Effects of nisin on *Staphylococcus aureus* count and physicochemical properties of Minas Frescal cheese

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ABSTRACT

The aim of this work was to evaluate the effects of nisin on in vitro and in situ *Staphylococcus aureus* counts. For in vitro experiment, milk was inoculated with $5.0 \log \text{cfu} \cdot \text{mL}^{-1}$ of *S. aureus* and nisin was added at concentrations of 0, 100, 200, 400, and $500 \text{ IU} \cdot \text{mL}^{-1}$. The main effect of the bacteriocin was lag phase extension from 0 h, for 0 and $100 \text{ IU} \cdot \text{mL}^{-1}$ to 8 h, when 200, 400, and $500 \text{ IU} \cdot \text{mL}^{-1}$ of nisin were used; however, log phase was not affected. Microbial growth rate was found to be exponential and around $0.11 \log \text{cfu} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$ for all treatments. For in situ experiments, 0, 400, and $500 \text{ IU} \cdot \text{mL}^{-1}$ of nisin were directly added to pasteurized milk previously inoculated with $5.0 \log \text{cfu} \cdot \text{g}^{-1}$ of *S. aureus*. Milk, curd, and whey were analyzed to *S. aureus* counts. Nisin at concentration of $500 \text{ IU} \cdot \text{mL}^{-1}$ was able to reduce *S. aureus* count in curd and whey, demonstrating nisin partition between both phases. Throughout storage at 4°C , *S. aureus* count increased for all treatments, but the bacterial grew slower when nisin was added in both concentrations, maintaining *S. aureus* count about 1.5 log cycles lower than the control, despite abusive initial *S. aureus* count. Therefore, nisin seems to play an important role in reducing *S. aureus* initial count in cheese made with highly contaminated milk. Nisin showed potential to be used as an additional, important hurdle to improve Minas Frescal cheese safety, without replacing good manufacturing practices.

Key words: bacteriocin, bacterial growth, high moisture cheese, milk, food poisoning

INTRODUCTION

Minas Frescal cheese is widely consumed in Brazil. It is a fresh, soft white cheese produced via enzymatic coagulation, resulting in a preservative-free cheese having high water activity, low salt content, and pH higher

than 5.0, and that does not undergo maturation. These features make Minas Frescal cheese highly perishable and its shelf life remarkably short, even under refrigeration (Silva et al., 2003; Malheiros et al., 2012; Magenis et al., 2014).

The physicochemical characteristics of Minas Frescal cheese increase the potential risk of pathogenic bacteria incidence, including *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus* (Carvalho et al., 2007). Staphylococcal food poisoning is often related to cheese consumption due to the presence of enterotoxins (Hennekinne et al., 2012), which in turn are probably caused by excessive human manipulation during cheese production.

The most efficient way of reducing the initial milk contamination and, consequently, producing a safer cheese is by improving the hygienic conditions during milking and cheese production (Poli et al., 2007). However, these practices have not been enough to ensure Minas Frescal cheese quality and safety because many studies have associated this cheese with food poisoning (Silva et al., 2003; Carvalho et al., 2007; André et al., 2008; Malheiros et al., 2012; Magenis et al., 2014).

Some studies have shown that bacteriocins can act as an additional hurdle to reduce pathogenic bacteria contamination in cheeses (Pinto et al., 2011; Malheiros et al., 2012). Bacteriocin action mechanism is particularly interesting when compared with traditional antibiotics due to their strong antibacterial activity against a broad microorganism spectrum and to the low bacterial resistance rates (Malheiros et al., 2012; Tong et al., 2014). Nisin, the most studied bacteriocin, is generally regarded as safe (GRAS) by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives. Nisin is a 34 amino acid peptide produced by *Lactococcus lactis* ssp. *lactis*, and it is antimicrobially effective against a wide range of gram-positive bacteria (Liu et al., 2015).

Many studies have shown that nisin is effective against *Staphylococcus* sp. (Hamama et al., 2002; Pinto et al., 2011; Pimentel-Filho et al., 2014; Zhao et al.,

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2014; Liu et al., 2015). However, to the best of our knowledge, few data are available regarding the effect of nisin against *S. aureus* in Minas Frescal cheese, which is a fresh, highly manipulated product and, thus, an important vehicle for the staphylococcal enterotoxin.

