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Probiotic traits of lactic acid bacteria isolated from aerial surfaces of pomegranate

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Abstract: Lactic acid bacteria isolated from aerial plant surfaces of pomegranate viz., *Lactococcus lactis* subsp. *cremoris* PB6, *Lactobacillus brevis* PFR77, *L. lactis* subsp. *cremoris* PFL9, *Enterococcus faecium* PB119 and *L. lactis* subsp. *lactis* PFL4 were tested for hemolysis, antibiotic susceptibility, gelatinase activity, tolerance to low pH and bile, resistance to digestive enzymes and antibacterial activity against human pathogens. All the isolates were non-hemolytic and non-gelatinolytic. These isolates were sensitive to clinically important antibiotics (amoxyclav, ampicillin, chloramphenicol, doxycycline, erythromycin, gentamycin, methicillin, penicillin G, Rifampicin and tetracycline), whereas resistance was recorded in case of norfloxacin and vancomycin. They could survive for more than 2h at pH 3 and the survival period of PB6, PFR77 and PFL9 was 3 h. Thus, the tolerance level of these LAB isolates was higher than the suggested values which indicate their good tolerance to stomach pH. In case of PB6, PFR77 and PFL9, around 80% of population survived in presence of 0.3% bile for 6 h. Also, they could survive at 1% concentration for 6 h. Thus, these isolates had good bile salt tolerance. All the isolates inhibited the growth of Gram positive and Gram negative bacterial pathogens with greater inhibition zones. PB6, PFR77 and PFL9 showed 60-70% survival in presence of trypsin whereas in presence of pepsin the survival decreased in between 50 and 60%. These isolates also showed good resistance to amylase and lipase. Thus, these LAB may be good candidates in formulation of probiotic preparations.

Key words: Probiotic; Lactic acid bacteria; *Lactobacillus*; *Lactococcus*; *Enterococcus*

Introduction

Lactic acid bacteria (LAB) are amongst the bacteria with ancient and broad applications because of their diverse metabolic capabilities. LAB are most commonly included under Generally Recognized as Safe (GRAS) (Salminen *et al.,* 1998) category, as there have been very few cases of infection due to LAB. LAB are used in the probiotic preparations due to their GRAS status and various technological properties. Probiotic is a live microbial feed supplement, when consumed in adequate amounts, beneficially affects the host animal by improving its intestinal microbial balance (WHO, Gaggia *et al.,* 2010). The beneficial properties of probiotic bacteria are, adherence to intestinal epithelium and antagonistic to pathogens, stimulation or suppression of immune response, anti-carcinogenic and anti-mutagenic activities, alleviation of lactose intolerance, reduction in serum cholesterol and reduction in blood pressure (Salminen *et al.*, 1998; Klaenhammer & Kullen 1999). The probiotic strains have to pass through many barriers for their final reach in the intestine to deliver beneficial effects. They must pass through highly acidic conditions in stomach and survive following exposure to bile secretions and digestive enzymes. LAB used in the probiotic preparations mainly involve species of *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus* and *Pediococcus* (Gaggia *et al.,* 2010). LAB are ubiquitous in the environment and their isolation is reported from vegetables (Carr *et al.,* 2002; Sathe *et al.,* 2007), aerial plant surfaces (Magnusson *et al.,* 2003), pickled cabbage (Laitila *et al.,* 2002), grass silage (Strom *et al.,* 2002), malted cereals (Rouse *et al.,* 2008) and also from soil (Chen *et al.,* 2005). In view of this background, LAB isolated from aerial surfaces of pomegranate were tested for their probiotic traits.

Materials and Methods

Isolation and identification of LAB

Pomegranate sample types such as flowers, buds, leaves and fruits were inoculated into 10ml sterile selective LAB broth (Hi-Media) followed by incubation at 30°C for 48 h. A loopful from this was streaked on sterile MRS agar and incubated at 30°C for 48 h. The isolates were identified by phenotypic characteristics and 16S rRNA gene sequencing (Gajbhiye *et al.,* 2012). The sequences were submitted to NCBI GenBank and their accession numbers are PB6 (JN792509), PFR77 (JN792499), PFL9 (JN792508), PB119 (JN792505), PFL4 (JN792510).

