Investigating the antimicrobial effects of encapsulated Nisin on the growth and binding ability of *Streptococcus mutans* bacteria

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Abstract

Introduction: Nisin is an antimicrobial peptide consisting of 34 amino acid residues. Due to the presence of lanthionine amino acids, it is classified as a lantibiotic and demonstrates effectiveness against numerous pathogenic microorganisms. Although its effectiveness is greatly reduced in various clinical and environmental settings due to its reaction with other compounds. New methods may be able to solve this problem in microcapsules and control their release. The use in toothpaste helps control factors that contribute to tooth decay, one of the most common diseases that humans face in their lifetime. The aim of this study was to investigate the effects of free and encapsulated nisin on *Streptococcus mutans* and its growth kinetics.

Method: Nisin was encapsulated in lecithin and alginate liposomes using the thin-layer hydration method. The size and distribution of nisin microliposomes were determined using scanning electron microscopy and Dynamic Light Scattering (DLS) techniques. To determine the minimum inhibitory concentration (MIC) of nisin microliposomes and free nisin on *S. mutans* at various concentrations ranging from 0.78 to 25 µg/ml. To investigate the growth kinetics, the growth rate or inhibition of *S. mutans* was studied by exposing it to concentrations equivalent to the MIC over a 50-hour period at a wavelength of 600 nm. The study compared the effects of nisin and nisin microcapsules to those of the antibiotics amoxicillin and penicillin.

Results: Microencapsulated nisin reduced the growth of planktonic strains of *S. mutans* more effectively than free nisin. Nisin microliposomes at a concentration of 6.25 µg/ml inhibited the growth of *S. mutans*, whereas the inhibitory effects of free nisin were observed at a concentration of 12.5 µg/ml in both clinical and standard strains. Additionally, there was a significant reduction in the production of lactic acid by *S. mutans*.

Conclusion: Based on the current findings regarding the inhibitory effects and durability of nisin micro encapsules, it is plausible that this compound could be utilized in combination with other methods to control tooth decay and enhance the anti-caries efficacy.

Keywords: Microliposome, Nisin, growth inhibitory effects, Oral pathogens, *Streptococcus mutans*.

Introduction

Streptococcus mutans is a type of bacteria commonly found in the human mouth. It is known for its role in causing dental cavities by breaking down sugars in the mouth and producing acids that can erode tooth enamel (Bowen et al., 2018). This bacterium thrives in a low-pH environment, which is why acidic foods and drinks can contribute to tooth decay. Research has shown that *S. mutans* is particularly adept at forming biofilms on teeth, making it difficult to remove through regular brushing and flossing (Aleti et al., 2015). Biofilms provide a protective barrier for the bacteria, enabling them to persist in producing acids and causing damage to the teeth. Preventing the growth of *S. mutans* is crucial for maintaining good oral health (He et al., 2019; Kazeminia et al., 2020). This can be achieved through regular brushing and flossing to remove plaque, as well as by limiting the intake of sugary foods and drinks. Additionally, using fluoride toothpaste and receiving regular dental cleanings can help reduce the risk of cavities caused by this bacterium (Ansari et al., 2017; Bowen et al., 2018). In recent years, researchers have been exploring alternative methods for controlling *S. mutans*, such as probiotics and antimicrobial agents. Probiotics containing beneficial bacteria can help restore a healthy balance in the mouth (Wu et al., 2020), while antimicrobial agents can target and eliminate harmful bacteria such as *S. mutans*. Understanding the role of *S. mutans* in tooth decay is crucial for maintaining optimal oral health. By practicing good dental hygiene habits and exploring new methods to control this bacterium (Petersen & Ogawa, 2016), individuals can reduce their risk of developing cavities and other dental problems.

Nisin is a polypeptide bacteriocin produced by certain strains of the Gram-positive bacterium Lactococcus lactis. It is a natural (Wei et al., 2006), food-grade antimicrobial agent that has been used as a preservative in various food products. Nisin is effective against a wide range of Gram-positive bacteria (van Staden et al., 2012), including food spoilage bacteria and pathogenic bacteria such as *Listeria monocytogenes* and *Clostridium botulinum*. The antimicrobial activity of nisin is attributed to its capacity to target the bacterial cell membrane, forming pores that result in cell leakage and ultimately cell death (Linville et al., 2021).