Therefore, with the aim of contributing to Minas Frescal cheese safety and extending its shelf life, the goal of this work was to evaluate the inhibitory effect of different nisin concentrations against *S. aureus* inoculated in Minas Frescal cheese during 30 d of storage.

MATERIALS AND METHODS

Nisin Preparation

For in vitro experiments, commercial nisin (Nisaplin, Danisco Brasil Ltda, Pirapozinho, Brazil) suspensions at 2.5% (wt/wt) in reconstituted (10% wt/vol) skim milk (RSM). For in situ experiments, nisin was directly added to milk during cheese manufacturing.

Culture and Growth Conditions

Staphylococcus aureus (ATCC 6538) cultures were used in this study. The stock culture was maintained at -80°C in brain heart infusion (BHI) broth (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ) containing 10% (vol/vol) of glycerol. Stock cultures were recovered in BHI broth and subsequently subcultured thrice in BHI at 37°C for 24 h.

Nisin In Vitro Antagonistic Effect Against *S. aureus*

Staphylococcus aureus was added to nisin suspensions, prepared in RSM at 10%, at concentrations of 0, 100, 200, 400, and 500 $\text{IU}\cdot\text{mL}^{-1}$ to obtain an initial count of $1 \times 10^5 \text{ cfu}\cdot\text{mL}^{-1}$. Systems were incubated at $37 \pm 2^{\circ}\text{C}$ during 24 h and *S. aureus* was counted throughout this period by using Petrifilm 3M Rapid *S. aureus* Count Plates (AOAC International, 2003; method 981.15). Previous studies have demonstrated that Petrifilm is suitable for *S. aureus* count in dairy products (Viçosa et al., 2010).

Nisin Antagonistic Effect Against *S. aureus* Inoculated in Minas Frescal Cheese

To evaluate nisin in situ effect on *S. aureus*, Minas Frescal cheese was produced with pasteurized milk containing 3.0% (wt/wt) of fat and added by commercial rennet and sodium chloride at 2.0% (wt/wt), according to the flowchart illustrated in Figure 1. Good manufacturing practices were followed to avoid cheese contamination.

Staphylococcus aureus was added to pasteurized milk to obtain an abusive initial count of $1 \times 10^5 \text{ cfu}\cdot\text{mL}^{-1}$. Based upon in vitro tests, nisin was added at concentrations of 400 and 500 $\text{IU}\cdot\text{mL}^{-1}$. Nisin was not added to control milk.

Microbiological analyses were carried out in milk, whey, and curd during cheese production as well as in cheese after 1, 7, 14, 21, and 30 d of storage. For each analysis, 25-g (or 25 mL) samples were diluted in 225 mL of 0.2% (wt/vol) peptone water. *Staphylococcus aureus* was counted in Petrifilm 3M Rapid *S. aureus* Count Plates (AOAC 981.15), according to the manufacturer's recommendations.

Data Analysis

The experiments were carried out 3 times in a completely randomized design. The decimal logarithms of *S. aureus* counts were evaluated by ANOVA, using the Tukey test at a significance level of $P < 0.05$ to compare mean values. The analyses were performed using the Software Analysis and Experimentation Group, version 8.0 (SAEG, Viçosa, Minas Gerais, Brazil).

RESULTS AND DISCUSSION

Nisin In Vitro Effect Against *S. aureus*

Culture medium composition is known to influence antimicrobial tests (Huys et al., 2002). Therefore, we chose reconstituted (10% wt/wt) skim milk (RSM) to investigate the nisin in vitro effect on *S. aureus*. Reconstituted skim milk is rich in proteins that can interact with nisin, reducing its effect. Inhibition of *S. aureus* inoculated in milk containing different nisin concentrations is shown in Figure 2.

In the absence of nisin, *S. aureus* grew immediately without a lag phase and reached a maximum population density (MPD) of 7.8 $\log \text{ cfu}\cdot\text{mL}^{-1}$ during 24 h in RSM ($\text{pH} = 6.5$). When 100 $\text{IU}\cdot\text{mL}^{-1}$ was added to RSM, a similar behavior was observed ($P > 0.05$). Pinto et al. (2011) also did not confirm the effect of low nisin concentration (100 $\text{IU}\cdot\text{mL}^{-1}$) against *S. aureus* in RSM (12% wt/wt).

