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ORIGINAL ARTICLE

Evaluation of anti-biofilm activity of *Lactobacillus rhamnosus* GG and Nisin on the expression of *aap*, *ica*-A and *ica*-D as biofilm-associated genes of *Staphylococcus epidermidis*

Mohammad Dalvand[1], Seyed Ali Mirhosseini[1], Kiumarss Amini[2], Soghra Khani[3], Hamideh Mahmoodzadeh Hosseini[1*], Kowsar Mansoori[4]

[1]Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2]Department of Microbiology, Faculty of Basic Sciences, Saveh Branch, Islamic Azad University, Saveh, Iran
[3]Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran
[4]New Hearing Technologies Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background and Objectives: In the present study, the anti-biofilm activity of *Lactobacillus rhamnosus* GG and Nisin was investigated on biofilm-forming abilities of *Staphylococcus epidermidis* strains and the expression of the biofilm-associated genes.
Materials and Methods: In this study, the standard strain of *L. rhamnosus* GG (ATCC 53103) and Nisin were used to assess their anti-microbial and anti-biofilm effects on *S. epidermidis* (RP62A).
Results: The MIC and MBC analysis showed that Nisin at 256 µg/mL and 512 µg/mL, and *L. rhamnosus* GG at 1×10^7 CFU/mL and 1×10^8 CFU/mL have anti-microbial activity compared to the negative control respectively. *L. rhamnosus* GG bacteria and Nisin inhibited the biofilm formation of *S. epidermidis* based on optical density of at 570 nm ($P<0.001$). The relative mRNA expression of *aap*, *icaA*, and *icaD* genes was significantly reduced compared to the negative control after treating *S. epidermidis* with sub-MIC of Nisin (0.44, 0.25 and 0.6 fold, respectively) ($P>0.05$). In addition, the relative expression of *aap* and *icaA* genes, but not *icaD* ($P>0.05$), was significantly lower than the negative control (0.62 and 0.7 fold, respectively) ($P>0.05$), after exposure to the sub MIC of *L. rhamnosus* GG.
Conclusion: Nisin and *L. rhamnosus* GG exhibit potent activity against biofilm-forming abilities of *S. epidermidis* and these agents could be utilized as an anti-biofilm agents against *S. epidermidis* infections.

Keywords: *Staphylococcus epidermidis*; Probiotic; *Lactobacillus rhamnosus* GG; Nisin; Biofilm

INTRODUCTION

Staphylococcus epidermidis is the most frequent commensal bacterium of human skin and is considered an opportunistic microorganism. Due to the increasing number of immunocompromised patients and recipients of biomedical implants, infections caused by this bacterium have dramatically increased. *S. epidermidis* is now one of the leading causes of hospital-acquired infections, including

*Corresponding author: Hamideh Mahmoodzadeh Hosseini, PhD, Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: +98-2182454825 Email: hosseini361@yahoo.com

catheter-associated infections and infections resulting from the use of external equipment (1).

The ability of this bacterium to form biofilms and colonize different surfaces is associated with its capability to cause life-threatening catheter-related bloodstream infections in immunocompromised patients. *S. epidermidis* possesses a range of genes that contribute to the biofilm phenotype, including the *ica* (icaABCD) gene, which produces Polysaccharide Intercellular Adhesive (PIA); the *Bap* (biofilm-associated protein) gene; the *Aap* (accumu- lation-associated protein) gene; and other genes that produce surface proteins and exopolymers essential for adhesion and biofilm formation on host cells and implanted devices (2, 3).

Considering the emergence of antibiotic-resistant *S. epidermidis* strains as a significant health concern, it is necessary to explore alternative novel approaches to control infections caused by this bacterium.

Lactobacillus rhamnosus is a facultatively anaerobic, heterofermentative, rod-shaped bacterium that can be found in different parts of the human body, including the gastrointestinal tract (4). *Lactobacillus rhamnosus* strain GG is one of the most well-studied probiotic strains and is generally recognized as safe (GRAS) by the Food and Drug Administration (5). Initially, *L. rhamnosus* GG was obtained from a fecal sample of a healthy adult person by Barry Golding and Sherwood Gorbach, and later, the abbreviation *L. rhamnosus* GG was used to refer to this strain (6).

