Functional Attributes of Lactic Acid Bacteria Isolates in Conjunction with Most Wanted Probiotic Properties

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I. Introduction

Now-a-days a high percent of the population in the world has gastrointestinal problems as a consequence of an improper diet, stress and unhealthy lifestyle1. The health benefits attributed to probiotic bacteria in literature have been categorized into two aspects nutritional and therapeutic2. According to FAO/WHO, probiotics are live organisms which when administered in adequate amounts may confer health benefits on the host3. As stated by studies probiotics are digestive bioregulators or direct fed microbials (DFMs) as they produce various enzymes that help in digestion of monogastric animals, phytases and lipases being the important ones4,5. Probiotics considered as alternatives to antibiotics4,6 are well known for their production of bacteriocins, organic acids etc4,7. The probiotic, with viability for a long period during storage and transport, resistant at low pH in the stomach and tolerance to bile concentrations, along with bacteriocin activity can be candidates for inclusion in fermented food industry. Development of fermented milk products containing a bacteriocingenic strain of Lactococcus lactis has broad potential applications8. Bacteriocins produced by LAB are of particular interest owing to their potential application in the food industry as natural preservatives with little or no effect on the normal microbiota of host7,9,10,11. The probiotics having multifaceted health benefitting attributes which include prevention and treatment of various types of diarrohea, alleviation of lactose intolerance, modulation of gut microbiota, immunomodulation, and or alleviation off allergies and atropic diseases12. Remarkable importance of probiotics in health develops a great interest to study fermented food products as a source of new probiotic isolates. Herein, we are using Kaladi a fermented cheese product made of cow's milk as the richest source of LAB.

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Fermentation by LAB helps in preservation of food, improve its nutritional value and enhances its sensory properties. The role of probiotics in production of organic acids and other antimicrobial compounds during fermentation process can make non dairy fermented products an ideal vehicle to deliver probiotics to consumers13. In the present study we are trying to observe tolerance of LAB isolated from fermented milk products to simulated gastric acid conditions.

II. Materials and Method

A. Isolation and Morphological Studies

1 g of sample from above stated edible sources was dissolved in 9 mL of 0.85% normal saline. de Man, Rogosa and Sharpe (MRS) medium (HiMedia, India) was used for isolation, purification and maintenance of LAB isolates. Samples were enriched in MRS broth for 24 h at 37°C. Samples were spread on MRS agar plates for single colony isolation and incubated in an anaerobic jar at 37°C for 48 h. In order to study single colony morphology spread plate technique was adopted.

B. Morphological, Physiological and Biochemical Characterization of the Isolates

The isolates were tested for biochemical characteristics. Grams staining and catalase test was carried out for all the isolates. Gas production from glucose was determined for 24 h grown cultures. Growth of cultures at different temperatures (30, 37, and 45°C) was carried out in MRS medium. Growth of the isolates at different NaCl concentrations (3.5% and 6.5%) was studied. The growth of gram positive cocci was checked on mannitol salt agar (MSA) medium (HiMedia, India).

C. Tolerance to Bile and Low pH

The relevant physiological concentration of human bile range from 0.3-0.5%24-26 but the mean intestinal bile salt concentration is believed to be 0.3%. To study bile and low pH tolerance, bacterial cultures grown in MRS broth were

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centrifuged at 10,000 rpm for 10 min at 4°C. For this purpose, cultures grown in MRS broth were inoculated in 10 mL phosphate buffer saline (PBS), the composition of PBS was NaCl: 9 g/L, Na2HPO4.2H2O (HiMedia, India): 9 g/L, KH2PO4 (SRL, India): 1.5 g/L, pH 6.2, which was further adjusted to pH 3.0 using 10 M HCl (Merk, India). The cultures were incubated in above stated medium for a period of 2 h at 37°C. PBS adjusted to pH 6.2 was taken as control. The survival rate percentage was calculated by dividing log cfu N1 by log cfu N0 and the multiplication factor was 100 percent, N1 represents the total viable count of LAB strains after treatment with pH 3.0 or 6.2 and N0 denotes the total count of LAB strains before treatment.

