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Distribution and Diversity of Nisin Producing LAB in Fermented Food

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Abstract

An attempt was made, to characterize natural antibiotics or lantibiotics from unconventional sources and its antibacterial spectrum against food borne pathogens and drug resistant bacteria. Six different traditional fermented foods i.e., fermented fish, fermented soybeans, *Soibum* (fermented bamboo shoots), milk, *idly* and *dosa* batter were used for the isolation of bacteriocin producing Lactic acid bacteria (LAB). Among all bacterial cultures isolated from the various sources, 129 cultures have found to produce antimicrobial compounds. Nisin specific reporter bacteria was utilized as biosensor to identify the Nisin like bacteriocin, where 10 cultures found to be positive Nisin producer. Identified Nisin like bacteriocin was partially concentrated by using ammonium sulphate followed by butanol extraction. Minimum inhibitory concentration (MIC) was analyzed against food borne pathogen and drug resistant bacteria. MIC of partially purified Nisin (pp-Nisin) of all the LAB isolates against food-borne pathogens are ranged between 0.5 and 92 µg/ml respected to various Gram-positive bacteria. Similarly, the drug resistant bacteria were also inhibited by pp-Nisin (MIC ranged between 15 and 175 µg/ml). All samples of ppnisin exhibited auto induction ability. Taxonomic identification of the nisin producers was done by whole genome sequencing which reveals that cultures belongs to *Lactococcus lactis* ssp. *lactis.* Also it was found that *Lactococcus lactis* ssp. *lactis* C2d and *Lactococcus lactis* ssp. *lactis* SP2C4 harbor *nis*A gene and *Lactococcus lactis* ssp. *lactis* FS2 (*L. lactis* FS2) harbor *nis*Q gene. The finding of this study highlights the first case of *L. lactis* FS2 isolated from fermented fish harbor *nis*Q gene. Antibacterial activity of pp-Nisin against drug resistant LAB is also reported.

Introduction

Nisin is an antibacterial polypeptide which belongs to the group of post-translationally modified peptide called lantibiotics which contain an unusual amino acid i.e., lanthionine [1]. Nisin is the most utilized antimicrobial peptides produced by some *Lactococcus lactis* strains, due to excellent features as a food preservative, including high activity against Gram-positive food pathogens [2]. Nisin binds to the lipid II (Precursor for peptidoglycan biosynthesis), which inhibit the biosynthesis of bacterial cell wall [3]. Nisin is considered as generally regarded as safe (GRAS) and the only bacteriocin approved as a food preservative, which explains its increasingly use in food industry [4]. Nowadays, ecosystems of health, environment and veterinary (food chain) are facing the spread of drug resistant microbiota, also developing countries are the hotspot for the same [5]. Recently researchers have shifted towards minimizing the risk of drug resistant bacteria by developing novel compounds and innovative concepts. Bacteriocins, especially the natural lantibiotics group considered as potential alternative to chemical antibiotics [6]. Bacteriocins possess novel mode of action, towards the drug resistant organism and specifically targeted for particular application [7].

The unique structure and mechanism of action makes the lantibiotics a potential weapon against drug resistant bacteria [8]. Nisin variants exhibited improved antimicrobial activity against VRE, MRSA [9, 10]. Till date 10 nisin variants are reported which are particularly different from each other in the amino acid pattern i.e., nisin A, nisin H, nisin Z, nisin O, nisin P, nisin F, sal D, nisin Q, nisin U and nisin U2 [9, 11]. Among all the Nisin variants under studied, the nisin A and Z are most frequently used and often marketed. The organization and sequences of nisin cluster and operon is different in each variant. These reports are mainly focus in the distribution of nisin like lantibiotics. Limited studies are available for the characterization of nisin variant from variety of sources. The LAB which has been reported till

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date for the production of nisin, are mainly from dairy based product. In this study an attempt was made for the rapid detection of nisin-like bacteriocin using reporter bacteria from different fermented food sources. Influence of cultural condition on the production of nisin was also investigated and MIC of ppnisin against food-borne pathogen and drug resistant bacteria were studied.

Materials and Methods

Media Chemicals and Reagents

All the media and chemicals, M17, MRS, BHI, Agar, 5-bromo-4-chloro3-indolyl-beta-D-galacto-pyranoside (X-Gal), Ortho-Nitrophenyl-β-galactoside (ONPG), Nisin, chloramphenicol used in this study were purchases from Hi Media Pvt. Ltd, Mumbai, India. Ammonium sulphate, sodium chloride and organic solvent such as butanol were purchases from Sisco Research Laboratory, Bangalore, India. All the chemical used in this study were of analytical grade.

