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Antibacterial Activity of *Leuconostoc lactis* Isolated from Raw Cattle Milk and its Preliminary Optimization for the Bacteriocin Production

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Abstract: Leuconocin, a bacteriocin like inhibitory substance produced by *Leuconostoc lactis* an isolate from fresh raw cattle milk was inhibitory against *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis* and interestingly to the gram-negative species like *Pseudomonas putida*, *E. coli* DH5α and *E.c oli* DH5α with pUC 18 vector. The inhibitory potential was confirmed both by spot assay and cut well agar assay as well with the cell-free supernatant of the test culture in Elliker's broth. MRS broth adjusted to pH to 7.0 and 6.8, respectively produced an inhibitory zone of 15-16 mm against *B. cereus*. This promising wild-type isolate was identified up to a species level by 16S rDNA-based PCR which showed a band at about 692 bp. The set of primer used appeared to be specific as it did not amplify the closely related species. The cell-free supernatant upon concentration by 5 fold (approximately) showed a much stronger biological activity and showed heat stability. This isolate thus appears to be novel as no bacteriocin so far has been reported from *Leuconostoc lactis*. Moreover, the bacteriocin was active against both gram-positive and gram-negative organisms.

Key words: Bacteriocin, bliss, inhibition, species identification, 16S rDNA, PCR

INTRODUCTION

Lactic Acid Bacteria (LAB) are widely used in the dairy industry for the production and preservation of fermented food products and they are well known as dairy starter organisms for their industrially important virtues (Ahn and Stiles, 1990; Daba *et al*., 1991; Roy *et al*., 2001). Amongst the various scions of lactic acid bacteria, the lactococci and leuconostocs are enviably placed for their multifaceted attributes as starter cultures. These food grade organisms primarily find their application in the preparation of fermented foods known for their exotic flavours and well documented health attributes. LAB play a crucial role in preservation and safety of the fermented foods as they inhibit both spoilage and pathogenic microflora (Gupta and Batish, 1992; Daba *et al*., 1991; Guinane *et al*., 2005). Their usefulness has also been realized and established in terms of probiotic and therapeutic potentials in the intestine (Batish *et al*., 1990; Wells and Mercenier, 2008; Ventura *et al*., 2009).

LAB produce a number of antimicrobial compounds including organic acids, hydrogen peroxide and neutral metabolites. Besides producing different acids *viz*., lactic, acetic and propionic, lactic acid bacteria furnish a wide range of inhibitory substances that prolong the time scale of preservation of the fermented products (Salminen *et al*., 1996; Batish *et al*., 1997). In addition to the above mentioned non-

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prtoeinaceous substances many strains of lactic acid bacteria also produce proteinaceous substances which show antagonistic activity against stains or species closely related to the producer. These substances are known as bacteriocins. However, a few bacteriocin and bacteriocin like inhibitory substances (BLISS) do exhibit antimicrobial prowess against a number of distantly related species and thus exert more or, less antibiotic like activity. The proteinaceous nature of these bacteriocins and BLISS implies their degradation in the gastrointestinal tracts of man and animals and this is why, these antimicrobial proteins produced by starter cultures are excellent candidates to improve the safety of various fermented foods (Piard *et al*., 1992; Cotter *et al*., 2005). Most bacteriocins are documented to be antagonistic against the gram positive bacteria. Nisin has been the most extensively studied bacteriocin produced by *Lactococcus lactis* . It is active against gram positive bacteria and appears as several types (A, B, C, D or E) that differ in both amino acid composition and biological activity (Hurst, 1981). Bacteriocins from *Carnobacteria*, *Enterococci*, *Lactococci*, *Leuconostocs* and *Pediococci* and their characteristics have been reviewed (Klaenhammer, 1988; Hastings *et al*., 1991; Biet *et al*., 1999). *Leuconostoc* species are lactic acid bacteria occurring in raw food stuffs fermented dairy products and wine fermentation. *Leuconostoc* species are known to produce bacteriocins that possess favorable characteristics for use as food biopreservatives (Tagg *et al*., 1976; Van Laack *et al*., 1992; Mathieu *et al*., 1993).

This study presents an attempt to identify a promising wild type lactic acid bacterial isolate from fresh raw cattle milk (pH 6.6) at the level of genus and species based on the results of preliminary biochemical tests and 16S rRNA based PCR amplification. This study also reports the inhibitory profile of the bacteriocin leuconocin against a few selected gram positive and gram-negative spoilage and pathogenic organisms and the inhibitory potential at a chosen environmental condition using various media as well as an effect of physical parameters on bacteriocin production.

