Isolation of antibacterial protein from *Lactobacillus* spp. and preparation of probiotic curd

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Abstract The study was aimed to isolate antagonistic lactobacilli and the molecules responsible for their antagonistic ability from curd. Preparation of probiotic curd and the ability of the selected lactobacilli to suppress the pathogen therein was also assessed. All the 116 isolates were identified as *Lactobacillus* spp. based on morphological, biochemical and curdling assays. Five of these lactobacilli (Lb-17, Lb-33, Lb-108, Lb-112, and Lb-N3) were found most promising to inhibit all test pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Shigella sonnei*). The cell-free culture supernatants of these five lactobacilli were recorded as thermo-tolerant when subjected to heat treatment at 100 °C for 20 min. The loss in the activity after protease treatment indicated the proteinaceous nature of the antimicrobial molecule present in the culture supernatants. Active protein (19 kDa) produced by lactobacilli was confirmed by SDS-PAGE followed by agar-overlay method. Antibiotic sensitivity assay revealed that the selected *Lactobacillus* spp. isolates were resistant to methicillin and vancomycin. Probiotic curd prepared by using Lb-108 and Lb-N3 was found to be superior to rest of the three isolates based on organoleptic tests and shelf-life.

Complete inhibition of all the test pathogens in curd was shown by Lb-108 and Lb-N3. Inhibition spectrum, production of thermostable protein and preparation of quality curd suggest Lb-108 and Lb-N3 as promising candidates to prepare probiotic curd.

Keywords Antibiotics · Antimicrobials · Bacteriocin · Lactic acid bacteria · Probiotics

Introduction

Potential benefits of probiotics like improved nutrition, growth and prevention of various gastrointestinal (GI) disorders have been well documented (Markowiak and Slizewska 2017; Verna 2010). These probiotics are known to introduce new and effective microbes to the GI tract to enhance beneficial microbial population, its maintenance and also amplify the gut mucosal barrier functions (Markowiak and Slizewska 2017; Zeng et al. 2008). The use of probiotics to prevent and treat a wide variety of ailments has gained favour in the past decades. There are increasing reports of the efficacy of probiotics in the treatment of diseases such as pouchitis (Gionchetti et al. 2003), diarrhoea (Guandalini and Stefano 2011) and irritable bowel syndrome (Whelan and Quigley 2013), etc. As one hundred trillion individual bacteria of 100 different types inhabit the intestine, it is challenging for probiotic strains to get established as gastrointestinal microflora. Thus, an organism that is to be used as a probiotic strain must compete with existing organisms and inhibit or kill the pathogenic bacteria in the intestinal milieu. Additionally, the probiotic strain is supposed to inhibit any pathogenic counterpart that is either already present there or may invade later. Lactobacilli are the most widely encountered organisms for

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probiotic use because they display diverse activities including inhibition of harmful intestinal pathogens and enhance beneficial microflora in the gut (Van Baarlen et al. 2013).

These microorganisms also ensure the safety and prolong the shelf-life of the product by the secretion of antimicrobial compounds such as bacteriocins, organic acids and hydrogen peroxide (Giraffa et al. 2010). Among these, bacteriocins are known to effectively inhibit the microbial growth in many food products and considered as bio-preservative (Mauriello and Villani 2012). Bacteriocins produced by the lactic acid bacteria (LAB) have been the subject of extensive studies in recent years because of their potential as novel and natural food preservatives (Balciunas et al. 2013; O'Connor et al. 2015). Application of bacteriocins in foods may be done either by inoculation of the food with bacteriocin producing LAB or by direct addition of the purified or semipurified bacteriocin. Once in the food, LABs are believed to be safe for consumption without any risk for human beings (Pilet and Leroi 2010). Consumption of dairy products such as probiotic curd, milk, ice-cream, and yoghurt is the most convincing approach to deliver these beneficial antibacterial proteins in the human gut (Champagne et al. 2011).

The present study was conducted to investigate the antibacterial potential of *Lactobacillus* spp. isolated from curd against intestinal pathogens and also to isolate the antibacterial compound produced by them. The potential isolates were further explored for the preparation of probiotic curd and to assess their effect on pathogen suppression when co-cultured in the curd.

