JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

MOLECULAR CHARACTERISATION OF PROBIOTIC *LACTOBACILLUS FERMENTUM* ISOLATED FROM HOME MADE CURD

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doi: 10.15414/jmbfs.2020.9.4.848-855

ARTICLE INFO

Received 8. 11. 2018
Revised 3. 7. 2019
Accepted 26. 8. 2019
Published 3. 2. 2020

Regular article

OPEN ACCESS

ABSTRACT

The present study was undertaken to explore the probiotic potential of Lactobacillus isolated from traditional homemade curd. A total of 32 lactic acid bacterial isolates were screened from homemade curd collected from various regions of chittoor district, Andhra Pradesh, India. Most of the bacterial isolates were found to be gram positive, catalase negative and were preliminarily evaluated for their probiotic potential like acid tolerance, bile tolerance, thermo tolerance, osmo tolerance and antibiotic susceptibility. Among all the 32 isolates, 9 isolates viz. L_1, L_2, L_3, L_4, L_5, L_6, L_7, L_8, and L_9 were found to be having promising probiotic activity were further screened for antagonism against enteropathogens. One of the isolate (*Lactobacillus* spp L_2) with significant probiotic properties, antagonistic activity was characterized by 16S rRNA sequence analysis and was identified as *Lactobacillus fermentum*. The bacteriocin produced by *Lactobacillus fermentum* was purified by a process of ammonium sulfate precipitation and chromatography techniques. The study revealed the possibility of using bacteriocin as a food preservative and the *Lactobacillus fermentum* strain as probiotic.

Keywords: Antimicrobial activity, 16S rRNA sequence analysis, *Lactobacillus fermentum*, phylogenetic tree, bacteriocin, purification

INTRODUCTION

Bacteriocins from Lactic Acid Bacteria (LAB) isolated from traditional foods of India have proven its applicability in biopreservation **(Jamuna and Jeevaratnam, 2005).** The curd is an Indian traditional fermented milk product that used as a main food sources in our routine life. The fermented milk products are probiotics that contain viable lactic acid bacteria (LAB) and its metabolic by-products act as an antioxidant, immune modulator and antimicrobial agents. Probiotics have become a major topic of lactic acid bacteria (LAB) research over the past 10 years, which include the genera of Lactobacillus and Bifidobacterim. There have been interests in commercial utilization of Lactobacillus strains isolated from traditional naturally fermented dairy products, which possess health-promoting effects. However to provide health benefits, Lactobacillus strains, which are mostly delivered in a food system, must overcome physical and chemical barriers in the gastrointestinal tract, especially acid and bile stresses **(Del piano *et al.*, 2006).** Thus, there is a growing interest worldwide in the use of probiotic bacteria for their various beneficial influences on animal and human health.

Lactobacilli are wide spread in nature and many species have found applications in the food molecular techniques involving gene sequencing are now the “gold standard”. Over the past decades a number of strains of LAB have been incorporated in a wide range of food products for human and animal nutrition. As the probiotic capacities are strain-dependent, methods for identifying LAB at the strain level are of great importance, especially for the quality control of approved strains, to avoid health risks and misleading claims, as well as for the description of new strains. Now a days the main focus for identification has moved from phenotypical to genotypical methods as the latter generate more sensitive and accurate results. Gram-positive lactobacilli belong to the general category of lactic acid bacteria. They are found in a variety of habitats, including the gastrointestinal tract and some strains are extensively used in food industry and pharmacy due to their healthful properties **(Walter *et al.*, 2000)**. Recently, many efforts have been made to search potential probiotic Lactobacillus strains from dairy products. For this purpose, simple and reliable identification methods are required **(Nakatawa, 1994; Heilig, 2002).** Conventional biochemical and physiological tests **(Verthier and Ehrlich, 1999; Kandler and Weiss, 1986)** clearly have some limitations in discriminating large number of isolates showing similar physiological characteristics. Therefore, many studies have focused on the application of molecular biology techniques for the rapid identification of Lactobacilli **(Andrighetto, 1998)**. The DNA– DNA hybridization technique has improved knowledge on taxonomic relationships between Lactobacillus species, but this technique is still time-consuming and labours- intensive **(Song *et al.*, 1999)**. Besides, various methods such as DNA sequencing, fatty acid analysis and RFLP have been established and applied to the identification of probiotics **(Yeung, 2002)**. Recently, rRNA genes have been generally accepted as the potential targets for identification and phylogenetic analysis of bacteria **(Amann, 1995; Burton *et al.*, 2003)**. Sequencing analysis of the 16 S r RNA genes has been used to determine the diversity and dynamics of LAB in food **(Chen and Hoover, 2003; Jung-Min, 2010)**.

Bacteriocins produced by this LAB are natural peptides that exert bactericidal activity against pathogenic bacteria and this fact creates the possibility of improving their characteristics to enhance their activity and spectra of action **(Saavedra *et al.*, 2004)**. In order to avoid confusion with therapeutic antibiotics, which can potentially illicit allergic reactions in humans and other medical problems, bacteriocins are produced by bacteria and are normally not termed as antibiotics **(Deraz *et al.*, 2005)**. Bacteriocin production could be considered as an advantage for food and feed producers in sufficient amounts and these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against related bacteria with nutritive demands for the same scarce resources **(Deegan *et al.*, 2006)**. Purification of the bacteriocins is helpful for the knowledge of the mechanism of action, structure and other characteristics, which helps to isolate the bacteriocin biosynthetic genes.

In recent times, the concept of biodiversity in a particular ecosystem has been articulated through the use of phylogenetic tree maps based on gene sequences. Owing to the considerable economical importance of LAB, many researchers are now actively working on these bacteria using an array of genetic tools. Moreover chromosomal genes of interest have been characterized providing a new insight into the genetic organization of LAB.

