J. Gen. Appl. Microbiol., **53**, 207–213 (2007)

Short Communication

Screening of probiotic properties of lactic acid bacteria isolated from *Kanjika*, an ayruvedic lactic acid fermented product: An in-vitro evaluation

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(Received July 31, 2006; Accepted April 27, 2007)

Key Words——*Kanjika*; lactic acid bacteria; *Lactobacillus coryniformis*; *Lactobacillus curvatus*; probiotic

Kanjika is a well known ayruvedic lactic acid-based fermented product. *Ayruveda*, the traditional medical science practiced in India can be traced to the pre-historic period. 'Sandhana Kalpana' is the term used for the process of fermentation using herbs, with medicinal properties. *Kanjika* (an Indian functional food, also abbreviated as *Kanji*), a probiotic product, is a lactic fermented rice product. It is prepared from raw material of plant origin and is devoid of dairy products. It is consumed either alone or in combination with other food items. It has been prescribed for a number of chronic diseases by Indian ayurvedic practitioners (Misra, 1993).

Lactic acid bacteria (LAB) are known for their probiotic properties (Isolauri et al., 2001; Ronka et al., 2003; Demirer et al., 2006). They should adhere to the intestinal mucosa and exhibit antagonistic activity against pathogenic microorganisms (Lan-szu and Bart, 1999). LAB are also reported to have the ability to control acute gastro-enteritis, which is of bacterial or viral origin. Several studies have shown that *Lactobacillus rhamnosus GG*, *Lactobacillus reuteri*, *Lactobacillus casei* and *Lactococcus lactis* can shorten the duration of diarrhea by approximately 1 day (Kaila et al., 1992; Shornikova et al., 1997; Sugita and Togawa, 1994). Lactobacilli have been shown to be antagonistic to *Helicobacter pylori* which are implicated in chronic gastritis, stomach carcinoma, gastric and duodenal ulcers and in gnotobiotic murine models (Aiba et al., 1998; Kabir et al., 1997; Midolo et al., 1995).

Ayruveda has been practiced for many years in India and *Kanjika* is a well known ayruvedic lactic acid fermentation product. However there have been no efforts on the isolation, identification and characterization of the LAB involved in the process of *Kanjika* preparation. The present study is the first report in this direction. The novelty of the present study is the isolation of potent probiotic LAB from a fermented product, *Kanjika*, and evaluation of probiotic properties of the isolates. The selected isolates can be used as starter cultures for dairy products and as probiotic powder for pharmaceutical applications. Seventeen strains from *Kanjika* were selected according to the colony characteristics and were tested by gram staining and for catalase activity. These isolates were further screened for

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their acid and bile salt tolerance, β-galactosidase activity, cholesterol lowering capacity and antimicrobial activity.

Kanjika was prepared according to Acharya (Acharya Sidh Nandan Misra, 1993). Broken rice was cooked in 1 L of water till the rice becomes soft. The resulting starchy liquid (*Kanji*) after straining was made up to 1 L, and cooled to room temperature. Finely chopped radish (50 g), ground mustard (25 g), rock salt (25 g) and mustard oil (12 g) were added to the 1 L *Kanji*, mixed well and fermented in a closed vessel at 37°C±2°C. Sampling was carried out up to 7 days of fermentation.

Appropriately diluted samples with saline, withdrawn at different stages of fermentation, were taken for enumeration of LAB in MRS agar. Based on the colony characteristics, 17 colonies were selected and subcultured in MRS broth. Isolated strains were stored in MRS broth with 20% glycerol at −80°C and subcultured every 6 months. Acid and bile tolerance was tested according to Park et al. (2002). 10^7–10^8 CFU/ml of bacterial suspension was inoculated to sterile MRS broth whose pH was adjusted to 2, 2.5 and 3 with 0.1 M HCl and MRS broth containing 0.3, 0.6, and 1% bile salt (w/v). Incubation was carried out at 37°C (4 h and 12 h) for acid and bile tolerance respectively. The method described by Karasova et al. (2002) was used to test the presence of β-galactosidase activity. Plates were incubated at 37°C for 2 days. Colonies producing β-galactosidase were blue in color.

