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RESEARCH ARTICLE

Probiotic Properties of *Lactobacillus plantarum*

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Abstract

Recently, probiotic lactic acid bacteria have been utilized as therapeutic supplements and food additives. Nowadays, the interest has been increased regarding the commercial utilization of probiotic LAB strains isolated from traditional fermented food products. Therefore, the present study was aimed to investigate the probiotic properties of lactic acid bacteria isolated from traditional food sources viz. dosa batter and sauerkraut. Total 7 lactic acid bacteria were obtained, 4 from dosa batter and 3 from sauerkraut, out of which one isolate from each food source were selected based on their broadest antagonistic spectrum. These strains were identified using 16S r RNA technique as *Lactobacillus plantarum* DB-2 (isolated from dosa batter) and *Lactobacillus plantarum* SK-3 (isolated from sauerkraut). The investigation of acid-bile tolerance, antibiotic sensitivity, auto-aggregation, co-aggregation, bacterial adhesion to hydrocarbons were confirmed. The results revealed normal growth of *L. plantarum* DB-2 and *L. plantarum* SK-3 in the presence of low pH, high bile salt concentration and ability to produce antimicrobial compounds viz. bacteriocin and H_2O_2. No gelatinase, lipase and hemolytic activity were observed. Natural susceptibility to the tested antibiotics was investigated. Thus, according to these results, *L. plantarum* DB-2 and SK-3 proved the good probiotic candidates as they survived during stress conditions posing to them and can be exploited for the preparation of nutraceutical products. This study also revealed the potential of using LAB and /or bacteriocin produced by them as food bio preservative to control food borne pathogenic bacteria in near future.

Keywords: *Lactobacillus plantarum*, probiotics, acid and bile tolerance strains, bacteriocin, dosa batter, sauerkraut, auto-aggregation, antibacterial activity.

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INTRODUCTION

Probiotics are commonly referred as health-promoting bacteria and have been shown to improve the intestinal microbial balance[1]. Probiotics are defined as 'Live microorganisms which have health benefits on host when taken in sufficient amount'[2]. Probiotic microorganisms are viable, non-pathogenic microorganisms which have the ability to restrict the growth of potential pathogenic microbes in the GIT, when reach in sufficient numbers and increase the multiplication of beneficial microbes and therefore, delivers the health benefit to the host[3]. LAB is designated as GRAS (generally regarded as safe) micro-organisms. Different geographical location across the world has diverse fermented foods which provide various lactic acid bacteria having potential probiotic properties[4]. The most commonly used probiotics belonging to the species of the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus,* etc. and the genus *Bifidobacterium*[5]. Nowadays, lactic acid bacteria are used in probiotic preparations because it inhibit the growth of harmful bacteria, enhance good digestion, modify the balance of intestinal microflora, improve immune function and improve resistance to infection[6].

A potential probiotic strain must fulfill certain selection criteria such as the ability to overcome gastric pH, the toxic effects of bile salts, phenol stability, antimicrobial production, antibiotic-resistance, and antagonistic activity and co-aggregation ability to decrease the ill-effects of pathogens. An appropriate alternative to antibiotic treatment is the use of probiotics with broad antagonistic potential. A good probiotic strain must possess the property of auto-aggregation before providing any health benefits. This property helps LAB to adhere to the intestinal epithelium and produce antimicrobial substances such as organic acid, hydrogen peroxide, and bacteriocins. Presently, traditional food products have been accepted for commercial purposes due to the probiotic significance. The probiotic strains survive the passage through the gastrointestinal tract and thus adaptable to intestinal conditions. This criterion helps the potential probiotic strain isolated from traditional food products to be used for industrial purposes other than those of animal origin[7].

The main objectives of this study were to screen the probiotic potential of *Lactobacilli* spp. isolated from traditional fermented dosa batter and sauerkraut for its safety by antibiotic susceptibility test and assessment of probiotic qualities such as acid-bile tolerance, auto-aggregation, hydrophobicity, co-aggregation, and antagonistic potential. Tolerance studies were carried out to signify the importance of survivability of the strains in the stomach and intestine. Bacteriocin production was also investigated against several pathogenic bacteria including *Escherichia coli IGMC, Staphylococcus aureus IGMC, Bacillus cereus CRI, P. aeruginosa IGMC, and S. pyogenes ATCC14289*.

MATERIALS AND METHODS

Collection of food samples

Both food items were prepared at home. The food samples were taken in a sterilized bag and stored at -4°C until use.

Strain isolation

1 g of food sample was added into 9 ml of normal saline. After homogenization, serial dilutions were prepared up to 10^{-9} with 0.85% (w/v) normal saline and 0.1 ml decimal of appropriate dilutions were plated onto de Man, Rogosa, Sharpe (MRS) agar medium (HiMedia)[8]. The agar plates were incubated at 35°C for 24 h under anaerobiosis. Morphologically different colonies were picked and re-streaked onto MRS agar plates up to purity. Pure strains (observed microscopically for homogeneity of cellular morphology) were preserved in glycerol stocks at -20°C.

Identification of strain

Phenotypic characterization

The morphological and cultural characteristics including gram staining[9] and colonial appearance were examined.

Biochemical characterization

Indole test, MR-VP test, citrate utilization test, sugar fermentation test, gelatin hydrolysis test, lipase activity, hemolytic activity, BSH activity were employed to identify the isolated lactic acid bacteria[10].

Genotypic characterization

Isolation of genomic DNA was done by following the method of[11]. Genomic DNA of the isolates was subjected to PCR for amplification

of small 16S r RNA genes using universal primers 27F and 1492R having expected product size of 1500 bp. After amplification, PCR products were visualized using ethidium bromide (Thermo Fisher Scientific) on 1.5% agarose gel (Sigma-Aldrich)[12]. These have got sequenced by Bioserve Biotechnologies (India) Pvt. Ltd. to identify the isolates. BLAST software from the Genbank was used for sequence alignment. Program CLUSTAL_X was used for the multiple sequence alignment. MEGA-6.0 was used for a construction of phylogenetic tree by neighbour joining method.

Safety assessment of LAB

Safety is an important criteria for bacterial strains intended to use in the food industry.

