Brazilian Journal of Microbiology 44, 4, 1199-1206 (2013)
ISSN 1678-4405

Research Paper

Molecular characterization of lactobacilli isolated from fermented *idli* batter

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Submitted: April 2, 2012; Approved: April 4, 2013.

Abstract

Lactic acid bacteria are non pathogenic organism widely distributed in nature typically involved in a large number of spontaneous food fermentation. The purpose of this study was to characterize the bacteriocinogenic lactobacilli from fermented *idli* batter which can find application in biopreservation and biomedicine. Eight most promising lactobacilli were chosen from twenty two isolates based on their spectrum of activity against other lactic acid bacteria and pathogens. The eight lactobacilli were characterized based on the various classical phenotypic tests, physiological tests and biochemical tests including various carbohydrate utilization profiles. All isolates were homo fermentative, catalase, and gelatin negative. Molecular characterization was performed by RAPD, 16S rRNA analysis, 16S ARDRA, and Multiplex PCR for species identification. RAPD was carried out using the primer R2 and M13. Five different clusters were obtained based on RAPD indicating strain level variation. 16S rRNA analysis showed 99 to 100% homology towards *Lactobacillus plantarum.* The restriction digestion pattern was similar for all the isolates with the restriction enzyme *Alu*I. The subspecies were identified by performing Multiplex PCR using species specific primer. Among the five clusters, three clusters were clearly identified as *Lactobacillus plantarum* subsp. *plantarum, Lactobacillus pentosus*, and *Lactobacillus plantarum* subsp. *argentoratensis.*

Key words: *Idli* batter, bacteriocin, *Lactobacillus plantarum* subsp*. argentoratensis*, *Lactobacillus plantarum, Lactobacillus pentosus*.

Introduction

Lactic acid bacteria (LAB) are non pathogenic organism widely distributed in nature. LAB have an important role in the preservation of foods and fermented products and are designated as GRAS (Generally regarded as safe). The genus *Lactobacillus* is the largest group among the *Lactobacteriaceae*, and contains over 100 species (Canchaya *et al.*, 2006). They are characterized as Gram-positive rods, anaerobic but aero tolerant, non-sporulating and catalase negative. They are commercially used as starter cultures in the manufacture of dairy products, fermented vegetables, fermented dough, alcoholic beverages, and meat products (De Vuyst *et al.*, 2007).

The primary antimicrobial effect exerted by lactobacilli is by the production of lactic acid and reduction of pH. However, there are other metabolic products such as hydrogen peroxide, diacetyls, propionic acid, acetic acid, carbon dioxide, reuterin, and bacteriocins reported to contribute to their antimicrobial activity (El-Ghaisha *et al.*, 2011). Lactobacilli also display antifungal activity (Yang and Chang, 2010). Lactobacilli living as commensal in the intestinal environment might possess probiotic activity (Zago *et al.*, 2011). They may have anti-tumoral activity (Paolillo *et al.*, 2009), reduce cholesterol level (Wang *et al.*, 2012), alleviate lactose intolerance, stimulate the immune system, and also they may be able to stabilize the micro flora of the gut (O'Flaherty and Klaenhammer, 2010). These various possible attributes of lactobacilli promote beneficial effects to human health. Thus, lactobacilli are now a focus of intensive research worldwide and new species are being reported.

In the majority of fermented foods, particularly traditional foods of India, the nature of fermentation is by LAB associated with the cereals and legumes (Agrawal *et al.*,

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2000). *Idli* batter is traditionally prepared from pre-soaked parboiled rice (*Oryza sativa*) and dehulled black gram (*Phaseolus mungo*) and allowed for a natural yeast-lactic fermentation for 18-30 hours, while *idlis* are made by steaming the fermented batter. However, the preparation of *idli* batter varies from region to region in south India especially, the proportion of rice and black gram as well as the duration of soaking and fermentation of the batter. Previous reports showed the prevalence of yeast such as *Saccharomyces cerevisiae, Debaryomyces hansenii*, and *Hansenula anomala* and the lactic acid bacteria such as *Leuconostoc mesenteroides, Streptococcus faecalis, Lactobacillus fermentum*, and *Pediococcus cerevisiae* in the fermented batter (Soni and Sandhu, 1989), although *L. mesenteroides* and *S. faecalis* are considered essential for leavening of the batter and for acid production (Mukherjee *et al.*, 1965; Purushothaman *et al.*, 1993). Recently, *Pediococcus pentosaceous* or *Enterococcus faecalis* in combination with yeast the *Candida versatilis* were tried as starter cultures for *idli* batter fermentation (Sridevi *et al.*, 2010). However, the lactobacilli isolated from *idli* batter have been least explored, as well as delineation of the isolates to sub-species level has not been reported.

