

Insights into the antagonism of *Lactobacillus fermentum* curd isolate against Gram-positive and Gram-negative pathogenic bacteria

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ABSTRACT

This communication characterizes the curd isolate of lactic acid bacteria having the capacity to antagonize human pathogenic bacteria. The commercially available curd, in sealed form in a plastic cup, was procured from Malda town market (West Bengal state, India) and processed microbiologically, using de Man Rogosa Sharpe medium, for the isolation of lactic acid bacteria. The pure bacteria culture obtained was identified, by phenotypic characterization through conventional methods, as *Lactobacillus fermentum*, and designated as LMEM 22. The *Lactobacillus fermentum* LMEM 22 curd isolate had mixed antibiotic susceptibility patterns, showing resistance (ZDI: ≤ 15 mm) to amikacin, ciprofloxacin, kanamycin, methicillin and vancomycin, sensitivity (ZDI: ≥ 21 mm) to ampicillin, amoxycylav, gentamycin, cefotaxime, imipenem, meropenem and tetracycline, and intermediate susceptibility (ZDI: 16 – 20 mm) to cfoxitin and trimethoprim. The *L. fermentum* LMEM22 antagonizes both gram-negative: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Salmonella enterica* serovar Typhi, and gram-positive: *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, bacterial pathogens, following agar overlay (ZDI: 17 ± 1.73 mm to 20 ± 1.00 mm, for gram-positive, and 18 ± 2.00 mm to 33 ± 2.65 mm for gram-negative bacteria) as well as agar-well diffusion (ZDI: 10.67 ± 2.08 mm to 12 ± 1.00 mm, for gram-positive, and 13.00 ± 2.65 mm to 18.00 ± 3.00 mm, for gram-negative bacteria) techniques. The overall bacteriocin activity (AU/ml) of *Lactobacillus fermentum* LMEM 22 for the test bacterial pathogens ranged 142.27 – 240.00, and the 'R' value ranged 5.5 – 13.5. This study underlines the usefulness of locally available lactic acid bacteria in designing the probiotic microorganisms for biotherapy.

KEY WORDS: LACTIC ACID BACTERIA, *LACTOBACILLUS FERMENTUM*, ANTAGONISTIC ACTIVITY, BACTERIOCIN ACTIVITY, PATHOGENIC BACTERIA

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INTRODUCTION

Among the lactic acid bacteria (LAB), *Lactobacillus* spp. are characteristically known as probiotics, meaning, as per the definition of FAO/WHO (2001), the 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'; LAB isolates are beneficial in many ways, but, essentially by restricting the toxigenic bacterial growth in the gut (Podolsky, 1998). The FAO/WHO (2007) suggested that the probiotic microorganisms must possess the capacity to display the antagonistic activity against bacterial pathogens. Among the large number of lactobacilli isolated from various fermented foods, 42 isolates showed activity against *Escherichia coli*, while 15 isolates had antibacterial activity against *Klebsiella pneumoniae* (Shehata *et al.*, 2016).

Nivien *et al.* (2016) isolated LAB from fermented milk, identified the isolates by phenotypic characterization and reported their antibacterial activity against the bacterial strains: *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. It has been reported that the lactobacilli, including *Lactobacillus fermentum*, had growth inhibitory action against gram-positive as well as gram-negative human pathogenic bacteria (Vuotto *et al.*, 2016). As per the report of Sharma *et al.* (2016), the LAB (*Pediococcus acidilactici* and *Lactobacillus casei*) isolated from milk cream and lassi had growth inhibitory activity against a number of gram-positive food borne bacteria.

Benavides *et al.* (2016) demonstrated that the *Lactobacillus fermentum* isolate from local ecological niche was sensitive to ampicillin, cefuroxime, tetracycline and amoxicillin/clavulanic acid and resistant to gentamycin and kanamycin, and the LAB was found inhibitory to *Escherichia coli* and *Salmonella* Typhimurium. As per the previous report (Halder *et al.*, 2017), four lactobacilli (*Lactobacillus animalis* LMEM6, *Lactobacillus plantarum* LMEM7, *Lactobacillus acidophilus* LMEM8 and *Lactobacillus rhamnosus* LMEM9) procured from different commercially available curd samples had antibacterial activity against gram-negative pathogenic bacteria, such as *Escherichia coli*, *Proteus vulgaris*, *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhi, and had resistance to vancomycin and amoxycylav.

