ANTIMICROBIAL ACTIVITY OF NISIN PRODUCED BY *LACTOCOCOCCUS LACTIS* SUBSP. *LACTIS* AGAINST MULTI DRUG-RESISTANT ORAL PATHOGENS.

Dr. Mukta Sharma

Professor, Department of Microbiology,

SBB Dental College & Research Centre, Ghaziabad (UP) India.

Abstract:

The development and spread of pathogenic bacteria that are resistant to the existing catalog of antibiotics is a major public health threat. Biofilms are complex, sessile communities of bacteria embedded in an organic polymer matrix which serve to further enhance antimicrobial resistance. Consequently, novel compounds and innovative methods are urgently required to arrest the proliferation of drug-resistant infections. Nisin is a lantibiotic widely used for the preservation of food and beverages. Recently, investigators have reported that nisin may have clinical applications for treating bacterial infections. The aim of this study was to investigate the effects of Nisin on multi drug resistant oral bacteria, isolated from human oral cavities. Chlorhexidine resistant *Streptococcus mutans*, penicillin resistant *Streptococcus sanguinis* and *Streptococcus gordonii*, tetracycline resistant *Streptococcus mitis*, metronidazole resistant *Lactobacillus acidophilus* and *Actinomyces israelii* were isolated from supragingigival biofilms. The minimum inhibitory concentrations and minimum bactericidal concentrations of taxonomically distinct oral bacteria were determined using agar and broth dilution methods. Nisin inhibited planktonic growth of oral bacteria at low concentrations (2.5–50μg/ml). Accordingly, it has been suggested that antimicrobial peptides could be used as novel natural inhibitors that can be used in formulations with synergistically acting antibiotic. This work highlights the potential therapeutic value of food grade nisin to inhibit the growth of oral bacteria and the development of biofims relevant to oral diseases.

Keywords: Nisin, Lactococcuslactis subsp. lactis, multi drug resistant, oral pathogens, biofilms.

Introduction:

The emanation and worldwide spread of multidrug resistant Gram-positive pathogens has commenced a search for alternative chemotherapeutic agents against these pathogenic bacteria. One class of agents that has received increasing attention recently is the Nisin, Nisin is a bacteriocin produced by a group of Gram's positive bacteria that belong to the Lactococcusand Streptococcus species. Over the past few decades, nisin has been used widely as a food biopreservative. Since then, many natural and genetically modified variants of nisin have been identified and studied for their unique antimicrobial properties.

Nisin belongs to a group of cationic peptide antimicrobials collectively called Type A(I) lantibiotics (Smith and Hillman, 2008). Nisin and other lantibiotics have gained considerable attention due to their potent and broad spectrum activity, low likelihood of promoting the development of bacterial resistance and low cellular cytotoxicity (Shin et al., 2015). In addition, researchers from interdisciplinary fields have bioengineered newer forms of nisin variants that have therapeutic potential for human diseases (Field et al., 2015).

In the present study, we isolatedchlorhexidine resistant Streptococcus mutans, penicillin resistant Streptococcus sanguinis and Streptococcus gordonii, tetracycline resistant Streptococcus mitis, metronidazole resistantLactobacillus acidophilus and Actinomycesisraelii.All the bacterial strains were isolated from supragingigival biofilm of the patients of Shree Bankey Bihari Dental College and Research Centre, Ghaziabad. All the oral isolateswere tested for the sensitivity to nisin, which was characterized from the isolated strain of Lactococcuslactissubsplactis CCSU1011 in a previous study.

Materials and Methods:

Bacterial Strains and Growth Conditions: Nisin producing Lactococcuslactis subsp. lactisCCSU 1011 was procured grown on modified MRS Agar. This study used Chlorhexidine resistant Streptococcus mutans,Penicillin resistant Streptococcus sanguinis and Streptococcus gordonii, Tetracycline resistant Streptococcus mitis, Metronidazole resistantLactobacillus acidophilus and Actinomycesisraelii.All the bacterial strains were isolated from supragingigival biofilm of the patients of Shree Bankey Bihari Dental College and Research Centre, Ghaziabad. These isolated strains were identified using different morphological, biochemical and molecular parameters and were grown on Brain Heart Infusion agar (BHI Agar, HiMedia Laboratories, and India) and cultured in BHI broth media.

