Isolation, Identification, and Antibiotic Susceptibility of nis$^+$ *Lactococcus lactis* from Dairy and Non-dairy Sources

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Abstract

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Eight isolates of *Lactococcus lactis* subsp. *lactis* were isolated and identified by phenotypic and molecular characterisation out of 23 isolates of lactic acid bacteria (LAB) from different dairy and non-dairy sources. Out of eight strains, four were obtained from dairy and four from non-dairy sources. All eight strains of *L. lactis* subsp. *lactis* were able to produce zones of inhibition against the *Lactobacillus acidophilus* NCDC 015. The antimicrobial agent produced by the isolates inhibited the growth of a range of related lactic acid bacteria and certain Gram positive food-borne microorganisms. The antimicrobial agent, i.e. nisin, produced by the strains was confirmed by PCR amplification of nisin gene sequences of 174 bp size. Antibiotic susceptibility test to 21 different types of antibiotics was evaluated. All the isolates were resistant to fosfomycin, cefepime, amikacin, kanamycin, neomycin, nalidixic acid, pipemidic acid, norfloxacin, sulphadiazine, colistin, polymixin, teicoplanin, nystatin, and amphotericin B but susceptible to ampicillin, erythromycin, spiramycin, spectinomycin, ciprofloxacin, rifampicin, and trimethoprim.

Keywords: *Lactococcus lactis* subsp. *lactis*; antimicrobial agent; nisin

Lactic acid bacteria (LAB), the industrially important food grade bacteria, are the part of the commensal intestinal flora of humans and animals (Vaughan *et al.* 2005; Klare *et al.* 2007). The application of LAB and their metabolites for the preservation and extension of shelf-life of food against spoilage has aroused the interest in their isolation and characterisation (Cleveland *et al.* 2001). Among lactic acid bacteria, *Lactococcus lactis* has extensively been employed in the food industry due to the preservative attributes of antimicrobial metabolites such as hydrogen peroxide, diacetyl, lactic acid and nisin (Lindgren & Dobrogosz 1990). Nisin is a 34 amino acid peptide produced by certain strains of *L. lactis* which exhibits a broad spectrum of inhibitory activity against several Gram-positive bacteria of the genera *Bacillus, Enterococcus, Listeria, Clostridium*, and *Staphylococcus* (Tagg *et al.* 1976; Chung *et al.* 1989; Klaenhammer 1993). This bacteriocin usually has no effect on Gram-negative bacteria, yeasts and moulds, although Gram-negative bacteria can be sensitised to nisin by permeabilisation of the outer membrane by sublethal heating, freezing and chelating agents (Delves-Broughton *et al.* 1996).

Besides, strains of *L. lactis* are frequently used on a large-scale as starter cultures in food industries for the manufacture of various fermented dairy products such as sour milk, cream, butter, fresh

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cheeses and many varieties of semi-hard cheeses (Prodelalova *et al.* 2005). In these applications, selected strains are used as food supplements that may favourably influence the intestinal flora of human and animal hosts, such as competitive exclusion of gastrointestinal pathogens, stimulation of immune responses or antimutagenic and anticancerogenic activities (Mercenier *et al.* 2003; Reid *et al.* 2003). Because of their long-time use in various food and feed preparations, *L. lactis* has been given the so-called GRAS (generally recognised as safe) status (Federal Register 1988). This indicates that *L. lactis* strains are food-grade organisms without imposing a health risk for the consumers or the environment. However, by the presence and expression of virulence genes and/or antibiotic resistance genes in food-associated LAB such as *L. lactis* strains on mobile genetic elements such as plasmids or (conjugative) transposons, these antibiotic resistance traits can potentially be transferred to the human or animal commensal flora and to pathogenic bacteria which are temporarily residing in the body of hosts and provide a survival benefit to the invading microorganisms. Under such circumstances it is difficult to eliminate the infection from the host body caused by pathogens. Therefore, the fermentative and nutritional LAB such as *L. lactis* that is consumed on a daily basis worldwide must be characterised to ensure the absence of acquired antimicrobial resistance properties so that these would be safe for human and animal consumption (Belen Florez *et al.* 2005; Liasi *et al.* 2009). Therefore, to contribute to the potential uses of *L. lactis* isolates in the food and dairy industries, the isolation, identification, antimicrobial activity, and antibiotic susceptibility determination of eight strains of *L. lactis* from dairy and non-dairy sources have been investigated.

