Original Research Article

Probiotic Property and Antibacterial Activity Analysis of Sour Curd Derived Lactobacilli Isolates by *in vitro* Methods

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ABSTRACT

There are massive bacteria in the curd, especially the lactic acid bacteria (LAB), which have been considered as probiotics in human and animals for a prolonged period. Probiotics have its importance in our daily life and native LAB are well known probiotics having antibacterial activity against potential pathogenic bacteria. Several authors from various regions all over the world have reported LAB to have been isolated from different fermented as well as non-fermented foods and beverages. The current study evaluated the probiotic potential LAB isolates from commercially available sour curd, Malda (West Bengal, India). A total of three types of sour curd (contained in plastic cup, plastic pouch and earthen pot) were tested and the commonest LAB isolates found in sour curd were *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactocaseibacillus rhamnosus*. Safety aspect of LAB isolates was screened using gelatinase and antibiotic susceptibility test. The commercially available sour curd-derived LAB isolates have been demonstrated as good probiotics which displayed excellent probiotic potentiality and antibacterial activity against both gram negative gam positive bacteria as well.

Keywords: Probiotic bacteria, Antibacterial activity, Safety profile, Sour curd

INTRODUCTION

Various fermented food products, which are yielded worldwide, significantly attribute to the diets of many people (Sharma et al. 2016). Fermentation plays an important role towards alteration of the biochemical composition of the food by breaking down a large organic molecule into smaller ones through bacterial and enzymatic action (Sharma et al. 2020). Kumar et at. 2022 stated that the consumption of probiotic bacteria fundamentally leads to the promotion of the health of the consumers. Lactic acid bacteria (LAB) are a momentous group of bacteria that are able to produce lactic acid through homo or heterofermentative metabolism (Mohamad et al. 2020). LAB are a various group of microbes comprised with Gram-positive, aerotolerant, acid-tolerant, catalase-negative and non-spore forming cocci or rod microorganisms with high tolerance at low pH, and take part a significant role in the process of food fermentation by inhibiting pathogenic bacteria (Coelho et al. 2022). Manifold LAB strains have been isolated from different sources of food such as cheese, yogurt, camel milk, bovine milk and also from the gastrointestinal tract of human (Kwun et al. 2020). Several researchers have reported that LAB are able to produce various beneficial metabolites such as essential amino acids, short-chain fatty acids, water-soluble vitamins, antimicrobial agents and digestive enzymes (Amin et al.

2020). Research conducted by Ricci et al. (2019) indicated that some LAB strains are probiotics, and others likely to be potential ones which are widespread in nature and can be applied in the food industry. Celik et al. (2021) stated that the isolation and identification of LAB strains aims to characterise the taxonomy of lactic acid bacteria, obtain potential beneficial and functional probiotic strains particularly from the genus of Lactobacillus and thereby ensuring the production of standard quality of fermented food products. Lactobacillus strains maintain a long history of safe use where they are responsible of the fermentation process together with associated starter cultures (Widyastuti et al., 2021). The most common types of probiotic LAB include different *Lactobacillus* sp including *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. casei* Shirota, *L. animalis* (Farahmand et al. 2021). Research conducted by Dowarah et al. (2018) stated that several in vitro studies display that the growth of food-borne pathogenic microorganisms is deterred by probiotic lactic acid bacteria. LAB plays a crucial role towards the treatment against life-threatening issues and infections caused by multiple drug-resistant pathogenic bacteria (Marco et al. 2017) due to its probiotic property. Cai et al. (2019) stated that fermentation products of probiotics can enhance the synthesis of various bioactive components with beneficial effects that strengthens the functional value and acceptability of food products. Probiotics isolated from dairy and non-dairy sources can also exhibit broad spectrum of antibacterial activity (Halder et al. 2017; Yang et al. 2020).

Based upon the above background, probiotic characterization in terms of stress tolerances, antibacterial activity test and antibiotic susceptibility testing are necessary task to know LAB's beneficial effects on various fermented milk products or any kind of fermented foods for human.

