


## Effect of nisin on the inhibition of the growth of *Staphylococcus aureus* and on the sensory properties of coastal cheese



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### Abstract:

*Staphylococcus aureus* is a common cause of food poisoning in humans. It is very common in dairy products made with raw milk, such as costeño cheese, an artisanal cheese common on Colombia's Caribbean coast. Costeño cheeses from Montería, Colombia, were analyzed to quantify their *S. aureus* load, the effect of added nisin in inhibiting *S. aureus* growth in this matrix and on product sensory characteristics. *Staphylococcus aureus* was isolated from commercial costeña cheeses and the minimum inhibitory concentration (MIC) of nisin versus this microbe quantified. Costeña cheese was made containing 0, 500 and 625 IU/kg added nisin, *S. aureus* counts done at 0 and 24 h storage, and the results analyzed using a random design with 57 experimental units. Sensory evaluation was done with a triangular test involving a panel of 40 tasters to identify any differences in the sensory characteristics of costeño cheese with and without 500 IU/kg added nisin. All the commercial costeño cheeses were contaminated with *S. aureus*, but all identified strains were inhibited by 500 IU/ml nisin

(MIC). Addition of nisin during cheese production reduced ( $P \leq 0.05$ ) *S. aureus* counts compared to a nisin-free control; at 24 h, nisin lowered *S. aureus* counts 2.3 log cycles in the 500 IU/kg nisin treatment and 1.9 log cycles in the 625 IU/kg treatment. Sensory characteristics did not differ ( $P \geq 0.05$ ) between costeño cheese containing 500 IU nisin/kg and a nisin-free control. Addition of 500 IU/ml nisin to costeño cheese can inhibit *S. aureus* growth without affecting sensory characteristics.

**Key words:** Bactericides, Minimum inhibitory concentration, *S. aureus*.

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Food safety consists of guaranteeing that food does not biologically, physically or chemically endanger the consumer; in other words, the absence of dangerous substances, the certainty of no adverse effects, the absolute opposite of risk<sup>(1)</sup>. A major biological risk in food are pathogenic microorganisms such as *Staphylococcus aureus*. This gram-positive bacterium is a normal species in the human bacterial flora, but can effectively grow in many foods (sliced meats, milk and derivatives, sauces, preserves, bakery products, and egg creams, among others), where it produces exotoxins that can result in staphylococcal poisoning with an intake as low as 100 ng toxin<sup>(2)</sup>.

Costeño cheese is a native food common on the Caribbean coast of Colombia. Produced artisanally from unpasteurized (raw) milk via enzymatic coagulation and dry salting, it is pressed, unripened, not acidified and white in color. Widely consumed in the region, the largely unsanitary conditions in which it is produced make it an important source of food poisoning<sup>(3)</sup>. Colombian Technical Standard (NTC) 750 stipulates that coagulase-positive *S. aureus* counts in costeña-type cheese must remain between 100 and 1,000 CFU/g<sup>(4)</sup>. Previous studies have identified unsafe *Staphylococcus* levels in costeña cheeses in Colombia. For example, 41.1 % of costeño cheese samples sold in Montería Municipality, Córdoba Department, were found to have unacceptable positive *Staphylococcus* coagulase values<sup>(5)</sup>. Another study identified *S. aureus* in 75 % of studied soft costeña cheeses and 25 % of semi-hard cheeses sampled in Valledupar Municipality, Cesar Department<sup>(6)</sup>.

Nisin (E234) is a FDA-approved food preservative classified as “generally recognized as safe” (GRAS) and is widely used to inhibit *S. aureus*. A polypeptide substance produced by

different strains of *Lactococcus lactis* and *Streptococcus lactis*, it acts against gram-positive bacteria, is stable in acidic pH and slightly heat-sensitive. Widely used in cheese production<sup>(7)</sup>, it has been frequently studied in this food matrix, especially against *S. aureus*<sup>(8)</sup>. Quantifying an antimicrobial's *in vitro* activity against a bacterial culture is done using broth or agar dilution techniques, such as minimum inhibitory concentration (MIC). This technique involves a series of tubes or plates containing broth or agar, respectively, to which the antimicrobial of interest is added in different concentrations. Each tube or plate is inoculated with a standardized suspension of the studied microorganism(s) and the MIC (expressed as µg/ml) measured based on the minimum bactericide concentration at which no bacterial development (i.e. turbidity) is observed<sup>(9)</sup>.