D. Survival of LAB Isolates In Simulated Gastric Conditions

An important prerequisite for potential probiotic bacteria is to survive through harsh gastrointestinal tract (GIT) conditions. Cells grown in MRS broth for 24 h at 37°C were harvested and incubated in gastric conditions mimicked by addition of pepsin (1:10000, Himedia, India) in PBS solution at pH 3.0. The sterile pepsin solution was prepared by filtering through 0.22 micro meter pore size filter membrane (Moxcare, India) and added to the sterilized PBS buffer solution with final concentration of 3g/L. The bacterial cultures grown in MRS medium at 37°C for 24 h were centrifuged (10000 rpm at 4°C for 10 min). The cell pellets were suspended in sterile saline (0.85% NaCl, w/v) solution and added to PBS (pH 3.0) supplemented with sterile solution of pepsin, incubated at 37°C for a time period of 0-3 h. The total viable count of LAB isolates in PBS solution and simulated gastric juice conditions was studied on MRS agar after an incubation period of 24 h at 37°C. The survival rate was calculated using the equation as given above.

E. Identification of inhibitory substance by agar well diffusion method

Methodology adopted for the identification of inhibitory substance from LAB isolates was same as described by Angmo et al. 2016. All the LAB isolates from Kaladi were grown under anaerobic conditions at 37°C for 24 h in MRS broth. Cells were harvested after centrifugation at 10,000 rpm for 10 min at 4°C, supernatants of samples were filter sterilized using 0.22 µm pore size syringe filters (Moxcare, India). This filter sterilized cell free supernatant was assayed as following: Fraction A, cell-free supernatant of each LAB isolates. Fraction B, consisted of pH neutralized supernatant (pH 7.0) for detection of inhibition by organic acids. Fraction C, was used in order to ensure the proteinaceous character of bacteriocin, pH neutralized supernatants were treated separately with proteinase K (HiMedia, India) and pepsin at final concentration of 1 mg/mL at 37°C for 2 h. This inhibitory activity of fraction A, B and C was observed using agar well diffusion method against selected pathogens (*Bacillus subtilis* MTCC 121, *Mycobacterium smegmatis* MTCC 994, *Staphylococcus aureus* MTCC 3160, *Proteus vulgaris* MTCC 426 and *Escherichia coli* MTCC 1652) obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Plates were incubated over night at 37°C for zone formation.

F. Statistical Analysis

All analyses were carried out independently in triplicate. All data expressed here are in the form of mean ± SE (standard error).

III. Results and Discussions

A. Isolation, Morphological and Biochemical Characterization of Isolates

100 LAB isolates were obtained from thirty different edible sources i.e fermented dairy and non-dairy products; maximum isolates were obtained from Kaladi (Fig. 1). Kaladi, served as an indigenous and most promising source for LAB isolates having antibacterial and bacteriocin activity. Twenty LAB isolates from Kaladi were screened as best strains which showed highest survival rate in simulated gastric conditions. These twenty LAB isolates were non motile, catalase negative and gram positive (Table 1). Gram-positive and catalase negative identified as lactic acid bacteria37. Growth of LAB cultures was studied at 30, 37 and 45°C, 37°C was found to be an optimum temperature for the growth of all the cultures (Fig. 2). Growth at different NaCl concentrations was observed. NaCl is an inhibitory substance which has been reported 38 to inhibit the growth of certain types of bacteria and the present results showed that all of the isolates had the ability to grow at 3.5% and 6.5% NaCl concentrations (Table 1). These findings imply with the earlier studies of Razdan et al that the isolates which are tolerant to NaCl have the ability to withstand gastrointestinal conditions. Isolates having cocci morphology were streaked on MSA medium and incubated for 24 h at 37°C under anaerobic conditions and no growth was observed. So it shows the absence of pathogenic genera Staphylococcus.

B. Resistance to Low pH And Tolerance To Bile

Being resistant to low pH (3.0) and showing high survival rate at low gastric pH is one of the prerequisite for use of LAB as dietary adjuncts39. It helps the strains to survive for longer periods in acidic foods without large reduction in their viable numbers. Although in stomach the pH can be as low as 1.0, but in most in vitro assays pH 3.0 has been preferred40. For selecting the cultures resistant to low pH, PBS having pH (3.0) was used. Fig. 3 shows the survival rate of twenty isolates under low pH conditions. All the strains showed resistance at pH 3.0 after an incubation period of 2 h and the survival rate was above 70

% except two gram positive rods designated as K.U (b) and K.B (1). The maximum survival rate (99.87%) was observed in case of a gram positive cocci designated as K.U (a) and a gram positive rod designated as K. All the LAB isolates had an optimum growth at 37°C but K.U (a) could survive at 45°C and a good growth rate was observed for isolate K at 45°C in comparison to K.U (a) (Fig. 2).The mean intestinal bile concentration is believed to be 0.3% w/v. Ox bile concentration of 0.3% is considered to be a crucial concentration to evaluate a bile-tolerant probiotic characteristics. A considerable variation in resistance to bile salts among the different species of Lactobacillus has been reported by Wang et al thus emphasizing the importance of assessing the bile tolerance of isolates in order to select a potential probiotic strain.. All isolates which showed survival rate greater than 70% at pH (3.0) were able to show survival rate above 50% at 0.3% bile salt concentration except K.P (c), a gram positive cocci, capable of growing at 45°C showed weak survival rate (35.42%) after an incubation period of 2 h in PBS solution with 0.3% bile salt concentration. The survival rate of this gram positive cocci at pH 3.0 was 99.73%. K.6, a gram positive cocci, showed maximum survival rate (99.55%) to bile salt and a high survival rate (99.36%) at pH 3.0 (Fig. 3).

