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Probiotic Properties of *Lactobacillus plantarum*

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ABSTRACT

The present study is to investigate the probiotic properties of lactic acid bacteria isolated from different food sources. Bacterial strains were screened for probiotic properties viz. tolerance to pH, bile salt, NaCl and phenol. They were also tested for auto-aggregation and hydrophobicity. They were also subjected to antibiotic susceptibility testing for their potential in reducing the negative effects of antibiotic therapy on host system. Out of 7 samples, 2 bacterial strains were tolerant to acid, bile salt, NaCl and phenol stress. Both the strains were identified by morphological, biochemical and molecular characterization. BLAST tool was used to compare the 16S rRNA sequences. Auto-aggregation and hydrophobicity were carried out and found the good auto-aggregation and hydrophobicity capability of both the strains. Bacteriocin produced by these two strains were investigated for their anti-bacterial potential against test pathogens. Among several selected bacterial strains *L.plantarum* SK3 and *L.plantarum* DB2 have good probiotic potential for further use in preparation of functional food. Currently research has been done to prepare functional food with health benefits along with overcoming nutritional deficiencies. These functional foods can be prepared by using lactic acid bacteria in fermentation. The lactic acid bacteria isolated in this study proved a good probiotic candidate as they survived during stress conditions posing to them. This study revealed the potential/possibility of using LAB and /or bacteriocin produced by them as food bio preservative to control foodborne pathogenic bacteria in near future.

Keywords: *Lactobacillus plantarum*, probiotics, acid tolerance and bile tolerance strains, hydrophobicity, auto-aggregation, anti-bacterial activity

Functional food has gained special recognition ascribed to increased understanding of health benefits related to such foods. Functional foods has the ability to provide essential nutrition and maintain innate immunity of the host. Fermented food with live cultures having beneficial effects on human health are considered to be a functional food. The consumption of fermented foods has gained the importance from the beginning of 20th century because of its probiotic characteristics. Food with health benefits were termed as 'Probiotics' by Fuller (1989) who gave its definition as 'live microbes which affect the host in a beneficial manner by improving and maintaining the microbial flora of the intestine'. Since then, the definition has been reformed many times. According to FAO/WHO (2001), 'Probiotics are living micro-organisms which has health benefits on host which when taken in sufficient amount'. Probiotic micro-organisms has the ability to restrict the growth of potential pathogenic microbes in the GIT and increase the multiplication of beneficial microbes. 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. A probiotic strain must fulfil some attributes like acid tolerance, bile tolerance, salt tolerance, phenol tolerance and antibiotic-resistance. Appropriate alternative to antibiotic treatment is 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. Bacteriocins are peptides, bactericidal to pathogens associated with food borne diseases and spoilage of food.

The objective of the study was to explore the probiotic potential of *Lactobacilli* spp. isolated from traditional fermented dosa batter and sauerkraut. Tolerance studies were carried out to signify the importance of survivalability 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° until use.

Strain isolation

1 g of food sample was added into 9 ml of normal saline. After homogenization, serial dilutions were prepared upto 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, India). The agar plates were incubated at 35° for 24 h under anaerobiosis. Morphologically different colonies were picked and re-streaked onto MRS agar plates upto purity. Glycerol stocks of strains were preserved at -20°.

Identification of strain

Phenotypic characterization

The morphological, cultural and biochemical characteristics including gram staining, catalase test and colonial appearance was determined.

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.

Genotypic characterization

Genomic DNA of the isolates were 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). 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 multiple sequence alignment. MEGA-6.0 was used for construction of phylogenetic tree by neighbour joining method.

Safety assessment of LAB

Safety is the 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. 24 h old culture were swabbed on MRS agar plates. Antibiotic impregnated discs (Himedia) were placed onto these inoculated plates. These plates were incubated at 37° for 24 h. Zone of inhibition was observed after 24 h.

Antibiotic used in this study were: Ampicillin 10, Amoxicillin 10, co-Trimoxazole 30, Cefotaxime 30, Cefuroxime 30, Gentamycin 10 and Tetracycline 303

Hemolytic activity

Hemolytic activity of both the strains were determined by spot inoculating overnight bacterial cultures on Blood agar plates followed by incubation of 24 h at 35°.