The present study objective was to apply the minimum inhibitory concentration technique to evaluate how nisin affects the development of *S. aureus* isolated from costeño cheese sold at licensed distributors in Montería Municipality, Córdoba Department, Colombia. Analyses were also done of *S. aureus* growth in costeña cheeses containing nisin, and the effects of nisin on costeña cheese sensory characteristics.

***S. aureus* isolation and identification.** Samples (250 g) of costeño cheese were acquired at points of sale in Montería and transported to the Food Microbiology Laboratory, University of Córdoba, Colombia. Serial dilutions were done, plated in duplicate on plates containing salt agar and mannitol, and incubated for 24 to 48 h at 37 °C<sup>(10)</sup>. Characteristic *S. aureus* colonies were confirmed by Gram stain, and the catalase and coagulase test<sup>(11)</sup>. Three of the isolated strains were analyzed by PCR<sup>(12)</sup>. Stored at 2 to 4 °C, the strains were activated with nutrient broth and cultured in Baird-Parker agar (BPA).

**Measuring MCI of nisin versus *S. aureus* strains isolated from Costeño cheese.** A McFarland scale was prepared as a turbidity standard to standardize density in microorganism preparations. The 0.5 scale was prepared with 0.18 M sulfuric acid and an aqueous solution of 0.048 M barium chloride equivalent to  $1.5 \times 10^8$  CFU/ml, with an optical density of 0.08 to 0.12 at 625 nm<sup>(13)</sup>. Three *S. aureus* colonies were added to tubes containing 5 ml Mueller-Hilton (MH) broth, incubated at 37 °C for 1 h, and absorbance and turbidity measurements taken. Those tubes exhibiting absorbance and turbidity measurements within the established range were considered standardized on the 0.5 McFarland scale, with approximately  $1.5 \times 10^8$  cells/mL<sup>(14)</sup>.

A nisin stock solution (NISIN(z) Qiqihaer Heilongjiang Province, China, from Amtech Biotech Laboratory, HS Code 2941909090) was prepared by diluting 1.0 g nisin in 100 ml 0.02 N HCl. The solution contained  $1.0 \times 10^4$  IU/ml with a pH near 2.0, which was adjusted to 3.21 with 0.5 N NaOH. This was diluted with MH broth to seven concentrations: 100, 200, 500, 1,000, 1,500, 2,000 and 2,500 IU/ml<sup>(15)</sup>. Broth alone was used as a control. Inoculum (10  $\mu$ L) was prepared with *S. aureus*, added to each tube, and the tubes incubated at 37 °C for 24 h with shaking at 182 rpm.

The lethality curve was built based on four concentrations:  $\frac{1}{2}$  MIC, MIC, 2xMIC and 4xMIC. Tube contents were cultured in triplicate on BPA plates at six incubation times (0, 2, 4, 8, 20 and 24 h) and the colonies counted. When bacterial count (CFU/ml) vs exposure time (hours) was graphed<sup>(11)</sup>, it showed a first-order exponential kinetics following the formula  $N_T = N_0 e^{-KT}$ ; applying logarithms produces  $\ln N_T = \ln N_0 - KT$ , the lethality rate (K). Clearing the previous equation allows calculation of the time it takes for the microorganism population to reduce by half, known as the lethality rate:  $T_{1/2} = \ln 2/K$ <sup>(16)</sup>.

