

The effects of nisin on the growth of milk-derived *Staphylococcus aureus* strains in the cheese

Süt kaynaklı *Staphylococcus aureus* suşları gelişimi üzerine peynirdeki nisin etkisi

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ABSTRACT

The use of bacteriocins in protecting the foods has significantly increased in recent years. Nisin is an important bacteriocin and is a food preservative additive safely used in food industry. In the present study, it was aimed to investigate the effect of nisin, which is an alternative to the synthetic additives, on the growth of milk-derived *Staphylococcus aureus* strains in the cheese. For this purpose, 100 different milk specimens were obtained, transferred using cold chain (at 0-4°C), and inoculated to Baird-Parker agar. At the end of inoculation process, 37 suspicious colonies were selected and coagulase-positive *Staphylococcus aureus* was detected. The strains obtained were passaged to the pre-enriched agars and the intensities of the strains were determined according to McFarland. Besides these strains, the coagulase-positive *Staphylococcus aureus* strains obtained from Etlik Veterinary Microbiology Institute were inoculated into each specimen at 10⁴ CFU mL⁻¹ concentration. The cheese production was made using milks containing the strains, which were isolated from the milk, and the standard strains at different concentrations. The specimens obtained from cheeses produced from milk containing using standard strains were divided into groups K, A, B, and C containing 0, 100, 200, and 400 IU mL⁻¹ nisin, respectively. Similarly, the specimens obtained from cheeses produced from milks containing milk-derived strains were divided into groups K1, A1, B1, and C1 containing 0, 100, 200, and 400 IU mL⁻¹ nisin, respectively. The procedures were triplicated. *Staphylococcus aureus* counting on the cheese specimens were performed on 1st, 6th, 12th, and 24th hours from curd to ball and on 5th, 10th, 20th, 30th, and 40th days of storage at +4°C. In conclusion, it was observed that the nisin inhibited the growth of all the *Staphylococcus aureus* strains. However, it was also determined that the inhibition of milk-derived *Staphylococcus aureus* strains were higher than the inhibition of standard strains.

Key Words: Nisin, Antimicrobial, Milk-derived *Staphylococcus aureus*, Bacteriocin, Cheese

ÖZ

Son yıllarda, gıdaların muhafazasında bakteriyosinlerin kullanımı artmıştır. Nisin önemli bir bakteriyosin olup gıda sektöründe güvenle kullanılan koruyucu gıda katkı maddesidir. Bu çalışmada süt kaynaklı *Staphylococcus aureus* suşlarının peynir içerisindeki gelişimi üzerine sentetik katkı maddelerine alternatif olarak süt endüstrisinde kullanılan nisin etkisinin araştırılması amaçlanmıştır. Bu amaçla, 100 farklı süt örneği temin edilmiş, soğuk zincir sağlanarak (0-4°C'de) Baird-Parker agara ekim yapılmış ve inkübasyon süresi sonunda 37 adet şüpheli koloni seçilerek koagülaz pozitif *Staphylococcus aureus* tespit edilmiştir. Elde edilen suşlar, ön zenginleştirme besiyeri içerisinde pasajlanmış ve suşların yoğunlukları McFarland'a göre belirlenmiştir. Bu suşların yanı sıra Etlik Veteriner Mikrobiyolojisi Enstitüsü'nden temin edilen koagülaz pozitif *Staphylococcus aureus* suşları ile her örneğe 10⁴kob/mL suş eklenmiştir. Sütten izole edilen suşlar ile standart suşları içeren sütler ile farklı konsantrasyonlarda nisin içeren peynir üretimi gerçekleştirilmiştir. Örnek grupları, standart suşları içeren sütlerden yapılan peynirler K, A, B ve C olarak sırasıyla ile 0, 100, 200, 400 IU mL⁻¹ nisin içeren gruplardan oluşmaktadır. Benzer şekilde, sütten izole edilen suşları

içeren sütlerden yapılan peynirler K1, A1, B1 ve C1 olarak sırasıyla ile 0, 100, 200, 400 IU mL⁻¹ nisin içeren gruptan oluşmaktadır. Çalışma üç tekerrür halinde dizayn edilmiştir. Peynir örneklerin *Staphylococcus aureus* sayımı telemeden kelleye kadar 1, 6, 12 ve 24. saatlerde ve +4C'de depolama süresi boyunca 5, 10, 20, 30 ve 40.günlerde yapılmıştır. Sonuç olarak, nisinin *Staphylococcus aureus*'un tüm suşlarının gelişimini inhibe ettiği görülmüştür. Ancak nisinin, süt orjinli *Staphylococcus aureus* suşları üzerine inhibisyonun, standart suşları üzerine sağladığı inhibisyonundan daha fazla olduğu tespit edilmiştir.

