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SHORT COMMUNICATION

Effects of Nisin and Temperature on Behavior of Enterotoxigenic *Staphylococcus aureus* in Model Cheeses

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Summary

The growth and enterotoxin synthesis of *S. aureus* as affected by different concentration of nisin (0, 1 and 2 µg/g) and storage temperatures (8 and 25°C) were studied in model cheeses manufactured with ultra-filtered milk. Microbiological analysis of the cheese samples were performed at 0, 1, 8, 15, 30, 45 and 60 days. Detection of the enterotoxins was done by an indirect double-sandwich ELISA technique using anti-enterotoxin monoclonal antibodies. Nisin concentration as low as 1 µg/g was found to have an inhibitory effect on the growth and enterotoxin production of *S. aureus*, besides the effect was more pronounced at 8°C than at 25°C.

Keywords: *S. aureus, Enterotoxin, Nisin, ELISA, Cheese*

Model Peynirler İçinde Enterotoksijenik *Staphylococcus aureus* Davranış Üzerine Nisin ve Sıcaklığın Etkisi

Özet

Staphylococcus aureus'un büyüme ve enterotoksin sentezi farklı nisin konsantrasyon düzeylerinden (0, 1 ve 2 µg/g) ve saklama sıcaklıklarından (8 ve 25°C) etkilendiği için ultra-süzülmüş süt ile üretilen model peynirler üzerinde çalışılmıştır. Peynir örneklerinin mikrobiyolojik analizi 0, 1, 8, 15, 30, 45 ve 60 gün üzerinden gerçekleştirilmiştir. Enterotoksin tespiti anti-enterotoksin monoklonal antikorlar kullanarak dolaylı çift-sandviç ELISA tekniği ile yapılmıştır. 1 µg/g gibi düşük bir nisin konsantrasyonunda *S. aureus*'un büyüme ve enterotoksin üretimi üzerinde bir inhibitör etkisi görülmüş olup, söz konusu etki 8°C'de 25°C'ye kıyasla daha belirgin olmuştur.

Anahtar sözcükler: *S. aureus, Enterotoksin, Nisin, ELISA, Peynir*

INTRODUCTION

Staphylococcal food poisoning (SFP) caused by ingestion of one or more preformed toxins are one of the most common causes of reported food-borne illnesses [1]. In the last few decades SFP has been economically one of the most important diseases in the world [2]. The annual number of SFP cases is 185.000 with about 1750 hospitalizations in the United States [3].

Staphylococcus aureus is normal flora on the skin and mucosae of animal and it is frequently associated to subclinical mastitis leading to contamination of milk and predispose for the public health to risk due to food poisoning [3,4]. The origin of contamination by *S. aureus* can be mastitic milk, the processing plant environment or from personel activity during food processing [5].

The European regulation set the upper limit for *S. aureus* in cheeses at a count of 10^5 colony-forming units (CFU) per gram. Above this limit, there is an obligation to determine whether enterotoxins are present (European Community Regulation No. 852-853/2004). Therefore, it is important to control *S. aureus* growth and enterotoxin production throughout the cheese-making process [6].

Nisin is a bacteriocin widely used as a food preservative particularly in cheese and generally regarded as safe (GRAS) [7]. It is produced by some strains of

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Lactococcus lactis and is active against most Gram-positive bacteria [8,9].

The objective of this study was to determine whether different concentrations of nisin (0, 1 and 2 µg/g) as well as storage temperatures (8 and 25°C) could alter the growth and enterotoxin production of *S. aureus* in model cheeses made from ultra-filtered milk.

MATERIAL and METHODS

Preparation and Enumeration of the Bacterial Inocula

Enterotoxigenic *S. aureus* ATCC 13565 (American Type Culture Collection, Rockville, USA) was used for the inoculation. The inoculum preparation and enumeration of the inocula were performed as previously described by Alomar et al.[6], and FDA/BAM [10]. Briefly, *S. aureus* was grown on a brain-heart infusion (BHI) agar (Merck) at 35°C for 24 h. One to two well-grown colonies on the plate were transferred to 5 ml of sterile BHI broth (Merck) and incubated at 35°C for 24 h. The bacterial suspension was precipitated by centrifugation at 6200 ×g for 15 min. The supernatant was discarded and the resultant pellet was washed with two consecutive steps using Ringer solution containing 0.05% (w/v) Tween 80 (Merck). The pellet was resuspended in Ringer solution and was diluted to obtain the final load of 5 log cfu/g of UF-retentate.

