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Influence of the salt concentration on action mechanisms of natamycin against microorganisms of importance in food manufacture

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Abstract

The objective of this study was to evaluate the influence of salt concentration on action of natamycin on important microorganisms in food manufacturing as this preservative has been used in immersion baths in several dairy products in the country. Strains of *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* were inoculated at different saline and peptone water concentrations and received natamycin treatments. These solutions were maintained at 12 °C and the behavior of the microorganisms evaluated at 0, 24 and 48 hours (T0, T1 and T2). Each microorganism was assessed in isolation as well as the association of *C. albicans* and *E. coli.* Under the conditions proposed by the research, it was possible to conclude that 0.025% natamycin has no efficacy on *C. albicans* inoculated at saline concentrations below 5%. The results obtained in *E. coli* counts suggest that natamycin may interfere with its development even at concentrations that may be considered low (0.1%) and at salinity conditions of 7.5% to 10%. The association of natamycin with sodium chloride potentiates its antimicrobial action, which can represent an economy and its use is amplified by the industries.

Keywords: preservatives; brine; pathogenic microorganisms.

Practical Application: This study generated knowledge for the practical application of natamycin in brine for cheeses. From this study it was possible to conclude that the association of natamycin with sodium chloride potentiates the antimicrobial action of natamycin, which may represent an economy, and its use be amplified by the industries.

1 Introduction

Natamicin is a macrolide antifungal polyene produced by strains of Streptomyces, as *S. natalensis*, *S. chattanoogensise*, *S. lydicus* (Atta et al., 2015), and is used in humans for topical treatment of bacterial and fungal keratitis, mouth, skin and vaginal infections, and in the food industry as a preservative for cheeses, salamis, yogurts, and, in some countries, juices and wines (Dalhoff & Levy, 2015).

This antimicrobial is part of the group of preservatives in the list of food additives authorized in the European Union and is approved by the US FDA for application on cheeses surface (European Food Safety Authority, 2009). The effect of natamycin on the development of bacteria, protozoa and viruses is considered to be inexpressive and, therefore, poorly studied (Ramos et al., 2012; Dalhoff & Levy, 2015). However, Atta et al. (2015) demonstrated that natamycin produced by *S. lydicus* showed effective action against *Candida albicans* and several bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and molds and yeasts.

In the Brazilian regulation regarding the production of cheeses (Brasil, 1996) natamycin is included as an additive for use as conservative surface with a maximum limit of 1.0 mg/dm^2 not detectable at 2 mm depth, being absent in the mass. Although the recommendation of the use of natamycin in cheese production is limited to spraying or immersion in aqueous solutions, with concentrations varying from 0.1% to 0.4% (Laurindo, 2017), in practice it is possible to verify its use as a coadjuvant in the treatment of brine for cheeses. Such use has become widespread, and the concentration used in brine tanks is usually 0.025% natamycin.

According to data from the Ministry of Health, milk and its derivatives are among the foods involved in food outbreaks in the country from 2000 to 2017 (Brasil, 2018). Some authors indicate that *E. coli* and *S. aureus* are among the main etiological agents involved in these outbreaks (Brasil, 2018) and both microrganisms have the capacity of to develop in environments with high salinity (How et al., 2013)

Other microorganisms such as yeast *Candida albicans* can also contaminate food by manipulation (Medeiros et al., 2012); this microorganism is an opportunistic pathogen which can proliferate when there is an imbalance of the microbiota in the environment also can develop in environments with low water activity (Tortora et al., 2017).

In July 2015 at the 38th Session of the Codex Alimentarius Commission in Geneva, was defined the need to revise the parameters of preservatives based on sorbates, propionates, nisin and natamycin. These preservatives are currently permitted in mozzarella-type cheese by the standards defined by Codex Standard for mozzarella (Food and Agriculture Organization

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of the United Nations, 2011). At the occasion, each country was asked to express its opinion on the technological justification for the use of natamycin in this cheese (Food and Agriculture Organization of the United Nations, 2015) demonstrating the great interest in the evaluation of this antimicrobial in the dairy industry.

