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ORIGINAL ARTICLE

Functional characterization and microencapsulation of probiotic bacteria from *koozh*

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Abstract *Koozh* is a traditional fermented millet beverage unique to south India. Analysis of six market samples of *koozh* for their microbial profile resulted in 69 isolates of presumptive lactic acid bacteria (LAB). They were grouped as *Leuconostoc sp*., *Enterococcus sp*., *Streptococcus sp*. and *Lactobacillus sp*. based on morphological characteristics and biochemical tests. Eight among them showed probiotic features: resistance to acid (2.5 pH for 6 h), resistance to 0.3 % ox bile, moderate hydrophobicity (40 %), antibacterial activity against 10 pathogens, susceptibility to 50 % of antibiotics tested. Sequencing of 16srDNA showed them to be five strains of *Enterococcus hirae* and one each of *Enterococcus facecalis*, *Bacillus amyloliquefaciens* and *Lactobacillus plantarum*. The probiotic isolates were encapsulated in skim milk powder by two different drying techniques: freeze drying and spray drying. The encapsulated probiotic isolates survived both in simulated gastric fluid and simulated intestinal fluid with high cell viability (98–99 %). Storage for 16 weeks at room temperature (27 °C), resulted in 2 log reduction, but better survival with only 1 log reduction was observed at 4 °C and was best at −20 °C. Survival of isolates was similar in both spray and freeze dried products.

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Introduction

The steadily growing research interest in probiotic microorganisms, has contributed to their recognition as health promoters that modulate the gut microbiota. The potential health benefits of probiotics include: bacteriocin production, alleviation of lactose intolerance, reduction of intestinal pathogens, hypocholesterolemic effect, stimulation of the immune system and prevention of—antibiotic associated diarrhoea, inflammatory bowel diseases and allergy (atopic eczema, food allergy).

Fermentation of millets increases bioavailability of nutrients while reducing the level of anti-nutrients through microbial activity. It decreases the carbohydrates, dietary fiber, fatty acid and increases the protein quality and B vitamins. Fermented whole grains have become the preferred choice of probiotics delivery vehicles as they offer buyers double benefits from probiotics along with whole grains. Fermented finger millet foods as probiotic carriers would enhance consumer health via the benefits from probiotics, calcium and bioactive components.

Lactic acid bacteria (LAB) are prevalent in most of the Indian cereal fermented foods and strains identified include *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Leuconstoc mesnetroides* (Tamang 2010). Although probiotic microbes have been isolated from many dairy products, not many Indian fermented millet foods have been explored as a potential source of probiotics.

Koozh, is a unique traditional millet food from the natural fermentation of finger or pearl millet. The fermentation is carried out on one occasion with raw millet slurry and second

on the cooked porridge (Ilango and Antony 2014). The organoleptic property is similar to porridge and depending on the butter milk added, provides the sour tang flavor. Studies on *koozh* are very limited. Kumar et al. (2010) have studied the microflora of *koozh* prepared from finger millet under laboratory conditions, with fermentation for 2 days without the addition of cooked broken rice and reported the presence of *Weisella paramesenteroides* with probiotic properties and *Lactobacillus fermentum* with antibacterial activity towards *Salmonella typhi*, *Vibrio parahaemolyticus* and *Listeria monocytogenes*. The objective of this work was to screen, isolate and identify the lactic acid bacteria (LAB) prevalent in six market samples of *koozh* and to assess their probiotic characteristics *in vitro*. The effect of encapsulation by spray and freeze drying on the probiotic characteristics of the isolates was also studied.

Materials and methods

Sample collection

Six samples of *koozh* were purchased from streets and village markets of Tamil Nadu. They were collected aseptically in wide mouthed sterile bottles and transported to the laboratory in a cooling box and used immediately for the isolation of microbes.

Microbiological analysis

The non-starter LAB (NSLAB) were isolated from aliquots of *koozh* samples by appropriate dilution and pour-plating, in MRS agar and M17 agar (HiMedia, Mumbai, India). The plates were examined after 24 h incubation at 35 °C. The colonies obtained were examined for morphological differences (shape, size, elevation, surface characteristics and edges). All isolated Gram positive, non-motile, catalase and oxidase negative isolates were stored at −80 °C in 30 % glycerol and sub-cultured twice by quadrant streaking separately on fresh MRS and M17 media.

All the NSLAB isolates were initially screened for their ability to produce acid by pour plating on MRS and M17 agar supplemented with 1 % $CaCO_3$ and incubated under anaerobic conditions at 35 °C for 24 h using the HiMedia's Gas pack systems. They were grouped based on the following criteria: ammonia utilization from arginine, methylene blue reduction, gas production from glucose, ability to grow at different temperatures (15 °C, 45 °C), pH (3.9, 9.6) and in different concentrations of sodium chloride (7 %, 10 %) (Schillinger and Lücke 1987). The ability of the NSLAB isolates to ferment the following carbohydrates: adonitol, arabinose, cellobiose, dextrose, dulcitol, fructose, galactose, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose was determined by using carbohydrate coated paper discs according to manufacturer's instructions (HiMedia, India).

Resistance of NSLAB to biological barriers *in vitro*

Five tests were conducted *in vitro* to assess the probiotic activity/character of the NSLAB isolates. The bacteria needs to be metabolically active and stable in the product, should survive the strong acidic conditions of upper digestive tract and alkaline conditions of small intestine of the host (Anal and Singh 2007).

The initial qualitative test for pre-selection of probiotic strains was acid tolerance (Hyronimus et al. 2000). The LAB isolates were grown in MRS and M17 broth at 37 °C in a static incubator overnight. An aliquot (0.1 mL) of the isolate was inoculated into 10 mL of MRS and M17 broth adjusted to a pH 2.5 with 3.0 M hydrochloric acid and incubated at 37 °C in aerobic static condition. One milliliter aliquots were taken at 0, 3 and 6 h intervals for identifying the cell viability by pour plating in MRS and M17 agar. The residual viable population was determined after 48 h of incubation at 37 °C. The results are expressed in logarithmic colony-forming unit per milliliter (log CFU mL^{-1}). The survival rate of the isolates was calculated according to the formula:

$$\text{Acid survival } \% = \frac{\text{Log number of viable cells survived } \left(\log \text{CFU mL}^{-1}\right)}{\text{Log number of initial viable cells inoculated} \left(\log \text{CFU mL}^{-1}\right)} \times 100$$

Presumptive LAB that had survived after 6 h incubation with growth reduction not exceeding 2 log CFU mL^{-1} were considered presumptive probiotic lactic acid bacteria (PPLAB) and further screened for probiotic characteristics.