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° for 24 h.

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 olive oil as a source of fatty acids. Plates were incubated at 35° for 24 h.

Assessment of probiotic attributes

Tolerance to low acid conditions

Selected isolates were grown in MRS broth at 37° overnight. 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° 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.

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

Effect of bile salts on the growth rate of isolates

Selected isolates were grown in MRS broth at 37° overnight. 0.3%, 1% and 2% (w/v) of bile salt (oxoid) was prepared and added to the 24 hour old active culture of selected isolates and incubated at 37° 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.

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

Tolerance to NaCl

Selected isolates were grown in MRS broth at 37° overnight. Equal aliquots were taken and added to MRS broth containing NaCl in different concentrations (4, 6, 8, 10 and 12%) followed by incubation at 37° for 24 h. Control was run alongside. 0.1 ml aliquot was taken 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} after 24 h.

$$\text{Survivalability \%} = \frac{\log CFU\ 4,6,8,10,12}{\log CFU\,0} \times 100$$

Phenol tolerance

Selected isolates were grown in MRS broth at 37° overnight. Equal aliquots were taken and added to MRS broth containing phenol in different concentrations (0.1, 0.2, 0.3 and 0.4%) followed by incubation at 37° for 24 h. Control was run alongside. 0.1 ml aliquot was taken 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} after 24 h.

$$\text{Survivalability \%} = \frac{\log CFU\ 0.1,0.2,0.3,0.4}{\log CFU\,0} \times 100$$

Survival in simulated in vitro digestion

The pH in human stomach ranging from 1 (during fasting) to 4.5 (after a meal). Ingestion of food can take upto 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.

Aggregation property: Autoaggregation

Selected isolates were grown in MRS broth at 37° overnight. After incubation, the broth were centrifuged at 10,000 rpm at 4° 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° for 5 h. Equal amount of aliquot was taken and absorbance was measured at OD_{600} at 0, 1, 2, 3, 4 and 5 h.

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

Adhesion property: Hydrophobicity

Selected isolates were grown in MRS broth at 37° overnight. After incubation, the broth were centrifuged at 10,000 rpm at 4° 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 hydrocarbons (xylene, toluene, chloroform 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.

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

Antibacterial activity of bacteriocin producing L.plantarum

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

The selected probiotic isolates were grown overnight in TGY medium; a bacteriocin producing medium. The isolates were centrifuged at 12,000 rpm at 4° 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. The wells in the pre-swabbed nutrient plates were cut with sterile borer and 20 µl of neutralized culture supernatant was placed into the wells.

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

Where *AU* = Arbitrary units / activity units of bacteriocin

H_2O_2 production

Quantitative estimation of hydrogen peroxide was done by inoculating the bacterial isolate into MRS broth (25 ml) at 35° 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, India) and incubated at 35° for 24 h. The plates were observed for white precipitates.

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 $P < 0.05$.

RESULTS 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 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 catalase negative and non-sporulating. Out of 7 isolates, SK-3 and DB-2 gave clear halos around the

indicator pathogenic organisms and were selected for further study.

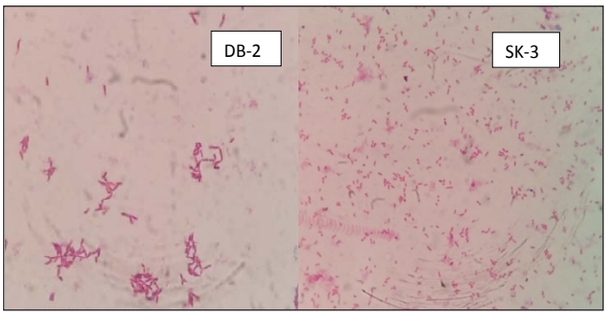

Fig. 1: Micrograph of isolates DB2 and SK3

Phenotypic characterization

Both strains appear transparent white, punctiform, flat with entire margins.

Biochemical characterization

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.

Genotypic characterization

Gel electrophoresis is shown in Fig. 2. 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 *Lactobacillus plantarum* SK3 and DB2 based on 16S rRNA gene sequences is shown in Fig. 3 and 4 respectively.