MATERIALS AND METHODS

Bacterial Cultures and Media

Bacterial strains used in this study are *Leuconostoc* sp. *Staphylococcus aureus, Escherichia coli* DH5α, *Pseudomonas putida and Enterococcus faecalis.* The *Leuconostoc* culture was maintained as frozen stocks held at -80°C in Elliker's Broth (Hi-Media, India) plus 18% sterile glycerol and in case of *E. coli* DHα, *Pseudomonas putida*, *Enterococcus faecalis* and *S. aureus*, BHI broths (Hi-Media) were used. *Leuconostoc* sp. was inoculated to Elliker's broth (pH 7.2) at 1% level and subcultured twice at 30°C before experiments.

Leuconostoc Isolates

The lactic acid bacteria were isolated from fresh raw cattle milk (cattle firm, Dehradun, India) by inoculating at the rate of 5% into yeast extract dextrose acetate medium followed by incubation at 30°C for 24 h. Appropriate dilutions were made from the curdled and the semi-curdled milks and plated on bromo cresol purple lactose agar (McKay *et al*., 1972) followed by incubation at 30°C for 24 h. Isolates tentatively identified as *Lactococci* based on microscopy and sugar utilization along with a reference culture of *L. lactis* subsp. *lactis* was maintained in sterile litmus milk (Peter *et al*., 1986) supplemented with 1% glucose and 0.3% yeast extract.

The isolates were grown in Tryptone dextrose yeast extract (pH 6.50) broth at 30°C/24 h as this favored growth of lactic acid bacteria. The grown culture was serially and appropriately diluted and pour plated on M 17 glucose agar to obtain about 80 to 120 colonies per plate. These colonies were selected randomly with sterile tooth picks and were subjected to replica plating against the indicator organisms namely *Bacillus cereus* and *Staphylococcus aureus.* The molten BHI soft agar (0.7%) medium (7 mL) seeded with 5 µL of an overnight culture of each of the two indicators was overlaid on

each plate. After overnight incubation, the plates were examined for the presence of circular and clear zones of inhibition around colonies of possible bacteriocin producers. The colony that produced a clear zone of inhibition of diameter 12 mm or more against the two indicators were streaked on the MRS medium from the master plate for the purification and subjected to a battery of biochemical tests.

Presumptive Identification

The leuconostoc isolates were further identified by microscopic examination and conducting a series of biochemical and physiological test based on Bergey's Manual of Determinative Bacteriology (Peter *et al*., 1986).

16 S rDNA Based Identification of the Strain by PCR

The total DNA isolated (Pospiech and Neumann, 1995) was subjected to PCR amplification by using the two oligonucleotides designed to identify the species of *Leuconostoc lactis*. The 16 S rDNA sequences of *Leuconostoc lactis*, *L. cremoris* and *L. mesenteroides* were compared. The two oligonucleotide (forward primer URRL -5' AGT GTT GTA GAG GTA AGT GGA ACT C 3') and (reverse primer 5' AGC GAT TCC GAC TTC GTG CAG TC 3') were chosen inside (positions 664 to 688 and 1334 to 1356, respectively) of the 1,499 bp sequence 16 S rDNA of *Leuconostoc lactis*. The PCR was performed at 94°C for 3 min, followed by 30 cycles 94°C/45 sec, 60 sec at 55°C / 60 and 90 sec at 72°C/90 sec and a 7 min extension period at 72°C using the PCR (Perkin-Elmer 2400). The DNA extracted from *Lactococcus lactis* sp. *lactis*, *Leuconstoc mesenteroides* subsp. *mesnteroides* NCIM 2947 and *E.coli* DH5α were also subjected to PCR using same set of primers and programme.

Assay for Antibacterial Activity

The antibacterial activity of the randomly picked up colonies including *Leuconotoc* sp. was determined by following the well agar diffusion method (Batish *et al*., 1990). Thirty milliliter of molten nutrient agar was transferred to the sterile petridish and allowed to solidify. A freshly grown (16 h) culture of *Staphylococcus aureus*, *B. cereus*, *E. faecalis*, *E. coli* DH5α and *Pseudomonas putida* was appropriately diluted in sterile 0.9% saline solution and an aliquot of 100 µL diluted cell suspension was plated on the dry hardened nutrient agar and spread uniformly over the agar surface with a sterile bent glass rod. Plates were dried at 30°C for 1 h and wells of 8 mm diameter were cut. The selected isolates were grown in Elliker's broth (Elliker *et al*., 1956) for 24 h to 72 h at 30°C and the cell free supernatant of each of the isolate was obtained by centrifugation (Hermle, Germany) at 12,000 rpm for 30 min at 4°C. The 100 µL supernatant treated and untreated was added separately into each well. The plates were at kept at 5-6°C/8 h to facilitate diffusion process of cell free supernatant fluid into the agar medium followed by incubation at 37°C for 12 h. The zone of inhibition indicating bacteriostatic activity around each well was measured in millimeter.

