Antimicrobial properties/Antimicrobial activity test for screening of isolates against pathogens

Agar spot assay

Lactiplantibacillus argentoratensis AKJ(Y) showed antimicrobial activity against all six pathogens and highest against *B. subtilis* MTCC 441. *Levilactobacillus brevis* AKJ (P) showed antagonistic activity against three pathogens (*E. coli* MTCC 730, *B. subtilis* MTCC 441 and *Candida albicans* MTCC 183) and highest in the case of *B. subtilis* MTCC 441. *Lactiplantibacillus argentoratensis* AKJ(Y) showed less antimicrobial activity against pathogens as compared to *Lactiplantibacillus argentoratensis* AKJ(Y) and *Levilactobacillus brevis* AKJ (P) (Figure 12). The antimicrobial effect shown by the isolates can be a barrier to the growth and propagation of pathogens. The antagonistic activity against pathogens might be because of bioactive compounds such as bacteriocin produced by the bacteria during the log phase of growth (**Iseppi *et al.*, 2021)**

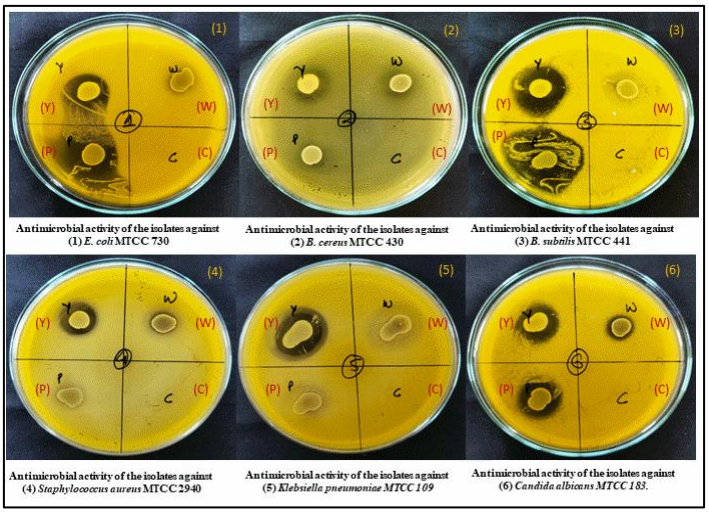


Figure 12 Antagonistic activity shown by *Lactiplantibacillus argentoratensis* AKJ(W) represented as (W), *Lactiplantibacillus argentoratensis* AKJ(Y) as (Y) and *Levilactobacillus brevis* AKJ (P) as (P) against six pathogens (1) *E. coli* MTCC 730 (2) *B. cereus* MTCC 430 (3) *B. subtilis* MTCC 441, (4) *Staphylococcus aureus* MTCC 2940, (5) *Klebsiella pneumoniae* MTCC 109 (6) *Candida albicans* MTCC 183.

Effect of bacteriocin-like proteins against pathogens

The results suggest that the antimicrobial activity shown by the crude extract of CFS was found to be effective against *E. coli* MTCC 730 and *Staphylococcus aureus* MTCC 2940 at varying degrees (Figure 13). However, the effect was pH dependent and showed high antimicrobial activity at lower pH. In the case of E.

coli, the antimicrobial activity of isolates was observed at pH 2, 3 and 4. Maximum activity was shown by (W) at pH 2 and 3 and (Y) at pH 2. In the case of S. aureus, CFS of isolates showed activity at pH 2 and 3 and maximum activity was demonstrated by (W) at pH 2. Lactate was taken as a control at 100% v/v concentration. The pH of unadjusted CFS was between 4.1 to 4.6. Results showed that antimicrobial activity was observed till pH 4 for all the isolates, but no activity was observed after neutralization to pH 5 and 6, which can be relatable with earlier works of Mariam *et al.*, 2014. This might be due to the combined synergistic effect of the bioactive peptide and low pH (**Mariam *et al.*, 2014**).