The anti-pathogenic mechanisms of *L. rhamnosus* GG include its ability to endure gastrointestinal stress, acid, and bile tolerance, high growth ability, adherence capacity to the intestinal epithelium, protection, and strengthening of the epithelial barrier, production of different antimicrobial substances, immunomodulatory effects, ability to produce biofilm, prevention of pathogen biofilm formation, competitive adhesion to host cells, inhibition of bacterial pathogen growth, and more (5, 7-9).

According to the mentioned characteristics of this strain, it has been applied to various disease states in clinical trials and has shown many benefits to the host. These include improvements in diarrhea in children, atopic diseases, anti-obesity, and respiratory pathology, lower depression and anxiety scores in women, reduced risk of colon cancer, and treatment of recurrent *Clostridium difficile*-induced colitis in children (10-13).

Nisin is an antimicrobial peptide produced by *Lactococcus lactis.* In 1969, FAO (Food and Agriculture Organization) and WHO (World Health Organization) approved the usage of this bacteriocin as a food preservative instead of a chemical one (14, 15). Nisin has gained considerable attention due to its potent and broad-spectrum activity, low likelihood of promoting the development of bacterial resistance, easy degradability by proteolytic enzymes in mammals, and low cellular cytotoxicity at antimicrobial concentrations (16, 17). Several studies have shown that the antimicrobial activity of Nisin is due to pore formation on the surface of cells, inhibition of cell wall biosynthesis, and anti-biofilm formation (16, 18, 19).

According to the information presented above and in order to explore alternative novel methods, the purpose of this study is to evaluate the effects of Nisin and *L. rhamnosus* GG on the biofilm-forming potential of *S. epidermidis* and the expression of biofilm-associated genes, including *icaA*, *aap*, and *icaD.*

MATERIALS AND METHODS

Sources and chemicals. *Lactobacillus rhamnosus* GG (ATCC53103) was obtained from the Pasteur Institute of Iran, and Nisin was purchased from Sigma Co. (St. Louis, MO, USA). *Staphylococcus epidermidis* (RP62A) was gifted by the Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences.

Determination of MIC and MBC of Nisin and *Lactobacillus rhamnosus* GG. The broth micro dilution method determined MIC and MBC concentrations according to the Mottaghiyan approach (20). MIC is considered the lowest concentration of an antimicrobial agent capable of inhibiting the visible growth of bacteria after overnight culture. To this purpose, Nisin and *L. rhamnosus* GG were treated or co-cultured with *S. epidermidis* to determine their MIC individually on *S. epidermidis.* Initially, 100 µL of *S. epidermidis* suspension in TSB ($0.4 \leq OD \leq 0.6$) was cultured in a 96-well plate. Next, 100 µL of Tryptic Soy Broth (TSB) containing probiotic bacteria (ranging from 1×10^5 to 1×10^8 CFU/mL) or 100 µL of TSB containing Nisin (ranging from 64 µg/mL to 1024 µg/mL in two-fold serial dilutions) was added to each well of the 96-well microtiter plate, followed by overnight incubation with gentle rotation at 37°C

using a shaker incubator. Bacterial growth was evaluated based on the broth's turbidity and the lowest concentration of probiotic or Nisin that prevented *S. epidermidis* growth was considered their MIC. The MBC was defined as the lowest concentration where no bacterial growth was observed. This was determined by aseptically sub-culturing the contents of wells from the MIC results for individual bacteria on antimicrobial-free agar.