Recently, the food industry showed an increasing interest in antimicrobial substances to enhance food safety and product shelf life, therefore the objective of this research was to verify the influence of the salt concentration on action mechanisms of natamycin against on the development of *E. coli*, *S. aureus* and *C. albicans*, as well as its effect on the association of *C. albicans* with *E. coli*, in order to verify their effectiveness under these conditions.

2 Material and methods

2.1 Inoculum preparation

For inoculum preparation *Staphylococcus aureus* strains ATCC12600, *Escherichia coli* ATCC 25922 and *Candida albicans* SC5314 were used, which were inoculated in 0.85% saline so as to obtain a degree of turbidity corresponding to tube 1 of the McFarland scale (Nefelobac®, Probac do Brasil), which represents a concentration of approximately 3.0 x 10^8 CFU mL. After the initial concentration was established, successive dilutions were carried out in order to reach the approximate concentration of 3.0 x 10^4 CFU/mL of the microorganisms tested. The natamycin used was Delvocid Plus® (Delvocid, DSM Food Specialties).

Evaluation of the effect of different combinations of concentrations of natamycin and saline solution on *S. aureus*, *E. coli* and *C. albicans*

Different combinations of concentrations of natamycin and saline solution were adopted for *S. aureus* and *E. coli*. For these species of microorganisms, the concentrations of natamycin tested were 0.00 (control), 0.025, 0.05 and 0.1% (w/v). Concentrations of saline solution were equivalent to 0.85, 2.00, 5.00, 7.00 and 10.00% (w/v). With respect to *C. albicans* and the association of *C. albicans* and *E. coli*, combinations 0.025% (w/v) of natamycin and concentrations of saline solution equivalent to 0.85, 2.00, 5.00, 7.00 and 10.00% (w/v) were evaluated.

Evaluation of the effect of different concentrations of natamycin on *S. aureus*, *E. coli* and *C. albicans* in peptone water

Different concentrations of natamycin in peptone water on *C. albicans*, *E. coli*, *S. aureus* and for association of *C. albicans* and *E. coli* were evaluated. For *E. coli* and *S. aureus*, the concentrations of natamycin equivalent to 0.00 (control), 0.025, 0.05 and 0.1% (w/v) were adopted. For *C. albicans* and association of *C. albicans* and *E. coli* the 0.025% (w/v) concentration of natamycin was evaluated.

2.2 Microbiological analyzes

The counts of the different species of microorganisms were carried out immediately after the exposure to the different treatments and after 24 and 48 hours, being kept at a temperature around 12 °C. For the counts of *S. aureus*, *E. coli* and *C. albicans* the Rida® Count Staph system was used. Aureus, Rida® Count E. coli/Coliform and Rida® Count Yest & Mold Rapid (RBiopharm AG, Darmstadt, Germany) were used for counting *S. aureus*, *E. coli* and *C. albicans*, respectively according to the manufacturer's recommendations. The dilutions adopted were 1:10 and 1:100. The results of the counts of the microorganisms surveyed were converted into log10.

2.3 Experimental design

A completely randomized design was used in triplicate. In the evaluation of the effect of natamycin on *S. aureus* and *E. coli*, a 4×5×3 factorial scheme was adopted: four concentrations of natamycin (0.00, 0.025, 0.05 e 0.1% (w/v)), five concentrations of saline solution (0.85; 2.00; 5.00; 7.50; 10.00% (w/v)) and three evaluation periods (0, 24 e 48 h). In the evaluation of the effect of natamycin on *C. albicans* and on *C. albicans* and associated *E. coli*, a 5×3 factorial scheme was adopted, with five concentrations of saline solution (0.85, 2.00, 5.00, 7.50, 10.00% (w/v)) and three evaluation periods (0, 24 and 48 h). Regression analysis was performed, and the graphs were plotted with Sigma Plot 10.0 software. Descriptive statistics were used in the analysis of the effect of natamycin on *S. aureus*, *E. coli* and *C. albicans* inoculated in peptone water.

