Incidence and preliminary characterization of Lactic acid bacteria as potential probiotic strains from an artisanal milk product, Chilika curd of Odisha

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Abstract: A traditional fermented milk product prepared from buffalo milk in Chilika, situated in the eastern coast of India, is known for its unique organoleptic properties, an exceptionally extended shelf-life and a special method of preparation by the ethnic community of that region. In order to study the potential probiotic lactic acid bacteria associated with this indigenous curd, a total of five isolates were identified and characterized for their probiotic properties. The isolates were identified as *Lactobacillus plantarum* SSU1, *Lactobacillus delbreuckii* subsp. *bulgaricus* SSU2, *Lactobacillus rhamnosus* SSU3, *Lactobacillus casei* SSU4 and *Lactobacillus rhamnosus* SSU5 by 16S rDNA gene sequencing. All the isolates showed good acid and bile tolerance ability, antibacterial activity against wide range of pathogens and presence of bacteriocin producing genes. Such strains with the probiotic attributes could be potential novel starter cultures for producing natural probiotic and various fermented functional food.

Keywords: Chilika curd, fermented buffalo milk, Lactic acid bacteria, probiotic, Plantaricin

Introduction

Milk and milk products are considered to be wholesome food due to the presence of whole array of nutrients such as proteins, fats, carbohydrates, vitamins and minerals. Milk being a good

substrate provides a perfect atmosphere for many microorganisms of which Lactic acid bacteria (LAB) are the predominant. LAB are a group of Gram positive, rod shaped, non- spore forming bacteria, mostly associated with various fermented food such as dairy products, beverages, meat and vegetables (Grosu-Tudor and Zamfir, 2012). They are known for the rapid acidification of substrate and production of acetic acid, ethanol, aromatic compounds, exo-polysaccharides and enzymes (Vuyst and Leroy, 2007). LAB and their food products are thought to confer a variety of important nutritional and therapeutic benefits and have many documented health promoting or probiotic effects in human (Parvez et al. 2006). Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (Joint FAO/WHO Expert Consultation 2002). LAB for use as probiotic cultures must be tolerant to acid and bile as these are the primary factors affecting strain selection (Hyronimus et al. 2000). LAB also exhibits vast antimicrobial property due to production of various organic acids, hydrogen peroxide and certain proteinaceous compounds called bacteriocins (Jamuna and Jeevaratnam, 2004). Bacteriocins produced by LAB have high thermal stress, wide pH range with no reported development of resistant bacteria (Perez et al. 2014) thus, making it highly useful for probiotic purpose.

Curd is one of the most popular milk products in India and forms a part of the regular diet of the common population. It is produced using the fermentation process in which LAB plays a key role. As curd is prepared in nearly every household, each region of the country has its own unique combinations of LAB flora used as starter culture in making of the indigenous curd of that region. Chilika curd is one such dairy food traditionally prepared with milk produced from buffaloes of Chilika lake area of Odisha state by the ethnic community of this region. Situated in the eastern coast of India and being the largest brackish water lagoon, the sea weeds of high salinity content serves as a special diet for the buffaloes of this region (Nanda et al. 2013). This makes the curd produced from the milk of these buffaloes very unique in its organoleptic properties. It is also known to have an exceptionally extended shelf life (Nanda et al. 2013). Thus, it is essential to study the consortium of LAB flora present in the Chilika curd responsible for its uniqueness and to recognize the probiotic properties of these LAB.

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Materials and Methods

Preparation of Chilika curd

The farmers of Chilika Buffalo Society follow a unique traditional technique for preparation of Chilika curd which is made in baskets of bamboo mats. The inner surface of these baskets is layered with inoculum of previously made curd and is sun dried. This process is repeated with multiple curd layers and intermittent sun treatment. Finally, boiled concentrated buffalo milk cooled to room temperature is added to these baskets covered with leaves and left undisturbed for the fermentation process to be completed.