Anahtar Kelimeler: Nisin, Antimikrobiyal, Süt kaynaklı *Staphylococcus aureus*, Bakteriyosin, Peynir

Introduction

Cheese may be described as a food structure including largely of casein, fat, and water having a relatively short shelf-life (Kaya-Özkök and Tacer-Caba 2019). The use of bacteriocins in protecting the foods and especially cheeses has increased in recent years. Bacteriocins are generally defined as the ribosomal peptides or proteins synthesized by the bacteria inhibiting or killing many microorganisms (Leroy and De Vuyst 2004; Cotter *et al.* 2005).

Since they can be easily digested in human gastrointestinal system, the bacteriocins attracted significant attention as safe food protectants (Mills *et al.* 2011). The use of bacteriocins as natural food protectants meets the demands of customers for high-quality and safe product containing no chemical protectant. Besides that, the use of bacteriocins as food additive might be limited because of the reasons such as the efficiency of pathogen elimination or the high costs (Chen and Hoover 2003). Moreover, in order to overcome both biological and economic concerns, the scientists continue investigating new and more effective bacteriocins and the interest in research on bacteriocins continued in recent years (Silva *et al.* 2018)

Nisin is an important bacteriocin and is the lantibiotic that has been most investigated and most commercialized among others (Paul Ross *et al.* 2002; Cotter *et al.* 2005). As the safely used food protectant additive, the nisin is licensed (E234) by the Food Additive Specialist Committee of Food and Agriculture Organization of United States (FAO) and World Health Organization (WHO) (FAO/WHO 2001; Favaro *et al.* 2015). To date, 8 species of nisin have been discovered and characterized: Nisin A, Z, F and Q produced by

Lactococcus lactis and Nisin U, U2, P and H produced by several *Streptococcus* species (O'Connor *et al.* 2015).

Nisin has antimicrobial activity against lactic acid bacteria, spore-forming bacteria and pathogens such as *Listeria* and *Staphylococcus*, and many gram-positive bacteria such as *Bacillus* and *Clostridium* (Chen and Hoover 2003).

Nisin is widely used in cheeses and pasteurized cheeses in order to replace nitrate for preventing the development of clostridia spores (Abee *et al.* 1995; Chen and Hoover 2003).

Arqués *et al.* (2011) reported that nisin decreased the concentration of *L. monocytogenes* and *S. aureus* in milk stored at cooling temperatures. In various studies, the addition of nisin to various cheese types (cottage cheese, cheddar, and ricotta cheeses) were examined and it was shown that the concentrations of *L. monocytogenes* and *S. aureus* were successfully reduced (Chen and Hoover 2003). In Minas Frescal cheese, the number of *S. aureus* decreased by 1.5 log CFU after adding nisin (Felicio *et al.* 2015).

In the present study, the main objective is to determine how resistance the milk-derived target microorganism to nisin in another milk product. *S. aureus*, the target microorganism, is a microorganism that widely exists on personnel, milking equipment, environment, and dairy animals. If the heat treatment is not properly performed while producing dairy product by using milk containing this microorganism, then the risk may arise. For this purpose, the growth of *S. aureus* in non-brined fresh white cheeses containing nisin was monitored and the effect of nisin was analyzed.

Material and Method

Material

The raw milk specimens were obtained from and around Sivas. Baird-Parker agar (Oxoid-CM0275) was used together with Egg Yolk Tellurite Emulsion (Oxoid-SR0047) for counting *S. aureus* in milk specimens and for the coagulase test. Mueller Hinton Broth (Oxoid-CM0405) was used for the reproduction of *S. aureus*. Nisin was obtained from Merck (N5764). The coagulase-(+) *S. aureus* strains (ATCC 25923) were obtained from Etlik Veterinary Microbiology Institute.