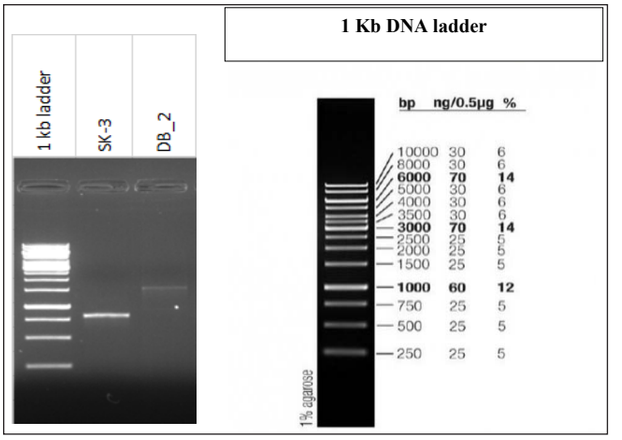

Fig. 2: Molecular identification of *Lactobacillus* strains by 16S rRNA gene technique

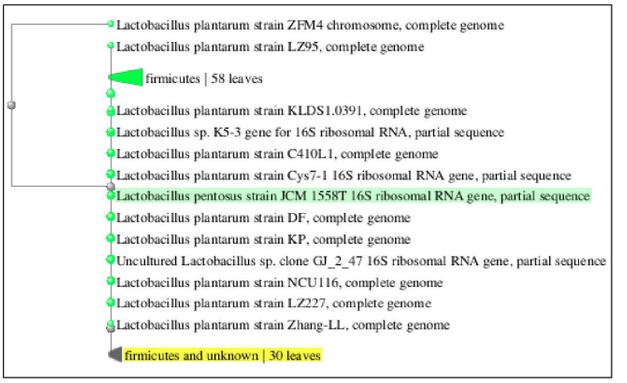

Fig. 3: Phylogenetic dendogram of strain SK3 and related lactic acid bacterial species based on 16S r RNA gene sequence similarity

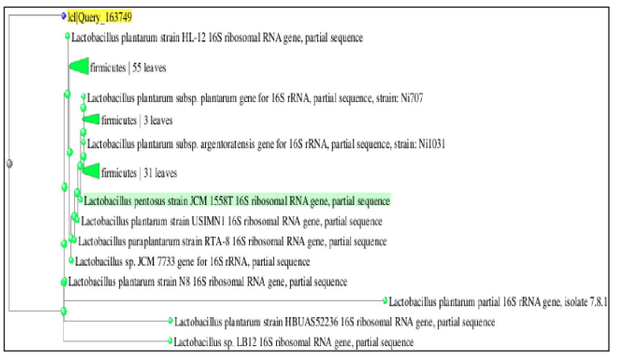

Fig. 4: Phylogenetic dendogram of strain DB2 and related lactic acid bacterial species based on 16S r RNA gene sequence similarity

Table 1: Acid tolerance of *L.plantarum* SK3 & DB2

pH	*L. plantarum*	Abs. at 0 h	log cfu/ ml	Abs. at 60 min	log cfu/ ml	Abs. at 120 min	log cfu/ ml	Abs. at 180 min	log cfu/ ml	Mean±SD of log cfu/ml	Survival Rate
1	SK3	0.056	8.089	0.055	8.079	0.055	8.056	0.050	7.989	8.053 ± 0.07	89.45
	DB2	0.051	8.037	0.052	8.008	0.046	7.903	0.029	7.627	7.893 ± 0.18	88.84
2	SK3	0.062	8.173	0.060	8.127	0.052	8.089	0.051	8.033	8.105 ± 0.05	90.03
	DB2	0.059	8.113	0.054	8.053	0.048	7.948	0.042	7.894	8.002 ± 0.09	88.31
3	SK3	0.094	8.741	0.093	8.696	0.090	8.672	0.091	8.660	8.692 ± 0.03	96.55
	DB2	0.078	8.475	0.077	8.447	0.075	8.429	0.073	8.409	8.440 ± 0.02	94.07
4	SK3	0.102	8.845	0.100	8.786	0.097	8.775	0.095	8.755	8.790 ± 0.03	97.64
	DB2	0.090	8.563	0.089	8.551	0.086	8.517	0.080	8.485	8.529 ± 0.03	95.06
5	SK3	0.103	8.851	0.101	8.831	0.102	8.843	0.101	8.823	8.837 ± 0.01	98.16
	DB2	0.095	8.631	0.094	8.610	0.093	8.594	0.092	8.583	8.604 ± 0.02	95.89
6.5	SK3	**0.099**	**8.893**	**0.101**	**9.014**	**0.102**	**9.033**	**0.104**	**9.071**	**9.002 ± 0.07**	**100**
	DB2	**0.098**	**8.853**	**0.097**	**8.981**	**0.099**	**9.012**	**0.103**	**9.045**	**8.972 ± 0.08**	**100**