Sampling and isolation of LAB

Random curd samples ($n = 3$) were collected aseptically from the local sellers covering regions in and around Chilika, Odisha. The samples were then transported to the laboratory in aseptic refrigerated condition. Each of these samples was homogenized. 1 ml of each sample was added to 9 ml of MRS (de Man, Rogosa and Sharpe) broth (Himedia) and incubated at 37°C for 48 h. Subsequently, dilution series of each enriched culture was prepared by using sterile normal saline solution (1:10; wt:vol) and from appropriate dilution, 0.1 ml was spread plated evenly on MRS plates. The plates were incubated anaerobically at 37°C for 48 h. Individual colonies were selected on the basis of morphology, Gram staining and catalase activity. The Gram positive and catalase negative isolates were transferred into MRS broth and incubated at 37°C for 48 h. The isolates were kept in MRS broth containing 20% (v/v) glycerol at - 80°C. Further analysis was carried out from the stored cultures.

Phenotypic characterization

The isolates were initially characterized based on Gram reaction, catalase activity, motility, carbohydrate fermentation and arginine hydrolysis (Marroki et al. 2011; Yu et al. 2012). The results are shown in Table 1.

Genotypic identification using 16S rDNA

Isolates showing varying phenotypic properties were selected for genotypic identification. Genomic DNA was extracted using overnight cultures of the selected isolates as described previously by Abed (2013). Amplification of the 16S rDNA was carried out using universal bacterial 16S rDNA primers; forward primer (5'-AAGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GGTTACCTTGTTACGACTT-3') amplifying 1500 bp amplicons as per Stanley et al. (1995). The PCR reaction mixture consisted of 100 ng of DNA template, 20 pmol of both forward and reverse primer, 2.5 mM of dNTPS, 1xPCR buffer, 0.75 units of Taq DNA polymerase and PCR water. The PCR was carried out under following conditions: initial denaturation at 94°C for 5 min,

followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min. Partial sequencing of the purified PCR products was done. The sequences of the isolates were deposited in GenBank database. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) program. The partial 16S rDNA sequences determined in the present study and those available in GenBank database were analyzed. Isolates were identified with $\geq 97\%$ identity.

Screening for probiotic properties

Acid and bile tolerance activity

The acid and bile tolerance of each isolate was tested according to the method described by Hyronimus et al. (2000) with some modifications. Active cultures of LAB isolates (1% v/v) with initial concentration of 10^6 cfu ml⁻¹ were used for both the tests. For acid tolerance test, the isolates were inoculated in 10 ml MRS broth with the pH adjusted to 2, 3 and 4 by using 1N HCL. Similarly, for bile tolerance tests, isolates were inoculated in MRS broth with 0.1%, 0.2% and 0.3% Ox bile (Himedia). All the test broths were incubated at 37°C for 24 h. The viable number of LAB at different pH and bile concentrations were enumerated by pour plate count on MRS agar plate incubated at 37°C for 48 h, compared to initial bacterial concentration and the percentage of survivability was calculated.

Antibacterial activity

The antibacterial activities of the strains were confirmed using agar well diffusion assay as described by Yang et al. (2012) with slight modifications. The isolates were tested against five food spoilage and pathogenic bacteria, *Vibrio parahaemolyticus* JF966211, *Bacillus cereus* ATCC 11778 (Himedia), *Aeromonas hydrophila* CAHH14, *Salmonella enteric* ATCC 35640 (Himedia) and *Staphylococcus aureus* ATCC 6538 (Himedia). Strains *Vibrio parahaemolyticus* JF966211 and *Aeromonas hydrophila* CAHH14 were obtained from Fish Health Management Division, ICAR- Central Institute of Fresh water Aquaculture, Bhubaneswar, India. Overnight cultures of the indicator strains were grown in nutrient broth (Himedia) and were lawn cultured in sterile Tryptic Soya Agar (TSA) (Himedia) plates with 6mm diameter wells. 50 µl of cell free supernatant of the isolates (10^8 cfu ml⁻¹) was placed in the wells in TSA plates. After 24 h of incubation at 37°C, the diameter of the zone of inhibition surrounding each agar well was measured.

