Insights Into The Broad Antibacterial Spectrum Of Bacteriocin Isolated From Probiotic *Lactobacillus Fermentum* LMEM22 Strain

Debashis Halder
University of Gour Banga, Malda-732103, India
Manisha Mandal
MGM Medical College, Kishanganj-855107, India https://orcid.org/0000-0002-9562-5534
Nishith Kumar Pal
NRS Medical College and Hospital, Kolkata-700014, India
Shyamapada Mandal (samtropmed@gmail.com)
University of Gour Banga, Malda-732103, India https://orcid.org/0000-0002-9488-3523

Research Article

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Abstract

This communication aims to validate the probiotic attributes of the *Lactobacillus fermentum* LMEM22 strain, safety profiling, 16S rRNA gene sequence analysis, and bacteriocin characterization. The bacteriocin of 13.1 kDa (based on the glycine-SDS-PAGE analysis) was isolated from *L. fermentum* LMEM22 curd strain (NCBI GenBank with accession No. MH380182), the identity for which was confirmed by 16S rRNA gene sequence analysis. The probiotic properties and safety profiles were authenticated *in vitro* systems. The zone diameter of inhibition from the *L. fermentum* LMEM22 bacteriocin action, following agar-well diffusion, ranged 19 – 23 mm and 17 – 24 mm, respectively, for gram-negative and gram-positive bacteria. On enzymatic treatment with proteinase K and trypsin the bacteriocin lost the bacterial growth inhibition capacity, and it was found insensitive against α-amylase action, authenticating its proteinaceous nature. The γ-haemolytic *L. fermentum* LMEM22 strain, for which gelatinase and DNase activities were negative, had tolerance to high sodium chloride concentration range (2 – 6.5%), low pH (2 – 4%), and bile salts (0.125 – 0.5%). This study, thus, authenticated the probiotic attributes of *L. fermentum* LMEM22 strain for safe consumption by the people, and the usefulness of bacteriocin isolated, as a valued protein antibiotic for the prevention and treatment of multidrug resistant bacterial infection.

Introduction

The antagonism of probiotic LAB (lactic acid bacteria) strains against human pathogenic bacteria has been reported to be due to their capacity to produce hydrogen peroxide, organic acids, such as lactic acid, and bacteriocins. The bacteriocins are antimicrobial peptides of low molecular weights and produced by gram-negative as well as gram-positive bacteria (Usui *et al.,* 2012; Bosak *et al.,* 2018); however, those produced by LAB remain the most valuable compounds in food and medicine (Dobson *et al.,* 2012), because the majority of such producers are 'generally regarded as safe' and designated as probiotics. Therefore, the one plausible opportunity to curb the ARPB (antibiotic resistant pathogenic bacteria) infections to humans is to deploy LAB strains, or to utilize bacteriocins produced by them, as protein antibiotics (Das and Goyal, 2014; Gupta *et al.*,, 2017). The probiotic potentiality assessment of different LAB strains takes an account of testing for physiological stressors tolerance, safety aspects and functionality (Halder *et al.*,, 2017), and before *in vivo* application, in order to get a benefit for a given health disorder or pathogenic infection to humans *in vitro* testing is mandatory in substantiating the preferred outcome (Gareau *et al.*,, 2010).

The gram-negative ARBP that pose severe global health threats include *Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, Salmonella enterica* serovar Typhi and *Proteus vulgaris,* while among the gram-positive bacteria, the MDR strains of *Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus* and *Listeria monocytogenes* cause threats to the patients' safety. Antibiotics remain the basis of all kinds of therapies in modern healthcare systems, such as enabling the treatment life-threatening bacterial infection to humans (Van Boeckel *et al.*,, 2014). However, the global emergence of multiple antibiotic resistant pathogenic bacteria necessitates for novel

antimicrobials safe for human usage. The bacteriocin producing probiotic lactobacilli have been proved to be safe and suitable for biotherapy because of their capacity to display antibacterial activity against clinically relevant bacteria, or spoilage as well as food-borne pathogenic bacteria (Das and Goyal, 2014). Many authors isolated and identified, following conventional phenotypic characterization as well as 16S rRNA gene sequencing, LAB strains from varied sources and characterized bacteriocins produced by them (Ge *et al.,* 2016; Oliveira *et al.,* 2017).Earlier, we have identified (by phenotypic characterization) a LAB strain, *Lactobacillus fermentum* LMEM22 procured from commercially available curd (West Bengal, India), checked the antibiogram and validated its antagonistic capacity against human pathogenic bacteria (Halder and Mandal, 2018). The current study was, thus, prompted to be undertaken in order to authenticate the probiotic attributes of the *Lactobacillus fermentum* LMEM22 strain through stressors tolerance, safety profiling, 16S rRNA gene sequence analysis, and bacteriocin characterization.

Materials And Methods

Bacterial strains and media

The LAB strain, which was isolated from curd, identified as *L. fermentum* LMEM22 by conventional phenotypic characterization (Halder and Mandal, 2018), and maintained in MRS broth as well as in MRS agar stab in freezing, was utilized in the instant study. The indicator bacteria used include the randomly selected gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, Salmonella enterica* serovar Typhi and *Proteus vulgaris)* and gram-positive (*Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus)* clinical bacterial isolates from the laboratory stock cultures, and *Listeria monocytogenes* MTCC657 standard strain, which were maintained in cystine tryptone agar stabs, in refrigeration.

