JOURNAL OF PURE AND APPLIED MICROBIOLOGY, August 2014. Vol. **8**(4), p. 3325-3334

Molecular Characterization of Potential Bacteriocin Producing Lactic Acid Bacteria and Its Efficacy against Food Borne Pathogens

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(Received: 16 November 2013; accepted: 24 December 2013)

A total of 135 samples from 6 different fermented food sources were used to evaluate the presence of bacteriocin producing lactic acid bacteria. 100 strains of lactic acid bacteria isolated from the samples were screened for antibacterial activity against *Staphylococcus aureus* NCIM2079, *Bacillus subtilis* NCIM2106, *Listeria monocytogenes* MTCC 657, *Enterococcus faecalis* MTCC 9845 and *Salmonella typhi* NHRC 001. The potential LAB strains showing activity against pathogenic organisms were identified as *Leuconostoc mesenetroides* VITMS1, *Leuconostoc mesenetroides* VITMS4, *Pediococcus pentosaceus* VITMS7, *Enterococcus mundtii* MVS1 and interestingly MVP5 strain was found to be Uncultured bacterium clone ncd36c11c1. *Leuconostoc mesenetroides* VITMS4, *Pediococcus pentosaceus* VITMS7 showed high antibacterial activity against *Listeria monocytogenes* with 1280 AU/mL and Staphylococcus aureus with 1280 and 640 AU/mL. Suggesting that these strains can be exploited for the production of potential bacteriocins to inhibit food borne pathogens.

Key words: Lactic acid bacteria, Screening, 16S rRNA, Antibacterial activity, Arbitrary units.

Interest in microorganisms as a component of biological diversity has been renewed in recent years. Lactic acid bacteria (LAB) are a group of Gram- positive, non spore forming, catalase negative and fastidious organisms grow under anaerobic condition, cocci or rods which produce lactic acid as the major end product during the fermentation of carbohydrate (Axelsson 2004). LAB are considered as "Generally Recognized as Safe" (GRAS) organisms and do not produce any health risk to man. LAB plays an important role in majority of food fermentation (dairy, vegetable, meat and fish) (Gulahmadov *et al*., 2009).

Many chemicals are being used for inactivation of food borne pathogens so as to preserve food products for longer time. Chemical preservatives show some undesirable side effects and toxic effects on human health (Sharma *et al*., 2006). In using chemical preservatives food borne diseases by food borne pathogenic bacteria occur frequently (Adak *et al*., 2002). A new technique involved for using preservative i.e biopreservatives to overcome the increase demand of consumers for faster, healthier ready-to-eat food without chemical preservatives. Biopreservatives offers the possibility of extending storage life of high quality food stuffs without the use of chemical preservatives (Stoyanova *et al*., 2006). LAB is well known for their production of peptides and proteins with antimicrobial proteins, known as bacteriocins. Bacteriocins caught much attention to researchers and industries as biopreservatives. Bacteriocins produced by lactic acid bacteria have received considerable attention in recent years, because of their possible use as biopreservatives in food processing industry with resultant reduction in the use of chemical preservatives (Van Reenen *et al*., 1998). Bacteriocins produced by these probiotic LAB use as a biopreservatives alter

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the composition of the gastrointestinal .flora. Bacterial pathogens like *E. coli*, *Bacillus subtilis*, *Salmonella typhi*, *Listeria monocytogenes*, *Enterococcus faecalis* and *Staphylococcus aureus* are inhibited by bacteriocin producing LAB. Research on antimicrobial substances, mainly bacteriocins, produced by LAB, has led to consideration of their use as natural preservatives in meat products (Castellano *et al*., 2004) .

The aim of the present research is to: (i) Screen as large as possible number of bacteriocin producing LAB active against bacterial pathogens; (ii) Identify the potential isolates by biochemical and molecular characterization; (iii) Calculate bacteriocin activity in arbitrary units (AU/mL) for the potential strains against *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus faecalis.*

MATERIALS AND METHODS

Samples

Sauerkraut (S) =15 samples, rice flour (RF) = 20 samples, cow milk (CM) = 25 samples, curd (C) =25 samples, milk cream (MC) =25 samples and mixed vegetable pickle (MVP) =25 samples were collected from the local market of Vellore, Tamil Nadu. The collected samples were stored at 4ºC for up to a maximum of 24 h before it was subjected for microbiological analysis.