Biofilm formation. To identify the effects of Nisin and *L. rhamnosus* GG on biofilm formation, the microtiter plate test (MPT) was employed with some modifications compared to the method described by Merritt et al. (21). Bacterial suspension in Muller-Hinton Broth (MHB) (Hi-Media Ltd., Mumbai, India) with a density of 0.5 McFarland was prepared. Then 100 µL of the suspension was added to separate wells in a 96-well microtiter plate. Next, 100 µL of MHB containing Nisin (ranging from 64 µg/mL to 1024 µg/mL in two-fold serial dilutions) or 100 µL of TSB containing probiotic bacteria (ranging from 1×10^5 to 1×10^8 CFU/mL) was added to each well. The plates were incubated overnight at 37°C. Next, the free-floating cells were removed, and the plate was washed using distilled water. Then, the wells were stained with 125 µL of 0.2% (w/v) crystal violet and incubated at room temperature for 15 min. The excess dye was rinsed with distilled water three times, and the plates were allowed to dry. Subsequently, 200 µL of absolute ethanol was added and incubated for an additional 15 min at room temperature. The contents of each well were transferred to new microtiter plate wells. Finally, the optical density of each well was measured at 570 nm. The negative controls consisted of all added reagents except for the bacterial suspension. The test was performed in triplicate. The mean absorbance at 570 nm was calculated. Merritt et al. explained that the strain is considered a biofilm producer if the optical density is higher than 0.2.

Expression levels of genes associated with biofilm formation. To evaluate the effects of Nisin and *L. rhamnosus* GG on the expression of some biofilm-associated genes (*aap*, *icaA*, and *icaD*), the Mottaghiyan approach (20) was used. *S. epidermidis* was treated with a sub-MIC amount of Nisin (128 µg/mL) and *L. rhamnosus* GG (1×10^6 CFU/mL) for 24 h. After that, RNA extraction was carried out using the CinnaPure kit (cat No: PR891620, Cinnagen Co., Tehran). The concentration of RNA samples was quantified via the Nanodrop2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Next, RNA samples were treated with DNase (Fermentase, Thermo Fisher Scientific, USA) based on the manufacturer's instructions, followed by cDNA synthesis using Reverse Transcriptase AMV at 25 U/µL (Roche Life Science). Target genes were relatively quantified using Q-Master Mix with SYBR Green I (Genetbio, Daejeon, Korea; cat. No: Q9210) by the Real-time PCR system (Applied Biosystems, Foster City, CA, USA). For each sample, the reaction mixture comprised the following components: 10 µL of 2X Prime Q-Master Mix with SYBR Green I (Genetbio CAT. NO: Q9210), 1 µL of each primer (final concentration 1 µM), 1 µL of Rox Dye, 5 µL of RNase-free water, and 2 µL of cDNA, in a final reaction volume of 20 µL. The primer sequences and cycling temperature are presented in Table 1. The expression level of the *gyr* gene was surveyed as an internal control. The ΔΔCT method that normalizes to a housekeeping gene was used for quantification.

Statistical analysis. Relative quantitative gene expression was analyzed using an independent *t*-test. Changes with a P-value of ≤0.05 were considered statistically significant.

RESULTS

MIC and MBC of Nisin and probiotic *Lactobacillus rhamnosus* GG on *Staphylococcus epidermidis* strains. To evaluate the inhibitory and bactericidal effects of Nisin and *L. rhamnosus* GG on *S. epidermidis* growth, MIC and MBC tests were performed using the broth microdilution method. The MIC and MBC values were calculated as the medians of the three experiments. In this study, *S. epidermidis* was exposed to serial concentrations of Nisin or *L. rhamnosus* GG. The results showed that the MIC values for Nisin and *L. rhamnosus* GG were 256 µg/mL and 1×10^7 CFU/mL, respectively. The optimal MBC results for Nisin and *L. rhamnosus* GG were also observed at 512 µg/mL and 1×10^8 CFU/mL, respectively.

Nisin and *Lactobacillus rhamnosus* GG efficiently eliminate *S. epidermidis* biofilms on microtiter plates. The biofilm-forming capacity of *S. epidermidis* was examined using a microtiter plate assay. As

Table 1. Primer sequences and thermal conditions of Real-time PCR analysis

Gene	Forward (F) (5'-3') Revers (R) (5'-3')	Denaturation Temp & Time		Annealing Temp & Time		Extension Temp & Time		Amplicon Size (bp)	Ref
Aap	F-AGAAACAAGCTGGTCAAG R- CTGCGTAGTTAAGAAAATC	90°C	30 s	56°C	40 s	72°C	60 s	117	(20)
icaA	F-TCTCTTGCAGGAGCAATCAA R- AGGCACTAACATCCAGCA	90°C	30 s	56°C	40 s	72°C	60 s	186	(20)
icaD	F-CCGGAGTATTTTGGATGTATTG R-TTGAAACGCGAGACTAAATGTA	90°C	30 s	56°C	40 s	72°C	60 s	197	(20)
gyr	F-CTTATATGAGAATCCATCTGTAGG R- AGAACAATCTGCCAATTTACC	90°C	30 s	56°C	40 s	72°C	60 s	154	(20)