Bacterial Cultures and Maintenance

All the bacterial strains, *Lactococcus lactis* NZ9700, *Kocuria rhizophila* ATCC9341, *Salmonella typhimurium* MTCC1251, *Escherichia coli* CFR02, *Enterococcus faecalis* MF3, *Staphylococcus aureus* MTCC96, were procured from IMTECH Chandigarh, India. *Listeria monocytogenes* Scott A was obtained from AK Bhunia Purdue University, USA. All the Drug resistant bacteria (aminoglycosides resistant bacteria) were our laboratory isolates [34]. All the LAB cultures obtained this study were grown in M17/MRS media at temperatures (15, 25, 30, 37 and 45 °C) under static condition. Food-borne pathogens were grown in BHI medium (37 °C shaking at 150 RPM) and the drug resistant lactic acid bacteria (LAB) were grown in MRS media (37 °C static condition). All the cultures used in this study were stored at − 20 °C in their cultivation media along with 40% glycerol.

Isolation of LAB Producing Bacteriocin

Different sources like *idly* batter, fermented fish, fermented soybeans, *Soibum* (fermented bamboo shoots), milk, *dosa* batter were used for the isolation of the bacteriocin producing LAB. These bacteriocin producing LAB were screened by overlaying the pour-plated LAB with the freshly grown indicator strain *Kocuria rhizophila* ATCC9341 as described by Nithya and Halami [12].

Antibacterial Activity Assay

The native isolates were grown in M17 broth (30 °C) for 16-18 h under static condition, followed by centrifuged at 8000 rpm for 10 min in 4 °C. Cell free supernatant (CFS) was collected and tested against indicator strain *K. rhizophila* ATCC 9341 by agar well diffusion methods as described by Nithya and Halami [12].

Chromogenic Plate Assay

The isolates which were showing antibacterial activity against *K. rhizophila* ATCC 9341 were further screened against the nisin specific reporter assay (*Lactococcus lactis* AUT1) with positive control *Lactococcus lactis* NZ9700, using the method as described by Burkard et al. [13]. The overnight grown (16 ± 2 h) native LAB isolates were spotted against reporter strain *L. lactis* AUT1, on the M17 agar plate supplemented with 50 µg/ml of X-Gal. The plates were incubated for 24–36 h at 30 °C until a blue coloration is observed around the point of inoculation of test culture on the lawn of reporter bacteria due to the induction of *lac*Z resulting in the production of β-galactosidase.

Bacterial Culture Identification

The bacterial cultures which were showing positive response to nisin specific reporter bacteria were subjected to detailed identification and characterization by conducting various tests as described in Bergey's Manual of Systematic Bacteriology [14]. Various microbiological tests carried out were Gram-staining, catalase and SEM analysis. Biochemical tests such as growth at different media (MRS and M17), temperature (15, 25, 30, 37 and 45 °C), pH values (5.5, 6, 6.5 and 7) and salt concentrations (1, 2 and 4%) were also performed. Media which is well suited for the highest production of bacteriocin was selected for further studies. Media with pH of 5.5, 6.0, 6.5 and 7 (adjusted by HCl or NaOH), also media with 1, 2 and 4% Sodium chloride (w/v) was prepared as described at Devi and Halami [15]. Antibacterial activity expressed as arbitrary unit per ml (AU/ml) and defined as the highest dilution of test sample exhibiting the zone of inhibition against the indicator *K. rhizophila* ATCC 9341.

Partial Purification of the Antimicrobial Compound

Extraction of antimicrobial compound from the CFS of native isolates was carried out initially by ammonium sulphate precipitation methods [16] followed by butanol extraction [17]. Ammonium sulphate was added to the CFS

to a different saturation level (0–80%) under constant stirring at 4 °C. The saturation point of ammonium sulphate was determined on the basis of maximum activity of the native isolates against the indicator organism *K. rhizophila* ATCC9341. The precipitate thus obtained was dissolved in phosphate buffer saline (pH 7.0) and was checked for its activity. Then the solution was mixed with butanol (1:1 v/v) and left at 4 °C under constant stirring for 1 h, followed by centrifugation and collection of upper layer. The butanol layer was lyophilized and the residue was further suspended in phosphate buffer (pH 7.0) and was tested for its activity.