Materials and methods

Isolation and identification of lactobacilli isolates

Isolation of lactobacilli was done from a total of 94 curd samples by spread plate method. Selected dilutions (10^{-2} to 10^{-6}) of curd samples were spread on MRS medium (De Man et al. 1960). Identification of the isolates was done by morphological, biochemical tests and curdling assay.

Assay for antibacterial activity

All the 116 isolates were evaluated for antibacterial potential against the five test pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Shigella sonnei*) by agar well diffusion assay. The isolates were inoculated in MRS broth and incubated on a rotary shaker at 100 rpm and 37 ± 1 °C for 48 h. After incubation the cultures were centrifuged at 10,000 rpm for 10 min to obtain cell-free culture

supernatant (CFCS). The CFCS of different isolates were examined for antibacterial activity against all the five pathogens. One hundred μ l of 24 h old broth cultures of the target bacterial pathogens was spread on Mueller–Hinton agar plates. The plates were incubated for 15–20 min for the adequate establishment of pathogens. Three to four wells were made in the seeded agar plates with the help of cork borer (6 mm dia.). Aliquots of 100 μ l of the CFCS of the different *Lactobacillus* spp. isolates were added to the different wells. The plates were incubated for 24 h at 37 ± 1 °C and then examined for the appearance of the zone of inhibition around each well (if any).

Antibiotic sensitivity of lactobacilli and test pathogens

Antibiotic susceptibility test of different lactobacilli and test pathogens was done by 'Kirby-Bauer's method (1966). For the assay, inocula of all 116 lactobacilli and test pathogens were prepared. The turbidity of cell suspensions was matched to 0.5% MacFarland solution to ensure uniformity. Further, the inoculum of the individual isolate was evenly spread on separate Mueller–Hinton agar plates. The plates were incubated for 15–20 min and then antibiotic discs of kanamycin, penicillin, rifamycin, cefotaxime, gentamycin, amoxicillin, methicillin, vancomycin, and tetracycline (HiMedia Pvt. Ltd., Mumbai, India) were applied on seeded agar plates in triplicates. Plates were incubated at 37 ± 1 °C for 24 h and diameter of zones was measured in each case to assess susceptibility or resistance against the antibiotic used.

Effect of physicochemical parameters on antibacterial property of culture supernatants

Stability to heat

The heat stability of the culture supernatant was observed at 100 °C for 20 min as per method described by Deraz et al. (2005). After treatment, the supernatants were again assayed for antibacterial activity as described above.

Susceptibility to trichloroacetic acid (TCA)

The culture supernatants were treated with 30 μ g ml⁻¹ solution of trichloroacetic acid (HiMedia Pvt. Ltd.) to assess the susceptibility to TCA. After treatment, the samples were incubated at 37 ± 1 °C for 24 h and assayed for antibacterial activity by the agar-well diffusion method.

Susceptibility to proteases

The culture supernatants were treated individually with $100 \mu\text{g ml}^{-1}$ solution of enzymes trypsin and proteinase K (HiMedia Pvt. Ltd.) to assess their susceptibility to these enzymes. After treatment, the samples were incubated at $37 \pm 1^\circ\text{C}$ for 30 min and assayed for the antibacterial activity (Deraz et al. 2005).

Isolation of the antimicrobial protein

All the *Lactobacillus* spp. isolates showing antibacterial activity were inoculated in MRS broth and incubated for 48 h at $37 \pm 1^\circ\text{C}$. After incubation, the cells were removed from the broth culture by centrifugation at 10,000 rpm for 10 min. The pH of the supernatant(s) was adjusted to 5.5 by adding 12% ammonium hydroxide (Merk, India) to negate the effect of lactic acid as well as other organic acids produced by lactobacilli. Protein from the supernatant was precipitated by fractional precipitation method by adding an increasing concentration (30, 60, and 90%) of ammonium sulphate (Merk, India) as described by Aroutcheva et al. (2001). Fractions of precipitated proteins of each supernatant of the different isolates thus obtained were assayed for the antimicrobial activity by the agar well diffusion method as mentioned above.