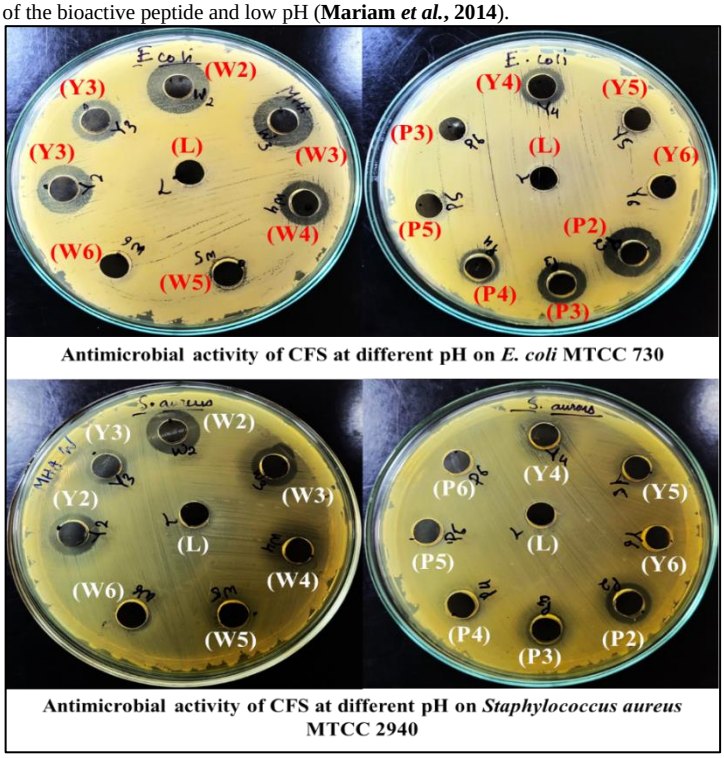


Figure 13 Antimicrobial activity of CFS adjusted to different pH. [W2, W3, W4, W5, W6 denoted for CFS of *Lactiplantibacillus argentoratensis* AKJ(W) at pH 2,3,4,5,6 respectively.; Y2, Y3, Y4, Y5, Y6 denoted for CFS of *Lactiplantibacillus argentoratensis* AKJ(Y) at pH 2,3,4,5,6 respectively; P2, P3, P4, P5, P6 denoted for CFS of *Levilactobacillus brevis* AKJ (P) at pH 2,3,4,5,6 respectively].

In vitro food safety assessment of the isolates

DNase activity

DNase test was conducted to determine if the strain produces a DNase enzyme, which could damage host DNA (**Somashekarain *et al.*, 2019**). Since, no defined zone was seen surrounding the smear (Figure 14), all test isolates demonstrated the absence of DNase activities, indicating that the isolates are suitable for consumption and use as probiotics.

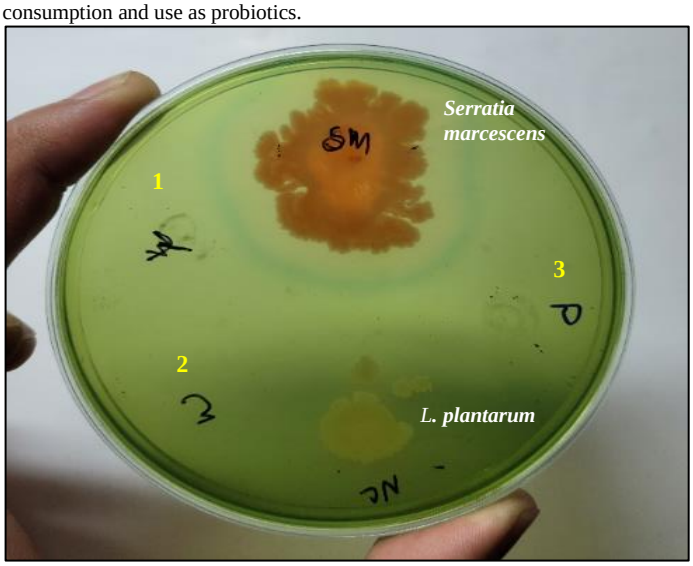


Figure 14 DNase test. Positive control as SM (*Serratia marcescens*), (1) *Lactiplantibacillus argentoratensis* AKJ(W), (2) *Lactiplantibacillus argentoratensis* AKJ(Y), (3) *Levilactobacillus brevis* AKJ (P), negative control (*L. plantarum*).

Haemolytic activity

Haemolysis activity is a safety assessment test to observe whether the isolate performs haemolysis (i.e., blood clotting)**.** Absence of haemolytic activity is one of the safety criteria used to choose probiotic microorganisms. (**FAO/WHO, 2002; Kowsalya, *et al.*, 2022; Lata *et al.*, 2023**). In the present study, the isolates did not show any haemolytic activity (i.e., gamma haemolysis), which matched with the negative control (i.e., *L. plantarum*) (Figure 15**)**. Earlier studies have also mentioned that LAB isolated from fermented foods showed no haemolysis activity (**Santini *et al.*, 2010**). The absence of haemolysis activity conferred the isolates to be safe.