Recently, Mahalot and Mandal (2018) have isolated LAB from locally available cow milk and goat milk samples showing sensitivity to most of the test antibiotics, while resistance was recorded for all isolates to methicillin, for *Lactobacillus* sp. G1 and *Lactococcus* sp. G2 to trimethoprim, while to vancomycin for *Lactobacillus* sp. G1 and *Lactobacillus* sp. C1. Since the good LAB are not even waived from antibiotic resistance phenomenon, many authors documented the status of various resist-

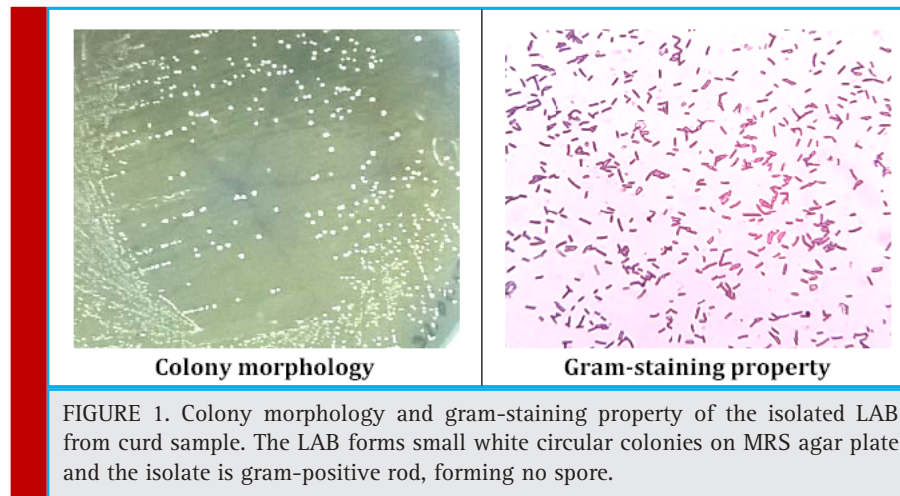
ances to antibiotics for safety profiling of native LAB isolates (Mandal *et al.*, 2017). This background prompted us to assess the broad spectrum antibacterial activity of lactic acid bacillus isolated from commercially available curd, and explore the antibiogram of the isolated *Lactobacillus*, through phenotypic characterization.

MATERIAL AND METHODS

A single cup of commercially available curd sample was procured from Malda town market (West Bengal, India), and processed microbiologically for the isolation of lactic acid bacteria (LAB), following the protocol mentioned earlier (Halder and Mandal, 2015): growth enrichment of LAB in MRS broth (Hi-Media, India), pure culture (single discrete colony isolation) of LAB on MRS agar (Hi-Media, India) plate, and storage of the LAB in MRS agar stab at 4°C for further processing. The isolated LAB (n=1), following Bergey's manual (Holt, 1984), as described earlier (Halder and Mandal, 2015), was subjected to phenotypic (gram-staining, colony morphology study and motility test) and biochemical (oxidase and catalase production) characterization. The non-motile non-spore forming gram-positive rod shaped bacteria (no cocci were found), showing negative results to oxidase and catalase tests, were subjected to IMViC, amino acid decarboxylation and sugar fermentation tests.

The antibiotic susceptibility of the LAB (LMEM 22) was executed by disc diffusion method (Bauer *et al.*, 1996), the details of which was described before (Halder and Mandal, 2016; Halder *et al.*, 2017). The antibiotic discs (Hi-Media, Mumbai, India) used in the study included amikacin (Ak: 30-µg/disc), amoxycylav (Ac: 30-µg/disc), ampicillin (Am: 10-µg/disc), ciprofloxacin (Cp: 5-µg/disc), cfoxitin (Cx: 30-µg/disc), cefotaxime (Ct: 30-µg/disc), cefotaxime/clavunilic acid (Cc: 30/10-µg/disc), gentamycin (Gm: 30-µg/disc), imipenem (Ip: 10-µg/disc), kanamycin (Km: 30-µg/disc), methicillin (Me: 5-µg/disc), meropenem (Mp: 10-µg/disc), tetracycline (Tc: 30-µg/disc), trimethoprim (Tm: 5-µg/disc) and vancomycin (Vm: 30-µg/disc). The results, in terms of ZDI (zone diameter of inhibition) values, were interpreted according to Liasi *et al.* (2009) and Vlkova *et al.* (2006), in order to label the test bacterial isolate as resistant (ZDI: ≤ 15 mm), sensitive (ZDI: ≥21 mm), or intermediately susceptible (ZDI: 16–20 mm).