Cholesterol assimilation was studied by a modified Searchy and Bergquist method (1960). LAB isolates were cultivated in MRS broth supplemented with cholesterol and cholesterol with 0.3% bile salt. The cultures were grown in this media for 12–24 h at 37°C. The amount of unassimilated cholesterol was estimated in the cell free culture broth. The uninoculated broth was used as the control (0% assimilation). For the detection of antimicrobial activity, an agar spot method (Jacobsen et al., 1999) was used. The spotted culture plates were incubated at 37°C for 24 h and overlayed with indicator pathogens (*Escherichia coli*, *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Staphylococcus aureus*) in soft agar. The probable inhibitory effect of the constituents of MRS agar was tested by overlaying the MRS agar plates (uninoculated with the LAB) with indicator pathogens in soft agar. The zone of inhibition in mm was measured after 12 h incubation. The selected acid-tolerant isolates of *Kanjika* were tested for susceptibility to antibiotics. Antibiotic susceptibility was determined semi quantitatively according to Charteris et al. (2000). Inhibition zone diameter was measured after 24 h incubation at 37°C. The results (mean of three determinations) are expressed in terms of resistance, moderate susceptibility and susceptibility according to interpretative standards described by Charteris et al. (1998a). *Lactobacillus plantarum* B 4496 was used as the control for comparative evaluation of all the probiotic properties. Selected acid tolerant isolates were identified according to the Schillinger and Lucke (1987) method. Biochemical characterization was carried out by checking the gas production from glucose in MRS broth without citrate using inverted Durham's tubes. Hydrolysis of arginine was tested in MRS broth containing 0.3% arginine and 0.2% sodium citrate instead ammonium citrate but without glucose and meat extract. Ammonia was detected using Nessler's reagent. Ability to ferment various carbohydrates was checked using MRS broth supplemented with filter-sterilized respective carbohydrate solutions to a final concentration of 0.5% w/v without glucose or meat extract and 0.004% chlorophenol red. Salt tolerance at different NaCl concentrations, and growth at different pH and temperatures were also studied. All the experiment was carried out in triplicate. The statistical significances and standard deviation were done using Microsoft excel (Version 5.0; Microsof Corp; Redmond, WA).

Percentage of survival of 17 *Kanjika* isolates at pH of 2 and 2.5 for 4 h incubation at 37°C was studied (Fig. 1). Of the 17 isolates tested, 6 showed little or no decrease in the viable count in comparison with the initial cell count at pH 2 and 2.5. Percentage survival of the *L. plantarum* was found to be 75% and 80% at pH 2 and pH 2.5 respectively. The acid tolerant isolates were further tested for their bile salt tolerance at different bile salt concentrations (0.3, 0.6 and 1% w/v) (Fig. 2). All the strains including *L. plantarum* except K7b were found to be tolerant up to 1% bile salts till 12 h incubation at 37°C. The percentage survival of K7b was found to decrease with an increase in bile salt concentration. The appearance of the characteristic blue colonies on MRS agar supplemented with X-gal and IPTG indicated the presence of β-galactosidase activity (Fig. 3). After 48 h of incubation, all isolates other than K7a and K7b showed the β-galactosidase activity. Assimilation of cholesterol by selected

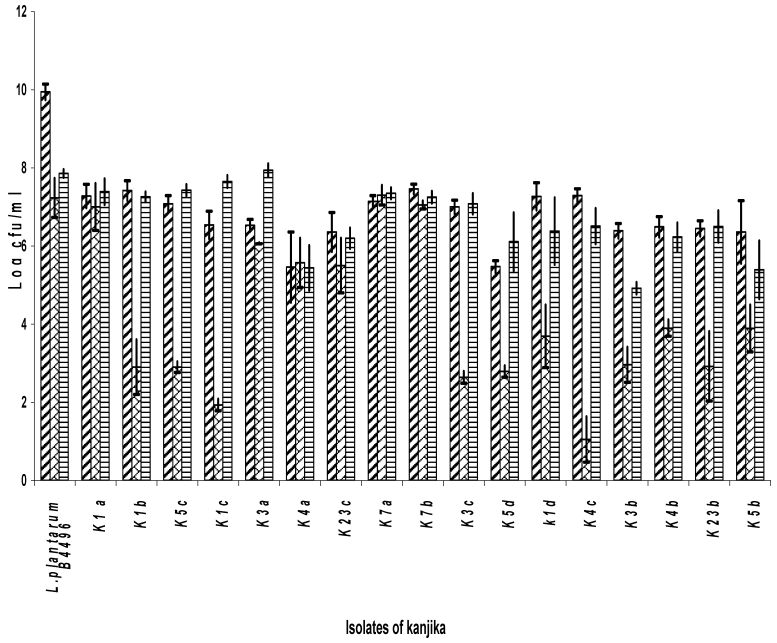

Fig. 1. Survivability of *Kanjika* isolates strain in MRS broth adjusted to pH 2 and pH 2.5. ▨ Initial conc.; ▩ pH 2; ▤ pH 2.5.