Resistance to bile was also assessed *in vitro*. The PPLAB isolates were grown in 3 mL MRS broth at 37 °C overnight. A 0.25 mL volume of the culture was inoculated into 25 mL of MRS broth supplemented with 0.3 % ox bile salts (test) and without (control). These were incubated at 37 °C and A_{600nm} measured every hour for 6 h. The growth curves were plotted and time needed to attain a 0.3 A_{600nm} was monitored for both test and control. The effect of bile inhibition "d" in terms of delay in growth of the isolate was calculated as the difference between control and test expressed in minutes (min). This difference is considered as the major factor for this arbitrary classification. According to Chateau et al. (1994), the PPLAB isolates may be classified as resistant strains (delay of growth: d is ≤15 min), tolerant isolates (d is >15 min and ≤40 min), weakly tolerant isolates (d is >40 min and ≤60 min) and sensitive (d is >60 min). Based on the isolates growth curve for 24 h, the difference in time between the control and test to

reach the stationary phase, considered as Lag Time (LT) was also recorded.

The adherence of bacterial cells was evaluated using the microbial adhesion test of hyrophobicity (MATH) (Doyle and Rosenberg 1995). The PPLAB isolates were incubated in MRS broth under aerobic conditions for 15 h at 37 °C. A 3 mL aliquot was centrifuged at 6000 rpm for 10 min, the supernatant removed and the pellet washed thrice with 50 mM K_2HPO_4 (Merck) of pH 6.5. The absorbance of the culture (A) was adjusted to 0.7 ± 0.2 A_{600nm}. A 3 mL aliquot of the washed culture (A) was added to 0.6 mL of n-Hexadecane and vortexed for 120 s. The tubes were left undisturbed at 37 °C for 30 min for phase separation. Without disturbing the upper phase, 0.7 mL of the lower aqueous phase was removed with a micropipette and A_{600nm} was measured (A_o). The decreased value of A_o due to cell partitioning into the hydrocarbon and aqueous phases was noted down and percentage of hydrophobicity (H%) was calculated using the formula: $H\% = (A - A_o/A) \times 100$.

The agar-well diffusion method as described by Schillinger and Lucke (1989) was used to determine the antibacterial activities. Ten microorganisms namely *Bacillus cereus* ATCC 14579, *Enterobacter aerogenes* MTCC 111, *Escherichia coli* MTCC 728, *Klebsiella pneumoniae subsp. ozaenae* MTCC 2653, *Listeria monocytogenes* MTCC 1143, *Micrococcus luteus* MTCC 4428, *Proteus vulgaris* MTCC 426, *Salmonella typhi* MTCC734, *Shigella flexneri* MTCC 1457 and *Staphylococcus aureus* ATCC 43300 were used for assessing antimicrobial inhibitory potential. The pathogenic bacteria were grown separately in nutrient broth at 37 °C for 18 h in aerobic environment and adjusted to 1.00 A_{600nm} before pour plating in MRS soft agar.

The cell free supernatants (CFS, 0.05 mL) of NSLAB isolates were dispensed in wells cut in the MRS agar containing the respective pathogens and incubated at 37 °C for 24 h and the zone of clearance noted. To analyze the nature of inhibition, the CFS was neutralized to pH of 6.50 ± 0.08 with 5 M NaOH and 14.4 mg of fresh catalase (HiMedia:2000–5000 unit mg^{-1}) added. The neutralized cell free supernatants (NCFS) were dispensed into fresh wells and assessed as above. A value of 1 mm or more was considered positive inhibition.

The antibiotics used were selected based on their frequency of use in the Chennai region. The PPLAB isolates were tested for resistance to various classes of 21 regional antibiotics in ready-to-use antibiotic impregnated paper discs (HiMedia). They were ampicillin, amoxyclav, penicillin-G, cefixime, cefuroxime, vancomycin, bacitracin, amikacin, gentamicin, azithromycin, streptomycin, erythromycin, chloramphenicol, tetracycline, norfloxacin, ciprofloxacin, nalidixic acid, gatifloxacin, gemiofloxacin and levofloxacin. Initially PPLAB isolates were grown for 24 h at 37 °C in a 3 mL MRS broth and the cell density adjusted to1.00 A_{600nm} and pour plated in 95 mm petriplates with 20 mL MRS agar. After solidification, four antibiotic discs were dispensed in equal corners of a plate according to manufacturer's instructions and incubated at 37 °C for 24 h. The diameter of inhibition was measured and compared with interpretive chart of Charteris et al. (1998) and the isolates classified as resistant (R), susceptible (S), or intermediate (I).

Genotype of probiotic LAB

Of the 69 isolates, PPLAB isolates that showed acid tolerance, bile resistance and antibacterial activity were represented as probiotic lactic acid bacteria (PLAB) submitted to Macrogen, Korea for sequencing. These genomic DNA were obtained by InstaGene™ Matrix (BIO-RAD). The primers 27F 5′ (AGA GTT TGA TCC TGG CTC AG) 3′ and 1492R 5′ (TAC GGY TAC CTT GTT ACG ACT T) 3′ were used for executing the PCR (Lane 1991). The PCR reaction was carried out with 20 ng of DNA as the template in a 30 μL reaction mixture by using a EF-Taq (SolGent, Korea): initiation of Taq polymerase at 95 °C for 2 minutes, 35 cycles of 95 °C for 1 min, 55 °C, and each of 72 °C for 1 min, ending it with a 10-min step at 72 °C. The amplification products were purified with a multi-screen filter plate (Millipore Corp., Bedford, MA, USA). The Sequencing reaction was performed using sequencing primer 518 F 5′ (CCA GCA GCC GCG GTA ATA CG) 3′ and 800R 5′ (TAC CAG GGT ATC TAA TCC) 3′ using the cycle sequencing kit PRISM BigDye Terminator v3.1. This DNA sample containing extension products were combined with Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, then kept on ice for 5 min and analyzed in ABI Prism 3730XL DNA (Applied Biosystems, Foster City, CA). The sequence was aligned with NCBI GeneBank database using the nucleotide homology search Blast N program. Then the blast program was used to compare with other homologous sequence to identify the origin and geographical distribution of the identified PLAB with other submitted sequence around the world.

Encapsulation and effect of Storage

The PLAB isolates were grown in 3 mL MRS broth at 37 °C overnight. A 100 μL aliquot of overnight grown cultures were inoculated in 50 mL of MRS broth for 15 h in an incubator shaker. The culture was pelleted by centrifugation at 1600×*g* for 10 min at 4 °C. The pellet was diluted with saline to 0.93 ± 0.03 A_{600nm} inoculated (1 % *v*/*v*) in 50 mL of 10 % skim milk powder (SMP) containing 0.5 % yeast extract (YE). Skim milk suspension (SMS) was kept in incubator shaker at 180 rpm, 37 °C for 6 h. This was then pour plated on MRS agar for cell count.