The antagonistic activity of the LAB LMEM 22 isolate from curd was determined against gram-negative (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Salmonella enterica* serovar Typhi) and gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*) bacterial pathogens,



following agar-well diffusion (Tagg, 1971; Halder *et al.*, 2017) and agar overlay (Shokryazdan *et al.*, 2018) methods, as described and interpreted earlier (Shokryazdan *et al.*, 2014, Halder *et al.*, 2017, Mandal and Halder, 2018).

The 'R' values, from the action of LMEM 22 isolate over the bacterial pathogens, were calculated applying the formula described elsewhere (Halder and Mandal, 2016), and interpreted according to the criteria mentioned earlier (Carasi *et al.*, 2014; Pisano *et al.*, 2014), while the bacteriocin activity of LAB (LMEM 22), in terms of arbitrary units per milliliter (AU/ml), was calculated following the formula put forwarded by Iyapparaj *et al.* (2013).

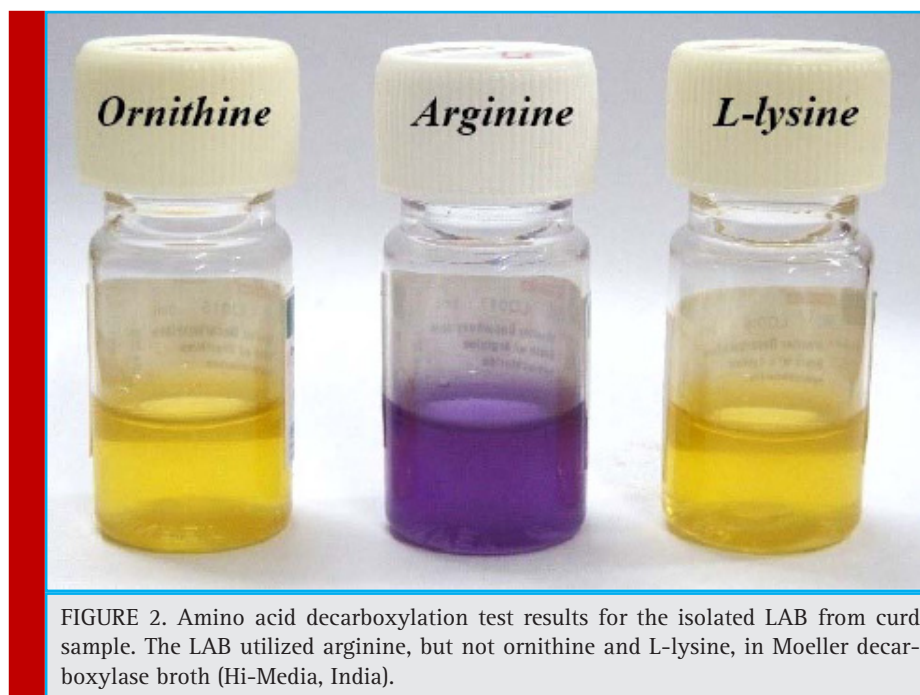
RESULTS AND DISCUSSION

As has been demonstrated by Iyapparaj *et al.* (2013), the morphologically identical bacterial colonies, procured from goat milk on the MRS agar plate, have been identified as *Lactobacillus* sp., on the basis of physical and biochemical characteristics, following Holt *et al.* (1984). In this study, a single isolate of non-motile non-spore forming gram-positive rod was procured from the curd sample (Figure 1), and the isolated bacteria (LMEM 22) was an hetero-fermentative strain, which in TSI test showed the production of acid as well as gas (CO₂). The LMEM 22 isolate showed negative test results for catalase and oxidase, and in IMViC test battery the isolates was positive for methyl red. The sugar fermentation pattern of LMEM 22 isolate is represented in Table 1, while the amino acid decarboxylation test results are depicted in Figure 2. Thus, following phenotypic and biochemical characterization the isolated LAB was identified as *Lactobacillus fermentum* LMEM 22. The LAB isolates procured from different fermented foods, including

