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ORIGINAL ARTICLE

# Effects of nisin on the expression of virulence genes of methicillin-resistant/sensitive *Staphylococcus aureus*

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## ABSTRACT

**Background and Objectives:** Few studies have considered potential benefits of probiotic bacteria and their derivatives on human and animal health. Nisin is an antimicrobial agent that is produced by lactobacilli and served as a preservative in foods. This study aims to investigate whether nisin suppresses or decreases the genes involved in the pathogenicity of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA).

**Materials and Methods:** MSSA and MRSA strains were cultured at the ¼, ½, and 1 × minimum inhibitory concentration (MIC) of nisin. Next, RNA extraction was performed at the mid-exponential stage of growth, and cDNA was synthesized. The expression of virulence factors was measured by qPCR, and the data were analyzed by the ΔΔCt formula.

**Results:** Depending on the incubation times and the *Lactobacillus* species, the MIC of nisin on MRSA and MSSA observed in 800 and 1600 mg/l, respectively. The qPCR assay showed the expression level of the *sea*, *agrA*, and *spa* genes decreased and the level of the *sae* gene increased at the sub-MIC of nisin, and no antagonism was observed. Concerning MRSA, the maximum downregulation rate was observed in the *sea* gene (up to 5.9 folds) while in MSSA, the maximum downregulation rate was noticed in the *agrA* gene (up to 10 folds).

**Conclusion:** Due to the high inhibitory effect of the sub-MIC of nisin on the expression of virulence factor genes in MRSA and MSSA, this compound could potentially reduce the virulence of *S. aureus.*

**Keywords:** Nisin; Methicillin-resistant *Staphylococcus aureus*; Methicillin-sensitive *Staphylococcus aureus*; Virulence factor; Food-borne

## INTRODUCTION

*Staphylococcus aureus* is an opportunistic pathogen that could greatly threaten human health due to its antibiotic resistance, mediated virulence genes, and invasion capability (1). *S. aureus* could contaminate food products during processing (2). It is the most common foodborne disease and one of the major concerns in the worldwide public health programs (3, 4). In the US, approximately half of annual

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1,000,000 hospitalizations and 50,000 deaths happened following food contamination by *S. aureus* (5). The most frequent case of staphylococcal food poisoning was reported to have occurred between 2006 and 2009 in China (6). In the past decades, antibacterial agents have improved human lives and brought about incredible well-being among creatures. Nevertheless, because of the wide utilization of antimicrobial agents at clinics, resistant bacteria have extended their survival (7-9).

*S. aureus* produces a large diversity of staphylococcal enterotoxins (SEs) that could cause enteritis. SEs belongs to a large family of enterotoxins that cause vomiting and diarrhea when ingested (10-13). Furthermore, *S. aureus* secret protein A (SpA) as secretary protein, that encoding some virulence genes (14). Accordingly, the *sea, spa* genes encode main virulence factors that are adjusted by accessory gene regulators (*agr*) and two components signal transduction system (*sae*) in reply to environmental stimuli (15, 16).

Nisin is an antimicrobial peptide (AMP) produced by some strains of *Lactococcus lactis.* In addition, it is a molecule that could potentially eliminate some pathogens and could be used as a preservative in the food industry (17). In fact, nisin particles form pores in the bacterial cell layers and disrupt membrane charges, thereby making the membrane unusable as an ATP transport system (18). Nisin performs a strong bactericidal action against a wide range of Gram-positive microorganisms and is a safe substance approved by the Joint Expert Committee on Food Additives (JECFA), the Food and Agriculture Organization (FAO), and the World Health Organization (WHO) (17, 19-21). Additionally, nisin is used in the cheddaring process over the world (22, 23).