Capsulated nisin refers to nisin that has been encapsulated or formulated with a delivery system, such as liposomes (Radaic et al., 2022), nanoparticles (Shin et al., 2016), or emulsions (Gao et al., 2022). The encapsulation of nisin can enhance some of its characteristics, such as protecting nisin from degradation by environmental factors like heat, pH, and proteases, thereby improving its stability and shelf-life (Zainodini et al., 2018). Additionally, enhanced antimicrobial activity can be achieved through encapsulation. This process can enhance the delivery of nisin to the target site, resulting in higher local concentrations and increased

antimicrobial efficacy (Radaic et al., 2020). Furthermore, encapsulation can regulate the release rate of nisin, enabling sustained or targeted delivery, which can be advantageous in specific applications (Quichaba et al., 2023). Both free nisin and encapsulated nisin have been shown to exhibit strong antimicrobial activity against a variety of Gram-positive bacteria, including food spoilage and pathogenic microorganisms (Shin et al., 2016). Therefore, the evaluation of the antimicrobial effects of encapsulated nisin against another Gram-positive bacterium is one of the primary objectives of this research. *S. mutans* was chosen as a significant pathogen in oral healthcare.

Material and Methods

Bacterial Strain and Growth Conditions

A standard bacterial strain of *Streptococcus mutans* PTCC-1683 from the Iran Scientific and Industrial Research Organization (PTCC) and a pathogenic strain isolated from an oral sample were identified and obtained using biochemical and microbiological tests. Bacteria were grown and maintained on blood agar plates (Quelab, Canada).

Preparation of Nisin

Nisin powder was purchased under the brand Pimaripro. Subsequently, the powder was dissolved in sterile distilled water and sterilized using a 0.45 μm microbiological filter. To prepare a nisin solution, first weigh 10 mg of nisin powder using a sensitive scale. Then, add 3% v/v glycerol, polyethylene, and phosphate buffer with pH levels of 6, 5.5, and 7.4. Finally, add 5 ml of the nisin solution. The mixture was then placed on a magnetic stirrer set at a speed of 500 rpm in a bain-marie at a temperature of 40°C (Narsaiah et al., 2014).

Nisin Encapsulation

Encapsulation of nisin in lecithin and alginate liposomes (Mertins et al., 2008) was achieved using the thin-layer hydration method. Briefly, 0.076 g of lecithin was dissolved in 10 mL of chloroform in a round-bottomed flask, and the organic solvent was evaporated to form a thin layer on the flask wall. Traces of organic solvents were removed by placing the sample in an oven for 18 hours. The dried lipid film was dispersed by adding phosphate buffer containing nisin. Then these mixtures were heated above their phase transition temperature (60°C). Sonication, aimed at reducing the size and homogenizing the liposomes, was carried out using a sonicator (40 kHz, Elma) for 30 minutes. The size of nanovesicles was determined using light scattering (DLS) and electron microscopy (Dominy et al.) (Teixeira et al., 2014).

Determination and Distribution of Particle Size

It was done by a particle size measuring device. The average size of the prepared particles, based on the volume diameter, was determined by this device after one hour of storage at a temperature of 4°C.

$$D[4,3] = \frac{\Sigma n_i - d_i^4}{\Sigma n_i - d_i^3}$$

di= particle diameter

D [4,3] = average volume diameter (average volume equivalent)) ,Teixeira et al., 2014(

The particle size distribution was also calculated using the following equation:

$$\text{Span}= \frac{D(90\%)-D(10\%)}{D\ (50\%)}$$

Determining the antimicrobial properties of nisin microcapsules

Effect of Free and Encapsulated Nisin

To determine the minimum inhibitory concentration of nisin microcapsules against the growth of clinical and standard *S. mutans*, the modified microdilution method (MIC) recommended by CLSI in a microplate was investigated (Jain et al., 2020). In this method, 96-well polystyrene plates were used. In this method, dilutions of 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 µg/ml were prepared from a microliposome stock. Subsequently, 20 microliters of a 0.5 McFarland suspension of prepared microbial strains in Mueller Hinton Broth culture medium were added to each well of the microplate. The contents were thoroughly mixed with the liquid inside the well, followed by the addition of 50 microliters of the sample to the side column and mixed. Afterward, the microplate was incubated for 18 hours at 35°C. Following this incubation period, the value of each concentration was measured using a microplate reader to determine the Minimum Inhibitory Concentration (MIC). The minimum microliposome concentration at which the optical density (Hagiwara et al.; Hagiwara et al., 2006) decreased was considered the minimum inhibitory concentration (MIC).