Molecular identity of lactic acid bacterium

The identity authentication of the bacteriocinogenic LAB strain was done by 16S rRNA gene sequence and phylogenetic analyses. The 16S rDNA (≈1.5 kb fragment) was PCR amplified (from the genomic DNA extracted from the LAB) and thereafter sequenced using 16S rRNA specific primers (forward primer: 5'-AGHGTBTGHTCMTGNCTCAS–3' and reverse primer: 5'-TRCGGYTMCCTTGTWHCGACTH–3') from Chromous Biotech Pvt Ltd, India.

The sequenced data (partial 16S rRNA gene sequence: 672 bp) was aligned using ClastalW (Thompson *et al.,*, 1994), and in the NCBI (National Center for Biotechnology Information) GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) the closest known relatives of the sequence obtained (from the test LAB strain) were determined through nucleotides homology search, with BLAST (Basic Local Alignment Search Tool), for nucleotide sequences (BLASTN).

The phylogenetic tree, based upon 16S rRNA gene sequences was constructed within the SeaView version–4 software (Gouy *et al.,* 2010),following maximum likelihood method with PhyML GTR model

(bootstrap with 1000 replicates). The partial 16S rRNA gene sequence of the test LAB strain has been deposited with the NCBI Genbank accession No. MH380182.

Probiotic characterization

The probiotic features of the test *L. fermentum* LMEM22 strain was substantiated through physiological stressors tolerance tests and safety profiling. The *L. fermentum* LMEM22 was subjected to probiotic characterization by performing bile salt and low-pH (acid) tolerance tests according to Liong and Shah (2005), and sodium chloride tolerance test following Chowdhury *et al.* (2012), with some alteration as cited before (Halder and Mandal, 2015; Halder *et al.*,, 2017).

In order to check the viability of the LAB at different incubation hours, the *L. fermentum* LMEM22 strain, with $\approx 5 \times 10^5$ CFU/ml (5.698 $\log_{10}$ CFU/ml) inocula, was allowed to grow in presence of varied concentrations of sodium chloride (2, 4 and 6.5 %) and bile salts (0.125, 0.25 and 0.5 %), and under acidic environmental condition (at pH: 2, 3 and 4) in MRS broth, up to 24 h, at 35 ^{o}C. Aliquots (each containing 100 µl), from the above mentioned cultures with different physiological stressors, were removed at 0, 2, 4, 6 and 24 h, mixed with MRS agar and pour plated, and following incubation at 35 ^{o}C for 24 h, CFUs were enumerated.

The safety properties of the LAB, *L. fermentum* LMEM22 strain, were validated by their hemolytic, gelatinase and DNase activity (Halder *et al.*,, 2017; Yadav *et al.*,, 2016).

Isolation and quantification of bacteriocin

Bacteriocin from the *L. fermentum* LMEM22 strain was extracted following Ismail *et al.* (2013), with modifications as explained below. Following subcultures of *L. fermentum* LMEM22 strain twice in MRS broth, 100 µl was inoculated into 25 ml of MRS broth, and after incubation at 37°C for 48 h, cell free supernatant (CFS) was prepared by centrifugation (at 10,000 rpm, for 10 minutes at 4°C) and syringe filtration. The CFS was treated with 60% ammonium sulfate at 4°C for 24 h to get precipitated the bacteriocin, which by centrifugation (at 12,000 rpm for 15 minutes at 4°C), was extracted. The isolated bacteriocin was processed by washing with sterilized double distilled water and centrifuging (at 10,000 rpm for 10 minutes at 4°C) for thrice, in order to remove the impurities in bacteriocin. At the final stage, pure bacteriocin pellet was mixed with phosphate buffer solution (1 ml, pH 7.2), with 0.6 % SDS, and stored at 4°C for further usage.

The bacteriocin yield was quantified spectrophotometrically, following Lowry *et al. (*1951), using bovine serum albumin as the standard.

SDS-PAGE analysis of bacteriocin

The molecular weight of bacteriocin isolated from *L. fermentum* LMEM22 strain was approximated by glycine-SDS-PAGE (glycine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis (Laemmli, 1970), using a vertical slab gel apparatus (Tarsons, India) with 7.5% stacking and 12.5 % separating gels, and high range protein molecular markers (Hi-Media, India). Following electrophoresis for 4 h at 130 V, the gel was subjected to Coomassie brilliant blue (Hi-Media, India) staining (for 30 min) and thereafter de-staining in 20% (v/v) methanol / 7.5% (v/v) glacial acetic acid until the bands were clearly visible. The molecular weight of *L. fermentum* LMEM22 bacteriocin was calculated from the relative mobility of the molecular weight markers in the gel.

Antibacterial activity of bacteriocin

The antibacterial activity of *L. fermentum* LMEM22 bacteriocin was determined by agar-well diffusion following Tagg and McGiven (Tagg and McGiven, 1971), using 66.67-µg bacteriocin per well (6 mm diameter), prepared on nutrient agar plate, which was swabbed with the overnight grown culture of indicator bacterial strains in nutrient broth, and the ZDIs were measured (in nearest whole in millimeter), in order to interpret the effectiveness of bacteriocin, following the criteria mentioned earlier[6], as less active, moderately active, or highly active through ZDIs ≤ 10 mm, 11—14 mm, and ≥ 15 mm, respectively.