In general, the classical protocols of morphological and biochemical characterizations of microbial cultures are in use to identify bacteriocinogenic culture. The development of PCR-based methods using random amplification of polymorphic DNA (RAPD) (Nigatu *et al.*, 2001), analysis of rRNA gene homology, amplified 16S rDNA restriction analysis (16S-ARDRA) (Rodas *et al.*, 2003), and species specific primers (Chagnaud *et al.*, 2001) have proved useful for identification of various species of lactobacilli. Thus, the present study was focused on isolation and characterization of bacteriocinogenic lactobacilli from fermented *idli* batter by both classical and PCR-based molecular methods to identify the isolates to sub-species level which may help to formulate starter culture as well as in the biological preservation of foods.

Materials and Methods

Isolation of lactobacilli

The *idli* batter was prepared from rice (*Oryza sativa*) and black gram (*Phaseolus mungo*), a legume. The ingredients were washed, soaked, grounded, and allowed to ferment overnight (Vijayendra *et al.*, 2010). The fermented *idli* batter was serially diluted with saline, plated on De Man Rogosa Sharpe (MRS) agar (Himedia, Mumbai, India) and incubated anaerobically at 37 °C for 24-48 h. The colonies on MRS agar which were milky white, circular, convex, elevated and non- pigmented were chosen and further sub cultured. The colonies were streaked on MRS agar to check for purity. The pure cultures were overlaid with glycerol and preserved for further study (Pal *et al.*, 2005).

Antimicrobial activity of the isolates

Various indicator strains (Table 1) for antimicrobial activity determination were obtained from the Microbial Type Culture Collection (MTCC) of the Institute of Microbial Technology, Chandigarh. The lactobacilli isolates were propagated in MRS broth and its cell free supernatant (CFS) from 48 h culture was collected. The CFS was ad-

Table 1 - Antimicrobial activity against LAB and various pathogens Diameter of the well is 6 mm.

Indicator strains	Inhibition zone in mm							
	JJ 18	JJ 22	JJ 24	JJ 29	JJ 30	JJ 55	JJ 58	JJ 60
Lactobacillus plantarum (MTCC 6161)	10	10	10	10	10	10	10	10
Lactococcus lactis subsp. lactis (MTCC 3038)	12	14	14	17	15	16	12	17
Lactobacillus fermentum (MTCC 1745)	12	10	10	13	12	13	12	12
Lactococcus lactis subsp. lactis (MTCC 440)	10	10	10	10	10	10	10	10
Leuconostoc mesenteroides subsp. *mesenteroides* (MTCC 107)	10	10	10	10	10	10	10	10
Lactococcus lactis subsp. chacetylactis (MTCC 3042)	17	18	18	18	18	18	17	18
Lactobacillus rhamnosus (MTCC 1408)	11	11	10	10	10	10	11	10
Brevibacterium casei (MTCC 1530)	12	11	11	12	11	11	11	11
Listeria monocytogenes (MTCC 657)	18	18	18	19	18	18	18	19
Staphylococcus aureus subsp. aureus (MTCC 737)	18	18	18	19	19	19	18	19
Aeromonas hydrophila subsp. *hydrophilia* (MTCC 1739)	16	16	16	16	17	15	15	17
Pseudomonas aeruginosa (MTCC 2295)	13	14	14	13	14	14	14	14
Micrococcus luteus (MTCC 106)	13	13	13	13	13	14	13	15
Bacillus cereus (MTCC 1272)	16	16	16	18	16	16	16	19
Vibrio parahaemolyticus (MTCC 451)	16	15	16	16	15	15	15	18
Bacillus subtilis (MTCC 619)	16	15	14	16	15	16	15	19

justed to pH 5 with 3N NaOH and the antimicrobial spectrum was carried out against various indicator LAB and pathogens by agar well diffusion method (Jamuna and Jeevaratnam, 2004). Similarly, the CFS treated with protease (1 mg/mL) (Sigma, Saint Louis, USA) for 2 h adjusted to pH 5 was also evaluated for the antimicrobial activity (Vijayendra *et al.*, 2010). The zone of inhibition (in mm) was measured against all the indicator strains (Jamuna and Jeevaratnam, 2004).