All bacterial species mentioned above were incubated at 37°C under appropriate atmospheric conditions. For each strain, a single colony was inoculated into 5 ml of culture medium (as indicated above) and incubated to exponential growth phase. For use in experiments, the optical density at 600 nm (OD) of each culture was adjusted approximately to 0.15 to correspond to a bacterial concentration of 10^9 CFU/ml in culture medium.

Nisin and nisin derivatives purification: Nisin and nisin derivatives were purified according to previously described protocols (M Sharma, 2017). Thebacteriocin producing strain Lactococcuslactis subsp. lactisCCSU 1011 was grown under optimized conditions. Cells were removed by centrifugation at 5,000 x g for 30 min at 4°C temperature, and the supernatant was filtered through a 0.45 µm pore size membrane filter. It was defined as cell free filterate (CFF). Ammonium sulphate was added to achieve 90 % saturation and allowed to precipitate for 24 h at 4°C. Precipitate was collected by centrifugation at 10,000 x g for 20 min and dialyzed on magnetic stirrer using a membrane with a molecular cut-off of 10,000 Dalton against 10 mM phosphate buffer (pH 7.0) for 72 h at 4°C temperature with frequent changes of fresh buffer. The nisin was then freezed and stored at -20°C for further use.

Dialysate was applied to the carboxymethyl- celluolose column which had been equilibrated with 10 mM glycine NaOH buffer (pH 8.0). After the column was washed in 10 mM glycine NaOH buffer (pH 8.0), the bacteriocin was eluted by a step gradient of 50, 100, 200, 400 mMNaCl in the same buffer. Fractions of 2ml were collected at a flow rate of 0.2 ml/min. The resulting

protein was concentrated and then loaded onto Sephadex G-50 column which was equilibrated with 10 mM phosphate buffer (pH 7.0). The sample was eluted with the same buffer at the flow rate of 0.05 ml/min. The active fractions were stored at -20°C.The purified peptides were subjected to mass spectrometric analysis to confirm their purity before use.

MIC and MBC of Oral Bacteria: The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of nisin against oral bacterial strains were determined using the Clinical and Laboratory Standards Institute (CLSI) standards with slight modifications as described below (Wiegand et al., 2008). A total volume of 200 μl with different concentrations of nisin and the bacterial culture suspended in BHI broth medium was added to a 96-well microplate. Of the total volume, each well contained 150 μl of bacterial culture and 50 μl of nisin. As mentioned above, the initial optical density of the bacterial cultures were calibrated approximately to 0.15 (OD600) to achieve 10^9 CFU/ml. The final working concentrations of nisin were 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 15, 25, 50, 100, 200 μg/ml. The microplates were incubated at 37°C for 24 h. The determined MIC was the lowest concentration of nisin that inhibited the visible growth of bacteria compared to the zero time point, indicated by an increase (≤0.05) in optical density (OD600). For determination of MBC, 10 μl of these bacterial samples were removed from wells that had bacterial concentrations equivalent to and higher than the MIC, and inoculated on appropriate agar plates. The MBC was defined as the lowest concentration of nisin that killed at least 99.9% of the bacteria in a given time.

Results and Discussion:

Procurement of nisin producing strain : Nisin producing Lactococcuslactis subsp. lactis CCSU1011 was procured from the Microbial culture collection of Department of Microbiology, CCS University, Meerut. This nisin producing strain was previously isolated by the researcher and was maintained in the culture collection centre of the university. This strain was sub-cultured and characterized before the study.