Antibiotic sensitivity test

Study of antibiotic resistance pattern is important for selection and evaluation of safe probiotic strain. The antibiotic susceptibility of two *L. plantarum* strains was examined by disc diffusion technique[13]. The 24 h old culture was swabbed on MRS agar plates. Antibiotic impregnated discs (HiMedia) were placed onto these inoculated plates. These plates were incubated at 37°C for 24 h. Zone of inhibition was observed after 24 h[14]. Resistance was assessed against Ampicillin (10μg), Amoxicillin (10μg), co-Trimoxazole (30μg), Cefotaxime (30μg), Cefuroxime (30μg), Gentamycin (10μg) and Tetracycline (30μg).

Hemolytic activity

Hemolytic activity of both the strains were determined by spot inoculating overnight bacterial cultures on Blood Agar plates (HiMedia) followed by incubation of 24 h at 35°C[15].

Gelatinase production

Gelatinase production was determined by streaking both the isolates on the MRS agar plates supplemented with 3% gelatin and the plates were incubated at 35°C for 24 h[16].

Lipase production

Lipase enzyme production was evaluated by streaking the 24 h old culture of both the isolates on the MRS agar plates supplemented with 1% Tween 80 as a source of fatty acids. Plates were incubated at 35°C for 24 h[17]. The lipolytic activity was detected by the appearance of an opaque zone around the colonies[18].

Assessment of probiotic attributes

Tolerance to low acid conditions

Selected isolates were grown in MRS broth at 37°C overnight. An equal amount of aliquot was taken and adjusted to pH 1.0, 2.0, 3.0, 4.0 and 5.0 with 5N HCl followed by incubation at 37°C for 3 h. Control was run alongside. 0.1 ml aliquot was taken every hour and enumerated by pour plate technique using 10-fold dilution using 0.1% peptone water. Simultaneously, the bacterial growth was monitored spectrophotometrically at OD_{600} at 0, 1, 2 and 3 h[19].

$$\text{Survivability \%} = \frac{\log \text{CFU } 1,2,3,4,5}{\log \text{CFU } 6.5} \text{ X } 100$$

Effect of bile salts on the growth rate of isolates

Selected isolates were grown in MRS broth at 37°C overnight. 0.3%, 1% and 2% (w/v) of bile salt (oxoid) was prepared and added to the 24 h old active culture of selected isolates and incubated at 37°C for 8 h. Control was run alongside. 0.1 ml aliquot was taken every hour and enumerated by pour plate technique using 10-fold dilution using 0.1% peptone water. Simultaneously the bacterial growth was monitored spectrophotometrically at OD_{600} at 0, 4 and 8 h[20].

$$\text{Survivability \%} = \frac{\log \text{CFU } 0.3,0.5,0.6,0.8,1,2}{\log \text{CFU } 0} \text{ X } 100$$

Survival in simulated in vitro digestion

The pH in the human stomach ranging from 1 (during fasting) to 4.5 (after a meal). Ingestion of food can take up to 4 h. Thus, the tolerance was assayed by determining the viable count in simulated gastric juice at different time intervals viz. 0,1 and 4 h[21].

Aggregation property

Autoaggregation

Selected isolates were grown in MRS broth at 37°C overnight. After incubation, the broth was centrifuged at 10,000 rpm at 4°C for 10 min. The pellet obtained was washed twice with PBS buffer solution and re-suspend in the same solution, followed by incubation at 37°C for 5 h. An equal amount of aliquot was taken and absorbance was measured at OD_{600} at 0, 1, 2, 3, 4 and 5 h[22].

$$\text{Autoaggregation \%} = 1 - (A_t / A_0) \times 100$$

Where A_t =Absorbance after incubation at 1, 2, 3, 4 and 5 h, A_0 = Absorbance at 0 h

Co-aggregation

Mixtures were made for both the isolates

with pathogenic bacteria viz. *Bacillus cereus* CRI, *Bacillus subtilis* MTCC 5981, *Clostridium perfringens* MTCC 1739, *Escherichia coli* IGMC, *Pseudomonas aeruginosa* IGMC, *Salmonella typhimurium* MTCC 3231 and *Staphylococcus aureus* IGMC at 1: 1 ratio. Probiotic bacterial cells and indicator microorganisms were kept as control and incubated at 35°C for 4 h. Absorbance (OD_{600}) was observed for mixture and each of individual strain[22]. Co-aggregation percentage was calculated by Handley's equation[23].

$$\text{Co-aggregation}(\%) = \frac{\left[\left[A_{(LAB)} + A_{(Pathogen)}/2\right]\right] - A_{Mixture}}{\left[A_{(LAB)} + A_{(Pathogen)}/2\right]} X100$$

Where A_{LAB} =Absorbance of lactic acid bacterial suspension

$A_{Pathogen}$ = Absorbance of indicator microorganisms

$A_{Mixture}$ = Absorbance of LAB suspension and indicator organisms

Adhesion property: Hydrophobicity

Selected isolates were grown in MRS broth at 37°C overnight. After incubation, the broth was centrifuged at 10,000 rpm at 4°C for 10 min. The pellet obtained was washed twice with PBS buffer solution and re-suspend in the same solution. 3 ml of cell suspension was added to 1 ml of each hydrocarbon (xylene, toluene, chloroform, n-hexadecane, n-octane and ethyl acetate). Absorbance (OD_{600}) was taken at 0 h and after vortexing both phases for 2 min. Incubation was done for 2 h and absorbance was taken again[24].

$$\text{Hydrophobicity } \% = \{(A_0 - A_t) / A_0\} \times 100$$

Where A_t = Absorbance at time t=2

A_0 = Absorbance at time t=0

Antibacterial activity of bacteriocin producing L.plantarum

0.1 ml of indicator strains (*Escherichia coli IGMC, Staphylococcus aureus IGMC, Bacillus cereus CRI, Pseudomonas aeruginosa IGMC, and Streptococcus pyogenes ATCC14289*) were swabbed onto sterilized nutrient agar plates.

The selected probiotic isolates were grown overnight in TGY medium; a bacteriocin producing medium[25]. The isolates were centrifuged at 12,000 rpm at 4°C for 15 min. The culture supernatant was collected in sterilized test tubes and was neutralized to pH 6.5 with 1N NaOH and catalase was added at the rate of 0.1 mg/ml.

Inhibitory activity of bacteriocin was observed by well diffusion method[13]. The wells in the pre-swabbed nutrient plates were cut with sterile borer and 20µl of neutralized culture supernatant was placed into the wells.

$$\text{AU/ml} = \frac{\text{Diameter of the zones of clearance (mm)/}}{\text{volume taken in well}} \times 1000$$

Where AU = Arbitrary units / activity units of bacteriocin

H_2O_2 production

Both the isolates were screened for Hydrogen Peroxide by Quantitative method[26]. It was done by inoculating the bacterial isolate into MRS broth (25 ml) at 35°C for 24 h. After overnight incubation, 0.1 M sulphuric acid (20 ml) was added to the broth and titrated against 0.1 N $KMnO_4$.