Isolation of bacteriocin producing LAB strain

For the isolation of LAB, each sample were aseptically weighed and homogenized for 2-5min. After homogenization, serial dilutions were prepared in ¼ Ringer's solution upto 10^{-7} for the enumeration of LAB on deMan-Rogosa –Sharpe (De Man *et al*., 1960) (Difco, Detroit, U.S.A) agar. The plates were incubated at 37º C for 24 h. Based on distinct morphology in color, size, elevation, shape (Greco *et al*., 2005; Bucio *et al*., 2006; Itoi *et al*., 2008) bacterial colonies were randomly picked from plates of all the samples containing 5-100 LAB isolates and subjected for initial identification by gram's reaction, catalase production. Pure culture maintained on MRS agar and stored at -20º C in MRS broth with 20% (W/V) glycerol for further use (Mathara *et al*., 2004).

Target organism

To study the antibacterial activity of bacteriocin from Lactic acid bacteria, the target bacterial strains used were *Listeria monocytogenes* MTCC 657, *Enterococcus faecalis* MTCC 9845, *Staphylococcus aureus* NCIM 2079 *Bacillus cereus* NCIM 2106 *Escherichia coli* NCIM 2931, and *Salmonella typhi* NHRC. All the test organism were grown in Tryptic soy broth supplemented with yeast extract (TSB+YE) at 30º C for 24 h. The strains were stored at -20º C in TSB broth containing 20% (W/V) glycerol, and subcultured twice before use in assays.

Bacteriocin bioassay

Screening isolates for antimicrobial activity by well diffusion assay

The antibacterial activity of selected bacterial isolates was tested against the 5 bacterial pathogens by a method described previously by (Tomé *et al*., 2006). All the Lactic acid bacterial cultures were grown in MRS broth at 37ºC for 24 h. Culture broths were centrifuged at 10,000 rpm for 20min, at 4º C. The pH of the cell free supernatant was adjusted to 6.5 with 1N NaOH to eliminate organic acids interruption. Then the pH adjusted cell free supernatant was treated with catalase 0.1mg/ml concentration and incubated at 37ºC for 1h and filter sterilized by 0.22µm membrane filter to eliminate hydrogen peroxide interruption. Wells were created on the TSB+YE agar plates and 100 ¼l of LAB culture supernatant obtained was added to each well and the plates were refrigerated for 4h to allow the radial diffusion assay of the compound contained in the supernatant prior to incubation for 24h at 37º C. The plates were examined for the presence or absence of clear zone of inhibition (mm) of the target organism and presented as + ve or - ve.

Characterization and Identification of Bacterial Isolate

Strains showing potential activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus faecalis* in initial screening were subjected to biochemical characterization and molecularly indentified using 16s r RNA gene sequence .

Biochemical Characterization

The growth of selected bacterial isolates at different temperature (10ºC, 37ºC and 45ºC) and pH (4.5 and 6.5) were determined by measuring the turbidity after 24h of incubation. All strains were also tested for salt tolerance, acid and gas production from glucose, Esculin hydrolysis, NH_3

production from arginine and Dextran production (Samelis *et al*.,1994), also tested for fermentation of D-xylose, galactose, D-fructose, glucose, lactose and mannose(Tserovska *et al*., 2002).

Molecular identification

16S r RNA Gene Amplification and Sequencing

16S r RNA gene sequence of LAB was amplified using forward primer as 5' - CWG RCC TAN CAC ATG SAA GTC - 3' and reverse primer as 5' - GRC GGW GTG TAC NAG GC - 3' and 16S r RNA gene sequences was characterized at (Amnion Bio Sciences Pvt. Limited) and the aligned sequence were compared with GenBank nucleotide database with BLAST (Altschul *et al*., 1990). Sequences were deposited at the GenBank database and under EMBL (European Nucleotide Archive). The 16s r RNA gene sequences determined were aligned along with the sequences of the type strains obtained from the GenBank using the CLUSTAL W program version 2.1(Chenna *et al*., 2003). A phylogenetic tree was constructed by neighbor- joining method (Saitou and Nei 1987) with the programme Tree view (Version 1.6.6).Individual branches in phylogenetic tree were determined by bootstrap analysis based on 1000 samplings (Felsenstein 1985).