**Evaluating different nisin concentrations during costeña cheese production.** An established protocol was used to produce costeña cheese<sup>(17)</sup>. Acidity, pH (potentiometry)<sup>(18)</sup> and *S. aureus* counts<sup>(11)</sup> were measured in filtered milk. This was heated to  $34 \pm 1$  °C and CaCL<sub>2</sub> (20 g/100 L milk) and Milkset liquid rennet (1.0 ml/10.0 L milk) added. The curd was cut into 1 to 2 cm squares and left to sit for 5 min. The whey was separated by gravity and salt added directly at 2.5 %. The salty curd was divided into three 500 g batches and nisin homogeneously distributed at one of the three studied concentrations (625, 500 and 0.0 IU/kg) in each batch. Each batch was individually molded and pressed, and samples taken from each and stored at  $4 \pm 2$  °C. Counts of *S. aureus* populations were done in triplicate at 0 and 24 h after production, following an established method<sup>(11)</sup>.

The resulting data were analyzed with a completely randomized design using 57 experimental units. The data was analyzed using the statistical software R Project version 3.1.1. An analysis of variance was run and a Duncan multiple range test ( $P < 0.5$ ) applied to identify significant differences between *S. aureus* counts in a control cheese with no nisin and those produced with the tested nisin concentrations.

**Triangular test of regular and nisin-containing costeña cheeses.** A sensory evaluation was done using forty untrained judges, aged 19 to 45 yr, to determine if sensory differences existed between a control costeño cheese without nisin and one containing 500 IU/kg nisin, the maximum permissible concentration<sup>(19)</sup>. Sensory evaluation was done using a group of

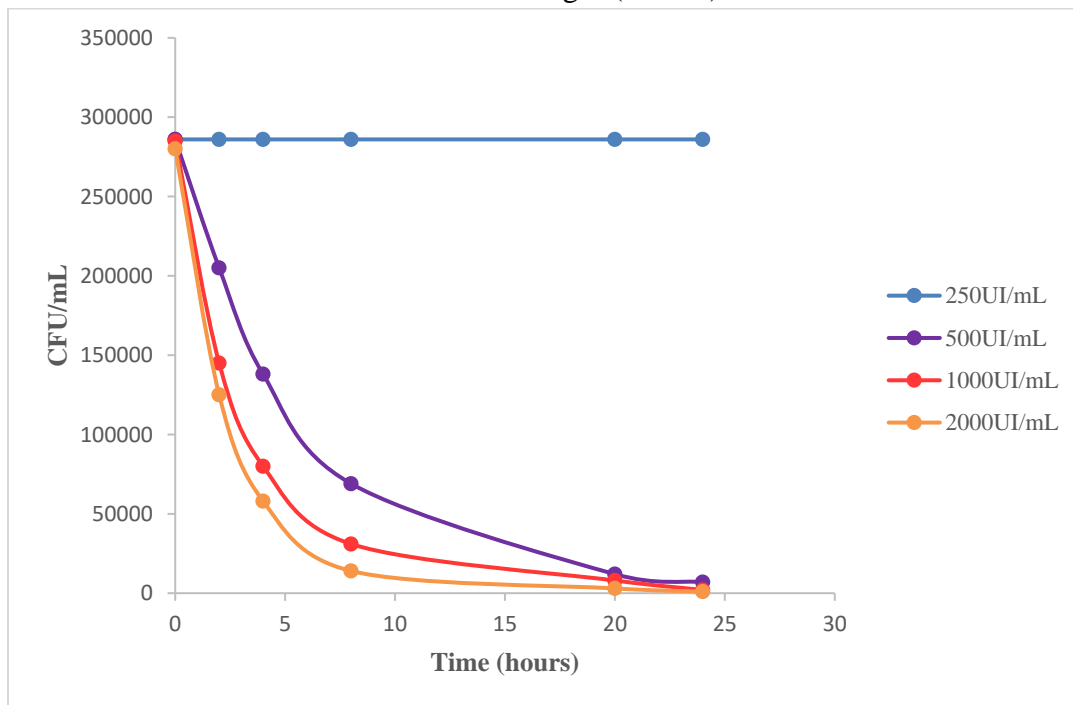
three cheese samples, two of which were the same, and the judge was asked to select which sample differed from the others. The number of correct answers was tabulated and compared in a table to identify the minimum number of correct answers and establish significance at different levels of probability in the triangular test (one-tailed,  $P= 1/3$ )<sup>(20)</sup>.