curd, have been identified earlier by Nigam *et al.* (2012), following phenotypic characterization of the bacteria. Currently, the treatment options with antibiotics are inadequate because of the escalating rate of emergence of antibiotic resistant pathogenic bacteria causing life-threatening infections to humans. Alternative to the antibiotics, which remains the mainstay of all therapy for bacterial infections (Van Boeckel *et al.*, 2014), probiotic lactobacilli have been found suitable for biotherapy with proven antibacterial activity (Iyapparaj *et al.*, 2013; Sing *et al.*, 2017).

Earlier, it has been reported that the curd isolates of *Lactobacillus animalis* LMEM6, *Lactobacillus plantarum* LMEM7, *Lactobacillus acidophilus* LMEM8 and *Lactobacillus rhamnosus* LMEM9, had bacterial growth inhibitory activity, having ZDIs 13.67 ± 0.58 – 29.50 ± 2.10 mm, by agar-well, and 11.33 ± 0.58 – 35.67 ± 2.52, by agar overlay, against human pathogenic bacteria, viz., *Escherichia coli*, *Proteus vulgaris*, *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhi (Halder *et al.*, 2017). The antibacterial activity of *Lactobacillus fermentum* LMEM 22 against gram-positive and gram-negative pathogenic bacteria, following agar-well diffusion method is depicted in Figure 3.

Gandevia *et al.* (2017) isolated, from cow milk, buffalo milk, goat milk and curd samples, a number of *Lactobacillus* species, including *Lactobacillus fermentum*, having the capacity to inhibit the growth of gram-positive bacteria, such as *Staphylococcus aureus* (ZDI: 8 – 17 mm) and *Bacillus cereus* (ZDI: 12 – 22 mm). The two *Lactobacillus fermentum* isolates from buffalo milk had ZDI of 19 mm, while the *Lactobacillus oris* conferred ZDI of 18 mm, against *Listeria monocytogenes* (Melia *et al.*, 2017). The broad spectrum antibacterial activity of *Lactobacillus fermentum* has been demonstrated earlier (Ilayajara *et al.*, 2011; Ramasamy and Suyambulingam, 2015; Podolsky, 1998), in which the LAB showed growth