Minimum Inhibition Concentration determination

All the native isolates of LAB were grown in M17 at 30 °C. Nisin like bacteriocin was concentrated using ammonium sulphate precipitation followed by butanol extraction, as described above. The lyophilized ppnisin thus obtained was dissolved in Mili Q water for further use. Partial purified nisin was tested against food-borne pathogen and drug resistant LAB. Minimum inhibition concentration (MIC) of ppnisin were determined against food-borne pathogens i.e. *Kocuria rhizophila* ATCC 9341, *Listeria monocytogenes* Scott A, *Enterococcus faecalis* MF3*, Staphylococcus aureus* MTCC96*, Escherichia coli* CFR02*, Salmonella thyphimurium* MTCC1251. Drug resistant bacteria used for these study were previously isolated LAB resistant to aminoglycosides: gentamicin (128–4096 µg/ml), kanamycin (128–4096 µg/ml) and streptomycin (128–4096 µg/ml) i.e. *E. faecalis* S1, *E. faecalis* MMO, *E. durans* CHB, *E. faecalis* 33-1, *E. faecalis* CSL2*, E. faecalis* MFO1*, E. faecalis* CSL3*, E. avium* CS31, *E. faecalis* A5, *E. faecalis* Chl(2), *E. faecalis* A40, *E. faecalis* 32-2 [34]. For MIC determination, food borne pathogens were grown in BHI broth at 37 °C under shaking condition and drug resistant LAB were cultivated in MRS broth at 37 °C under static condition. Next day, all the test cultures was diluted in a fresh medium till the OD_{600} value comes to 0.001. Different concentration of ppnisin were incorporated and test was carried out in 96 well ELISA plate, allowing FBPs and LAB to grow under shaking and static condition for 10–12 h. The OD600 was recorded using ELISA plate reader (Thermo Scientific). MIC was calculated wherein the lowest concentration of ppnisin (µg/ml) exhibited no growth of the indicator bacteria [17].

β-gal Induction Assay

For β-galactosidase activity, the ppnisin was use. Freshly grown reporter strains of *L. lactis* AUT1 were inoculated into 50 ml of M17 broth and allowed it to grow at 30 °C until it reached an OD_{600} values comes to 0.5. Different concentrations of ppnisin (0, 5 &10 µg/ml) of the native isolates was added in 2 ml each of the reporter strains and incubated for 1 h at 30 °C. Cell pellet was collected after incubation and resuspended in 1 ml of working buffer (20 mM β-mercaptoethanol, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCL and 1 mM MgSO4, pH 7.0) and assayed for β-galactosidase activity [17]. For statistical analysis, one-way analysis of variance was used (Microsoft excel) and statistical significant ($P < 0.05$). Each value represents the mean value ± SD from three measurements. Commercial nisin were used as positive control.

Identification of LAB by Whole Genome Sequencing

As from the study, the cultures which is more potent has been selected and subjected for whole genome sequencing at the facility of Genotypic Technology Pvt. Ltd., Bangalore. Whole genome sequencing was done and sequence homology was identified using BLASTN search tool. Multiple sequence alignment was performed by using Clustal W program [18]. The nucleotide sequences of the *Lactococcus lactis* ssp. *lactis* SP2C4 (*L. lactis* SP2C4), *Lactococcus lactis* ssp. *lactis* C2D (*L. lactis* C2D), and *Lactococcus lactis* ssp. *lactis* FS2 (*L. lactis* FS2) have been deposited at the GenBank database. ANI value has been measured using EzBioCloud. Phylogenetic analysis of whole genome sequence was done using EzBioCloud and Neighbor-joining phylogenetic tree with p-distance model was constructed by MEGA X software [35, 36].

Nucleotide Sequence and Accession Number

Accession number of *Lactococcus lactis subsp. lactis* 1O-1 (AP012281), 14B4 (CP028160), G423 (CP024958), IL1403 (AE005176). The complete genome sequence has been deposited in GenBank database under the accession number: *L. lactis* C2d (Accession: NZ_SAXH01000001), *L. lactis* FS2 (Accession: NZ_VANM01000001) and *L. lactis* SP2C4 (NZ_JACCJA010000000).

Results and Discussion

Screening of Nisin Producer (Lactic Acid Bacteria)

Overall 683 cultures have been isolated from various types of ethnic fermented foods. Out of this, 129 cultures are showing inhibition zone against *K. rhizophila* ATCC 9341 (Table 1). All the 129 isolates were showing zone of Inhibition in the range of 16–25 mm against *K. rhizophila* ATCC 9341. These positive cultures were checked for nisin producer by nisin- specific reporter bacteria (*L. lactis* NZ9800. AUT1) with positive control *L. lactis* NZ9700 [19]. Reporter bacteria is the genetically engineered bacteria which facilitate the easy and attractive tool for discovering novel classes

Table 1 Evaluation of nisin producing LAB by cell reporter assay which were obtained from fermented foods