3 Results and discussion

C. albicans was submitted to treatments in different salt solutions and in peptone water added 0.025% natamycin, which according to observations in industries corresponds to the concentration used in the brines. However, only from inoculation in 5% saline solution, and in 48 hours no yeast development was observed (Figure 1 and Table 1). When the yeast was inoculated in peptone water, no elimination was observed at any of the evaluated times, only a tendency towards reduction indicating that the presence of organic matter in the cheese brine may interfere with the antimicrobial action (Table 2).

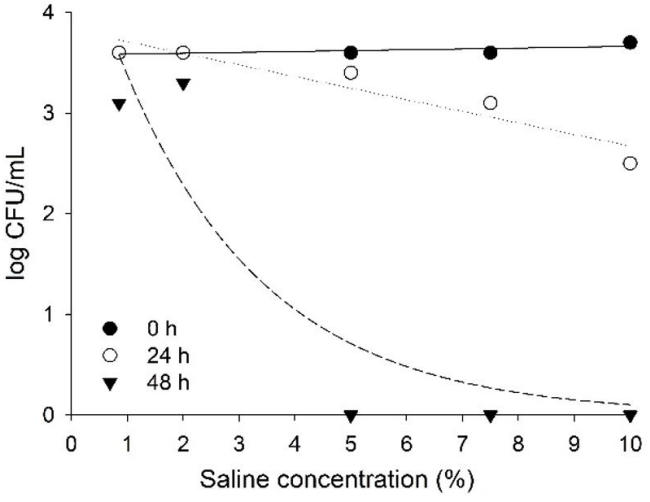

Figure 1. Counts (log CFU/mL) of *C. albicans* after addition of natamycin (0.025%), at 0, 24 and 48 h and different salt concentrations.

Most of the fungi have developmental capacity under low water activity (Soares et al., 2017) which can be achieved of addition of sodium chloride as carried out in this research. However, in the evaluation of the effect of natamycin 0.025% on an association of *C. albicans* with *E. coli*, no development of the yeast was observed after 24 hours, either when inoculated in saline solution or in peptone water (Figure 2A and Table 3).

According to Tortora et al. (2017), *C. albicans* is an opportunistic pathogen that can proliferate when the bacterial microbiota of the medium is suppressed. In the assay containing only *C. albicans*, development was observed that was inhibited by natamycin from 48 hours when in saline solution greater than 5%. However, in the association of *C. albicans* with *E. coli*, the presence of the bacteria in the medium prevented the growth of yeast. This result corroborates the study of Peleg et al. (2010), in which interactions between *C. albicans* and bacteria were reported and have been associated with reduced fungal viability, which can be attributed to the secretion of antifungal molecules by bacteria, the direct transfer of toxins from bacteria to fungal cells or by the depletion of nutrients.

These interactions, added to the action of natamycin on *C. albicans* and *E. coli*, lead to a reduction in the final count of these microorganisms. Natamycin has an antimycotic action because of its preference for ergosterol, a sterol present especially in the cell membrane of fungi and little found in bacteria (Ciesielski et al., 2016).

The *E. coli* counts in this association showed reductions greater than one log cycle only at the evaluations after 48 hours when inoculated in 2.0%, 5.0% and 7.5% saline solutions and no growth of the yeast was observed when in saline solution 10.0%

Table 1. Adjusted regression equations and previous correlation coefficient to the *C. albicans* count (log CFU/mL) after addition of natamycin (0.025%), for 0, 24 and 48 h at different salt concentrations.

Exposure period (h)	Adjusted regression equation	R^2
0	$\hat{y}=3.58$	-
24	$\hat{y}=3.82-0.12^{*}X$	0.90
48	$\hat{y}=4.99e^{-0.39^{*}X}$	0.85

*Significant at 5% probability.

Table 2. Mean results obtained in the evaluation of the effect of natamycin on the counts (log CFU/mL) of *Staphylococcus au*reus, *Escherichia coli* and *Candida albicans* inoculated in peptone water.