1 ml of $KMnO_4$ = 1.070 mg of H_2O_2

BSH activity

Isolates were cultivated in MRS agar medium supplemented with 0.5% sodium salt of taurocholic acid (HiMedia) and incubated at 35°C for 24 h. The plates were observed for white precipitates[27].

Statistical Analysis

All the experimental results were recorded as mean ± SD (Standard Deviation). For every observation, 3 determinations were used. Analysis of variance (ANOVA) was calculated by using one-way analysis. Duncan's multiple range test was employed for calculating significant differences between mean. Results were statistically significant at $P<0.05$.

RESULT AND DISCUSSION

Isolation and identification of strain

Total 7 lactic acid bacteria were isolated, 4 from dosa batter and 3 from sauerkraut. All 7 isolates were Gram-positive as examined by Gram's staining method under an oil-immersion microscope (Fig. 1). 3 out of four dosa batter isolates were confirmed as rods while 1 was confirmed as coccus and all 3 isolates of sauerkraut were confirmed as rods as revealed by microscopic examination. All 7 isolates were non-sporulating. Out of 7 isolates, SK-3 and DB-2 gave clear halos around the indicator pathogenic organisms with

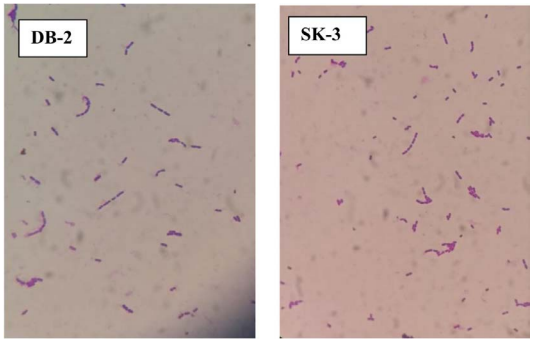

Fig. 1. Micrograph of *L. plantarum* DB-2 and SK-3

widest antimicrobial spectrum and were selected for further study. *P. pentosaceus* VTCC-B-601 showed effective antimicrobial effect when tested against foodborne pathogens *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 19430, *Pseudomonas aeruginosa* ATCC 27853 and *Micrococcus luteus* ATCC 10240[28].

Phenotypic characterization

Both strains appear off-white, mucoid, raised with entire margins (Fig. 2).

Biochemical characterization

All the isolates were catalase negative, not able to utilize citrate, no casein hydrolysis, no urease production, no indole production, no lipase production and no hemolysis zone were observed (Table 1).

Genotypic characterization

Gel electrophoresis is shown in Fig. 3. Analysis of the 16S rRNA sequences revealed that lactic acid bacteria isolated from dosa batter and sauerkraut showed 99% and 100% homology with *Lactobacillus plantarum* NCIMB 700965 respectively. The 16S rRNA gene sequence was submitted to Genbank and assigned accession number MK246167 and MK246169 for isolate SK3 and DB2 respectively. Neighbour-joining phylogenetic tree of *L. plantarum* DB-2 and SK-3 based on 16S rRNA gene sequences is shown in Fig. 4.

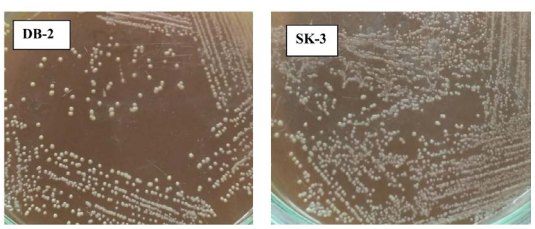

Fig. 2. Phenotypic appearance of *L. plantarum* DB-2 and SK-3

Safety assessment of selected isolates

L. plantarum SK-3 and *L. plantarum* DB-2 exhibited 75% and 62.5% sensitivity towards the antibiotics used in this work. *L. plantarum* SK-3 showed resistance towards Cefotaxime

Table 1. Biochemical characterization of *L.plantarum* DB-2 and SK-3.

L. plantarum	Gram staining	Catalase reaction	Sugar fermentation test	Indole test	MR-VP test	Citrate utilization test	H_2S production	Casein hydrolysis	Lipase production	Gelatin hydrolysis	Hemolytic activity
SK3	Rod +	-	A^+G^-	-	+,-	-	-	-	-	-	-
DB2	Rod +	-	A^+G^-	-	+,-	-	-	-	-	-	-