Method

100 different milk specimens were obtained from and around Sivas and transferred to the laboratory by using cold chain transfer. The inoculation into Baird-Parker agar was performed using Egg Yolk Tellurite Emulsion for *S. aureus* counting (37°C, 24-48h). *S. aureus* was detected in 37 of these 100 specimens and then taken to coagulase test. Among 37 specimens, 9 were found to be coagulase-(+) *S. aureus*. The verification of strains was made using API test

(API 20E, Oxoid). The pre-enrichment was performed by adding 10 mL Mueller Hinton Broth to 0.1 mL of strain and the intensities of strains were determined according to McFarland (Oxoid, R20410, Equivalence Turbidity Standard 0.5, rotary speed: 200 rpm, 37°C, 24h). The number of coagulase-(+) *S. aureus* was set to be 1.2×10^4 CFU mL⁻¹.

10^4 CFU mL⁻¹ was taken from coagulase-(+) *S. aureus* strains isolated from milk and characterized using molecular method and then inoculated to nisin-containing milk specimens at concentrations of 0, 100, 200, and 400 IU mL⁻¹ to K1, A1, B1, and C1 groups, respectively. The coagulase-(+) *S. aureus* strains (ATCC 25923) obtained from Etlik Veterinary Microbiology Institute were dosed at 10^4 CFU mL⁻¹ and then inoculated into milk specimens containing 0, 100, 200, and 400 IU mL⁻¹ nisin (K, A, B, and C groups, respectively).

Using the milk specimens containing nisin at different concentrations and coagulase-(+) *S. aureus* strains obtained from different sources, the cheese production was performed as illustrated in Figure 1.

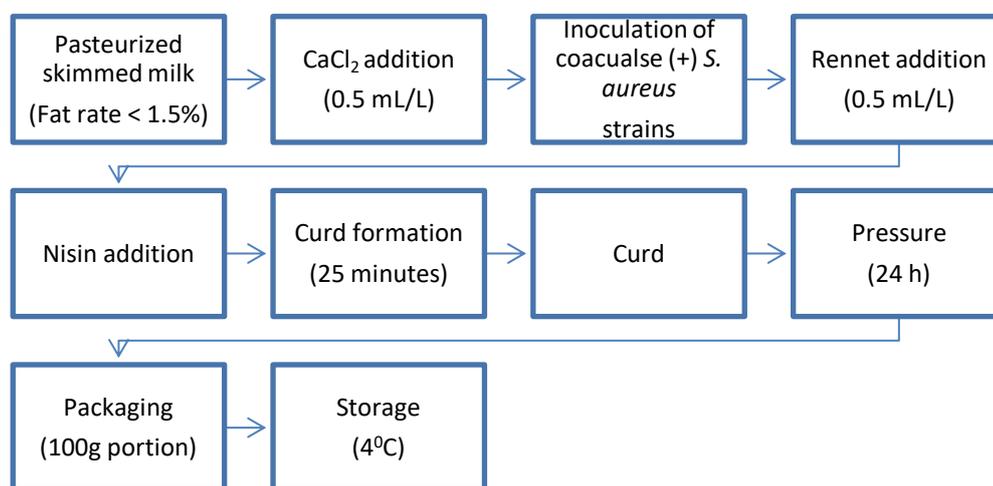


Figure 1. Cheese production

Şekil 1. Peynir üretimi

The non-brined fresh white cheese specimens were stored at +4°C till 40th day because of the limitation by microbial growth. The number of *S. aureus* (log CFUg⁻¹) in the period between curd and ball formation (1st, 6th, 12th, and 24th hours) and the number of *S. aureus* (log CFU g⁻¹) during

the storage at +4°C (5th, 10th, 20th, 30th, and 40th days) were determined.

The analyses were three times with 2 parallels. The data obtained were statistically analyzed with ANOVA test using SAS package software.

Results and Discussion

The number of *S. aureus* (log CFU g⁻¹) in the period between curd and ball formation (1st, 6th, 12th, and 24th hours) and the number of *S. aureus*

(log CFU g⁻¹) during the storage at +4°C (5th, 10th, 20th, 30th, and 40th days) in 8 different cheese specimens were determined and showed in Tables 1 and 2.