MATERIAL AND METHODS

Source of sample. A variety of samples of dairy (13) and non-dairy (21) sources were collected for isolation. Among the dairy samples, cow milk (5), buffalo milk (5), goat milk (2), and cheddar cheese (1) from an experimental dairy of the National Dairy Research Institute, Karnal (Haryana), India and among non-dairy sources, cattle hair (5), cattle dung (3), cattle fodder (5), and poultry beet (8) were collected from a local herd of Varanasi (UP).

Bacterial strains. Bacterial strains such as *Lactococcus, Lactobacillus* and *Leuconostoc* strains were grown in MRS (De Man, Rogosa, Sharpe) broth (Himedia Lab. Pvt. Ltd., Mumbai, India) at 30°C for 24–48 hours. *Bacillus, Enterococcus, Staphylococcus* and *Micrococcus* strains were grown in nutrient broth and *Escherichia coli* in Luria-Bertani broth (both Himedia Lab. Pvt. Ltd., Mumbai, India) and incubated at 37°C for 24 hours. The strains were maintained as glycerol stocks in their respective broth at –20°C.

Phenotypic and molecular characterisation of *L. lactis.* The isolation of Gram-positive cocci, catalase negative, homo-fermentative, and acid producing isolates of LAB was carried from dairy and non-dairy samples according to the method of Aneja (2005) and Mirhosaini *et al.* (2006). The catalase test was carried out by the application of 3–4 drops of 3% (v/v) H_2O_2 to bacterial colonies and catalase activity was confirmed by the observation of gas bubbling on bacterial colonies. Homo-fermentative isolates were identified by inoculating 100 µl inoculum (2–3 × 10^6 CFU/ml) in glucose broth (5 ml) containing inverted Durham tubes. The tubes were incubated at 30°C for 48 h and CO_2 evolution from glucose was observed as gas accumulation in Durham tubes. The identification of isolates for acid production through fermentation was carried out qualitatively by spreading 100 µl inoculum (1.5–2.0 × 10^5 CFU/ml) on MRS agar plates containing acid indicator BCP (0.004%) and non-diffusible buffer $CaCO_3$ (1%). The plates were incubated at 30°C for 24–48 h for acid producing LAB, which was characterised as yellow coloured bacterial colonies with yellow zones on purple medium.

The litmus milk reduction and the growth occurrence at different temperatures (10, 40, and 45°C) were carried out as suggested by Corroler *et al.* (1998) and Aneja (2005). The litmus milk reduction test was performed by inoculating 1% (3.5–4 × 10^6 CFU/ml) inoculum in 5 ml of sterilised litmus milk broth and incubated at 30 ± 1°C for 24–48 hours. The formation of pinkish band and white solid curd in litmus milk broth without gas evolution was observed for *L. lactis*.

The isolates were tested for the ability to ferment various carbohydrates (glucose, sucrose, lactose, maltose, mannose, xylose, glycerol, fructose, and mannitol) as described by Nomura *et al.* (1999) and Gunasekaran (2002). The carbohydrate broth (5 ml) in tubes was inoculated by 1% (v/v) inoculum

(1.2–2.4 × 10^6 CFU/ml). The tubes were incubated at 30 ± 1°C for 24–48 hours. The fermentation of carbohydrates was observed by the acid production in broth resulting in the change of the indicator from red to yellow. The isolates thus identified as members of the genus *Lactococcus* were further characterised at a subspecies level by performing the arginine hydrolysis test and growth at pH 9.2 and 4% (w/v) NaCl (Klijn *et al.* 1995; Gunasekaran 2002). The activity of arginine hydrolase was observed by stab inoculation of a loopful of *L. lactis* colonies in arginine agar containing phenol red (0.001% w/v) in screw capped tubes. The tubes were incubated at 30 ±1°C for 24–48 h and the release of ammonia from arginine hydrolysis by the activity of arginine hydrolase was observed by a change in the colour of the indicator from yellow to magenta. Further, the phenotypically identified strains were also identified by gene sequencing of a fragment (> 500 bp) of 16S rRNA (B27F 5'-AGA GTT TGA TCC TGG CTC AG-3') and (U1492R 5'-GGT TAC CTT GTT ACG ACT T-3') as proposed by Lane (1991). The GenBank accession numbers were allocated by NCBI for the sequences of 16S rRNA of strains of *L. lactis* subsp. *lactis.*

***Total titratable acidity of** L. lactis.* The total titratable acidity of *L. lactis* isolates was quantitatively estimated as % lactic acid by a titrimetric method using an aliquot of 5 ml broth culture, 0.1 mol/l NaOH and phenolphthalein as indicator (Ranganna 2004). The appearance of pink colouration of broth indicated the neutralisation of total acid by NaOH. The titre value of NaOH was noted and total acidity as % lactic acid (equivalent weight 90) was calculated by the following formula:

Total acidity (%) = (titre value × normality of alkali × volume made up × equivalent weight of acid × 100)/(aliquot taken × weight/volume of sample × 1000)

***Antimicrobial activity of** L. lactis.* The antimicrobial activity was estimated by disc diffusion and critical dilution methods (Schillinger & Lucke 1989). The cell free supernatant (pH 3.92–4.06) after 18 h of incubation at 30°C was neutralised up to pH 6 with 4 mol/l NaOH to inactivate any inhibitory activity by low pH. The titres of antimicrobial agents released in supernatant (pH 6) were quantified by spotting 5 µl of twofold serial diluted supernatant over the sterile paper discs on *Lactobacillus acidophilus* NCDC 015 as indicator strain (2.2 × 10^6 ± 0.1 CFU/ml) seeded MRS soft agar plates (0.6%) and incubation was carried out at 30°C for 24 hours. The titres of antimicrobial agents in supernatant were calculated and expressed in arbitrary units (AU/ml) (Barefoot & Klaenhammer 1983). Further, the correlation of antimicrobial activity with growth in terms of specific inhibitory activity (AU/10^4 CFU) was estimated by the ratio of antimicrobial activity (AU/ml) to the growth (CFU/ml) of test strains.

***Inhibitory spectrum of antimicrobial agents of** L. lactis.* The cell free supernatant (pH 3.92–4.06) after 18 h of incubation at 30°C was neutralised up to pH 6 with 4 mol/l NaOH to inactivate any inhibitory activity by low pH. The inhibitory spectrum of antimicrobial agents in supernatant (pH 6) was tested by an agar well diffusion method (Tagg & McGiven 1971) against a wide range of indicator strains comprising LAB and food-borne Gram-positive and Gram-negative bacteria. In the method, an aliquot of 100 µl of supernatant was placed in wells on indicator strain (2.3–2.6 × 10^6 CFU/ml) seeded MRS soft agar (0.6%) plates and incubated at 30°C for 24 h for the zone of clearance.

***PCR amplification of nisin gene in strains of** L. lactis.* To confirm the nisin as antimicrobial agent in supernatant the genomic DNA of all test strains and nis^+ reference strain *L. lactis* subsp. *lactis* NCDC 094 was extracted by the classical proteinase K-phenol-chloroform extraction as reported by Ho *et al.* (1991). The PCR amplification of nisin gene was carried out by designing a primer pair for the nisin A/Z structural gene (174 bp) from the published sequences of *L. lactis* subsp. *lactis* NIZOR5 acquired from the NCBI public databases. The primer pair (forward 5'-ATG AGT ACA AAA GAT-3' and reverse 5'-TTA TTT GCT TAC GTG AA-3') complementary to the sequences occurring proximally to 5' and 3' ends of the nisin A/Z structural gene was used to amplify the nisin gene.

The reaction mixture of 25 µl containing 5–10 ng of genomic bacterial DNA, 2.5 µl of 10X Taq buffer, 1.5mM $MgCl_2$, 10 pmole of each primer, 100µM of each dNTP mixture, and 3U Taq DNA polymerase was prepared for each PCR reaction. The amplification reaction involved initial denaturation at 94°C for 5 min, 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension step consisting of 72°C for 10 minutes. The DNA ladders (Bangalore Genei Pvt. Ltd., Peenya, India) of 50 bp ranging from 50 bp to 2000 bp and of

100 bp ranging from 100 bp to 1000 bp were used to measure the size of amplified PCR products (174 bp) of the nisin gene.

Determination of antibiotic susceptibility. The antimicrobial susceptibility test of eight strains of *L. lactis* was performed by disc diffusion method or Kirby-Bauer technique (Bauer *et al.* 1966). The inoculum was prepared by inoculating MRS broth and incubating it at 33 ± 0.5°C until it reached the equivalent turbidity of 0.5 McFarland standards (usually 2–6 h). For inoculation a swab stick was dipped into the inoculum and squeezed on the wall of the test tube to discard extra inoculum. The surface of MRS agar plates was lightly and uniformly inoculated by a cotton swab.

A total of 21 different types of commercially available antibiotic discs (Himedia Lab. Pvt. Ltd., Mumbai, India) were placed on the surface of seeded MRS agar plates and incubated at 33 ± 0.5°C for 24–48 hours. The diameter of inhibition zones was measured including the diameter of discs and calculated from the means of five determinations. Results were graded as resistant (+++), intermediate (++) and sensitive (+). Resistance was defined as the absence of inhibition zone around the discs. If the diameter of the inhibition zone was smaller than 10 mm or in the range of 10–35 mm, the strain was classified as intermediate sensitive or sensitive, respectively, to the antibiotic tested. The reference strain *L. lactis* subsp. *lactis* NCDC 094 was used as control.