Hemolytic activity of LAB

LAB isolate was grown in MRS broth for 48h at 30°C and spotted on blood agar plate containing human blood. The plates were incubated at 37°C for 48 h and hemolysis was recorded. *Acinetobacter hemolyticus* and *E. coli* were used as positive and negative controls, respectively.

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Antibiotic susceptibility of LAB

Antibiotic susceptibility test was performed by standardized disc diffusion method on MRS agar. LAB cell suspension (10^6 cfu ml^{-1}) was spread on MRS agar plate separately and antibiotic disc (hexadiscs) (HiMedia, India) was placed onto it. The plates were incubated at 4°C for 2 h for pre-diffusion and then transferred to 30°C incubator for 48 h. Results were recorded by measuring inhibition zone diameters and expressed as sensitive, intermediate or resistant according to the Clinical and Laboratory Standards Institute (CLSI 2007) and as described by Charteris *et al.,* (1998). All LAB isolates were tested for their sensitivity towards various antibiotics listed in table 1.

Gelatinase activity

Hydrolysis of gelatin by LAB was tested using nutrient gelatin medium. Hydrolysis was detected as liquefaction of the test medium and uninoculated remains solid.

Low pH tolerance

The pH of MRS broth was adjusted to 2, 3, 4 and 6 with 2N HCl (Chang *et al.,* 2010). Ten milliliters of MRS broth of each pH was dispensed in tubes and inoculated separately with LAB cell suspension to a final concentration of approximately 10^8 cfu ml^{-1}. The total viable count was determined on MRS agar at an interval of 1 h for 6 h and the survival (%) was calculated.

Tolerance to bile

MRS broth was supplemented with oxbile to a final concentration of 1, 0.5 and 0.3% (w/v) (Chang *et al.,* 2010). Ten milliliters of MRS broth of each concentration was inoculated separately with LAB cell suspension to a final concentration of approximately 10^8cfu ml^{-1}. The total viable count was determined on MRS agar at an interval of 1 h for 6 h and the survival (%) was calculated.

Resistance to digestive enzymes

Tolerance to digestive enzymes viz., pepsin, trypsin, amylase and lipase was determined (Charteris *et al.,* 1998). Trypsin, amylase and lipase solutions were prepared in phosphate buffered saline (pH 7) and pepsin solution was prepared in citrate buffer (pH 2.5) to a final concentration of 0.01% (w/v). Ten µl of LAB cell suspension (10^8 cfu ml^{-1}) was mixed with 1 ml of enzyme preparation and incubated for 4h at 37°C. Subsequently, the solutions were diluted and bacterial population was determined by plating on MRS agar. The total survival (%) was calculated.

Antagonistic activity against bacterial human pathogens

Culture supernatants from LAB were tested for the inhibition of human bacterial pathogens viz., *E. coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* by well diffusion assay method. The inhibition zone diameters were recorded following incubation.

Statistical analysis

The data was analyzed for significant difference with Post Hoc Test (Tukey's HSD procedure) using SPSS 18. A 95% confidence level was used for the analysis so that $P \leq 0.05$ were considered to be statistically significant.

Results

LAB isolates were analyzed for the safety characteristics such as hemolytic and gelatinolytic activities. None of the tested LAB isolates caused hemolysis on blood agar. *Acinetobacter hemolyticus* was used as positive indicator that showed β-hemolysis and *E. coli* was used as negative indicator that showed no hemolysis (Figure 1). Also these LAB isolates were non-gelatinolytic. All LAB isolates were sensitive to clinically important antibiotics (amoxyclav, ampicillin, chloramphenicol, doxycycline, erythromycin, gentamycin, methicillin, penicillin G, Rifampicin and tetracycline), that were tested except for norfloxacin and vancomycin, where strains have shown resistance. A difference in the response to other antibiotics by LAB isolates was noticed (Table 1). All the LAB isolates could survive for more than 2h at pH 3; however, the population of PB6 and PFL9 was higher. At pH 2, the survival period of PB6, PFR77 and PFL9 was 3 h (P>0.05) (Figure 2). The normal pH of gastric juice in an empty stomach is 0.9 to 1.5. In bile tolerance test it was noticed that around 80% of population of PB6, PFR77 and PFL9 survived in presence of 0.3% bile for 6 h (P>0.05). Also, they could survive at 1% concentration (which is 3 times higher) for 6 h. Thus, these LAB had good bile salt tolerance (Figure 3).