Table 2: Bile tolerance of *L. plantarum* SK3 & DB2

Bile salt concentration (%)	*L. plantarum*	Abs. at 0h	log cfu/ ml	Abs. at 4h	log cfu/ ml	Abs. at 8h	log cfu/ ml	Mean ± SD of log cfu/ml	Survival Rate
0.3	SK3	0.094	8.717	0.093	8.688	0.092	8.664	8.689 ± 0.02	96.67
	DB2	0.076	8.445	0.074	8.411	0.085	8.346	8.400 ± 0.05	93.86
0.5	SK3	0.087	8.522	0.079	8.495	0.077	8.451	8.489 ± 0.03	94.44
	DB2	0.071	8.397	0.068	8.334	0.063	8.294	8.341 ± 0.05	93.20
0.6	SK3	0.048	7.948	0.033	7.716	0.023	7.049	7.571 ± 0.46	84.23
	DB2	0.040	7.842	0.041	7.515	0.021	6.903	7.420 ± 0.47	82.91
0.8	SK3	0.030	7.635	0.028	7.584	0.022	6.944	7.387 ± 0.38	82.18
	DB2	0.031	7.673	0.027	7.536	0.019	6.803	7.400 ± 0.44	82.69
1	SK3	0.027	7.408	0.026	7.225	0.020	6.857	7.163 ± 0.28	79.69
	DB2	0.026	7.394	0.024	7.181	0.020	6.856	7.143 ± 0.27	79.81
2	SK3	0.024	7.017	0.020	6.806	0.015	6.505	6.776 ± 0.25	75.38
	DB2	0.021	6.944	0.020	6.805	0.012	6.380	6.709 ± 0.29	74.96
Control	SK3	**0.098**	**8.853**	**0.102**	**9.033**	**0.104**	**9.079**	**8.988 ± 0.11**	**100**
	DB2	**0.096**	**8.791**	**0.100**	**9.017**	**0.103**	**9.041**	**8.949 ± 0.13**	**100**

Safety assessment of selected isolates

Antibiotic sensitivity

L.plantarum SK3 and *L.plantarum* DB2 exhibited 75% and 62.5% sensitivity towards the antibiotics used in this work. *L.plantarum* SK3 showed resistance towards Cefotaxime CTX (30µg) and Cefoxitin CX (30 µg) whereas *L.plantarum* DB2 showed resistance towards Cefoxitin CX (30µg), Co-trimoxazole COT (30µg) and Gentamycin GEN (10µg) (Table 6 and Fig. 5).

Hemolytic activity

Nil 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.