'+' indicates positive reaction; '-' indicates negative reaction

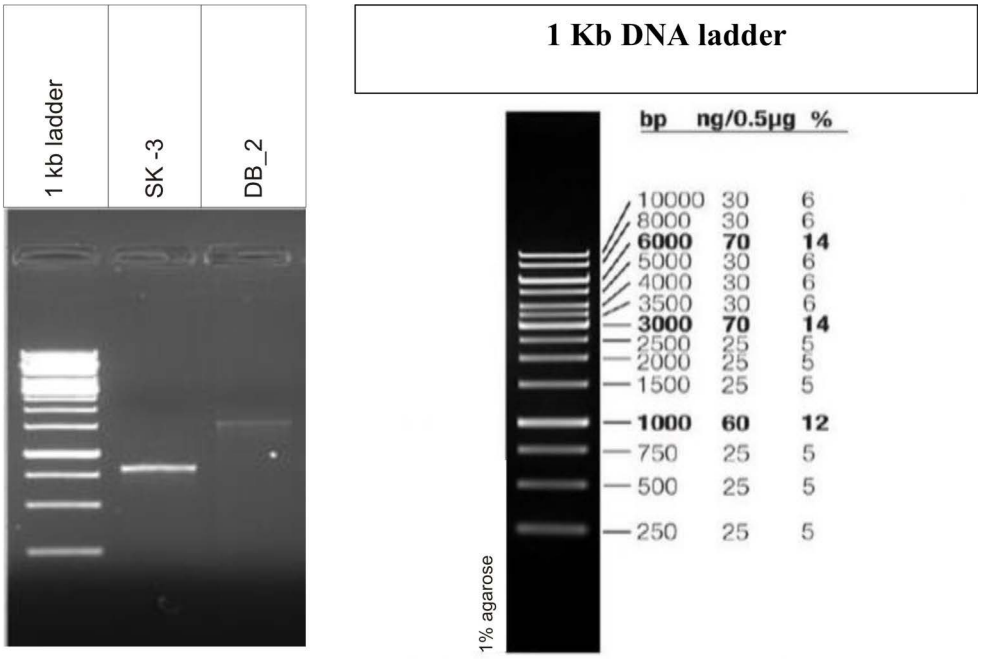

Fig. 3. Molecular identification of *Lactobacillus* strains by 16S rRNA gene

CTX (30µg) and Cefoxitin CX (30µg) whereas *L. plantarum* DB-2 showed resistance towards Cefoxitin CX (30µg), Co-trimoxazole COT (30µg) and Gentamycin GEN (10µg) (Table 2 and Fig. 5). For some preventive and therapeutic purposes in controlling intestinal infections, the resistance of probiotic strains to some antibiotics could be used. Resistance of probiotic strains to antibiotics elucidating their potential in minimizing the negative effects of antibiotic therapy on the host bacterial ecosystem[29]. No hemolytic activity was shown by both the isolates as no clear zones were observed around the colonies on blood agar medium showing in Fig. 6. Both isolates showed a negative response for gelatinase as well as lipase production, showing in Fig. 7. Positive hemolytic activity (ability to breakdown red blood cells) halt the underlying epithelial layer whereas positive gelatinase activity (ability to hydrolyse gelatin) breakdown the protective lining of the GIT. Absence of hemolytic, gelatinase and lipase activity makes a strain non-virulent, indicating its selection for probiotic strain[30].

Probiotic properties

Tolerance to low acid conditions

A successful probiotic possesses the property of tolerating harsh acidic conditions of stomach and gut. Probiotic bacteria must pass through the stomach where the pH can be as low as 1.5 to 2, before reaching the intestinal tract[31]. In this study, we were able to obtain the isolates that were able to grow at minimum tested pH of 1.0 after 60 and 120 min of incubation, respectively (Table 3). Lactic acid is produced by lactic acid bacteria during fermentation metabolism thus revealed its ability to survive in the acidic environment of the stomach. However, the pH inside the gut is 2-4 in normal conditions and may reach up to pH 1 during fasting. For industrial use in food preparations, the organisms must survive the lowest possible pH[32]. Therefore, tolerance to low pH by *L. plantarum* DB-2 and *L. plantarum* SK-3 disclosed their survival under low acidic conditions. Moreover, different probiotic bacteria exhibit different resistance to acidic conditions and this feature is species and strain dependent[33]. Tolerance to acidic condition helps the *Lactobacilli* to reach the small intestine and thus contribute in balancing the intestinal microflora.

Tolerance to bile salts

Bile salts are surface-active, amphipathic agents having potent antimicrobial activity. They act as detergent thus disrupts the cell membranes[34]. Bile acids are products of cholesterol metabolism and synthesized in the liver. It is secreted in the conjugated form (either with glycine or taurine)

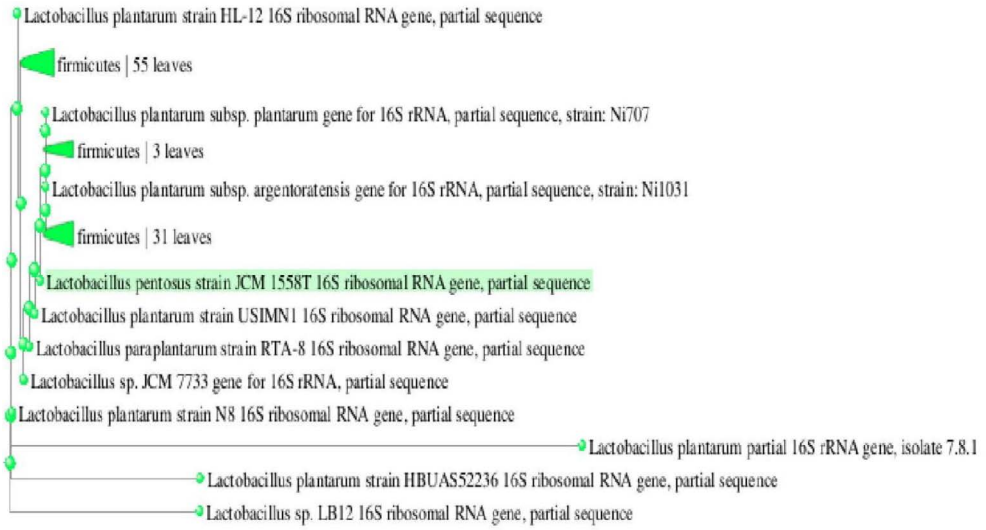

Fig. 4. (A) Phylogenetic dendogram of strain DB2 and related lactic acid bacterial species based on 16S r RNA gene sequence similarity

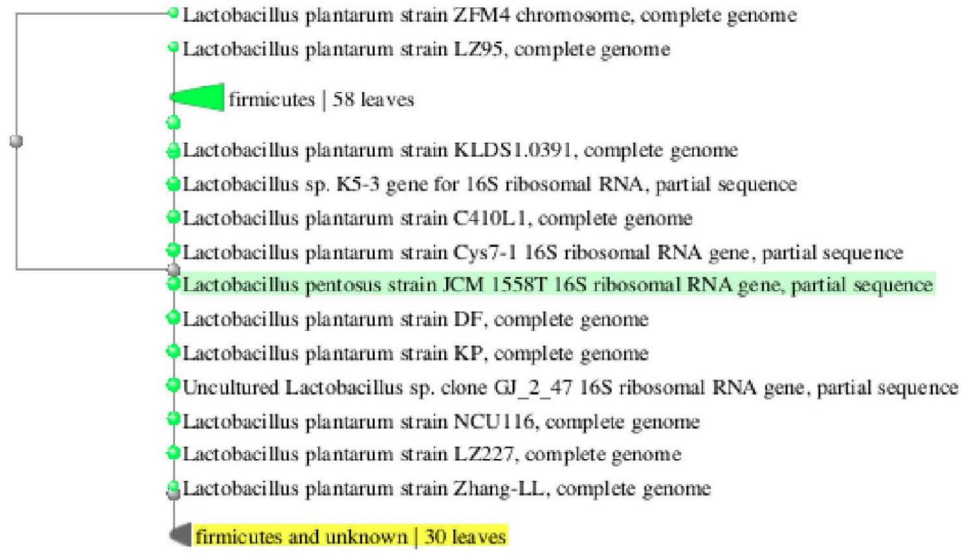

Fig. 4. (B) Phylogenetic dendogram of strain SK3 and related lactic acid bacterial species based on 16S r RNA gene sequence similarity