**Isolation, selection and identification of *S. aureus* from costeña cheese.** *Staphylococcus aureus* was isolated from all the studied costeño cheese samples. Eighteen (18) strains were identified with characteristics typical of *S. aureus* colonies (round, surrounded by a bright yellow area). The isolated strains characterized by cellular, colonial, and biochemical morphology corresponded to *S. aureus*. The PCR analysis identified three strains as having the genes that code for *S. aureus* enterotoxin.

**MIC versus *S. aureus* strains isolated from costeña cheese.** The nisin concentration that best inhibited *S. aureus* was 500 IU/ml. At 24 h incubation no turbidity was observed at this concentration, while turbidity was present in the 100 and 200 IU/ml concentrations. When the range of concentrations was extended from 500 to 1,125 IU/ml, inhibition was also observed at 625 IU/ml.

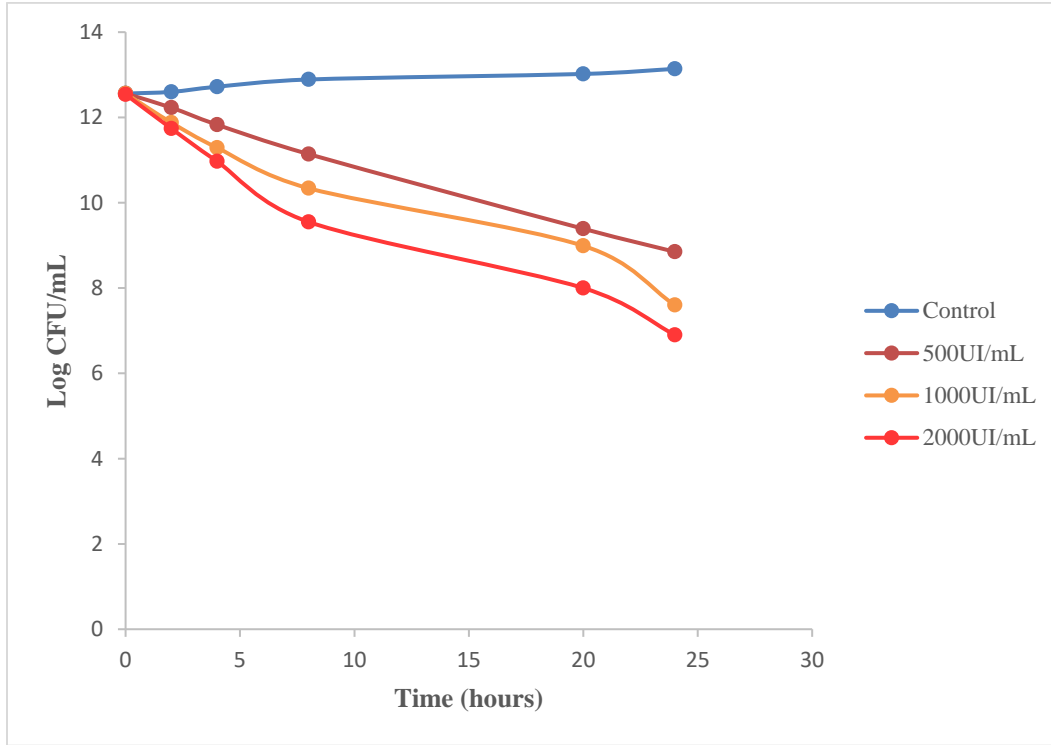
**Lethality curve.** The lethality curve clearly illustrates the relationship between nisin concentration and bactericide activity. Of the four tested nisin concentrations, 250 IU/ml did not inhibit *S. aureus* (Figure 1).

**Figure 1:** Lethality curve of *Staphylococcus aureus* growth versus nisin concentration in Baird-Parker agar (IU/mL)



Cell counts decreased logarithmically when *S. aureus* was challenged with nisin at concentrations at or above the MIC (500 IU/mL)(Figure 2), indicating that nisin meets the criterion of a bactericide<sup>(21)</sup>. Maximum bactericide activity at the MIC was attained at 20 h, with a 3.7log (CFU/ml) decrease in bacterial load.