For the determination of MICs of *L. fermentum* LMEM22 bacteriocin, against the indicator bacteria, broth dilution method was used as mentioned elsewhere (Wiegand *et al.,*, 2008), utilizing random bacteriocin concentrations of 30—200 µg/ml, in nutrient broth. Inoculation of indicator bacteria, from overnight grown nutrient broth cultures, was done; all incubations were at 35 ^{o}C for 24 h. The lowest bacteriocin concentration, which was required to inhibit the visible growth of test pathogenic bacteria, was defined as the MIC.

Enzymatic sensitivity of bacteriocin

The enzymatic effect on the bacteriocin isolated from *L. fermentum* LMEM22, against protease K, trypsin, and α-amylase, was evaluated following the protocol of Ge *et al (*Ge *et al.,* 2016). The test bacteriocin was mixed with each of the enzymes (10 mg/ml) and incubated at 37 ^{o}C for 24 h, and afterward the mixture was boiled at 80 °C for 5 min for enzyme inactivation. The *L. fermentum* LMEM22 bacteriocin without any enzymatic treatment was used as the control, in determining the residual bacteriocin activity (RBA), following agar-well diffusion against gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes)* as well as gram-negative (*Acinetobacter baumannii* and *Escherichia coli)* human pathogenic bacteria. The RBA was computed based on the ratio of bacterial growth inhibition (in terms of ZDI) with treatment and with the control (Ge *et al.,* 2016).

Results

Based upon the 16S rRNA gene sequence and phylogeny analysis, the LAB strain of curd origin, LMEM22, has been found to be closest to *Lactobacillus fermentum* hani.A ton 2 KM214424, and was identified as *Lactobacillus fermentum* LMEM22 (Fig. 1), the NCBI GenBank accession No. for which is MH380182. The antibacterial bacteriocin producing γ-haemolytic *L. fermentum* strain LMEM22 (Fig.1), for which gelatinase and DNase activities were negative, had tolerance to high sodium chloride concentration (2 %—6.5 %) and temperature (15 ^{o}C—42 ^{o}C) range, acidic milieu (pH: 2.0—4.0) and bile salts (0.125—0.5 %), as represented in Table 1.

A single band of 13.1 kDa bacteriocin, based on the glycine-SDS-PAGE analysis, was isolated from the *Lactobacillus fermentum* strain LMEM22, which possessed antibacterial capacity (Fig. 2); the 13.1 kDa LMEM22 bacteriocin yield has been recorded as 1.656 mg/ml.

The LMEM22 bacteriocin had antibacterial activity against gram-negative (*Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica* serovar Typhi and *Proteus vulgaris)* and gram-positive (*Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus* and *Listeria monocytogenes)* human pathogenic bacteria displaying respective ZDI (zone diameter of inhibition) values of 19—23 mm and 17—24 mm (Fig. 3), by agar-well diffusion. The minimum inhibitory concentration (MIC) values, as have been determined by agar dilution, ranged 50—199 µg/ml, for gram-negative bacteria, and 41–132 µg/ml, for gram-positive bacteria (Fig. 4).

The effect of enzymes on the *L. fermentum* LMEM22 bacteriocin, in terms of the antibacterial activity, has been represented in Table 2. When treated with enzymes, proteinase K and trypsin, the bacterial growth inhibition capacity (in terms of ZDI) of *L. fermentum* LMEM22 bacteriocin, against gram-positive: *Staphylococcus aureus* and *Listeria monocytogenes* as well as gram-negative: *Acinetobacter baumannii* and *Escherichia coli,* test bacteria was reduced by 3—5 mm (both for gram-positive and gram-negative bacteria) compared to the untreated bacteriocin activity, while mostly the bacteriocin activity was unaffected by α-amylase.

Discussion

This study authenticates the probiotic competency of a native *L. fermentum* LMEM22 strain from curd by means of safety profiling and functionality (stability against selective physiological stress and antibacterial efficacy) testing, and the identity confirmation, as well through 16S rRNA gene sequencing. The sequence determination of 16S rRNA genes of the genus *Lactobacillus* provides precise information on their phylogeny and identification (Vandamme *et al.*,, 1996). The 16S rRNA gene analysis, using the universal primers (forward: 5'-AGAGTTTGATCCTGGCTCAG–3' and reverse: 5'-TACGGCTACCTTGTTACGACTT–3'), of two heterofermentative lactobacilli isolates showed 99 % similarity to *L. fermentum,* while the two homofermentative isolates had similarity with *L. plantarum,* as has been demonstrated by Patil *et al. (*2010). A set of forward primer: 5'-AGHGTBTGHTCMTGNCTCAS–3' and reverse primer: 5'-TRCGGYTMCCTTGTWHCGACTH–3' has been utilized in the PCR amplification of 16S rRNA in order to recognize the identity of LAB strains isolated from a variety of sources (Roy and Rai,

2017). A non-spore forming gram-positive, but catalase and oxidase negative rod shaped LAB strain, LMEM22, which was conventionally identified as *L. fermentum* LMEM22 (Das and Goyal, 2014), has been subjected to molecular based identification, in this study, by 16S rRNA gene sequencing and phylogeny analysis. The identity of the test strain, being closest to *Lactobacillus fermentum* hani.A ton 2 KM214424, was confirmed as *L. fermentum* LMEM22 (the NCBI GenBank accession number for which is MH380182).