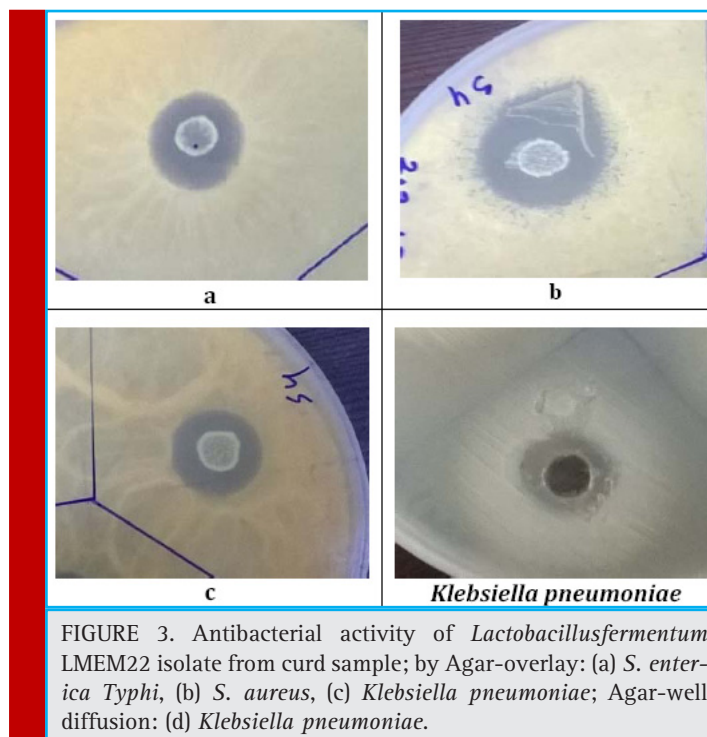


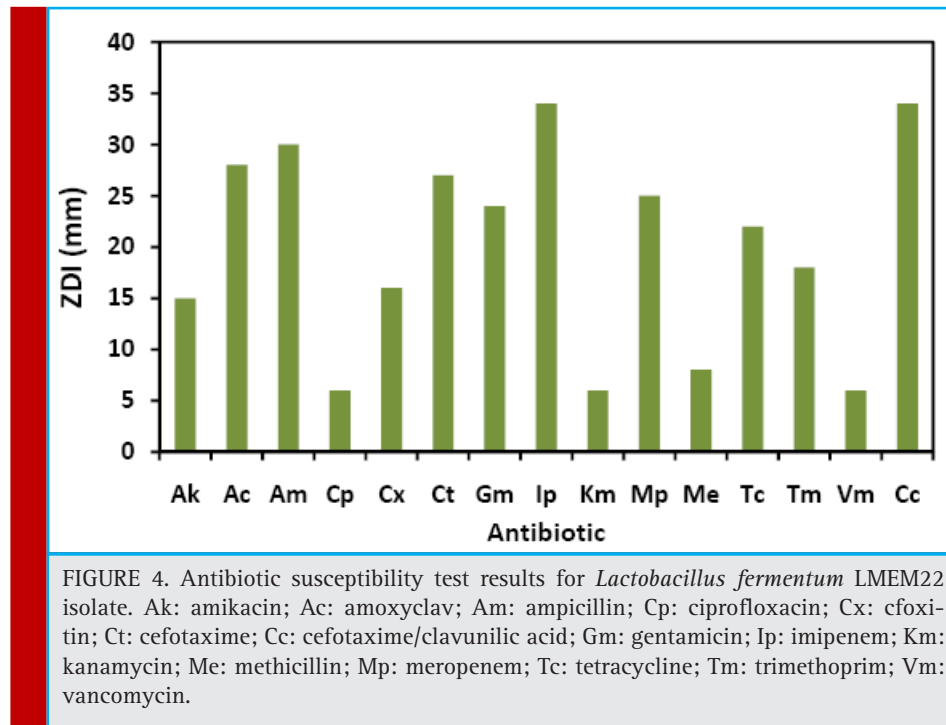
inhibitory activity against gram-positive (*Staphylococcus aureus*, *Enterococcus* spp., *Streptococcus* spp., *Bacillus subtilis*) and gram-negative (*Proteus* spp., *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*) bacterial pathogens.

Kang *et al.* (2017) reported the enhancing killing of *Staphylococcus aureus* strains by *Lactobacillus salivar-*

ius except *Staphylococcus aureus* ATCC 25923 strain, the growth of which was fully killed by *Lactobacillus fermentum*.

Sharma *et al.* (2016) isolated two lactic acid bacteria: *Pediococcus acidilactici* and *Lactobacillus casei* from milk cream and lassi, respectively, which had antibacterial activity against a large number of potential





food-borne bacteria, viz., *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Clostridium perfringens* and *Bacillus cereus* having respective ZDIs range from 12 mm to 20 mm and from 13 mm to 19 mm. In the current study, the agar-well diffusion had ZDIs of 10.67 ± 2.08 mm (*Staphylococcus aureus*) to 12 ± 1.00 mm (*Bacillus cereus*), for gram-positive, and 13.00 ± 2.65 mm (*Escherichia coli*) to 18.00 ± 3.00 mm (*Salmonella enterica* Typhi), for gram-negative bacteria (Table 2). The *Lactobacillus fermentum* LMEM 22 isolate, following agar overlay method, also had antibacterial activity against the indicator bacterial strains (Figure 3), display-

ing ZDIs from 17.00 ± 1.73 mm (*Listeria monocytogenes*) to 20.00 ± 1.00 mm (*Enterococcus faecalis*), for gram-positive, and from 18.00 ± 2.00 mm (*Escherichia coli*) to 33 ± 2.65 mm (*Acinetobacter baumannii*) for gram-negative bacteria (Table 3).

