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Original Article

The effect of nisin on biofilm forming foodborne bacteria using microtiter plate method

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Abstract

Some of pathogenic and food spoilage bacteria can attach on food contact surfaces and form a biofilm, the source of contamination of foods. Biofilm is a functional consortium of microorganisms attached to the surface and is embedded in the extracellular polymeric substances (EPS) produced by the microorganisms. Biofilms due to special structure and EPS are more resistant to antimicrobial agents. Thus control of biofilm formation in food processing is important. Nisin is a peptidic bacteriocin that is used for biocontrol of biofilm formation. The aim of the present study was to assess the effect of various concentration of nisin on biofilm formation of *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella enteritidis*. The reduction percent of biofilms was obtained using microtiter plate method and ELISA reader machine. Also, bactericidal effect of nisin was determined by Triphenyl Tetrazolium Chloride. The results indicated that 4×10^3 IU/ml nisin is more effective on biofilm of *S. enteritidis* (87%) than *L. monocytogenes* (57%) and *Staph. aureus* (30%) with significant difference ($P<0.05$).

Keywords: Antimicrobial; Biofilm; Extracellular polymeric substances; Microbial attachment; Microtiter plate method; Nisin

INTRODUCTION

Microbial biofilms are attracting attention of scientists in different areas such as the medical field, aquatic environment, food processing industries, etc. Microbial biofilms may be detrimental and undesirable in food processing premises. The formation of biofilms by some pathogenic bacteria such as S. enteritidis (1,2), *L. monocytogenes* (3) and *Staph. aureus* (4,5) have been reported. Such biofilms could be continuous sources of contamination of foods and medical products in contact with them. That may also lead to spoilage of foods or transmission of foodborne diseases (6). The increased resistance of biofilm cells to antibacterial agents and sanitizers has also been observed (7,8). Different species of microorganisms may possess diverse ability to attach or form biofilm on different surfaces. For example, biofilms can exist on all types of surfaces in food plants and medical devices ranging from plastic, wood, glass, metals and food products (8).

In recent years, in order to increase food safety, new approaches such as using bacteriocins to control pathogenic micro-organisms have been developed. Nisin is a small antimicrobial peptide that acts against many foodborne and spoilage bacteria (9), especially spore forming bacteria, and is adsorbed on various surfaces and added to packaging films (10). Nisin is considered a safe food antimicrobial agent, and has been approved

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as a generally recognized as safe (GRAS) additive (11).

Dawson et al. evaluated antimicrobial activity of nisin–adsorbed silica and corn starch powders against *L. monocytogenes* (12). Also, Boziaris and Nychas (13), Hampikyan and Ugur (14) studied the effect of nisin on *L. monocytogenes*. Millette et al. evaluated the potential of palmitoylated based-alginate film containing nisin to control *S. aureus* on round beef steak (15).

Pitts et al. using microtiter plate method measured the removal and killing efficacy of antibiofilm agents. This rapid screening method is sensitive enough to elucidate concentration-response relationships as well as differences between species responses to treatments. In this technique, crystal violet is suitable for measuring the amount of biofilm, but not its activity, so crystal violet staining could be used to measure removal of bacteria but not disinfection (16). Thus in this research respiratory dye triphenyl tetrazolium chloride (TTC) (17,18) was used to measure active metabolism by bacteria that survived disinfection.

The purpose of this study was devised to evaluate the effect of different nisin concentrations on biofilm forming pathogenic bacteria *S. enteritidis*, *Staph. aureus* and L. *monocytogenes* by microtiter plate method.

MATERIALS AND METHODS

Bacterial strains and culture media

In this study *Staph. aureus* was isolated from slicer of meat processing plant (4), *L. monocytogenes* RITCC 1293 serotype 4a and S. enteritidis RITCC 1624 were obtained from Razi Institute, Tehran, Iran. *L. monocytogenes*, *S. enteritidis* and *Staph. aureus* subcultured on PALCAM (Merck), XLD (Merck) and Baird Parker (Merck), respectively.

Nisin

100 mg of nisin (Sigma Nisaplin 2/5%) was solubilized in 10 ml 0.02 N HCl to give the concentration of 10^4 IU/ml (40 IU=1 g). Then the solution was sterilized by filtration through 0.45 filters and was stored at -20 ^{o}C (14).