Antagonistic activity is another vital characteristic of probiotic bacteria. This character was also demonstrated by these LAB. They inhibited the growth of Gram positive and Gram negative pathogens with greater inhibition zones as shown in table 2 and Figure 4. LAB are also exposed to several digestive enzymes during passage from oral cavity to intestine. Thus, tolerance to digestive enzymes viz., pepsin, trypsin, amylase and lipase was tested. PB6, PFR77 and PFL9 showed 60-70% survival in presence of trypsin (P>0.05) whereas in presence of pepsin the survival decreased in between 50 and 60%. These LAB also showed good resistance to amylase and lipase too (Figure 5).

Table 1: Antibiotic sensitivity pattern of antimicrobial lactic acid bacteria

Antibiotic (µg per disc)	*L. lactis* subsp. *cremoris* PB6	*Lact. brevis* PFR77	*L. lactis* subsp. *cremoris* PFL9	*E. faecium* PB119	*L. lactis* subsp. *lactis* PFL4
Amoxyclav (30)	S	S	S	S	S
Ampicillin (10)	S	S	S	S	S
Ceftazidime (30)	R	R	R	R	I
Cephalothin (30)	R	R	R	I	I
Cephoxitin (30)	R	R	R	R	S
Chloramphenicol (30)	S	S	S	S	S
Ciprofloxacin (5)	I	I	S	R	S
Clindamycin (2)	R	R	R	R	S
Co-Trimoxazole (25)	S	R	S	R	R
Doxycycline (30)	S	S	S	S	S
Erythromycin (15)	S	S	S	S	S
Fosfomycin (200)	R	R	R	R	S
Fusidic acid (30)	R	R	R	I	I
Gentamycin (10)	S	S	S	S	S
Linezolid (30)	S	S	S	R	R
Methicillin (5)	S	S	S	S	S
Nitrofurantoin (300)	S	R	R	R	R
Norfloxacin Nx (10)	R	R	R	R	R
Ofloxacin (5)	I	R	R	R	R
Oxacillin (1)	I	R	R	S	S
Piperacillin (100)	S	R	S	S	S
Penicillin G (10 units)	S	S	S	S	S
Rifampicin (5)	S	S	S	S	S
Teicoplanin (30)	R	S	R	I	R
Tetracycline (30)	S	S	S	S	S
Vancomycin (30)	R	R	R	R	R

S, Sensitive; I, Intermediate; R, Resistant

Table 2: Inhibition of human pathogenic bacteria by lactic acid bacteria

LAB isolate*	Inhibition zone diameter** (mm) produced against				
	Escherichia. coli	*Salmonella typhi*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Staphylococcus aureus*
PB6	35	18	18	14	15
PFR77	35	17	17	12	14
PFL9	35	18	19	15	15
PB119	35	18	18	15	15
PFL4	24	12	14	11	12

* Cell free supernatant prepared from 48 h old MRS broth, inoculated with respective LAB; *Determined by well diffusion agar technique

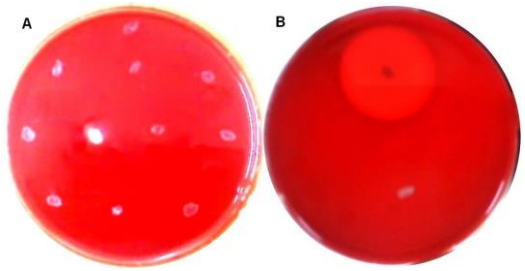

Figure 1: Test for hemolysis by LAB isolates (A). β-hemolysis (B, top) by *Acinetobacter hemolyticus* and no hemolysis by *E. coli* (bottom) on blood agar