The bacteriocin activity of the isolated LAB, *Lactobacillus fermentum* LMEM 22, has been shown in Table 2, while the Table 3 depicts the 'R' values of the isolated LAB. Iyapparaj *et al.* (2013) isolated *Lactobacillus* sp. MSU3IR strain, which against pathogenic bacteria: *Staphylococcus aureus* and *Pseudomonas aeruginosa*, displayed higher bacteriocin activity (393.2 ± 2.61 to 556.0 ± 5.34 AU/ml) in MRS medium, compared to the activity value (341.2 ± 2.36 to 473.2 ± 3.96 AU/ml) as recorded in *Lactobacillus* selection broth. Earlier, the 'R' values of curd lactobacilli strains ranged 3.00 - 13.17 mm, while the bacteriocin activity, in terms of arbitrary units (AU/ml), ranged 155.60 - 293.33, against MDR *Klebsiella pneumoniae* clinical isolates (Mandal and Halder, 2018).

In another study, the probiotic lactobacilli had excellent antibacterial activity against gram-negative human pathogenic bacteria (*Escherichia coli*, *Proteus vulgaris*, *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhi) displaying 'R' values ranging from 3.17 ± 0.29 to 15.33 ± 1.26 mm, and the bacteriocin activity ranging from 233.34 ± 45.54 to 280.56 ± 83.67 AU/ml (Halder *et al.*, 2017). As per the report of Shehata *et al.* (2016), among nine isolates of LAB, one (*Lactococcus lactis* subsp. *lactis*) had strong activity (1600 AU/ml)

Table 1. Sugar fermentation test results for the isolated LAB from curd sample

Sugars	Utilization	Sugars	Utilization
Adonitol	-	D-Melezitose	+
Arabinose	W	Raffinose	+
Cellobiose	+	Rhamnose	+
Dextrose	+	D-Ribose	+
Esculin	-	Salicin	-
Glucose	+(g)	Sorbitol	+
Lactose	+	Sucrose	+
Mannitol	+	Trehalose	+
Mannose	+	Xylose	+
Melibiose	+	D-Galactose	+

+: Strong fermentation; w: week fermentation -: No fermentation

Table 2. Agar-well diffusion test results in terms ZDI (mm) and the calculated bacteriocin activity (Au/ml) of *Lactobacillus fermentum* LMEM22 against gram-positive and gram-negative indicator bacterial strains.

Indicator strains	Bacterial isolates	ZDI range (Mean ± SD)	Bacteriocin activity
Gram-negative	<i>E. coli</i>	10-15 (13.00±2.65)	173.33
	<i>Pr. vulgaris</i>	12-14 (13.33±1.15)	177.73
	<i>A. baumannii</i>	16-20 (17.33±2.31)	231.07
	<i>Ps. aeruginosa</i>	15-16 (15.67±0.58)	208.93
	<i>K. pneumoniae</i>	14-17 (15.67±1.53)	208.93
	<i>S. enterica Typhi</i>	15-21 (18.00±3.00)	240.00
Gram-positive	<i>B. cereus</i>	11-13 (12.00±1.00)	160.00
	<i>E. faecalis</i>	10-13 (11.67±1.53)	155.60
	<i>S. aureus</i>	9-13 (10.67±2.08)	142.27
	<i>L. monocytogenes</i>	10-13 (11.67±1.53)	155.60

Au/ml: arbitrary units per milliliter

against *Klebsiella pneumoniae*, while, four isolates had bacteriocin activity of 800 AU/ml against *Escherichia coli* (for *Lactobacillus paracasei*), *Streptococcus pyogenes* (for *Lactobacillus gasseri*), *Staphylococcus aureus* (for *Lactobacillus rhamnosus*) and *Salmonella senftenberg* (for *Lactobacillus gasseri* RM28). The current investigation demonstrates the capacity of antibacterial activity of *Lactobacillus fermentum* LMEM 22 for the indicator microorganisms, consisting of both gram-positive and gram-negative pathogenic bacteria, with an overall bacteriocin activity (AU/ml) of 142.27 – 240.00 and the ‘R’ values of 5.5 – 13.5 mm.