Based upon the physiological stressor tolerance (bile salts, low-pH and sodium chloride) and safety profiles (γ-haemolysis and inept to gelatinase and DNase production), the *L. fermentum* LMEM22 has been substantiated as a potential probiotic strain, following the criteria mentioned earlier (Gareau *et al.*„ 2010; Halder *et al.*„ 2017). In addition, a vital probiotic feature that signifies the safe consumption of indigenous LAB strain is the antibiotic sensitivity (to avoid the risk of resistance transferability) and the intrinsic resistance (chromosomally conferred due to point mutation) as well (Georgieva *et al.,* 2015; Jose *et al.*„ 2015), for co-administration of probiotic with antibiotic (Mombelli and Gismondo, 2000). The isolated *L. fermentum* LMEM22 strain had been found to be safe, on the basis of non-transferable nature of antibiotic resistance (Ammor et al., 2008; Imperial and Ibana, 2016), and thus, the current study validated the single-strain based (*L. fermentum* LMEM22) probiotic benefit for people in our part of the globe (Malda, West Bengal state, India).

In order to muddle through the escalating incidence of antimicrobial resistance (AMR) among human pathogenic bacteria a class of protein antibiotics, the bacteriocins, produced especially by probiotic LAB strains, has been paid an immense attention, because of their stupendous capacity to antagonize the human pathogenic bacteria, including the WHO priority pathogens (*Mycobacterium tuberculosis, Clostridium difficile* and *Staphylococcus aureus)* too (Cotter *et al.,* 2015; WHO, 2017). The bacteriocin of 9.5 kDa from cow milk derived LAB strain, *Lactobacillus plantarum,* had antibacterial activity, having ZDI (zone diameter of inhibition) values 11—21 mm, against *Escherichia coli, Enterococcus faecalis, Listeria monocytogenes* and *Staphylococcus aureus (*Sankar *et al.,* 2012). The antibacterial activity of LAB bacteriocin has been reported earlier. It has been shown that the 1,77 kDa bacteriocin MN047A, isolated from *Lactobacillus crustorum* MN047 had broad spectrum antibacterial activity against gram-positive bacteria: *Staphylococcus aureus* isolates (ZDI: 20—23 mm)*, Enterococcus faecalis (*ZDI: 22 mm) and *Listeria monocytogenes (*ZDI: 22 mm), and gram-negative bacteria: *Escherichia coli (*ZDI: 21 mm), *Salmonella spp. (*ZDI: 20—21 mm), (ZDI: 21 mm), *Enterobacter sakazakii* isolates (ZDI: 19—21 mm), and the bacteriocin had MICs of 165 and 305 µg/ml against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922, respectively (Yi *et al.*„ 2016). The subtilosin A bacteriocin has been reported to display broad range of MICs, from 1.25 µg/ml, for *Shigella sonnei* ATCC 25931, *Enterobacter aerogenes* ATCC 13408, *Klebsiella pneumoniae* UMN5 and *Streptococcus pyogenes* ATCC 19615 strains, to >200 µg/ml, for *Bacillus cereus* ATCC 10876, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 25933, *Salmonella enterica* Typhi ATCC 12048 strains (Shelburne *et al.*„ 2007). As per the report of Sharma *et al.* (2018), a bacteriocin of ≈6.5 kDa from *Bacillus subtilis* GAS101, has been found to be the potential inhibitor of *Staphylococcus epidermidis* and *Escherichia coli.* The bacteriocin of 2.7 kDa isolated from *Weissella confusa* A3 strain had antibacterial

activity against gram-positive (*Bacillus cereus* ATCC14579 and *Micrococcus luteus* ATCC10240) and gram-negative (*Escherichia coli* UT181 and *Pseudomonas aeruginosa* PA7) bacteria, displaying MICs of 9.25 µg/ml and 18.5 µg/ml, respectively, while the ZDIs ranged 7.98 - 11.83 mm (Goh and Philip, 2015). Elayaraja *et al.(*2014) demonstrated the antibacterial activity of 21 kDa *Lactobacillus murinus* AU06 bacteriocin against gram-positive (*S. aureus, Micrococcus* sp., *Bacillus licheniformis, Enterococcus faecalis, Listeria monocytogenes)* and gram-negative (*Escherichia coli, Pseudomonas aeruginosa)* bacteria, with ZDIs 10—28 and 18—22 mm, respectively. The bacteriocin, paracin 1.7 of ≈10 kDa, produced by *L. paracasei* HD1–7 strain, had a wide spectrum of growth-inhibitory effect on *Staphylococcus, Micrococcus* and *Bacillus (*gram-positive bacteria) as well as *Proteus, Escherichia, Enterobacter, Pseudomonas* and *Salmonella* (gram-negative bacteria), as has been reported by Ge *et al.* (2009). The ≈13 kDa *L. fermentum* LMEM22 bacteriocin, in the current study, had broad antibacterial capacity (against bacteria of clinical relevance) displaying ZDIs of 17—23 mm and MICs of 41—199 µg/ml.