This study was designed to screen the potential probiotic bacteriocinogenic *Lactobacillus* spp from homemade curd sample that can able to inhibit enteropathogens and also the *Lactobacillus* spp was identified using 16S rRNA sequence analysis. The purification of bacteriocin from identified *Lactobacillus fermentum* may exert beneficial effects for mankind.

MATERIAL AND METHODS

Isolation and Identification of Bacterial strains

Curd samples were collected under aseptic conditions and were serially diluted, 100 µl of 10^{-6} dilution from each sample was plated in triplicate on DeMan Rogosa Sharpe (MRS) agar and incubated at 37 °C for 24 h **(De Man *et al*., 1960)**. The colonies on MRS agar plates were examined morphologically and microscopically. The colonies were streaked on MRS agar to check the purity. The pure cultures were preserved using 20 % glycerol for further study **(Pal *et al*., 2005).**

Screening of probiotic properties

To study the probiotic properties of *Lactobacillus* spp, the cultures were grown in MRS broth by changing pH, temperature, bile and salt concentrations **(Chesson *et al*., 2002)**.

Acid tolerance

Tolerance of isolated *Lactobacillus* strains to acidic pH was determined by growing the strains at 37ºC for 24 – 48 h in acidic MRS broth adjusted to four different pH values (pH 2, 3, 4 and 5) and by maintaining pH7.0 as control **(Xanthopoulos *et al*., 2000;Papamanoli *et al*., 2003)**. After 24 h of incubation, the growth was measured at 600 nm using spectrophotometer and the survival (viable number) of the *Lactobacillus* strains were enumerated by pour plate counts using two fold serial dilutions **(Conway *et al*., 1987 and Brashears *et al*., 2003)**.The experimental analysis was carried out in duplicates. The results are shown as the average of two replicates. A reference strain was used to consider as positive control.

Thermo tolerance

To study the thermo tolerance, the isolated bacterial cultures were grown in MRS broth and incubated at different temperatures like 30ºC, 40ºC, 50ºC and 60ºC for 24 - 48 h. The cultures grown at 37 ºC considered as control. After incubation, the growth was measured at 600 nm to select the thermo tolerant *Lactobacillus* strains and the survival rate of the *Lactobacillus* strains were enumerated by pour plate counts using two fold serial dilutions. The experiments were carried out in duplicates. The results are shown as the average of two replicates.

Bile tolerance

To determine the effect of bile on the growth of the *Lactobacillus* spp, strains were cultivated in MRS broth enriched with different concentrations of bile salt (sodium thioglycolate) 1%, 2% and 3% at 37ºC for 24 h. MRS broth without bile was considered as control. Bacterial growth was monitored by measuring absorbance at 600nm using spectrophotometer, simultaneously the viable counts of the *Lactobacillus* spp were expressed in colony forming units per millilitre (cfu/ml) **(Succi *et al*., 2005)**. The experiment was repeated twice and each reading represents the average of two replicates.

Osmo tolerance

To determine osmo tolerance of *Lactobacillus* spp strains were grown in MRS broth with different concentrations of NaCl like1%, 1.5%, 2%, 2.5% and 3% respectively and incubated at 37 ºC for 24 h. After incubation, the cell turbidity was measured at 600 nm **(Axelsson, 1998; Erkkila and Petaja, 2000)** and the survival rate of the *Lactobacillus* strains were enumerated by pour plate counts in colony forming units per mililiter **(Tsai *et al*., 2005)**. The *Lactobacillus* spp grown in MRS broth with 0 % NaCl was considered as control. The experimental analysis was carried out in duplicates with the average of two replicates.

Detection of antimicrobial activity of the selected Lactobacillus isolates

The inhibitory activity of the selected *Lactobacillus* isolates was determined using agar well diffusion assay against the enteropathogens *Escherichia coli, Enterococcus faecalis, Pseudomonas fluorescence, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhimurium* and *Proteus mirabilis* **(Subramanyam *et al*., 2014).** The test bacteria were inoculated in nutrient broth and incubated at 37 °C for 24 h. Cell free supernatant of the isolates was obtained by centrifugation at 5000Xg at 4 °C for 20 min and was collected for bacteriocin assay. Supernatant was adjusted to pH7 with 1N NaOH to nullify the acid activity. The cell free supernatant thus obtained was used for detection of antibacterial activity. The petridishes containing MRS agar was seeded with the selected Lactobacillus isolates. Wells were created on the seeded agar using incinerated agar borer. 50 µl of the supernatant was filled into the wells of the inoculated plates and incubated for 24 h at 37 °C and the inhibition zones around the wells was measured. Antimicrobial activity was determined as arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition.

Characterization and Identification of the Strain

The strain with probiotic properties and antagonistic activity was identified by 16S r RNA sequence analysis. The genomic DNA from selected bacterial culture was extracted according to the method described by **Park *et al*., (2010).** 16S r RNA gene was amplified by polymerase chain reaction (PCR) using the forward primer F (5'-AGAGTTTGATCMTGGCTCAG3') and reverse primer R (3' AAGGAGGTGWTCCARCC) 5'. Amplification was performed in a DNA thermal cycler at 95 °C for 3 min, followed by 30 cycles at 94 °C of 30 s, 1 min at 52 °C and 90 s at 72 °C with a final extension of 72 °C for 5 min. The nucleotide sequences obtained has been deposited in the EMBL database. Search of nucleotide sequence homology was done using the Blast algorithm **(Altschul *et al*., 1990)** and then the sequences were aligned using ClustalW **(Thompson *et al*., 1994)**. Phylogenetic analysis was performed using MEGA software **(Tamura *et al*., 2013).** The evolutionary distances were computed using the Maximum Composite Likelihood method **(Tamura *et al*., 2004).**

Purification of bacteriocin

Ammonium sulfate precipitation

For the isolation of bacteriocin, the bacterial strain was propagated in MRS broth at 37 ºC for 24 h. The cells were removed by centrifugation at 10,000 rpm, for 30 min at 4 ºC and the cell free supernatant was collected. The supernatant fluid was then precipitated with ammonium sulphate of 10 %, 20 %, 30 %, 40 %, 50 % and 60 % saturation for overnight at 4 ºC with gentle stirring till the level of saturation occurs **(Harris *et al*., 1989)**. A floating precipitate was formed. After centrifugation the precipitate was resuspended in 25 ml of potassium phosphate buffer (pH 6.0). The protein precipitate was concentrated and dialyzed in a tubular cut-off membrane, against buffer for 24 h. The obtained dialysate was used as partially purified sample.