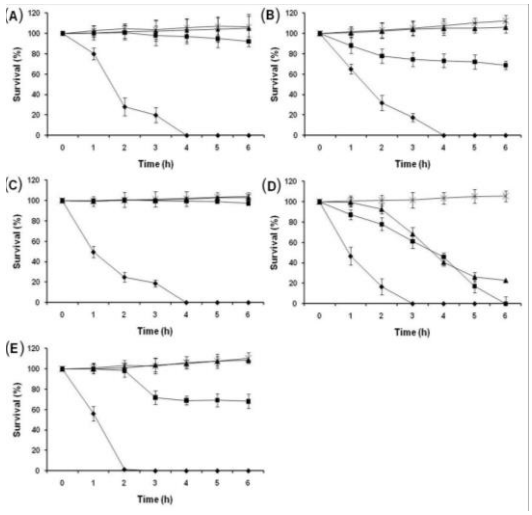

Figure 2: Survival of *L. lactis* subsp. *cremoris* PB6 (A), *Lact.brevis*PFR77 (B), *L. lactis* subsp. *cremoris* PFL9 (C), *E. faecium* PB119 (D) and L. *lactis* subsp. *lactis* PFL4 (E) at pH2 (♦), pH3 (■), pH4 (▲) and 6 (×). Each data point represents mean of three replications and bars extending from means represent standard errors of that mean

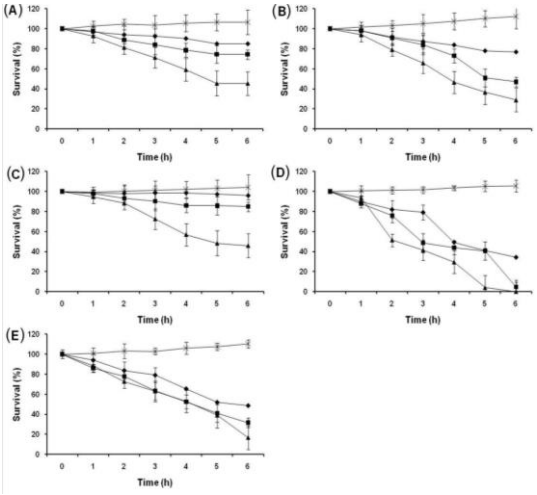

Figure 3: Survival of *L. lactis* ssp. *cremoris* (A), *Lact. brevis* (B), *L. lactis* subsp. *cremoris* (C), E. *faecium* (D) and *L. lactis* subsp. *lactis* (E) in MRS broth supplemented with 1% (▲), 0.5% (■), 0.3% (♦) bile salt and MRS (×). Each data point represents mean of three replications and bars extending from means represent standard errors of that mean

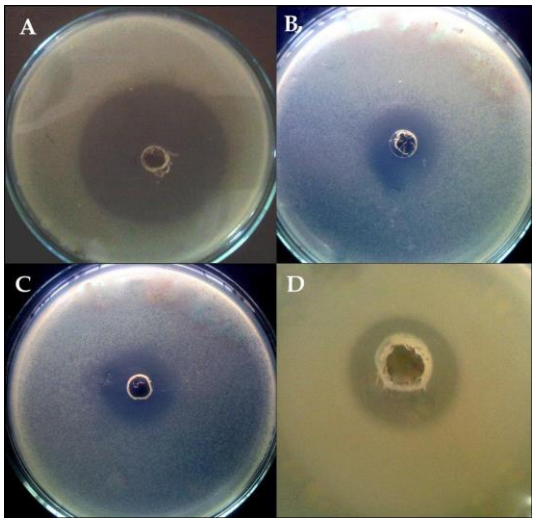

Figure 4: Inhibition of *E. coli* (A), *S. typhi* (B), *P. aeruginosa* (C) and *K. pneumoniae* (D) by culture supernatant of *Lactococcus lactis* subsp. *cremoris* PB6

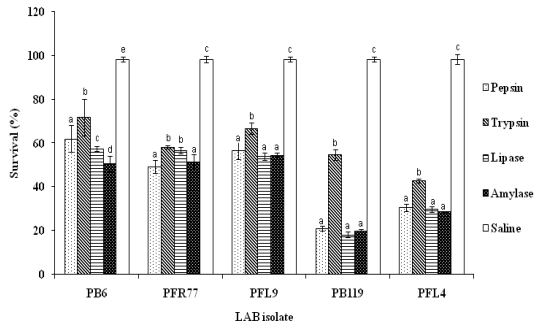

Figure 5: Survival of *L. lactis* subsp. *cremoris*, *Lact. brevis*, *L. lactis* subsp. *cremoris*, *E. faecium* and *L. lactis* subsp. *lactis* in presence of digestive enzymes. Each data point represents mean of three replications and bars extending from means represent standard errors of that mean. Values with different letters in a column for each cultural condition, are significantly different ($P<0.05$) according to Tukey HSD test. Columns with same letters for each isolate are not significant