C. Survival of LAB isolates in simulated gastric conditions

The ability to survive in the gastric conditions is one of the main desirable characteristics required for a probiotic. Isolated bacterial strains were checked for their ability to withstand the proteolytic enzymes like pepsin. This test has shown that all the isolates obtained from the Kaladi had a good simulated gastric condition survival property, despite the variation in degree of viability among the isolates (Fig. 3). Among all the isolates, K.R (c) showed the highest tolerance to the simulated gastric conditions with the survival rate of 99.87%. The results indicate that in simulated gastric environment the gastric juice will have negligible effect on the viability of probiotic bacteria isolated from Kaladi.

D. Identification Of Inhibitory Substance By Agar Well Diffusion Method

In many cases, the antimicrobial activity shown by LAB might be due to the production of organic acids, but the pH neutralization resulted in the loss of this effect. So in this study antibacterial activity of the LAB isolates was observed after the supernatants were neutralized with 1 N NaOH. The antibacterial activity was checked against standard strains obtained from MTCC, Chandigarh. Gram-positive indicator species included *Bacillus subtilis*, *Mycobacterium smegmatis* and *Staphylococcus aureus*, Gram-negative indicator species included *Proteus vulgaris* and *Escherichia coli*. The antimicrobial activity of fraction B was determined by measuring the diameter of zone of inhibition, the maximum zone of inhibition was observed in case of *Lactobacillus* strains designated as K.R (a), K.U (b) and K.P (a) against *Proteus vulgaris* and *E.coli* respectively. Four LAB isolates K.J (b), K.R (c), K.1 (a) and K.1 (b) had shown antimicrobial inhibitory effect (fraction A) against all pathogens because of the acidification of the medium as they had lost their inhibitory effect on neutralization (fraction B) (Table 2). The antagonistic effect of LAB towards pathogenic strains studied was due to production of one or more antimicrobially active metabolites such as organic acids or bacteriocin[31] which is in accordance with our studies. In case of K.U (b), *Lactobacillus* isolate had an inhibitory effect against all the pathogenic strains before and after neutralization. But reports have shown that production of lactic acid with further lowering of the pH, in case of *Lactobacillus* strains inhibits the growth of bacterial pathogens and sometimes even kills them[31,51]. Maximum inhibitory zone i.e 1.0 cm and above, was observed in case of (fraction A) by strains designated as K.U (a), K.P (c), K.R (a), K.R (c), K.6 against *Mycobacterium smegmatis*; four strains designated as K.U (a), K.U (b), K.P (a) and K against *Escherichia coli*; K.U (b) against *Staphylococcus aureus* and K.U (a), K.J (b), K.R (a), K.R (c), K, K.1 (a), K.1 (b) against *Proteus vulgaris*. Whereas no LAB strain have shown maximum inhibition against *Bacillus subtilis*. In case of fraction C, there is no inhibition zone formation. This proves proteinaceous character of bacteriocin and therefore it can be broken down by gastric juices, thus making it completely safe for human consumption. Out of the 20 isolates only one of the isolates, a gram positive rod designated as K.B (1) did not show any inhibitory activity against any of the standard strains studied although lactic acid production (4.80±0.025 mg/mL) for K.B (1) was observed after 24 h. Lactic acid production by K.2 isolate was highest (30.59±0.030 mg/mL), it had shown inhibitory effect (fraction A) against all the pathogenic strains although a very weak zone of inhibition (0.1±0 cm) was observed in case of *Proteus vulgaris*. In case of four strains K.J (b), K.R (c), K.1 (a) and K.1 (b) which have shown antimicrobial inhibitory effect against all standard pathogenic strains (fraction A) but inhibitory activity was lost (fraction B) on neutralization. The lactic acid production for the isolates K.J (b), K.R (c), K.1 (a) and K.1 (b) was 27.38±0.005, 19.47±0.005, 12.84±0.040, 26.62±0.035 mg/mL respectively. Thus showing that an inhibitory activity was the result of lactic acid production. Bacteriocinogenic LAB isolated from Kaladi may serve as a potential source to play a significant role as starter cultures, co-cultures, or bioprotective cultures, to improve the quality of food in food industry.