Sepharose column chromatography

The partially purified bacteriocin was further purified by conventional gel filtration chromatography using sepharose G-50 column, **(De courcy, 2004)**. The dialysed sample was injected into a sepharose fast-flow column which was pre-equilibrated with 20 mM sodium phosphate buffer (pH 6.8). The absorbed bacteriocin was eluted with phosphate buffer at a flow rate of 0.5 ml/min for 24 h. Each fraction was carried out for antimicrobial activity using *Staphylococcus aureus, Enterococcus faecalis, Proteus mirabilis, Salmonella typhi* and *Escherichia coli* as indicator strains. The fractions with high antimicrobial activity were pooled and the concentrated fraction further purified by HPLC.

HPLC

The final purification step was performed with HPLC run. Bioactive fraction F2 obtained from column chromatography was subjected to high performance liquid chromatography **(Guyonnet *et al*., 2000)**. The sample was slowly injected into the HPLC system and it was passed through the C18 column. Hexane and ethyl acetate was used as mobile phase and the bacteriocin was eluted at the flow rate of 1 ml min−1. The absorption was measured at 220 nm.

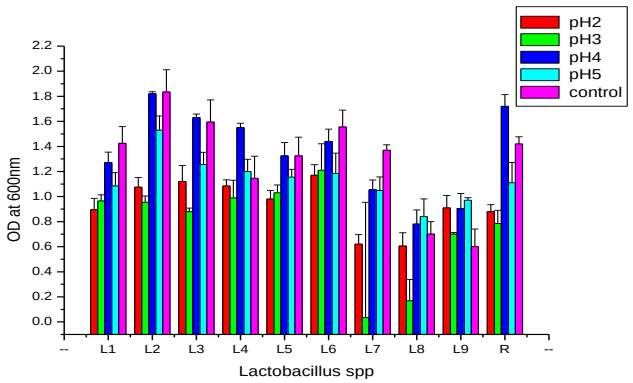
RESULTS

Isolation and screening of Lactobacillus strains for probiotic properties

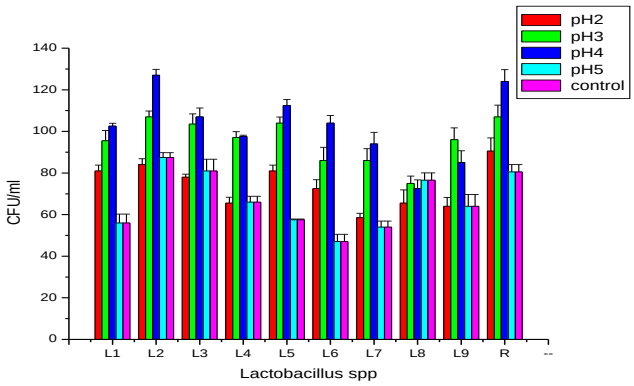
About 32 isolates of *Lactobacillus spp*. were isolated from home made curd and were characterised as non-motile, catalase negative, Gram positive rods. All the thirty two strains were evaluated for their probiotic properties of acid, thermo, bile, osmo tolerance, *in vitro* antimicrobial activity and antibiotic susceptibility.

Acid tolerance

Tolerance level of all *Lactobacillus* spp to acidic environment was found significantly variable. The growth pattern and the survival rate of the *Lactobacillus* spp, under acidic conditions at pH 2, 3, 4, 5 is illustrated in Fig 1 (a) and (b). All the thirty two strains had shown limited growth towards acidic conditions at pH 4. Maximum growth of 1.82 O.D was recorded for L_2 strain of *Lactobacillus* at pH value 4.0, followed by L_3(1.63), L_1(1.27), L_6(1.44), L_8(0.78) and L_9 (0.90) isolates, the remaining *Lactobacillus* isolates shown fair growth. The decline in the growth was noticed at pH 2.0 and 3.0. the maximum growth of 1.72 O.D value at pH 4 and the lowest growth of 0.78 at pH 3 was obtained to the reference strain. As shown in fig 1 (a), as the pH value increases beyond 4, growth of all the cultures decreased correspondingly. Regarding the cell viability, highest viable count of 127 CFU/ml at pH 4.0 was found with *Lactobacillus* spp of L_2 followed by *Lactobacillus* spp of L_5, L_3, L_6, L_1 and L_4 and the number of viable cells of these *Lactobacillus* spp reduced at pH value 5.0. The *Lactobacillus* spp L_2 was found to survive well at pH 4 followed by decrease in the number of viable cells at pH 5.0



(a)

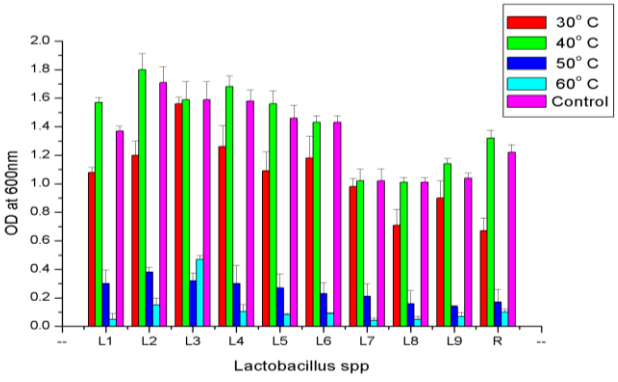


(b)

Figure 1 (a) Growth of *Lactobacillus* spp in acidic pH , (b)Viable count of *Lactobacillus* spp in acidic pH