The SMS was spray dried in a bench top drier (JISL SprayMate, India) with a inlet temperature of 140 °C and

outlet temperature of 40 °C at a pressure of 2 psi and feed rate of 14 rpm to obtain spray dried probiotic (SDP) powder. The SMS was freeze dried in a laboratory scale lyophilizer (SP Scientific, VirTis) at condensing temperature of −80 °C and vacuum pressure of 50 mTorr to get freeze dried probiotic (FDP) powder. The moisture of SDP and FDP (PLAB isolates encapsulated with skim milk powder) was analyzed using a moisture analyzer (Sartorius- MA35). Three batches of the encapsulated PLAB isolates were prepared.

The FDP and SDP were separately stored at temperatures of −20, 4, 27 and 37 °C in air tight containers. The survival was evaluated by pour plating samples on MRS agar every week for 1, 2, 4, 6, 8 and 16 weeks and survival percentage was calculated.

Probiotic efficacy of encapsulated probiotic isolates

The encapsulated PLAB stored at different temperatures were subjected to simulated gastric and intestinal fluid to assess their viability in such environments. Samples were rehydrated before checking viability by pour plate method in MRS agar. Samples (0.5 g) of FDP/SDP were added to test tubes containing 4.5 mL of pre-warmed (37 °C) sterile saline and incubated for 1 h at 37 °C.

The simulated gastric fluid (SGF) was primed by mixing saline with 0.32 mg mL^{-1} pepsin and adjusting it to pH 2 (Anal and Singh 2007); 0.45 mL of SGF was added with 0.5 g of FDP or SDP and incubated at 37 °C for 2 h. The samples were retrieved at 30, 60, 90, 120 min for pour plating on MRS agar.

The simulated intestinal fluid (SIF) was prepared by mixing pH 7.4 saline with 0.1 % pancreatin and 3.6 % *w/v* bile (Borza et al. 2010). As mentioned above, 0.45 mL of SIF was mixed with 0.5 g FDP or SDP and incubated for 4 h at 37 °C. These samples are again retrieved at 120, 240 min and pour plated on MRS agar to check survival of the isolates.

Statistical analysis

Each of the experiments was performed in triplicates. The difference in mean and rank correlation was evaluated by using Graph Pad 5.01 (SanDiego, Ca, USA) software.

Results and discussion

Phenotyping and characterization of NSLAB isolates

A total of 69 NSLAB were obtained from *koozh* samples—44 from MRS and 25 from M17 media. All isolates were Gram positive, catalase negative, non-motile and acid producing. Methylene blue reductase test proved positive for all except for nine isolates which were identified as *Streptococcus sp*. The morphological characteristics revealed more number of cocci (48) than rods (21). Based on these and other biochemical characteristics, the isolates were grouped as hetero-fermentative (19 of *Leuconostoc sp*., 5 of *Lactobacillus sp*.) and homo-fermentative (20 *Entercoccocus sp*., 9 *Streptococcus sp*. and 16 *Lactobacillus sp*.) as presented in Table 1.

LAB have been reported in traditional fermented cereals like *kisra* from sorghum (Mohammed et al. 1991), *pozol* from maize (Ampe et al. 1999), *tape* from finger millet (Sujaya et al. 2001) and *borde*, from a mixture of maize and wheat (Abegaz 2007). LAB isolated in *kodo ko jaanr* made from finger millet and *selroti* made from rice were grouped based on biochemical tests (Yonzan and Tamang 2010). Geetha and Kalaichelvan (2013) have reported the predominance of LAB in their study where finger millet, pearl millet, sorghum and maize were made separately into *koozh* by fermenting for 20 h in the laboratory.

Resistance of NSLAB to biological barriers *in vitro*

The relationship of probiotic microorganisms to human health are well reviewed over the last decade. The required screening for effective PLAB demands that they withstand conditions of GIT (gastro intestinal tract) *in vitro* and *in vivo*.

The six *koozh* samples contained 26 acid tolerant isolates (growth up to 5 log CFU mL^{-1} after 6 h, pH 2.5) with survival of 70 % or more. Only eight of the 26 LAB survived up to 6 log CFU mL^{-1} after 3 h and 4 log CFU mL^{-1} after 6 h in the acidic condition and the decrease in growth for these isolates after 6 h was <2 log CFU mL^{-1}. The eight isolates considered as presumptive probiotic lactic acid bacteria (PPLAB) showed survival exceeding 80 and 60 % at pH 2.5 for 3 h and 6 h respectively (Table 2).

The microbial stability of LAB may be increased in the acidic environment of *koozh* (pH 4.5). Further, Hung (2004) opines that rice may be considered as a good substrate for a probiotic carrier that enhances survival and stability of the LAB against gastric acid damage. Therefore, *koozh* made from finger millet and rice may be a unique food matrix that supports the resistance of NSLAB to biological barriers.

The acid tolerance of the eight PPLAB may be understood in terms of the individual strains (Table 2). In the presence of high amount of lactate and proton in the environment, *L. plantarum* maintains a proton (pH) charge gradient inside and outside the cell, tolerating low pH. The *Enterococcus sp*. are generally slow acid producers but certain strains of *Entercoccus* like *E. hirae* are acid tolerant through maintenance of cytoplasmic pH by amplification of the proton translocating membrane bound ATPase (Papadimitriou et al. 2007).

While seven of PPLAB, were bile resistant, one *Enterococcus sp*. (Sha 1) was bile tolerant (Table 2). Based on the