The commercially available sour curd contained in plastic pouch, plastic cup and earthen pot have gained high demand for their daily consumption in our locality and due to availability and regular consumption of such packaging curds, these have been considered as important variable and hence we have given due importance and taken keen interest on such respect. Therefore, the purpose of this research is to analyze the sour curd microbiome through in-vitro study in terms of probiotic characterization, antibacterial activity and antibiogram, in our part of the globe,

MATERIALS & METHODS

Three types of sour curd samples (contained in plastic cup, plastic pouch and earthen pot) collected from local vendor from Malda district, West Bengal, India, were utilized in the current study. In order to isolate the LAB, de Man, Rogosa and Sharpe (MRS) broth (Hi-Media, Mumbai, India) was inoculated with three kinds of sour curd and after incubation for 48 hrs. at 37 °C, single isolated colonies were procured on MRS agar (Hi-Media, India) plate, from each of the curd samples, by streak dilution of the broth culture (Halder and Mandal, 2018; Halder and Mandal, 2015).

The shape, size, margin, opacity and hue of the colonies of isolated LAB were documented. Morphological and biochemical characterization of the isolates were performed following the standard protocol Taye et al. (2021). To study the biochemical properties, catalase, oxidase, citrate utilization, nitrate reduction, indole production, methyl-red (MR), Voges–Proskauer (VP), urease, Nitrate were adopted following Bergey's manual (Holt and Krieg, 1984).

The sodium chloride, pH, bile salt, and temperature tolerance tests were performed to determine the probiotic characteristics of LAB isolates. The bile salt and pH (acid) tolerance were tested at an interval of 24, 48 and 72 hrs. respectively (Liong and Shah, 2005), and to sodium chloride (NaCl), following the protocol of Chowdhury et al. (2012), with slight modifications as mentioned elsewhere (Halder and Mandal, 2015). Briefly, the isolated lactobacilli were grown (for 24 hrs. at 37 °C) in sodium chloride containing (of 2, 4, 6% and 8%) MRS broth, and then, the growth of lactobacilli, following subculture of the MRS broth cultures on MRS agar (for 24 h at 37 °C), showed their tolerance to sodium chloride. Like-wise bile salt (0.25%, 0.50% and 1.00%), pH (2, 3 and 4) and temperature (for 19, 40 and 60 °C) tolerance tests were also performed to assess their mode of stress tolerance level.

The antibacterial activity of three lactobacilli, isolated from sour curd was determined by agar-well diffusion method, against the indicator strains of pathogenic bacteria (procured from clinical samples including urine, threat swab and pus), both Gram negative: Pseudomonas aeruginosa, and Gram positive: Staphylococcus aureus. The agar-well method was done according to Tagg and McGiven, 1971. On the surface of nutrient agar plate swabbed with indicator bacterial broth culture, wells (of 6 mm diameter) were prepared, and bacteriocin of the three isolated lactobacilli were loaded in the wells (20 µL/well, 40 µL/well and 60 µL/well). Following 24 hrs. incubation at 37 ◦C, ZDI (zone diameter of inhibition) values (nearest whole) were recorded, and interpreted as less active, moderately active and highly active with ZDIs ≤10 mm, 11–14 mm, and ≥15 mm, respectively. The antagonistic activity of the test lactobacilli in arbitrary unit per mL (AU/mL) was also calculated using the formula mentioned elsewhere (Iyapparaj et al. 2013).

The safety profile of the sour curd isolates was determined by their gelatine liquefaction and antibiotic susceptibility test. Gelatine liquefaction test was performed using nutrient gelatin media (following 24 hrs. incubation at 37 °C then freezing at 4 °C in alternative manner up to 6 days and checking the liquefaction of gelatin media) followed by the protocol of Dela Cruz and Torres (2012), to confirm the capacity of isolated lactobacilli to hydrolyse gelatine by producing gelatinase. All the isolated LAB were tested against antibiotics (Hi-Media, India): amikacin (Ak: 30-µg/disc), ampicillin (Am: 10-µg/disc), gentamycin (G: 10-µg/disc), tetracyclines (Te: 30-µg/disc) and vancomycin (Vm: 30-µg/disc), using the disc diffusion method (Bauer et al., 1966), as per the Clinical and Laboratory Standards Institute (CLSI) criteria (Clinical and Laboratory Standards Institute (CLSI), 2020).

RESULT

Three lactic acid bacteria, one from each curd samples, were isolated: LMEM401A (from commercial plastic cup curd), LMEM402B (from commercial plastic pouch curd), LMEM403A (from commercial earthen pot curd). All isolates were gram-positive rod shaped and were negative to oxidase and catalase and tests, and thus recognized as *Lactobacillus.* Two isolates were rod shaped and remaining one was round shaped. The isolated bacteria (LMEM401A, LMEM402B and LMEM403A), which in TSI test revealed the production of acid slant and acid butt. LMEM401A and LMEM403A were negative for IMViC test except LMEM402B which showed positive test result for methyl red. Following cultural, morphological, gram staining and biochemical tests, the isolates were identified as *Lactocaseibacillus rhamnosus* LMEM401A, *Lactococcus lactis* LMEM402B, *Lactobacillus acidophilus* LMEM403A. Colony morphology, gram staining and biochemical test results are shown in Fig. 1 and Table 1 respectively.