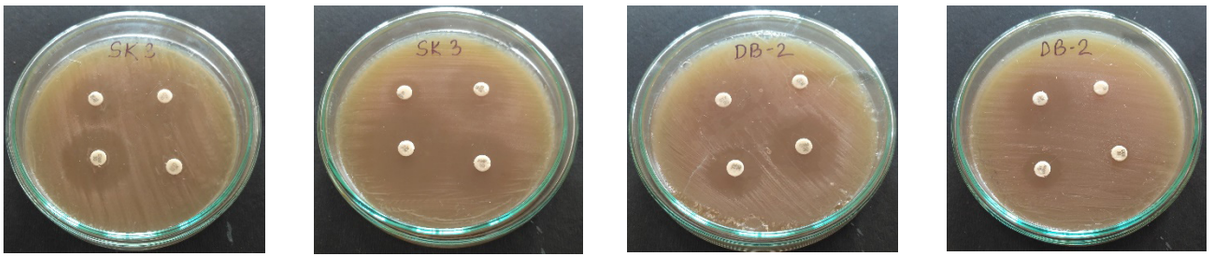

Fig. 5: Antibiotic susceptibility testing

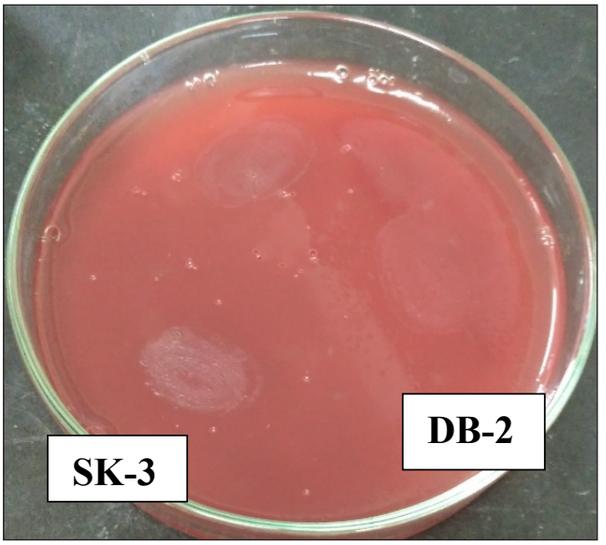

Fig. 6: Hemolytic activity of *L.plantarum* SK3 and DB2

Enzymatic activity

Both isolates showed negative response for gelatinase as well as lipase production, showing in Fig. 7.

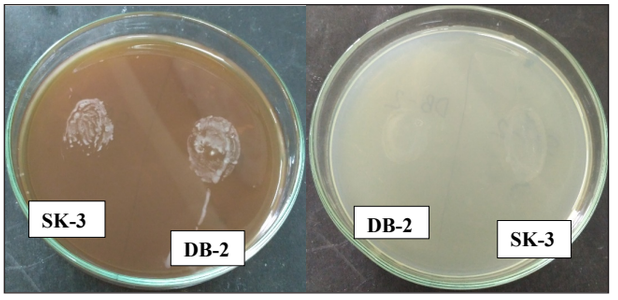

Fig. 7: Gelatinase and Lipase activity of *L.plantarum* SK3 and DB2

Absence of hemolytic, gelatinase and lipase activity makes a strain non-virulent, indicating its selection for probiotic strain.

Probiotic properties

Tolerance to low acid conditions

A successful probiotic possess the property of tolerating harsh acidic conditions. *L. plantarum* DB2 and *L.plantarum* SK3 were tested for survival in acidic conditions at different pH levels. Both grew well at minimum tested pH of 1.0 after 60 and 120 min of incubation. Lactic acid is produced by lactic acid bacteria during fermentation metabolism thus revealed its ability to survive in acidic environment of stomach.

Tolerance to low pH by *L. plantarum* SK3 and *L.plantarum* DB2 revealed in Table 1.

Tolerance to bile salts

Bile salts are surface active agents having potent antimicrobial activity. They act as detergent thus disrupts the cell membranes. Small intestine have low concentration of bile salts between 0.2-2 percent. Both the strains of *L.plantarum* (SK3 and DB2) showed good survival after 8 h of incubation. The result of assessment are summarized in Table 2.

Tolerance to NaCl

L.plantarum SK3 and DB2 grew significantly better under different concentrations of salt condition. Result are summarized in Table 3.

Table 3: NaCl tolerance of *L. plantarum* SK3 & DB2

NaCl concentration (%)	*L. plantarum*	Abs. at 0h	log cfu/ml	Abs. at 8h	log cfu/ml	Abs. at 24h	log cfu/ml	Mean ± SD of log cfu/ml	Survival Rate
4	SK3	0.095	8.740	0.091	8.620	0.057	8.056	8.472 ± 0.36	94.15
	DB2	0.094	8.739	0.084	8.515	0.050	7.996	8.416 ± 0.38	93.81
6	SK3	0.092	8.637	0.085	8.518	0.054	8.008	8.387 ± 0.33	93.20
	DB2	0.086	8.539	0.078	8.475	0.055	8.012	8.342 ± 0.28	92.98
8	SK3	0.075	8.426	0.070	8.363	0.049	7.948	8.245 ± 0.25	91.63
	DB2	0.074	8.418	0.069	8.342	0.042	7.907	8.222 ± 0.27	91.65
10	SK3	0.068	8.336	0.062	8.278	0.038	7.816	8.143 ± 0.28	90.49
	DB2	0.069	8.301	0.061	8.267	0.034	7.778	8.115 ± 0.29	90.45
12	SK3	0.056	8.025	0.048	7.948	0.035	7.783	7.918 ± 0.12	87.99
	DB2	0.049	7.952	0.036	7.795	0.029	7.627	7.791 ± 0.16	86.84
Control	**SK3**	**0.099**	**8.868**	**0.103**	**9.045**	**0.105**	**9.082**	**8.998 ± 0.11**	**100**
	DB2	0.097	8.848	0.101	8.992	0.104	9.075	8.971 ± 0.11	100