Assessment of Different Media for Bacteriocin Production

Different growth media namely MRS (Rogosa *et al*., 1951), Elliker's broth, Chalmer's media, TYD broth, Trypticase soy broth, Yeast glucose broth were dispensed in 10 mL aliquots and inoculated at the rate of 1% with the test culture and incubated at 28°C for 48 h. The cell free supernatant from each growth was prepared as described previously and tested for inhibition against the indicator organism *Staphylococcus aureus* by cut well agar assay.

Effect of Temperature, pH and Period of Incubation

The test organism was inoculated in Elliker's broth in different tubes and the tubes were incubated at 28, 30, 37 and 42°C for 48 h and the supernatants were tested for antibacterial activity against

S. aureus. The impact of pH on bacteriocin synthesis was assessed by adjusting the pHs of the broth media to 5.5, 6.0.6.5, 7.0 and 8.0. Likewise the effect of incubation period was determined by incubating the broth tubes for different time intervals.

Measurement of Growth

The growth was measured in terms of Optical Density (OD) at 620 nm using a spectrophotometer (Jasco-UV Visible, Japan).

RESULTS AND DISCUSSION

Lactic Acid Bacteria (LAB) are a large group of phylogenetically related, lactic acid producing bacteria used primarily in the production of fermented food stuffs. They encompass many genera, including *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Enterococcus*, *Oenococcus* and *Carnobacterium* (Stiles and Holzapel, 1997). The ever increasing use of specialized strains of LAB requires careful attention to strain identification (Cuisick and O'Sullivan, 2000). While classical morphological of biochemical identification methods still have an important role in preliminary strain diagnosis, they are not definite enough. The gram positive cocci isolated from raw cattle milk showed inhibitory activity against some food spoilage causing and pathogenic bacteria when grown in Elliker's broth and MRS broth. Morphological and biochemical tests including sugar fermentation tests (data not shown) indicate that the wild type isolate belonged to genus *Leuconostoc*. However, a few deviations from the normal biochemical characteristics such as delayed and very scanty growth at 6.5% (w/v) NaCl after 24 h, no growth at pH 9.6 and arabinose negative and sorbitol postive characteristics prompted us to verify this promising strain by 16S rDNA based PCR. Therefore, identification of the bacteriocin producing isolate upto species level was confirmed by 16Sr DNA based gene amplification.

The total DNA isolated after the quantitation at 260 nm was subjected to PCR amplification by using the two oligonucleotides designed to identify the species of *Leuconostoc* isolate. Representative results of all the strains tested for PCR specificity with the selected primer are shown in Fig. 1. The wild type strain *Leuconostoc* sp. exhibited a PCR fragment of size 692 bp on 1% agarose gel. No DNA amplification was observed in strain *Lactococcus lactis*, *E . coli* DH5α (Fig. 2) and in other strain of *L. mesenteroides* (NCIM 2947). When the temperature of annealing was reduced from 56.5 to 54.5°C neither any change in the band position was observed nor did any extra weak band appear (Fig. 2). So, the PCR primers used in this study were able to identify as *Leuconostoc lactis* strain.

The test culture *L. lactis* was able to exhibit a comparatively wider spectrum of antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas putida*, *E. coli*. DH5α and *E. coli* DH5α with pUC 18 vector.

Effect of Different Media

The data on the effect of different media on production of bacteriocin by *Leuconostoc lactis* has been compiled in Table 1. The maximum zone of inhibition of 20 mm diameter was recorded when the culture was grown in Elliker's broth. This was followed by MRS broth and TDY broth where the zone diameter of 16 mm each was observed. No activity could be observed in all other media tested. As far as the growth of the organism was concerned the optical densities of 0.22 and 0.21 were obtained in case of Elliker's broth and MRS broth broths, respectively. However in yeast glucose broth where the growth was comparable to Elliker's broth (0.22) no biological activity was found.