Bacteriocin activity assay

Arbitrary units (AU) of bacteriocin activity were calculated according to Yamamoto *et al*., 2003. Cell-free supernatant was serially diluted two fold with phosphate buffer (10mM, pH 6.5). Solidified TSB+YE agar plates were swabbed with the overnight culture of target strain. After 30 min of drying, wells were made using the sterile borer and 10µl of each diluted sample was added. The plates were incubated at 37ºC for 24 h and the titer was defined as the reciprocal of the highest dilution (2n) that resulted in inhibition of the indicator lawn. Thus, the arbitrary unit (AU) of antibacterial activity per milliliter was defined as

$$AU/mL = \frac{2^{\circ} \times 1000ul}{10\mu l}$$

Where N= dilution number with smallest zone of inhibition.

RESULTS AND DISCUSSION

Isolation of bacteriocin producing LAB strains

In the present research work, potential bacteriocin producing lactic acid bacteria were isolated from the different sources such as sauerkraut, cow milk, curd, milk cream, rice flour

Table 1. Characterization of isolates based on morphology

S. No	Sample No	Gram's Reaction	Shape
1	MSS01	Gram +ve	Cocci in chains,pairs,tetrads
2	MSS02	Gram +ve	Bacilli in long rods
3	RFVS01	Gram +ve	Cocci in chains,pairs
4	RFVS02	Gram +ve	Cocci in chains,pairs,tetrads
5	CMVS01	Gram +ve	Bacilli in single long rods
6	CMVS02	Gram +ve	Bacilli in short rods, pairs
7	CMVS03	Gram +ve	Bacilli in single long rods
8	CVS01	Gram +ve	Bacilli in long rods, chains
9	CVS02	Gram +ve	Bacilli in short rods
10	MVPVS01	Gram +ve	Cocci in chains
11	MVPVS02	Gram +ve	Cocci in chains,pairs
12	MVPVS03	Gram +ve	Bacilli in long rods, chains
13	MVPVS04	Gram +ve	Cocci in chains, pairs, tetrads
14	MVPVS05	Gram +ve	Cocci in pairs, tetrads
15	MVPVS06	Gram +ve	Cocci in pairs
16	MVPVS07	Gram +ve	Cocci in chains, pairs, tetrads
17	MCVS01	Gram +ve	Bacilli in short rods
18	MCVS02	Gram +ve	Bacilli in long rods
19	MCVS03	Gram +ve	Bacilli in long rods
20	MCVS04	Gram +ve	Cocci in chains, pairs, tetrads
21	MCVS05	Gram +ve	Bacilli in single long rods
22	MCVS06	Gram +ve	Cocci in chains, pairs

and mixed vegetable pickle. Preliminary 100 bacterial strains were isolated and named. Predominantly 30 isolates (named MVPVS01- 30) were found in mixed vegetable pickle followed by the less relative abundance of bacteria in milk cream with 26 isolates (named MCVS01-26), cow milk with 15 isolates (named CMVS01-15), rice flour (named RFVS01-12) and curd (named CVS01-12) with 12 isolates. Comparatively less bacteria were found in sauerkraut with 5 isolates (namedMSS01-05).These isolates were selected based on their differences in colony morphology, color, elevation and shape given in Fig.1.Out of 100 bacterial strains 22 strains were characterized as lactic acid bacteria based on the catalase and Grams reaction. All the 22 isolates were found to be catalase negative. In which 11 Strains were found to be Gram positive cocci in chains, pairs and 11 strains showed Gram positive bacilli given in Table.1. From the results it was evident that high incidence of bacteriocin producing lactic acid bacteria in mixed vegetable pickle and milk products as reported by (Jie *et al*., 2012; Rodriguez *et al*., 2000).