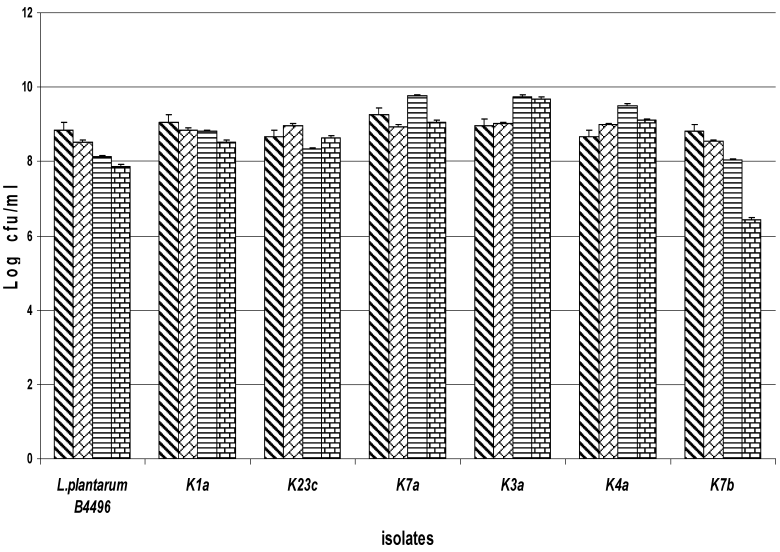

Fig. 2. Effect of high bile concentration on growth of isolates at 37°C for 12 h. ▨ Initial conc.; ▩ 0.3%; ▤ 0.6%; ▦ 1%.

Kanjika isolates in the presence and absence of 0.3% bile in the spent broth (24 h incubation) is shown in Fig. 4. The assimilation of cholesterol by K3a was found to be 34% in the absence of bile salts, whereas in the presence of bile salt (0.3%), it was found to be 58%. *L. plantarum* B 4496 was found to assimilate 25% and 45% cholesterol in the presence and absence of 0.3% bile, respectively.

The antimicrobial property of six selected acid-tolerant *Kanjika* isolates was evaluated against well-known food-borne pathogens such as *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Yersinia enterocolitica*. All the selected isolates showed good antimicrobial property giving a 10 to 20 mm (dia) inhibition zone against all tested pathogens (Table 1). The selected acid-tolerant isolates were tested for antibiotic susceptibility by the Charteris et al. method (1998b). All the strains were

resistant to karamycin, metronidazole, vancomycine, co-trimoxazole and polymyxin-B antibiotic and moderately susceptible to ampicillin, erythromycin and streptomycin; however they were susceptible to chloramphenicol (Table 2). The data on phenotypic, physiological and biochemical characterization of the isolates are presented in Table 3. All the three isolates lacked gas production from glucose. Isolate K1a was found to be weakly positive towards ammonia production and the other two isolates (K23c and K3a) negative towards ammonia production. K3a was a thermophile while K1a and K23c were mesophiles. All the three isolates were found to ferment only glucose. Based upon the above mentioned biochemical characteristics, the isolates have been identified as follows; K3a: *Lactobacillus delbrueckii*, K1a: *Lactobacillus curvatus* and K23c: *Lactobacillus coryniformis.* Identification of the above isolates was further confirmed by Microbial Type Culture Collection, IMTECH, Chandigarh, India.

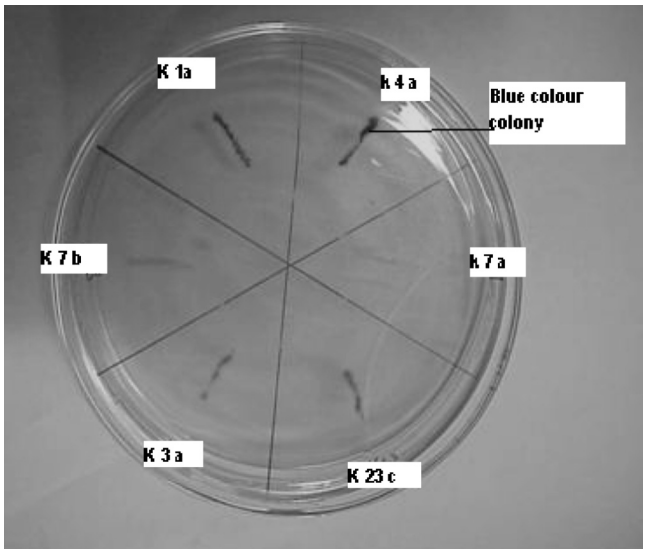

Fig. 3. β-Galactosidase activity assay by screening method showing characteristic Blue color colony formation.