This clearly points to the phenomenon that although the biomass buildup is one of the important parameters for the production of the bacteriocin yet the synthesis is also dependent on the other inducing factors of the medium. In case of TDY broth the growth of *L. lactis* was less (OD 0.16) then

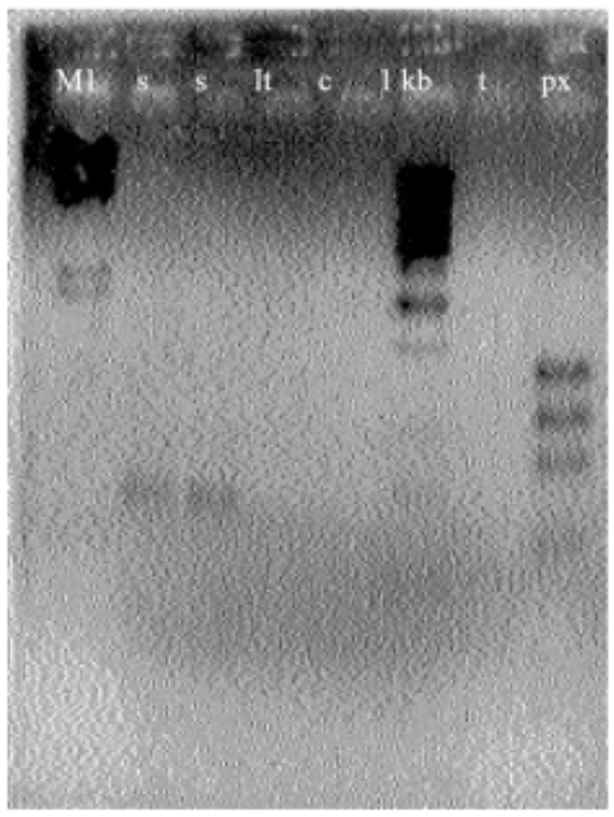

Fig. 1: Lane1: λ Hind III digest, Lane 2 (s): Amplified product l0 µL loaded from 1st PCR, Lane 3(s): Amplified product 10 µL loaded from 2nd PCR from *Leuconostoc mesenteroides*, Lane 4 (lt): No amplification from *Lactococcus lactis* NCIM 2063. Lane 5 (c): No amplification from negative control; Lane 6, 1 kb ladder, Lane 7 (t): No amplification from *E. coli* DH5α Lane (px) ΦX174- Hae-III digest. as a marker

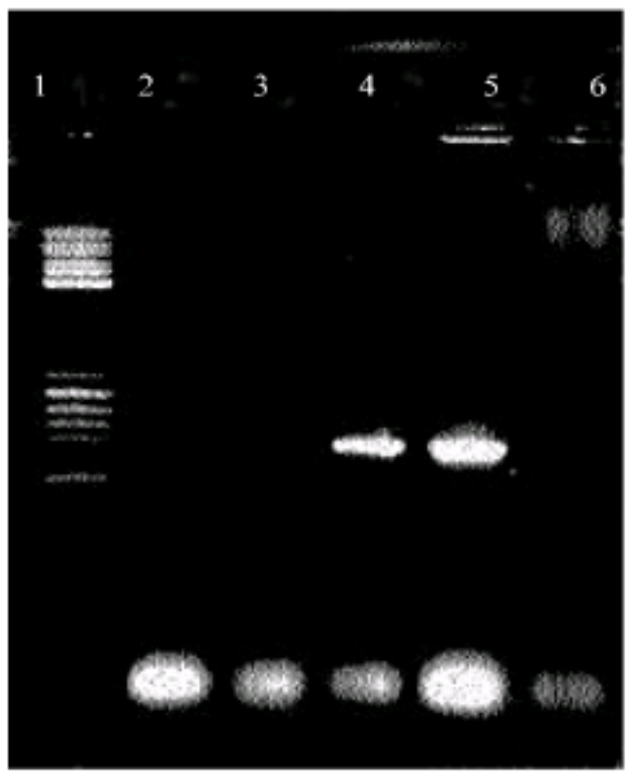

Fig. 2: Lane 1: λ-Hind III digest, no amplification from *Lactococcus lactis* in the lane 2 and 3. The lane 4 and 5 show the PCR products of size 692 bp from *Leuconostoc lactis* and *Leuconostoc mesenteroides* NCIM2947 and c (negative control)

that observed in case of Yeast glucose broth, however, there was moderate bacteriocin synthesis (zone diameter 16 mm) (Table 1). The growth of the test organism in other two media namely Trypticase soy broth and Charmer's medium was poor and no antagonistic activity could be observed. Good