Microtiter plate method

Biofilm elimination potential of nisin

The wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid were filled with 200 µl of each bacterial suspension. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 h at 37 ^{o}C. Then, the content of each well was aspirated, and each well was washed three times with 250 µl of sterile physiological saline. The plates were vigorously shaken in order to remove all non-adherent bacteria. Thereafter, 200 µl of different concentrations of nisin were added to each well but not to the control. After one hour, nisin was removed from wells and microtiter plate wells were washed five times with sterile distilled water to remove loosely associated bacteria and remaining nisin. Then, plates were stained for 5 min with 0.2 ml of 2% crystal violet (bioMerieux) per well. Excess stain was rinsed off by placing the plate under running tap water. Then the plates were air dried, the bounded dye to the adherent cells was resolubilized with 160 µl of 33% (v/v) glacial acetic acid per well and incubated at 30 ^{o}C for 15 min. The optical density (OD) of each well was measured at 492 nm using an automated Statfax ELISA reader. Ultimately, biocide efficiency or reduction percent of biofilms were obtained using following formula (3, 19, 20):

Reduction Percent= $\left[\frac{(C-B)-(T-B)}{(C-B)}\right]\times 100$

C=mean OD of control wells, B=mean OD of negative controls, T=mean OD of test wells

Bactericidal effect of nisin on biofilms

This method was performed similar to assessment of biofilm elimination potentional of nisin, but in this case TTC dye was used instead of crystal violet and after the last incubation of plates for 2 hour, the OD of each well was measured at 450 nm (17).

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. P values of <0.05 were considered significant.

RESULTS

The elimination potentional and bactericidal effect of nisin on biofilms of *S. enteritidis*, *Staph. aureus* and *L. monocytogenes* are given in Figure 1 and 2, respectively. Approximately 93% of bacterial biofilms were eliminated by high concentration of nisin (Fig. 1). As shown in Figure 1, the elimination potential of 4×10^4 IU/ml nisin on *S. enteritidis*, *Staph. aureus* and *L. monocytogenes* was 93, 65 and 77%, respectively (with no significant difference). The acceptable concentration of nisin (4×10^3 IU/ml) in food indicated the highest antibacterial effect on biofilms of *S. enteritidis*, *L. monocytogenes* and *Staph. aureus* respectively (P<0.05) (Fig. 3)

DISCUSSION

Incomplete elimination of biofilms would cause regrowth of the remaining biofilms on surfaces that have some living cells. This phenomenon will return microorganisms to the microbial biofilm state, repairing their structures and contaminating systems. Therefore, knowing quantity of cells within biofilms which are alive and can multiply, after using antimicrobial agents, is necessary.

In general, the formation of bacterial biofilms is believed to take place over at least three stages: a reversible adsorption step, primary adhesion of microorganisms to the surface, and colonization. The rates of these processes vary widely depending on the environmental conditions and the type of microorganisms, but the adhesion and colonization stages are considered to be relatively slow compared to the first step of cell adsorption. In principle, it should be possible to retard, if not prevent, the formation of biofilms on substrates by using materials to which bacteria cannot initially attach, and such a material or surface coating would be of considerable commercial interest (21). Frank and Chmielewski demonstrated that the type and topography of food contact surface play a significant role in the inability to decontaminate a surface (22).

Food packaging materials were only used to provide a barrier and had only protective functions. However, various kinds of active substances can now be incorporated into the packaging material to improve its functionality and give it new or extra functions. Such active packaging technologies are designed to extend the shelf life of foods, while maintaining their safety and nutritional quality (23). Common antimicrobial chemicals that can be incorporated into a packaging material are propionic acid, peroxide, ozone, chlorine oxide, eugenol, cinnamaldehyde, allyl isothiocyanate, lysozyme, nisin, and EDTA (24). This study has focused on effects of different concentrations of nisin on biofilm forming bacteria. The antimicrobial activity of nisin is based on pore formation in the cytoplasmic membrane of target organisms.

In this study bactericidal effect of different concentrations of nisin on bacterial biofilms had no significace.Also, the effects of nisin on various bacterial biofilms were equal (Fig. 2).