Discussion

Several guidelines are suggested to claim a bacterial strain as potential probiotic strain (Ganguly *et al.,* 2011). Among them, tolerance to gastrointestinal conditions is a prerequisite for screening. LAB are widely used in several foods as starter culture organisms due to their technological properties. It has a long history of safe use, but in rare cases, they could cause clinical infections like bacterimia and endocarditis (Snydman, 2008). *Lactococcus* sp. may cause poor α-hemolysis (Casalta & Montel, 2008). Gelatinase enzyme is considered as a virulence factor as it may hydrolyze collagens that initiate inflammatory response (Barbosa *et al.,* 2010). However, in present investigation, all the LAB were non-hemolytic and non-gelatinolytic.

After meal, the food passes from oral cavity to stomach where it is retained for approximately 2 to 4 h and then passes into intestine (Martini *et al.,* 1987). Therefore, generally LAB are incubated for 4h at pH 2 and 3. The tolerance level suggested for probiotic organism is 2h at pH 3 (Usman & Hosono, 1999). Thus, the tolerance level of these LAB isolates was higher than the suggested values which indicate that these LAB have good tolerance to stomach pH. The concentration of bile salt in human intestine is 0.03 to 0.3% (Lee & Salminen, 1995) and according to the suggested guidelines, the survival in 0.1% bile is considered as good tolerance. All the LAB isolates thus have good tolerance to bile too.

LAB may contain transferable antibiotic resistance genes (Mathur & Singh, 2005) thus, it is recommended to determine the antibiotic resistance pattern of LAB. Lactobacilli have natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamycin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim and vancomycin (Danielsen & Wind, 2003).

Inhibition of enteropathogenic bacteria is an extra advantage associated with probiotic strains. Few strains of LAB have potential to fight gastrointestinal pathogenic bacteria such as *Helicobacter pylori*, *E. coli* and *Salmonella* (De Vuyst & Leroy, 2007). Inhibitory activity of *Lact. buchneri* and *L. lactis* subsp. *lactis* against *E. coli* and *Staphylococcus aureus* has been demonstrated (Zeng *et al.,* 2010). Asahara *et al.,* (2010) have confirmed the anti-infectious activity of *Lact. casei* against multi-drug resistant *S. enterica*. Commercial fermented

milk products that contain probiotic strains viz., *Lact. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, decrease the survival of *Mycobacterium avium* subsp. *paratuberculosis* (Van Brandt *et al.,* 2011). Bacteriocins from LAB also have antagonistic effect on *Helicobater pylori*, pathogen that causes gastritis and peptic ulcers (Kim *et al.,* 2003). *E. faecium* isolated from milk shows inhibitory activity towards *Listeria* species due to production of multiple bacteriocins (Chanos & Williams, 2010). In agreement to this, these LAB had broad spectrum of activity against enteropathogens that may be attributed to organic acids or bacteriocins (Zeng *et al.,* 2010). Several studies have demonstrated the tolerance of probiotic LAB to digestive enzymes (Morandi *et al.,* 2013).

Several research publications described the low pH tolerance and bile salt tolerance in case of species of *Lactobacillus* (Prins *et al.,* 2010). Zeng *et al.,* (2010) have demonstrated that *Lact. buchneri* possess probiotic properties of cholesterol reduction, acid and bile tolerance. Several LAB isolated from kimchi have probiotic properties, suitable for use as starter culture in yogurt fermentation (Chang *et al.,* 2010). Morandi *et al.,* (2013) described the probiotic potential of *E. lactis*. *E. faecium* have been used as probiotic culture in Cheddar cheese (Giraffa, 2003). According to Kimoto-Nira *et al.,* (2010), *L. lactis* is a probiotic bacterium with immunomodulatory activity, resistance to simulated gastrointestinal stress, including the presence of lysozyme, low pH, and bile.

Conclusion

Tolerance to gastrointestinal conditions is a prerequisite for screening of LAB with probiotic potential. In present study, LAB isolates have been demonstrated to possess safety characters and several desirable traits which include tolerance to low pH, high concentration of bile salts and digestive enzymes. Thus, entire antimicrobial LAB isolates with potent technological properties can be used in probiotic preparations and related dairy processes. This may be the first report of evaluation of probiotic traits of LAB of pomegranate origin.

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