Source	Bacterial Culture	Activity against *Kocuria rhizophila* ATCC 9341 (mm)	Mode of action by reporter *Lactococcus lactis* AUT1 (Nisin Producer)
Soya product	*Lactococcus* sp. SP2C1	16	+ + +
	Lactococcus sp. SP2C2	18	+ + +
	Lactococcus sp. SP2C4	20	+ + +
	Lactococcus sp. SP2C9	19	+ + +
Fermented fish	*Lactococcus* sp. FS1	17	+ + +
	Lactococcus sp. FS2	20	+ + +
Dosa batter	*Lactococcus* sp. 11	19	+ + +
Milk	*Lactococcus* sp. C2d	21	+ + +
Idly batter	*Lactococcus* sp. A6	17	+ + +
Soibum	*Lactococcus* sp. S16	16	+ + +
Positive	*Lactococcus lactis* NZ9700	16	+ + +

+ + + represent positive nisin producer

of cell wall inhibitors [20]. Among 129 cultures, 10 cultures were showing positive response to nisin specific reporter strain NZ9800. AUT1 (Table 1). All the 10 isolates showed positive results similar with control, indicating blue ring which is formed when reporter bacteria senses nisin at sub-lethal concentration in its environment produced by the test cultures and induce β-galactosidase (Supplementary Fig. I).

Spieβ et al. [19] reported that nisin specific reporter NZ9800. AUT1 (NZ9800 containing Pnz8148 P*nis*A-lacZ Cm^r) can be used for the rapid detection of nisin production and facilitate to measures auto-induction capacity. Also, Pascale et al. [20], reported that reporter bacteria (*B. subtilis* expressing *Enterococcus faecium Van*RS genes and a *Van*H-*lac*Z fusion) is a powerful tool for discovering antibacterial compound.

Characteristics of Native Isolates and Effect of Cultural Conditions on the Nisin Production

All the native LAB were Gram-positive, catalase negative and cocci in shape were established by SEM analysis (Supplementary Fig. 2). Nisin like bacteriocin production of all the LAB isolates were studied using different medium i.e. MRS, M17 and incubated at different temperature (15, 25, 30, 37 and 45 °C). It has been found that the production of nisin is high in M17 media as compared to MRS when kept at 30 °C. All the isolates were able to grow and produce nisin at all temperature, but the production is high at 30 °C, which might be due to the wider adaptability of LAB isolates to environment. It was observed that the best medium for the production of nisin is M17 and temperature at 30 °C which is selected for the further studies.

Following this, all the isolates were allowed to grow for 10, 20, 25, 30 h at static condition at 30 °C. It has been found that nisin production is high when incubated for 15-20 h. All the nisin producers strain were checked for the stability at different pH (5.5, 6, 6.5, 7) and different salt concentration (1%, 2%, 4%). It has been found that all the cultures retained its activity in the pH range from 5.5–7 and salt concentration up to 4%. The LAB isolate *Lactococcus* sp. SP2C1, *Lactococcus* sp. SP2C2, *Lactococcus* sp. SP2C4, *Lactococcus* sp. SP2C9, *Lactococcus* sp. FS1, *Lactococcus* sp. FS2 and *Lactococcus* sp. 11 were able to grow and produce high nisin at pH 6.5 whereas LAB isolates *Lactococcus* sp. C2d and *Lactococcus* sp. A6 were able to produce high nisin at pH 6 and LAB isolates S16 at pH 7. Also all the cultures except LAB isolates *Lactococcus* sp. 11 were able to grow and produce nisin at 4% salt concentration. The optimum production of nisin was found to be at temperature 30 °C pH 5.5–7 and NaCl 1–4%. The production of bacteriocin (nisin) is greatly influenced by the nutrients, temperature, time, pH, NaCl concentration [21]. It was reported that the production of nisin like bacteriocin by *Lactococcus lactis* is high when grown in M17 media at 30 °C and optimal pH is 6 [22]. In this study, it was found that optimal pH for higher production of nisin vary, these might be due to the sources from which the cultures has been isolated and their adaptability to the different environment [23]. Yang and Ray [24] reported that *Lactococcus lactis* grown under optimum environment for 16 h facilitates high bacteriocin production.

Partial Purification of Nisin-Like Bacteriocin

The nisin like bacteriocin produced by the test cultures was recovered by stepwise extraction procedures. Precipitation of bacteriocin using ammonium sulphate showed maximum activity of 20-26 mm against *K. rhizophila* ATCC 9341. That was much higher than the crude sample. Inhibitory activity of the nisin like bacteriocin at various saturation levels ammonium sulphate was checked and it was found that maximum activity is in the resolved precipitate with 50% saturation. Purification was further carried out by butanol extraction. Inhibitory activity of the Nisin like bacteriocin at 50% saturation levels of ammonium sulfate against *K. rhizophila* ATCC9341 is presented in Fig. 1 (a) followed by butanol extraction of all the LAB isolates shown in Fig. 1 (b), also ppnisin were checked by the nisin reporter, shown in Fig. 1 (c). In the past, ammonium sulphate and butanol has been successfully used for the precipitation and extraction of protein, respectively [16, 17].