Since nisin is a preservative used in the food industry and *S. aureus* is a major foodborne pathogen, this study aims to survey the effects of nisin on the expression of some virulence genes (*sea, spa, agrA,* and *sae* genes) of methicillin-resistant/susceptible *Staphylococcus aureus.*

## MATERIALS AND METHODS

**Nisin preparation.** A nisin stock was prepared by dissolving 50 mg of the nisin powder (Sigma-Aldrich Darmstadt, Germany) in 5 ml of 0.02 N HCl (22). The diluted nisin was vortexed, sterilized by filtration (pore sizes of 0.22 µm and a standard bacterial membrane filter), and diluted at an appropriate concentration to perform the tests.

**Preparation of bacteria and growth conditions.** *S. aureus* strains (ATCC 33591) (resistant to methicillin, MRSA) and *S. aureus* (ATCC 6538) (susceptible to Methicillin, MSSA) were prepared from the Persian Type Culture Collection (PTCC), Iran (https://irost.org/ptcc/ptccdb/bacteria).

The MIC of two strains was determined by the microbroth dilution technique (22). These strains were cultured on Mueller Hinton (MH) broth at 37°C in a shaking incubator in 50 ml falcon tubes until their bacterial concentration reached logarithmic phases equal to the optical density (OD) of 600 nm (1 McFarland turbidity or $3\times10^8$ CFU/mL). Next, nisin was added to the MH broth cultures (inoculated with MRSA and MSSA) at the ¼, ½, and 1 × minimum inhibitory concentration (MIC) in the mid-exponential phase at 37°C. After 6 and 12 h, the bacteria were harvested by being centrifuged at 12000×g/10 min. In addition, the supernatants were removed, and the pellets ($2\times10^8$ CFU/ mL) were used for RNA extraction. The control media (without nisin) were cultured with two strains alongside the tests.

**RNA extraction.** RNA extraction was carried out by the rapid bacterial RNA isolation kit (BIO BASIC CANADA INC.) according to the manufacturer's instructions. For this purpose, the total RNA was solved in 30 µl of RNase-free water. Next, the extracted RNA was treated with DNase I (Cinna Gene, Iran). In addition, the quantity and quality of the RNA were confirmed by A260/A230 and A260/A280 ratios (Denovix, NanoDrop, USA). Besides, the integrity of the RNA sample was determined in a 1% agarose gel (the data were not shown). Next, the cDNA was synthesized from total RNA by reverse transcription using Pars Tous kit (Iran).

**Quantitative PCR and statistical analysis.** The expression ratio of the virulence genes of *S. aureus* was determined by qPCR based on the primers listed in Table 1. The qPCR was performed by the SYBR-Green method (SYBR Premix Ex Taq™ Takara, Japan) for each gene in a triplicate form with the reaction mixture. The reaction mixture was at the final concentration of 20 µL, including 10 µL of SYBR Green Mix (Takara Bio®, Japan), 5ng of cDNA, 0.1

**Table 1.** primers used to evaluate the expression of genes on *S. aureus.*

| Gene | Primer sequence (5 → 3) | Ref |
|---|---|---|
| *sae* | F CTGCCAAAACACAAGAACATGATAC | (40) |
| | R ATTTACGCCTTAACTTTAGGTGCAGAT | |
| *agrA* | F TGATAATCCTTATGAGGTGCTT | (41) |
| | R CACTGTGACTCGTAACGAAAA | |
| *sea* | F ATGGTGCTTATTATGGTTATC | (42) |
| | R CGTTTCCAAAGGTACTGTATT | |
| *spa* | F GCGCAACACGATGAAGCTCAACAA | (43) |
| | R ACGTTAGCACTTTGGCTTGGATCA | |
| 16srRNA | F GCTGCCCTTTGTATTGTC | (44) |
| | R AGATGTTGGGTTAAGTCCC | |

mM of primers, and deionized water. Next, PCR amplification was performed in 96 well-plates (ABI, France) using Applied Biosystems 7500 and Step One plus. The cycling parameters for qPCR included initial denaturation at 95ºC/5 min followed by 40 cycles, denaturation at 95ºC/30 sec, annealing at 60ºC/30 sec, and the extension at 72ºC/30 sec. The 16S rRNA housekeeping gene was used as the internal control (Table 1). The amplification efficiency of each sample was calculated by reconstruction of the linear log phase from the initial plateau phase of the PCR reaction (24). Besides, the expression ratio (fold) of the genes was computed using the ΔΔCT formula. In the end, statistical analysis was carried out using GraphPad Prism software version 5.0 (GraphPad Software, Inc., CA, USA).