Table 1: Growth and bacteriocin production by *Leuconstic lactis* in different media after 48 h at 26°C

Medium	Growth (OD at 620 nm)	Zone of inibition dia. (mm)	Final pH of the spent media
Elliker's broth	0.22	2	5.20
MRS	0.21	16	5.20
Yeast glucose broth	0.22	No activity	4.90
Tryptone dextrose yeast extract broth (TDY)	0.16	16	5.30
Trypticase soya broth	0.13	No activity	5.80
Chalmer's medium	0.08	No activity	5.30

Data presented after three trials

Table 2: Growth and bacteriocin production by *L. lactis* at 28°C at different time intervals in Elliker's broth

Incubation period (h)	Growth (OD at 620 mm)	Zone of inibition dia. (mm)	Final pH of the spent medium
12	0.10	No activity	5.60
24	0.16	No activity	5.40
36	0.20	14	5.20
48	0.21	20	5.10
60	0.22	20	4.90
72	0.22	16	4.70
96	0.22	-	5.10

bacteriocin synthesis in Elliker's broth, MRS broth and TDY broth indicate that some of the nutritional factors present in these media were lacking in other media and these factor may be present in tryptone or peptone which are one of constituent in the former media. Also it can be notice that the test organism lowered the pH of the spent medium which fell in the range 4.9-5.2 owing to the production of acids. In case of Yeast glucose broth the pH decreased to 4.9 but there was no inhibition of indicator organism thus indicating that inhibition was not due to decrease in pH (Table1).

Since, the maximum synthesis of bacteriocin occurred in Elliker's broth, this medium was further used to study the production of antimicrobial substance at varying time intervals of incubation (Table 2). The data showed that the biological activity was clearly manifested not before 24 h and dependent solely on the cell density and its accumulated metabolites. After 48 h of incubation the culture free supernatant showed a distinct zone of inhibition against *Staphylococcus aureus* (Table 2, Fig. 3). It has also been observed that incubation at 28 and 30°C for 48 h are the most suitable conditions for production of maximum bacteriocin as evident from the Fig. 4. The incubation temperature of 37°C and beyond resulted in drastic reduction in the biological activity after 72 h of growth and at 96 h almost no biological activities were recorded.

Ahn and Stiles (1990) have reported that *Leuconostoc mesenteriodes* isolated from vacuum packaged meat produced a bacteriocin named leucocin A in MRS broth. The results of the present study are in partial agreement with the above findings in the fact that *L. mesenteriodes* also synthesized bacteriocin in MRS broth; however, the improved production was noticed in Elliker's broth. Also the bacteriocin first appeared after 36 h but the peak production occurred after 48 h (Fig. 4). This observation conforms to that of Daba *et al*. (1991) who reported that the biosynthesis of mesenterocin by *L. mesenteroides* took place at early stationary phase. Synthesis of Sakacin A, bacteriocin from *Lactobacillus sake* 706 occurred optimally at 25°C (Schillinger, 1990). Similar results have been reported in an earlier study conducted by Reddy and Ranganathan (1985). In most cases the optimum temperature for growth is also the optimum for bacteriocin synthesis.

The optimum temperature for growth of *Leuconostoc lactis* was between 25 and 28°C where growth absorbance at 620 nm was 0.20 to 0.21. The maximum production of bacteriocin occurred at 28°C where a zone diameter of 21 mm was obtained (Fig. 1). However, no significant difference was observed at 25 and 30°C where zones of diameter of 20 mm in each case were observed. Initial pH of the medium had an effect on growth of the test organisms as well as on bacteriocin production. Appreciable bacteriocin synthesis occurred in media with initial pH in a range of 6.0 to 7.0, with maximum production at pH 6.5 to 7.0 where a zone diameter of 21 mm against the indicator organisms

Fig. 3: The inhibition zone produced by cell free supernatant from the test culture *Leuconostoc lactis* after 48 h of incubation in Elliker's broth. The indicator organism is *Staphylococcus aureus*