**Figure 2:** Surviving cell count (Log CFU/g) vs time in hours (IU/mL)



The lethality rate (CFU/hour) showed that cell counts decreased proportionally over time (Table 1), suggesting that as nisin concentration increased so did microorganism mortality.

**Table 1:** Lethality rate and *Staphylococcus aureus* population reduction at evaluated nisin concentrations

Nisin (IU/ml)	K (CFU/hours)	T <sub>1/2</sub> (hours)
500	0.154	4.50095572
1000	0.184	3.76710424
2000	0.205	3.38120576

**Evaluation of different nisin concentrations in costeño cheese production.** The milk used as raw material in the costeña cheese production had pH (6.8) and acidity (0.16 % lactic acid) within established parameters, but *S. aureus* counts exceeding 10,000 CFU/mL<sup>(22)</sup>. Inclusion of nisin during production lowered counts ( $P<0.05$ ) at both the 625 and 500 IU/kg concentrations (Table 2). In the control treatment without added nisin, counts increased notably to levels above  $10^3$  CFU/g at both 0 and 24 h, which represents risk of food poisoning. At zero hours, the 625 IU/kg treatment exhibited the lowest ( $P<0.05$ ) average count, followed by the 500 IU/kg treatment. The same held true at 24 h, although the count in the control without nisin had actually increased. Both nisin concentrations effectively controlled *S. aureus* counts in the evaluated costeña cheeses.

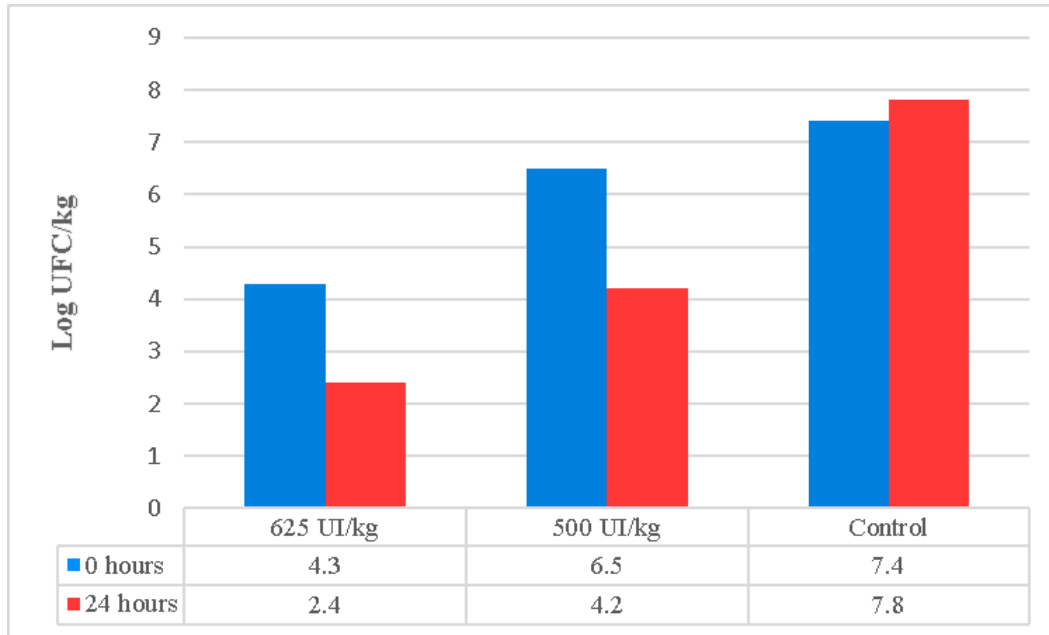
**Table 2:** Average *Staphylococcus aureus* counts in three costeño cheese production lots at zero and 24 h storage

Average counts (CFU/g) $\pm$ SD					
Nisin (IU/kg)	Zero hours		24 hours		Reduction (B/A,%)
625	69.9 $\pm$ 49.1	<i>a</i>	11.1 $\pm$ 9.2	<i>a</i>	15.9
500	658 $\pm$ 254	<i>ab</i>	64.4 $\pm$ 53.6	<i>a</i>	9.8
NO nisin	1669 $\pm$ 609	<i>c</i>	2403 $\pm$ 738	<i>b</i>	

<sup>abc</sup> Different letters indicate difference ( $P<0.05$ ).