Screening isolates for antibacterial activity by well diffusion assay

The isolates were screened for their antibacterial activity against *L. monocytogenes*, *S. aureus*, *E. faecalis*, *B. subtilis*, and *S. typhi* given in Table.2. Isolates MSS01, MVPVS04, MVPVS05, MVPVS07 and MCVS04 showed high activity against major food borne pathogens *L. monocytogenes*, *S. aureus*, *E. faecalis* and *B subtilis* given in Fig.2. Mayr- Harting *et al*., 1972 have shown that 128 lactic acid bacteria tested for antimicrobial activity and 12 strains were proved to be active against target organisms. Thus in our study, out of 22 strains 5 strains showed good activity against most of the target organism used. Only 2 isolates (RFVS2, MVPVS06) were active against Gram negative bacteria *S. typhi* and 7 isolates showed no activity against any of the target bacteria used. At the end of the screening process, based on the ability to antagonize most of the pathogens used in the assay MSS01, MVPVS04, MVPVS05, MVPVS07 and MCVS04 were selected for further studies.

Table 2. Screening of isolates for Antibacterial activity by Well diffusion assay

S. No	Sample No	Activity against the test organism				
		L. monocytogenes	*S. aureus*	*E. faecalis*	*B. subtilis*	*S. typhi*
1	MSS01	+++	++	++	+	-
2	RFVS01	-	+	+	+	-
3	MSS02	-	-	-	-	-
4	RFVS02	-	++	+	+	++
5	CMVS01	-	-	-	-	-
6	CMVS02	-	-	-	-	-
8	MVPVS01	-	++	+	++	-
9	MVPVS02	-	+	+	++	-
10	MVPVS03	-	+++	+	+	-
11	MVPVS04	+++	+++	++	++	-
12	MVPVS05	++	-	+	-	-
13	MVPVS06	-	++	+	++	+
14	MVPVS07	++	++	-	++	-
15	MCVS01	+	-	+	-	-
16	MCVS02	-	-	+	-	-
17	MCVS03	-	+	+	+	-
18	MCVS04	+++	+++	++	++	-
19	MCVS05	-	-	+	-	-
20	MCVS06	-	-	+	+	-
21	CVS01	-	-	-	-	-
22	CVS02	-	-	-	-	-

+++ = Diameter of zone of inhibition >5mm
++ = Diameter of zone of inhibition>3mm
+ = Diameter of zone of inhibition < 2mm
- = No zone of inhibition

Characterization and Identification of bacterial isolates

Biochemical Characterization

All the 5 bacterial isolates were found to be Gram+ve cocci, catalase negative, produce acid with gas production from glucose also grown luxuriously at 10ºC, 37 ºC as well as pH 6.5 but less growth was found at 45 ºC. Only MCVS04 grown very well at pH 4.5 and had the ability to tolerate 2%, 4% but comparatively less growth at 6% salt concentration.

The isolates MVPVS04, MCVS04, had the ability to ferment glucose, galactose, lactose, also utilized citrate as sole source of carbon, failed to hydrolyze arginine, and dextran production was found to be negative. For MVPVS07 arginine and esculin hydrolysis was found to be positive. MSS01 also utilized esculin and had the ability to ferment

Table 3. Morphological and Biochemical characterization of potent bacteriocin producing strains

Characters Gram stain reaction	MVPVS04 +ve cocci	MVPVS05 +ve cocci	MVPVS07 +ve cocci	MCVS04 +ve cocci	MSS01 +ve cocci
Catalase activity	-	-	-	-	-
Acid production from glucose	+	+	+	+	+
Gas production from glucose	+	+	-	+	+
NH_3production from arginine	-	-	+	--	+
Esculin hydrolysis	-	-	+	-	+
Dextran production	-	-	-	-	-
Growth at temperature					
10ºC	+	+	+	+	+
37 ºC	+	+	+	+	+
45 ºC	-	-	+	-	-
Growth in a medium with Nacl 2%	-	-	-	+	-
4%	-	-	-	+	-
6%	-	-	-	W+	-
Growth at pH4.5	-	-	-	+	-
6.5	+	+	+	+	+
Carbohydrate utilization pattern of bacteriocin producing LAB isolates					
D-Xylose	-	-	W+	-	+
Galactose	+	+	+	+	+
Fructose	-	-	+	-	-
Glucose	+	+	+	+	-
Lactose	+	+	-	+	+
Mannose	-	-	+	-	+
Citrate Hydrolysis	+	+	-	+	-

W+ - Weak growth.