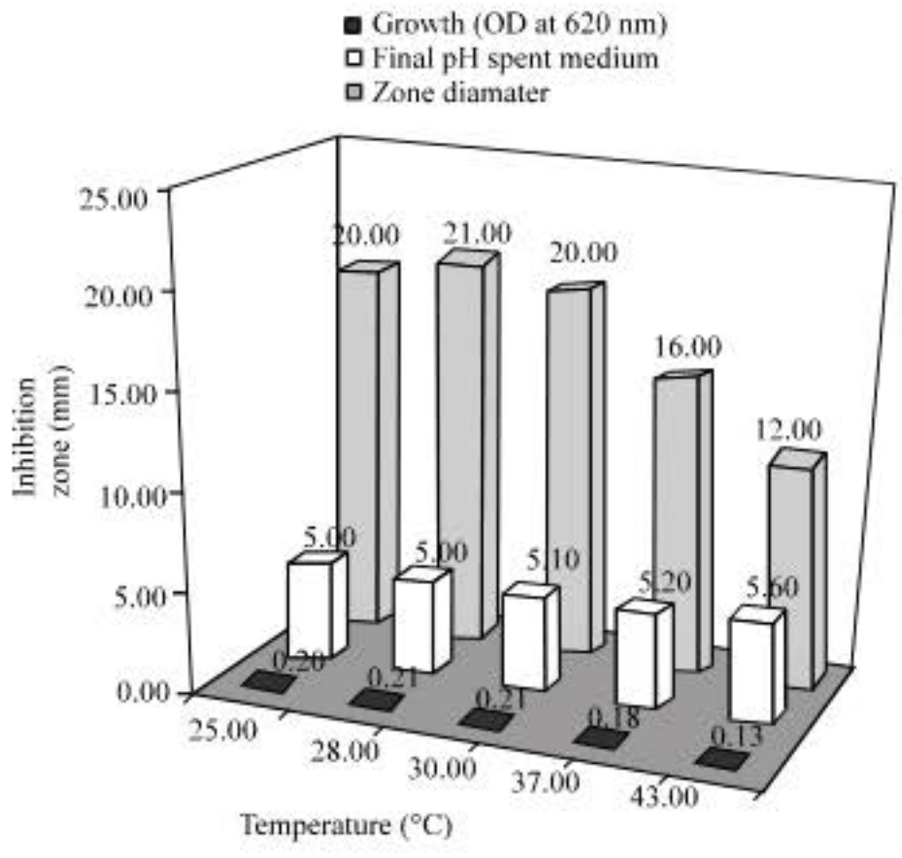

Fig. 4: Growth of becteriocin by *Leuconocstoc lactis* at different temperature incubation in Elliker's broth (initial pH of medium, 7.2; incubation period 48 h)

were recorded (Fig. 5). The bacteriocin production was drastically reduced at the initial of pH 5.5 and 8.0 with zone diameter of 12 and 14 mm, respectively (Fig. 5). This may be due to slower growth rates of the organisms at these suboptimal pH values. These results are similar to those reported by Van Laack *et al.* (1992) who observed that maximum synthesis of carnosin, a bacteriocin produced by *Leuconostoc carnosum* LA44 A occurred at initial pH of 6.50 and temperature 25°C.

The novelty this organism claims is that it has the potential to inhibit the gram negative organisms like *Pseudomonas putida* and *E. coli* DH5α apart form inhibiting S. *aureus, B. cereus* and *E . faecalis*. The incubation period of 48 to 72 h at 30°C confirm to be optimum for the demonstration