The antibiotic susceptibility test results for *Lactobacillus fermentum* LMEM 22 isolate is shown in Figure 4. One of the most important probiotic features, defining safe for human consumption, of lactic acid bacteria is being their antibiotic sensitivity, and the intrinsic resistance (chromosomally conferred from point muta-

tion) property as well (Georgieva *et al.*, 2015). As has been demonstrated by Benavides *et al.* (2016), the isolated *Lactobacillus fermentum* was sensitive to ampicillin (ZDI: 28 mm), cefuroxime (ZDI: 30 mm), tetracycline (ZDI: 24 mm) and amoxicillin/clavulanic acid (ZDI: 26 mm) and resistant to gentamycin and kanamycin, and the LAB was found inhibitory to *Escherichia coli* (ZDI: 13 mm) and *Salmonella* Typhimurium (ZDI: 12 mm). As per our earlier report the curd lactobacilli had sensitivity to majority of the test antibiotics displaying a common resistance to Vm (Halder and Mandal, 2016). In the instant case, the isolated LAB: *Lactobacillus fermentum* LMEM 22 showed resistance to Ak, Cp, Km, Me and Vm (ZDI: ≤15 mm; range: 6 – 15 mm), and such resistances are intrinsic as well as non-transferable (Bamidele *et al.*, 2017; Imperial and Ibana, 2016).

The *Lactobacillus fermentum* LMEM 22 was sensitive to Ac, Am, Cc, Cx, Gm, Im, Mp and Tc (ZDI: ≥21 mm;

Table 3. Agar overlay test results in terms ZDI (mm) and the calculated ‘R’ values for *Lactobacillus fermentum* LMEM22 against gram-positive and gram-negative indicator bacterial strains

Indicator strains	Bacterial isolates	ZDI range (Mean ± SD)	‘R’ value (mm)
Gram-negative	<i>E. coli</i>	16-20 (18.00±2.00)	6.00
	<i>Pr. vulgaris</i>	21-26 (23.33±2.52)	8.67
	<i>A. baumannii</i>	31-36 (33.00±2.65)	13.50
	<i>Ps. aeruginosa</i>	23-28 (26.00±2.65)	10.00
	<i>K. pneumoniae</i>	20-25 (22.67±2.52)	8.34
	<i>S. enterica Typhi</i>	20-25 (22.00±2.65)	8.00
Gram-positive	<i>B. cereus</i>	16-19 (17.67±1.53)	5.84
	<i>E. faecalis</i>	19-21 (20.00±1.00)	7.00
	<i>S. aureus</i>	18-20 (18.67±1.56)	6.34
	<i>L. monocytogenes</i>	15-18 (17.00±1.73)	5.50

SD: standard deviation; ZDI: zone diameter of inhibition.

range: 22 – 34 mm), and intermediately susceptible to Cx and Tm with ZDIs of 16 and 18 mm (ZDI criteria range: 16 – 20 mm); the LAB, while, showed resistance (ZDI: ≤ 15 mm) to Ak, Cp, Km, Me and Vm. Thus, the isolated LAB, in this study, has been found to be safe, on the basis of lack of transferable antibiotic resistance property (Ammor *et al.*, 2008; Imperial and Ibana, 2016), and this LAB might be useful in single-strain based probiotic formulation benefiting a large number of local population, in this part of the globe. It has been reported that multi-strain/multi-general probiotics might exhibit limited functional property for universal usage, requiring probiotics alternatives development and/or personalized probiotic approaches (Zmora *et al.*, 2018; Suez *et al.*, 2018).

CONCLUSION

The *Lactobacillus fermentum* LMEM22, which was isolated from locally available commercial curd, showed antagonistic activity against gram-positive as well as gram-negative pathogenic bacteria, with overall bacteriocin activity (AU/ml) of 142.27 – 240.00, and the 'R' value of 5.5 – 13.5, and (based upon the report available in literatures, too) there is no risk of transferable antibiotic resistance in the LAB. Thus, the isolated LAB might be useful as broad spectrum antibacterial biotherapeutics, and such native LAB isolate might be consumed alone, in place of antibiotic therapy, or can be used (based upon the antibiogram of the native LAB) in probiotic-antibiotic combination therapy. However, further studies are needed to validate the probiotic attributes of the isolated LAB, including its molecular identity as well as the antibiotic resistance management.

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