Table 1. Phenotypic and molecular characterization of *Lactococcus lactis* subsp. *lactis*

Characteristic	Isolate								RS
	LC14	LC23	LC18	LC28	LC25	LC29	LC30	LC27	
Isolation source	cow milk	buffalo milk	goat milk	cheddar cheese	poultry beet	cattle dung	cattle hair	cattle fodder	
Phenotypic characterisation									
Gram stain	+	+	+	+	+	+	+	+	+
Cell shape	coccus								
Catalase test	–	–	–	–	–	–	–	–	–
Cultural characteristics	white colony, circular with entire margin, no surface growth and granular growth at sediment								
Cell arrangement	chain/double/single								
Fermentation type	H	H	H	H	H	H	H	H	H
Growth at or in									
10°C and 40°C	+	+	+	+	+	+	+	+	+
45°C	–	–	–	–	–	–	–	–	–
4% NaCl & pH 9.2	+	+	+	+	+	+	+	+	+
Litmus milk reduction test	+	+	+	+	+	+	+	+	+
Arginine hydrolysis test	+	+	+	+	+	+	+	+	+
Fermentation of									
Glu/Lac/Suc/Mal/Fruc/Man	+	+	+	+	+	+	+	+	+
Xyl/Gly/Mann	–	–	–	–	–	–	–	–	–
Molecular characterisation									
Accession no. (16S rRNA, > 500 bp)	JQ31971	JQ319712	JQ319713	JQ319714	JQ319715	JQ31971	JQ319717	JQ319718	
Functional characterisation									
Total titratable acidity (% lactic acid)	0.98	0.36	0.68	0.70	0.92	0.75	0.61	0.66	0.78
	CD ($P < 0.05$) ($n = 3$) = 0.14[a]								
Specific inhibitory activity (AU/10^4 CFU)	0.39	0.20	0.53	0.70	0.21	0.17	0.27	0.24	0.40
	CD ($P < 0.05$) ($n = 3$) = 0.13[a]								
Identified as	*Lactococcus lactis* subsp. *lactis*								

+ = positive; – = negative; H – homo-fermentative; Glu – glucose; Lac – lactose; Suc – sucrose; Mal – maltose; Fruc – fructose; Man – mannose; Xyl – xylose; Gly – glycerol; Mann – mannitol; RS – reference strain (*L. lactis* subsp. *lactis* NCDC 094); values are the mean of three ($n = 3$) replications; superscript a represents statistically significant data; CD – critical difference

Statistical analysis. Analysis of variance and critical difference were performed after logarithmic transformation of all values according to the procedure of Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

The phenotypic and molecular characterisation of *L. lactis* subsp. *lactis* from dairy and non-dairy sources is presented in Table 1. Eight isolates of *Lactococcus lactis* subsp. *lactis* were isolated and identified out of 23 isolates of lactic acid bacteria (LAB) from a total of 34 different dairy and non-dairy samples. Out of eight strains, four were obtained from dairy (cow milk, buffalo milk, goat milk, and cheddar cheese) and four from non-dairy (poultry beet, cattle hair, cattle dung, and cattle fodder) sources. From the phenotypic characterisation, all tested strains were Gram-positive cocci, arranged as single/double/chains, catalase negative and homo-fermentative. The isolates exhibited the growth at 4% NaCl, pH 9.2, 1 and 40°C but they were inhibited at 45°C. The isolates reduced litmus milk to a white curd with pinkish band and hydrolysed the arginine to ammonia. Based on these characteristics and on the basis of carbohydrate fermentation profiles, the isolates were phenotypically identified as *L. lactis* subsp. *lactis*. Further, the maximum sequence similarity of a fragment of 16S rRNA of eight strains with the GenBank reference strain *L. lactis* subsp. *lactis* KF147 confirmed the identification at a molecular level.