Treatments / Weather		Peptone water		
		0h	24h	48h
C. albicans	Natamycin 0.025%	3.6	3.5	3.0
	Control	4.2	4.8	Countless
S. aureus	Natamycin 0.025%	4.2	4.8	Countless
	Natamycin 0.05%	4.4	4.9	Countless
	Natamycin 0.1%	4.0	4.2	4.6
	Control	4.5	5.9	6.7
E. coli	Natamycin 0.025%	4.5	5.9	6.3
	Natamycin 0.05%	4.5	5.8	6.0
	Natamycin 0.1%	4.5	5.8	6.0
C. albicans / *E.coli*	Natamycin 0.025%	4.0/4.9	0/4.7	0/4.5

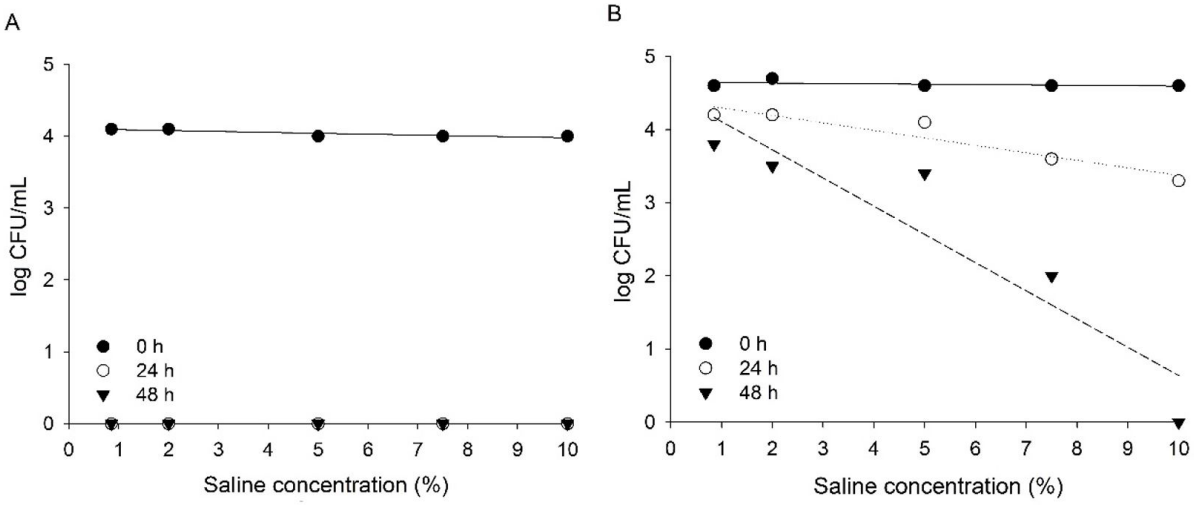

Figure 2. Counts (log CFU/mL) of *C. albicans* (A) and *E. coli* (B) when in association with and after addition of natamycin (0.025%), at 0, 24 and 48 h, at different saline concentrations.

(Figure 2B and Table 3). When the association was performed in peptone water, there was no reduction in *E. coli* counts (Table 2).

In the trials performed with *S. aureus*, no reduction was observed in any of the evaluated treatments and in the inoculations in peptone water, the counts increased significantly (Table 2).

Table 3. Adjusted regression equations and respective determination coefficients for the counts (log CFU/mL) of *C. albicans* (A) and *E. coli* (B) when associated and exposed to natamycin (0.025%), for 0, 24 and 48 h in different concentrations.

Species	Period of exposure (h)	Adjusted regression equation	R^2
C. albicans	0	$\hat{y}=4.10$	-
	24	$\hat{y}=0$	-
	48	$\hat{y}=0$	-
E. coli	0	$\hat{y}=4.65$	-
	24	$\hat{y}=4.40-0.10^{*}X$	0.90
	48	$\hat{y}=4.50-0.39^{*}X$	0.86

*Significant at 5% probability.