In this study, control sample absorption (negative control: including 5 µl of bacteria + 195 µl of broth culture medium), sample absorption (difference in turbidity absorption of the treatments that include nisin nanoliposomes and free nisin in the culture medium before incubation and turbidity after incubation), and the following formula were used to calculate the inhibition percentage.

$$\frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

In the formula above, the absorption of the control sample was (A blank) and the absorption of the treated sample was (A sample).

Effect of Free and Encapsulated Nisin on Growth Kinetics

Free and encapsulated nisin (0.5 mg/mL) was added to tubes containing 10 mL of Tryptic Soy Broth (TSB). Bacterial cells were added to achieve an initial count of 10^4 CFU/ml. For standard conditions and the treatment of each active substance, the tubes should be incubated for 0, 2, 4, 8, 24, 30, and 48 hours at 35°C. From 2 to 8 hours of incubation (every 2 hours), and every 4 hours thereafter, the control and treated samples were measured for the density of bacterial cells at 600 nm using a spectrophotometer. To evaluate the impact of free and completely enclosed nisin, 500 microliters from the tubes were transferred to sterile plates. Mueller Hinton Agar was then added to the culture medium, which was incubated for 18 hours at 35°C. The number of grown colonies was counted and compared with the control sample (growth without nisin microcapsules treatment), and the results were reported as CFU/ml (Kumar et al., 2022). Amoxicillin and penicillin G antibiotics were also found to be effective against *S. mutans* in this research study.

Antibiofilm Assay

The Crystal Violet (CV) assay was utilized to assess the inhibition of biofilm formation. After an overnight culture of bacteria in TSB, the suspension with an OD600nm of 1.28 at a concentration of 1% (v/v) was inoculated into TSB broth containing 1% sucrose. A 96-well plate was prepared with serially diluted bound and free nisin treatments starting from 12.5 µg/ml. The total volume was adjusted to 150 µL per well by adding the *S. mutans* mixture. Water and 0.01% chlorhexidine were added to the negative and positive control groups, respectively. Plates were cultured at 35°C and incubated in 5% CO_2. After 72 hours, the plates were washed with PBS and fixed with ethanol for 30 minutes. After washing with PBS, the microplate wells were incubated with 0.1% crystal violet for 20 minutes, then washed with water, and dissolved in ethanol. The optical density (Hagiwara et al., 2010) at 595 nm was measured to quantify biomass. This process was repeated three times, and the results were reported as the average absorption.

Results

Investigating the production of nisin microliposomes by DLS method

The size distribution of nisin liposomes in terms of dispersion percentage is shown in Figure 1. Liposomes loaded with nisin had an average diameter of 1100-1700 nm. The entrapment efficiency of nisin in liposomes was 87%.