To further test the proteinaceous nature of the substance responsible for inhibition, 10 µl of the trypsin enzyme (Sigma, Madrid) solution (10 mg ml⁻¹ in distilled water) was added to the cell free supernatant of the isolates which was then subjected to well diffusion assay (Ben Omar et al. 2008). Absence of zone of

inhibition indicated the proteinaceous nature of inhibitory substances.

Screening for known plantaricin gene

All the isolates were tested for the presence of plantaricin genes (plnA, plnB, plnC, plnD, plnEF, plnI, plnJ, plnK, plnG and plnN) as described by Ben Omar et al. (2008). PCR amplification was carried out under following conditions: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, annealing at appropriate temperatures (Table 2) for 1 min, extension at 72°C for 30 s and final extension at 72°C for 5 min. The PCR products were electrophoresed in 2% agarose gel and 1× TBE buffer (Sambrook et al. 1989).

Statistical analysis

Statistical analysis was conducted using the computerized software Statistical Package for Social Sciences (SPSS) version 18.0. Data are reported as means $\pm$ SE. Statistical significant difference was determined using Tukey's Method with One-Way ANOVA. Differences were considered significant at $P < 0.05$.

Results and Discussion

Phenotypic characterization and genotypic identification

In the present study, the isolates were phenotypically characterized using 21 biochemical tests including Gram's reaction,

Table 1 Phenotypic characteristics of the LAB isolates

Characteristics	Isolates				
	<i>L. plantarum</i> SSU1	<i>L. delbreuckii</i> <i>bulgaricus</i> SSU2	<i>L. rhamnosus</i> SSU3	<i>L. casei</i> SSU4	<i>L. rhamnosus</i> SSU5
Gram's Reaction	+	+	+	+	+
Shape	R	R	R	R	R
Catalase	-	-	-	-	-
Motility	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-
L-arabinose	-	-	-	-	-
D - glucose	+	+	+	+	+
Esculin	+	+	+	+	+
Galactose	+	-	-	+	-
Inositol	-	-	-	-	-
Inulin	+	+	+	-	+
Lactose	+	-	+	+	+
Maltose	+	-	-	+	+
Mannitol	+	-	-	-	-
Melezitose	+	-	-	+	-
Melibiose	-	-	-	-	-
Raffinose	-	-	-	-	-
Ribose	+	-	+	+	-
Salicin	+	+	+	+	-
Sorbitol	-	-	-	-	-
Sucrose	+	-	+	+	+
Trehalose	-	-	-	+	+

R: rod; - : negative; + :positive

catalase activity, motility and carbohydrate utilization tests. Almost all the strains isolated from curd in MRS agar were considered to be LAB based on their positive Grams reaction, absence of motility, absence of spore formation and absence of catalase activity. The fermentation profile revealed that the isolates assimilated variously a panel of carbohydrates that reflected their enzymatic and genetic potentials along with their phenotypic heterogeneity and diversity. The LAB genomes are predicted to carry a large number of carbohydrate transport and utilization genes that display substantial variations among strains (Ceapa et al. 2015; Maji et al. 2016).

Of the phenotypically characterised strains, 5 rod shaped isolates showing varying phenotypic characters were presumptively determined as derivatives of the genus *Lactobacillus* and subjected to genotypic identification. The partial sequencing of 16S rDNA gene identified the isolates as *Lactobacillus plantarum* SSU1, *Lactobacillus delbreuckii* subsp. *bulgaricus* SSU2, *Lactobacillus rhamnosus* SSU3, *Lactobacillus casei* SSU4 and *Lactobacillus rhamnosus* SSU5 with accession numbers as KF971888, KF971889, KF971890, KF971891 and KF971892 respectively. Nanda et al. (2013) have also reported the isolation of *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* from this indigenous curd. Every region has its typical sets of lactoflora in the indigenous fermented product of that region and *Lactobacillus* is considered to be the predominant genus in almost all dairy products. A study on naturally fermented yak milk found *L. delbreuckii* subsp. *bulgaricus* and *S. thermophilus* as the predominant microflora in the product (Sun et al. 2010). Bettache et al. (2012) also reported the dominance of *Lactobacilli*