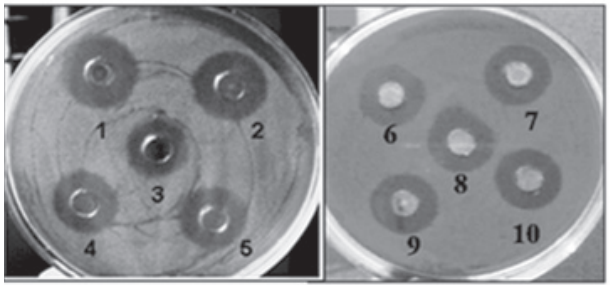

Figure1. Inhibitory activity of cell free supernatant of *Lactococcus lactis* subsp. *lactis* against *Lactobacillus acidophilus* NCDC 015

Strain: 1 – LC14, 2 – LC23, 3 – LC18, 4 – LC28, 5 and 10 – *Lactococcus lactis* subsp. *lactis* NCDC 094, 6 – LC25, 7 – LC29, 8 – LC30, 9 – LC27

The identified strains exhibited statistically significant production of acid (0.36–0.98% lactic acid) and specific inhibitory activity (0.17–0.70 AU/10^4 CFU) against *Lactobacillus acidophilus* NCDC 015 (Table 1). The inhibitory spectrum of the antimicrobial agents produced by isolates of *L. lactis* against different Gram-positive and Gram-negative

Table 2. Inhibitory spectrum of *L. lactis* subsp. *lactis* (n = 8) against Gram-positive and Gram-negative bacteria

Indicator strain	Strain								RS
	LC14	LC23	LC18	LC28	LC25	LC29	LC30	LC27	
Lactobacillus plantarum NCDC 021	+++	+++	+++	+++	+++	+++	+++	+++	+++
Lactobacillus brevis NCDC 01	+++	+++	+++	+++	+++	+++	+++	+++	+++
Lactobacillus acidophilus NCDC 015	+++	+++	+++	+++	+++	+++	+++	+++	+++
Lactobacillus casei NCDC 017	+++	+++	+++	+++	+++	+++	+++	+++	+++
Leuconostoc mesenteroides NCDC 029	+++	+++	+++	+++	+++	+++	+++	+++	+++
Leuconostoc dextranicum NCDC 023	+++	+++	+++	+++	+++	+++	+++	+++	+++
Micrococcus luteus ATCC 10261	+++	+++	+++	+++	+++	+++	+++	+++	+++
Staphylococcus aureus NCDC 237	++	++	++	++	++	++	++	++	++
Bacillus subtilis NCDC 215	++	++	++	++	++	++	++	++	++
Bacillus stearothermophilus NCDC 206	++	++	++	++	++	++	++	++	++
Enterococcus faecalis NCDC 114	++	++	++	++	++	++	++	++	++
Bacillus cereus ATCC 10876	+	+	+	+	+	+	+	+	+
Enterococcus faecium NCDC 124	+	+	+	+	+	+	+	+	+
Escherichia coli NCDC 135	–	–	–	–	–	–	–	–	–
L. lactis subsp. *lactis* NCDC 094	–	–	–	–	–	–	–	–	–

NCDC – National Collection of Dairy Culture; ATCC – American Type Culture Collection; RS – *L. lactis* subsp. *lactis* NCDC 094; iinhibition zone (mm): +++ = 11–12 mm; ++ = 6–10 mm; + = 1–5 mm; – = no inhibition

bacteria is presented in Table 2. The inhibition zones created by the isolates against *L. acidophilus* NCDC 015 are shown in Figure 1. All isolates demonstrated maximum inhibitory effect against *Lactobacillus plantarum*, *Lactobacillus brevis, Lactobacillus acidophilus, Lactobacillus casei, Leuconostoc mesenteroides, Leuconostoc dextranicum*, and *Micrococcus luteus* followed by intermediate inhibition that was observed against *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Bacillus stearothermophilus, Enterococcus faecalis*, and *Enterococcus faecium*. However, no inhibitory activity was observed against nis$^+$ *L. lactis* subsp. *lactis* NCDC 094 and *Escherichia coli* NCDC 135 (Table 2). *Escherichia coli* NCDC 135 is a Gram-negative bacterium and Gram-negative bacteria are resistant to nisin because of far less permeable cell walls than Gram-positive bacteria. Therefore, no inhibition was observed against *Escherichia coli* NCDC 135 (Delves-Broughton 2005). Further, according to Koponen (2002), the lactococcal cells may have some immunity mechanisms by which cells possess the ability of protecting themselves against a nisin produced by their own metabolism. Both the cell wall constitution and the membrane lipid composition have been demonstrated to be involved in nisin action as well as nisin resistance. The inhibitory action of these antimicrobial peptides can vary between different genera, species of the same genera, identical species and even identical cultures under different environmental conditions. This might be the reason that no inhibitory activity was observed against nis$^+$ *L. lactis* subsp. *lactis* NCDC 094.