Table 1 Biochemical classification of NSLAB isolated from *koozh*

Groups	1[a]	2[a]	3[a]	4[a]	5[a]	6[b]	7[b]	8[b]	9[c]	10[d]	11[d]	Total
No of Isolates	4	7	2	2	4	3	14	3	9	5	16	69
Cell shape	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Bacillus	Bacillus	
NH3	+[e]	+	+	+	+	+	+	+	+	+	+	69
Gas from glucose	+	+	+	+	+	−	−	−	−	+	−	32
Methylene blue	+	+	+	+	+	+	+	+	−	+	+	60
15 °C	+	+	+	+	−	+	+	+	+	+	+	65
45 °C	+	+	+	+	+	+	+	+	+	+	+	69
9.6Ph	+	+	−	+	+	+	+	−	+	+	+	65
3.9Ph	+	−	+	−	+	+	+	+	−	–	+	52
2.5pH at 6th hour	4/4[f]	0/7	0/2	0/2	2/4	3/3	06/14	1/3	2/9	1/5	07/16	26
7 % NaCl	¾	7/7	2/2	1/1	4/4	3/3	14/14	3/3	7/9	4/5	15/16	63
10 % NaCl	0/4	0/7	0/2	0/1	0/4	0/3	03/14	0/3	0/9	5/5	02/16	10
Adonitol	0/4	0/7	0/2	0/2	0/4	0/3	0/14	0/3	0/9	0/5	0/16	0
Arabinose	4/4	7/7	2/2	2/2	2/4	3/3	14/14	3/3	9/9	5/5	16/16	67
Cellobiose	4/4	7/7	2/2	2/2	4/4	3/3	14/14	3/3	9/9	5/5	16/16	69
Dextrose	4/4	7/7	2/2	2/2	4/4	3/3	14/14	3/3	9/9	5/5	16/16	69
Dulcitol	0/4	0/7	2/2	0/2	0/4	0/3	0/14	0/3	0/9	0/5	0/16	02
Fructose	4/4	7/7	2/2	0/2	4/4	3/3	14/14	3/3	9/9	5/5	16/16	69
Galactose	4/4	7/7	2/2	2/2	4/4	0/3	14/14	3/3	9/9	5/5	16/16	66
Inositol	0/4	0/7	0/2	0/2	0/4	0/3	0/14	0/3	0/9	0/5	0/16	0
Inulin	0/4	0/7	0/2	0/2	0/4	0/3	0/14	0/3	0/9	0/5	0/16	0
Lactose	4/4	7/7	2/2	2/2	4/4	3/3	11/14	1/3	0/9	5/5	16/16	60
Malltose	4/4	7/7	2/2	2/2	4/4	3/3	14/14	3/3	9/9	5/5	16/16	69
Mannitol	4/4	7/7	0/2	0/2	0/4	0/3	14/14	0/3	9/9	5/5	16/16	55
Mannose	4/4	7/7	2/2	2/2	4/4	3/3	14/14	3/3	9/9	5/5	16/16	69
Melibiose	4/4	7/7	2/2	2/2	4/4	3/3	14/14	3/3	9/9	4/5	16/16	68
Raffinose	0/4	0/7	2/2	2/2	0/4	0/3	14/14	0/3	7/9	5/5	14/16	44
Rhamnose	0/4	0/7	0/2	0/2	0/4	0/3	08/14	0/3	0/9	5/5	16/16	29
Salicin	4/4	7/7	2/2	2/2	4/4	3/3	13/14	3/3	9/9	5/5	16/16	68
Sorbitol	0/4	0/7	0/2	0/2	0/4	0/3	05/14	3/3	0/9	0/5	09/16	17
Sucrose	4/4	7/7	2/2	2/2	4/4	3/3	14/14	3/3	9/9	5/5	16/16	69
Trehalose	4/4	7/7	2/2	2/2	4/4	3/3	14/14	3/3	9/9	5/5	16/16	69
Xylose	4/4	5/7	2/2	2/2	4/4	3/3	14/14	3/3	9/9	0/5	0/16	46
					19			20	9	5	16	

[a] Groups 1 to 5 belonged to *Leuconostoc sp*

[b] Groups 6 to 8 as *Enteroccocus sp*

[c] Group 9 as *Streptococcus sp*

[d] Group 10 and 11 as *Lactobacillus sp*

[e] +: Positive, −: Negative

[f] No of positive strains/Total no of strains

growth curve of PPLAB in the presence of bile for 24 h, the difference in time between the control and test to reach the stationary phase, considered as Lag time (LT) was variable. An overall 6 h for *Enterococcus sp.* of Sha 1, Sha 8, Sha 22, Sha 24, Sha 28, Sha 35, 4 and 2 h for *Lactobacillus sp*., Sha 31 and Sha 7 respectively suggested varied ability to adapt to the bile environment in the intestine.

Bile is much more detrimental than low pH as it has a detergent like effect during intake, but the capacity of PPLAB to survive the bile stress (*in vitro*) may help them reach the intestine alive in large quantities in order to provide probiotic effect to the host.

The hydrophobicity percentages (H%) of PPLAB were classified according to Riveros et al. (2009) (Table 2). The

Table 2 Acid tolerance, bile tolerance and hydrophobicity of PPLAB isolates from *koozh*

Strains	Isolate	Acid survival % at pH 2.5		Growth delay between control and $Oxbile_{min}$		Hydrophobicity %	
		3 h	6 h	Resistant[a]	Tolerant[b]	Low	Medium
Enterococcus sp.	Sha1	95.77 ± 03.59	83.99 ± 05.77	–	22.0	–	41 ± 2.82
	Sha8	93.32 ± 03.36	69.80 ± 20.60	8.0	–	–	44 ± 2.12
	Sha22	88.59 ± 03.05	66.89 ± 19.24	0.5	–	04 ± 0.70	–
	Sha24	82.49 ± 09.34	71.45 ± 12.93	4.0	–	06 ± 0.70	–
	Sha28	91.39 ± 07.33	61.25 ± 23.14	1.0	–	07 ± 0.70	–
	Sha35	81.12 ± 10.05	73.01 ± 11.88	0.0	–	10 ± 1.41	–
Lactobacillus sp.	Sha7	91.01 ± 02.15	73.30 ± 22.90	2.0	–	–	49 ± 4.24
	Sha31	90.19 ± 02.40	69.17 ± 06.39	13.5	–	04 ± 0.70	–

[a] Growth delay ≤15 min

[b] Growth delay >15 min and ≤40 min

value of hydrophobicity values provide an estimate of the hydrophobic interaction that may exist between the host intestine and the PPLAB external surface structure, providing greater ability to adhere to epithelial cells of the host.

While five *Enterococcus sp.* showed a low range from 4–7 %, the remaining showed an intermediate range from 45 49 %. The *Lactobacillus sp*. Sha 7 isolate had comparatively greater H% and therefore might have a greater ability to adhere in the host intestine, due to the protein present for surface anchoring as observed by Kumar et al. (2011), who documented adhesion of *L. plantarum* strains to human intestinal HT-29 cell line.

The hydrophobic outer surface of bacteria is the first direct physical contact to the host that would provide a probable value of the bacterial adherence to the GIT. The hydrophobicity of half of the PPLAB in this study did not exceed 40 %, suggesting variability in the level of expression of cell surface proteins.