During 24 h storage, average *S. aureus* populations increased in the control treatment without nisin, but were 3.62 logarithmic cycles lower in the 500 IU/kg treatment and 5.4 cycles lower in the 625 IU/kg treatment. (Figure 3).

**Figure 3:** Average *Staphylococcus aureus* count in costeña cheeses containing different nisin concentrations and a nisin-free control



**Sensory test.** The panel of judges found no differences ( $P<0.05$ ) between costeña cheese made with or without nisin, suggesting that addition of 500 IU/kg nisin to the cheese did not appreciably change sensory properties.

The presence of *S. aureus* in the studied costeña cheese samples showed that the population that consumes this product is at risk of food poisoning. This microorganism is most likely present in costeña cheese due to lack of milk pasteurization and poor hygiene practices during cheese handling, distribution and sale. In Colombia, 881 foodborne disease outbreaks were reported in 2018, cheeses being involved in 19.4 % of them. In cases where the etiological agent was identified, 12 % of the outbreaks were caused by *S. aureus*<sup>(23)</sup>. Previous studies of costeña cheese shipped in Córdoba department have found the product unfit for human consumption due to a significant microbiological load associated with deficiencies in product handling, production and storage facilities, and sales conditions<sup>(5)</sup>.

In the present study, most of the points where cheese was acquired exhibited clearly unsafe conditions during product distribution, storage and sale; very few establishments refrigerated the product. These conditions very probably directly affect costeño cheese microbiological quality. Review and monitoring of artisanal cheese production have been suggested since *S. aureus* contamination in artisanal cheeses can originate from the skin, mouth or nostrils of food handlers, from raw milk, and from processing and environmental conditions<sup>(24)</sup>.



Previous studies evaluating food safety in artisanal cheeses made with raw milk in various locations all found the presence of *S. aureus* to be associated with insufficient food safety practices<sup>(25-28)</sup>. Specifically, in artisanal cheeses in which starter cultures were not used *S. aureus* had no competing bacterial flora (e.g. lactic acid bacteria) and exhibited a significant risk of toxin production if initial counts were high enough<sup>(29)</sup>.

In the present results 500 IU/ml nisin MIC was required to inhibit *S. aureus*, which agrees with previous studies of artisanal cheeses. In Brazil, evaluation of nisin in *S. aureus*-contaminated artisanal cheese from Araxá (Minas Gerais) stored for 10 d found inhibition to be highest at 400 and 500 IU/ml nisin<sup>(30)</sup>; in addition, 100 IU/ml nisin concentrations reduced a logarithmic cycle in microorganism count during 60 day's storage, without changing attributes such as color and texture<sup>(31)</sup>. Adding 500 IU/ml nisin to the milk used in production of fresh cheese in Minas Gerais produced 2 log cycle reductions in *S. aureus* counts in curd and whey, a 1.5 logarithmic cycle reduction in cheese at 30 d refrigerated storage, and extended the latent phase in *S. aureus* growth in milk by 8 h<sup>(32)</sup>. A study of costeña cheese made with pasteurized milk and inoculated with *S. aureus* ATCC 29213 recorded a MIC of 500 IU/ml for nisin<sup>(33)</sup>.

Nisin's antibacterial effect is probably due to depolarization of the cytoplasmic membrane as part of a dual-action process in which it first forms pores in the microorganism membrane and then acts as a detergent destabilizing it<sup>(34)</sup>. This occurs in three steps. First, nisin's positive charge interacts with the cell wall's negative charge; second, it binds to a peptidoglycan carrier molecule from the cytoplasm to the cell wall (called lipid II), preventing synthesis; third, the nisin molecules bind to the lipid II to fix and bind to the cell membrane and form pores, causing cell death<sup>(7)</sup>.