The maximum ppnisin concentration recovered after partial purification of the LAB isolates *Lactococcus* sp. SP2C1: 556.34 IU/ml, *Lactococcus* sp. SP2C2: 598.22 IU/ml, *Lactococcus* sp. SP2C4: 648.16 IU/ml, *Lactococcus* sp. SP2C9: 608.16 IU/ml, *Lactococcus* sp. FS1: 770.22 IU/ml, *Lactococcus* sp. FS2: 728.04 IU/ml, *Lactococcus* sp. 11: 608.16 IU/ml, *Lactococcus* sp. C2d: 652.04 IU/ml, *Lactococcus* sp. A6: 508.16 IU/ml, *Lactococcus* sp. S16: 598.16 IU/ml and for the commercial nisin is 900 IU/ml.

Antibacterial Spectrum of Partially Purified Nisin Against Food Borne Pathogen (FBP) and Drug Resistant Bacteria

In this study, evaluation of ppnisin like bacteriocin against food-borne pathogens and aminoglycosides resistance bacteria was done, (Table 2). MIC of ppnisin of all the LAB isolates against *K. rhizophila* ATCC 9341 was found in the range 0.5–2.5 µg/ml*; List. monocytogenes* Scott A (15–42 µg/ml)*; E. faecalis* MF3 (55–92 µg/ml. However, higher concentration of ppnisin was required in case of *St. aureus* MTCC96 (> 1000 µg/ml), *Es. coli* CFR02 (> 5000 µg/ml) and *S. thyphimurium* MTCC1251 (> 5000 µg/ml) which might be due to mode of action against only Gram positive bacteria [25]. Thus the result obtained indicated that ppnisin of LAB isolates has a broad spectrum antibacterial activity and *Lactococcus* sp. SP2C4 and *Lactococcus* sp. FS2 were found to be more potent as compared to other isolates.

MIC of ppnisin of all the isolates were checked against aminoglycoside resistant LAB i.e. native LAB isolates resistant to apramycin, streptomycin, neomycin, amikacin, spectinomycin, kanamycin and gentamicin shown in Table 3. The MIC range of ppnisin of all the LAB isolates against *Enterococcus faecalis* S1 (15–45 µg/ml); *E. faecalis* MMO (35–75 µg/ml); *E. durans* CHB (1) (40–89 µg/ml); *E. faecalis* 33-1 (40–87 µg/ml); *E. faecalis* CSL2 (55–110 µg/ml); *E. faecalis* MF01 (100–135 µg/ml); *E. faecalis* CSL3 (70–98 µg/ml); *E. avium* CS31 (70–116 µg/ml); *E. faecalis* A5 (80–175 µg/ml); *E. faecalis* Chl (2) (15–35 µg/ml);

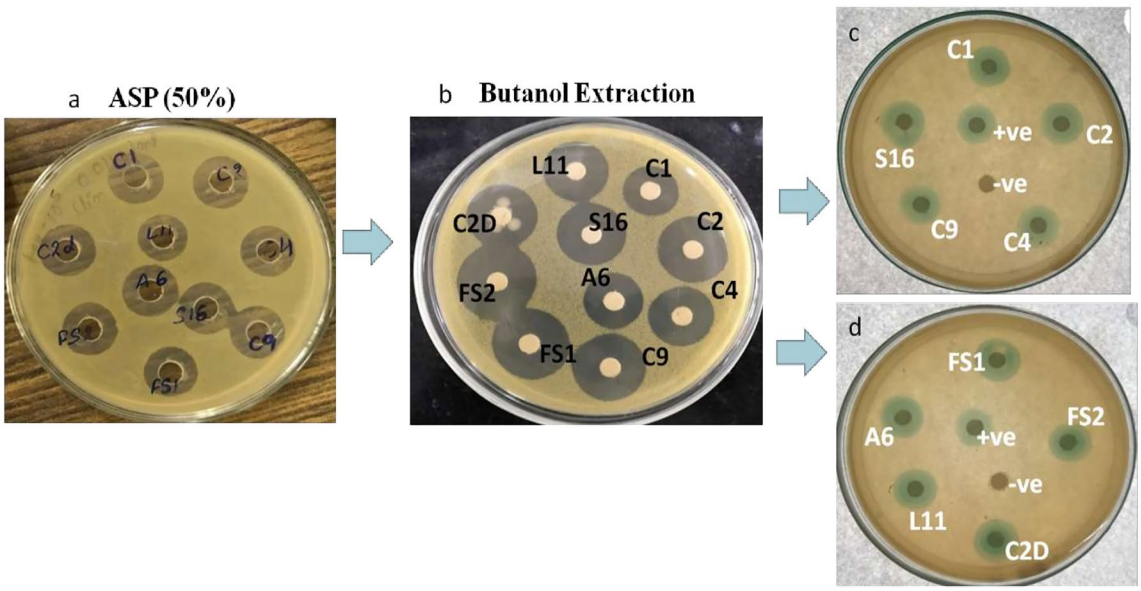

Fig. 1 **a** Antimicrobial activity of ammonium sulphate precipitate nisin against *Kocuria rhizophila* ATCC9341; **b** Antimicrobial activity of butanol extract of nisin against *Kocuria rhizophila* ATCC9341; **c** and **d** ppnisin were checked against nisin specific reporter AUT1