Table 1. The number of *Staphylococcus aureus* between curd formation and ball formation in cheeses produced from milk containing different concentrations of nisin and *Staphylococcus aureus* (log CFU/g)

Çizelge 1. Farklı oranlarda nisin ve *Staphylococcus aureus* içeren sütlerden elde edilmiş peynir örneklerinin telemeden kelle edinceye kadar *Staphylococcus aureus* sayısı (log kob/g)

Group Grup	1 st hour 1. saat	6 th hour 6. saat	12 th hour 12. saat	24 th hour (1 st day) 24. saat (1. gün)
K(control)	3.7	3.9	5.3 ^{a.k}	5.8 ^{a.k}
K1(control)	3.6	3.9	5.3 ^{a.k}	5.8 ^{a.k}
A	3.3	3.1	4.9 ^{a.k}	5.3 ^{a.k}
A1	3.5	3.7	4.1 ^x	4.7 ^{a.k}
B	3.6	3.9	4.2 ^x	4.5 ^{a.k}
B1	3.2	3.0	3.0 ^y	3.2 ^x
C	3.5	3.6	4.7 ^k	4.6 ^{a.k}
C1	3.1	3.0	3.0 ^y	3.1 ^x

a superscript indicates statistically significant difference in the same row ($p < 0.01$); superscripts y, x, and k indicate statistically significant difference in the same column ($p < 0.01$).

a üstel harfi aynı satırdaki istatistiksel olarak farkı ($p < 0,01$); y, x ve k üstel harfleri aynı sütündeki istatistiksel olarak farkı ($p < 0,01$) ifade etmektedir.

K: Including standard strain, K1: Including isolated strain A: Including standard strain and 100 IU/mL nisin, A1: Including isolated strain and 100 IU/mL nisin B: Including standard strain and 200 IU/mL nisin, B1: Including isolated strain and 200 IU/mL nisin C: Including standard strain and 400 IU/mL nisin, C1: Including isolated strain and 400 IU/mL nisin

K: Standart suş içeren örnek, K1: İzole edilen suş içeren örnek A: Standart suş ve 100 IU/mL nisin içeren örnek, A1: İzole edilen suş ve 100 IU/mL nisin içeren örnek B: Standart suş ve 200 IU/mL nisin içeren örnek, B1: İzole edilen suş ve 200 IU/mL nisin içeren örnek C: Standart suş ve 300 IU/mL nisin içeren örnek, C1: İzole edilen suş ve 300 IU/mL nisin içeren örnek

In cheese specimens with initial *S. aureus* number of 3.7 log CFU g⁻¹, the number of *S. aureus* increased in the period between curd formation and ball formation. At the end of 24h period, the highest number of *S. aureus* (5.8 log CFU g⁻¹) was observed in nisin-free cheese specimens (control), whereas the lowest number of *S. aureus* (3.1 log CFU g⁻¹) was observed in C1 cheese specimens produced from milk containing 400 IU mL⁻¹ nisin and coagulase-(+) *S. aureus* isolated from milk and characterized using molecular method.

Comparing the effect of nisin on milk-derived coagulase-(+) *S. aureus* strains and ATCC 25923 strains, it was determined that the nisin was more effective on the milk-derived *S. aureus* strains. For every time interval, it was determined that the number of *S. aureus* decreased as the amount of nisin increased and that the number of *S. aureus* increased as the time elapsed.

The change in the number of *S. aureus* during the

storage of specimens at +4°C is presented in Table 2. The number of *S. aureus* in cheese specimens obtained from milk containing *S. aureus* obtained from different sources and containing various concentrations of nisin has changed during the storage and, at the end of 40th day, the highest number of *S. aureus* (7.9 log CFU g⁻¹) was observed in nisin-free specimen inoculated with ATCC 25923 strain (K). Although nisin inhibited the growth of *S. aureus*, the number of *S. aureus* generally increased during the storage.

It was determined that the number of *S. aureus* decreased as the nisin content of cheese specimens increased. The lowest number of *S. aureus* (3.1 log CFU g⁻¹) was found in C1 cheese specimen containing 400 IU mL⁻¹ nisin and inoculated with milk-derived *S. aureus* strain (Figure 2).

Table 2. The number of *Staphylococcus aureus* in cheese specimens produced from various concentrations of nisin and *Staphylococcus aureus* during storage at 4°C (log CFU/g)Çizelge 2. Farklı oranlarda nisin ve *Staphylococcus aureus* içeren sütlerden elde edilmiş peynir örneklerinin 4°C'de muhafaza sırasındaki *Staphylococcus aureus* sayıları (log kob/g)