All the three isolates *L. rhamnosus* LMEM401A, *L. lactis* LMEM402B and *L. acidophilus* LMEM403A were resistant to 2%, 4%, 6% and 8% NaCL. *L. rhamnosus* LMEM401A had resistance at pH 2, 3, 4 while *L. lactis* LMEM402B showed weakly tolerant at pH 3 and sensitive at pH 4, on the other hand *L. acidophilus* LMEM403A showed sensitivity at pH 3 and 4 both. In respect of bile salt tolerance test, at 0.25 % bile salt concentration, all the three isolated lactobacilli were resistant and at 1%, only *L.*

rhamnosus LMEM401A was weakly tolerant. In connection with temperature tolerance test, all three isolates possessed weakly tolerance at 19°C while *L. rhamnosus* LMEM401A and *L. lactis* LMEM402B had resistant capacity to 40 °C but *L. acidophilus* LMEM403A was weakly tolerant. At 60°C all were non-tolerant. The tolerance test results to different stresses (low-pH, sodium chloride, temperature and bile salts) for the isolated lactobacilli are represented in Table 2.

The antibacterial activity, following agar-well diffusion (based on the ZDIs values obtained around the bacteriocin-loaded wells on the agar plates), of the lactobacilli isolates against the indicator bacteria is represented in Table 3, Fig 2. The *L. acidophilus* LMEM403A had highest activity, with ZDI of 23 mm, against gram positive *S. aureus* while *L. lactis* LMEM402B had highest antibacterial activity, with ZDI of 21 mm, against gram negative bacteria *Pseudomonas aeruginosa*. Among the curd lactobacilli, the *L. rhamnosus* LMEM401A had the highest antibacterial activity (275 AU/mL), while remaining two isolates *L. lactis* LMEM402B and *L. acidophilus* LMEM403A showed less activity (250 AU/mL) than *L. rhamnosus* LMEM401A against gram positive indicator strain *S. aureus.* On the other hand, *L. lactis* LMEM402B showed the highest activity (275 AU/mL) against gram negative indicator bacteria *P. aeruginosa*, while the other two isolates *L. rhamnosus* LMEM401A and *L. acidophilus* LMEM403A had lowest activity (200 AU/mL). So, the overall values ranged from 200- 275 AU/mL (Table 4, Fig. 2)

All the isolated LAB had resistance to Vm, and *L. lactis* LMEM402B had Ak resistance, in addition to the Vm. All the isolated lactobacilli showed sensitivity to Tc, while sensitivity to Ak and Am was shown by *L. acidophilus* LMEM403A; The intermediately susceptibility (IS) to Gm was recorded for *L.* rhamnosus 401A and *L. acidophilus* LMEM403A. The antibiogram results for the lactobacilli are shown in Table 5.

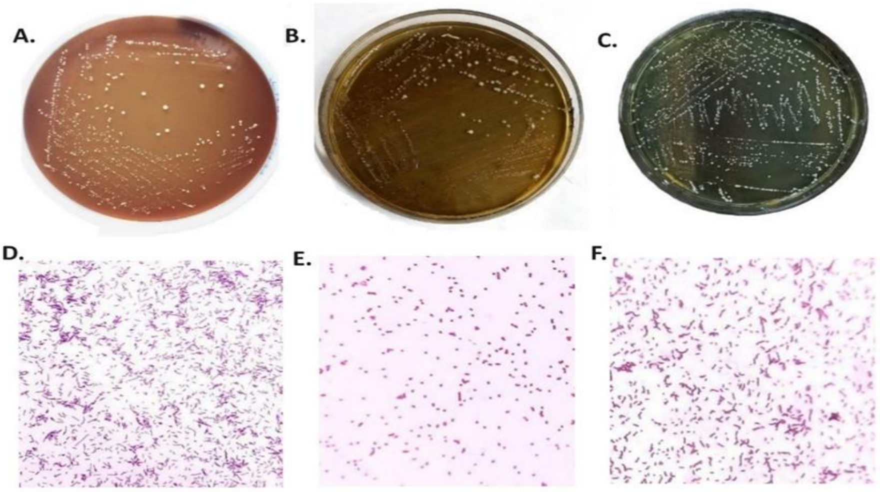

Figure 1. Colony morphology test results of isolated lactobacilli; A: LMEM401A, B. LMEM402B, C. LMEM403A and gram staining test results of LAB isolates; D: LMEM401A, E: LMEM402B, F: LMEM403A