Table 4: Phenol tolerance of *L. plantarum* SK3 & DB2

Phenol concentration (%)	*L. plantarum*	Abs. at 0h	log cfu/ml	Abs. at 4h	log cfu/ml	Abs. at 8h	log cfu/ml	Mean ± SD of log cfu/ml	Survival Rate
0.1	SK3	0.073	8.409	0.071	8.383	0.068	8.311	8.367 ± 0.05	93.44
	DB2	0.050	7.952	0.049	7.944	0.036	7.795	7.897 ± 0.08	88.68
0.2	SK3	0.067	8.328	0.063	8.283	0.059	8.178	8.263 ± 0.07	92.28
	DB2	0.049	7.985	0.047	7.915	0.045	7.866	7.922 ± 0.05	88.96
0.3	SK3	0.062	8.232	0.060	8.184	0.059	8.170	8.195 ± 0.03	91.52
	DB2	0.049	7.971	0.048	7.920	0.046	7.857	7.916 ± 0.05	88.89
0.4	SK3	0.057	8.041	0.055	8.004	0.050	7.956	8.000 ± 0.04	89.34
	DB2	0.039	7.852	0.034	7.754	0.030	7.695	7.767 ± 0.07	87.22
Control	SK3	**0.099**	**8.899**	**0.102**	**8.966**	**0.103**	**8.998**	**8.954 ± 0.05**	**100**
	DB2	0.098	8.851	0.100	8.914	0.101	8.951	8.905 ± 0.05	100

Tolerance to phenol

Both strains SK3 and DB2 showed good tolerance to phenol concentrations. *L.plantarum* SK3 showed 93.44%, 92.28%, 91.52% and 89.34% survival rate at 0.1, 0.2, 0.3 and 0.4% phenol concentration and *L.plantarum* DB2 showed 88.68%, 88.96%, 88.89% and 87.22% at 0.1%, 0.2%, 0.3% and 0.4% phenol concentration respectively, mentioning in Table 4.

Tolerance to simulated gastric conditions

A probiotic strain must survive during the transit in gastrointestinal tract. The survival of *L.plantarum* SK3 and *L.plantarum* DB2 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* SK3 and *L.plantarum* DB2 showed good survival at pH 2.0 (log CFU/ml 7.623 and 5.647) after 1 h of incubation whereas *L.plantarum* DB2 did not show the survival after 4 h of incubation in pepsin at pH 2.0 (Table 5). *L.plantarum* SK3 and *L.plantarum* DB2 resist the effects of pepsin and pancreatin during the transit in

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

Survival percentage (%)					
Gastrointestinal juices		**1 h**		**4 h**	
	L.plantarum	**log CFU/ml**	**% Survival**	**log CFU/ml**	**% Survival**
Pepsin (pH 2.0)	**SK-3**	7.623 ± 0.27	71.73	5.312 ± 0.03	49.99
	DB-2	5.647 ± 0.28	58.37	—	—
Pepsin (pH 3.0)	**SK-3**	8.047 ± 0.15	75.72	6.545 ± 0.15	61.59
	DB-2	6.366 ± 0.18	65.80	4.395 ± 0.07	45.43
Pancreatin (pH 8.0)	**SK-3**	9.318 ± 0.07	87.69	8.689 ± 0.22	81.77
	DB-2	8.509 ± 0.06	87.95	7.419 ± 0.40	76.69

log CFU/ml = Mean ± SD where n=3; % Survival = (log CFU 4th or 1st h / log CFU 0th h) × 100.