Group	5 th day	10 th day	20 th day	30 th day	40 th day
Grup	5. gün	10. gün	20. gün	30. gün	40. gün
K(control)	5.7 ^{b,k}	7.3 ^{a,k}	7.5 ^{a,k}	7.6 ^{a,k}	7.9 ^{a,k}
K1(control)	6.9 ^{a,k}	7.1 ^{a,k}	7.3 ^{a,k}	7.7 ^{a,k}	7.3 ^{a,k}
A	7.1 ^k	6.8 ^k	7.2 ^k	7.5 ^k	7.4 ^k
A1	7.0 ^k	6.9 ^x	7.1 ^k	7.3 ^k	7.5 ^k
B	5.7 ^x	4.9 ^x	5.6 ^y	6.1 ^x	6.1 ^x
B1	4.1 ^y	3.9 ^x	4.3 ^y	4.5 ^y	3.8 ^y
C	5.9 ^{b,x}	6.1 ^{b,k}	6.4 ^{a,x}	6.9 ^{a,x}	6.1 ^{b,x}
C1	4.3 ^{a,y}	4.1 ^{a,x}	3.2 ^{b,y}	3.3 ^{b,y}	3.1 ^{b,y}

a and b superscripts indicate statistically significant difference in the same row ($p < 0.01$); superscripts y, x, and k indicate statistically significant difference in the same column ($p < 0.01$).

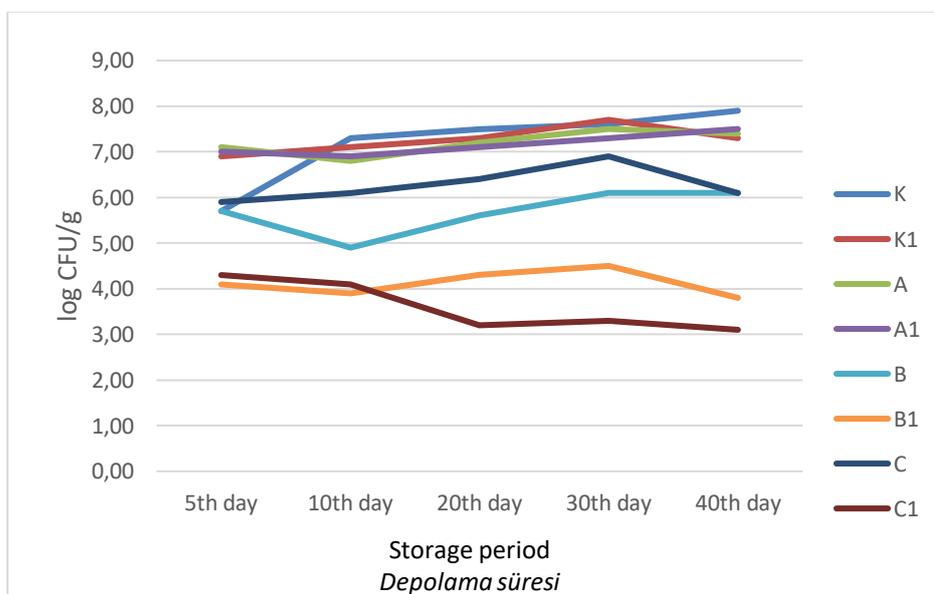
a ve b üssel harfleri aynı satırdaki istatistiksel olarak farkı ($p < 0,01$); y, x ve k üssel harfleri aynı sütündeki istatistiksel olarak farkı ($p < 0,01$) ifade etmektedir.

K: Including standard strain, K1: Including isolated strain A: Including standard strain and 100 IU/mL nisin, A1: Including isolated strain and 100 IU/mL nisin B: Including standard strain and 200 IU/mL nisin, B1: Including isolated strain and 200 IU/mL nisin C: Including standard strain and 400 IU/mL nisin, C1: Including isolated strain and 400 IU/mL nisin

K: Standart suş içeren örnek, K1: İzole edilen suş içeren örnek A: Standart suş ve 100 IU/mL nisin içeren örnek, A1: İzole edilen suş ve 100 IU/mL nisin içeren örnek B: Standart suş ve 200 IU/mL nisin içeren örnek, B1: İzole edilen suş ve 200 IU/mL nisin içeren örnek C: Standart suş ve 300 IU/mL nisin içeren örnek, C1: İzole edilen suş ve 300 IU/mL nisin içeren örnek

In Food Additive Code of Turkish Food Codex, the amount of nisin allowed in cheese is limited to 12.5 mg kg⁻¹ or mg L⁻¹. Amer et al. (2003), added 10 and 12 ppm nisin to Damietta cheese and kept at room temperature (+30°C) and cold storage (+4°C). Then, they counted the number of *S. aureus* for 8 weeks. The initial number of *S.*

aureus was found to be 5.61 log CFU g⁻¹. At the end of 8-week period, the number of *S. aureus* in control group containing no nisin was found to be 4.59 log CFU g⁻¹, whereas the numbers of *S. aureus* in cheese specimens containing 10 and 12.5 ppm nisin were found to be 2.69 and 2.3 log CFU g⁻¹, respectively.