Figure 15 γ hemolysis exhibited by (a) *Lactiplantibacillus argentoratensis* AKJ(W), (b) *Lactiplantibacillus argentoratensis* AKJ(Y), (c) *Levilactobacillus brevis* AKJ (P) and (e) *L. plantarum* (negative control); α haemolysis shown by (d) *S. marscences* (positive control)

Antibiotic susceptibility/ Antibiotic resistance

Antibiotic susceptibility is an essential safety requirement for potential probiotic bacteria since they can occasionally become carriers of antibiotic-resistance genes that can be passed on to pathogens (**Murugan and Mishra, 2022**). According to studies, *Lactobacillus sp.*, show differences in antibiotic susceptibility depending on strain, species type, antibiotics, and other nutritional factors (**Charteris *et al.*, 2001; Klayraung *et al.*, 2008**). In our present study, all three isolates showed varying results for antibiotic susceptibility (Figure 16, and Table 4). Antibiotic susceptibility test revealed that *Lactiplantibacillus argentoratensis* AKJ(W) is resistant to 7 and susceptible to 13 antibiotics. In contrast, *Lactiplantibacillus argentoratensis* AKJ (Y) is resistant to 6 and sensitive to 14 antibiotics. *Levilactobacillus brevis* AKJ (P) showed resistance to 4 and was susceptible to 16 antibiotics. Based on the number of antibiotic resistances of each isolate, MAR index value was determined. The highest value for MAR index was shown by *Lactiplantibacillus argentoratensis* AKJ (W), which was 0.35. *Lactiplantibacillus argentoratensis* AKJ (Y) and *Levilactobacillus brevis* AKJ (P) showed MAR index values of 0.3 and 0.2, respectively. This indicates that the isolates *Lactiplantibacillus argentoratensis* AKJ(W) and *Lactiplantibacillus argentoratensis* AKJ (Y) are resistant to multiple antibiotics. There are two aspects of antibiotic resistance in case of probiotic bacteria. Antimicrobial resistance may be either natural or acquired (**Delgado *et al.*, 2007**). If the resistance is encoded in the chromosome, there is less chance of transmission to pathogenic bacteria. But if the resistance is acquired or is encoded in the plasmid, then there is more possibility for transfer of antibiotic resistance to pathogens through horizontal gene transfer in the gut. However, a probiotic should not contain acquired and transferable genes for antibiotic resistance (**Siangpro *et al.*, 2023**). In our study, all three isolates showed resistance to certain antibiotics.

The resistance of isolates towards β-lactam antibiotics Ampicillin, Methicillin, Cefotaxime, Ceftazidime and Penicillin G in case of *Lactiplantibacillus argentoratensis* AKJ(W), Ceftazidime and Penicillin G in case of *Lactiplantibacillus argentoratensis* AKJ (Y) and *Levilactobacillus brevis* AKJ (P) might be due to the presence of *bla* gene (**Stefanska *et al.*, 2021**). Resistance to Nalidixic acid (quinolones) shown by *Lactiplantibacillus argentoratensis* AKJ (Y) can be explained as intrinsic resistance due to the enzymatic inactivation of the antibiotic rendering it useless to bind to the target site, which is reported earlier in *Lactobacillus* for 16srRNA of 30S ribosomal subunit (**Gotcheva et al, 2002; Delgado *et al.*, 2007; Murugan and Mishra, 2022**). Resistance to polymyxin B and colistin is basically because they are known to have little to no activity against gram-positive bacteria (**Vidal and Thompson, 1987**). Thus, resistance of isolates Fluconazole, Miconazole can be further confirmed by screening the resistance genes for these antibiotics.

Nevertheless, the positive aspect of antibiotic resistant probiotics is that they can be co-administered with antibiotics (**Siangpro *et al.*, 2023**) and in fact, might have synergistic activity against pathogens reported earlier (**Biswas, *et al.*, 2017**). In addition, resistance and susceptibility are not the only factors determining the risk of a probiotic strain. The ability of isolate to transfer resistance gene is the real deciding factor and need to be tested as for antibiotic resistance genes before commercial application (**Divisekera *et al.*, 2019; Somashekaraiah *et al.*, 2019**). Apart from that, risk factor is less for LAB as they are found naturally in fermented foods which have been consumed by people since ages and have been considered as GRAS.