Determination of molecular weight of active fractions

SDS-PAGE

The precipitated protein fractions showing antibacterial activity against the target pathogens were then resolved by SDS-PAGE using 13.5% separating gel. Protein markers (HiMedia Pvt. Ltd.) along with isolated samples were loaded on a gel that was run on 100 V (Laemmli 1970). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (HiMedia Pvt. Ltd.) and destained overnight by 30% sodium chloride solution (Merk, India).

Agar-overlay method

Identification of the active protein was done by agar overlay method. The electrophoresed gels were placed in sterile petriplate and overlaid by molten MHA medium seeded with the target pathogens. The plates were incubated at $37 \pm 1^\circ\text{C}$ for overnight and then observed for zones of inhibition if any.

Preparation of probiotic curd and its quality assessment

Preparation of curd

For the preparation of probiotic curd using selected lactobacilli, 20 ml milk was taken in Erlenmeyer flask and sterilized by autoclaving. Five flasks were inoculated individually with broth cultures of different *Lactobacillus* spp. isolates. Un-inoculated milk prepared likewise served as control. All flasks were then incubated at $37 \pm 1^\circ\text{C}$ for 24 h. After incubation, the curd was assessed for texture, odour, and taste after every 24 h till 120 h.

Estimation of acidity (Horral Ellikar test)

In order to assess the acidity of curd prepared by the use of five selected isolates, 20 ml of sterilized milk was taken in five different Erlenmeyer flask and was inoculated with 300 μl of each lactobacilli culture individually. These flasks were then incubated in a water bath at $37 \pm 1^\circ\text{C}$ for 3.5 h. After incubation, few drops of phenolphthalein (HiMedia Pvt. Ltd.) were added and titrated against 0.1 N NaOH (Ranganna 1986). The appearance of pink colour was recorded as the end point.

AMC production (Vogues Proskauer test)

Production of acetyl methyl carbinol by the selected isolates was done as described by Atherton and Newlander (1987). For this, 2.0 ml of the starter culture of each isolate was taken in test tubes and 2.0 ml of 40% KOH solution (HiMedia Pvt. Ltd.) was added to it. A pinch of α -naphthol (HiMedia Pvt. Ltd.) powder was added to the above solution. The content of the tubes was mixed gently and tubes were then placed in dark for 30 min and observed for the formation of characteristic ring.

Methylene blue reduction test

The selected lactobacilli were inoculated separately in test tubes containing pre-sterilized milk and incubated at $37 \pm 1^\circ\text{C}$ for 24–48 h. After incubation, 1.0 ml of methylene blue (HiMedia Pvt. Ltd.) was added to each tube and mixed gently. The tubes were incubated in water bath at $37 \pm 1^\circ\text{C}$ for 6 h and observed for methylene blue reduction by a change in colour (blue to white) at regular intervals of 30 min (Atherton and Newlander 1987).

Pathogen suppression and quality assessment

To examine the suppression of pathogens in probiotic curd prepared by the use of selected isolates, twenty five flasks (50 ml each) containing 20 ml milk were sterilized by autoclaving. An aliquot of 100 µl of each pathogen culture was added to each set of five flasks and incubated for 5–10 min. After pre-incubation, 2.0 ml of broth culture of different lactobacilli was added to each flask and incubated at 37 ± 1 °C till 120 h. The texture of the curd was examined at regular intervals of 24 h till 120 h. One gm of curd from each flask was taken to prepare serial dilutions (up to 10^{-7}). An aliquot of 100 µl of 10^{-2} and 10^{-3} dilutions was spread on the deoxycholate citrate agar plates and incubated at 37 ± 1 °C for 24–48 h. The plates were observed for characteristic growth of pathogens after incubation.

Data analysis

The data recorded during the study was subjected to Duncan's multiple range test (DMRT) and two way ANOVA to calculate the significance by the magnitude of F value ($p = 0.01$).

Results and discussion

Isolation and identification of *Lactobacillus* spp.