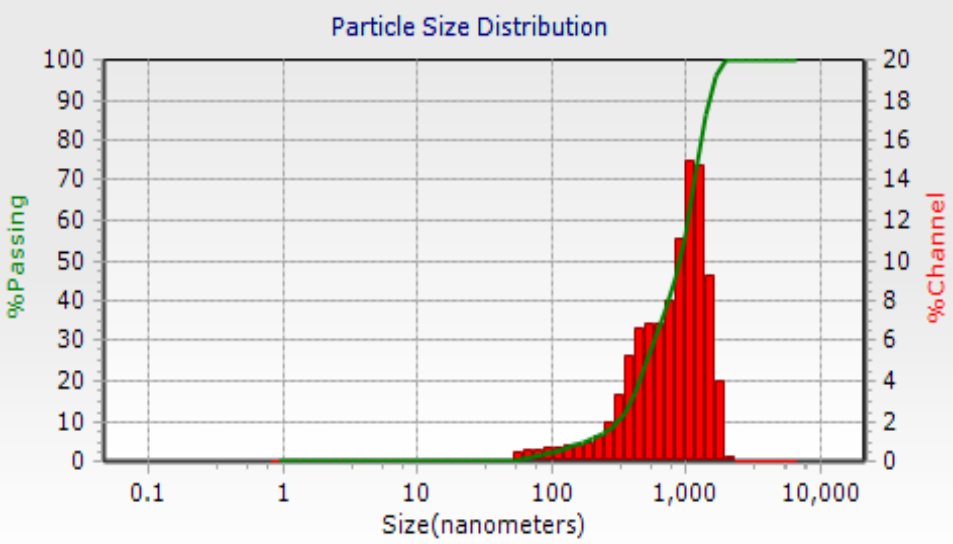

Fig 1. Size distribution of nisin microcapsules

According to the TEM image, nisin microliposomes were formed and the size distribution was very appropriate and formed in the range of 850 nm and were mostly spherical with an average size of 95 nm. The microcapsules confirmed the proper production and distribution of microliposomes containing nisin and the results are consistent. Therefore, in general, it should be acknowledged that the synthesis was successful and the process resulted in the formation of microliposomes (Fig. 2).

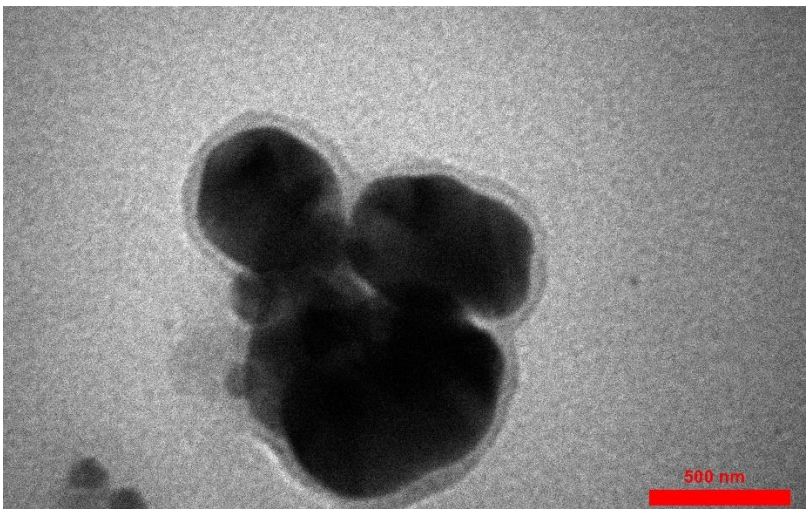

Fig 2. TEM image of microliposomes containing nisin

Determination of MIC and Comparison of Nisin and Nisin Nanoliposomes in Clinical and Standard Bacteria *S. mutans*

In this study, the microliposome itself caused turbidity when added to the bacterial culture medium. The growth values before and after bacterial inoculation and incubation were measured using a microplate reader at wavelengths of 440 and 630 nm. The minimum inhibitory concentration of bacteria was investigated and compared before and after incubation

with nisin and nisin microliposomes separately. The lowest dilution of treatments that inhibited the growth of each bacterium was considered the minimum inhibitory concentration (MIC). According to Table 1, the MIC of free nisin in both clinical and standard strains was 12.5 µg/mL. In the case of enclosed nisin (microencapsulated nisin), the minimum inhibitory concentration (MIC) was 6.25 µg/mL. Hence, the increase in nisin microcapsules somewhat resulted in an elevation in growth inhibition. According to figure 3, which displays the growth percentage ratio of nisin and nisin microcapsules on *S. mutans* bacteria, the effectiveness of nisin microcapsules was higher than that of nisin at all concentrations. The percentage of growth of nisin microcapsules on *S. mutans* bacterium increases starting at 3.12 µg/mL, which decreases the concentration of the population. The inhibition percentage of *S. mutans* increased with the growth of nisin at all concentrations. The most significant difference in the inhibition percentage of growth between nisin and nisin microcapsules was observed at a concentration of 12.5 µg/mL in the clinical sample.