Ramos et al. (2012), in a study with edible films, stated that natamycin has no action against bacteria. *S. aureus* is a Gram positive bacterium and its cell wall consists of many layers of peptideoglycan that form a thick and rigid structure that can prevent the action of some antimicrobials on the microorganism (Tortora et al., 2017). However, Atta et al. (2015) demonstrated that an antimicrobial compound produced by *Streptomyces lydicus* and classified as natamycin showed activity against Gram positive bacteria such as *S. aureus*, *Micrococcus luteus* and *Bacillus subtilis*. In action on *E. coli*, reductions of up to one log cycle in the counts after 24 hours were observed in the treatments with addition of natamycin 0.025, 0.05 and 0.1% from 7.5% saline solution (Figure 3B and Table 4). In these same treatments after 48 hours, reductions greater than one log cycle were observed from treatments in 7.5% saline solution (Figure 3C and Table 4).

When the bacterium was inoculated in peptone water, none of the concentrations of natamycin evaluated, was able to promote reduction of the counts, including increases ≥ 1.5 log cycles in the final counts (Table 2), suggesting that the availability of substrate may accelerate bacterial metabolism and render antimicrobial action unfeasible. According to information obtained from the manufacturer it is recommended to add 5-10% salt to the immersion bath to limit bacterial growth on the cheese shell. However, the