Table 2 Minimum inhibitory concentration of partially purified nisin against food borne pathogen

Bacterial cultures name	*Kokuria rhizophila* ATCC9341	*Listeria monocytogenes* Scott A	*Staphylococcus aureus* MTCC96	*Escherichia coli* CFR02	*Salmonella Thyphimurium* MTCC1251	*Enterococcus faecalis* MF3
Lactococcus sp. SP2C1	2	42	> 1000	> 5000	> 5000	80
Lactococcus sp. SP2C2	1.5	35	> 1000	> 5000	> 5000	65
Lactococcus sp. SP2C4	1.5	34	> 1000	> 5000	> 5000	92
Lactococcus sp. SP2C9	1	25	> 1000	> 5000	> 5000	84
Lactococcus sp. FS1	1.5	30	> 1000	> 5000	> 5000	85
Lactococcus sp. FS2	1	25	> 1000	> 5000	> 5000	64
Lactococcus sp. 11	2	40	> 1000	> 5000	> 5000	80
Lactococcus sp. C2d	1.5	28	> 1000	> 5000	> 5000	76
Lactococcus sp. A6	2.5	30	> 1000	> 5000	> 5000	70
Lactococcus sp. S16	2.5	38	> 1000	> 5000	> 5000	74
Commercial Nisin	0.5	15	> 1000	> 5000	> 5000	55

MIC is reported in µg/ml

E. faecalis A40 (15–45 µg/ml); *E. faecalis* 32-2 (15–50 µg/ml). The result obtained shows the MIC of ppNisin from all the LAB isolates has a broad spectrum of antibacterial activity against a wide range of drug resistant bacteria. Out of all the LAB isolates, MIC of ppNisin from *Lactococcus* sp. SP2C4, *Lactococcus* sp. C2D, *Lactococcus* sp. SP2C1 and *Lactococcus* sp. FS2 is higher. Antibacterial activity of ppnisin against drug resistant bacteria is reported for the first time in the study. Halami [17] reported that lantibiotics have the ability to inhibit the growth of drug resistance bacteria. Similarly, Fuchs reported that Lantibiotics have the ability to inhibit MRSA and *E. faecalis* [26]. Lantibiotic group of bacteriocin can be an alternative to conventional antibiotics because lantibiotic have a high potency and low toxicity, and nisin has obtained a GRAS status [4, 27].

β-Galactosidase Induction Assay of the Ppnisin

Lantibiotic group of bacteriocin bind to the undecaprenyl pyrophosphate and inhibit its dephosphorylation resulting in blocking its recycling and cell wall biosynthesis [28]. *L. lactis* NZ9800.AUT1 reporter strain are sensitive and more specific biosensor for Nisin like lantibiotics than any other biosensor available [19]. Thus, the native LAB isolates which exhibited positive results against *L. lactis* NZ9800.AUT1 were checked for the induction ability of β-Galactosidase activity against reporter strain *L. lactis* NZ9800.AUT1, which helped to differentiates nisin-like lantibiotics from other group of cell wall acting lantibiotics. All the ppnisin was checked for induction by β-gal activity against the reporter strain shown in Fig. 2.

All the ppnisin of LAB isolates exhibited β-Gal activity in the range 47.54–84.35 Miller units. Among all the ppnisin of the LAB isolates *Lactococcus* sp. SP2C1, *Lactococcus* sp. SP2C4 and *Lactococcus* sp. FS2 exhibited higher β-Gal activity of about 75–82 Miller Units. From the results, it was observed that all the isolates showed remarkable difference for its auto-induction activity to nisin-like lantibiotics. Bacitracin producing *Bacillus* spp. and other antibiotics were checked for the auto-induction ability and were found different mode of actions [28]. Spieß et al. [19] also reported the auto-induction ability of nisin-like lantibiotics especially for studying specificity with mutagenized nisin variants. Similarly, *Bacillus* isolates were checked for induction of β-Gal activity against reporter strain reported for their stress-based classifications [28].