Table 1. Determination of the MIC of microencapsulated nisin against *S. mutans*

							MIC		
	Concentration (µg/ml)	0*	0.39	0.78	1.56	3.12	**6.25**	**12.5**	25
microencapsulated Nisin	Clinical *S. mutans*	0.991	0.944	0.478	0.377	0.245	**0.097**	0.071	0.045
	Bacteria reduction percentage after 18 hours of treatment	-	4.741	50.85	61.95	75.27	**90.21**	92.83	94.45
	S. mutans PTCC 1683	0.828	0.798	0.766	0.333	0.174	**0.041**	0.087	0.064
	Bacteria reduction percentage after 18 hours of treatment	-	6.65	13.64	64.45	78.98	**95.04**	74.1	91.65
Free Nisin	Clinical *S. mutans*	0.922	0.978	0.812	0.529	0.319	0.098	**0.061**	0.074
	Bacteria reduction percentage after 18 hours of treatment	-	6.07	11.93	42.62	65.40	89.37	**93.38**	91.97
	S. mutans PTCC 1683	0.936	0.811	0.621	0.341	0.199	0.145	**0.088**	0.021
	Bacteria reduction percentage after 18 hours of treatment	-	13.35	33.55	63.56	78.73	84.50	**90.59**	97.75

*** Turbidity of negative control (no treatment)**

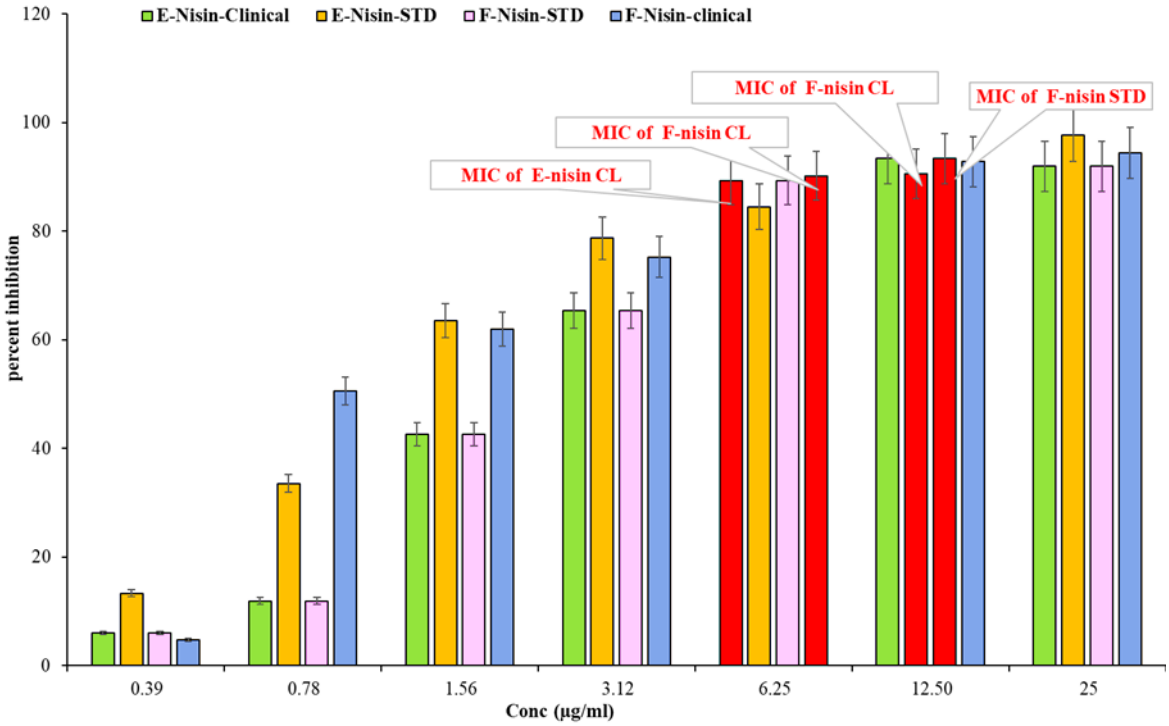

Fig 3. the growth percentage ratio of Nisin and Nisin microcapsules on *S. mutans* bacteria

Investigating the effects of nisin on the growth pattern of *S. mutans*

In order to investigate the antimicrobial sensitivity of bacteria to a concentration equal to the MIC, growth kinetics under nisin treatment and nisin microcapsules. Additionally, we analyzed the growth and adaptation patterns of bacteria at a wavelength of 600 nm in comparison to specific concentrations. The investigation results showed that standard *S. mutans* bacteria were more sensitive than clinical *S. mutans* and displayed higher susceptibility to high concentrations of nisin microcapsules compared to other strains. In Figure 4, the growth curves of *S. mutans* under various combinations of antibiotics are presented and can be analyzed.