from the gall bladder to duodenum (500-700 ml/day). Bile acids play an important role in the digestive process (emulsification of fat). Bile concentration of intestine is 0.3% w/v. The small intestine has a low concentration of bile salts between 0.2-2 percent[35]. It is of great importance to evaluate the ability of probiotic strains to tolerate bile acids as these can act as antimicrobial molecules and thus influence the intestinal microflora. In this study, concentrations of 0.3%, 0.5%. 0.6%, 0.8%, 1% and 2% bile salts were used and their effect on growth rate of isolates was studied. Both the strains, *L. plantarum* DB-2 and *L. plantarum* SK-3 showed good survival after 8 h of incubation (Table 4). When the concentration of bile salt was increased up to 2%, the decrease was observed in the viable counts of isolates. It was considered that the biological cell membranes are

composed of lipids and fatty acids and bile salts increase the permeability of these membrane.

Tolerance to simulated gastric conditions

Probiotics must have the ability to survive passage through the stomach and small intestine as they are usually administered orally. Therefore, the survival of the probiotic strain in simulated conditions establishing in the stomach and the duodenum was examined by incubating selected cultures in MRS supplemented with pepsin (pH 2.0 and 3.0) and pancreatin (pH 8.0) for 4 h at 35°C. The survival of *L. plantarum* DB-2 and *L. plantarum* SK-3 at pH 2.0 and 3.0 containing pepsin (stomach conditions) and pH 8.0 containing pancreatin (intestinal conditions) was observed for different time intervals upto 4 h. *L. plantarum* DB-2 and *L. plantarum* SK-3 showed good survival at pH 2.0 (log CFU/ml 3.711 and 4.879) after 1 h of incubation whereas both the isolates did not show the survival after 4 h of incubation in pepsin at pH 2.0. At pH 3.0 of simulated gastrointestinal juice, both the strains survived after 4 h of incubation (log CFU/ml 5.798 and 3.711). Both isolates exhibited good survival at pH 8.0 of simulated gastrointestinal juice (log CFU/ml 7.437 and 7.920) (Table 5). Probiotic bacteria exert their health-promoting effects as metabolically viable active cells when they reach the colon by first surviving the transit through stomach followed by the intestine[36]. Ability to resist gastrointestinal conditions should be tested as survival through the GI tract is an important criterion for the selection of probiotic lactic acid bacteria. The simulated

Table 2. Antibiotic sensitivity of *L. plantarum* SK3 and DB2

Antibiotics used	*L. plantarum* SK-3	*L. plantarum* DB-2
Ampicillin AMP (10μg)	*S	S
Amoxicillin AX (10μg)	S	S
Cefotaxime CTX (30μg)	*R	S
Cefoxitin CX (30μg)	R	R
Cefuroxime CXM (30μg)	S	S
Co-trimoxazole COT (30μg)	S	R
Gentamycin GEN (10μg)	S	R
Tetracycline TE (30μg)	S	S
% survivalability	75	62.5

*Sensitive/Resistant

Table 3. Acid tolerance of *L. plantarum* DB-2 & SK-3

pH		Incubation time (min)												
		Absorbance*				Cell survival (log CFU/ml)**					%Cell survival***			
		0	60	120	180	0	60	120	180	Mean	60	120	180	Mean
1	DB-2	0.051	0.050	0.009	0.001	8.037	8.008	2.704	0.000	4.687	89.16	30.00	00.00	39.72
	SK-3	0.056	0.055	0.011	0.002	8.089	8.079	2.731	0.000	4.724	90.95	89.62	30.23	70.26
2	DB-2	0.059	0.054	0.017	0.008	8.113	8.053	3.399	2.711	5.569	89.66	37.71	29.97	52.44
	SK-3	0.062	0.060	0.023	0.009	8.173	8.127	3.501	2.712	5.628	91.90	90.15	38.75	73.60
3	DB-2	0.078	0.077	0.075	0.073	8.475	8.447	8.429	8.409	8.440	94.05	93.53	92.96	93.51
	SK-3	0.094	0.093	0.090	0.089	8.741	8.696	8.672	8.660	8.692	98.29	96.47	96.00	96.92
4	DB-2	0.090	0.089	0.086	0.080	8.563	8.551	8.517	9.485	8.779	95.21	94.50	93.80	94.50
	SK-3	0.102	0.100	0.097	0.095	8.845	8.786	8.775	8.755	8.790	99.46	97.47	97.14	98.02
5	DB-2	0.095	0.094	0.093	0.092	8.631	8.610	8.594	8.583	8.604	95.86	95.36	94.89	95.37
	SK-3	0.103	0.101	0.102	0.101	8.851	8.831	8.843	8.923	8.862	99.52	97.96	97.89	98.45
6.5	DB-2	0.097	0.098	0.099	0.103	8.853	8.981	9.012	9.045	8.972	100	100	100	100
	SK-3	0.099	0.103	0.105	0.107	8.893	9.014	9.033	9.071	9.002	100	100	100	100

*Absorbance: Mean of results from three different experiments
**log CFU/ml: Mean of results from three different experiments
***Survivability: (log cfu/ml $pH_{1, 2, 3, 4, 5}$/ log cfu/ml $pH_{6.5}$)