Nisin

Nisin containing 2.5% active nisin was obtained from Sigma-Aldrich Inc. (United Kingdom, EC 215-807-5). Nisin stock solution was prepared with 0.02 mol l^{-1} HCl (pH 1.6) and was filter sterilized through a 0.45 µm sterile, disposal and non-pyrogenic syringe filter.

Manufacture of the Model Cheeses

Model cheeses were prepared in the pilot-plant of the Tabriz Pegah Dairy Processing Plant (Tabriz, Iran). The ultrafiltered milk with volume concentration factor of 5.1 kg milk to 1.0 kg UF-retentate was inoculated with approximately 5 log cfu/g of *S. aureus*. Nisin concentrations of 0, 1 and 2 µg/g was added and mixed thoroughly with the UF-retentate. Rennet 0.002% (w/w) was mixed with water and with 10 ppm anti-foaming and 15 ppm anti-sticking agents (Danapak, Elteknik Landbrugsvej, Denmark) were added to each cheese container and homogeneously mixed. In the sealing machine, 3% (w/w) salt was added onto the parchment paper on the top of cheese and then by using aluminum foil, the container was sealed. The samples were kept for 1 day at 37°C for pre-ripening (day 1) and 2 weeks at 8°C for further ripening (days 8 and 15). Then cheese samples were stored for 45 days at 8 and 25°C (days 30, 45 and 60). Experiments at two different temperatures were designed to determine retention of viability and enterotoxin production of *S. aureus*.

Enterotoxin Assay

Staphylococcal enterotoxin detection was performed by the RIDASCREEN SET kit (R-Biopharm, Darmstadt, Germany), an indirect double-sandwich ELISA technique using antienterotoxin monoclonal antibodies, with a minimum detectable limit of 0.50 to 0.75 ng toxins per gram sample. Enterotoxin assay was performed according to the kit manufacturing. The Ridascreen Kit results of the OD colored enzymatic reactions was determined at 450 nm using a microplate reader (Tecan, Sunrise, Durham, NC, USA).

Statistical Analysis

The experiment was repeated in triplicate and analysis of the variance (ANOVA) by mean of Duncan's test was performed on the microbial counts as well as quantity of enterotoxin.

RESULTS

Effect of Nisin on S. aureus Growth in Model Cheeses Stored at 8°C

Here, it was revealed that growth of *S. aureus* influenced by different concentrations of nisin (0,1 and 2 µg/g) in the model cheeses stored at 8°C *(Fig. 1)*. During the pre-ripening of control samples (no nisin) at 37°C, *S. aureus* increased significantly ($P<0.05$) exceeding the degree at inoculation time. After the growth period of *S. aureus*, we observed a stabilization of the number and then a slight decrease during the storage period (from 15 days to 60 days).

According to the results nisin had a significant ($P<0.01$) inhibitory effect on the *S. aureus*. In the model cheeses containing 1 µg of nisin/g cheese the number of *S. aureus* decreased within the first day of pre-ripening. For *S. aureus*, by the increase of nisin concentration to 2 µg/g, the growth of organism at 8°C was significantly affected and the $\log_{10}$ cfu/g of the organism reached $<10^1$ cfu/g.

Effect of Nisin on S. aureus Growth in Model Cheeses Stored at 25°C

In this study, the different concentration of nisin did not affect the growth of *S. aureus* at 25°C *(Fig. 2)*. The number of the organism almost reached the same as that was at the first day of the study. However, at the first day of fermentation, there was a significant difference ($P<0.01$) between in the model cheese with and without nisin for the number of *S. aureus*.