The protein/peptide nature of LAB bacteriocin has been specified previously through enzyme (proteolytic) sensitivity testing, wherein the disbanding of antibacterial capacity of such protein antimicrobials by enzymes (proteinase K, chymotrypsin and trypsin) validated their (bacteriocins) proteinaceous nature (Xiraphi *et al.*„ 2008; Ge *et al.,* 2016). The bacteriocin of *L. fermentum* sl36 strain isolated from goat milk, which inhibited the growth of food-borne bacteria (*Enterococcus faecalis, Listeria monocytogenes, Listeria innocua, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli)*„ lost the antibacterial activity, against gram-positive strains, after treatment with trypsin (Mitjans *et al.*„ 2018). The antimicrobial bacteriocin Lac-B23, of ≈6.73 kDa from L. plantarum J23 strain, demonstrated complete loss of anti-Listeria monocytogenes activity on being treated with proteinase K, trypsin, and proteinase E, indicating the complete disruption of bacteriocin Lac-B23, by proteolytic enzymes, due to its proteinaceous nature (Zhang *et al.*„ 2018).As has been reported by Todorov *et al. (*2013), the *Lactobacillus sakei* strains ST22Ch, ST153Ch and ST154Ch (from fermented meat products), containing bacteriocin of 3 kDa, 10 kDa and 3 kDa, respectively (in tricine/SDS-PAGE), which inhibited the growth of gram-positive (*Enterococcus* spp., *Staphylococcus* spp., *Listeria* spp. and *Streptococcus* spp.) and gram-negative (*Escherichia coli, Pseudomonas* spp. and *Klebsiella* spp.) bacteria, demonstrating a decrease of antibacterial action with proteinase K and pronase treatment of the bacteriocin, but not due to α-amylase treatment. The bacteriocins of *L. plantarum, L. pentosus* and *L. paracasei* displaying growth inhibitory effect against *Salmonella* and *S. aureus* with ZDIs 13.08±0.15—15.22±0.13 mm, lost their antimicrobial action after treatment with proteolytic enzymes (proteinase K, pepsin, and papain), as has been reported by Ren *et al. (*2018). In the current study, on enzymatic treatment with proteinase K and trypsin, the *L. fermentum* LMEM22 bacteriocin activity has been found to be decreased when tested against gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes)* as well as gram-negative (*Acinetobacter baumannii* and *Escherichia coli)* indicator bacteria, and the bacteriocin was found insensitive against α-amylase activity, authenticating its proteinaceous nature, which was in accordance to the report of the earlier authors (Ge *et al.,* 2016; Fahim *et al.*„ 2017).

Conclusion

The isolated *L. fermentum* LMEM22 bacteriocin (13.1 kDa) of proteinaceous nature had broad spectrum antibacterial activity against an array of gram-positive (ZDIs: 17—24 mm; MICs: 41—132 µg/mL) and gram-negative (ZDIs: 19—23 mm; MICs: 50—199 µg/ml) bacteria of clinical importance, and the bacteriocinogenic LAB *L. fermentum* LMEM22 strain accomplished the probiotic attributes along with the indispensable safety profiles so as to be safe for human consumption, at least in our developing part of the globe. However, in order to resolve whether the beneficial effect is shaped enough by the live form of *L. fermentum* LMEM22 strain of curd origin and/or the bacteriocin produced by this LAB further studies (animal model/clinical trials) are warranted for global usage.

Declarations

Conflicts *of interest:* Authors declared that they have no conflicts of interests.

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Author's contributions: Debashis Halder: performed experimental work; Manisha Mandal: designed the study, standardized the protocols, co-wrote; Nishith Kumar Pal: designed the study, identified the target pathogenic bacteria; Shyamapada Mandal: designed the study, standardized the protocols, wrote and discussed the paper.

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Tables

Table 1 Stressors tolerance of *L. fermentum* LMEM22 stain

Physiological stressors	Level of stressors exposed	log_{10} CFU/mL at different hours of incubation				
		0	2	4	6	24
pH	2.0	5.699	7.736	7.619	7.761	7.968
	3.0	5.699	7.806	7.829	7.952	8.085
	4.0	5.699	7.848	7.867	7.92	8.255
Bile salts (%)	0.125	5.699	8.049	8.118	8.204	8.38
	0.25	5.699	7.928	7.968	7.997	8.304
	0.5	5.699	7.903	7.92	7.982	8.267
NaCl (%)	2	5.699	8.043	8.061	8.091	8.476
	4	5.699	8.024	8.043	8.085	8.338
	6.5	5.699	8.003	8.024	8.067	8.331

CFU: colony forming unit

Table 2 Effect of enzymes on the antibacterial activity *L. fermentum* LMEM22 bacteriocin

Treatment against gram-positive bacteria	*E. coli*		*A. baumannii*	
	ZDI (mm)	% activity	ZDI (mm)	% activity
Proteinase K	16	76.19	20	86.96
Trypsin	16	76.19	20	86.96
α-amylase	18	85.71	22	95.65
Control	21	100	23	100
Treatment against gram-positive bacteria	*S. aureus*		*L. monocytogenes*	
	ZDI (mm)	% activity	ZDI (mm)	% activity
Proteinase K	20	83.33	12	70.59
Trypsin	21	87.5	13	76.47
α-amylase	23	95.83	16	94.18
Control	24	100	17	100