Classical characterization of bacteriocinogenic isolates

Growth was assayed in MRS broth at various pH (3.5, 4.5, 8.5, and 9.5), temperatures (15, 37, and 45 °C), and salt concentrations (4, 6.5, and 10% of NaCl) (Pal *et al.*, 2005). Gelatin hydrolysis, catalase production, starch hydrolysis, acetoin production, ammonia production, carbon dioxide production, slime production, homo-hetero fermentation were analyzed for the isolates as described by Pal *et al.* (2005). Carbohydrate utilization profile was determined using HiCarbo kit (Himedia, Mumbai, India). The optical nature of the isomer of lactate was also determined.

RAPD analysis

Genomic DNA was isolated by the procedure as described by de Los Reyes-Gavilan *et al.* (1992). RAPD analysis was carried out using the primers R2 5'-GGCGACCACTAG 3' and M13 5' GAGGGTGGCGGTTCT-3' (Bonomo *et al.*, 2008). The PCR cocktails (50 µL) consisted of 50 pM of the primer, 50 ng of DNA, 1x Taq DNA polymerase buffer, 2 U of Taq polymerase, 0.4 mM of each dNTP, and 3 mM of $MgCl_2$ (Genei, Bangalore, India). Amplification conditions were initial denaturation at 94 °C for 5 min, 40 cycles of 94 °C for 1 min, annealing at 38 °C for R2 and 40 °C for M13 for 45 s, and elongation at 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min (Bonomo *et al.*, 2008). The pattern was analyzed by running in 1.5% agarose gel electrophoresis with DNA ladder (Sigma, Saint Louis, USA).

Molecular characterization by 16S rRNA gene analysis

Amplification of 16S rRNA gene was performed from genomic DNA of the isolates using universal primers fD1 (5'-GAGTTTGATCCTGGCTCA-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3'), as described by Naik *et al.* (2008). PCR cocktails (50 µL) contained 50 pM of primer, 50 ng of genomic DNA, 1x Taq DNA polymerase buffer, 1 U of Taq DNA polymerase, 0.2 mM of each dNTP, and 1.5 mM $MgCl_2$. Amplification was performed in a DNA thermo cycler at 94 °C for 3 min, followed by 30 cycles of 10 s at 94 °C, 1 min at 56 °C and 30 s at 72 °C with an extension of 72 °C for 5 min. Purified PCR products were sequenced with automated DNA sequencer with specific primers using the facility at Macrogen Inc. (Macrogen Inc., Seoul, Korea). Phylogenetic analysis for the isolates was performed for the isolates using MEGA software v5.05 (Yu *et al.*, 2011).

16S ARDRA

Restriction digestion of PCR amplified product was performed with the restriction enzyme *Alu*I for overnight at 37 °C in 20 µL volumes of incubation buffer containing 5 U of the restriction enzyme and adequate DNA (Rodas *et al.*, 2005). The pattern was analyzed by running in agarose gel electrophoresis with 100 bp DNA ladder (Sigma, Saint Louis, Missouri, USA).

Multiplex PCR assay

A multiplex PCR assay was performed with the *recA* gene-based primers paraF (5'-GTC ACA GGC ATT ACG AAA AC-3'), pentF (5'-CAG TGG CGC GGT TGA TAT C-3'), planF (5'-CCG TTT ATGCGG AAC ACC TA-3'), and pREV (5'-TCG GGA TTA CCA AAC ATC AC-3'), as described by Torriani *et al.* (2001). PCR cocktails (50 µL) contained 0.25 mM of primers, 50 ng of genomic DNA, 1x Taq DNA polymerase buffer, 1 U of Taq DNA polymerase, 0.2 mM of each dNTP, and 1.5 mM $MgCl_2$. PCR were performed with initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 10 s, and elongation at 72 °C for 30 s, and final extension at 72 °C for 5 min (Torriani *et al.*, 2001). The PCR products were visualized in agarose gel electrophoresis with 100 bp ladder.

Accession number

These sequence data have been submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under accession number JN573601 to JN573608.