GI tract and thus could be a potential probiotic for use in functional food formulations.

Aggregation property: 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* SK3 showed 89.65% aggregation while *L.plantarum* DB2 showed 85.18% after 24 h of incubation, mentioning in Table 7. 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.

Table 6: Antibiotic sensitivity of *L.plantarum* SK3 and DB2.

Antibiotics used	***L.plantarum* SK3**	***L.plantarum* DB2**
Ampicillin AMP10	*S	S
Amoxicillin AX 10	S	S
Cefotaxime CTX 30	*R	S
Cefoxitin CX 30	R	R
Cefuroxime CXM 30	S	S
Co-trimoxazole COT 30	S	R
Gentamycin GEN 10	S	R
Tetracycline TE 30	S	S
% survivalability	75	62.5

**Sensitive/Resistant.*

Table 7: Estimation of auto-aggregation of selected *Lactobacillus* strai**ns**

		Absorbance	**Percentage (%)**
0h	SK3	0.058	
	DB2	0.054	
1h	SK3	0.045	22.41
	DB2	0.047	12.96
2h	SK3	0.031	46.55
	DB2	0.035	35.18
3h	SK3	0.026	55.17
	DB2	0.028	48.14
4h	SK3	0.013	77.58
	DB2	0.017	68.51
5h	SK3	0.006	89.65
	DB2	0.008	85.18

Adhesion property: Hydrophobicity

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 whereas low hydrophobic towards polar solvent, ethyl acetate. 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. The results of microbial adhesion to hydrocarbons are mentioned in Fig. 8.

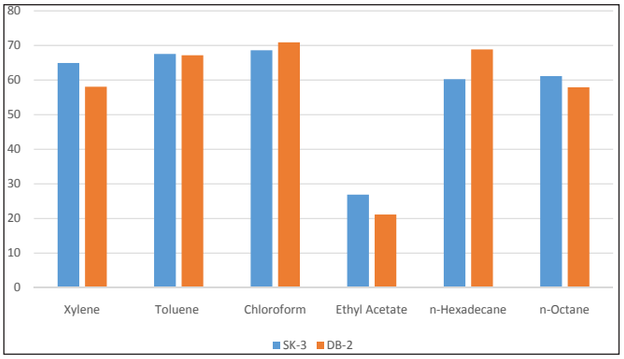

Fig. 8: Comparison of hydrophobicity of *L.plantarum* SK3 and DB2

Antibacterial activity of bacteriocin producing L.plantarum

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* SK3 and *L.plantarum* DB2 with 745 AU/ml and 710AU/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. On the basis of bacteriocin production, both the isolates have the potential to be used as a safe bio-preservative in food products. Results are shown in Fig. 9.

H_2O_2 production

Probiotic isolates *L.plantarum* SK3 and *L.plantarum* DB2 were screened for production of hydrogen peroxide. *L.plantarum* SK3 and *L.plantarum* DB2 have been reported to produce 0.52 g L^{-1} and 0.56 g L^{-1} respectively. In this study, *L.plantarum* SK3 and *L.plantarum* DB2 have been reported to produce H_2O_2 as an antimicrobial agent against food spoilage pathogens. 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.