ZDI: zone diameter of inhibition

Figures

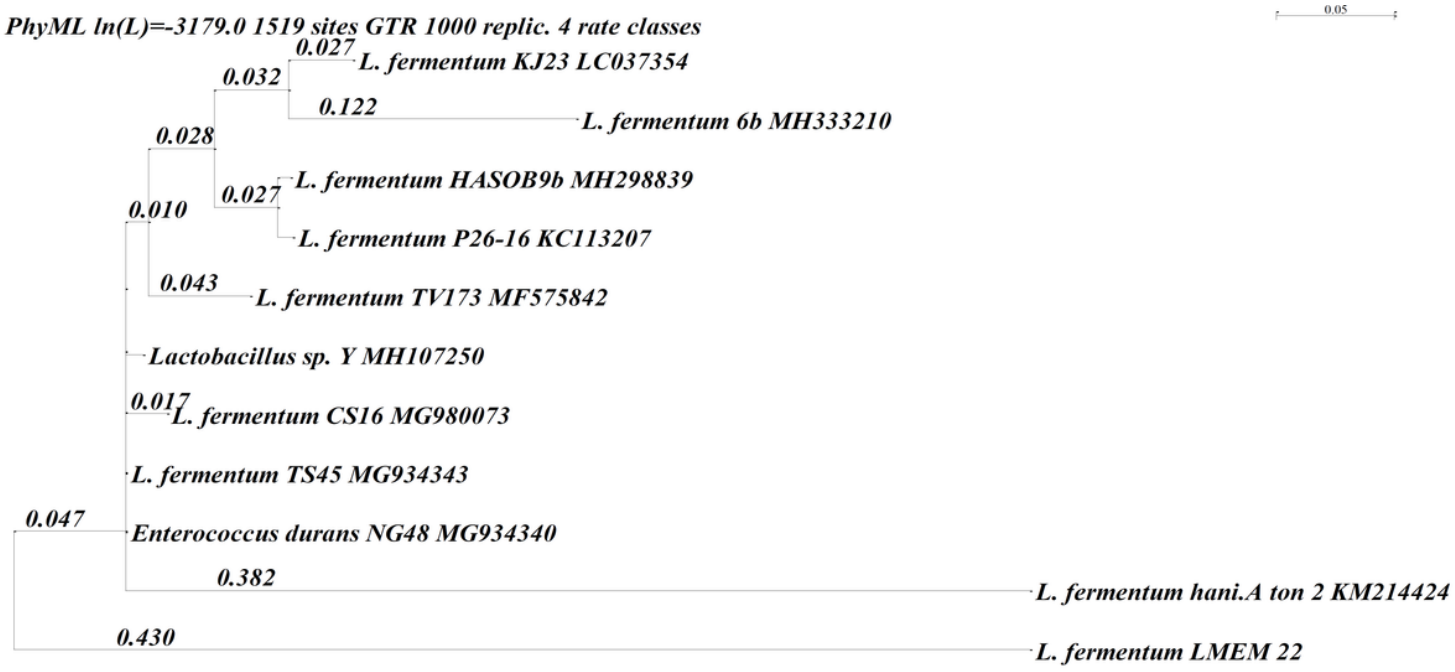

Figure 1

The 16S rRNA gene sequence based phylogenetic tree for Lactobacillus fermentum LMEM22 strain compared with the sequences of closely related reference bacterial strains retrieved from NCBI GenBank database.