Table 1. Biochemical test results of LAB isolates

Sample code	Cat	Oxi	TSI	Cit	Gel	Ind	Ure	MR	VP	Nit
LMEM401A	-	-	Y/Y	-	-	-	-	-	-	-
LMEM402B	-	-	Y/Y	-	-	-	-	+	-	-
LMEM403A	-	-	Y/Y	-	-	-	-	-	-	-
Cat: Catalase; Oxi: Oxidase; Cit: Citrate; Gel: Gelatinase; Ind: Indole; Ure: Urease; MR: Methyl Red; VP: Voges-Proskauer; Nit: Nitrate.										

Table 2. Physiological stress tolerance test results for isolated lactobacilli

LAB Strains	NaCl (%)				pH			Bile salt (%)			Temperature (°C)		
	2	4	6	8	2	3	4	0.25	0.5	1	19	40	60
L. rhamnosus LMEM401A	+	+	+	+	+	+	+	+	+	W	+	+	−
L. lactis LMEM402B	+	+	+	+	+	W	–	+	−	−	+	+	−
L. acidophilus LMEM403A	+	+	+	+	+	−	−	+	−	−	+	W	−
"+": resistant/tolerant; "−": sensitive/non-tolerant; "w": weakly tolerant.													

Table 3. Antibacterial activity of lactobacilli isolates against the indicator bacterial pathogens following agar-well diffusion method.

	ZDI (mm)					
Pathogenic bacteria	***Staphylococcus aureus***			***Pseudomonas aeruginosa***		
Bacteriocin concentration (µl)	**20**	**40**	**60**	**20**	**40**	**60**
L. rhamnosus LMEM401A	7	11	16	6	8	16
L. lactis LMEM402B	7	10	18	6	11	21
L. acidophilus LMEM403A	8	10	23	6	8	19

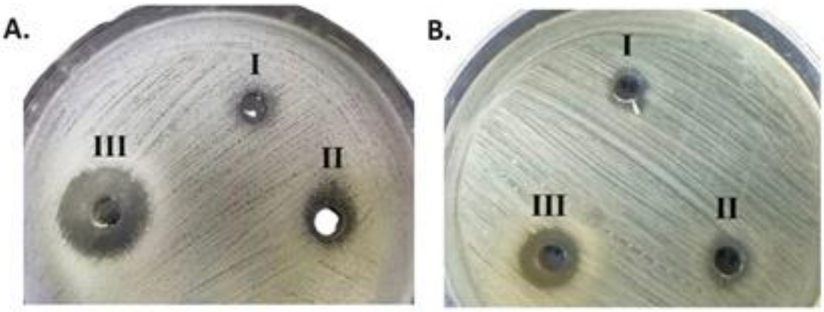

Figure. 2. Agar-well diffusion test results in terms of ZDI (mm) and the calculated bacteriocin activity (Au/mL) of *Lactobacillus acidophilus* LMEM403A against A. *Staphylococcus aureus*; I: 20 µl, II: 40 µl, III: 60 µl and B. *Pseudomonas aeruginosa*; I: 20 µl, II: 40 µl, III: 60 µl.

Table 4. The calculated bacteriocin activity (AU/ml) of isolated lactobacilli against gram-positive and gram-negative indicator bacterial strains.

	Indicator stains	LAB Isolates	AU/mL
Gram-positive	***Staphylococcus aureus***	*L. rhamnosus* LMEM401A	275
		L. lactis LMEM402B	250
		L. acidophilus LMEM403A	250
Gram-negative	***Pseudomonas aeruginosa***	*L. rhamnosus* LMEM401A	200
		L. lactis LMEM402B	275
		L. acidophilus LMEM403A	200
AU/mL: arbitrary units per milliliter			

Table 5. Antibiotic susceptibility test results of isolated lactobacilli.