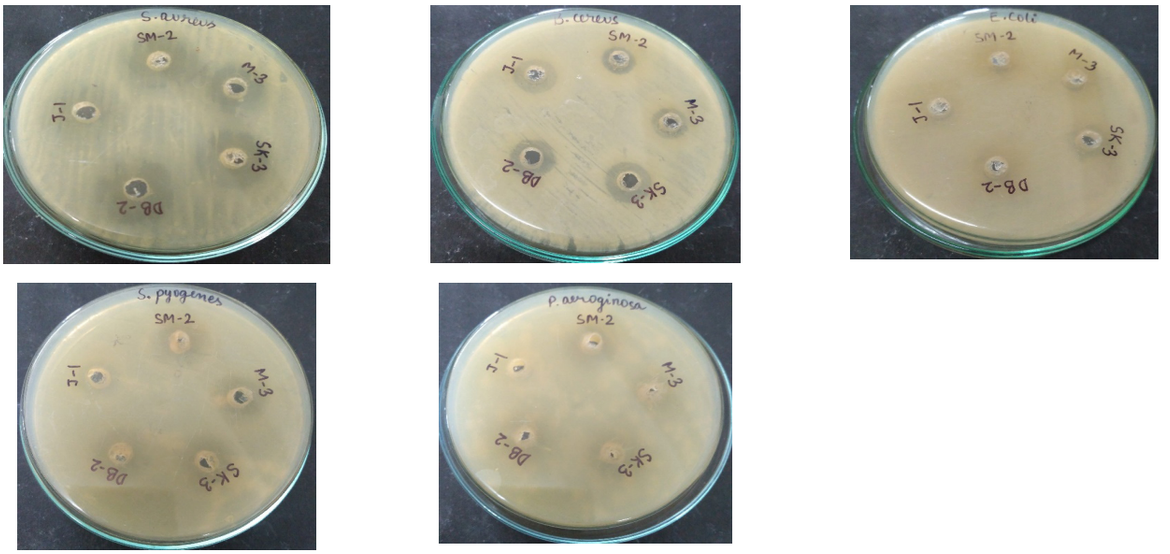

Fig. 9: Inhibitory activity of crude bacteriocin against five pathogenic organisms

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. 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. 10 exhibited the BSH activity of both the isolates.

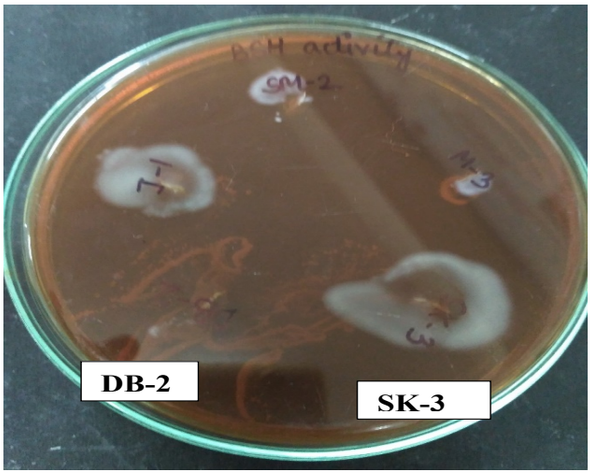

Fig. 10: Plate showing BSH activity of *L.plantarum* SK3

CONCLUSION

In the present study, *Lactobacillus plantarum* were isolated from dosa batter and sauerkraut. Various biochemical tests were performed for identification. None of the strains showed positive result towards gelatinase production, lipase production and hemolytic activity. 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. Survival and persistence of the probiotic bacteria during the transit through the GIT need to be considered for providing health benefits to the host. Once the bacteria reach the stomach, they enter the upper GIT where bile is secreted. Bile salts are surface active agents and amphipathic molecules. Bile acids are products of cholesterol metabolism and synthesized in liver. It is secreted in conjugated form (either with glycine or taurine) from gall bladder to duodenum (500-700 ml/day). Bile acids play an important role in digestive process (emulsification of fat). Bile concentration of intestine is 0.3% w/v. The average time of food transit through the small intestine varies generally from 1-4 hour. pH of small intestine is about 8.0. Presence of bile salts and pancreatin makes adverse conditions for survival in small intestine. Thus, strains selected for use as probiotic bacteria should possess acid-tolerant and bile-resistant qualities for providing health benefits. The presence of bacteriocin in both strains against six indicator organisms showed having a probiotic potential. The multi-drug resistance need to be solved with bacteriocin producing lactic acid bacteria. Interaction of the bacterial strain with itself (clumping of the cell) determines the auto-aggregation capability. Probiotic bacteria should adhere to the enterocytic cellular lines of oral cavity and GIT in order to exhibit their beneficial effects. Bacterial aggregation depends on the amount of biofilm production which helps in adhesion of the cell. Exact mechanism is not known of autoaggregation. 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. All the probiotic attributes tested in this study revealed the safe status of both the isolates for further use in food and fermentation industry. However, further evaluation of their beneficial effects on human beings will promote the application of both the strains in pharmaceutical and cosmetic industry.

Compliance with Ethics Requirements

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Conflict of interest

Conflict of interest declared none.

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

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