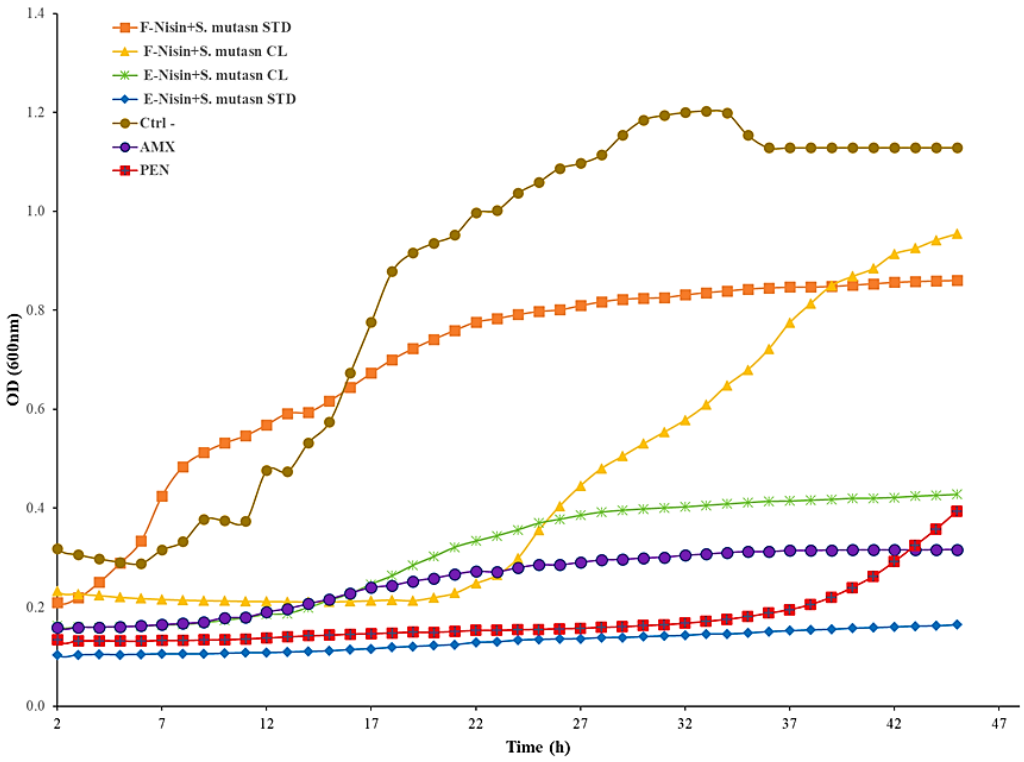

Fig 4. the growth curve of clinical and standard *S. mutans* strains, treated with nisin microcapsules and free nisin, in comparison to amoxicillin and penicillin antibiotics.

Based on the results obtained from the growth patterns of clinical and standard *S. mutans* treated with free and encapsulated nisin, similarities were observed in the growth patterns under free nisin treatment in both clinical and standard strains. In Figure 4, the growth pattern of the strains treated with encapsulated nisin showed more pronounced inhibitory effects. This can be attributed to the increased potency and prolonged duration of the encapsulated nisin's effects. Based on similar research on different target bacteria, it can be concluded that encapsulation enhances the stability and durability of nisin, leading to significant antimicrobial effects. To ensure more comprehensive results and investigations, obtaining relevant licenses is necessary.

Antibiofilm Investigation

Inhibition of biofilm formation caused by various concentrations of nisin and microencapsulated nisin has been demonstrated (see Figure 5). Absorption of biofilms increased in proportion to the matrix volume. The effect of encapsulated nisin on standard *S. mutans* at a concentration of 25 µg/ml resulted in the lowest absorption, with an inhibition rate of 87% compared to the control group and 97% compared to chlorhexidine. In other samples, nisin encapsulated in the clinical sample exhibited the highest inhibitory effect and binding

power. According to the growth kinetics demonstrated in the previous test, the inhibitory rate increased with each dilution.