in the microflora of Dhan, a traditional butter. Angmo et al. (2016) identified and characterized *Lactobacillus brevis*, *Lactobacillus plantarum* and *Lactobacillus fermentum* as probiotic lactic acid bacteria from chhurpe, a dried cottage cheese and chhang, a barley based beer of Ladakh. Also, *Lactobacillus* was found to be the dominant lactic acid bacteria isolated from various traditional fermented dairy products of Turkey (Erginkaya et al. 2018).

Acid and bile tolerance activity

LAB are known for their probiotic characteristics and their use in probiotic products. Acid and bile tolerance are important traits in characterizing the probiotic property of a strain. In order to reach the hindgut in an active and functional form and exert their beneficial properties, the strains should tolerate and survive in extremely stressed conditions like low pH and high bile concentration in the upper parts of gastrointestinal tract (Maji et al. 2016; Zhang et al. 2016). In the present study, most of the isolates showed good survivability even after 24 h of exposure to pH 3 and pH 4 (Table 3) which is in consistent with earlier findings (Grosu-Tudor and Zamfir, 2012). However, not all strains showed similar tolerance capability as survivability at low pH is strain specific (Tokatl et al. 2015) and is an adaptation to environmental conditions (Grosu-Tudor and Zamfir, 2012). *L. casei* SSU4 showed maximum significant ($P < 0.05$) viability at pH 3 and pH 4 compared to other isolates. Also, none of the isolates survived at pH 2 for such a long time whereas *L. delbreuckii* subsp. *bulgaricus* SSU2 did not survive at pH 3 but showed

Table 2 PCR primers used for detection of Plantaricin genes

Target Genes	Primers	Annealing Temperature(°C)	Size(bp)
<i>plnA</i>	F: GTACAG TAC TAATGG GAG R: CTT ACG CCAATC TAT ACG	53	450
<i>plnB</i>	F: TTCAGAG GCAAGC CTAAAT GAC R: GCCACT GTAACACCATGAC	51.5	165
<i>plnC</i>	F: AGCAGATGAAAT TCG GCAG R: ATAATC CAA CGG TGC AAT CC	49.5	108
<i>plnD</i>	F: TGAGGACAAACA GAC TGG AC R: GCATCG GAAAAA TTG CGG ATAC	53	414
<i>plnEF</i>	F: GGCATA GTT AAAATT CCC CCC R: CAG GTT GCC GCAAAAAAAG	53.2	428
<i>plnI</i>	F: CTC GAC GGT GAAATT AGG TGT AAG R: CGT TTATCC TAT CCT CTAAGC ATT GG	52.5	450
<i>plnG</i>	F: TGC GGT TAT CAG TAT GTC AAAG R: CCT CGAAAC AAT TTC CCC C	52.8	453
<i>plnN</i>	F: ATT GCC GGG TTA GGT ATC G R: CCT AAA CCA TGC CAT GCAC	51.9	146
<i>plnJ</i>	F: TAACGACGG ATT GCT CTG R: AAT CAA GGAATT ATC ACATTA GTC	51	475
<i>plnK</i>	F: CTG TAA GCA TTG CTAACC AAT C R: ACT GCT GAC GCT GAAAAG	52.9	246

100% survivability at pH 4. Similarly, in bile tolerance test, all the isolates showed 100% survival at 0.1%, 0.2% and 0.3% bile after 24h of exposure except *L. delbreuckii* subsp. *bulgaricus* SSU2 which showed no survival rate at 0.2% and 0.3% bile concentration (Table 3). Earlier studies have reported high survivability rate of *L. plantarum* and *L. brevis* in 0.3% and 0.5% bile concentrations (Grosu-Tudor and Zamfir, 2012; Tokatl et al. 2015).