Table 4. Activity spectra of bacteriocin produced by lactic acid bacterial cultures

Indicator Organism	Activity of bacteriocin –producing bacteria (AU/mL)				
	Leuconostoc mesenetroides VITMS4	Uncultured bacterium	*Pediococcus pentosaceus* VITMS7	*Leuconostoc mesenetroides* VITMS1	*Enterococcus mundtii* MVS1
Staphylococcus aureus	640	640	1280	320	320
Bacillus subtilis	320	160	640	0	0
Listeria monocytogenes	1280	0	1280	1280	640
Enterococcus faecalis	160	320	320	80	160

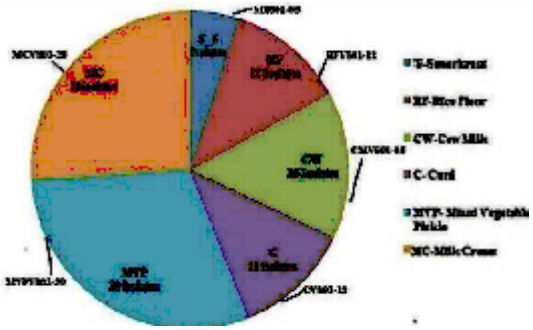

Fig. 1. Relative abundance of LAB isolates from food products

xylose, galactose, glucose, lactose and mannose. Based on the biochemical characterization of the isolates given in Table 4 and comparing the results with the Bergy's manual of systematic bacteriology (Sharpe 1979), the MVPVS04, MCVS04, MVPVS05 were identified as genus *Leuconostoc*, MVPVS07 as genus *Pediococcus* and MSS01 as *Enterococcus*.

Molecular identification of 5 potent bacteriocin producing LAB strain

16s rRNA was exported to the database and checked for homology alignment. Based on

A) B) C) D) E)

Fig. 2. Bacteriocin activity assay A) MVPVS05 against *Listeria monocytogenes*, B) MVPVS07against *Listeria monocytogenes*, C) MVPVS04 against *Listeria monocytogenes*, D) MCVS04 against *Listeria monocytogenes*, E) MSS01 against *Listeria monocytogenes*

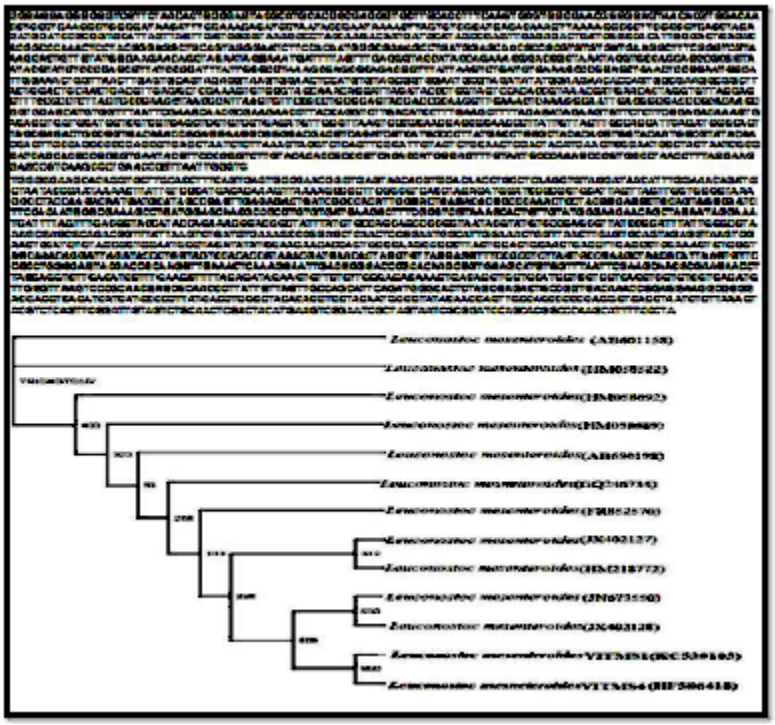

Fig. 3. The neighbor-joining method cladogram showing a phylogenetic relationship between *Leuconostoc mesenetroides* VITMS1, VITMS4 and other related *Leuconostoc mesenetroides* based on the 16s rRNA gene sequence analysis. The numbers at branching point refer to bootstrap values based on 1000 re-samplings. The branch lengths in the cladogram are not to scale