All the 116 isolates were found to be Gram-positive, catalase positive, VP negative, oxidase negative, methylene reductase positive, large rods in chains producing no gas from glucose fermentation. The colonies formed by these isolates on MRS agar were small and yellow/white in colour, typical of lactobacilli. The selected 5 isolates, when tested for their ability to curdle milk were found to form a smooth textured curd. Based on these properties and source, these isolates were considered to belong to *Lactobacillus* spp. Erdogrul and Erbilir (2006) isolated two lactobacilli from cheese and probiotic preparation and identified them as *Lactobacillus casei* and *Lactobacillus bulgaricus* respectively. Hoque et al. (2010) isolated *Lactobacillus* spp. from two regional yoghurts in Bangladesh, which were identified based on their colony morphologies and some biochemical tests.

Antibacterial activity of *Lactobacillus* spp. isolates

Out of 116 lactobacilli, 11 isolates showed antibacterial activity. Among these, five isolates (Lb-17, Lb-33, Lb-108, Lb-112, and Lb-N3) were found to be the best with 10–17 mm zone of inhibition against the five target

pathogens viz., *S. aureus*, *E. coli*, *S. sonnei*, *K. pneumoniae*, and *S. typhi* (Table 1). When we compared the performance of all five *Lactobacillus* spp. isolates with each other by DMRT test, it showed no significant difference in the antibacterial activities of Lb-17, Lb-33 and Lb-112. However, Lb-N3 and Lb-108 showed significantly higher inhibition of maximum three pathogens. Both the isolates were therefore selected for further investigation. Numerous studies suggest the lactobacilli to be good antagonists against different pathogens (Chowdhury and Islam 2016; Kumari et al. 2016; Zhao et al. 2015). The antibacterial activity of the lactobacilli might be due to the secretion of antibacterial metabolites such as bacteriocins, casein-derived peptides etc. *Lactobacillus pentosus* has been reported to inhibit *E. coli* where pentocin 31-1 was found responsible for its inhibitory action (Liu et al. 2008). Similarly, Gautam and Sharma (2009) reported antibacterial activity of bacteriocin producing *Lactobacillus* spp. against *L. monocytogenes* and *S. aureus*. Therefore, in the present study, the antibacterial activity of lactobacilli might be due to the secretion of any such protein or some other metabolite. It was therefore considered worthwhile to further investigate and find out the metabolite responsible for the antibacterial property of these selected isolates.

Antibiotic sensitivity of lactobacilli isolates and test pathogens

Lactobacillus spp. isolates, Lb-17, Lb-33, and Lb-112 were found to be resistant to methicillin and vancomycin whereas Lb-108, Lb-N3 were resistant to vancomycin only (Fig. 1a). Duskova and Karpiskova (2013) reported vancomycin and trimethoprim resistance in lactobacilli and penicillin resistance in *E. coli* and *K. pneumoniae*. Pathak and Dutta (2016) observed resistance of *Lactobacillus acidophilus* against penicillin and neomycin. The resistance of the lactobacilli to vancomycin has earlier been reported due to the presence of D-Ala-D-lactate in their peptidoglycan instead of normal dipeptide D-Ala-D-Ala (Kumari et al. 2016). Results showed that all the test pathogens displayed variable sensitivity pattern to antibiotics. *Staphylococcus aureus* was sensitive to rifamycin and tetracycline and resistant to penicillin and methicillin. However, *E. coli*, *K. pneumoniae*, *S. sonnei* and *S. typhi* were sensitive to cefotaxime and resistant to kanamycin (Fig. 1b). The findings showed that pathogens were susceptible to specific antibiotics whereas lactobacilli exhibited resistance. Such resistant lactobacilli may be good candidates when the patient has to undergo antibiotic therapy for severe illness as such organisms may survive better in the intestine and may also help the host to recover from the infection by secretion of inhibitory compounds.