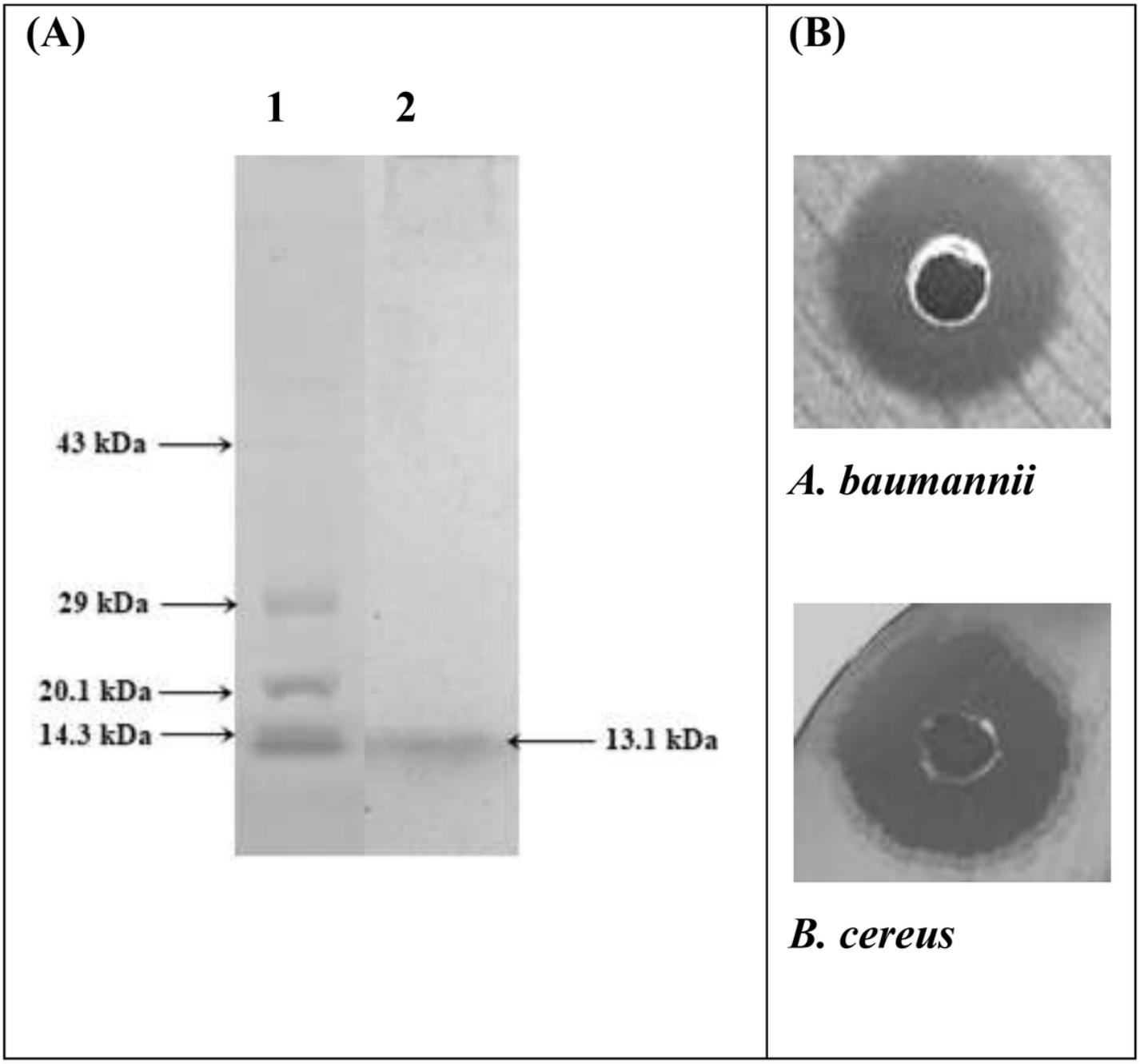

Figure 2

Molecular mass approximation and antibacterial activity of bacteriocin isolated from L. fermentum LMEM22. (A) SDS-PAGE image: lane 1: protein molecular weight standards; 2: L. fermentum LMEM22 bacteriocin, (B) L. fermentum LMEM22 bacteriocin antibacterial activity against A. baumannii and B. cereus by agar-well diffusion.