Results and Discussion

The present study deals with molecular characterization of bacteriocinogenic LAB isolates from fermented *idli* batter. This study forms a broader objective to obtain a uniform consortium of strains having many beneficial properties as starter culture for commercial purposes. There were 22 lactobacilli isolates, which were Gram positive and catalase negative, of which 8 isolates (JJ 18, JJ 22, JJ 24, JJ 29, JJ 30, JJ 55, JJ 58, JJ 60) showing maximum zone of inhibition against other LAB and various Gram positive and Gram negative pathogens were chosen for the study (Table 1). The ability of the CFS of our isolates to inhibit certain other LAB organisms indicated that these isolates are probably bacteriocinogenic in nature. Moreover, the CFS treated with protease showed no zone of inhibition (Figure 1) depicting that the activity is due to a proteinaceous substance (Vijayendra *et al.*, 2010). Our isolates showed good inhibition of pathogens like *Bacillus cereus* and

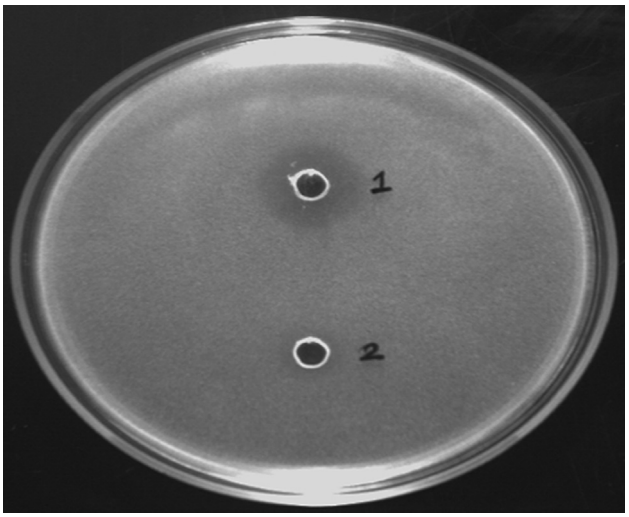

Figure 1 - Antimicrobial activity of JJ 18 against *Staphylococcus aureus*. (1) CFS adjusted to pH 5 (2) CFS treated with protease.

Staphylococcus aureus which were earlier reported to be common contaminants in *idli* batter fermentation (Jama and Varadaraj, 1999). Additionally, the lactobacilli showed potent inhibition against *Listeria monocytogenes* and *Escherichia coli* which are common food pathogens (Luo *et al.*, 2011). An earlier report has demonstrated that addition of bacteriocin, plantaricin LP84 from *Lactobacillus plantarum* NCIM 2084 to *idli* batter exerted antagonistic effect against organisms like *Staphylococcus aureus, Bacillus cereus* and *Escherichia coli* (Jama and Varadaraj, 1999). In the present study, the native lactobacilli isolated from *idli* batter exhibited good antimicrobial activity against the above mentioned food pathogens (Table 1) suggesting their use as protective cultures in food industry. The inhibitory potential of these isolates against other pathogens like *Aeromonas, Pseudomonas, Micrococcus*, and *Bacillus* (Table 1) suggests its application in biopharmaceutical industry.

The 8 lactobacilli were characterized by classical methods. All the isolates were Gram positive and were negative for slime production. Various physiological tests were carried for the lactobacilli. The 8 lactobacilli showed good growth at 15, 37, and 45 °C whereas no growth was observed at 10 °C, indicating their mesophilic character (Kandier and Weiss, 1996). The lactobacilli were able to tolerate salt concentration of 6.5%, but were unable to grow at 10%. The lactobacilli were able to grow in acidic as well as in alkaline pH. The similarity among 8 lactobacilli in phenotypic and physiological tests may probably be due to the fact that the isolates were from similar ecological niche. The biochemical properties such as catalase production, gelatin, starch, and arginine hydrolysis were negative for all the 8 lactobacilli. All the isolates were negative for acetoin production with the sole exception of the strain JJ 60. All the bacilli were homo-fermentative exhibiting DL lactic acid configuration (Table 2). The conventional characterization of the isolates showed that the isolates could be *Lactobacillus plantarum* (Kandier and Weiss, 1996; Pal *et al.*, 2005). However, this characterization is not sufficient to distinguish the sub-species of *Lactobacillus plantarum.* Therefore, PCR based molecular tools were carried out to identify the sub-species.