Antibacterial activity

All the isolates showed antibacterial activity against the food spoilage and pathogenic bacteria. The inhibitory zones varied between the ranges of 11 mm to 17 mm in diameter (Table 4) with *L. casei* SSU4 showing the best results. These findings are in

Table 3 Acid and bile tolerance capability of the isolates

Isolates	Initial viable count	Viable count at pH 2 after 24h (CFU mL ⁻¹)	Viable count at pH 3 after 24h (CFU mL ⁻¹)	Viable count at pH 4 after 24h (CFU mL ⁻¹)	Viable count at 0.1% bile after 24h (CFU mL ⁻¹)	Viable count at 0.2% bile after 24h (CFU mL ⁻¹)	Viable count at 0.3% bile after 24h (CFU mL ⁻¹)
<i>L. plantarum</i> SSU1	7.87 ± 0.01	Nil	4.43 ± 0.23 ^a	5.38 ± 0.1 ^a	6.26 ± 0.13 ^a	7.31 ± 0.16 ^a	7.14 ± 0.18 ^a
<i>L. delbreuckii</i> subsp. <i>bulgaricus</i> SSU2	7.81 ± 0.05	Nil	Nil	6.06 ± 0.17 ^a	7.68 ± 0.04 ^b	Nil	Nil
<i>L. rhamnosus</i> SSU3	7.86 ± 0.02	Nil	4.9 ± 0.15 ^a	5.66 ± 0.08 ^a	7.61 ± 0.06 ^b	7.45 ± 0.08 ^a	7.41 ± 0.06 ^a
<i>L. casei</i> SSU4	7.9 ± 0.05	Nil	5.71 ± 0.05 ^b	7.6 ± 0.11 ^b	7.78 ± 0.04 ^b	7.71 ± 0.05 ^a	7.56 ± 0.08 ^a
<i>L. rhamnosus</i> SSU5	7.76 ± 0.08	Nil	4.59 ± 0.1 ^a	6.48 ± 0.1 ^a	7.5 ± 0.1 ^b	7.47 ± 0.09 ^a	7.4 ± 0.1 ^a

Values are mean of triplicate and presented as means ± S.E; In columns, parameters of isolates with different letters (a, b) are significantly different ($P < 0.05$)

Table 4 Antibacterial activity of the isolates

Isolates	Zone of inhibition (in mm) ± SEM against <i>V. parahaemolyticus</i>	Zone of inhibition (in mm) ± SEM against <i>B. cereus</i>	Zone of inhibition (in mm) ± SEM against <i>A. hydrophila</i>	Zone of inhibition (in mm) ± SEM against <i>S. enterica</i>	Zone of inhibition (in mm) ± SEM against <i>S. aureus</i>
<i>L. plantarum</i> SSU1	12.2 ± 0.12	12.8 ± 0.30	10.3 ± 0.27	9.6 ± 0.27	11 ± 0.19
<i>L. delbreuckii</i> subsp. <i>bulgaricus</i> SSU2	14 ± 0.11	11.1 ± 0.16	15 ± 0.23	13.1 ± 0.13	10.3 ± 0.27
<i>L. rhamnosus</i> SSU3	15.3 ± 0.13	13.1 ± 0.22	11.6 ± 0.36	10.5 ± 0.23	11.1 ± 0.36
<i>L. casei</i> SSU4	16 ± 0.09	15 ± 0.19	10.6 ± 0.27	11.8 ± 0.36	13.7 ± 0.32
<i>L. rhamnosus</i> SSU5	17 ± 0.23	13 ± 0.09	12.8 ± 0.13	12.4 ± 0.28	9.5 ± 0.23