The acidity of CSF of isolates (pH of 4.49–4.58) contributed to zones of inhibition in the range of 2 to 4 mm against ten pathogenic microorganisms (Table 3). The PPLAB NCFS inhibited pathogens to a lesser extent than CFS, indicating that acidity contributed significantly to the antibacterial activity along with hydrogen peroxide and / or antimicrobial compounds produced. The PPLAB NCFS could inhibit 50 80 % of the pathogens, while the CFS inhibited 10–90 % with three of the eight CFS showing ZI > 2 mm against 10–40 % of the pathogens. No significant difference ($p < 0.05$) between ZI of CFS and NCFS were noted in all pathogens except *M. luteus*, *E coli*, *P. vulgaris* and *S. flexneri* . It was interesting to note that CFS and NCFS of *Enterococcus sp*. (Sha 35 and Sha 26) and *Lactobacillus sp*. (Sha 31) showed similar antimicrobial activity in terms of number of total pathogens inhibited (Table 3). The CSF and NCSF of NSLAB efficiently inhibited *E. coli*. While NCFS of all PPLAB inhibited *S aureus*, *K. pneumonia* and *L. monocytogenes* they could not inhibit *M. luteus* and *S. flexneri.*

The *Enteroccocus sp*. showed a broad antibacterial spectrum due to the presence of its natural heterologous protein. The PPLAB identified as *Enterococcus sp*. effectively inhibited the *E. aerogenes* (MTCC 111) proving the inhibitory potential against taxonomically closely related species. As in the present study, *L. plantarum* demonstrated antimicrobial activity *in vitro* and was also found *in vivo* systems (Kumar et al. 2011) with a wide range of inhibitory spectrum, including decreasing pathogen by trans-epithelial migration (Michail and Abernathy 2003).

The prerequisite for efficient LAB lies in their ability to counteract pathogenic organisms in the human host. The criterion for LAB to function as probiotic on ingestion is to interact with enteric pathogens and antagonise them, aiding in host defense. Lactic acid bacterial fermentation produces inhibitory compounds like organic acid, diacetyl, nitric oxide, hydrogen peroxide and antibacterial proteins. The probable mechanism for antimicrobial action may perhaps be the organic acid present in CFS that could have caused acidification of cytoplasm, collapsing the electrochemical proton gradient eventually altering the lipoteichoic acid (LTA) of the cell membrane, leading to cell disruption. Otherwise, it can also be suggested that certain LAB may be deficient in electron transport chains that cause an incomplete diminution of oxygen to hydrogen peroxide which are accumulated intracellular and released to inhibit other neighboring microbes. These synergistic inhibitory activity, are neutralized with addition of NaOH to the organic acid. Correspondingly the hydrogen peroxide after addition of catalase is converted to water. Certain NCFS had shown more antimicrobial activity than CFS, indicating they are potentially strong bacteriocin producers.

Table 3 Antimicrobial activity of PPLAB strains from *koozh* against pathogens

Strains	Isolate	*B. cereus*		*E. aerogenes*		*E. coli*		*K. pneumonia*		*L. monocytogenes*		*M. luteus*		*P. vulgaris*		*S.aureus*		*S. flexneri*		*S. typhi*		Total Inhibition %	
		Zone of Inhibition (ZI) mm																					
		CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS	NCFS	CFS [b]	NCFS [c]
Enterococccsu sp.	Sha 1	0	10	0	0	32	15 [a]	27	10 [a]	0	12	0	0	0	0	27	12 [a]	0	0	0	11	30	60
	Sha 8	22	0	0	10	23	15 [a]	0	12	20	12	0	0	0	11	10	15	0	0	0	10	10	70
	Sha 22	0	0	45	15 [a]	32	15 [a]	30	12 [a]	42	11 [a]	25	0	25	0	22	14 [a]	22	0	22	0	90	50
	Sha 24	0	11	22	13 [a]	25	16 [a]	0	12	0	11	20	0	0	0	0	13	25	0	61	11 [a]	80	70
	Sha 28	40	11 [a]	50	19 [a]	47	14 [a]	0	12	0	13	19	0	32	0	18	11	15	0	32	10	50	70
	Sha 35	45	12 [a]	55	14 [a]	15	14 [a]	35	13 [a]	53	19 [a]	55	0	52	11 [a]	12	12	18	0	80	10	80	80
Lactobacillus sp.	Sha 7	30	11 [a]	0	20	11	15 [a]	25	13 [a]	0	14	27	0	25	11 [a]	05	15 [a]	0	0	0	15	40	80
	Sha 31	0	10	73	21 [a]	55	14 [a]	32	12 [a]	32	13 [a]	50	0	42	11 [a]	32	12 [a]	16	0	30	0	80	70

[a] Indicate a significant difference between CFS and NCFS ($p<0.01$)

[b] PPLAB CFS which produced ZI > 2 mm against indicator strains

[c] NCFS which produced ZI > 1 mm against indicator strains

In the current antibiotic study (Table 4) intrinsic resistance to GI and G II antibiotics were identified in *Enteroccocus sp.* and to G III in *L. plantarum*, which according to Ahn et al. (1992) is due to the presence of β lactam, cat gene and tet (S) plasmid. LAB antibiotic resistance has been documented in many traditional fermented foods, similar to the commercial products (Temmerman et al. 2003). But resistance of PPLAB to antibiotics may be beneficial if the host is under antibiotic therapy, providing dual protection to the host.

The antimicrobial activity of isolates against specific pathogens could help prevent the development of antibiotic resistance strains. The main aim of these PPLAB is to reach the distal ileum and colon in large amounts to facilitate adhesion, colonization and reduction of pathogens *in vivo*. As most of them have been satisfied *in vitro* these isolates could be considered as probiotic LAB (PLAB).

Genotyping for identification of bacteria and its geographical distribution

Molecular characterization through isolating ribosomal RNA is an essential taxonomic tool and is an unswerving method for identifying the genera of lactic acid bacteria to species, subspecies and strain levels.

All the eight PLAB strains displaying probiotic characteristics as per FAO and WHO (2006) criteria were partially sequenced for 16S ribosomal DNA and identified as *E. hirae* - Sha 1, Sha 8, Sha 22, Sha 24, Sha 28; *E. faecalis* - Sha35; *B. amylolquefaciens* -Sha 31 and *L. plantarum* - Sha 7. When compared with other similar genetic sequences, strains were from fermented foods, agriculture, livestock and feces widely distributed in Eurasia (Table 5): 8 from China and India; 4 each from Japan and Spain; 3 in Iran, Korea and Demark; 2 in Thailand; 1 in Indonesia, Taiwan, Serbia, Egypt, South Africa, France and UK.

Characteristics and storage effects of encapsulated PLAB

The SDP and FDP containing encapsulated LAB had low moisture content of 6 ± 1 %, contributing to storage stability. The colour of SDP was found to be very similar to skim milk powder but the FDP had darkened colour due to the prolonged duration of drying. SEM analysis (Fig. 1a) indicated larger particle size in FDP compared to the SDP due to the coalescence of water and particle bridging.