the alignment results MVPVS04 was found as *Leuconostoc mesenetroides*, MVPVS07 as *Pediococcus pentosaceus*, MCVS04 as *Leuconostoc mesenetroides* and MSS01 as *Enterococcus mundtii*. Interestingly one strain MVPVS05 was found as uncultured bacterium clone ncd36c11c1. Based on the 16s rRNA showed 99% similarity with *Leuconostoc mesenetroides*, *Pediococcus pentosaceus* and *Enterococcus mundtii*. The 16s rRNA sequences of representative strains of each group have been submitted to EMBL and Gene Bank Database the accession numbers given were KC539103 for *Leuconostoc mesenetroides* VITMS1, HF586418 for *Leuconostoc mesenetroides* VITMS4, JX123479 for *Enterococcus mundtii* MVS1 and KC692718 for *Pediococcus pentosaceus* VITMS7. Fig.3, 4 and 5 shows the phylogenetic tree based on 16s r RNA sequences of *Leuconostoc mesenetroides* VITMS1, VITMS4, *Enterococcus mundtii* MVS1 and *Pediococcus pentosaceus* VITMS7. Lee, K and Lee, Y, 2010; Tamang *et al*., 2005 suggested the presence of *Leuconostoc mesenetroides, P. pentosaceus, P. acidilactici* in mixed vegetable pickle and milk cream. In our study also we have isolated and identified *Leuconostoc mesenetroides* VITMS1, *Leuconostoc mesenetroides* VITMS4, *Pediococcus pentosaceus* VITMS7 from mixed vegetable pickle and milk cream. Hence it conforms that, these bacteria were predominantly present in mixed vegetable pickle and milk cream which play the vital role in the preservation of food products. Giraffa and Garminati 1997 suggested that *Enterococcus faecalis* and *Enterococcus faecium* were often reported in milk and dairy products where as in our work we have isolated *Enterococcus mundtii* from sauerkraut which proved that these bacteria were widely distributed in other food sources too. Interestingly in molecular characterization one strain (MVPVS05) was found to be unidentified bacteria isolated from mixed vegetable pickle, which has the potential to inhibit the target food borne pathogens like *Staphylococcus aureus*, *Bacillus subtilis*, and