Table 1 Antibacterial activity of different *Lactobacillus* spp. isolates against test pathogens

Pathogens	Zones of inhibition (mm) produced by different lactobacilli				
	Lb-17 ^b	Lb-N3 ^d	Lb-33 ^a	Lb-108 ^c	Lb-112 ^c
<i>S. aureus</i>	13.00 ^{ns}	13.00 ^{ns}	15.00*	17.00*	13.00 ^{ns}
<i>E. coli</i>	11.00 ^{ns}	13.00*	10.00 ^{ns}	12.00*	12.00*
<i>K. pneumoniae</i>	12.00*	11.00 ^{ns}	10.00 ^{ns}	13.00*	12.00*
<i>S. typhi</i>	11.00 ^{ns}	12.00*	11.00 ^{ns}	11.00 ^{ns}	11.00 ^{ns}
<i>S. sonnei</i>	11.00 ^{ns}	12.00*	11.00 ^{ns}	11.00 ^{ns}	11.00 ^{ns}

Duncan's multiple range test was used to analyze the mean of different treatments. Significant differences among the zones of inhibition produced by different lactobacilli were identified by comparing their differences on or above the computed Rp value (i.e. 1.440). Data represents average of three replicates. *Significant; ns: Non-significant

Fig. 1 **a** Antibiotic sensitivity pattern of *Lactobacillus* spp. isolates (Data are mean of three replicates, vertical bar in the column representing error bars with 5% value); **b** Antibiotic sensitivity pattern of different test pathogens (Data are mean of three replicates, vertical bar in the column representing error bars with 5% value, *represents minimum error); **c** CFCS of lactobacilli showing antibacterial activity after TCA treatment; **d** Identification of

antimicrobial protein by SDS-PAGE (Lane M—Broad range protein weight marker 14.3 to 97.4 kDa, Lane 1 & 2—Active proteins produced by *Lactobacillus* spp. isolates Lb-N3 and Lb-108 respectively). [K—Kanamycin, P—Penicillin, Rif—Rifamycin, Ce—Cephalexime, G—Gentamycin, Am—Amoxycillin, M—Methicillin, Va—Vancomycin, T—Tetracycline]

Effect of physicochemical parameters on antibacterial activity

Results revealed that CFCS of all the selected isolates, when subjected to heat treatment at 100 °C for 20 min, retained their antibacterial activity. The antagonistic activity of Lb-17 and Lb-108 against *E. coli* remained unaffected. In addition, no change in the activity of Lb-33 against *S. sonnei* and *S. typhi* was observed after heat treatment. Likewise, Lb-112 showed no change in activity against *S. aureus* after heat treatment. Also, slight reduction or increase in activity was recorded in some CFCS against one or two of the test pathogens. However, most of these differences were statistically non-significant (Table 2). A bacteriocin-like substance isolated from LAB isolates showed heat stable antibacterial activity against *L. innocua* and *E. coli* at 80 and 100 °C, respectively (Rumjuankiat et al. 2015; Yang et al. 2012). Heat stability of metabolites may ensure antibacterial activity and good shelf-life of probiotic products even after heating during their preparation or preservation processes.

The CFCS of selected lactobacilli possessing antibacterial activity when subjected to TCA treatment revealed that their antibacterial activity remained unaffected (Fig. 1c). However, the bioactive supernatants of different lactobacilli lost their antibacterial activity when subjected to proteinase K and trypsin treatments. The results suggest that the activity was due to some proteinaceous metabolite. Similarly, Ravi et al. (2011) reported the proteinaceous nature of the antimicrobial metabolite in their study. Such metabolites can be considered as valuable biopreservatives in food industries after purification.

Isolation of active protein fractions

Active protein from CFCS of all the five lactobacilli was precipitated by ammonium sulphate fractionation. The fraction obtained after treatment with 90% ammonium sulphate depicted antibacterial activity. No activity was observed in the 30 and 60% protein fractions of any of the *Lactobacillus* spp. isolates. Our study gets support from the findings of Yamato et al. (2003) who precipitated low molecular weight antibacterial metabolite from *Lactobacillus acidophilus* (YIT 0154) with a high concentration (80%) of ammonium sulphate. Similarly, Rajaram et al. (2010) also found a low molecular weight antibacterial metabolite from a marine bacterium *L. lactis*. Precipitation of active protein at a higher concentration of ammonium sulphate in our study also indicates that it is a low molecular weight protein.