Thermo tolerance

All the isolated *Lactobacillus* spp shown moderate growth and cell viability at 40ºC, but the *Lactobacillus* spp of L_2 yielded maximum growth of 1.8 OD, with the viable count of 114 CFU/ml at 40ºC, followed by other isolates L_1(1.57), L_3(1.59), L_4(1.68),L_5(1.56), L_6(1.43) and L_7(1.07). The *Lactobacillus* spp L_2 was still resistant at 50 ºC with 0.38 O.D value and 40 CFU/ml and it rapidly lost the growth at 60 ºC. The remaining *Lactobacillus* spp shown fair growth at 50 ºC. Figure 2 (a) and (b) represent the growth rate and viable count of *Lactobacillus* spp at different temperatures. The above results revealed that the nine *Lactobacillus* strains of L_1 to L_9 are found to be tolerant to 50 ºC temperature with good growth and cell viability. There is gradual decrease in the growth as the temperature increased.



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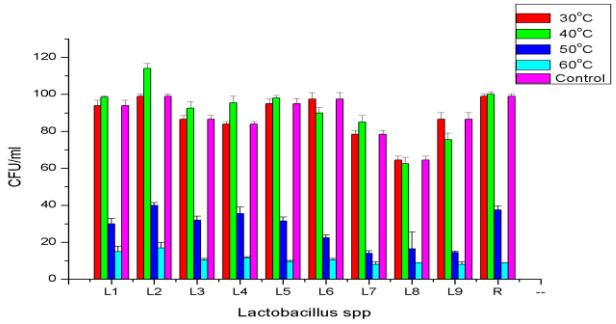


(b)

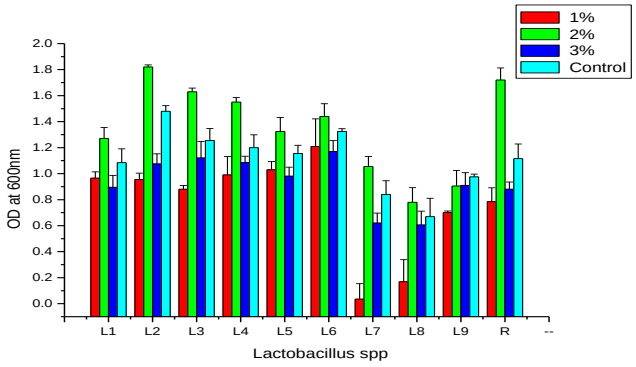
Figure 2 (a) Growth of *Lactobacillus* spp at different temperatures, (b) Viable count of *Lactobacillus* spp at different temperatures

Bile tolerance

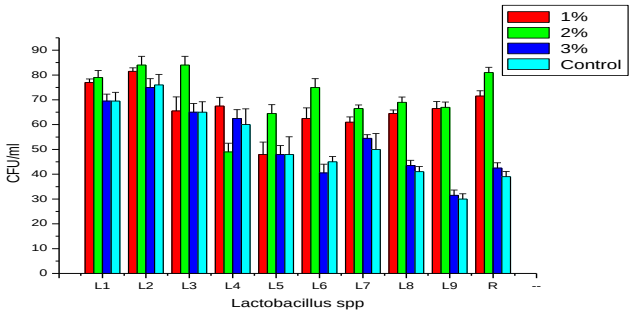
Effect of bile salt on the isolated *Lactobacillus* spp at different concentrations (sodium thioglycolate) was evaluated (Fig 3 (a) and (b)). Maximum growth of 1.82 OD value was obtained for *Lactobacillus* spp of L_2, followed by the isolates of L_3, L_4 and L_6 with the OD values of 1.63, 1.55 and 1.44 respectively at 2% level of bile concentration. The highest viable count of 84 CFU/ml was found with *Lactobacillus* spp L_2 and L_3 at 2 % bile concentration followed by *Lactobacillus* spp of L_1, L_6, L_8 and L_4. The growth and the viable counts of *Lactobacillus* spp L_3, L_4, L_8 and L_6 was decreased as the bile salt concentration increased to 3 % or decreased to 1 %.

Osmo tolerance

Tolerance to salt concentration was tested as an additional indicator for survival of the bacterial strain. The *Lactobacillus* spp L_2 exhibited maximum growth at 2 % NaCl concentration with 1.93 O.D value , whereas the isolates of L_3, L_4, L_5 and L_6 shown 1.59, 1.14, 1.32 and 1.55 O.D values respectively when compared to the other isolates. The results are shown in fig 4 (a) and (b). Further the growth and the viable count started to decline significantly at 2.5 % and 3.0 % concentration of NaCl with *Lactobacillus* spp L_2 isolate. The results indicates that all the five tested concentrations of salt influenced the growth and viability of *Lactobacillus* isolates L_{11} to L_{32}. The viable count of the *Lactobacillus* spp L_2 was found to be greater at 2 % NaCl concentration of 104 CFU/ml followed by L_3, L_1 and L_5 isolates. The results clearly indicates that the strain L_2 has maximum tolerance to 2 % NaCl concentration followed by L_4, L_3, L_5, L_6 and L_9.