shown in Fig. 1A, increasing the concentration of Nisin bacteria prevented the biofilm formation *of S. epidermidis* strains, as indicated by the optical density at 570 nm. Our results also revealed that *L. rhamnosus* GG treatment could reduce *S. epidermidis* biofilm formation *in vitro* (Fig. 1B).

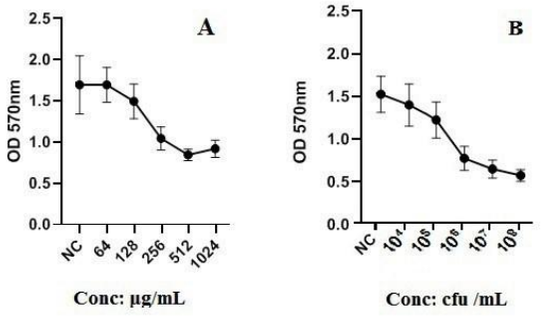

Fig. 1. The effects of Nisin (A) and *L. rhamnosus* GG (B) treatment on biofilm-formation ability of *S. epidermidis* clinical isolates and on their anti-biofilm activities. Means and standard deviation of three independent experiments are shown.

Significant differences were observed between the untreated group and the Nisin or *L. rhamnosus* GG-treated groups ($P<0.001$). The highest biofilm inhibition rates were achieved with Nisin at a concentration of 512 µg/mL and with *L. rhamnosus* GG at a concentration of 1×10^8 CFU/mL against *S. epidermidis.*

Biofilm-associated gene expression analysis. All extracted RNA was subjected to DNase treatment to remove genomic DNA before cDNA synthesis. RT-PCR was performed for each RNA sample to confirm the absence of genomic DNA. As seen in Fig. 2, there is only one single product for each target gene.

Specific primer pairs were used to assess the genes of interest using real-time qRT-PCR. As illustrated in Fig. 3, each reaction shows a single melt curve, confirming the optimal experimental design.

The RT-qPCR data showed that the expression ratio of the *aap*, *icaA* and *icaD* gene was significantly reduced after Nisin treatment compared to the untreated group (0.44, 0.25 and 0.6 fold, respectively) ($P>0.05$). Quantitative gene expression analysis was performed at 1×10^6 CFU/mL of the probiotic, based on the MIC assay results on *S. epidermidis.* In the present study, all biofilm-forming bacteria treated with *L. rhamnosus* GG showed reduced gene expression in biofilm production. The relative mRNA expression of the *aap* and *icaA* gene was significantly reduced (0.62 and 0.7 fold, respectively) compared to the negative control ($P>0.05$). *Lactobacillus rhamnosus* GG did not appear to affect the gene expression levels of *icaD* ($P<0.05$).

DISCUSSION

Staphylococcus epidermidis, due to its various virulence factors and unique features, such as its potential ability in biofilm formation and colonization on different surfaces, is considered the most important cause of nosocomial infections. In recent decades, this bacterium has posed many challenges in the treatment process due to the increase in the number of immunocompromised patients, the rise in medical device interventions, and the emergence of methicillin-resistant *S. epidermidis* strains, all of which are associated with the growing elderly population. Therefore, finding new alternative approaches to control *S. epidermidis* infections seems necessary. Considering that *L. rhamnosus* GG can prevent the production of biofilm by pathogenic bacteria and the