Bauer et al., described nisin inhibitory

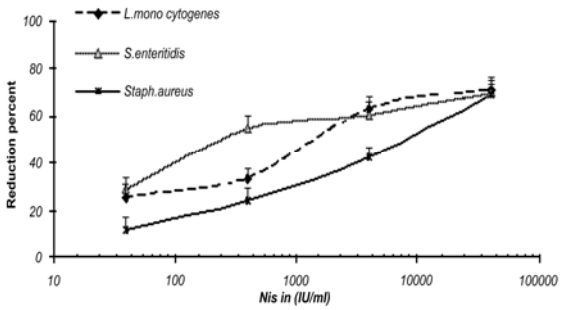

Fig. 1. Biofilm elimination potentional of nisin on *L. monocytogenes*, *S. enteritidis* and *Staph. aureus* (n=3).

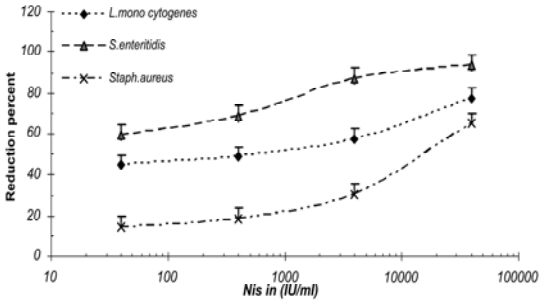

Fig. 2. Bactericidal effect of nisin on *L. monocytogenes*, *S. enteritidis* and *Staph. aureus* (n=3).

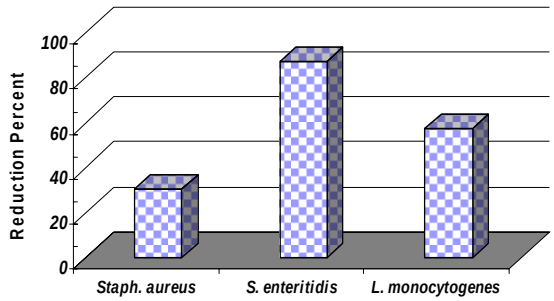

Fig. 3. The effect of 4×10^3IU/ml concentration of nisin on elimination of biofilms.

effect on the formation of *Oenococcus oeni* biofilms on stainless steel surfaces and reported the successful use of nisin, as well as pediocin PD-1 and plantaricin 423, to remove biofilms from stainless steel surfaces (25). In our study, 4×103 IU/ml concentration of nisin was effective to remove biofilms but not to kill the cells. The elimination and bactericidal effect of 4×103 IU/ml concentration of nisin on S. enteritidis, was about 90% and 60%, respectively (P<0.05) (Fig. 1 and 3). Although, there were not significant differences on elimination and bactericidal effect of 4×104 IU/ml concentration of nisin between various tested bacteria.

Han and Floros summarized the applications of antimicrobial packaging including nisin-containing packaging (26). Nisin-containing packaging materials have had potential applications to prevent growth of many foodborne pathogenic bacteria and their spores, *C. botulinum* and *L. monocytogenes* (27). These films reduced *L. mono-cytogenes* in skim milk by 3 logs (cfu/ml) after 48 h (28) and L. *plantarum* in peptone water to below detection level (29). Bowers et al., found no suppression of *L. monocytogenes* growth but a reduced adhesion rate of the bacterium to silica surfaces that had nisin adsorbed (30). Carballo and Arajjo evaluated the effect of the adsorption of nisin on materials used in food industry on their superficial characteristics and on the adhesion of *L. monocytogenes* to them (31). They conclude that the highest roughness of rubber justifies the biggest amounts of adsorbed nisin onto this material and this would be the cause of the highest reduction in bacterial adherence. In our experiments, as shown in figure 2 and 3, elimination and bactericidal effect of nisin on *L. monocytogenes* biofilms were nearly 57% and 62%, respectively. These results exhibited reduction effect of nisin on *L. monocytogenes* biofilms. Our results revealed that elimination and bactericidal effect of nisin had no significant differences between *Staph. aureus*, *S. enteritidis* and *L. monocytogenes*. However, the highest effect of nisin was on *S. enteritidis* biofilms. *Salmonella enteritidis* is an important food-borne enteric pathogen and remains an important cause of gastroenteritis in human world-wide (32). Also, exceeding from the acceptable concentration of nisin had no significant effects on these biofilm cells. Development of biofilms on many surfaces is a potential

source of contamination of foods that may lead to spoilage or transmission of foodborne pathogens.

CONCLUSION

From the results of this study it can be concluded that the changes induced in the surface of materials influence bacterial adhesion, so the modification of food contact materials, including their treatment with nisin is a promising method for the reduction of bacterial attachment and biofilm formation.

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