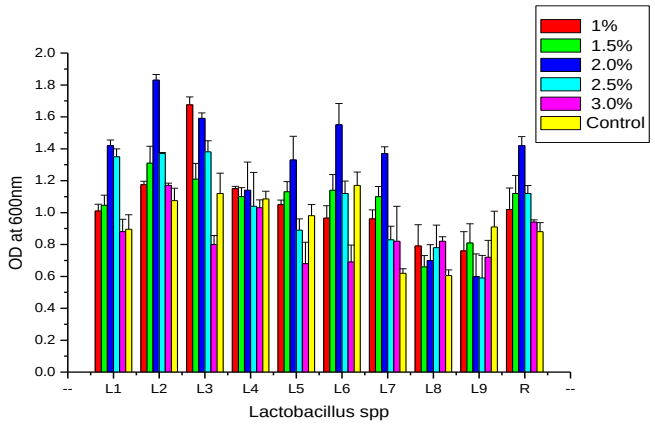


(a)



(b)

Figure 3 (a) Growth of *Lactobacillus* spp in bile salt, (b) Viable count of *Lactobacillus* spp in bile salt



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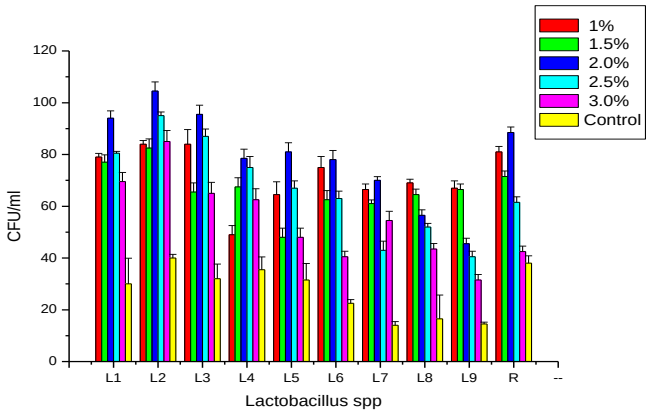

(b)

Figure 4 (a) Growth of *Lactobacillus* spp in different NaCl concentration, (b) Viable count of *Lactobacillus* spp in different NaCl concentration

Screening of *Lactobacillus* isolates for bacterial activity

The selected nine *Lactobacillus* isolates having probiotic properties were able to inhibit the growth of some enteropathogenic organisms by agar well diffusion assay (Fig 5 A-K). To study the antimicrobial properties of bacteriocin the pH of the cell free supernatant (CFS) of the nine *Lactobacillus* strains was neutralized with 1 N NaOH solution in order to eliminate acids like lactic acid and then sterilized by boiling. The CFS was treated with catalase of 5 mg/ml to completely eliminate possible inhibitory effect of hydrogen peroxide. Inhibitory activity of the neutralized cell free supernatant of *Lactobacillus* spp L_2 was able to inhibit the growth of *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Proteus mirabilis*, the antimicrobial activity of CFS of *Lactobacillus* spp L_3 could inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*, and *Lactobacillus* spp L_4 has the ability to inhibit *Staphylococcus aureus*, whereas the *Lactobacillus* spp L_5 inhibit *Proteus mirabilis, Escherichia coli* and *Salmonella typhimurium*, *Lactobacillus* spp L_6 possess antimicrobial activity against *Salmonella typhimurium*, while *Lactobacillus* spp L_1, L_7, L_8 and L_9 did not show any antimicrobial activity against the enteropathogens. The results are presented in (Tab1). Bacteriocin capable of inhibiting the growth of gram negative strains is very obscure; however the strain L_2 exhibited strong antagonism to gram positive and gram negative pathogenic bacteria than the other strains. Hence strain L_2 was selected for further identification.

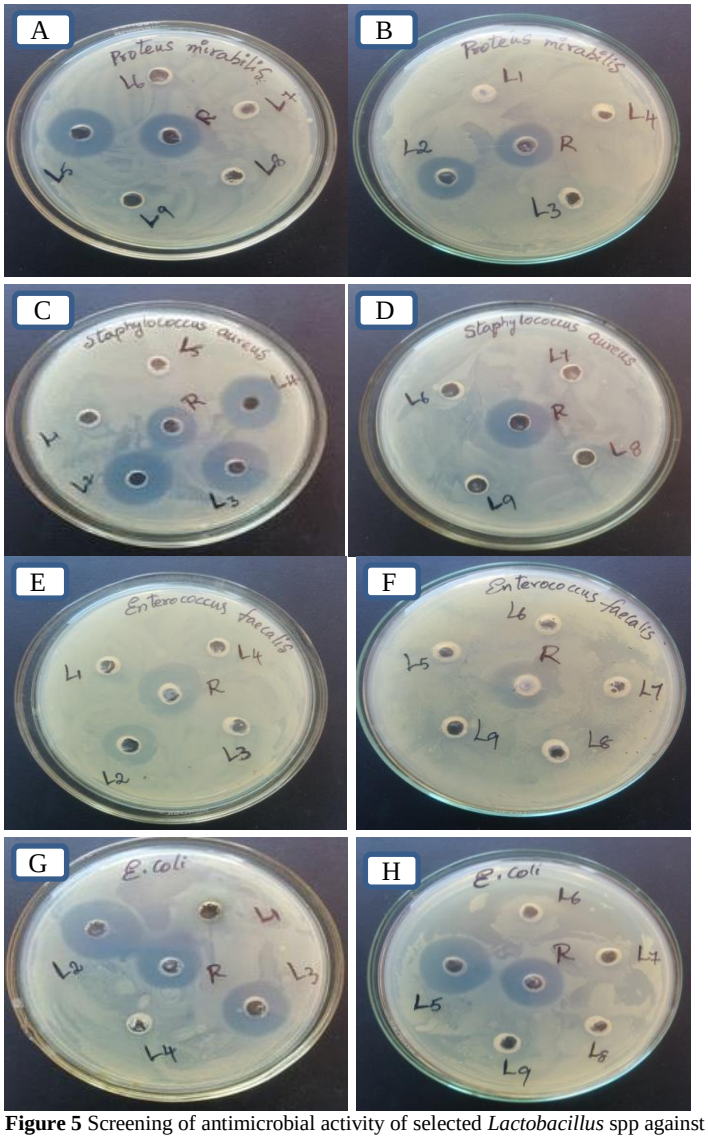

Figure 5 Screening of antimicrobial activity of selected *Lactobacillus* spp against enteropathogens. L_1, L_2, L_3, L_4, L_5, L_6, L_7, L_8, L_9 – *Lactobacillus* spp; R- Reference strain. A & B: The *Lactobacillus* spp L_2, L_5 showing inhibition zones against *Proteus mirabilis;* Figure C &D: The *Lactobacillus* spp L_2, L_3, L_4 showing inhibition zones against *Staphylococcus aureus:* E & F: *Lactobacillus* spp L_2 show inhibition zones against *Enterococcus faecalis*; G & H- *Lactobacillus* spp L_2, L_3, L_5 showing inhibition zones against *Escherichia coli*. I, J and K showing inhibition zone against *Salmonella typhi* by *Lactobacillus* spp L_2, L_5, L_6.