Separation and identification of the active protein fraction by SDS-PAGE

A single prominent band of a molecular weight of 19 kDa was observed in all the samples after destaining the gel. When the electrophoresed gel was overlaid with the indicator bacteria, a clear inhibition zone was observed in the indicator lawn corresponding to 19 kDa fragment (Fig. 1d). Similarly, Zhao et al. (2015) reported a bacteriocin produced by *Lactobacillus acidophilus* XH1, having a molecular weight of 16 kDa which was active against *E. coli* and *S. aureus*. Another bacteriocin LS1 with 10 kDa molecular mass was purified by Busarcevic et al. (2008) from *Lactobacillus salivarius* BGHO1. The antibacterial

Table 2 Effect of heat treatment on antibacterial activity of *Lactobacillus* spp. isolates

Treatment	<i>S. aureus</i>		<i>E. coli</i>		<i>K. pneumoniae</i>		<i>S. sonnei</i>		<i>S. typhi</i>		Grand Mean
	HT	C	HT	C	HT	C	HT	C	HT	C	
Lb-17	13.00*	15.00	11.00 ^{ns}	11.00	12.00 ^{ns}	10.00	11.00*	13.00	11.00*	13.00	12.00
Lb-33	15.00 ^{ns}	16.00	10.00 ^{ns}	11.00	10.00 ^{ns}	11.00	11.00 ^{ns}	11.00	11.00 ^{ns}	11.00	11.70
Lb-N3	13.00 ^{ns}	14.00	13.00*	11.00	11.00 ^{ns}	12.00	12.00*	17.00	12.00 ^{ns}	11.00	12.60
Lb-108	17.00 ^{ns}	17.00	12.00 ^{ns}	12.00	13.00 ^{ns}	11.00	11.00 ^{ns}	11.00	11.00 ^{ns}	11.00	12.60
Lb-112	13.00 ^{ns}	13.00	12.00*	15.00	12.00 ^{ns}	10.00	11.00 ^{ns}	15.00	11.00*	15.00	11.80
Mean	14.20	15.00	11.60	12.00	11.60	9.00	11.20	13.40	11.20	12.20	
Mean (b)	14.60		11.80		10.30		12.30		11.70		
SEM	HT: 0.69, C: 0.10, HT*C: 0.24		HT: 0.74, C: 0.092, HT*C: 0.20		HT: 1.16, C: 0.12, HT*C: 0.43		HT: 0.68, C: 0.11, HT*C: 0.26		HT: 0.58, C: 0.11, HT*C: 0.25		
CD at 1%	HT: 1.18, C: 0.74, HT*C: 1.67		HT: 1.28, C: 0.81, HT*C: 1.81		HT: 1.68, C: 1.06, HT*C: 2.37		HT: 1.24, C: 0.78, HT*C: 1.76		HT: 1.21, C: 0.76, HT*C: 1.71		

Data represents the average of three replicates

HT: Heat treatment at 100 °C, C: Control, *Significant; ns: non-significant

Table 3 Quality assessment of the probiotic curd prepared by using selected lactobacilli

Isolates	No. of days	Smooth curdling	Thick curdling	Odor	Taste	CFU
Lb-33	1			Fruity	Sweet	1.25×10^4
	2	+		Fruity	Sweet	1.0×10^4
	3	+		Sour	Sour	TNC
	4			Sour	Sour	TNC
	5		+	Sour	Sour	TNC
Lb-112	1	+		Creamy	Sweet	Nil
	2	+		Creamy	Sweet	Nil
	3			Creamy bitter	Bitter	4.0×10^4
	4		+	Pungent	Bitter	4.5×10^4
	5		+	Pungent	Bitter	Nil
Lb-N3	1	+		Fruity	Sweet	1.0×10^3
	2	+		Fruity	Sweet	Nil
	3	+		Fruity	Sweet	Nil
	4	+		Creamy	Sweet	1.0×10^2
	5			Creamy bitter	Bitter	Nil
Lb-17	1			Creamy	Sweet	6.0×10^2
	2			Creamy bitter	Bitter	7.0×10^2
	3			Creamy bitter	Bitter	3.0×10^4
	4		+	Creamy bitter	Bitter	Nil
	5		+	Sour	Bitter	Nil
Lb-108	1	+		Sour	Fruity	Nil
	2	+		Fruity	Fruity	Nil
	3	+		Fruity	Fruity	TNC
	4			Creamy	Creamy	1.0×10^2
	5			Creamy bitter	Creamy bitter	Nil