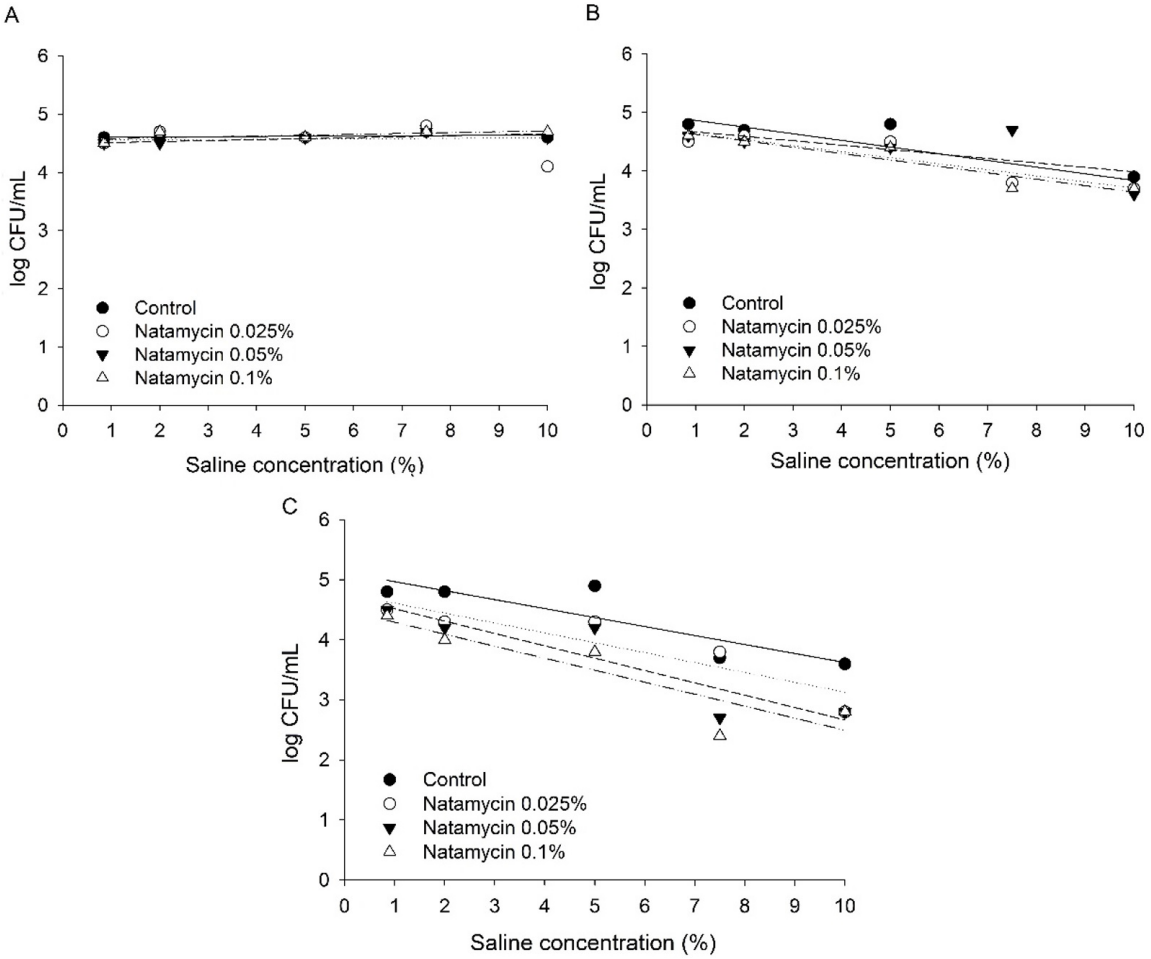

Figure 3. Counts (log CFU/mL) of *E. coli.* after addition of natamycin at 0 (A), 24 (B) and 48 h (C) and different saline concentrations.

Table 4. Adjusted regression equations and respective determination coefficients for *E. coli* counts (log CFU/mL) after addition of natamycin (0, 0.025, 0.05, and 0.1%) in 0 (A), 24 (B) and 48 h (C) at different salt concentrations.

Period of exposure (h)	Concentration of natamycin (%)	Adjusted regression equation	R2
0	Control –0	$\hat{y}$=4.60	-
	0.025	$\hat{y}$=4.36	-
	0.050	$\hat{y}$=4.49	-
	0.100	$\hat{y}$=4.57	-
24	Control –0	$\hat{y}$=4.98	-
	0.025	$\hat{y}$=4.75-0.10*X	0.82
	0.050	$\hat{y}$=4.75-0.08*X	0.43
	0.100	$\hat{y}$=4.74-0.11*X	0.88
48	Control –0	$\hat{y}$=5.12	-
	0.025	$\hat{y}$=4.77-0.16*X	0.82
	0.050	$\hat{y}$=4.72-0.21*X	0.82
	0.100	$\hat{y}$=4.49-0.20*X	0.81

*Significant at 5% probability.

results showed that at the recommended concentration (0.025%), natamycin had an effect on the bacterium only when in 7.5% saline solution after 24 hours indicating that only immersion may not be efficient to limit the bacterial development referred to. According to Atta et al. (2015) the natamycin produced by *S. lydicus* presents effective antimicrobial action against *E. coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*.

Unlike *S. aureus*, the effect of natamycin on *E. coli* may be due to the fact that this bacterium is Gram negative and has a cell wall formed by one or a few layers of peptideoglycan and an outer membrane that does not provide a barrier to all substances in the environment, being more permeable due to the presence of porins that form channels that allow the passage of certain molecules, being even more susceptible to mechanical rupture (Tortora et al., 2017).

E. coli is not a bacterium considered halotolerant, however several studies have demonstrated its capacity to develop tolerance to extreme saline concentrations. Hrenovic & Ivankovic (2009) carried out research with the objective of verifying the resistance of this bacterium in marine environments, when coming from domestic sewage or effluent from treatment stations, noting that the elimination of the bacteria occurred only in concentrations of 20% salt in 72 hours and 30% in 48 hours, maintaining multiplication activity in salt concentrations above 5%. How et al. (2013) demonstrated the ability of *E. coli* to adapt to different salt concentrations (from 0% to 10%) and after 60 passages to be able to increase its tolerance by 1%, multiplying in environments with 11% saline concentration.

4 Conclusion

Under the conditions proposed by the research, it was possible to conclude that 0.025% natamycin has no efficacy on *Candida albicans* inoculated at saline concentrations below 5%. When yeast was associated with *Escherichia coli* showed susceptibility to the proposed treatments, however the reduction in counts may be due to the low capacity of the same to develop when in association with other microorganisms. The results obtained in the *E. coli* counts showed that natamycin may interfere with its development, even at concentrations considered low (0.1%) and at salinity conditions of 7.5% to 10%. Thus, the association of natamycin with sodium chloride potentiates its antimicrobial action, which can represent an economy and its use is amplified by the industries.

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