Rehydration is an important step in dried powders for assessing the final probiotic product that also depends on temperature of the solution. Rehydration in a slow manner would decrease the osmotic shock. The main effect of spray drying is to increase the solubility of the mixture, to decrease clogging in the spray nozzle, eventually increasing the survival rate greater than control. Encapsulating materials provide resistance against damage to the PLAB, preventing significant

Table 4 Antibiotic susceptibility profiles of PPLAB from *koozh*

Antibiotics	*Enterococcus sp.*						*Lactobacillus sp.*	
	Sha 1	Sha 8	Sha 22	Sha 24	Sha 28	Sha 35	Sha 7	Sha 31
Group I								
Ampicllin	S	S	S	S	S	S	S	S
Amoxyclav	S	S	S	S	S	S	S	S
Pencillin G	R	R	MS	R	R	R	R	R
Cefuroximin	R	R	R	R	MS	R	R	R
Cefixime	R	R	R	R	R	R	R	R
Vancocmycin	S	R	R	MS	R	R	S	R
Bacitracin	R	MS	R	R	R	R	R	R
Group II								
Amikacin	R	R	MS	R	R	R	R	R
Gentamycin	R	S	MS	R	R	R	R	R
Streptomycin	R	R	R	R	R	R	R	R
Azithromycin	S	S	S	S	S	S	S	S
Erythromycin	R	R	R	R	R	MS	R	MS
Chloramphenicol	S	S	S	S	R	S	S	S
Tetracycline	S	S	R	S	S	MS	S	S
Group III								
Norofloaxicin	MS	R	R	MS	MS	R	R	R
Ciprofloaxicin	S	R	R	R	MS	R	S	R
Nalidixic acid	R	R	R	R	R	R	R	R
Levoflaxicin	S	S	R	MS	R	R	MS	MS
Gemoflaxicin	MS	S	MS	R	MS	R	MS	MS
Galtifloaxin	MS	MS	MS	R	S	MS	S	MS
Overall Resistance %	45	50	55	60	55	65	50	55

R resistant, *MS* moderately susceptible, *S* susceptible

drying effects while reducing structural and physiological damage to the cells.

Storage temperature is an important parameter for cell survival. Only a 2 log reduction was observed when spray dried *L. plantarum* was stored for 16 weeks at 27 °C. Storage survival of all PLAB isolates was better at 4 °C and at −20 °C at the end of 16 weeks than ambient temperatures (27 °C, 37 °C) (Fig. 1b). The decrease may be attributed to disruption membrane and cell deactivation dependent final moisture concentration.

The freeze dried *L. plantarum* as in the case of spray dried was stable with good survival after 16 weeks storage at low temperature (4 °C, −20 °C) compared to ambient (Fig. 1b). Survival of freeze dried PLAB isolates was poorer compared to spray dried isolates, due to the week long preparation compared to SDP. But for starter probiotic cultures freeze dried strains are preferred due to storage stability.

In our study *Enterococcus sp.* counts declined rapidly than the *Lactobacillus sp.*, however Ivanova et al. (1998) observed that the inhibitory activity of *Streptococcus* at 4 °C was not affected for more than 2 months and in frozen state was not influenced for 6 months. Following spray drying, the lipid oxidation during storage changes lipid cell membrane composition. The limitation of drying process was the loss of viability during its storage even though no spectacular losses were seen during processing. The composition of the encapsulated powder, oxygen content and glass transition temperature have an important effect on the survival of the dried powder. It is to be noted that medium used for encapsulating probiotics for food product preparation would not provide precise protection during storage of microbial cells. The probiotic storage life could be increased by the use of better encapsulating agent, suitable packaging material and vacuum packaging.

Viability of PLAB after encapsulation

During spray drying and freeze drying (Table 6) LAB counts reduced from 10^9–10^{10} CFU mL^{-1} to 10^7–10^8 CFU mL^{-1} suggesting good retention of viability after encapsulation. The SDP resulted in 0.10 ± 0.06 log reduction with overall survival of 98 % (significant difference, $p < 0.05$). Similarly FDP had a log reduction of 0.9 ± 0.3 with overall survival of 91 % (significant difference,

Table 5 Molecular identification of PLAB and their sequence analog of geographical distribution

Isolate no.	Species Identified	Source Code	Query Length [bp]	Similarity [%]	Few e.g., of analogous gene code	Query Length [bp]	Source	Country
Sha 1	*Enteroccocus hirae*	KF040093	970	98	KC699174	1430	Ewe bulk tank milk	Spain
					LC035115	1459	Wild boar- *Sus scrofa*	Thailand
					LC027236	1657	Commercial swine feces	Thailand
Sha 8	*Enteroccocus hirae*	KF040095	983	95	LC027236	1657	Commercial swine feces	Thailand
					LC035114	1461	Wild boar - *Sus scrofa*	Thailand
					KF183510	1454	Fish processing waste	India
					KP662076	1535	Fermented Fresh Pepper	China
					EU919863	1487	Cock- poultry	China
					JQ411243	1493	Sicilian Pig breed- Suino Nero Dei Nebrodi	Italy
					EU722743	1477	Human Feces	UK
					FN822766	1478	Leaf of northern bedstraw	Finland
					KF040098	1421	Ewe bulk tank milk	Spain
Sha 22	*Enteroccocus hirae*	KF040096	993	95	KC699174	1430	Ewe bulk tank milk	Spain
Sha24	*Enteroccocus hirae*	KF040097	1015	95	KC699174	1425	Ewe bulk tank milk	Spain
Sha 28	*Enteroccocus hirae*	KF040098	1001	94	KC699166	1430	Ewe bulk tank milk	Spain
Sha 35	*Enteroccocus faecalis*	KF040099	981	No match found at species level				
Sha 31	*Bacillus amyloliquefaciens*	KF386011	439	99	KF702296	885	Corn sheath of Saffron	India
					KF018921	964	Fish	India
					KJ567097	1517	Ginger rhizosphere soil	India
					KM853035	1429	Tea phylloplane	India
					KM406427	779	Lemon	India
					KP973969	1377	Rhizosphere of Perenial herb *Mimosa sp*.	Indonesia
					KJ716497	1024	Edible mushroom- *Agaricus bisporus*	China
					KP261025	1020	Moldy corn	Taiwan
					KM922581	1359	Fermented soybean paste	Korea
					KP273195	901	Baker's yeast	Egypt
Sha 7	*Lactobacillus plantarum*	KF040094	1004	93	KJ802485	1539	Infant stool sample	India
					JX183220	1509	Goat Milk and Cow	India
					CP004082	3203964	Healthy newborn fecal	China
					KR149356	1489	ZhenJiang aromatic vinegar- Fumigated vinegar	China
					KJ764641	1521	Kefir	China
					KM005165	1489	Silage	China
					KC166237	1493	Tibetan kefir grains	China
					KF583521	1492	Intestine of Rainbow trout-salmon fish	China
					LC042605	1492	Pickle vegetable	Japan
					AB601179	1525	Italian rye pasture	Japan
					AB713901	1489	Mixed pasture of Timothy and orchard grass	Japan
					AB819501	1489	Intestinal mucosal content of bivalvia	Japan
					KM670024	1493	Vagina	Korea
					KM670021	1499	Oyster	Korea
					KC113208	1472	Traditional Salted Crab Poo-Khem	Thailand
					KM495889	1499	Siahmazgi cheese	Iran
					KP090130	1452	Vaginal tract of healthy woman	Iran
					KF472174	1527	Traditional dairy product	Iran
					HE962114	1315	Travnik young cheeses, sweet creams and sweet kajmaks	Serbia
					JX409637	984	Piglet feces	Denmark
					JX409633	970	Fermented liquid feed	Denmark