Identification of LAB Isolates

The native LAB isolates *Lactococcus* sp. (C2D, SP2C4, FS2) which are showing more potent was selected and subjected for whole genome sequencing. It was found that, their sequences are showing high similarity with *Lactococcus lactis* subsps. *lactis.*

Phylogenetic tree was constructed based on the whole genome sequence to understand the phylogenetic relationship among *Lactococcus lactis* strains which showed a close relationship with *L. lactis* subsp. *lactis* 1O-1, 14B4, G423 and IL1403 shown in Fig. 3. This observation suggests that the isolates C2D, SP2C4 and FS2 belonged to *L. lactis* subsps. *lactis.*

We have examined the average nucleotide identity (ANI) value between *L.lactis* C2D, *L. lactis* SP2C4, *L. lactis* FS2 with that of *L. lactis* subsp. *lactis.* The ANI comparison between the genome of *L. lactis* FS2 with that of *L. lactis*

Table 3 Minimum inhibitory concentration of partially purified nisin against aminoglycosides resistant LAB

Cultures name	*E. faecalis* S1	*E. faecalis* MMO	*E durans* CHB (1)	*E. faecalis* 33–1	*E. faecalis* CSL2	*E. faecalis* MF01	*E. faecalis* CSL3	*E. avium* CS31	*E. faecalis* A5	*E. faecalis* Chl (2)	*E. faecalis* A40	*E. faecalis* 32–2
Lactococcus sp. SP2C1	35	50	75	52	95	120	92	95	150	26	25	50
Lactococcus sp. SP2C2	45	75	87	83	110	125	84	92	175	35	40	50
Lactococcus sp. SP2C4	32	50	56	54	83	105	86	86	120	25	25	30
Lactococcus sp. SP2C9	25	50	65	56	95	115	85	102	125	30	40	50
Lactococcus sp. FS1	41	62	83	86	90	128	90	116	140	30	42	50
Lactococcus sp. FS2	28	45	52	49	72	118	79	96	115	25	25	50
Lactococcus sp. 11	31	47	58	55	95	135	98	85	165	20	32	30
Lactococcus sp. C2d	35	47	52	52	76	100	80	90	175	28	30	30
Lactococcus sp. A6	32	55	83	87	80	130	90	98	115	22	35	40
Lactococcus sp. S16	37	73	89	84	70	125	98	87	135	35	45	40
Commercial Nisin	15	35	40	40	55	100	70	70	80	15	15	15

MIC is reported in µg/ml

subsp. *lactis* strains,1O-1 and 14B4 showed results of 98.86% and 98.03%, indicating that *L. lactis* FS2 belongs to the species *L. lactis* subsp. *lactis*. Similarly, *L. lactis* C2d showed 98.89% and 98.09% similarity with *L. lactis* subsp. *lactis* strains, 1O-1 and G423. Also, *L. lactis* SP2C4 showed 97.97% similarity with *L. lactis* subsp. *lactis* strains IL1403. This depict that *L. lactis* FS2, *L. lactis* C2D and *L. lactis* SP2C4 belongs to the species *L. lactis* subsp. *lactis*. It has been reported that Taxonomic affiliation of new genomes can be verified using average nucleotide identity analysis [29].

For the biosynthesis of nisin, cluster of several genes are involved. There are total 11 genes namely *nis*ABTCIPRKFEG which make up the *nis* operon. Each gene contributes toward the production of Nisin [30]. There are 10 variants of nisin reported till date which shows difference in their structural gene [31, 32]. In this study, we found *L. lactis* C2d and *L. lactis* SP2C4 harbor *nis*A gene and *L. lactis* FS2 harbor *nis*Q gene. Genes which are involved in NisinQ production are clustered in the order *nis*QBTCIPRKFEG, this cluster were aligned with the *L. lactis* 61–14 which were known to produce NisinQ, [11]. Beside its similarity, source of isolation is different as compared to the Nisin Q producer *Lactococcus lactis* 61–14, which has been isolated from the river water in Japan and Nisin Q producer in this study has been isolated from fermented fish. Also gene which are involved in NisinA production are clustered in the order *nis*ABTCIPRKFEG, this cluster was aligned with the *Lactococcus lactis* subsps. *lactis* CV56 and *Lactococcus lactis* subsps. *lactis* NCDO895 which are known to produce nisin A [33]. *Lactococcus lactis* subsps. *lactis* CV56 has been isolated from healthy women and *Lactococcus lactis* subsps. *lactis* NCDO895 from dairy starters and *Lactococcus lactis* SP2C4 and C2D were isolated from fermented soyabean and cow milk, respectively, although the isolates are showing high similarity, the source of isolation is different.