RAPD analysis was performed initially to cluster the isolates using two different primers R2 and M13. JJ 18, JJ 22, JJ 29 and JJ 30 having similar pattern in the RAPD analysis belonged to a single group, while JJ 24, JJ 55, JJ 58, and JJ 60 having different patterns clustered in to different groups (Figure 2). Thus, five different clusters were clearly observed based on the RAPD analysis. The sugar utilization pattern was also different for all the five groups indicating strain level variation among these isolates (Table 2).

The 16S rRNA were analyzed for the five different clusters of isolates. The PCR products were sequenced and were subjected to nucleotide BLAST. The isolates showed 99 to 100% homology towards *Lactobacillus plantarum.* Multiple sequence alignment was carried out by CLUSTAL W and later phylogenetic analysis was performed using software MEGA v5.05. All the isolates were phylogenetically closely related to *Lactobacillus plantarum* and *Lactobacillus pentosus* (Figure 3). Thus, other molecular methods were carried out to clearly identify the species.

The different clusters obtained as a result of RAPD indicated strain level variation among the isolates. As 16S ARDRA is a rapid and reliable tool for strain identification, the same was performed with *Alu*I restriction enzyme. *Alu*I is generally used in differentiating *Lactobacillus* species (Rodas *et al.*, 2003). The digestion pattern was similar for all the 8 lactobacilli (Figure 4). The results showed high homology of the ribosomal genes (Rodas *et al.*, 2005). Generally, the *Lactobacillus pentosus* and *Lactobacillus plantarum* are genotypically closely related and show high homology in the 16S rRNA gene sequence (Rodas *et al.*, 2003). However, the differential utilization of carbohydrates by the isolates (Table 2) further prompted us to carry

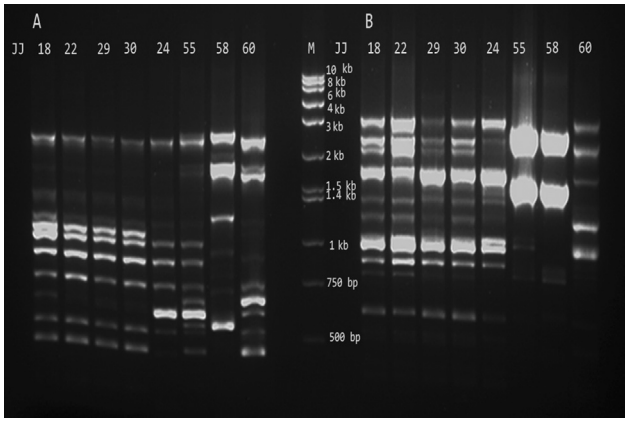

Figure 2 - RAPD Analysis using the primer R2 (A) and primer M13 (B). M is the 500 bp marker.

Table 2 - Phenotypic, physiological and biochemical characterization of the isolates.

	JJ 18	JJ 22	JJ 24	JJ 29	JJ 30	JJ 55	JJ 58	JJ 60
Cell form	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus
Gas from glucose	-	-	-	-	-	-	-	-
Homo-hetero fermentation	Ho	Ho	Ho	Ho	Ho	Ho	Ho	Ho
Growth at temperature								
10 °C	-	-	-	-	-	-	-	-
15 °C	+	+	+	+	+	+	+	+
37 °C	+	+	+	+	+	+	+	+
45 °C	+	+	+	+	+	+	+	+
Growth at pH								
3.5	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+
9.5	+	+	+	+	+	+	+	+
Salt tolerance								
4%	+	+	+	+	+	+	+	+
6.5%	+	+	+	+	+	+	+	+
10%	-	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-
Slime from sucrose	-	-	-	-	-	-	-	-
Acetoin production	-	-	-	-	-	-	-	+
Isomers of lactic acid	DL	DL	DL	DL	DL	DL	DL	DL
Arginine hydrolysis	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	+	+	+	+
Carbohydrate utilization[a]								
Lactose	-	-	-	-	-	+	-	+
Galactose	-	±	±	-	±	±	-	+
Trehalose	-	+	-	-	-	+	+	+
Melibiose	+	-	-	-	-	±	+	-
L-Arabinose	-	-	-	-	-	-	-	±
Inositol	+	-	-	-	-	+	+	-
Sorbitol	+	-	-	-	-	+	+	-
Melezitose	-	-	-	-	-	+	+	+
α-methyl mannoside	-	-	-	-	-	-	±	-