The inhibitory activity exhibited by *L. lactis* isolates was expected to be a peptidic antibacterial agent such as nisin. Therefore, the presence of nisin in supernatant as antimicrobial agent was confirmed by amplifying 174 bp fragment of the *nis* A/Z structural gene of isolates' genomic DNA. The PCR amplified product of nisin gene in isolates and reference strain *L. lactis* subsp. *lactis* NCDC 094 are shown in Figure 2. The amplification of 174 bp PCR products (lane 1 to 7 and 10) in isolates was identical to that of a reference nis$^+$ strain of *L. lactis* subsp. *lactis* NCDC 094 (lane 12). No amplification was observed with the genomic DNA of negative control *Enterococcus faecalis* NCDC 114 (lane 8), *Leuconostoc mesenteroides* NCDC 029 (lane 9), and *Micrococcus luteus* ATCC 10261 (lane 11). Study on antimicrobial nisin produced by *L. lactis* isolates in supernatant has led to their potential use as bio-preservatives that may be used to inhibit the growth of pathogenic microorganisms in the food industry. This antimicrobial agent can be applied either as purified chemical agents or as viable cultures in the case of fermented products.

Lactic acid bacteria (LAB) in fermented products generally act as a reservoir of antimicrobial-resistance genes that could be transferred to other pathogenic microorganisms either through the food chain or in the gastrointestinal tract of human and animals. Transfer of resistance to antimicrobial agents is also an essential mechanism of *L. lactis* (Belen Florez *et al.* 2005; Herreros *et al.* 2005). Therefore, before a strain of LAB can be used as a feed additive, it must undergo antibiotic resistance screening to ensure its safe application. In this regard the results of susceptibility studies of the *L. lactis* isolates tested against 21 different types of antibiotics are presented in Table 3. All isolates

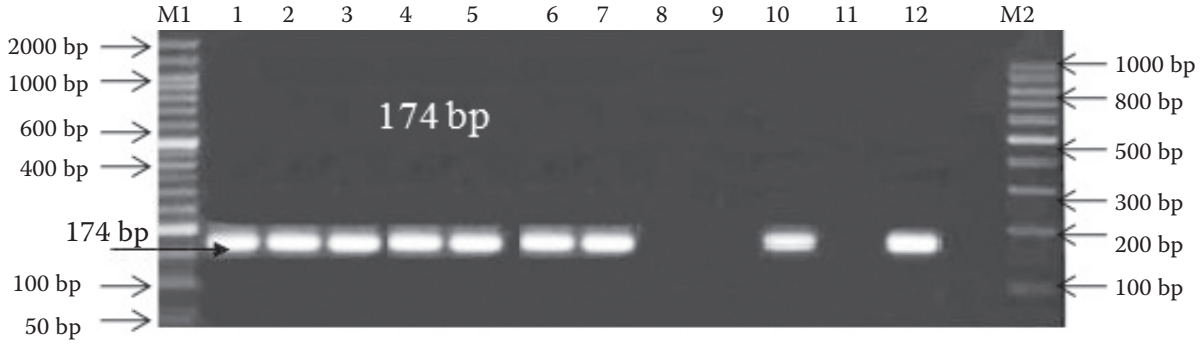

Figure 2. PCR amplification of nisin gene (*nis A/Z*) in the strains of *Lactococcus lactis* subsp. *lactis*

Lane 1–7 and 10 = nis$^+$ strains (1 – LC14, 2 – LC23, 3 – LC18, 4 – LC28, 5 – LC25, 6 – LC29, 7 – LC30, 10 – LC27); lane 12 = *Lactococcus lactis* subsp. *lactis* NCDC 094 (positive control); lane 8–9 and 11 = negative control; 8 = *Enterococcus faecalis* NCDC 114; 9 = *Leuconostoc mesenteroides* NCDC 029; 11 = *Micrococcus luteus* ATCC 10261; M1 – 50 bp DNA ladder; M2 – 100 bp DNA ladder

were susceptible to ampicillin of the β-lactam group of antibiotics, erythromycin and spiramycin of the macrolide group, spectinomycin of aminoglycosides, ciprofloxacin, and rifampicin of quinolones and trimethoprim (sulphonamides). In addition, the isolates were also resistant to a large number of antibiotics which include fosfomycin and cefepime (β-lactam group), amikacin, kanamycin, and neomycin (aminoglycosides), nalidixic acid, pipemidic acid, and norfloxacin (quinolones), sulphadiazine (sulphonamides), colistin, and polymixin (polypeptide), teicoplanin (glycopeptides), and nystatin and amphotericin B of the antifungal group of antibiotics.