LAB Strains	R (ZDI: ≤15mm)	IS (ZDI: 16- 20 mm)	S (ZDI: ≥ 21 mm)
L. rhamnosus LMEM401A	Vm: 7	Gm: 19; Am: 20	Am: 26; Tc: 22,
L. lactis LMEM402B	Vm: 6; Ak: 14	Gm: 16	Am: 42; Ak: 38, Tc: 30
L. acidophilus LMEM403A	Vm: 7	Gm: 20	Ak: 28; Am: 29; Tc: 34
Ak: amikacin; Am: ampicillin; Gm: gentamicin; Tc: tetracycline; Vm: vancomycin; IS: intermediately susceptible; R: resistant; S: sensitive; ZDI: zone diameter of inhibition.			

DISCUSSION

As has been demonstrated by Taye et al. (2021), the morphologically identical bacterial colonies, procured from cow milk and different milk products on the MRS agar plate, have been identified as *Lactobacillus* sp, on the basis of physiological and biochemical features, following Patil et al. (2010). In this study, three non-motile non-spore forming gram-positive rod-shaped bacteria were procured from three types of the curd samples (Fig. 1) and following phenotypic and biochemical characterization the isolated lactobacilli were identified as *L. rhamnosus*, *L. lactis* and *L. acidophilus*. The LAB isolates procured from different fermented foods including traditional fermented and dried buttermilk, have been identified earlier by Hassan et al. (2023) following morphological and biochemical characterization of the bacteria.

Now a days, the scope for treatment with antibiotics is not adequate in consequence of the fluctuating rate of emergence of antibiotic resistant pathogenic bacteria causing life-threatening infections to humans. Probiotic lactobacilli have been considered suitable for biotherapy with proven antibacterial activity (Sing et al. 2017) as alternative measures to the antibiotics, which exist in the mainstay of all therapy for bacterial infections (Murugaiyan et al. 2022).

Earlier, Halder et al. (2017) stated that the curd isolates of *L. plantarum*, *L. animalis*, *L. rhamnosus* and *L. acidophilus* had antibacterial activity, having ZDIs from 13.65–30 mm approximately by agar-well, against human pathogenic bacteria, like *Escherichia coli*, *Acinetobacter baumannii*, *Proteus vulgaris* and *Salmonella enterica* serovar Typhi. In the current study, the agar-well diffusion had ZDIs 10–22 mm (for gram-positive Staphylococcus aureus bacteria) and 9–21 mm (for gram-negative bacteria *Pseudomonas aeruginosa*) for all three LAB isolates (Table 3), (Fig. 2). As per the report of Halder et al. (2018), the capacity of antibacterial activity of *Lactobacillus fermentum* for both the gram-positive and gram-negative pathogenic bacteria, with an overall bacteriocin activity (AU/ml) of 140–240 approx. The current investigation demonstrates the capacity of antibacterial activity of *L. rhamnosus* LMEM401A, *L. lactis* LMEM402B and *L. acidophilus* LMEM403A for the indicator microorganisms, consisting of both gram-positive and gram-negative pathogenic bacteria, with an overall bacteriocin activity (AU/ml) of 200–275.

The most important probiotic features of lactic acid bacteria considered to have safe for human consumption, is being their antibiotic sensitivity, and the intrinsic resistance property as well (Duche et al. 2023). Halder et al. (2018) stated that the *Lactobacillus fermentum* was sensitive to Cx, Gm, Im, Mp, Ac, Am, Cc, and Tc with ZDIs of approx 21-34 mm, intermediately susceptible to Cx and Tm with ZDIs of approx. 18 mm and showed resistance to Km, Me, Ak, Vm and Cp (ZDI: ≤15 mm), while in the current study, all the isolated LAB had resistance to Vm and and *L. lactis* LMEM402B had Ak resistance, in addition to the Vm. While sensitivity to Ak and Am was shown by *L. acidophilus* LMEM403A; The intermediately susceptibility to Gm was recorded for *L. acidophilus* LMEM403A and *L. rhamnosus* LMEM 401A. Thus, in this study, the isolated lactobacilli, has been found to be safe, on the basis of lack of transferable antibiotic resistance property (Anisimova et al. 2022). In current study isolated lactobacilli showed probiotic potential characteristics as well as high antibacterial property and for which they are highly beneficial for human consumption.

CONCLUSION

In the current study, three *Lactobacillus* isolated from commercially available sour curds were found to have probiotic characteristics. The isolated LAB showed high antibacterial activity that can be acted as broad-spectrum antibiotic and lactobacilli so isolated were also fit for human consumption due to their probiotic properties. The study also evaluated that isolated lactobacilli strains can have potentialities for food industries as effective probiotic cultures with health benefits.

Declaration by Authors

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