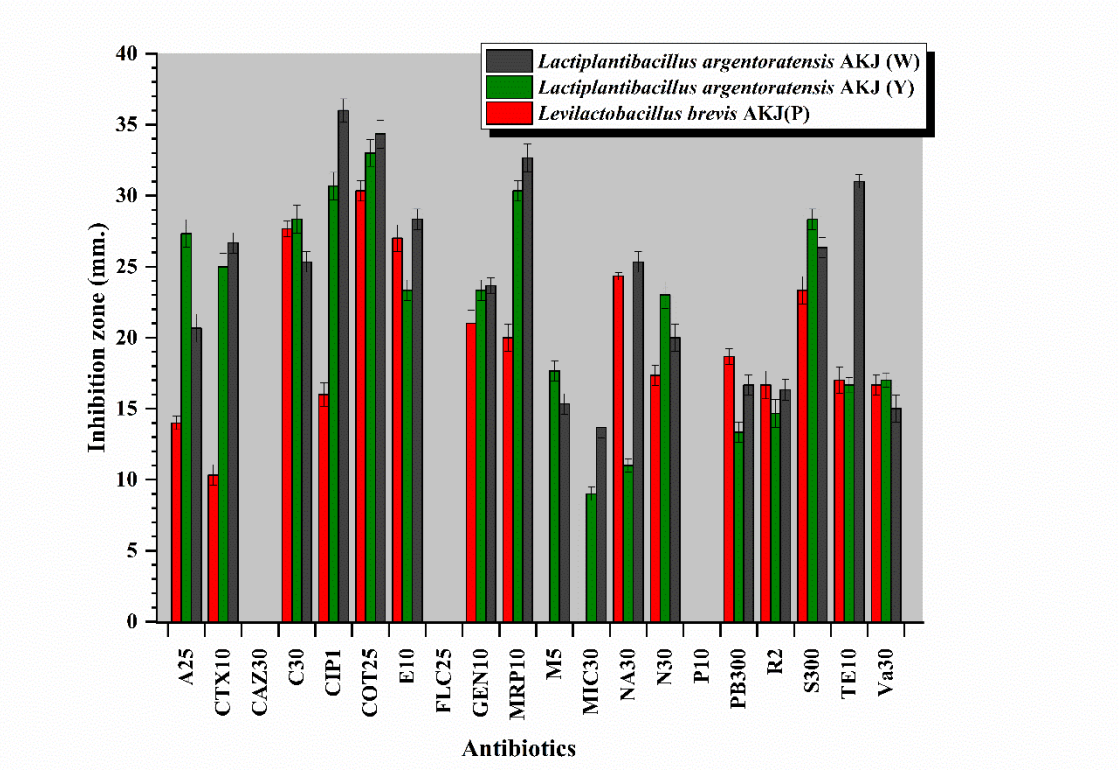


Figure 16 Antibiotic susceptibility test of *Lactiplantibacillus argentoratensis* AKJ(W), *Lactiplantibacillus argentoratensis* AKJ(Y) and *Levilactobacillus brevis* AKJ (P) against 20 known antibiotics. Error bars represent the standard deviation of mean values ($p < 0.05$).

Table 4 Susceptibility in relation to inhibition zone diameter (mm)

Antibiotics	**Isolates (S- Susceptible; I- Intermediate; R- Resistant)**			
Name	Disc symbol with concentration (in µg)	*Lactiplantibacillus argentoratensis* AKJ (W)	*Lactiplantibacillus argentoratensis* AKJ (Y)	*Levilactobacillus brevis* AKJ (P)
Ampicillin	A25	R	S	S
Cefotaxime	CTX10	R	S	S
Ceftazidime	CAZ30	R	R	R
Chloramphenicol	C30	S	S	S
Ciprofloxacin	CIP1	I	S	S
Co-trimoxazole	COT25	S	S	S
Erythromycin	E10	S	S	S
Fluconazole	FLC25	R	R	R
Gentamycin	GEN10	S	S	S
Meropenem	MRP10	S	S	S
Methicillin	M5	R	I	I
Miconazole	MIC30	R	R	R
Nalidixic acid	NA30	S	R	S
Neomycin	N30	I	S	S
Penicillin G	P10	R	R	R
Polymyxin B	PB300	I	R	I
Rifampicin	R2	I	I	I
Streptomycin	S300	S	S	S
Tetracycline	TE10	I	I	S
Vancomycin	Va30	I	I	I

Legend: Susceptible: ≥20; Intermediate: 15- 19; Resistant: ≤14.