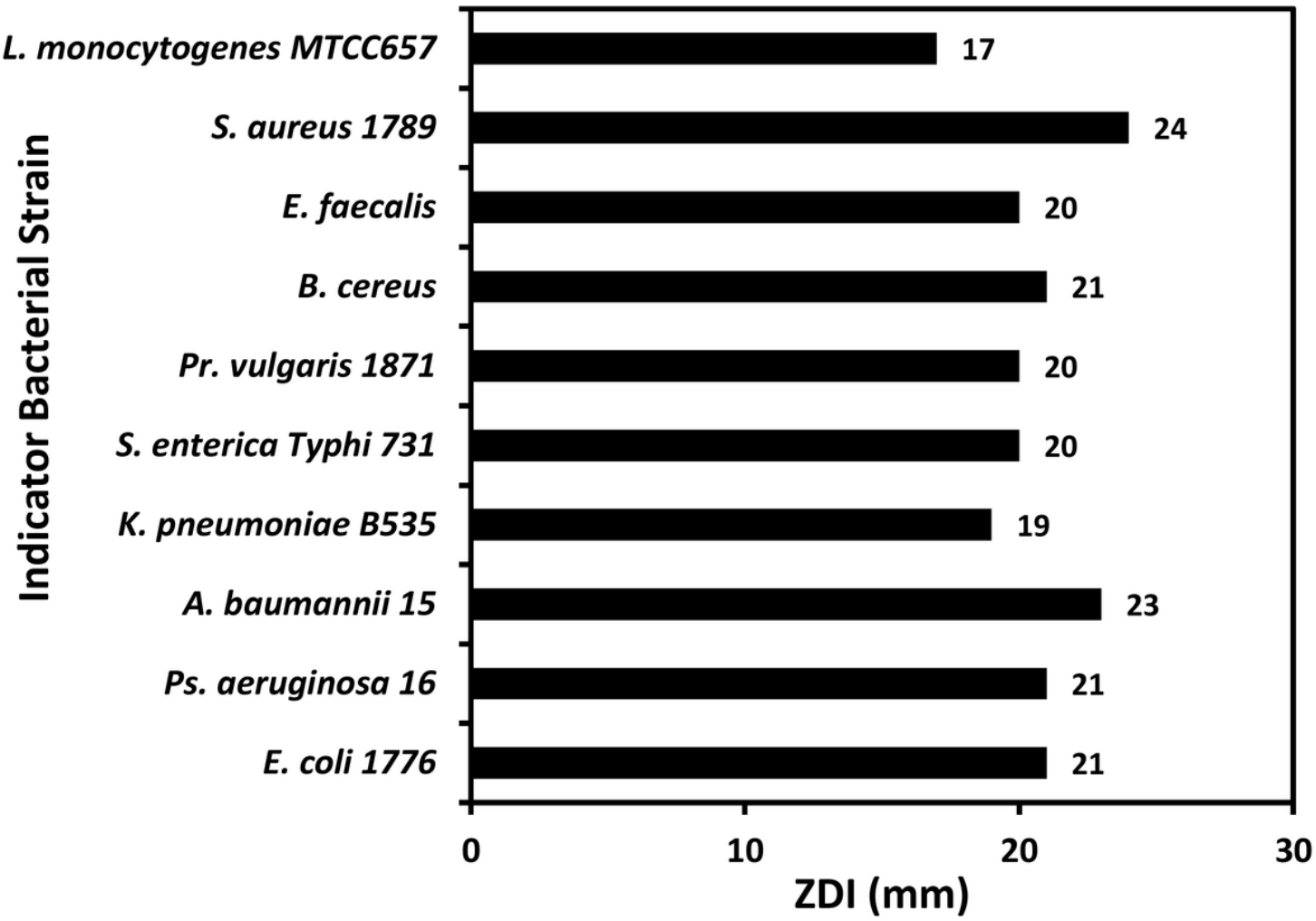

Figure 3

Agar-well diffusion test results of L. fermentum LMEM22 bacteriocin action against indicator bacterial strains; ZDI: zone diameter of inhibition.

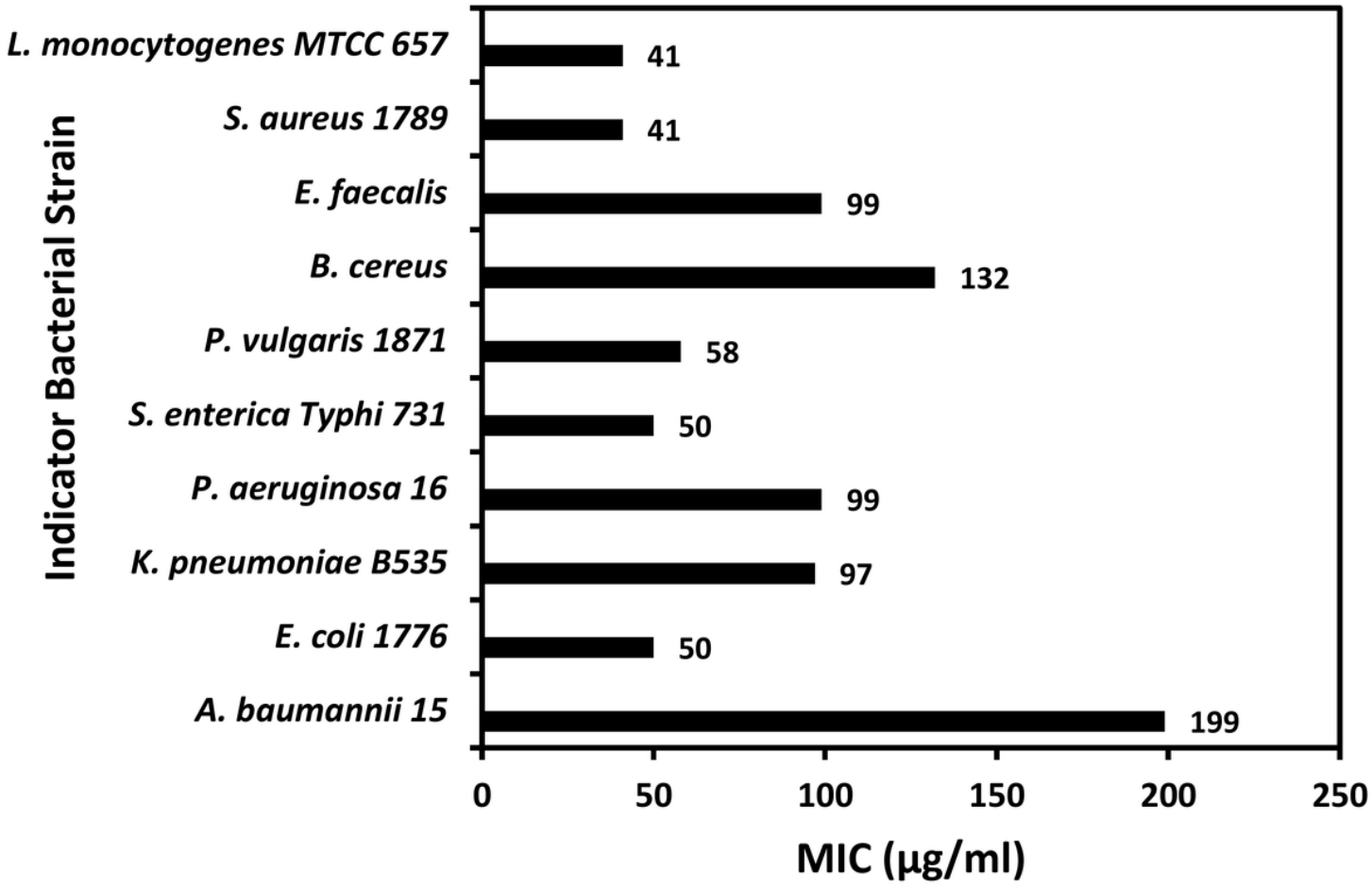

Figure 4

Minimum inhibitory concentration (MIC) values of L. fermentum LMEM22 bacteriocin against indicator bacterial strains.