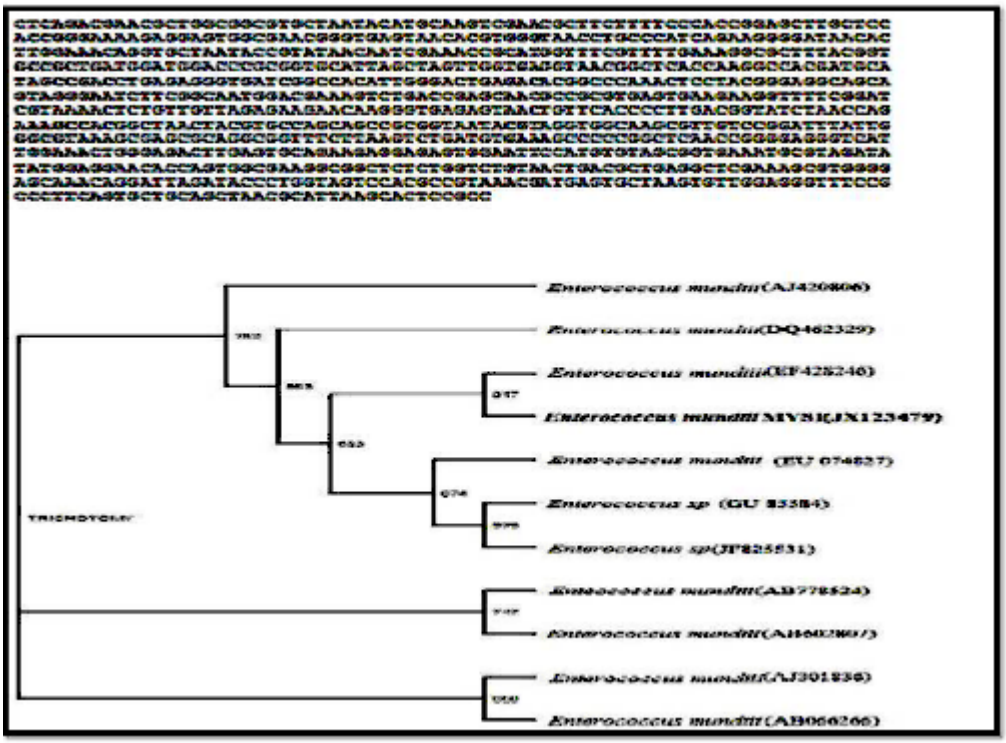

Fig. 4. The neighbor-joining method cladogram showing a phylogenetic relationship between *Enterococcus mundtii* MVS1and other related *Enterococcus mundtii* based on the 16s rRNA gene sequence analysis. The numbers at branching point refer to bootstrap values based on 1000 re-samplings. The branch lengths in the cladogram are not to scale

Enterococcus faecalis. Hence we suspect this organism may be new and novel bacteria for which extensive molecular characterization like RNA-RNA hybridization and other molecular tools to be used for the identification.

Bacteriocin activity assay

Leuconostoc mesenetroides VITMS1, VITMS4, *Enterococcus mundtii* MVS1, *Pediococcus pentosaceus* VITMS7 and MVPVS05 were subjected to bacteriocin activity assay against 4 target organism (*S. aureus, L. monocytogenes, E. faecalis* and *B subtilis*) were given in Fig. 6 and calculated arbitrary units were given in Table 4 which shows that *Leuconostoc mesenetroides* VITMS4, *Pediococcus pentosaceus* VITMS7, *Leuconostoc mesenetroides* VITMS1 showed

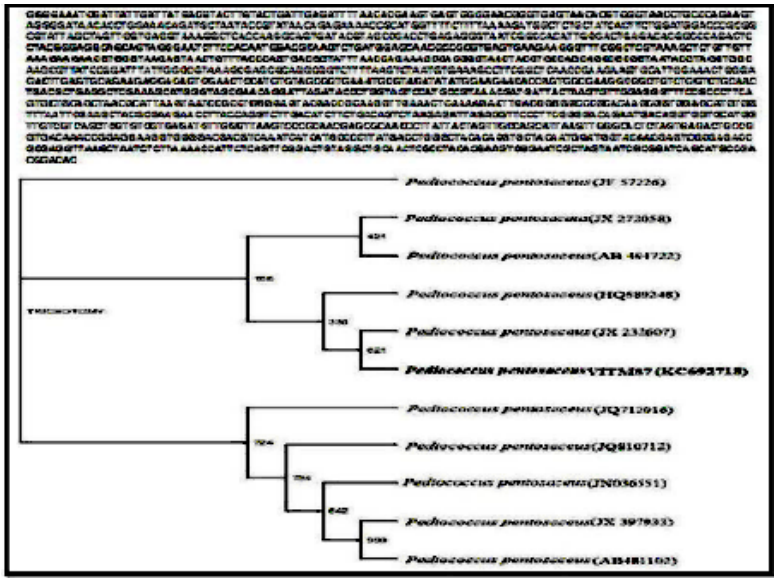

Fig. 5. The neighbor-joining method cladogram showing a phylogenetic relationship between *Pediococcus pentosaceus* VITMS7and other related *Pediococcus pentosaceus* based on the 16s rRNA gene sequence analysis. The numbers at branching point refer to bootstrap values based on 1000 re-samplings. The branch lengths in the cladogram are not to scale