Conclusion

The identified bacteriocin sources are unconventional than marketed products and considered as natural antibiotic or lantibiotics. All the identified nisin-like bacteriocin showed antimicrobial effects against food-borne pathogen and certain drug resistant bacteria hence can be used for bio-preservation of food and health products. Nisin Q is more potent among all identified Nisin-like bacteriocin so further characterization should be required as bio-preservative. Apart from these, other than aminoglycoside resistant bacteria should be considered as drug resistant species for checking the

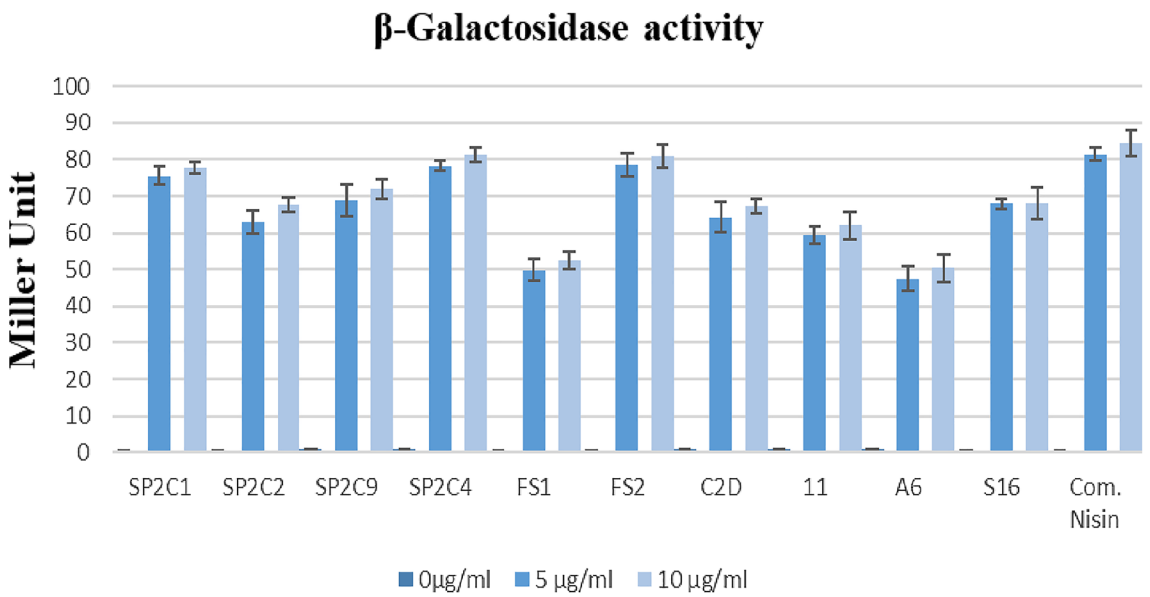

Fig. 2 β-Gal activity of ppnisin of all the LAB isolates against nisin specific reported starin. Error bars represent the SD of 10 separate ppnisin measure in triplicate. Commercial Nisin as a control and negative, bacteriocin added

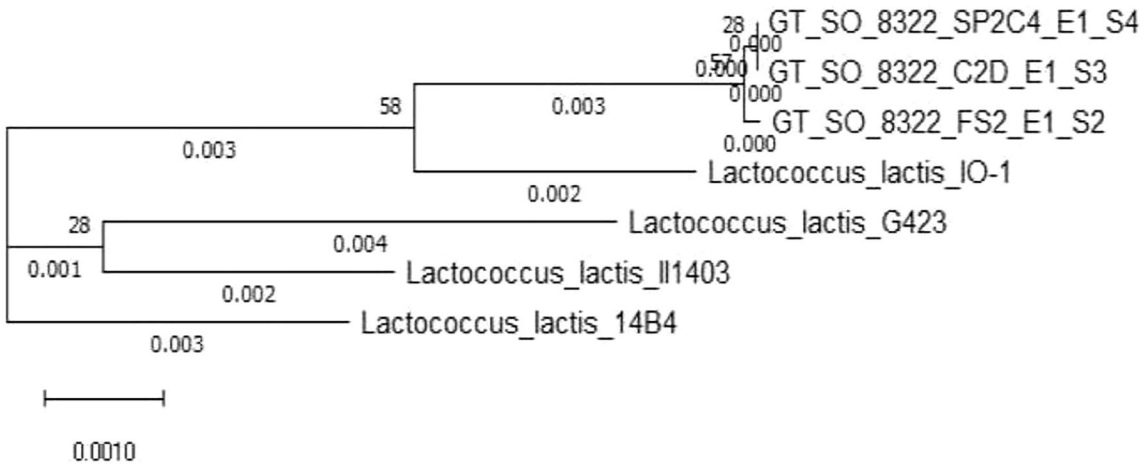

Fig. 3 Construction of phylogenetic tree using EzBioCloud by Neighbor-joining method for complete genome sequence

antimicrobial effect of nisin-like bacteriocin. The present study can be the first milestone for bio-preservation techniques in all ecosystems.

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