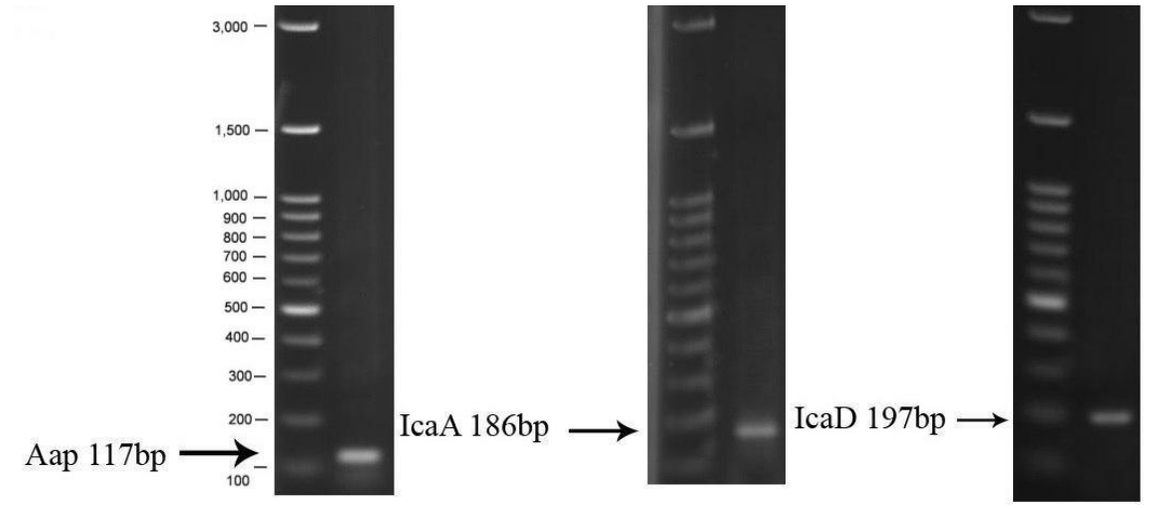

Fig. 2. Evaluating the specificity of gene amplifications.

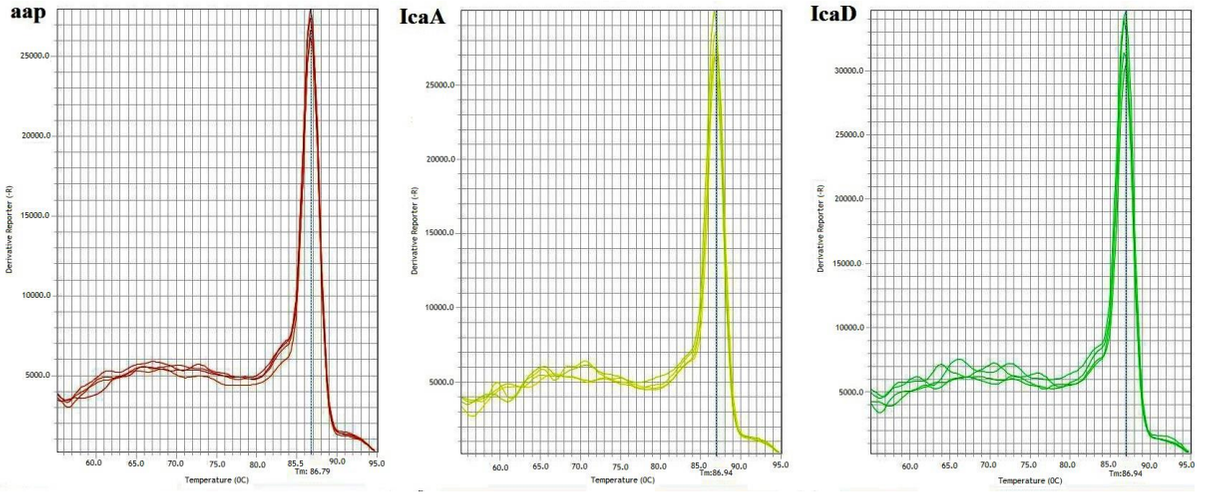

Fig. 3. Melting curve analysis from a Real-time PCR assay for *Aap*, *IcaA* and *IcaD* gene expression in *S. epidermidis* isolates.

anti-biofilm effects of Nisin have already been proven, this study aims to evaluate, for the first time, the antibiofilm activity of Nisin and *L. rhamnosus* GG on *S. epidermidis* and the expression of biofilm-associated genes, including *icaA*, *aap*, and *icaD*. To study the effect of Nisin on the target genes, the MIC of this bacteriocin on *S. epidermidis* was initially determined, and then the effects of Nisin and *L. rhamnosus* GG on *aap*, *icaA*, and *icaD* were assessed. The same steps were carried out for *L. rhamnosus* GG.