Among the 21 tested antibiotics, *L. lactis* isolates were observed to be resistant to 14 different types of antibiotics. Several reports have also suggested the resistance of *L. lactis* to aminoglycosides, sulphonamides, β-lactam, polypeptide and quinolone groups of antimicrobial agents (Liasi *et al.* 2009). In the present study, all isolates showed the resistance towards Gram-negative spectrum antibiotics (nalidixic acid, pipemidic acid, and norfloxacin) and aminoglycoside antibiotics (amikacin, kanamycin, and neomycin). Similar observations were also reported by Termmerman *et al.* (2003), Zhou *et al.* (2005) and Liasi *et al.* (2009).

Table 3. Susceptibility spectrum of *L. lactis* subsp. *lactis* (n = 8) to 21 different types of antibiotics

Antibiotic	Concentration/disc	Strain								RS
		LC14	LC23	LC18	LC28	LC25	LC29	LC30	LC27	
β-Lactams										
Fosfomycin	50 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Ampicillin	25 µg	+	+	+	+	+	+	+	+	+
Cefepime	30 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Macrolides										
Erythromycin	15 µg	+	+	+	+	+	+	+	+	+
Spiramycin	30 µg	+	+	+	+	+	+	+	+	+
Aminoglycosides										
Kanamycin	30 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Amikacin	30 µg	++	++	++	++	++	++	++	++	++
Spectinomycin	100 µg	+	+	+	+	+	+	+	+	+
Neomycin	30 µg	++	++	++	++	++	++	++	++	++
Quinolones										
Nalidixic acid	30 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Pipemidic acid	30 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Norfloxacin	10 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Ciprofloxacin	30 µg	+	+	+	+	+	+	+	+	+
Rifampicin	15 µg	+	+	+	+	+	+	+	+	+
Sulphonamides										
Sulphadiazine	30 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Trimethoprim	30 µg	+	+	+	+	+	+	+	+	+
Polypeptides										
Colistin	25 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Polymixin	100U	+++	+++	+++	+++	+++	+++	+++	+++	+++
Glycopeptide										
Teicoplanin	30 µg	+++	+++	+++	+++	+++	+++	+++	+++	+++
Antifungal										
Nystatin	100 U	+++	+++	+++	+++	+++	+++	+++	+++	+++
Amphotericin B	100 U	+++	+++	+++	+++	+++	+++	+++	+++	+++

RS – reference strain (*L. lactis* subsp. *lactis* NCDC 094); +++ = resistant; ++ = intermediate; + = sensitive

CONCLUSION

It can be concluded that our strains of nis$^+$ *L. lactis* subsp. *lactis* exhibited a specific spectrum of inhibitory activity against a wide range of microorganisms. Therefore, these strains of *L. lactis* isolated from dairy and non-dairy sources may be included in the industrially important culture collection and may be recommended as starter culture for the manufacture of fermented foods and to provide safety against the microflora tested in the present investigation either as viable cells or purified form of antimicrobial agents.

References

Aneja K.R. (2005): Experiments in Microbiology, Plant Pathology and Biotechnology. New Age Publishers, New Delhi.

Barefoot S.F., Klaenhammer T.R. (1983): Detection and activity of lactacin B: a bacteriocin produced by *Lactobacillus acidophilus.* Applied and Environmental Microbiology, **45**: 1808–1815.

Bauer A.W., Kirby W.M.M., Sherris J.C., Turck M. (1966): Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology, **45**: 493–496.

Belen Florez A., Delgado S., Mayo B. (2005): Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Canadian Journal of Microbiology, **51**: 51–58.

Chung K.T., Dickson J.S., Crouse J.D. (1989): Effect of nisin on growth of bacteria attached to meat. Applied and Environmental Microbiology, **55**: 1329–1333.

Cleveland J., Montville T.J., Nes I.F., Chikindas M.L. (2001): Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology, **7**: 11–20.

Corroler D., Mangin I., Desmasures N., Gueguen M. (1998): An ecological study of lactococci isolated from raw milk in the Camembert cheese registered designation of origin area. Applied and Environmental Microbiology, **64**: 4729–473.

Delves-Broughton J. (2005): Nisin as a food preservative. Food Australia, **57**: 525–527.

Delves-Broughton J., Blackburn P., Evans R.J., Hugenholtz J. (1996): Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek, **69**: 193–202.

Federal Register (1988): Nisin preparation: Affirmation of GRAS status as a direct human food ingredient. Federal Register, **53**: 11247–11251.

Gunasekran P. (2002): Laboratory Manual in Microbiology. New Age Publishers, New Delhi: 47–91.