Nisin concentration of 200 $\text{IU}\cdot\text{mL}^{-1}$, however, was effective against *S. aureus* as lag phase was increased to 8 h. After this adaptation period, cells grew and achieved a MPD of 7.6 $\log \text{ cfu}\cdot\text{mL}^{-1}$ during 36 h. This result demonstrates that nisin was effective both in increasing the *S. aureus* lag phase and in reducing bacteria growth in RSM even at the optimum inoculation temperature.

When nisin concentration was enhanced to 400 and 500 $\text{IU}\cdot\text{mL}^{-1}$, different *S. aureus* growth patterns were observed if compared with lower nisin concentrations.

Figure 1. Schematic flowchart of Minas Frescal cheese production.

Initially, a reduction of 1.3 log cycles in bacteria count occurred until 8 h of incubation, showing evidence of nisin bactericidal effect. However, after 16 h, *S. aureus* achieved a count that compensated the initial reduction caused by nisin, and MPD ($7.2 \log \text{cfu} \cdot \text{mL}^{-1}$) was reached until 36 h. For control and all nisin concentrations, log phase showed a similar behavior ($P > 0.05$) and the exponential microbial growth rate was almost the same (around $0.11 \log \text{cfu} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$). These results indicate that the main nisin effect is lag phase extension, which is in accordance with a previous report (Kiss et al., 2007).

In another work, $10,000 \text{ IU} \cdot \text{mL}^{-1}$ of nisin was able to reduce *S. aureus* counts by $4.68 \log \text{cfu} \cdot \text{mL}^{-1}$ in milk after 4 h of incubation at 37°C , followed by bacteria

surviving cell regrowth after 24 h. This behavior was attributed to the presence of nisin-resistant bacteria (Arqués et al., 2011). Grisi and Gorlach-Lira (2005) used a nisin concentration close to that used in the present experiment and found that this bacteriocin was bacteriostatic up to 8 h for *S. aureus* growth in BHI broth. However, after the lag period, the multiplication rate was similar to that in the absence of nisin, which is the behavior also associated with resistant cells. On the other hand, according to Gallo et al. (2007), the survivors are not necessarily resistant to nisin. They may also have escaped from nisin action due to insufficient bacteriocin availability.

Because only 400 and $500 \text{ IU} \cdot \text{mL}^{-1}$ of nisin demonstrated potential to inhibit *S. aureus* mainly by in-

Figure 2. Activity of different nisin levels against *Staphylococcus aureus* (initial count: 1×10^5 cfu·mL⁻¹) inoculated in reconstituted (10%) skimmed milk: 0 (■), 100 (●), 200 (▲), 400 (▼), or 500 (○) IU·mL⁻¹.

creasing its lag phase, we selected these concentrations to apply in Minas Frescal cheese production (in situ experiment). It is worth emphasizing that both nisin concentrations are in agreement with the Food and Agriculture Organization/World Health Organization Codex Committee on milk and milk products, which accepted nisin as a food additive for processed cheese at a concentration of 12.5 mg of pure nisin/kg of product (Ross et al., 2002).

S. aureus Count in Milk, Whey, and Curd During Minas Frescal Cheese Production

The presence of different constituents as well as the production steps may affect both bacteria cell and bacteriocin distributions when milk is curdled into curd and whey. It is very important to consider nisin distribution because its presence in curd is an indicative of its effect against *S. aureus* and, as a consequence, of cheese shelf life. Nisin had been used to prolong cheese shelf life in previous studies (Kykkidou et al., 2007; Mitra et al., 2010). Table 1 presents the nisin effect against *S. aureus* in milk, whey, and curd.

In all production steps, *S. aureus* count was higher when nisin was absent. No difference ($P > 0.05$) was found between nisin concentrations of 400 and 500 IU·mL⁻¹, concentrations that led to reductions of 0.75 and 0.91 log cfu·mL⁻¹, respectively, on *S. aureus* count. Results higher than ours were found for 400 IU·mL⁻¹

of nisin, which was able to inhibit 26.2% of *S. aureus* in raw milk. However, the initial count studied was lower (6.03×10^2 cfu·mL⁻¹) than that used in our experiment (Takahashi et al., 2003).