Table 5 (continued)

Isolate no.	Species Identified	Source Code	Query Length [bp]	Similarity [%]	Few e.g., of analogous gene code	Query Length [bp]	Source	Country
					JX409627	978	Unsupplemented whey permeate silage	Denmark
					KC351898	1555	Wine made up of grillo grapes	Italy
					JX426120	1502	Natural wine	Italy
					KC416990	1491	Wheat flour	Italy
					JX025073	1559	Wine of the Rhone Valley	France
					JX968494	1548	Barley malt	South Africa

$p < 0.05$). Survival of strains was better ($p < 0.05$), after spray drying (0.10 ± 0.06 log reduction, survival of 98 %) compared to freeze drying (0.9 ± 0.3 log reduction, survival of 91 %), due to homogenous film formation, emulsification and product formation in less time. But freeze drying may help to retain biological properties of foods, e.g., fermented freeze dried soy milk had relatively lower loss of antioxidant activities than the spray dried product (Rivera-Espinoza and Gallardo-Navarro 2010). Spray-dried starter cultures cannot be used for direct inoculation in dairy fermentation due to increased lag phase before the onset of growth (Boza et al. 2004). *L. plantarum* showed good survival after spray drying at 160 °C, 3 bars psi (Golowczyc et al. 2011). Rice soyabean gruel fermented with *L. plantarum* when spray dried produced a functional starchy food (Nguyen et al. 2007). *L. plantarum* when lyophilized with alginate, fermented meat faster than unencapsulated cells (Kearney et al. 1990). *E. faecalis* demonstrated heat and oxidative tolerance during spray drying (Santivarangkna et al. 2007).

Survival of encapsulated PLAB in simulated fluid

All freeze dried PLAB (FDP) were tolerant to gastric conditions (pH 2.0, pepsin). These isolates showed little change after 2 h in SGF, although a slight decrease was recorded in the last 30 min (Fig. 2a). Survival of encapsulated PLAB in gastric simulation was better when spray dried than freeze dried (Fig. 2a). Encapsulated *L. plantarum* Sha7 survived better compared to the rest of the strains.

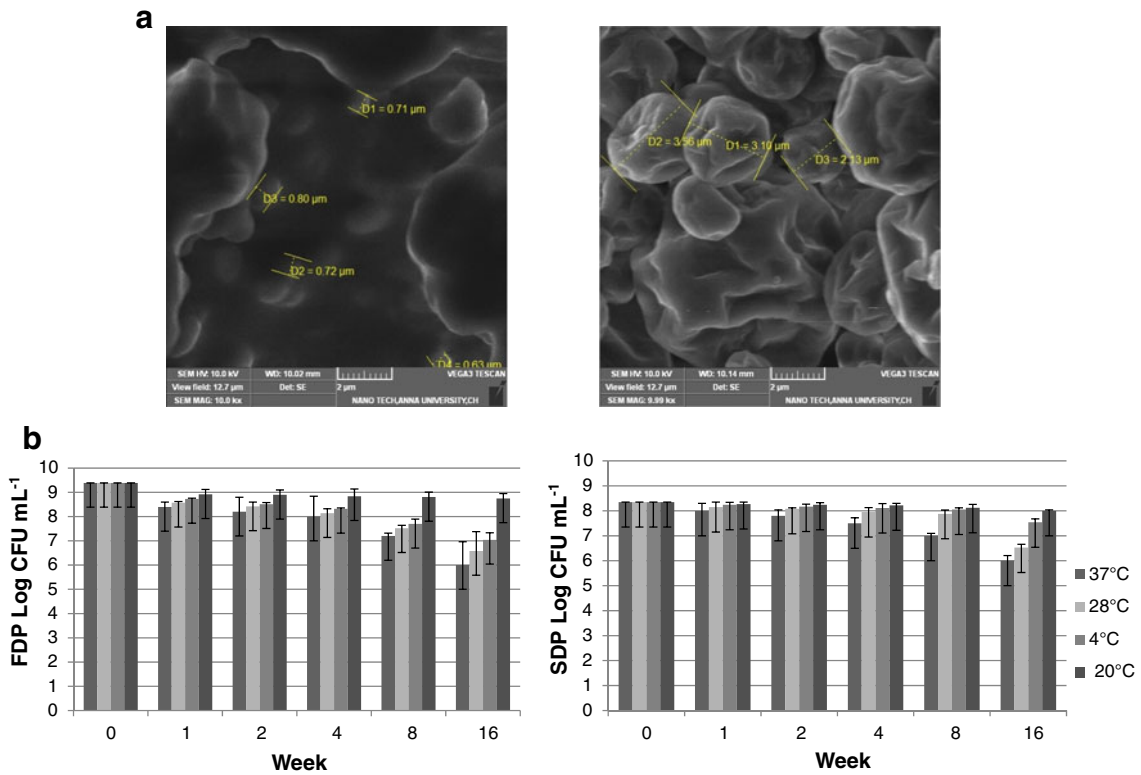

Fig. 1 FDP and SDP of Sha 7- *L. plantarum* **a** SEM analysis and **b** Storage

Table 6 Viability of PLAB isolates before- after freeze drying (FDP) and spray drying (SDP)