Herreros M.A., Sandoval H., Gonzalez L., Castro J.M., Fresno J.M., Tornadijo M.E. (2005): Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from armada cheese (a Spanish goats' milk cheese). Food Microbiology, **22**: 455–459.

Ho S.A., Hoyle J.A., Lewis F.A., Seker A.D., Cross D., Mapstone N.P., Dixon M.F., Wyatt J.I., Tompkins D.S., Taylor G.R., Quirke P. (1991): Direct polymerase chain reaction test for detection of *Helicobacter pylori* in humans and animals. Journal of Clinical Microbiology, **29**: 2543–2549.

Klaenhammer T.R. (1993): Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiology Review, **12**: 39–86.

Klare I., Konstabel C., Werner G., Huys G., Vankerckhoven V., Kahlmeter G., Hildebrandt B., Muller-Bertling S., Witte W., Goossens H. (2007): Antimicrobial susceptibilities of *Lactobacillus, Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. Journal of Antimicrobial Chemotherapy, **59**: 900–912.

Klijn N., Weerkamp A.H., De Vos W.M. (1995): Detection and characterization of lactose-utilizing *Lactococcus* spp. in natural ecosystems. Applied and Environmental Microbiology, **61**: 788–792.

Koponen O., Tolonen M., Qiao M., Wahlstrom G., Helin J., Saris P.E.J. (2002): Nis B is required for the dehydration and NisC for the lanthionine formation in the post-translational modification of nisin. Microbiology, **148**: 3561–3568.

Lane D.J. (1991): 16S/23S rRNA sequencing. In: Stackebrandt E., Goodfellow M. (eds): Nucleic acid Techniques in Bacterial Systematics. John Wiley and Sons, New York: 115–175.

Liasi S.A., Azmi T.I., Hassan M.D., Shuhaimi M., Rosfarizan M., Ariff A.B. (2009): Antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu. Malaysian Journal of Microbiology, **5**: 33–37.

Lindgren S.E., Dobrogosz W.J. (1990): Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiology Review, **87**: 149–164.

Mercenier A., Pavan S., Pot B. (2003): Probiotics as biotherapeutic agents: Present knowledge and future prospects. Current Pharmaceutical Design, **9**: 175–191.

Mirhosaini M., Nahvi I., Kasra Kermanshahi R., Tavassoli M. (2006): Isolation of bacteriocin producing *Lactococcus lactis* strain from dairy products. International Journal of Dairy Science, **1**: 51–56.

Nomura M., Kimoto H., Someya Y., Suzuki I. (1999): Novel characteristic for distinguishing *Lactococcus lactis*

subsp. *lactis* from subsp. *cremoris*. International Journal of Systemic Bacteriology, **49**: 163–166.

Prodelalova J., Spanova A., Rittich B. (2005): Application of PCR, rep-PCR and RAPD techniques for typing of *Lactococcus lactis* strains. Folia Microbiologica, **50**: 150–154.

Ranganna S. (2004): Handbook of Analysis and Quality Control for Fruits and Vegetable Products. 2nd Ed. Tata McGraw-Hill Publishing Company Ltd., New Delhi.

Reid G., Jass J., Sebulsky M.T., McCormick J.K. (2003): Potential uses of probiotics in clinical practice. Clinical Microbiology Review, **16**: 658–672.

Schillinger U., Lucke F.K. (1989): Antibacterial activity of *Lactobacillus sake* isolated from meat. Journal of Applied Bacteriology, **70**: 473–478.

Snedecor G.W., Cochran W.G. (1967): Statistical Methods. I. Regression. II. Two Way Classifications. 6th Ed. The Iowa State University Press, Ames: 141–144, 299–338.

Tagg J.R., Dajan A.S., Wannamaker L.W. (1976): Bacteriocins of Gram-positive bacteria. Bacteriology Reviews, **40**: 722–756.

Tagg J.R., McGiven A.R. (1971): Assay system for bacteriocins. Journal of Applied Microbiology, **21**: 943.

Temmerman R., Pot B., Huys G., Swings J. (2003): Identification and antibiotic susceptibility of bacterial isolates from probiotic products. International Journal of Food Microbiology, **81**: 1–10.

Vaughan E.E., Heilig H.G.H.J., Ben-Amor K., De Vos W.M. (2005): Diversity, vitality and activities of intestinal lactic acid bacteria and *Bifidobacteria* assessed by molecular approaches. FEMS Microbiology Review, **29**: 477–490.

Zhou J.S., Pillidge C.J., Gopal P.K., Gill H.S. (2005): Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. International Journal of Food Microbiology, **98**: 211–217.

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