Table 1 Inhibitory activity of the isolated *Lactobacillus* spp

Indicator strains	*Lactobacillus* spp									
	L_1	L_2	L_3	L_4	L_5	L_6	L_7	L_8	L_9	R
Escherichia coli	-	+	+	-	+	-	-	-	-	+
Enterococus faecalis	-	+	-	-	-	-	-	-	-	+
Pseudomonas fluorescence	-	-	-	-	-	-	-	-	-	-
Pseudomonas aerugenosa	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	+	+	+	-	-	-	-	-	+
Salmonella typhimuriun	-	+	-	-	+	+	-	-	-	+
Proteus mirabilis	-	+	-	-	+	-	-	-	-	+

Legend: - indicates no inhibition; + indicates Inhibition

Molecular identification of bacteriocinogenic Lactobacillus spp

16S rRNA gene sequence of the selected bacteriocinogenic *Lactobacillus* strain (L_2) was compared with the sequence available in GenBank database (http://www.ncbi.nlm.nih.gov) using BLAST software and sequence was deposited in the NCBI GenBank. Based on the alignment results, the *Lactobacillus* spp L_2 (KF836431) showed 99 % sequence homology with *Lactobacillus fermentum*. The phylogenetic tree was constructed based on neighbour joining tree method as illustrated in Fig 6. The phylogenetic tree revealed that the bacterial strain is closely related to *Lactobacillus fermentum.*

Nucleotide sequence accession number: The 16S rRNA gene sequence of the *Lactobacillus fermentum* strain was submitted to GenBank and given accession no. KF836431.

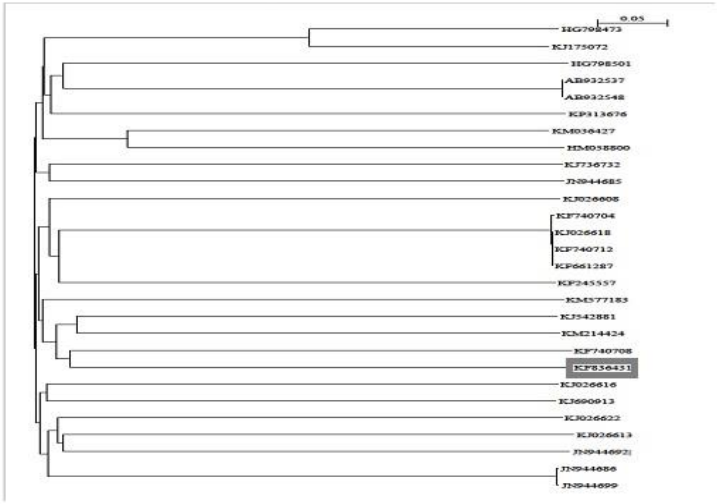

Figure 6 The phylogenetic position of the *Lactobacillus fermentum* strain among neighboring species.

Purification of bacteriocin produced by *Lactobacillus fermentum*

The cell free culture supernatant was precipitated with 40 % ammonium sulphate and the bacteriocin protein precipitate was collected by centrifugation and dialysed against potassium phosphate buffer for overnight and subsequently purified by conventional gel filtration chromatography using sepharose G-50 column equilibrated with sodium phosphate buffer. The bacteriocin fractions of F1, F2, F3, F4 and F5 were obtained by eluting with 0.05 M sodium phosphate buffer and all the five fractions were evaluated for their antibacterial activity against indicator strains *Staphylococcus aureus, Enterococcus faecalis, Proteus mirabilis, Salmonella typhi* and *Escherichia coli*. The highest bacteriocin activity was observed with F2 fraction of 6400 AU/ml for *Staphylococcus aureus,* 3200 AU/ml for *Enterococcus faecalis,* 1600 AU/ml for *Proteus mirabilis,* 8100 AU/ml for *Salmonella typhi* and 2700 AU/ml for *Escherichia coli* (Tab 2). The bioactive fraction F2 with highest antimicrobial activity was further purified by HPLC analysis.

HPLC

The chromatogram of bacteriocin produced by *Lactobacillus fermentum* revealed an active peak at the retention time of 2.73 minutes as shown in Fig 7.The standard bacteriocin used as a reference sample for this study also revealed a single active peak at a retention time of 2.73 minutes (Fig 8). The efficiency of the bacteriocin purification steps were presented in (Tab 3). The recovery yield of bacteriocin by ammonium sulphate precipitation was about 75.5 % and the total activity was approximately 1600 AU/ml and the purification reached to 1.16 folds. The total activity of bacteriocin after gel filtration and HPLC was 900 AU/ml and 300 AU/ml. The purification increased to 5.68 and 10.44 folds respectively by gel filtration and HPLC, but the yield recovery is only 29.5 % and 14 %.