Figure 2. The number of *Staphylococcus aureus* in cheese specimens produced from various concentrations of nisin and *Staphylococcus aureus* during storage at 4°C (log CFU/g)Şekil 2. Farklı oranlarda nisin ve *Staphylococcus aureus* içeren sütlerden elde edilmiş peynir örneklerinin 4°C'de muhafaza sırasındaki *Staphylococcus aureus* sayıları (log kob/g)

K: Including standard strain, K1: Including isolated strain A: Including standard strain and 100 IU/mL nisin, A1: Including isolated strain and 100 IU/mL nisin B: Including standard strain and 200 IU/mL nisin, B1: Including isolated strain and 200 IU/mL nisin C: Including standard strain and 400 IU/mL nisin, C1: Including isolated strain and 400 IU/mL nisin

K: Standart suş içeren örnek, K1: İzole edilen suş içeren örnek A: Standart suş ve 100 IU/mL nisin içeren örnek, A1: İzole edilen suş ve 100 IU/mL nisin içeren örnek B: Standart suş ve 200 IU/mL nisin içeren örnek, B1: İzole edilen suş ve 200 IU/mL nisin içeren örnek C: Standart suş ve 300 IU/mL nisin içeren örnek, C1: İzole edilen suş ve 300 IU/mL nisin içeren örnek

Pinto *et al.* (2011) added 0, 100, and 500 IU mL⁻¹ nisin to Serro cheese and followed both the number of *S. aureus* and physicochemical changes in the cheese specimens during the maturation period. Without any physicochemical change, nisin played effective role in decreasing the number of *S. aureus* in Serro cheese. When compared to the control group, the number of *S. aureus* decreased by 1.2 and 2.0 log units after 7th day in cheese specimens containing 100 and 500 IU mL⁻¹ nisin.

Sudagidan and Yemenicioğlu (2012) investigated the effects of nisin and lysozyme on the growth inhibition and biofilm formation capacity of 25 *S. aureus* strains isolated from raw milk and cheese. Nisin could inhibit the growth of all *S. aureus* strains when at high concentration (12.5 µg mL⁻¹), whereas it cannot prevent the biofilm formation at inhibitory concentrations.

It was reported that nisin is more active at low pH values (Delves-Broughton 1996; Benkerroum and Sandine 2010) and most effective in foods with low protein and fat rates and at pH < 6.0 (Okereke and Montville 1991). For this reason, it was reported in several studies that pH decreases and nisin's inhibitory effect increases as the nisin-containing cheese matures. Thus, nisin seems play no important role in decreasing the number of *S. aureus* in cheese produced from highly contaminated milk (Aktürkoğlu and Erol 1999).

Using pasteurized milk, in which they inoculated 5 log CFU/mL *S. aureus* strain, Felicio *et al.* (2015) produced cheese containing nisin at different concentrations (0, 200, 400, and 500 IU/mL) and then they stored the cheeses at +4°C. They reported that the number of *S. aureus* increased in all the applications but, in cheese produced from milk containing 500 IU/mL nisin, they reported the number of *S. aureus* to be 1.5 log units than in control.

When compared to the data reported in literature, it was determined in the present study that nisin played effective role in growth of all the *S. aureus* strains and the inhibition increased as the nisin concentration increased.

Conclusion

The effect of nisin, which is an important bacteriocin, on the growth of milk-derived *S.*

aureus strains and *S. aureus* strains obtained from laboratory was investigated. The nisin was added to cheeses, which were produced from milks containing these strains, and the number of *S. aureus* was counted during the shelf life of cheese.

In this study, it was observed that nisin inhibited the growth of all the *S. aureus* strains. However, it was determined that the nisin was more effective on the cheese samples produced from the milks inoculated with milk-derived *S. aureus* strains. Within this context, it can be stated that the nisin, which is widely used in many foods, inhibits milk-derived *S. aureus* strains and the effect of nisin on *S. aureus* varies between the origins.

Conflict of Interest: The authors declare that they have no conflict of interest.

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