Isolate	Strain	Freeze Drying log CFU mL−1		FDP log Reduction	Spray Drying log CFU mL−1		SDP log Reduction
		Before	After		Before	After	
E. hirae	Sha1	8.97 ± 0.03	8.58 ± 0.14	9.73 ± 0.20	9.63 ± 0.15	9.63 ± 0.15	0.10 ± 0.12
	Sha8	9.12 ± 0.20	8.59 ± 0.15	9.03 ± 0.03	8.85 ± 0.14	8.85 ± 0.14	0.18 ± 0.17
	Sha22	9.34 ± 0.05	8.67 ± 0.10	9.87 ± 0.08	9.85 ± 0.03	9.85 ± 0.03	0.02 ± 0.10
	Sha24	10.29 ± 0.08	9.30 ± 0.33	9.43 ± 0.03	9.39 ± 0.20	9.39 ± 0.20	0.04 ± 0.24
	Sha28	10.38 ± 0.08	9.19 ± 0.03	9.36 ± 0.05	9.20 ± 0.10	9.20 ± 0.10	0.16 ± 0.16
E. faecalis	Sha35	10.41 ± 0.12	9.04 ± 0.05	9.66 ± 0.00	9.47 ± 0.17	9.47 ± 0.17	0.19 ± 0.17
L. plantarum	Sha7	9.00 ± 0.00	8.35 ± 0.17	9.54 ± 0.12	9.39 ± 0.05	9.39 ± 0.05	0.15 ± 0.17
B. amyloliquefacians	Sha31	10.27 ± 0.03	9.18 ± 0.2	9.30 ± 0.08	9.17 ± 0.33	9.17 ± 0.33	0.13 ± 0.17

Encapsulated PLAB obtained from spray drying and freeze drying showed significant rise in cell count in SIF up to 2 h due to the protective coating of skim milk that supports growth (Fig. 2b). SDP and FDP had thrived in simulated fluids (skim milk). Our SIF results were similar to Teixeira et al. (1994) where there was no significant difference in the viability of spray dried and freeze dried isolates of *L. bulgaricus*.

In the GIT and food products the viability and survivability of probiotics decreases due to exposure to environmental factors: organic acids, hydrogen ions, molecular oxygen and antibacterial components (Mortazavian et al. 2007).

Spray drying also resulted in isolates with tolerance to both gastric pH and enzyme with excellent survival in the second hour. *L. plantarum* isolated from *kefir* grains and encapsulated by spray drying did not lose its viability and acidifying activity or its capacity to adhere to intestinal walls (Golowczyc et al. 2011). In all the PLAB isolates cell numbers per gram of the spray/ freeze dried powder was 10^8 to 10^9 cells, which is above the recommended probiotic dosage of 10^7–10^8 CFU g^{-1} laid down by FAO and WHO (2002) indicating their potential for application in food products.

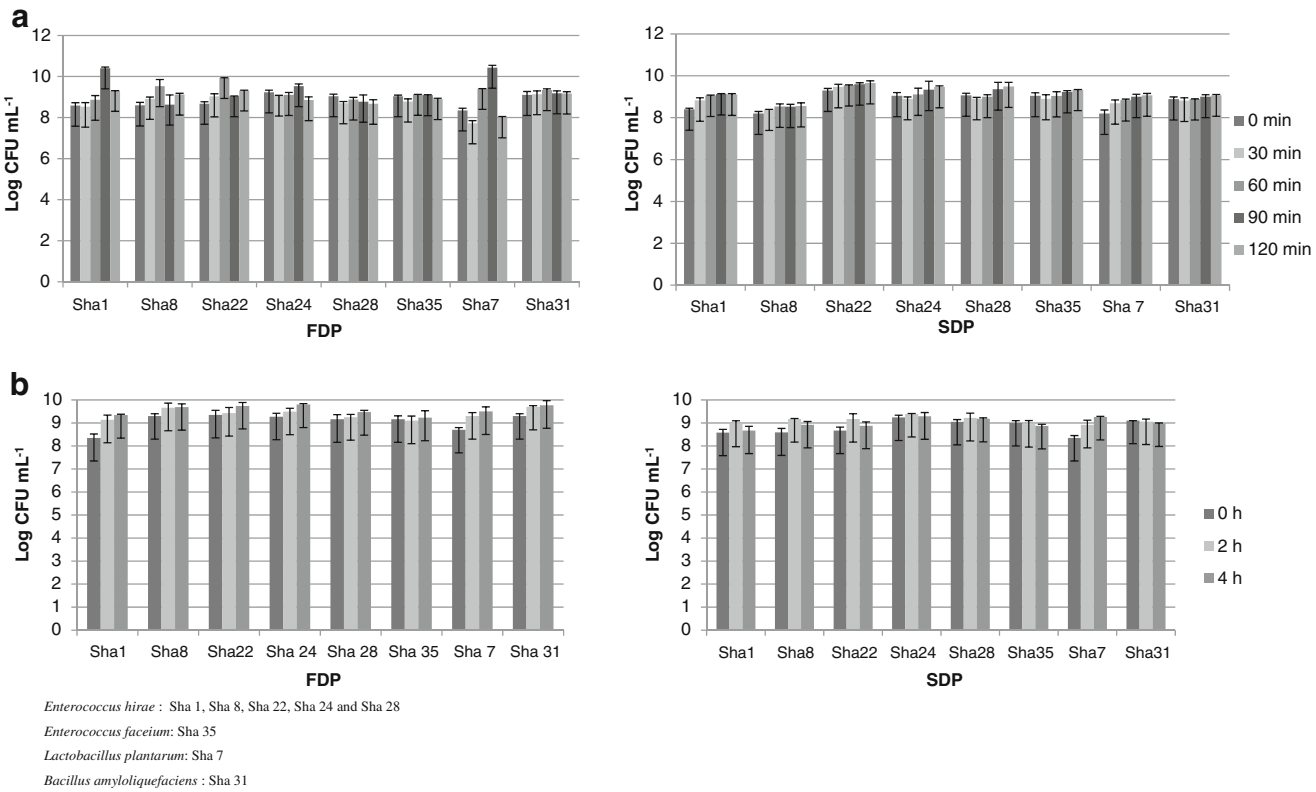

Fig. 2 FDP and SDP survival of Sha 7- *L. plantarum* **a** SGF and **b** SIF

Conclusion

Thus *koozh* a traditional millet fermented food is a source of a diverse group of NSLAB, largely strains of *Leuconostoc sp*., *Enterococcus sp*. and *Lactobacillus sp*. Further, eight PLAB strains among the 69 isolates, were acid and bile resistant with antimicrobial activity *in vitro* demonstrating their probiotic nature. As a value added product SDP performed better than FDP in simulated gastric and intestinal fluids and was stable for 16 weeks when stored at low temperature. They may be used as starter cultures in cereal based or other fermented foods and/or as a source of bacteriocins. *In vivo* evaluation can identify their specific application in promoting/protecting health of consumers. The outcome of this study underscores the need to modernize and commercialize traditional fermentation processes on a scientific basis. This will ensure the quality and safety of such foods and enable consumers to derive the health benefits.

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