Of the 17 isolates, 6 were found to be tolerant after 4 h incubation to low pH (pH 2 and 2.5). In general, studies have been directed towards the survivability of LAB isolates at pH 2.5 rather than pH 2 (Charteris et al., 1998b; Goldin et al., 1992; Hirayama and Rafter, 2000; Jacobsen et al., 1999). However in the present study, 6 out of 17 *Kanjika* isolates showed more than 90% survivability, at pH 2 and pH 2.5 for 4 h incubation. Of the above 6 acid-tolerant isolates, the isolate K3a identified as *Lactobacillus delbrueckii* showed 100% tolerance to pH 2 and 2.5 at 4 h incubation. The result indicates that isolate K3a exhibits better acid tolerance compared to that of *Lactobacillus GG* and *Lactobacillus plantarum* as cited in literature. These acid-tolerant isolates were further evaluated for other probiotic properties. According to Gilliland et al. (1984) 0.3% bile is considered to be the crucial concentration to evaluate bile-tolerant probiotic LAB. Among 6 acid-tolerant strains, 5 strains were found to survive at the tested bile salt concentrations (0.3, 0.6 and 1%) for 12 h. However the survival of the one of the isolates (K7b), among the six acid-tolerant isolates was found to decrease with an increase in bile salt concentration from 0.3–1% at 12 h incubation. The isolates studied

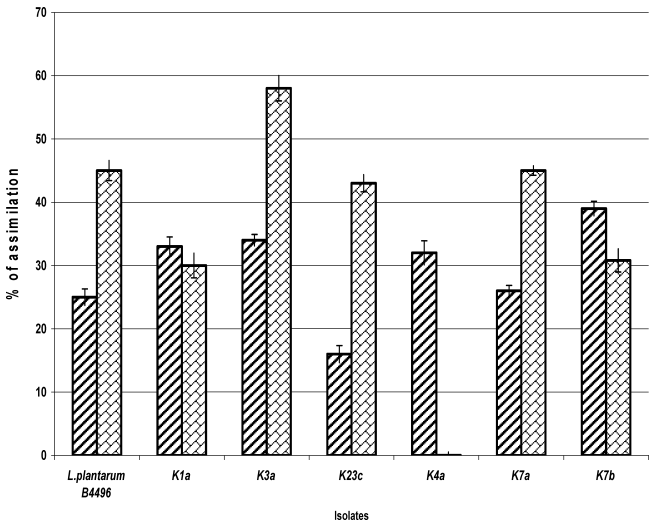

Fig. 4. Assimilation of cholesterol in presence and absence of bile salt (0.3%) at 24 h incubation. ▨ MRS+cholesterol; ▨ MRS+cholesterol+bile salt 0.3%.

Table 1. Antibacterial activities of selected *Kanjika* strains towards pathogenic microorganism.

Kanjika strains	Pathogenic bacteria (Zone of inhibition[a])				
	E. coli MTCC 108	*B. cereus* F 4810	*L. monocytogenes* Soctta	*Y. enterocolitica* MTCC 859	*SEA* FRI 722
L. acidophilus B-4496	++	++	++	++	++
$K_{23}3$	++	+++	+++	+++	++
K_71	++	++	+++	+++	++
K_72	++	++	++	++	++
K_11	++	++	++	+++	++
K_41	++	++	++	++	++
K_31	++	++	++	++	++

Note: [a]++, between 1–10 mm of inhibition; +++, 11–20 mm of inhibition; *SEA*: *Staphylococcus aureus*.

Table 2. Antibiotic susceptibility testing for the *Kanjika* isolates.