IV. CONCLUSIONS

Isolated LAB strains from Kaladi have probiotic properties that make them potential candidates for probiotic applications. These isolates have the ability to resist low pH, tolerate simulated gastric conditions, high

bile and NaCl salt concentration. The isolates were good lactic acid producers (data not shown) and exhibited effective inhibition against the gram positive and gram negative pathogens, whose antimicrobial activity was bacteriocinogenic in nature. The bacteriocin like inhibitory compound produced by LAB obtained from fermented milk product Kaladi may be used to combat the growth of pathogenic microorganisms in the food industry.

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Table I. Morphological, physiological and biochemical characteristics of isolates

S.No.	Strain code	Cell Shape	Gram staining	Motility	Catalase	3.5% NaCl	6.5% NaCl	Growth 30°C	37°C	45°C
1	K.U (a)	Cocci	+	_	+	+	+	+	+	+
2	K.U (b)	Rods	+	_	+	+	+	+	+	+
3	K.J (a)	Cocci	+	_	+	+	+	+	+	+
4	K.J (b)	Cocci	+	_	+	+	+	+	+	+
5	K.J (c)	Cocci	+	_	+	+	+	+	+	+
6	K.P (a)	Rods	+	_	+	+	+	+	+	+
7	K.P (c)	Cocci	+	_	+	+	+	+	+	+
8	K.R (a)	Rods	+	_	+	+	+	+	+	+
9	K.R (b)	Cocci	+	_	+	+	+	+	+	+
10	K.R (c)	Rods	+	_	+	+	+	+	+	+
11	K.B (1)	Rods	+	_	+	+	+	+	+	+
12	K.A	Cocci	+	_	+	+	+	+	+	+
13	K.M	Cocci	+	_	+	+	+	+	+	+
14	K	Rods	+	_	+	+	+	+	+	+
15	K.1 (a)	Coccobacilli	+	_	+	+	+	+	+	+
16	K.1 (b)	Cocci	+	_	+	+	+	+	+	+
17	K.2	Cocci	+	_	+	+	+	+	+	+
18	K.4	Rods	+	_	+	+	+	+	+	+
19	K.5	Cocci	+	_	+	+	+	+	+	+
20	K.6	Cocci	+	_	+	+	+	+	+	+

(+): positive, (-): negative

Table II. Inhibitory activity by LAB isolates (zone of inhibition in cm)

S. No.	Strain code	*Bacillus subtilis*		*Mycobacterium smegmatis*		*Escherichia coli*		*Staphylococcus aureus*		*Proteus vulgaris*	
		F-A	F-B	F-A	F-B	F-A	F-B	F-A	F-B	F-A	F-B
1.	K.U (a)	0.4±0	-	1.0±0	0.4±0.02	1.0±0	0.4±0	0.8±0	0.2±0.01	1.2±0	0.6±0
2.	K.U(b)	0.6±0	0.2±0	0.8±0	0.6±0.02	1.0±0	1.0±0	1.0±0	0.4±0	0.7±0	0.7±0.01
3.	K.J (a)	0.4±0	-	0.6±0	-	0.4±0	0.4±0	0.6±0	0.2±0	0.8±0	0.5±0.01
4.	K.J (b)	0.4±0	-	0.8±0	-	0.1±0	-	0.6±0	-	1.1±0	-
5.	K.J (c)	0.4±0	-	0.6±0	-	0.2±0	0.1±0	0.4±0	-	0.7±0	0.6±0
6.	K.P (a)	0.3±0	0.3±0	0.8±0	0.6±0	1.0±0	1.0±0	-	-	0.8±0	0.8±0.01
7.	K.P (c)	0.6±0.01	-	1.0±0	-	0.2±0	-	0.8±0	-	0.6±0	0.2±0
8.	K.R (a)	0.5±0.02	0.3±0	1.0±0	0.6±0.03	-	-	0.6±0	0.6±0	1.2±0	1.2±0
9.	K.R (b)	0.4±0.02	-	0.8±0	0.1±0.01	0.6±0	0.6±0	0.6±0	0.4±0	0.8±0	-
10.	K.R (c)	0.6±0	-	1.2±0	-	0.8±0	-	0.6±0	-	1.0±0	-
11	. K.B (1)	-	-	-	-	-	-	-	-	-	-
12.	K.A	0.4±0	-	0.4±0	0.4±0	0.1±0	0.1±0	0.6±0	-	0.6±0	0.6±0.02
13	. K.M	-	-	-	-	-	-	0.4±0	-	0.1±0	-
14.	K	0.7±0	0.7±0	0.8±0	0.04±0	1.0±0	-	0.8±0	0.2±0.02	1.2±0	-
15.	K.1 (a)	0.4±0	-	0.8±0	-	0.1±0	-	0.8±0	-	1.2±0	-
16.	K.1 (b)	0.3±0	-	0.8±0	-	0.6±0	-	0.6±0	-	1.0±0	-
17.	K.2	0.5±0	-	0.6±0.01	0.06±0	0.8±0.01	0.8±0	0.8±0	-	0.1±0	-
18.	K.4	0.6+0	0.2±0	0.8±0	0.1±0	0.2±0	-	0.4±0	0.2±0	0.4±0	0.4±0
19.	K.5	0.2±0	-	0.6±0.02	0.6±0	0.8±0	0.4±0	0.4±0	-	0.8±0	0.8±0.02
20.	K.6	0.4±0	-	1.0±0	-	0.4±0.02	-	0.8±0	-	0.6±0.01	0.6±0.0