Table 2 Antimicrobial activity (AU/ml) of sepharose column eluted fractions of F1 to F5

	Antimicrobial activity (AU/ml)				
Number of Fractions	***Staphylococcus aureus***	***Enterococcus faecalis***	***Proteus mirabilis***	***Salmonella typhi***	***Escherichia coli***
1	200	400	200	400	300
2	6400	3200	1600	8100	2700
3	800	400	800	900	200
4	1600	800	400	300	900
5	300	300	400	300	300

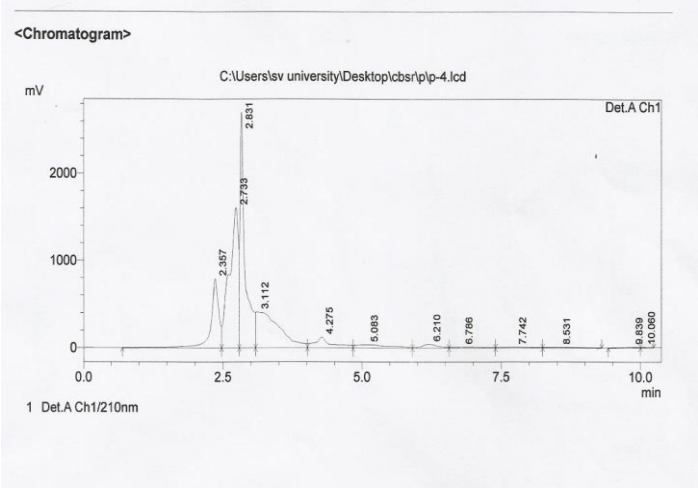

Figure 7 HPLC chromatogram of the purified active fraction of bacteriocin

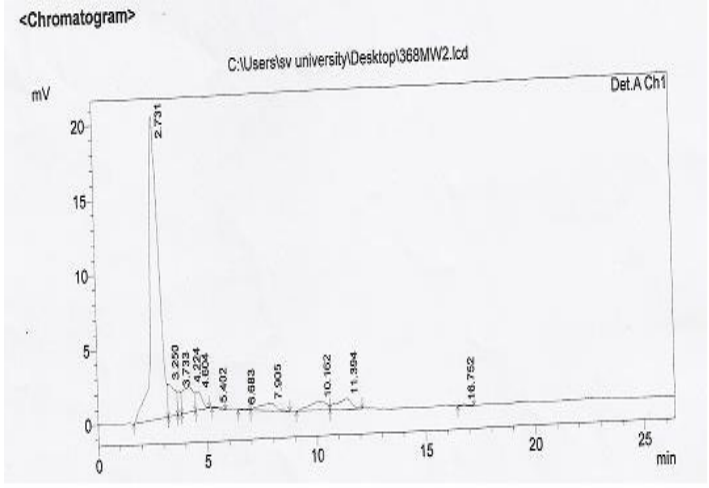

Figure 8 HPLC chromatogram of the standard bacteriocin

Table 3 Steps involved in purification of bacteriocin from *Lactobacillus fermentum*

Purification Stage	**Volume (ml)**	**Protein concentration (mg/ml)**	**Total activity (AU /ml)**	**Purification Fold**	**Recovery (%)**
Culture supernatant	100	0.32	2700	0.0	100
Ammonium sulphate precipitation	10	0.15	1600	1.16	75.5
Column chromatography	5	0.0214	900	5.68	29.5
HPLC chromatography	1	0.012	300	10.44	14

DISCUSSION

The beneficial effects of live bacteria like probiotics, on human health are increasingly being promoted during the last few years. Most probiotics are Lactic Acid Bateria isolated from the dairy products. According to the FAO/WHO guidelines (Joint FAO/WHO, 2002), prospective probiotics must fulfil certain criteria and should be selected through a defined process. The selection criteria for LAB to be used as 'probiotics' in food, include the ability to produce antimicrobial substances towards pathogens, withstand transit through the gastrointestinal tract, modulate immune responses association with health benefits. In this investigation, we have carried out the identification and evaluation of probiotic potential of the Lactobacillus strains isolated from home made curd, characterization of bacteriocinogenic Lactobacillus strain and also purification of the antimicrobial compound produced by a naturally fermented curd isolate of lactic acid bacteria, *Lactobacillus fermentum*.

About 32 isolated strains of *Lactobacillus* spp were screened for the probiotic properties such as acid, thermo, bile and osmo tolerance. All the thirty two strains showed moderate to good tolerance towards acid, thermo, bile and osmo conditions. Results revealed that maximum growth was recorded in case of *Lactobacillus* spp L_2 (*Lactobacillus fermentum*) at pH 4.0, whereas least growth was investigated in *Lactobacillus* spp L_8 and L_9. **Martini *et al.*, (1987)** also reported that the tolerance at pH 4 is significant as ingestion with food or dairy products raise the pH in stomach to 4 or higher. The *Lactobacillus* spp L_2 shown greater viable count at pH 4.0 followed by *Lactobacillus* spp of L_5, L_3, L_6, L_1 and L_4 and the number of viable cells of these *Lactobacillus* spp decreased at pH 5.0. All the *Lactobacillus* spp shown moderate growth at 40ºC, but the *Lactobacillus* spp L_2 exhibited highest growth and viable count at 40ºC, followed by other isolates L_1, L_3, L_4, L_5, L_6 and L_7. **Temmerman *et al.*, (2001)** also reported the highest viable count of the bacteria, in dried probiotic supplements tested. The effect of bile salts on the survivability of different Lactobacillus strains depends on the concentration and the specific properties of the strains. It is well known that bile-salt concentration in the gut is not static, ranging from 1.5% to 2% (w/v) in the first hour of digestion, and decrease afterwards to around 0.3% (w/v) **(Noriega *et al.*, 2004)**. In this study, we used 1%, 2% and 3% of sodium thioglycolate to study the bile tolerance of Lactobacillus strains. Similar studies have been used by other researchers **(Brashears *et al.*, 2003)**. According to our findings, *Lactobacillus fermentum* L_2 showed a strong bile tolerance with maximum growth and highest viable count at 2% bile concentration. Since bile salts disorganize the structure of the cell membrane, it is toxic for living cells **(Margolles *et al.*, 2003)**. Therefore, bile tolerance is considered as an important characteristic of the Lactobacillus which enables it to survive, grow and exert its action in gastrointestinal transit. Lactobacillus strains which could grow and metabolize in normal physical bile concentration could survive in gastrointestine. NaCl is an inhibitory substance which antagonizes the growth of certain types of bacteria, interestingly in our study the *Lactobacillus* spp of L_2 shown tolerance at 2% NaCl concentration with an viable count of 104 CFU/ml. **Elizete and Carlos, (2005)** isolated lactobacilli from gastrointestinal tract of swine were tolerable to 4-8% NaCl. **Veera jothi *et al.*, (2012)** also reported the tolerance of *Lactobacillus* VJ 15 and VJ32 at 2 % NaCl concentration in the medium. In our study out of 32 *Lactobacillus* spp isolated, only nine strains of L_1, L_2, L_3, L_4, L_5, L_6, L_7, L_8 and L_9 possessing excellent acid, thermo, bile and osmo tolerance were selected for the screening of their antimicrobial activity towards a broad range of target microorganisms. Interestingly, L_2 strain showed strong inhibition against *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Proteus mirabilis*. This strain was further identified as *Lactobacillus fermentum* by 16S rRNA analysis. **De Vuyst *et al.*, (1996**) also reported that many proteinaceous antibacterial compounds from *Lactobacillus amylovorus* showed stimulation and broad inhibitory spectra, against Gram-positive and Gram-negative bacteria. These findings are consistent with those of the present study. The production of bacteriocins by such strains can provide additional benefits concerning their application in the food industry. The inhibitory activity of *Lactobacillus fermentum* against different enteropathogens is a feature of interest for further investigation. This extends the investigation further into the characterization of the isolate (L_2) and purification of the bacteriocin produced by this isolate.