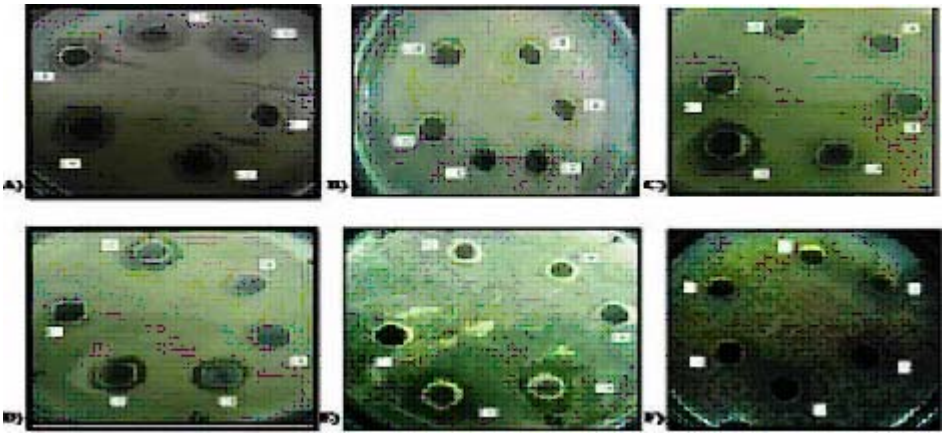

Fig. 6. (A) *Leuconostoc mesenetroides* VITMS4 against *Listeria monocytogenes*, B) *Leuconostoc mesenetroides* VITMS4 against *Staphylococcus aureus*, C) *Leuconostoc mesenetroides* VITMS7against *Listeria monocytogenes*, D) *Leuconostoc mesenetroides* VITMS7against *Staphylococcus aureus*, E) *Leuconostoc mesenetroides* VITMS1 against *Listeria monocytogenes*, F) *Enterococcus mundtii* MVS1 against *Listeria monocytogenes*

maximum activity units of 1280 AU/mL to *L monocytogenes*, and least activity to *L monocytogenes* were found for *Enterococcus mundtii* MVS1with 640 AU/mL. *Pediococcus pentosaceus* VITMS7, *Leuconostoc mesenetroides* VITMS4 showed maximum activity units of 1280, 640 AU/mL to *S aureus*, and least activity to *S aeureus* were found for *Leuconostoc mesenetroides* VITMS1, *Enterococcus mundtii* MVS1 with 320 AU/mL. Compared to *L. monocytogenes* and *S. aureus* the *E faecalis* was the least inhibited organism with maximum 160 AU/mL. Only *Leuconostoc mesenetroides* VITMS4 and *Pediococcus pentosaceus* VITMS7 inhibited *B. subtilis* with 320,640 AU/mL. Therefore our result clearly indicates that *Leuconostoc mesenetroides* VITMS4 and *Pediococcus pentosaceus* VITMS7 has the potential to inhibit the major food borne pathogens like *S. aureus* and *L monocytogenes.* Mayr- Harting *et al*.,1972 have shown that the bacteriocin extracted from LAB showed activity against *L. monocytogenes* and *S. aureus* which was found to be 200, 400AU/ mL. In the present research work the bacteriocin extracted from *Leuconostoc mesenetroides* VITMS4, *Pediococcus pentosaceus* VITMS7 showed very good activity against *L. monocytogenes* and *S. aureus* which was found to be 1280,640 AU/ mL. From this result it was evident that the bacteriocin produced by our strain was more active than the results reported earlier. *S. aureus* and *Listeria sp*. are often present in fresh tissues, because the slaughtering process does not include a bactericidal step. Listeria species have been found in meat and meat products (Johnson *et al*., 1990). The growth of *S. aureus* in foods shows many potential public health hazards, due to enterotoxin produce by many strains of *S. aureus* that cause food poisoning. Meat and meat products are commonly associated with staphylococcal food poisoning (Roberts 1982; Varnam and Sutherland 1995). *Leuconostoc mesenetroides* VITMS4, *Pediococcus pentosaceus* VITMS7 isolated in this present research can be extensively used to combat or mitigate the contamination of the serious pathway to inhibit *L. monocytogenes* and *S. aureus* in the food products.

CONCLUSION

In conclusion, the present research reveals the bacteriocin production potential of *Leuconostoc mesenetroides* VITMS4 and *Pediococcus pentosaceus* VITMS7 to antagonize the major food borne pathogens. Hence, these strains can be exploited in food preservation.

ACKNOWLEDGEMENTS

The authors are grateful to the management of VIT University, Vellore for providing us the facilities to conduct the research work.

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