Antibiotic	Disc. conc. (μg)	Interpretative zone diameter (mm)					
		K3A	K1A	K23C	K4A	K7A	K7B
Penicillin-G	10	18(R)	24(MS)	28(S)	19(R)	20(MS)	25(MS)
Karamycin	30	08(R)	11(R)	00(R)	10(R)	08(R)	10(R)
Metronidazole	5	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Vancomycin	30	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Ampicillin	10	20(MS)	25(MS)	22(MS)	19(MS)	24(MS)	23(MS)
Chloramphenicol	30	18(S)	28(S)	23(S)	22(S)	23(S)	18(S)
Streptomycin	10	08(R)	14(MS)	14(MS)	00(R)	13(MS)	12(MS)
Co-trimoxazole	25	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Tetracycline	30	18(MS)	19(S)	17(MS)	14(R)	18(MS)	17(MS)
Erythromycin	15	16(MS)	21(MS)	21(MS)	19(MS)	20(MS)	22(MS)
Rifampicin	5	18(MS)	20(S)	20(S)	16(R)	18(MS)	24(S)
Polymyxin-B	300	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)

Susceptibility expressed as (R), resistant; (MS), moderately susceptible; (S), susceptible.

under the present investigation showed better survivability, up to 1% bile salt concentration, and unlike the probiotics reported earlier including *L. plantarum*. LAB probiotics are reported to reduce lactose intolerance symptoms by accelerating the digestion of lactose (Sanders, 1993). β-Galactosidase enzyme plays a major role in the digestion of the milk sugar, lactose, in humans. Out of the 6 selected acid-tolerant isolates, 4 isolates, namely K1a, K3a, K23c, K4a, tested positive for β-galactosidase activity.

Hypercholesterolemia increases the risk of coronary heart disease in humans (Hirayama and Rafter, 2000). The present study showed that the *Kanjika* isolate K3a, has the ability to reduce cholesterol up to 60% in the presence of 0.3% bile in 24 h, whereas it can also reduce cholesterol up to 40% in the absence of bile in 24 h. Isolates K1a and K23c were found to reduce the cholesterol up to 30% in 24 h incubation, thus indicating the potential of these isolates for therapeutic uses. The antagonistic activity exhibited by various species of probiotic may be due to the production of any one or more of reuterin, bacteriocin like nicin, lactic acid and hydrogen peroxide (El-Ziney and Debevere, 1998). The 6 selected acid-tolerant *Kanjika* isolates were investigated for their antimicrobial properties against food-borne pathogenic bacteria. All the six selected

Table 3. Morphological, physiological and biochemical characterization of K1a, K3a, and K23c.

Characteristics		K1a	K3a	K23c
Morphology		Rod (slightly curve)	Rod	Cocco bacilli
Gram's reaction		+	+	+
Catalase test		−	−	−
Methyl red test		+	−	+
Gas production from glucose		−	−	−
NH_3 from arginine		+ (W)	−	−
Growth at	15°C	+ (W)	−	−
	25°C	+	+	+
	37°C	+	+	+
	45°C	−	+	−
pH	4.0	+ (W)	+	−
	5.0	+	+	+
	6.8	+	+	+
	8.0	+	+	+
Growth on NaCl (%)	2.0	+ (W)	−	+ (W)
	6.5	−	−	−
	10.0	−	−	−
Growth under anaerobic condition		+	+	+
Carbohydrate fermentation				
Adonitol		ND	ND	ND
Arabinose		−	−	−
Dulicitol		−	−	−
Dextrose		+	+	+
Lactose		−	−	−
Maltose		−	−	−
Rhamnose		−	−	−
Raffinose		−	−	−
Sucrose		−	−	−
Xylose		−	−	−

(+) growth, +(W) weak growth, (−) no growth, (ND) no data.

isolates showed inhibition against all the tested pathogens. Isolates K23c, K7a and K1a showed significant inhibition against *L. monocytogenes* and *Y. enterocolitica.*

The present study details the in vitro evaluation of the lactic acid bacteria isolated from *Kanjika*. The study indicates that the isolate K3a (*L. delbrueckii*) exhibits potential probiotic properties. Other isolates like K1a (*L. curvatus*) and K23c (*L. coryniformis*) also exhibited major probiotic properties but were found to be susceptible to 3 antibiotics, which is not desirable. Isolate K4a showed promising probiotic properties except cholesterol assimilation. It has been possible to isolate lactic acid bacteria exhibiting a profound degree of probiotic properties such as acid tolerance, bile salt tolerance, antimicrobial activity against food borne pathogens, β-galactosidase activity, antibiotic susceptibility and cholesterol assimilation from *Ayruvedic* fermented products.

Acknowledgments

The authors are grateful to the Life-Science Research Board, DRDO, for funding the project. Praveen Kumar Reddy is thankful to CSIR for the senior research fellowship. The authors thank the Director, CFTRI, for supporting the work.

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