CFU counts represent the average of three replicates

TNC: Too numerous to count

CFUs were counted by using Formula = $\frac{\text{No. of colonies} \times \text{Dilution factor}}{\text{Amount of sample}} \times 100$

(+) : Positive result, (–) : negative result

protein of a molecular weight 19 kDa isolated in the present study might also be a low molecular weight bacteriocin.

Preparation of probiotic curd and its quality assessment

Effect of prolonged incubation on the quality of the curd

Results of the assays revealed remarkable differences in the quality of the curd prepared with selected lactobacilli isolates. Lb-108 and Lb-N3 exhibited the best texture with fruity odour and sweet taste throughout the incubation period, till 120 h. It suggests that these isolates may probably be useful for the preparation of probiotic curd with improved shelf-life which is generally 2–3 days only at room temperature. However, other isolates showed sour to bitter taste after prolonged incubation (Table 3). The improvement in the sensory properties and shelf-life of the

probiotic yoghurts has also been reported by Yeganehzad et al. (2007) who also recommended the use of *Lactobacillus acidophilus* to prepare probiotic curd. The curd is considered as a part of the healthy diet in India. It has been found as a suitable vehicle for the delivery of the beneficial microbes in the gut of host (Balamurugan et al. 2014). But short shelf-life of curd remains a problem as it becomes very sour and is not liked by the consumers. Therefore, preparation of probiotic curd using such isolates may be helpful in enhancing the shelf-life and quality of curd.

Quality assessment of probiotic curd

There are a number of factors responsible for the viability of a probiotic curd including oxygen content, acidity and water activity, etc. (Reid et al. 2007). The acidity of a product affects the range of organisms that can survive and grow in it. The isolates Lb-17, Lb-33, Lb-108 and Lb-N3 produced 0.36% acidity, almost equivalent to standards

Table 4 Effect of *Lactobacillus* spp. isolates on the growth of different pathogens co-cultured in probiotic curd

Isolates	No. of days	<i>K. pneumonia</i>				<i>S. sonnei</i>				<i>S. typhi</i>				<i>E. coli</i>				<i>S. aureus</i>			
		Curdling		CFU		Curdling		CFU		Curdling		CFU		Curdling		CFU		Curdling		CFU	
		Sm	Th			Sm	Th			Sm	Th			Sm	Th			Sm	Th		
Lb-17	1	+	–	Nil		+	–	TNC		+	–	Nil		+	–	7.0×10^3		+	–	Nil	
	2	+	–	Nil		+	–	2.0×10^4		+	–	Nil		–	+	Nil		–	+	Nil	
	3	+	–	Nil		+	–	Nil		–	+	Nil		–	+	6.0×10^2		–	+	Nil	
	4	+	–	Nil		–	+	Nil		–	+	Nil		–	+	Nil		–	+	Nil	
	5	+	–	Nil		–	+	Nil		–	+	Nil		–	+	Nil		–	+	Nil	
Lb-33	1	+	–	3.0×10^4		+	–	3.0×10^4		+	–	1.2×10^4		+	–	TNC		+	–	Nil	
	2	+	–	7.0×10^3		+	–	3.0×10^4		+	–	1.0×10^4		+	–	TNC		+	–	Nil	
	3	+	–	1.25×10^4		+	–	1.0×10^4		–	+	1.0×10^4		–	+	4.0×10^4		+	–	Nil	
	4	–	+	Nil		+	–	1.2×10^4		–	+	Nil		–	+	Nil		–	+	Nil	
	5	–	+	Nil			+	Nil		–	+	Nil		–	+	Nil		–	+	Nil	
Lb-108	1	+	–	Nil		+	–	2.0×10^4		+	–	Nil		+	–	Nil		+	–	Nil	
	2	+	–	3.0×10^4		+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil	
	3	+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil	
	4	+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil	
	5	+	–	Nil		+	–	Nil		–	+	Nil		+	–	Nil		+	–	Nil	
Lb-112	1	+	–	Nil		+	–	TNC		+	–	6.0×10^2		+	–	Nil		+	–	Nil	
	2	+	–	Nil		+	–	2.0×10^4		+	–	7.0×10^2		+	–	Nil		+	–	Nil	
	3	–	+	1.2×10^4		+	–	2.0×10^4		–	+	Nil		–	+	Nil		+	–	Nil	
	4	–	+	Nil		–	+	2.5×10^4		–	+	Nil		–	+	Nil		+	–	Nil	
	5	–	+	Nil		–	+	Nil		–	+	Nil		–	+	Nil		–	+	Nil	
Lb-N3	1	+	–	Nil		+	–	Nil		+	–	4.0×10^3		+	–	Nil		+	–	Nil	
	2	+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil	
	3	+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil	
	4	+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil	
	5	–	+	Nil		+	–	Nil		+	–	Nil		+	–	Nil		+	–	Nil	