Table 4. Bile tolerance of *L. plantarum* DB-2 & SK-3

Bile salt conc.		Incubation time (h)									
		Absorbance*			Cell survival (log CFU/ml)**				%Cell survival***		
(%)		0	4	8	0	4	8	Mean	4	8	Mean
0.3	DB-2	0.076	0.074	0.070	8.445	8.411	8.346	8.400	93.27	92.31	92.79
	SK-3	0.094	0.093	0.092	8.717	8.688	8.664	8.689	96.18	95.42	95.80
0.5	DB-2	0.071	0.068	0.063	8.397	8.334	8.294	8.341	92.42	91.73	92.08
	SK-3	0.087	0.079	0.077	8.522	8.495	8.451	8.489	94.04	93.08	93.56
0.6	DB-2	0.040	0.036	0.021	7.842	7.765	6.903	7.503	86.11	76.35	81.23
	SK-3	0.048	0.033	0.023	7.948	7.716	7.049	7.571	85.42	77.64	81.53
0.8	DB-2	0.031	0.027	0.019	7.673	7.536	6.803	7.337	83.57	75.24	79.41
	SK-3	0.030	0.028	0.022	7.635	7.584	6.944	7.387	83.95	76.48	80.22
1	DB-2	0.026	0.024	0.020	7.394	7.181	6.856	7.143	79.63	75.83	77.73
	SK-3	0.027	0.026	0.020	7.408	7.225	6.857	7.163	79.98	75.52	77.75
2	DB-2	0.021	0.020	0.012	6.944	6.805	6.380	6.709	75.46	70.56	73.01
	SK-3	0.024	0.020	0.015	7.017	6.856	6.505	6.792	75.89	71.64	73.77
Control	DB-2	0.096	0.100	0.103	8.791	9.017	9.041	8.949	100.0	100.0	100.00
	SK-3	0.098	0.102	0.104	8.853	9.033	9.079	8.988	100.0	100.0	100.00

*Absorbance: Mean of results from three different experiments
**log CFU/ml: Mean of results from three different experiments
***Survivability: (log cfu/ml 0.3, 0.5, 0.6, 0.8, 1, 2% bile salt/ log cfu/ml 0% bile salt)

gastrointestinal and pancreatic digestions have been tested because of the independent action of strains to each of them and lead to a suitable global selection of probiotic bacteria[37]. Variation in acid resistance of probiotic lactic acid bacteria during the transit through gastrointestinal tract might be because of the changing pH values of the gastric juice (pH 2.0 to pH 3.5) depending on food components and feeding time[38]. In the present study, both lactic acid bacteria resisted the effects of pepsin and pancreatin during the transit in GI tract, therefore could be suggested as a potential probiotic candidate for further use in food preparations to improve the health of the gut.

Aggregation property

Autoaggregation

Interaction of the bacterial strain with itself (clumping of the cell) determines the auto-aggregation capability. Probiotic bacteria should

Table 5. Resistance of isolate SK-3 and DB-2 to simulated gastrointestinal juices

Gastro-intestinal		Isolates Incubation time (h)									
		Absorbance*			Cell survival (log CFU/ml)**				% Cell survival***		
juices		0	1	4	0	1	4	Mean	1	4	Mean
pH 2	DB-2	0.015	0.009	0.003	5.218	3.711	0.000	2.976	41.16	00.00	20.58
	SK-3	0.016	0.010	0.003	5.580	4.879	0.000	3.486	54.01	00.00	27.00
pH 3	DB-2	0.025	0.021	0.018	7.099	6.901	5.798	6.599	76.55	64.17	70.36
	SK-3	0.022	0.015	0.009	6.917	5.218	3.711	5.282	57.76	40.87	49.32
pH 8	DB-2	0.047	0.032	0.027	7.922	7.689	7.437	7.683	85.29	82.31	83.80
	SK-3	0.060	0.053	0.047	8.125	8.128	7.920	8.058	89.98	87.23	88.60
Control	DB-2	0.098	0.100	0.102	8.858	9.015	9.035	8.969	100.0	100.0	100.0
	SK-3	0.098	0.102	0.104	8.853	9.033	9.079	8.988	100.0	100.0	100.0

*Absorbance: Mean of results from three different experiments
**log CFU/ml: Mean of results from three different experiments
***Survivability: (log cfu/ml $pH_{2, 3, 8}$/ log cfu/ml $pH_{6.5}$)

adhere to the enterocytic cellular lines of oral cavity and GIT in order to exhibit their beneficial effects[39]. Bacterial aggregation depends on the amount of biofilm production which helps in adhesion of the cell[40]. The exact mechanism is not known of autoaggregation. Autoaggregation was investigated on the basis of sedimentation rate. The sedimentation rate was observed over 5 h of incubation. The ability of strains to auto-aggregate increased with increasing incubation time. *L. plantarum* DB-2 and *L. plantarum* SK-3 showed 97.6% and 98.2% aggregation after 5 h of incubation (Table 6). *L. plantarum* ST16Pa showed aggregation percentage as 37.05% after 60 min incubation[41]. The observed auto-aggregation could be due to cell surface component as they were not lost after washing and suspending of the cells in phosphate saline buffer[42].

Co-aggregation

As autoaggregation is the important property for adhesion to the mucoid lining of epithelial cells. In the same manner, co-aggregation posing a barrier in preventing the colonization of pathogenic microorganisms[43,44]. Co-aggregation is the process of joint aggregation of probiotic and pathogenic bacteria[45]. Probiotics have the ability to coaggregate with pathogenic microorganisms, inhibit their growth and finally kill them by secreting antimicrobial compounds which directly attack the cells of pathogenic bacteria[46]. *L. plantarum* SK-3 exhibited higher co-aggregation ability with pathogenic bacteria (71.25%) and the lower co-aggregation potential with pathogens was exhibited by *L. plantarum* DB-2 (59.34%). *L. plantarum* DB-2 and *L. plantarum* SK-3 showed highest co-aggregation potential against *S.aureus* (77.13% and 84.00%, respectively) (Table 7). The effective co-aggregation potential of probiotic bacteria against Gram-positive bacteria depend on the same cell wall morphology. Both have a thick peptidoglycan layer and bond get stronger by their hydrophobic nature[47]. The co-aggregation ability of lactic acid bacteria portrayed the high potential to kill undesirable microorganisms as they produce antimicrobial substances in close proximity to pathogenic bacteria. Lactic acid bacteria having co-aggregation potential have a significant role in human gut as they inhibit the growth of pathogenic strains by coaggregate with them in the gastrointestinal tracts[48]. During the process

Table 6. Estimation of auto-aggregation of selected *Lactobacillus* strains

Isolates	OD_{600} #						**Autoaggregation (%)					
	1h	2h	3h	4h	5h	Mean	1h	2h	3h	4h	5h	Mean
L. plantarum DB-2	0.023	0.020	0.017	0.016	0.012	0.017	95.4	96.0	96.6	96.8	97.6	96.48
L. plantarum SK-3	0.020	0.017	0.015	0.013	0.009	0.014	96.0	96.6	97.0	97.4	98.2	97.04

*Autoaggregation in terms of sedimentation rate

#OD_{600} = Mean of the results from three different experiments

**Autoaggregation % = 1- ($A_{t=1, 2, 3, 4 \text{ and } 5h}$/ $A_{0h)}$ x 100

Table 7. Evaluation of Co-aggregation ability of *L. plantarum* DB-2 and SK-3 with test indicators