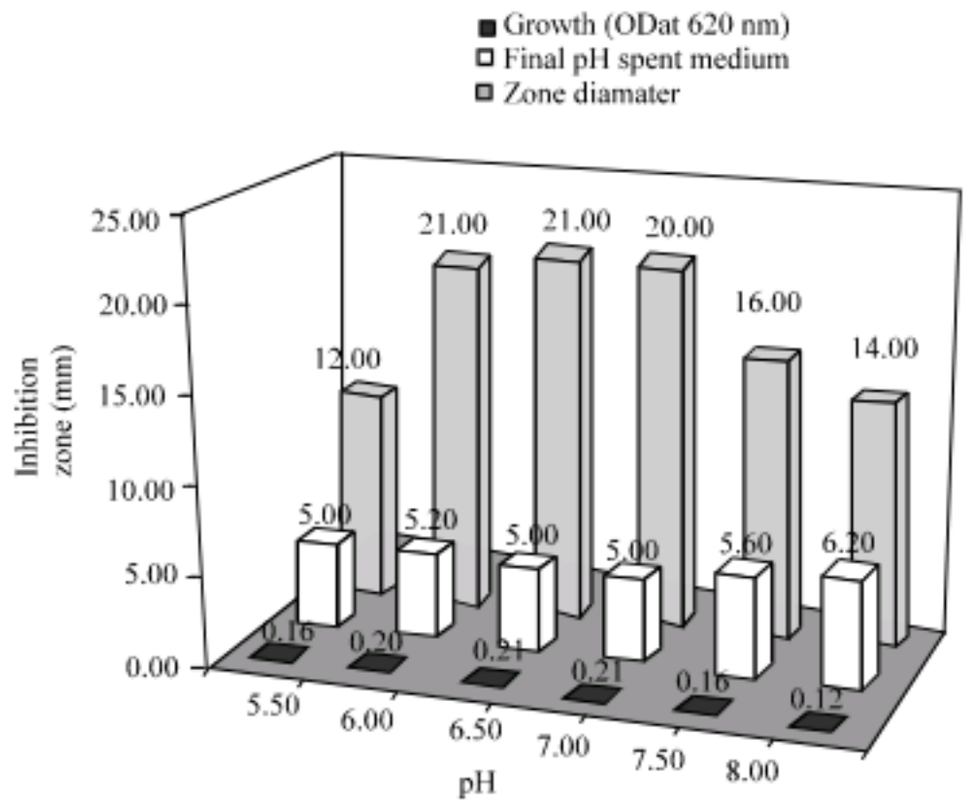

Fig. 5: Effect of different initial pH of the medium on growth and bactorian by *Leuconocstoc lactis* Elliker's broth (temperature 28°C and incubation period 48 h)

Table 3: Cell free supernatant obtained after hour at 30°C. Zone of inhibition (mm) on nutrient agar plates selete after 16 h of incubation at 37°C

Incubation period (h)	*B. cereus*	*E. coli* DH5α	*E. coli* DH5α pUC18	*Pseudomonas putida*
24	15	15	14	11
48	18	17	17	12
72	18	17	17	12
96	Negligible	Negligble	Negligible	Negligible
120	-	-	-	-

-: Indicates no biological activity detected

of its bacteriocinogenic potential. The concentrated supernatant (approximately five times), pH 6.6 and heat-treated at 55 and 63°C for 60 min also showed unequivocally the strong inhibition against all the indicator organisms used in the present study (Table 3). A few bacteriocin have been reported from *Leuconostoc* species namely mesenteriocin 5, leuconocin S, leucocin A and carnocin which are produced by *L. mesenteroides*, *L. paramesenteroides*, *L. gelidium* and *L. carnosum*, respectively (Klaenhammer, 1988; Nettles and Barefoot, 1993). These bacteriocins have been reported to inhibit lactic acid bacteria, *Listeria monocytogenes* and a few Gram positive and Gram negative bacteria. The bacteriocin could show activity against some potential spoilage causing and pathogenic Gram positive and Gram negative bacteria. However, further investigations on its antibacterial spectrum, physico-chemical characteristics and genetic determinants are required in order to assess its full potential as food preservative. The data altogether indicate the protein purification and its stability study in detail and it's over expression in an appropriate vector may give us a good food-grade bacteriocin for food and feed biopreservation.

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REFERENCES

Ahn, C. and M. Stiles, 1990. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. J. Applied Bacteriol., 60: 302-310.

Batish, V.K., L. Ram and S. Grover, 1990. Studies on environmental and nutritional factors on production of antifungal substance by nutritional factors on production of antifungal substance by *Lactobacillus acidophilus* R. Food Microbiol., 7: 199-206.

Batish, V.K., U. Roy, L. Ram and S. Grover, 1997. Antifungal attributes of lactic acid bacteria. A review. Crit. Rev. Biotechnol., 17: 209-225.

Biet, F., Y. Cenatiempo and C. Fremaux, 1999. Characterization of pFR18, a small cryptic plasmid from *Leuconostoc mesenteroides* ssp. *mesenteroides* FR52 and its use as a food grade vector. FEMS. Microbiol. Lett., 179: 375-383.

Cotter, P.D., C. Hill and R.P. Ross, 2005. Bacteriocins: Developing innate immunity for food. Nat. Rev. Microbiol., 3: 777-788.

Cuisick, M.S. and D.J. O'Sullivan, 2000. Use of a single, triplicate arbitrarily primed-pcr procedure for molecular fingerprinting of lactic acid bacteria. Applied Environ. Microbiol., 66: 2227-2231.

Daba, H., J.F. Pandian, R.E. Gooselin, J. Simiard Huang and C. Lacroix, 1991. Detection and activity of bacteriocin produced by *Leuconostoc mesenteroides*. Applied Environ. Microbiol., 57: 350-355.