CFU counts represent the average of three replicates

Sm: Smooth curdling, Th: thick curdling. CFU were counted at each 24 h time interval till 5 days

TNC: Too numerous to count. $CFU = \frac{\text{No. of colonies} \times \text{Dilution factor}}{\text{Amount of sample}} \times 100$

+: Positive result, –: no reaction

(i.e. 0.35% acidity) after 3.5 h of incubation whereas, Lb-112 showed 0.35% acidity. It was encouraging to note that all the five isolates showed a positive result for AMC production with the formation of the characteristic ring after addition of α -naphthol and 40% KOH in starter culture after 2 h of incubation in dark. AMC is known to impart an appealing odour to the curd. It was found that all the selected isolates reduced methylene blue as observed by a change in colour from blue to white. Among them, Lb-33 and Lb-112 reduced methylene blue within 1 h while reduction time for Lb-17, Lb-108 and Lb-N3 was 2–3 h. Unlike the milk, reduction time was less in case of our curd as inoculum was added in milk for effective curdling and therefore reduction time remained early in comparison to sterile milk (Atherton and Newlander 1987). The organism inducing early curdling and late reduction time may be a

suitable candidate for the preparation of a curd with superior quality and longer shelf-life. These isolates with all these parameters may, therefore, be used for the preparation of a quality probiotic curd.

Effect of lactobacilli on growth of different pathogens

Isolates Lb-108 and Lb-N3 showed 100% suppression of all the test pathogens (*S. aureus*, *E. coli*, *K. pneumoniae*, *S. sonnei* and *S. typhi*), whereas Lb-33 was least active against all the test pathogens except *S. aureus* (Table 4). Tharmaraj and Shah (2009) reported similar findings with a reduction in pathogenic population when LAB and pathogens were co-cultured in culture media. This might be due to the pH reducing ability of lactic acid bacteria, thus creating a hostile microenvironment after the production of

organic acids, not favourable for the growth of pathogens (Syngai et al. 2016). Lactobacilli showing antagonistic activity when co-cultured in the curd could be used to decrease the pathogenic microbes present in human gut by their consumption as a probiotic product (Masoumikia and Ganbrov 2015).

Conclusion

Out of the repository of 116 *Lactobacillus* spp. isolates, five most promising isolates were identified with good antibacterial property. A 19 kDa thermostable protein was found responsible for their antibacterial activity. On the basis of inhibition spectrum, production of thermostable protein and preparation of quality curd Lb-108 and Lb-N3 were found to be the best isolates. Such isolates after clinical trials may be recommended as potential candidates for the production of probiotic curd.

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Compliance with ethical standards

Conflict of interest Authors declared no conflict of interest.

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