(+) indicates growth/sugar utilization, (-) indicates no growth/no sugar utilization and (±) indicates weak positive growth.
[a]All isolates gave positive for the sugars maltose, sucrose, salicin, ribose, cellobiose, inulin, fructose, dextrose, and mannose, while negative for the sugars rhamnose, xylose, raffinose, glycerol, glucosamine, dulcitol, mannitol, adonitol, α methyl glucoside, xylitol, ONPG, D- arabinose, citrate, malonate, and sorbose.

out sub-species level identification by other PCR method, using *recA* gene as it can also be used as a phylogenetic marker (Ghotbi *et al.*, 2011; Torriani *et al.*, 2001). The *recA* gene codes for a small protein (352 amino acids in *Escherichia coli*) implicated in homologous DNA recombination, SOS induction, and DNA damage-induced mutagenesis (Torriani *et al.*, 2001). Multiplex PCR with *recA* gene-derived primers was carried out to identify the sub-species of the lactobacilli.

The isolate JJ 24 was identified as *Lactobacillus plantarum* subsp. *argentoratensis* based on its amplicon around 318 bp and 120 bp. Moreover, JJ 24 could not metabolize melizitose which is a key factor in identification of *Lactobacillus plantarum* subsp. *argentoratensis*. JJ 58 was

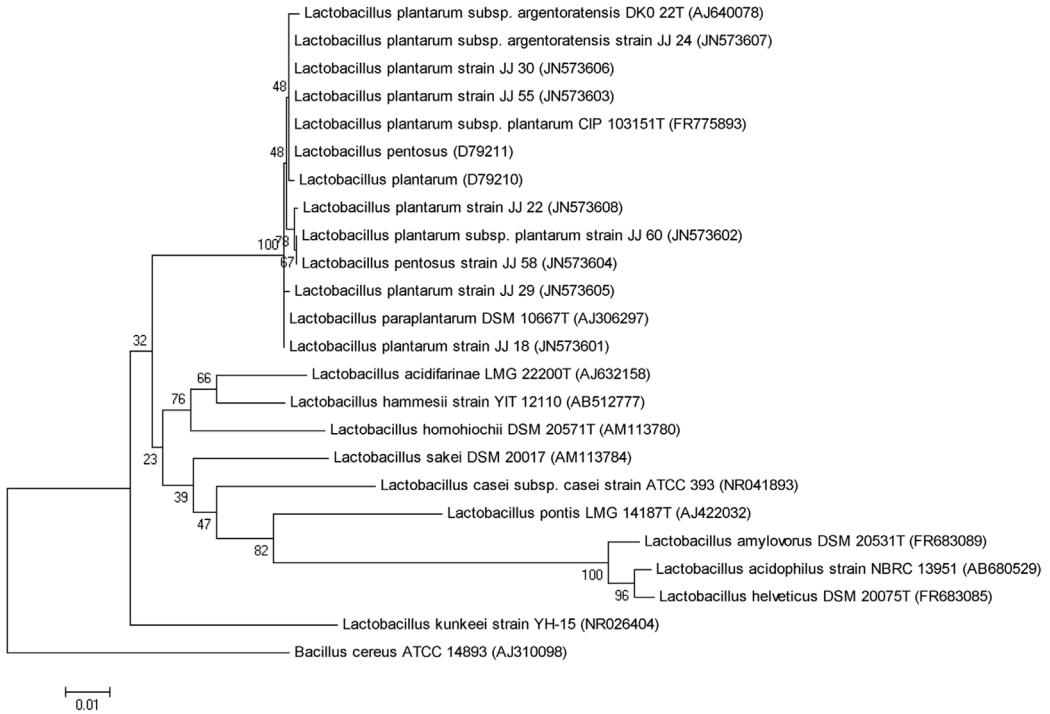

Figure 3 - Phylogenetic tree with the 16S rRNA gene using the MEGA v5.05 program by neighbor-joining (NJ) method.