Partially purified Nisin : The bacteriocin produced by Lactococcuslactis subsp. lactisCCSU 1011 was precipitated using ammonium sulphate (90% saturation) followed by dialysis. The dialyzed fraction yielded 11.76% (approx) protein which showed two fold increase in bacteriocin activity. Resuspendednisin was applied to a carboxymethyl cellulose cation exchange column (equilibrated with glycine NaOH buffer, pH 8.0) and eluted by a step salt gradient 50,100,200,400 mMNaCl where the purity increased 68 fold while the nisin activity enhanced only 4 fold. The fractions which showed nisin activity were collected and then applied onto a Sephadex G-50 column. Active fractions were collected again and defined as partially purified nisin. After gel filtration, the yield of nisin was about 2.35% (85 fold purity) but the specific zactivity was enhanced to 128000 AU/mg from the initial activity of 1505 AU/mg. This purified nisin was used to check the antibacterial activity against oral bacteria.

Antimicrobial Activity of Nisin on Oral Biofilm Colonizers : The MICs and MBCs of nisin on oral bacterial species are listed in Table 1. The MIC and MBC of nisin ranged from 2.5 to 50 μg/ml and 15 to 200 μg/ml, respectively (Table 1)

Chlorhexidine resistant Streptococcus mutans, a cariogenic Gram-positive bacteria, showed less sensitivity to nisin when compared to the two penicillin resistant commensal organisms Streptococcusgordonii and Streptococcussanguinis (Figure 1; Table 1). Although, a similar study
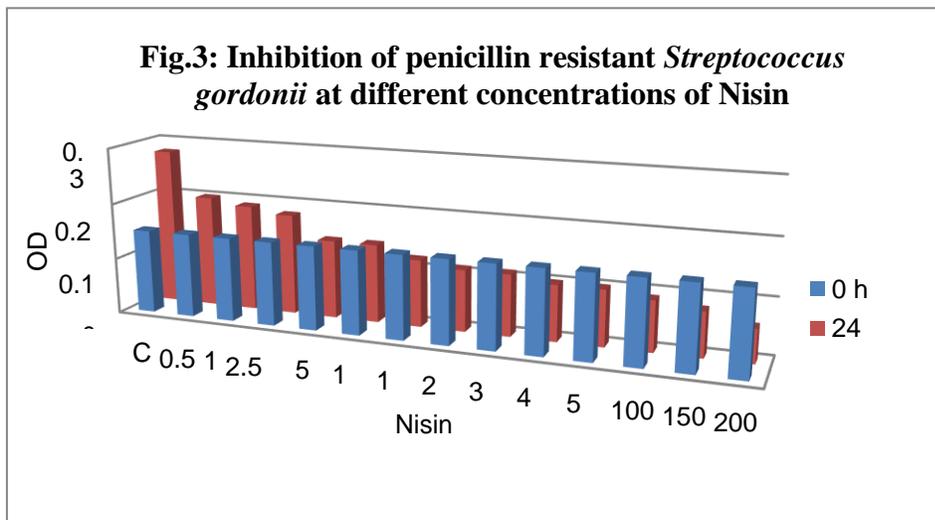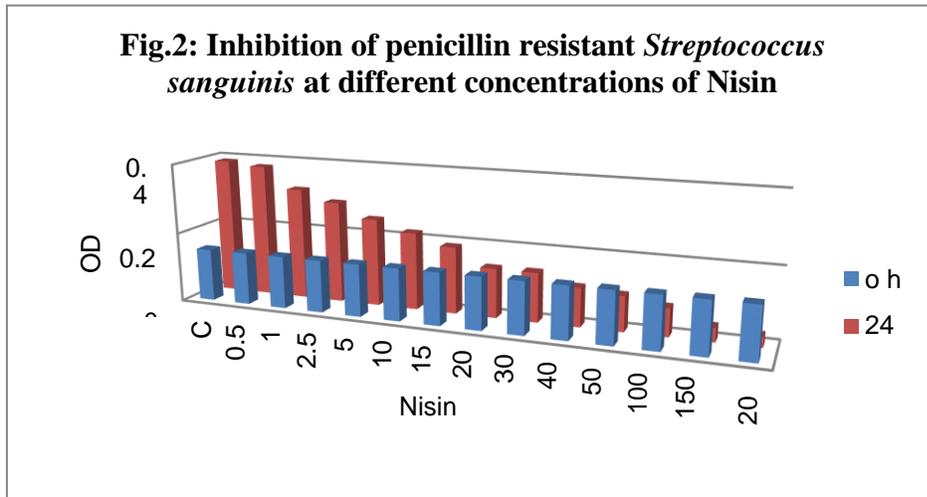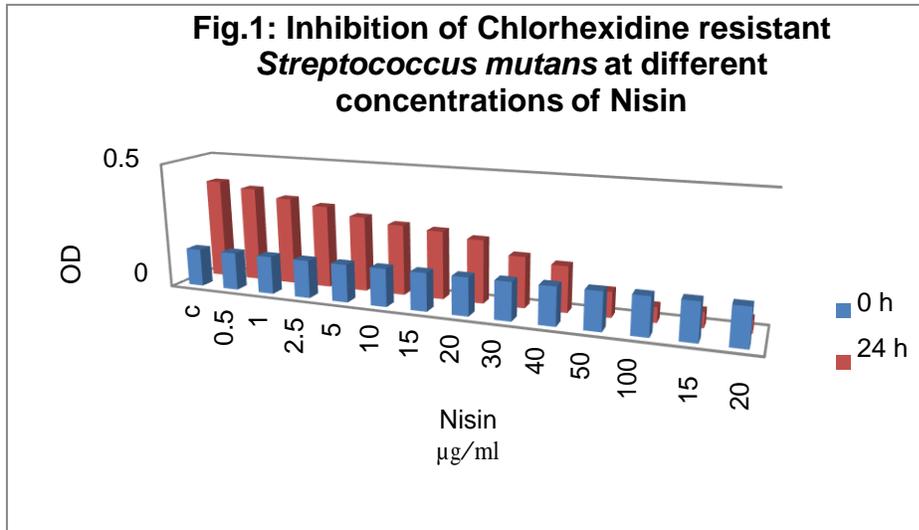
showed the inhibition of Streptococcus mutans with MIC 20 µg/ml, but this isolated chlorhexidine resistant S. mutans showed the MIC 40 µg/ml.Tong and colleagues demonstrated that nisin A can inhibit the growth of cariogenic bacteria, including Streptococcus mutans. Scanning electron microscopy confirmed that nisin exerted bactericidal activity by forming small pores on the surface of cells (Tong et al., 2010).
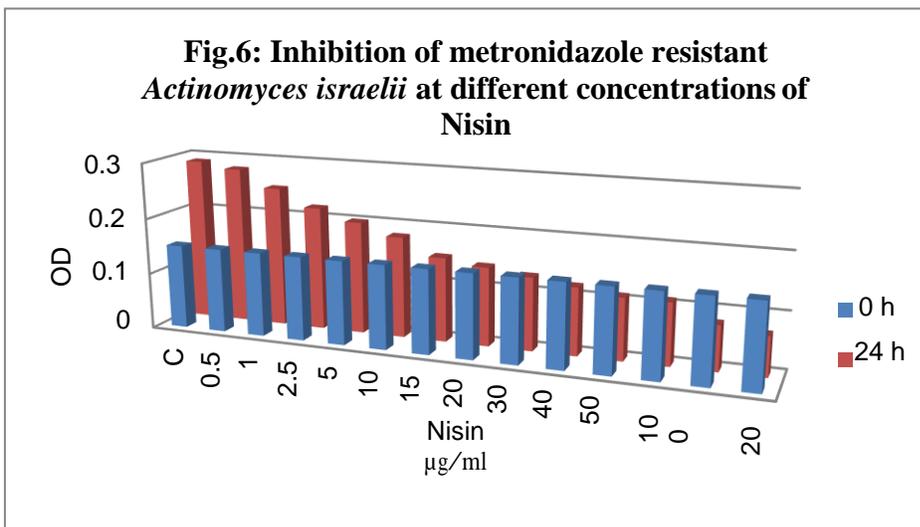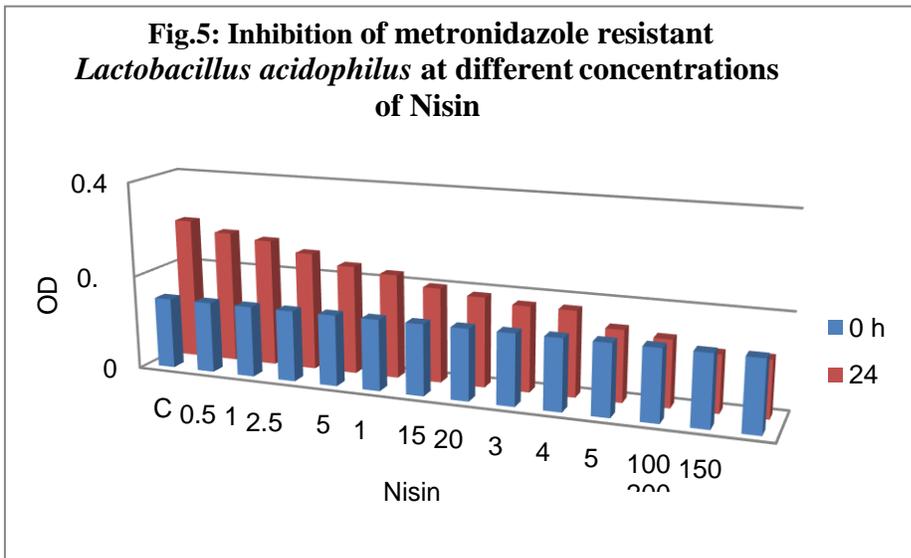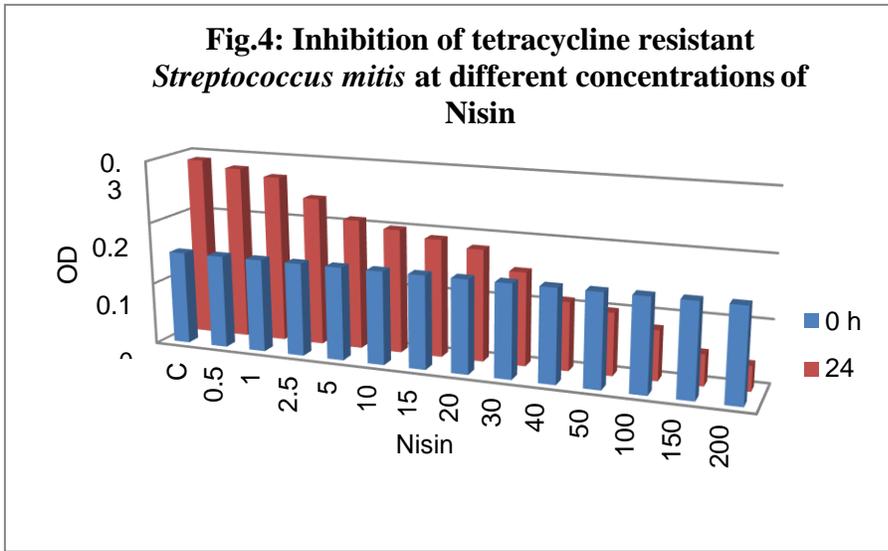
Penicillin resistant Streptococcus sanguinis and Streptococcus gordonii showed more sensitivity to nisin as compared to S. mutans, with MIC 15 µg/ml and 2.5 µg/ml, respectively (Fig.2&3). Present study investigated that penicillin resistant Streptococcus gordonii was the most sensitive strain to nisin.Tong et al., also reported that nisin A has been shown to inhibit the growth of Gram-positive oral bacteria such as Streptococcus sanguinis, Streptococcus sobrinus and Streptococcus gordonii (Tong et al., 2010). In addition, nisin also exerted antimicrobial activities against the tetracycline resistant Streptococcus mitis with MIC 20 µg/ml, which was isolated from a chronic dental caries (Fig.4). Metronidazole resistant Lactobacillus acidophilus(Fig.5) and Actinomycesisraelii(Fig.6) demonstrated the MIC 50 µg/ml and 10 µg/ml respectively. A. israelii, a saprophytic component of the endogenous flora of the oral cavity, cause a suppurative granulomatous, inflammatory lesions was the second most nisin sensitive strain of the present investigation.