Nisin concentration affected ($P < 0.05$) *S. aureus* count in whey and curd, provided the reduction of approximately 2.0 log cfu·g⁻¹ led by 500 IU·mL⁻¹ of nisin. This confirmed the nisin bactericidal effect against *S. aureus* in cheese. It is important to emphasize that even though part of the nisin added to milk diffused to whey, the remaining nisin in curd was enough to achieve *S. aureus* counts lower than that required by Brazilian legislation for Minas Frescal cheese (2.7 log cfu·g⁻¹). Pinto et al. (2011) also verified the nisin effect on *S. aureus* on Minas Traditional cheese curd, contributing to a low initial count.

S. aureus Count in Minas Frescal Cheese

Minas Frescal cheese is classified as a high moisture cheese. It has low salt content and pH near 6.0 (Carvalho et al., 2007). Therefore, it is expected that *S. aureus* count increases during storage. Figure 3 shows the nisin effect on *S. aureus* growth throughout Minas Frescal cheese storage.

After only 1 d of storage, *S. aureus* counts in all treatments were higher than that established by Brazilian legislation, indicating that cheese molding, turning, and packaging steps are critical to bacterial growth.

Although *S. aureus* count increased during storage, nisin inhibited bacterial growth in Minas Frescal cheese. Even after 30 d, *S. aureus* count was lower for both nisin concentrations, which were not different ($P > 0.05$), indicating that the bacteriocin was effective against this microorganism in the cheese matrix. It is known that nisin interacts with some food constituents such as proteins and lipids, reducing nisin action against bacterial development (Aly et al., 2012). Additionally, for nisin to be effective, it needs to diffuse through the medium and reach bacterial colonies, and cheese matrix may hamper nisin diffusion, decreasing its efficacy. In this experiment, we found that cheese

Table 1. *Staphylococcus aureus* count (log cfu·mL⁻¹) as a function of nisin concentration in different steps of Minas Frescal cheese production

Step	Nisin concentration, IU·g ⁻¹ or mL ⁻¹		
	0	400	500
Milk	6.30 ^a	5.55 ^b	5.39 ^b
Whey	5.48 ^a	4.50 ^b	3.40 ^c
Curd	4.59 ^a	2.91 ^b	2.49 ^c

^{a-c}For each step, mean values followed by different letters within a row were different ($P < 0.05$) as indicated by Tukey test.

Figure 3. *Staphylococcus aureus* growth in Minas Frescal cheese as a function of storage time at 4 °C, in the presence of 0 (■), 400 (○), or 500 (▲) IU·mL⁻¹ of nisin.

protein-lipid 3-dimensional structure did not avoid nisin action on bacteria.

In spite of nisin action, the main contribution of bacteriocin addition to Minas Frescal cheese seems to be the reduction on initial *S. aureus* count. It was clear that bacterial count increased during storage, but the growth was slower in cheese samples containing bacteriocin. *Staphylococcus aureus* growth could be attributed to the high pH of Minas Frescal cheese (which ranged from 6.6 to 5.9 during storage), given that nisin is more effective in low pH (Tong et al., 2014). Nisin could also have interacted with casein (Aly et al., 2012), resulting in less bacteriocin available to inhibit bacterial growth. Additionally, it is known that variant resistant cells can appear from nisin-sensitive strains (Garde et al., 2004). Therefore, some *S. aureus* cells could have survived and been selected, leading to reduced nisin effect during cheese storage.

In another study, nisin at 600 IU·g⁻¹ combined with bovicin was also used in Minas Frescal cheese having an initial *S. aureus* count of 10⁴ cfu·g⁻¹. This bacteriocin mix was able to reduce only 1 log cycle until 15 d of storage at 4°C. After this period, bacteria regrew (Pimentel-Filho et al., 2014).

Our results clearly demonstrate that nisin could be an important tool to avoid enterotoxin production in Minas Frescal cheese, especially if initial *S. aureus* count is low. Therefore, nisin should be used as an additional hurdle in Minas Frescal cheese production, but it cannot replace good manufacturing practices.

CONCLUSIONS

Nisin was shown to be effective in controlling *S. aureus* growth both in milk and Minas Frescal cheese. During cheese production, this bacteriocin was distributed either in whey or in curd. Nisin seems to increase the lag phase of *S. aureus* inoculated in milk. Higher nisin concentrations (400 and 500 IU·mL⁻¹) reduced *S. aureus* growth in Minas Frescal cheese, but the main effect was the reduction on *S. aureus* initial count in the tested cheese. Nisin has potential to be used as an additional hurdle to improve Minas Frescal cheese safety and preservation. It should be used together with good manufacturing practices to achieve the best outcome.

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