Cumulative probiotic score of isolates

Potential probiotic properties of a bacteria were expressed in terms of score following a method as described by **Gautam and Sharma, 2015**. The following equation is used to calculate probiotic potential,

$$\text{Probiotic potential} = \frac{\text{observed score}}{\text{maximum score}} \times 100$$

Table 5 Cumulative probiotics attributes of the isolates

Probiotic characteristics	Indication	Score		
		Lactiplantibacillus argentoratensis AKJ (W)	***Lactiplantibacillus argentoratensis*** AKJ (Y)	***Levilactobacillus brevis*** AKJ (P)
Acidity tolerance	Resistant =1 Sensitive = 0	1	1	1
Bile salt tolerance	Resistant =1 Sensitive = 0	1	1	1
Phenol tolerance	Resistant =1 Sensitive = 0	1	1	1
Antioxidant property	Positive =1 Negative = 0	1	1	1
Cell surface hydrophobicity test (CSH)	Positive =1 Negative = 0	1	0	1
Autoaggregation of cells	Positive =1 Negative = 0	1	1	1
Adhesion onto HEK 293 cell line	Positive =1 Negative = 0	1	1	1
Antagonistic activity against pathogens	<3 = 0.25 3-5 = 0.50 5-20 = 0.75 20 = 1	0.25	0.50	0.50
DNase test	Positive =1 Negative = 0	1	1	1
Haemolysis test	Positive =1 Negative = 0	1	1	1
Antibiotic sensitivity test	Antibiotic-Resistant =0 Antibiotic Sensitive = 1	1	1	1
Effect of crude extract of bacteriocin-like proteins against pathogens	Positive =1 Negative = 0	1	1	1
Total points	**12**	**11.25**	**11.50**	**11.50**
Total score		**11.25/12**	**11.50/12**	**11.50/12**

From the results of cumulative probiotic potential (Table 5), the probiotic potential for *Lactiplantibacillus argentoratensis* AKJ (W) was 93.75 %, for *Lactiplantibacillus argentoratensis* AKJ (Y) and *Levilactobacillus brevis* AKJ (P) it was 95.83 %. Commercially available probiotics have a probiotic score within 75- 85 % range. Our study results show that the LAB isolates follow the criteria of **FAO/WHO (2002)** (**Gautam and Sharma, 2015**). Thus, these isolates can be a potential candidate to be used as probiotics in food and pharmaceuticals.

CONCLUSION

Food products sold as functional foods claim to include a plethora of microorganisms that enhance the characteristics of hosts' natural microflora and have positive effects on the host **(Succi *et al.*, 2005)**. This has increased interest among consumers and researchers toward functional foods and nutraceuticals for their inclusion into regular diets. *Aduwa ko jaanr* is found to have probiotic properties due to the predominance of LAB and is safe for consumption as they meet the parameters of GRAS organisms. Recently, fermented foods have grabbed the attention of people due to various benefits, including improved consumer health, changed texture, unique aroma and enhanced nutritional value of the product. The results of probiotic properties such as high tolerance to low pH (pH 2), bile salt concentration (0.5%), phenol concentration (0.6%), good cell attachment property (i.e. cell surface hydrophobicity, autoaggregation, adhesion onto HEK 293 cell line), good radical scavenging property, antimicrobial activity against both Gram-positive (*S. aureus*, *B. subtilis* and *B. cereus*), Gram-negative (*E. coli*, *K. pneumoniae*), and fungal (*Candida albicans*) pathogens and tolerance to different conventional (antibacterial and antifungal) antibiotics leads to conclude from the present study that the fermented beverage, *Aduwa ko jaanr* is a sustainable beverage with health benefitting properties. Its consumption being safe with the process of manufacturing as economic and traditional, as well as the end product being recycled are indicators of this being a sustainable beverage. For food safety concerns, DNase, hemolytic, and antibiotic susceptibility tests revealed all three potential isolates, giving negative results, making the beverage safe to consume. The properties shown by isolates from *Aduwa Ko Jaanr* are the potential for developing functional foods with novel health benefits. They can be utilized as dietary supplements to create non-dairy drinks with probiotic properties. However, it is crucial to demonstrate this advantageous effect in in-vivo research, which is the next phase of the present work.

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