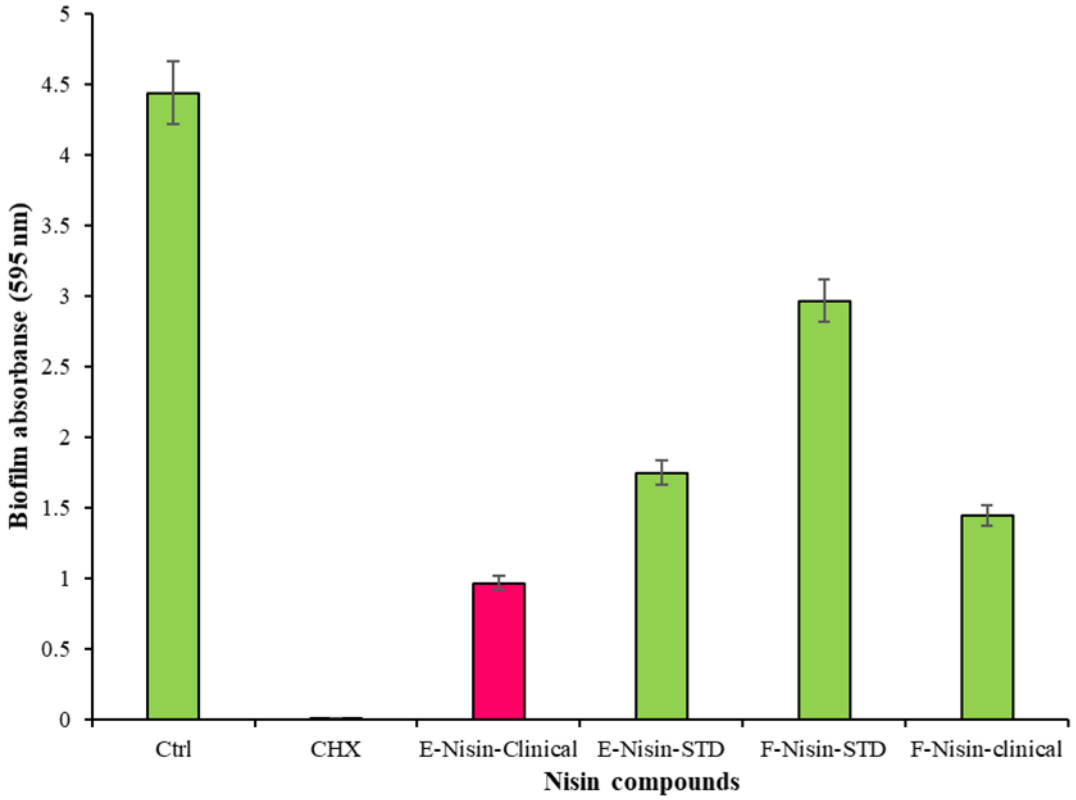

Fig 5. Assay of the biofilm caused by *S. mutans* in the presence of nisin and nisin microcapsules. Significant differences from the control (untreated) are shown compared to CHX (chlorhexidine).

Discussion

Nisin was approved as the only food preservative bacteriocin as well as a safe food additive by the World Health Organization in 1969 (Delves-Broughton, 1993). This material is currently used as a food biopreservative in barrier technology, meaning that it should not be used as the only barrier to prevent the growth or survival of foodborne pathogens. Liposome encapsulation technologies may allow the preservation of antimicrobial activity by protecting the antimicrobial from cross-reactions with food components (Taylor et al., 2008). Also, since antimicrobial compounds can be present in both the aqueous phase and the lipid phase of liposomes, a complementary effect can be expected, with both short-term (by release of encapsulated nisin) and long-term (excretion of membrane-stabilized nisin) effects. It creates antibacterial properties (Popa et al., 2022). Consistent with the present study, Taylor et al. (2008) reported that nisin-containing liposomes did not inhibit the growth of L. monocytogenes over the entire experimental period (48 h). However, these authors, by measuring the optical

density (Hagiwara et al.) at 630 nm, found a significant inhibition (approximately 60%) of pathogens against positive controls after 48 h of incubation at 32 °C in ambient BHI observed with dual power. The combined use of nisin (free or encapsulated) with low temperature, which is usually used routinely in the food industry and laboratories, (about 7 °C) is effective in controlling the growth of *L. monocytogenes* during 14 days, and the importance of the technological concept Strengthen the barrier to food quality (Taylor et al., 2008).