Indicators	OD_{600} #			**Co-aggregation (%)		
	L. plantarum		Mean	*L. plantarum*		
	DB-2	SK-3		DB-2	SK-3	Mean
Bacillus cereus	0.340	0.230	0.285	57.50	71.25	64.38
Bacillus subtilis	0.382	0.214	0.298	52.25	73.25	62.75
Clostridium perfringens	0.292	0.221	0.257	63.50	72.38	67.94
Escherichia coli	0.322	0.234	0.278	59.75	70.75	65.25
Pseudomonas aeruginosa	0.412	0.265	0.339	48.50	66.88	57.69
Salmonella typhimurium	0.346	0.318	0.332	56.75	60.25	58.50
Staphylococcus aureus	0.183	0.128	0.156	77.13	84.00	80.57

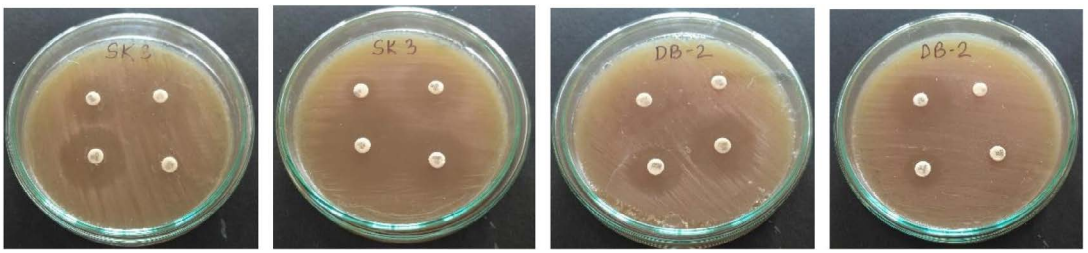

Fig. 5. Antibiotic susceptibility testing

of co-aggregation, lactic acid bacteria secretes antimicrobial substance in higher concentration and thus maintaining the environment around the pathogens[49] which behaves as an important host defence mechanism in the gut against the foodborne infections[50]. *L. plantarum* Lp-115 exhibited higher co-aggregation with *E. sakazakii* and good co-aggregation ability with *S. aureus*[51].

Adhesion property: Hydrophobicity

Cell surface hydrophobicity is the non-specific interaction between host and bacterial cells. Cell surface property of lactic acid bacteria is the key component for adhesion. Initially, the interaction is weak but gets stronger by adhesion process, mediated by cell surface proteins and lipoteichoic acids[52,53]. Bacterial adhesion to xylene, toluene, chloroform, ethyl acetate, n-Hexadecane and n-Octane was tested to study the Lewis acid-base characteristics of the bacterial cell surfaces. Out of the six solvents, chloroform is a monopolar acidic solvent, ethyl acetate is monopolar basic solvent, xylene, toluene, n-Hexadecane and n-Octane is the non-polar solvent. Determination of bacterial adhesion to xylene is a valid qualitative phenomenological approach[54]. The result of this study showed that the probiotic strains exhibited strong hydrophobicity towards non-polar solvents viz., chloroform, xylene, toluene, n-hexadecane and n-octane, exhibiting hydrophobic cell surface, which is a highly desirable probiotic trait. Electron donor and electron acceptor properties of bacteria were also regarded as a measure of hydrophobicity obtained with chloroform and ethyl acetate[55]. All the selected isolates proved to be a strong electron acceptor and weak electron donor as they exhibited strong affinity towards basic solvents such as ethyl acetate and low affinity

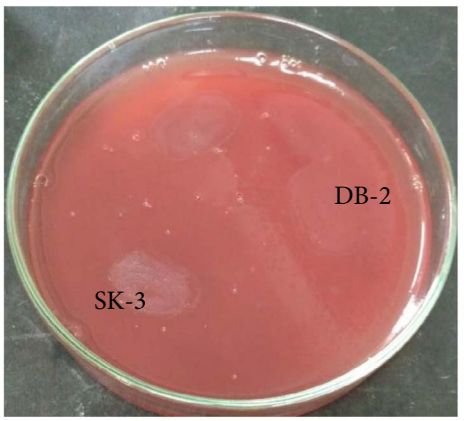

Fig. 6. Hemolytic activity of *L. plantarum* DB-2 and SK-3

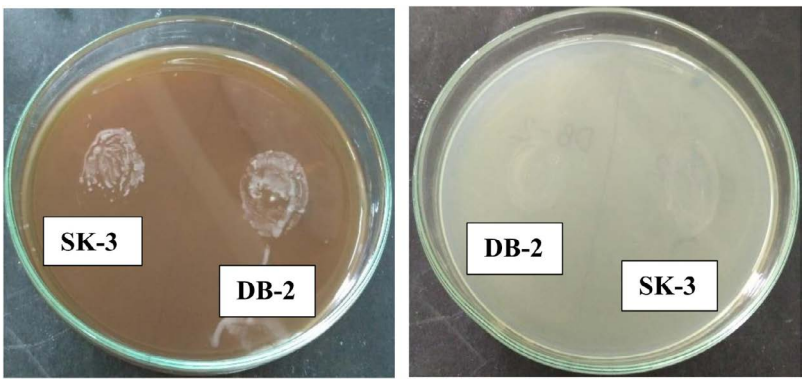

Fig. 7. Gelatinase and Lipase activity of *L. plantarum* DB-2 and SK-3

towards acidic solvent such as chloroform. *L. plantarum* DB-2 and *L. plantarum* SK-3 exhibited highest adhesion towards toluene (85.6% and 87.6%, respectively and lowest adhesion towards chloroform (66.0% and 80.8%, respectively) (Table 8). Higher hydrophobicity is required for the colonization and adhesion of bacteria to the epithelial cells of the gut[56]. It was hypothesized that the presence of S-layer proteins on the cell wall of *lactobacilli* which have high isoelectric point showed strong affinity towards non-polar solvent. It has been suggested that cell surface properties play key role in autoaggregation as well as hydrophobicity. Adherence to epithelia helps in evaluating the surface hydrophobicity towards the non-polar and polar solvent. A good probiotic must possess high autoaggregation and strong hydrophobicity.