Enterotoxin Production

The production of enterotoxin affected by nisin and temperature in model cheeses was determined. These factors had a significant effect ($P<0.01$) on staphylococcal

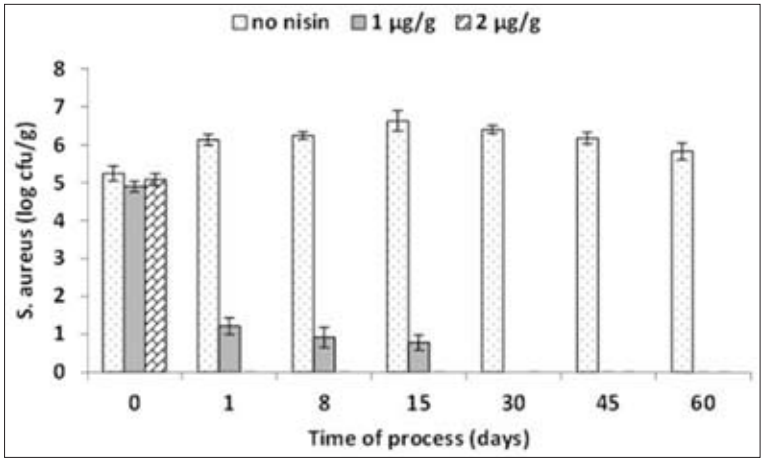
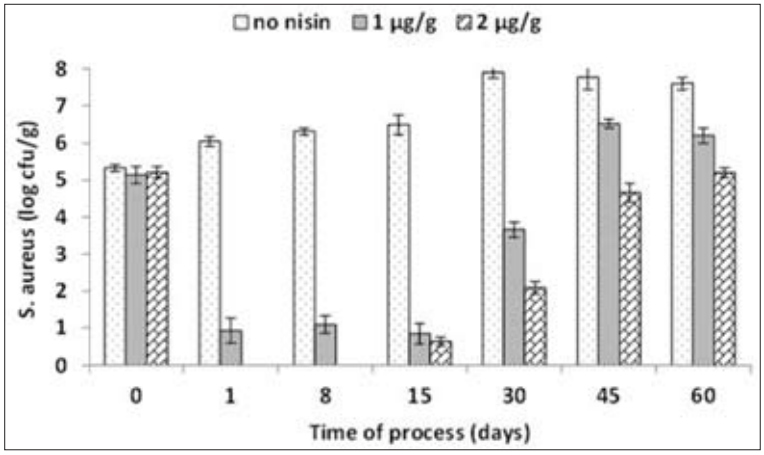


Fig 1. Effect of different concentrations of nisin on the growth of *S. aureus* during 60 days of storage at 8°C in model cheeses

Şekil 1. 60 gün boyunca 8°C'de muhafaza edilen model peynirlerde farklı konsantrasyonlardaki nisinin *S. aureus*'un gelişimi üzerine etkisi

Fig 2. Effect of different concentrations of nisin on the growth of *S. aureus* during 60 days of storage at 25°C in model cheeses

Şekil 2. 60 gün boyunca 25°C'de muhafaza edilen model peynirlerde farklı konsantrasyonlardaki nisinin *S. aureus*'un gelişimi üzerine etkisi



enterotoxin production throughout the ripening and storage. Staphylococcal enterotoxin was not detected in any of the cheese samples treated with nisin and or stored at 8°C. Enterotoxin A was detected in the nisin-free samples in quantities of 1.23, 1.28 and 1.11 ng/g during storage at 25°C in the 30, 45 and 60 days, respectively.

DISCUSSION

The model cheeses were produced based on a critical population density of *S. aureus*. Enterotoxin A was detected in model cheeses when counts of *S. aureus* were 7.9 log cfu/g. This result is in agreement with previous data [11], indicating that positive enterotoxin Camembert-type cheese made with artificially inoculated raw goats' milk had *staphylococci* counts higher than 10^6 cfu *S. aureus*/g.

According to the results, enterotoxin synthesis was inhibited by 1 µg of nisin per gram of cheese. However, during storage at 25°C, nisin degradation and *S. aureus* resuscitation occurred, there was no detectable enterotoxin in nisin treated samples (1 or 2 µg/g). In a previous work, diarrheal enterotoxin production of vegetative cells and spores of Bacillus cereus strain F3802A/84 in gravy was inhibited by adding 1 and 5 µg of nisin per ml at 15°C, respectively [12].

Decrease of nisin levels during storage was found to be temperature dependent. Nisin level remain relatively stable during refrigerated storage, but loss is faster at ambient temperatures [13]. Higher nisin levels are needed for products stored at high ambient temperatures for longer periods. Nisin may be degraded during storage by proteolytic enzymes derived from microbial, plant, or animal cells within the food [14].

The results obtained from this study indicates that the growth of *S. aureus* at 25°C is followed by enterotoxin production which yields a higher risk of food poisoning due to consumption of the cheeses. Those can be prevented by adding 1 µg of nisin per gram cheese.

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