## RESULTS

**MIC.** The MICs of nisin on *S. aureus* strains ATCC 6538 and ATCC 33591 were 800 mg/mL and 1600 mg/ml, respectively (Table 2).

***S. aureus* (ATCC 33591, MRSA).** According to the results, the transcriptions of the *sea*, *spa*, and *agrA* genes were significantly down-regulated after treatment at the ¼, ½ and 1×MIC of nisin in a 6-hour incubation time span; accordingly, upon an increase in the incubation time, the ratio of gene expression decreased significantly from 1.07 to 28.8 folds compared to the control. In addition, the gene expression level of the *sea* and *spa* genes decreased in the 6-hour incubation time span ($P < 0.05$) in comparison to the 12-hour incubation time span (Fig. 1). In contrast, the expression of the *sae* gene significantly increased at the ½ and 1×MIC of nisin compared to the control. However, there was no significant increase in the expression of the *sae* gene at ¼ MIC of nisin (Fig. 1a). In total, the maximum significant reduction belonged to the *sea* gene (28.8 folds) at the MIC of nisin.

**Table 2.** Minimal Inhibitory Concentration of nisin on *S. aureus* strains

| ***S. aureus* strains** | **MIC of bacteriocins (mg/mL)** |
|---|---|
| | Nisin |
| *S. aureus* (ATCC 33591) | 1600 |
| *S. aureus* (ATCC 6538) | 800 |

***S. aureus* (ATCC 6538, MSSA).** The alteration to the gene expression pattern in MSSA was similar to that in MRSA. Besides, the expression ratios of the *sea*, *agrA*, and *spa* genes significantly decreased in 6- and 12-h post-treatment time spans in comparison to the control at the ¼, ½, and 1 MIC of nisin, which ranged from 1.2 to 9.1 folds, respectively (Fig. 2). Similarly, the significant increase in the expression of the *sae* gene was observed in the presence of the ½ and 1 MIC of nisin (Fig. 2b). However, the increase in the incubation time did not significantly affect the expression ratios of the *sae* gene at the ½ of MIC of nisin. In addition, the maximum reduction, being up to 10 folds, was observed in the *agrA* gene (Fig. 2c).

## DISCUSSION

*S. aureus* is an important pathogen associated with foodborne diseases, which is easily transferred by foods (22). Staphylococcal food poisoning and enteritis are associated with the ingestion of pre-excreted exotoxins of *S. aureus* in foods, not with the ingestion of *S. aureus* itself (4, 25). Research shows that nisin could effectively form some pores in the bacterial cell wall and interrupt the cell membrane function (26). Some studies confirm that nisin could potentially be used as a natural preservative for some therapeutic purposes, especially in treating stomach ulcers, dermatitis, and gut infections (27, 28). In addition, surveys report that the antimicrobial activity of nisin in an animal model could control respiratory tract infections caused by *S. aureus* (29).