Elliker, P.R., A. Anderson and G.H. Hannesan, 1956. An agar culture medium for lactic acid streptococci and lactobacilli. J. Dairy Sci., 39: 1611-1612.

Guinane, C.M., P.D. Cotter, C. Hill and R.P. Ross, 2005. Microbial solutions to microbial problems: Lactococcal bacteriocins for the control of undesirable biota in food. J. Applied Microbiol., 98: 1316-1325.

Gupta, R.K. and V.K. Batish, 1992. Genetic evidence for plasmid encoded lactoccin production in *Lactoccus lactis* subsp. *lactis* 484. Curr. Microbiol., 24: 231-238.

Hastings, J.W., M. Sailer, K. Johnson, K.L. Ray, J.C. Vederas and M.E. Stiles, 1991. Characterization of leuconocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidium*. J. Bacteriol., 173: 7491-7500.

Hurst, A., 1981. Nisin. Adv. Applied Microbiol., 27: 85-123.

Klaenhammer, T.R., 1988. Bacteriocins of lactic acid bacteria. Biochimie, 70: 337-349.

Mathieu, F., I.S. Suwandhi, N. Rekhif, J.B. Milliere and G. Lefebvre, 1993. Mesenterocin 52, a bacteriocin produced by *Leuconostoc mesenteroides* ssp, *mesenteroides* FR 52. J. Applied Bacteriol., 74: 372-379.

McKay, L.L., K.A. Baldwin and E.A. Zottola, 1972. Loss of lactose metabolism in lactic streptococci. Applied Microbiol., 23: 1090-1096.

Nettles, C.G. and S.F. Barefoot, 1993. Biochemical characteristics of bacteriocins of food associated lactic acid bacteria. J. Food Prot., 50: 338-356.

Peter, N.H.A.S., N.S. Mair, M.E. Sharpe and J.G. Holt, 1986. Bergey's Manual of Determinative Bacteriology. Vol. 2, Williams and Wilkins, Baltimore.

Piard, S.C., P.M. Muriana, M.J. Desmazeaud and T.R. Klainhammer, 1992. Purification and partial charcterization of lactacin 481, a lanthionine containing bacteriocin produced by *Lactococca lactis* subsp. lactis NRT481. Applied Environ. Microbiol., 58: 279-284.

Pospiech, A. and B. Neumann, 1995. A versatile quick-prep of genomic DNA from Gram-positive bacteria. Trends Genet., 11: 217-218.

Reddy, N.S. and B. Ranganathan, 1985. Effect of time, temperature and pH on growth and production of antimicrobial substance by *S. lactis* sub sp. *diacetylactis* S1-67-C. Milchwissenschaft, 40: 346-347.

Rogosa, M., J.A. Mitchell and R.F. Wiseman, 1951. A selective medium for the isolation and enumeration of oral and feacal Lactobacilli. J. Bacteriol., 62: 132-133.

Roy, U., S. Grover, S. Neelakantan and V.K. Batish, 2001. Mode of inhibition of *Apergillus flavus* IARI antifungal substance produced by *Lactococcus lactis* sub sp. lactis CHD28). J. Food Sci. Technol., 38: 489-492.

Salminen, S., M. Laine, A. Wright von, J. Vuopio-Varkilb, T. Korhonen and T. Mattila-Sandholm, 1996. Development of selective criteria for probiotic strains to assess their potential in functional foods: A Nordic European approach. Biosci. Microflora, 15: 61-67.

Schillinger, U., 1990. Bacteriocins of Lactic Acid Bacteria. Academic Press Inc., Newark, DE, USA., pp: 55-74.

Stiles, M.E. and W.H. Holzapel, 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol., 36: 1-29.

Tagg, H.G., A.S. Dajani and L.W. Wannamaker, 1976. Bacteriocin of gram-positive bacteria. Bacteriol. Rev., 40: 722-756.

Van Laack, R.L.J.M., U. Schillinger and W.H. Holzapel, 1992. Characterization and partial purification of a bacteriocin produced by *Leuconostoc carnosum* LA44A. Int. J. Food Microbiol., 16: 183-195.

Ventura, M., S. O'Flahesty, M.J. Claesson, F. Turroni, T.R. Klaenhammer, D. Sinderen and P.W. O'Toole, 2009. Genome-scale analysis of health promoting bacteria: Probiogenomics. Nat. Rev. Microbiol., 7: 61-67.

Wells, G.M. and A. Mercennier, 2008. Microbial delivery of therapeutic molecule using lactic acid bacteria. Nat. Rev. Microbiol., 6: 349-362.