The utilization of bacteriocins or bacteriocin-producing bacteria in livestock is a field with enormous possibilities for both research and commercialization. We can easily say that there has been very limited research in this area, but in recent years the number of investigators has dramatically increased. As more countries develop antibiotic-limiting policies, the need for alternative antimicrobial will probably be the main driving force to continue identifying novel bacteriocins and testing existing ones. Because of the relative specificity of bacteriocins as compared with antibiotics, it can be anticipated that the identification of broader spectrum bacteriocins will be active research endeavour.

The 16S rRNA gene sequencing has been performed for the selected potential bacteriocinogenic Lactobacillus spp (L_2). The PCR product was sequenced and subjected to nucleotide BLAST. **Dickson *et al.*, (2005)** used a novel species specific PCR assay for identifying *Lactobacillus fermentum*. It would be desirable to construct a phylogenetic tree using 16S rRNA data and compare it with the BLAST sequences. There are reports identifying *Lactobacillus* strains by oligonucleotide probes or PCR assays **(Ward 1999; Schleifer *et al.*, 1995).**

Since bacteriocins are secreted into the culture medium, most strategies start with a step to concentrate bacteriocins from the culture free supernatant, using ammonium sulfate precipitation **(Yang *et al.*, 1992)**. In our study, we have purified bacteriocin from the cell free supernatant of *Lactobacillus fermentum* (L_2 strain) by three-step protocol, using ammonium sulphate precipitation (40% w/v), sepharose G-50 column chromatography and HPLC **(Tiwari and Srivastava, 2008 b)**. In the initial step of purification, the cells were removed from the culture broth by centrifugation. The cell free supernatant was concentrated by ammonium sulphate precipitation, where the bacteriocin was concentrated at 40% saturation and then the filtrate was dialyzed, pooled and concentrated for further purification, the partially purified dialysate was applied on sepharose G- 50 column chromatography and the fractions obtained (F1 – F5) were assayed for antibacterial activity. The active fraction F2 obtained in gel filtration chromatography was found to be effective with highest antimicrobial activity against the pathogens *Staphylococcus aureus, Enterococcus faecalis, Proteus mirabilis, Salmonella typhi* and *Escherichia coli*. The bioactive fraction F2 was further subjected to HPLC chromatography and has been successful in the purification of bacteriocin from *Lactobacillus fermentum* (L_2 strain). **Gong *et al.*,(2010)** and **Meizhong Hu *et al.*, (2013)** also employed these techniques for the purification of plantaricin 163, plantaricin MG. HPLC results revealed a single peak eluted at 2.73 minutes at 220 nm. The recovery (% yield) of bacteriocin was decreased particularly in HPLC. Similar results were reported by many authors that high loss of bacteriocin occurs during purification processes **(BogovicMatijasic and Rogels, 1998)**. The bacteriocin purified from *Lactobacillus fermentum* showing strong antagonistic effect against the enteropathogens indicated its usefulness in the preservation of different food products enhancing their shelf life.

Probiotic properties and bacteriocin production are two of the several selection criteria that must be met by probiotics **(Corr *et al.*, 2007)**. Diseases associated with enteropathogens such as intestinal abscesses and acute diarrhoeal diseases are rarely reported. In contrast, our strain might be proven to be beneficial as intestinal LAB and possibly also as probiotics; however further research is necessary to confirm whether bacteriocins from probiotic lactic acid bacteria are among the compounds responsible for inactivation of pathogens in urogenital tracts.

These attempts were thus expected to select a promising isolate of *Lactobacillus* possessing better probiotic potential as well as elucidation of good bacteriocin activity and production. This may further useful in the preservation of different food products enhancing their shelf life and also these biomolecules can be applied as biopreservatives, as nutraceuticals as well as for therapeutics in treating gastro intestinal diseases.

CONCLUSION

The results of the present study clearly suggest that the bacteriocin producing *Lactobacillus fermentum* with probiotic properties could potentially used in biopreservation of food as well as probiotic compounds. The studies on *Lactobacillus fermentum* deserve further investigation to elucidate its potential health benefits. However, there is still a long way to go for this strain to work as a probiotic for industry. In this regard, many efforts will be done to develop new scaffolds for effective antimicrobial peptides from natural product biosynthetic pathway.

Acknowledgements: The author Dr. N.S. Meera is grateful acknowledge to thank UGC PDF, New Delhi for financial assistance under the scheme SC/ST Post Doctoral Fellowship for the year 2017-18. Ref. No. F./31-1/2017/PDFSS-2017-18-AND-14173.

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