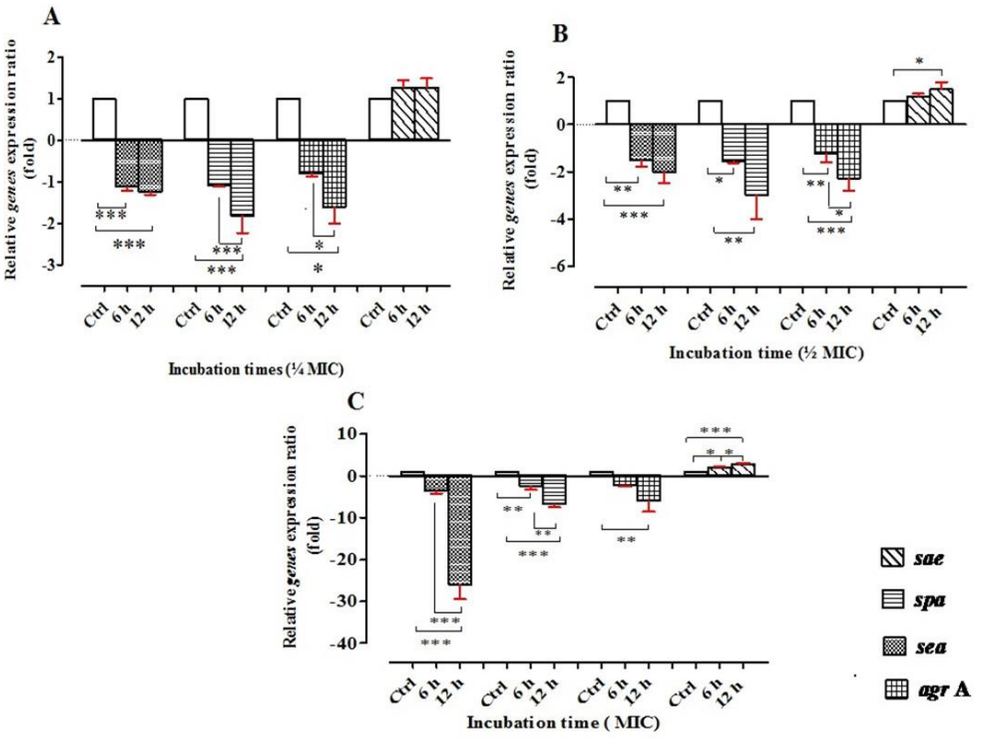


**Fig. 1.** Gene expression of *S. aureus* (ATCC 33591) in the present of different concentrations of Nisin. (A) ¼ MIC concentration (B) ½ MIC concentration C) MIC concentration in two different incubation times. Every data is an average of three independent experiments performed in triplicate and reported as M ± SD.