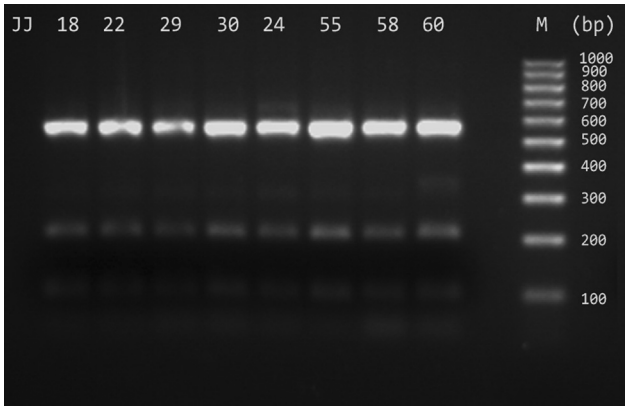

Figure 4 - 16S-ARDRA using *Alu*I restriction enzyme for the isolates. M is the 100 bp marker.

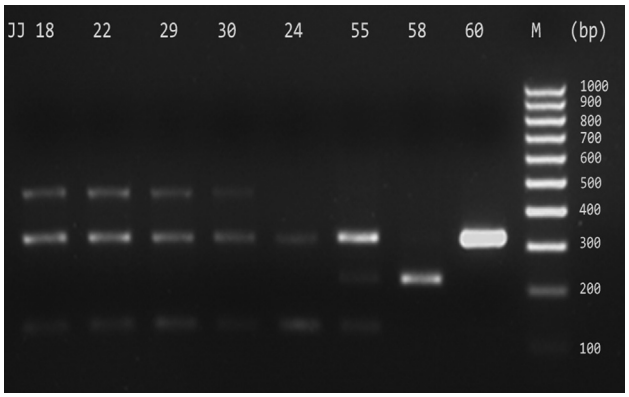

Figure 5 - Multiplex PCR using *recA* species specific primer. JJ 18, JJ 22, JJ 29 and JJ 30 were *Lactobacillus plantarum* with subspecies unidentified. JJ 24 is *Lactobacillus plantarum* subsp. *argentoratensis.* JJ 55 *is Lactobacillus plantarum* with subspecies unidentified. JJ 58 is *Lactobacillus pentosus*. JJ 60 is *Lactobacillus plantarum* subsp. *plantarum.* M is the 100 bp marker.

identified as *Lactobacillus pentosus* based on its amplicon around 218 bp and JJ 60 as *Lactobacillus plantarum* subsp. *plantarum* based on its amplicon around 318 bp. JJ 55 had an additional amplicon around 200 bp in addition to 318 bp and 120 bp. The carbohydrate utilization profile of JJ 55 was almost similar to JJ 58, except for lactose utilization. Probably, JJ 55 must be closely related to *Lactobacillus pentosus.* Similarly, JJ 18, JJ 22, JJ 29, and JJ 30 had an additional amplicon above 400 bp in addition to 318 bp and 120 bp (Figure 5). Based on the sugar utilization profile, JJ 18, JJ 22, JJ 29, and JJ 30 were not able to metabolize melizitose which is a key character in identifying *Lactobacillus plantarum* subsp. *argentoratensis* (Torriani *et al.*, 2001). The sugar utilization profile of other sugars was also similar to that of JJ 24 (Table 2). Probably, the four isolates JJ 18, JJ 22, JJ 29 and JJ 30 were closely related to *Lactobacillus plantarum* subsp. *argentoratensis.* Therefore, it is evident that in addition to various classical physiological, biochemical and sugar utilization profile, a combination of molecular methods can be used successfully for sub-species level identification of *Lactobacillus* isolates from fermented *idli* batter.

Bacteriocins produced by *Lactobacillus plantarum* are the subject of intense research because of their antibacterial activity against food borne pathogens and are being employed directly for preservation of food. LAB are natural food isolates which can be exploited in food industry as a tool to control undesirable bacteria. *Lactobacillus plantarum* is a versatile lactic acid bacterium, which is encountered in a range of environmental niches including dairy, meat and vegetable fermentations.

Conclusion

The bacteriocinogenic lactobacilli explored in this study can facilitate arriving at a consortium of lactobacilli as standard inoculums (starter cultures) to prepare *idli* batter having many beneficial effects. Further studies elucidating their probiotic and beneficial properties may pave way for commercial preparation of *idli* batter, biological preservation of foods, and biomedical applications.

Acknowledgments

The present study was supported by the grants received from Defence Research Development Organization (DRDO), New Delhi (ERIPR/ER/996). Ms. P. Jayaprabha Agaliya, JRF thanks DRDO for the financial support.

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