Oral microbiome studies have identified 500-700 species of microbes living in the oral cavity (Bowen et al., 2018). When the natural microbiome becomes unbalanced, it is likely to create a favorable environment for the growth of pathogenic bacteria, which ultimately leads to oral diseases (Cagetti et al., 2013). Tooth decay, one of the primary oral diseases alongside periodontitis, occurs when acid produced as a byproduct by microbes attached to the tooth surface damages the tooth enamel. The World Health Organization emphasized that dental caries affects approximately 60–90% of schoolchildren and most adults worldwide, and it is one of the primary causes of natural tooth loss in the elderly . In some clinical trials and animal experiments, fluorine compounds such as chlorhexidine and triclosan affect bacterial membranes and enzymes, inhibiting biofilm formation by disrupting bacterial metabolism (Forssten et al., 2010). Although chemical treatment methods with broad-spectrum antimicrobial effects are often chosen for periodontal disease, these methods can be toxic and may lead to side effects with prolonged use. Excessive exposure to fluoride can actually result in dental and skeletal damage and fluorosis (He et al., 2019). A growing interest in utilizing natural products to treat oral diseases has emerged to tackle these issues, and numerous studies have been published (Bowen et al., 2018). Here, we investigated the antibacterial effects of free and liposome-encapsulated nisin antibiofilm against the standard *S. mutans* strain with PTCC code 1683 and a clinical strain. First, we investigated the antimicrobial effects of free and encapsulated nisin against planktonic S. mutans. We observed a peak lethality of 95%, which remained above 90% even after a 50% dilution (12.5 μg/mL; see Fig. 3). Notably, at all treatment concentrations of nisin microcapsules, no significant difference in efficacy was observed compared to chlorhexidine, which is one of the most commonly used agents for the treatment of dental caries (Aleti et al., 2015; He et al., 2019). When the growth curves were examined over time, *S. mutans* exhibited a lag phase during the first 2 hours, followed by an exponential growth phase between 2 and 6 hours (Figure 4). In addition, standard *S. mutans* treated with nisin microcapsules did not exhibit an exponential growth phase, and the number of viable cells and uptake remained constant. Therefore, nisin microcapsules inhibited the

growth and division of *S. mutans*, preventing an increase in viable count and demonstrating antimicrobial activity.

Biofilm is one of the main pathogenic characteristics of Staphylococcus mutans and is an important factor in the development of dental caries (Dewhirst et al., 2010). Biofilms are composed of a complex and multidimensional structure of glucans and fructans synthesized during the glucose metabolism of *S. mutans.* This multidimensional glucan structure makes the biofilm thicker and stronger, playing a decisive role in plaque maturation (Cagetti et al., 2013). *S. mutans* formed a robust biofilm when cultured on a polystyrene surface with sucrose for 72 hours (data not shown). The effects of microcapsules and free nisin on a biofilm were then investigated under the same conditions. The antibiofilm effect of the microcapsules varied depending on the concentration (12.5-25 μg/mL), with a maximum efficacy of 97% at a concentration of 25 μg/mL (Figure 5). Interestingly, the results differed from those of antimicrobial activity, which also showed a dose-dependent correlation. In the concentration range of 12.5-25 mg/ml, the effectiveness of nisin microcapsules in inhibiting biofilm formation increased with each additional dilution. This suggests that some insoluble substances, which differ from the substances responsible for the antimicrobial effects of nisin microcapsules compared to the effectiveness of free nisin on clinical and standard strains, are the main suppressors of biofilm formation. Since *S. mutans* adherence occurs due to "pathogenicity determinants," protein expression in biofilm-forming strains of the bacterium differs from that of planktonic *S. mutans* (Dewhirst et al., 2010).

This study clearly shows that nisin microcapsules exhibit antimicrobial and antibiofilm activity. They inhibit the attachment and persistence of S. mutans, a caries pathogen that is more potent than other oral bacterial species. These results strongly support the hypothesis that nisin microcapsules can help prevent tooth decay by persisting in environments such as the mouth.

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