SEM : standard error of mean

Fig. 1 Agarose gel electrophoresis of PCR amplification products of bacteriocin, Plantaricin genes; (a) plnA, 450 bp, Lane 1 to 5 & plnB, 165 bp, Lane 6 to 10 (b) plnC, 108 bp, Lane 1 to Lane 5 & plnD, 414 bp, Lane 6 to 10 (c) plnEF, 428 bp, Lane 1 to 5 & plnI, 450 bp, Lane 6 to 10 (d) plnG, 453 bp, Lane 1 to 5 & plnN, 146 bp, Lane 6 to 10 (e) plnJ, 475 bp Lane 1 to 5 & plnK, 246 bp, Lane 6 to 10 in isolates; *L. plantarum* SSU1 (Lanes 1 & 6); *L. delbreuckii subsp. bulgaricus* SSU2 (Lanes 2 & 7); *L. rhamnosus* SSU3 (Lanes 3 & 8); *L. casei* SSU4 (Lanes 4 & 9); *L. rhamnosus* SSU5 (Lanes 5 & 10); M: Marker

agreement with earlier studies where similar *in vitro* antagonisms against several food spoilage pathogens have been reported by using *Lactobacillus* spp (Jamuna and Jeevaratnam, 2004; Yang et al. 2012; Zhang et al. 2016). The antimicrobial activity of the LAB isolates is thought to be multi factorial and is due to the synergistic effect of production of organic acids (acetic acid or lactic acid) and strain specific metabolites or non-lactic acid molecules, bacteriocins, etc (Maji et al. 2016).

During preliminary screening for bacteriocin production, all the strains showed no zone of inhibition after addition of trypsin indicating the proteinaceous nature of the inhibitor. Bacteriocin, the proteinaceous compound produced by LAB usually inhibits closely related species (Ben Omar et al. 2006). In the present study, however, the isolates showed inhibitory activity against a wide range of Gram positive as well as Gram negative bacteria, which is a very desirable probiotic property. A few earlier reports

have also shown antagonistic activity of *Lactobacillus* against Gram negative bacteria such as *V. parahaemolyticus*, *E. coli*, *S. typhi*, *S. enterica*, *P. fluorescens*, and *P. putida* (Gong et al. 2010; Chowdhury et al. 2012; Zhang et al. 2016).

Detection of known plantaricin gene

The entire plantaricin gene cluster consisting of *plnA*, *plnB*, *plnC*, *plnD*, *plnEF*, *plnI*, *plnJ*, *plnK*, *plnG* and *plnN* genes as described in *L. plantarum* C11 (Diep et al. 1996) was detected in *L. plantarum* SSU1. Also the isolates *L. delbreuckii* subsp. *bulgaricus* SSU2, *L. rhamnosus* SSU3, *L. casei* SSU4 and *L. rhamnosus* SSU5 showed the presence of all plantaricin genes (Fig. 1). Ben Omar et al. (2008) reported plantaricin genes in *L. fermentum* isolated from the same source as *L. plantarum*. Hurtado et al. (2011) also reported the presence of plantaricin in closely related species *L. plantarum* and *L. pentosus*. Bacteriocin production is frequently associated with mobile genetic elements such as plasmids which are common in LAB that may facilitate the transfer of genes between species and strain sharing the same niche. Also, some closely related species may have similar characteristic for survival in the same environmental conditions (Hurtado et al. 2011). Further confirmatory test are needed to be able to select the above mentioned strains of *Lactobacillus* as probiotic organisms.

Conclusions

The present study demonstrates the presence of diverse *Lactobacillus* species with promising probiotic potential in the indigenous curd unique to the Chilika lake area of the state of Odisha, India. It is to mention that, the Chilika Lake happens to be the largest brackish water lake of Asia. Further elucidated study of this artisanal product shall fully explore it as a potential source for such probiotic bacteriocinogenic strains which can contribute towards formulation of functional foods with health beneficial properties.

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