Our results demonstrated that Nisin can diminish the expression of *aap*, *icaA*, and *icaD* genes in *S. epidermidis* bacteria. The gene products of the *icaADBC* locus are responsible for synthesizing polysaccharide intercellular adhesin (PIA), the main molecule for intercellular adhesion in *S. epidermidis*. Additionally, the *aap* gene synthesizes the accumulation-associated protein, which is the main protein involved in self-aggregation and biofilm formation (2). However, biofilm formation is more complex than being solely governed by these genes, and suppressing these genes alone does not guarantee the inhibition of biofilm formation (22).

Twomey and his colleagues showed that Nisin A, at a MIC concentration of 3.75 µMol, can control *S. epidermidis* 28 strain, and at 7.5 µMol, it can control *S. epidermidis* 53 strain. Additionally, Nisin A significantly reduces the amount of biofilm formation by *S. epidermidis* on all surfaces (23). This study's results were consistent with the current study's re-

sults, and the difference in MIC concentrations was due to the use of different *S. epidermidis* strains in the two studies. Another study by Field et al. indicated that Nisin and its derivatives, alone and in combination with classical antibiotics, have antibiofilm effects against *S. aureus* and *S. pseudintermedius* (19). The effects of Nisin on *aap, icaA,* and *icaD* in other microorganisms have also been studied, such as the study by Pimentel-Filho et al., which showed that Nisin reduces *icaD* expression in *Staphylococcus aureus* (24). Furthermore, a study conducted on the inhibitory effect of Nisin on biofilm generation by *Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella enteritidis* demonstrated that Nisin can reduce biofilm formation by all studied species (25).

We provided novel insights regarding the inhibitory effect of *L. rhamnosus* GG on the expression of biofilm-associated genes of *S. epidermidis.* In this study, we showed that *L. rhamnosus* GG reduced the expression of the *aap* and *icaA* genes. Other studies align with our results, but they focus on other bacteria. For example, the results by Saidi et al. indicated that the cell-free supernatant (CFS) extracts of *L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469 cultures significantly increased the expression levels of the *cidA, hld,* and *icaR* genes, but significantly downregulated the *sarA* and *icaA* genes in *S. aureus.* Consequently, the CFSs of both *Lactobacillus* spp. significantly reduced cell surface hydrophobicity, initial attachment, and biofilm formation in *S. aureus* (26). Another study showed that commercially available *Lactobacillus* strains, such as *L. rhamnosus* GG, reduced the biofilm formation of *S. mutans* clinical isolates (27).

Similarly, Lee et al. demonstrated that *L. rhamnosus* GG exerted an anti-biofilm activity by decreasing the expression of the *gtfs* gene, which is involved in the synthesis of the exopolysaccharide matrix crucial for biofilm formation, in *S. mutans* (28). Additionally, *L. rhamnosus* GG has other mechanisms to prevent biofilm formation by pathogenic bacteria. For instance, it has been shown that *L. rhamnosus* GG expresses lectin-like molecules capable of suppressing *Escherichia coli* and *Salmonella* biofilm formation (29).

The current study is the first to report the inhibitory effect of *L. rhamnosus* GG on biofilm formation and the expression of biofilm-associated genes of *S. epidermidis,* and there are no other studies in this area. Furthermore, considering the antibiofilm effect of Nisin on *S. epidermidis,* it can be suggested that Nisin and *L. rhamnosus* GG could be viable and safe treatment options for controlling and preventing infections associated with *S. epidermidis* in the future.

CONCLUSION

Here, we have demonstrated a possible mechanism for the inhibitory impact of Nisin and *L. rhamnosus* GG on *S. epidermidis* biofilm formation by directly dampening the expression of genes involved in biofilm formation. Based on these findings, Nisin and *L. rhamnosus* GG could be utilized as new therapeutic alternatives or as a complement in combination with classical antibiotics for treating bacterial skin infections or systemic infections. Moreover, Nisin and *L. rhamnosus* GG could also be effective inhibitors of biofilms that form on biomedical implants or hospital equipment.

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