Antibacterial activity of bacteriocin producing *L. plantarum*

The antagonism exhibited by lactic acid bacteria occur due to the production of volatile short chain fatty acids such as lactic acid, acetic acid, propionic acid, hydrogen peroxide and specific inhibitory substances such as bacteriocin[57]. The bacteriocin activity of both isolates were measured using serial two-fold dilutions of acid neutralized and catalase treated cell-free culture supernatant against *S. aureus, B. subtilis, E. coli, S. pyogenes* and *P. aeruginosa.* The activity was lost after treatment with trypsin, this suggests that the activity was solely because of the bacteriocin production. Maximum bacteriocin production was observed during 18 h growth cycle of *L. plantarum* SK-3 and *L. plantarum* DB-2 with 745 AU/ml and 710 AU/ml against test indicator *S. aureus* followed by 685 AU/ml and 680 AU/ml against *S. pyogenes*, 455 AU/ml and 545 AU/ml against *E.coli*, 490 AU/ml and 440 AU/ml against *P. aeruginosa,* 410 AU/ml and 400 AU/ml against *B. cereus*, respectively (Fig. 8). Bacteriocins from *L.gasseri* inhibited various food borne pathogens[58]. The multi-drug resistance need to be solved with bacteriocin-producing lactic acid bacteria[59]. Both probiotic strains could be exploited for their implementation in controlling foodborne pathogenicity and safe bio-preservation of food products using natural antimicrobial agent i.e. bacteriocin as this study demonstrated the ability of lactic acid bacteria to inhibit the growth of foodborne pathogens through the production of bacteriocin.

H_2O_2 production

Probiotic isolates *L. plantarum* DB-2 and *L. plantarum* SK-3 were screened for production of hydrogen peroxide. *L. plantarum* DB-2 and *L. plantarum* SK-3 have been reported to produce 0.56 g L^{-1} and 0.52 g L^{-1} respectively. Normally, hydrogen peroxide is produced by vaginal

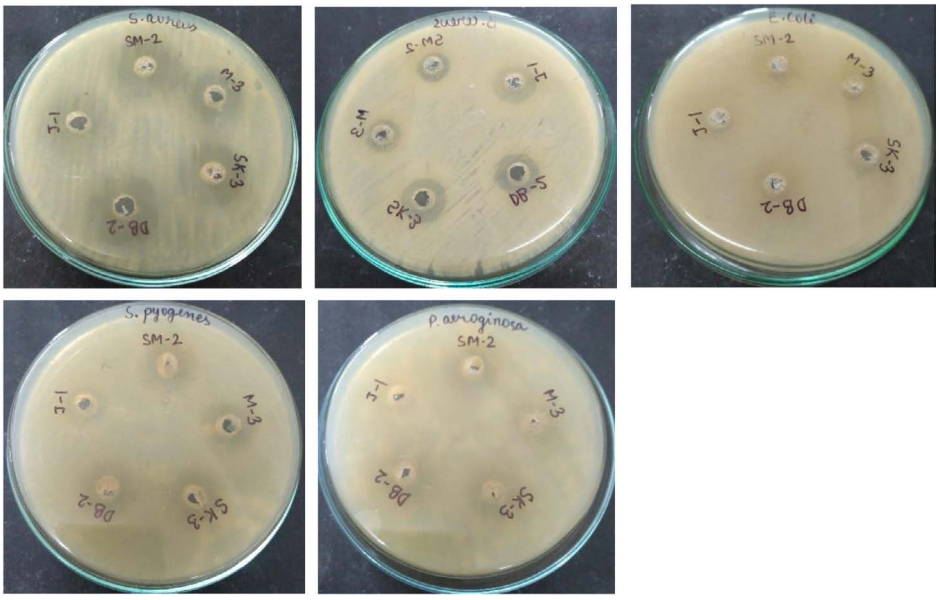

Fig. 8. Inhibitory activity of crude bacteriocin against five pathogenic organisms

Table 8. Adhesion of L. plantarum DB-2 and SK-3 to different hydrocarbons

Isolates	OD_{600}*						% Hydrophobicity**						
	Xylene	Toluene	Chloroform	n-Octane	n-Hexadecane	Ethyl Acetate	Mean	Xylene	Toluene	Chloroform	n-Octane	n-Hexadecane	Ethyl Acetate	Mean
L. plantarum DB-2	0.091	0.072	0.170	0.080	0.096	0.083	0.098	81.8	85.6	66.0	84.0	80.8	83.4	80.2
L. plantarum SK-3	0.083	0.062	0.096	0.082	0.086	0.075	0.080	83.4	87.6	80.8	83.6	82.8	85.0	83.9

lactobacilli isolates but may also be associated with intestinal lactobacilli[60]. In this study, *L. plantarum* DB-2 and *L. plantarum* SK-3 have been reported to produce H_2O_2 as an antimicrobial agent against food spoilage pathogens. Thus, this attribute can be used beneficially in improving vaginal health and in preventing urogenital infections. It can be hypothesized that the antagonism of these strains depends on lactic acid, H_2O_2 and bacteriocin thus exhibiting its potential and safe use as a bio-preservative in the food and fermentation industry.

BSH activity

BSH catalyses the deconjugation of bile salts and deconjugated bile salts have lower solubility at low pH and thus precipitate as a result of the fermentative metabolism of lactic acid bacteria[61]. Removal of cholesterol from the medium (broth) in the presence of bile might be ascribed to co-precipitation with deconjugated bile salts[62]. In this study, isolate SK-3 deconjugated the bile acids (growth was observed on plates) while isolate DB-2 was unable to conjugate the bile acids (no growth was observed on plates) (Fig. 9).

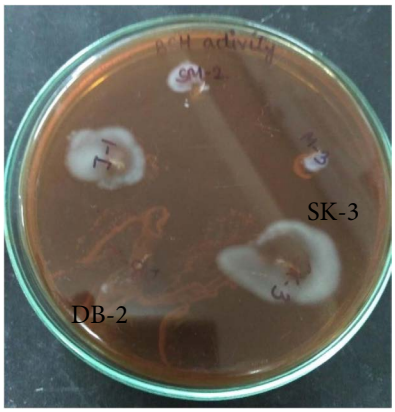

Fig. 9. Plate showing BSH activity of *L. plantarum* SK-3

All the probiotic attributes tested in this study revealed the safe status of both the isolates for further use in the food and fermentation industry. However, further evaluation of their beneficial effects on human beings will promote the application of both the strains in the pharmaceutical and cosmetic industry.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

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All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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