All results are mean value ± standard error (S.E.); F-A=Fraction A (supernatant), F-B= Fraction B (pH neutralized supernatant)

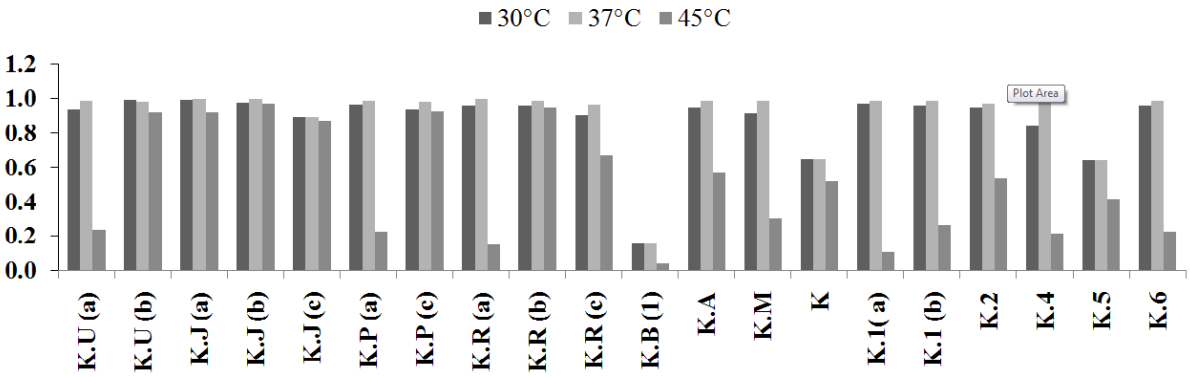

Fig. I: Growth Of Isolates At Different Temperature

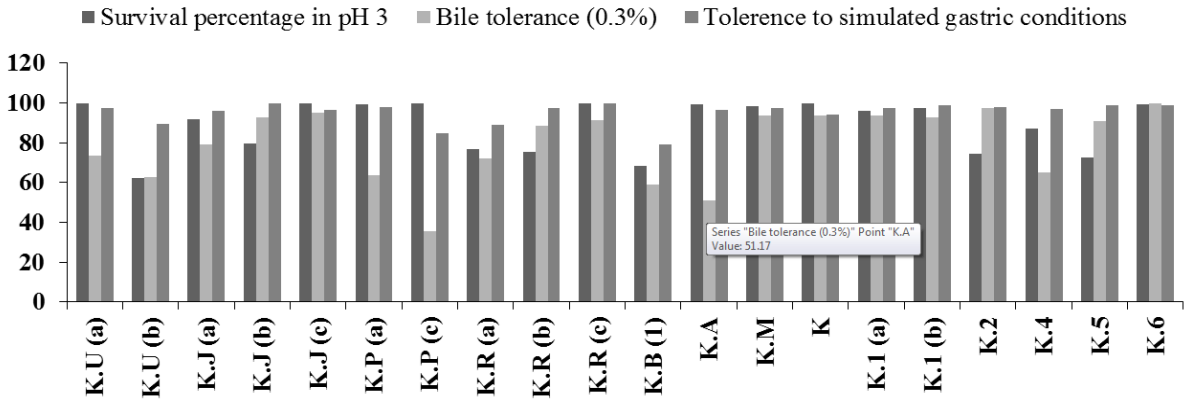

Fig. II: Lab Isolates Showing Different Probiotic Properties