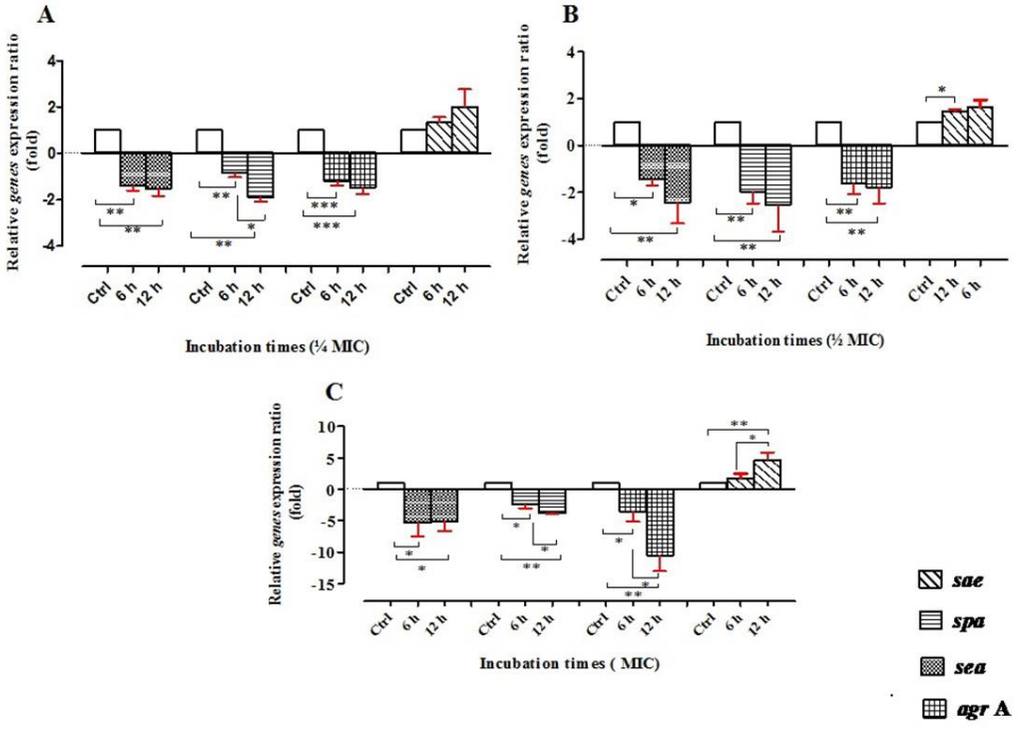


**Fig. 2.** Gene expression of *S. aureus* (ATCC 6538) in the present of different concentrations of Nisin. (A) ¼ MIC concentration (B) ½ MIC concentration C) MIC concentration in two different incubation times. Every data is an average of three independent experiments performed in triplicate and reported as M ± SD.

The use of nisin, as a successful therapy alternative to antibiotics, was studied in women with staphylococcal mastitis (30, 31). In addition, nisin is currently used as a sanitizer against some mastitis etiologic agents, i.e. species *Streptococcus* and *Staphylococcus*, in veterinary medicine (32, 33).

Numerous genes encoding virulence factors are directed to the assortment of intracellular and extracellular stimuli (34). Research indicates that sub-inhibitory concentrations of antimicrobial agents strongly influence the transcription of exoprotein-encoding genes, which could interfere with the interpretation of one administrative gene in *S. aureus* (35). In the present experiment, the *agrA*, *sea*, and *spa* genes were down-regulated, except the *sae* gene at ¼, ½, and × 1 MIC of nisin. Based on the present research results, being consistent with the investigation by Zhao et al. the reduction in gene expression was shown to be associated with capsule polysaccharide synthesis, cell-wall synthesis (the accessory gene regulator (*agr*) system) and the *sea* gene (enterotoxins), while up-expression was displayed in siderophore transporters regulated by the *sae* system. In a study, 274 out of 601 genes experienced a decline in expression, and 327 genes were up-regulated (36). The *agr* two-component regulatory system involves an interactive hierarchal regulative cascade among *agr*, *sae*, and *sar* genes as well as other regulatory gene products (37, 38). Since the expression levels of *sae*, and hemolysin are positively controlled by *agr* (1A, 3A), it is assumed that the influence of sub-inhibitory concentrations of nisin could depend in part on the inhibition of the *agr* two-component system. For instance, one could refer to the effect of the sub-inhibitory concentrations of nisin on *spa* expression. Therefore, it is concluded that nisin could inhibit global regulators, thereby resulting in a decrease in the secretion of virulence-related exoproteins. The *agr* locus has control over *sea* expression (37). Therefore, it is clear that the effects of nisin on *sea* secretion could be mediated by interactions with *agr* systems (36). According to the research conducted by Azizkhani et al. Zataria multiflora Boiss has an inhibitory expression effect on the enterotoxin genes (*sea* and *agrA*) of *S. aureus* (39). However, the effect of regulatory genes on *S. aureus* remains unclear, unlike that of *agrA* on *sea* production.

The expression results of the *sea*, *agrA*, and *spa* genes showed that the decreasing effect of nisin on gene expression intensified by an increase in the incubation time. Similarly, according to Dosler et al. nisin peptides exhibit rapid inhibition within the 1-4 h against MRSA strains (36). Zhao et al. stated that after 1 h from the post-treatment of nisin, the expression of virulence genes was altered (37). The results of the present study confirm that an increase in the incubation time has a significant effect on the virulence factor genes, which is considered a positive feature for addictive genes like nisin.

## CONCLUSION

The present study showed that nisin down-regulates expression *agrA* gene, thereby reducing the virulence factor genes of *S. aureus* and probably producing enterotoxins. It should be noted that *S. aureus* is the most significant agent in food poisoning. Thus, nisin is added to foods as a preservative to protect the body from poisoning. As a matter of fact, it should not be added to foods so much as to inhibit bacterial growth, but to the extent that it will decrease some part of gene expression at sub-MICs.

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