Table.1: Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of antibiotics resistant oral isolates.

S.N.	Antibiotic resistant oral isolates	MIC	MBC	MBC/MIC
1	Chlorhexidine resistant Streptococcus mutans	40	150	3.75
2	Penicillin resistant Streptococcus sanguinis	15	100	6.67
3	Penicillin resistant Streptococcus gordonii	2.5	15	6
4	Tetracycline resistant Streptococcus mitis	20	100	5
5	Metronidazole resistant Lactobacillus acidophilus	50	150	3
6	Metronidazole resistantActinomycesisraelii	10	40	4

MBC/MICratioof >4 indicatesthatnisinhasabacteriostaticeffect.MBC/MICratio of <4 indicatesthatnisinhasabactericidaleffect(PankeyandSabath,2004).

Fig.1: Inhibition of Chlorhexidine resistant *Streptococcus mutans* at different concentrations of Nisin

Fig.2: Inhibition of penicillin resistant *Streptococcus sanguinis* at different concentrations of Nisin

Fig.3: Inhibition of penicillin resistant *Streptococcus gordonii* at different concentrations of Nisin

Fig.4: Inhibition of tetracycline resistant *Streptococcus mitis* at different concentrations of Nisin

Fig.5: Inhibition of metronidazole resistant *Lactobacillus acidophilus* at different concentrations of Nisin

Fig.6: Inhibition of metronidazole resistant *Actinomyces israelii* at different concentrations of Nisin

Discussion:

Our data demonstrate that nisin had a wide and powerful bactericidal effect often accompanied by rapid lysis against a large panel of Gram-positive bacterial pathogens deliberately selected oral pathogens which include some widespread multi drug resistant oral pathogens, such as chlorhexidine resistant Streptococcus mutans,penicillin resistant Streptococcus sanguinis and Streptococcus gordonii, tetracycline resistant Streptococcus mitis andmetronidazole resistant Lactobacillus acidophilus and Actinomycesisraelii. All these drug resistant bacteria were isolated from the Outpatient Department of the SBB Dental College, Ghaziabad.The data presented here demonstrate the potential for nisin as an antimicrobial and anti-biofilm agent against oral pathogens. The peptide structure of nisin is characterized by the presence of five intra-molecular rings formed by the thioether amino acids lanthionine and 3-methyllanthionine (Wiedemann et al., 2001). Due to its unique chemical features, it has been hypothesized that nisin has various modes of antimicrobial action (Peschel and Sahl, 2006) and exerts multiple antimicrobial activities based on the interaction with multiple cellular targets (Bierbaum and Sahl 1985; Peg and Sahl, 2002)

However, when used in a clinical setting, there is potential risk of developing nisin resistance.There have been a few examples of lantibiotic resistance noted in laboratory settings, where certain bacteria have been reported to possess innate anti-lantibiotic mechanisms. For example,nisinase is a dehydro peptide reductase that can inactivate nisin (de Freire Bastos et al., 2014; Draper et al., 2015). Nisinase activity has been associated with Lactococcus lactis sub-species cremoris, Enterococcus faecalis, and Staphylococcus aureus (Carlson and Bauer, 1957) Lactobacillus plantarum (Kooy, 1952), Streptococcus thermophilus (Alifax and Chevalier,1962), Clostridium botulinum (Rayman et al., 1983),. Thus, future characterization of specific genetic or protein components that may contribute to nisin resistance is needed to understand any potential resistance issues in clinical settings.

Although all the multi drug resistant oral isolates were sensitive to nisin, but Streptococcus gordonii was the most sensitive strain requiring only 2.5 µg/ml to inhibit the growth, followed by Actinomyces israelii which require 10 µg/ml of nisin. Among all the isolates least sensitive strain was Lactobacillus acidophilus. In our study, nisin at low concentrations <10 μg/ml inhibited the growth of these periodontal pathogens. (Table.1). Thus, our findings suggest that nisin can inhibit the growth of multi drug resistant oral pathogens. The work presented here demonstrates that nisin is a promising candidate for development as an oral therapeutic anti- biofilm agent. Further investigation of the clinical role of nisin in modulating the microbiome of the biofilm community and its immunomodulatory role in human oral cells are necessary to determine its potential as a therapeutic or prophylactic agent against oral diseases.

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