Microbial lethality in response to nisin concentration was exponential in the present results, with first order kinetics due to linearity between the survivors-*versus*-treatment time logarithm. The curve slope symbolizes the rate at which cells die and is measured in CFU/time. The mathematical model that best estimates the slope, and determines CFUs at each time and concentration, optimally represented the results as comparable to the different kinetics of antimicrobials and antibiotics<sup>(35)</sup>. The kinetic parameters and mathematical models describing microbial population inactivation in thermal, pressure and electromagnetic processes are known<sup>(36)</sup>. Growth, survival or inactivation models have also been described for various microorganisms exposed to natural antimicrobials in various food matrices, and include information lag phase duration, generation time, maximum population density and exponential growth rate<sup>(37)</sup>. For example, the primary model is reported to describe *S. aureus* inhibition in different types of meat<sup>(38)</sup>.

Although previous research exists on nisin addition in pasteurized costeña cheese to inhibit *S. aureus*, raw milk was used in the present study because pasteurization has been linked to destruction of enzymes, decreased mineral content and elimination of geographically distinct microorganisms which differentiate cheese color and texture, and even probiotic potential<sup>(39)</sup>. In the raw milk used to produce costeña cheese, initial *S. aureus* concentrations exceeded maximum limits in Colombia; the presence of *S. aureus* in dairy products indicates contamination from handling or disease in cattle due to mastitis<sup>(40)</sup>.

Addition of nisin to costeño cheese during production lowered *S. aureus* counts at both zero and 24 h storage. This demonstrated an inverse relationship between nisin concentration and *S. aureus* counts over time, beginning during production and continuing during storage. This coincides with viability studies of *S. aureus* exposed to nisin for 1 to 24 h which report significant ( $P < 0.01$ ) reductions in microorganism population within the first hour of nisin exposure<sup>(41)</sup>. Similar behavior has been reported in artisanal cheese from Araxá (Minas Gerais-Brazil) in which *S. aureus* was most inhibited at 8 h in milk containing nisin and this inhibition was concentration-dependent<sup>(31)</sup>. As a food matrix, costeña cheese has considerable salt content, which supports nisin's antimicrobial effect, but it also contains lipids, which can interfere with the effect, probably due to its amphipathic behavior<sup>(42)</sup>. The greater bactericidal action of nisin in costeña cheese soon after production may be related to different factors: rapid diffusion of nisin when applied directly to food<sup>(43)</sup>; acquired or adaptive microbial resistance of *S. aureus* developing during exposure to nisin<sup>(44)</sup>, and degradation of nisin over time by food enzymes<sup>(45)</sup>. Both the nisin concentrations (625 and 500 IU/kg) added to the studied costeña cheeses significantly reduced (2.3 and 1.9 logarithmic units, respectively) *S. aureus* concentrations at 24 h. However, 500 IU/kg is the highest concentration recommended in food systems<sup>(18)</sup>.

Sensory evaluation comparing costeña cheese with and without 500 IU/kg added nisin showed an absence ( $P > 0.05$ ) of perceived organoleptic differences. Similar results have been reported when adding 5 mg/kg nisin to costeño cheese<sup>(46)</sup>. Because nisin is classified as GRAS, it can safely be used in food systems to lower *S. aureus* counts without affecting consumer acceptance.

Costeña cheese samples sold in Montería, Colombia, were found to harbor high *S. aureus* counts. Addition of 500 IU/ml nisin inhibited *S. aureus* strains isolated from these cheeses, with a maximum reduction of 3.7 Log (CFU/ml) at 20 h incubation. Lethality rate was 0.154 CFU/h, with a 4.5 h population reduction time. Compared to a control, no differences in sensory properties were observed in costeña cheeses containing 500 IU/kg nisin. Addition of nisin to artisanal costeña cheese could potentially reduce *S. aureus* populations and consequently reduce the risk of food poisoning in exposed consumers.

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