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Use of Bacterial Cellulose Incorporated with the Antimicrobial Nisin for Cheese Packaging.

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ABSTRACT

Listeriosis is a disease caused by Listeria monocytogenes bacteria that can cause miscarriage. It is important to control this bacterium's growth on foods, mainly dairy products and meats. Nisin is a bacteriocin widely used for this control, and it can be used in active food packaging. In this study, bacterial cellulose (BC) was produced and impregnated with nisin at four concentrations (10000 IU mL-1, 5000 IU mL-1, 2500 IU mL-1 and 1000 IU mL-1) during 0, 2, 4 and 6 hours. The most efficient nisin concentration was determined on Tryptone Soy Agar (TSA). BC films with and without incorporation of nisin at 2500 IU mL-1 after 4 hours of exposure were used to pack Minas Frescal cheese. After 7 days of storage, the use of nisin in BC films reduced the bacterial load of Listeria monocytogenes by 1 log CFU g-1. Bacterial cellulose demonstrated potential applicability in antimicrobial packaging films.

Keywords: Biofilm, Active packaging, Nisin, *Listeria*, Listeriosis, Food disease

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INTRODUCTION

Cheese is widely consumed around the world. However, during storage, contamination with bacteria, mold and yeast can lead to the development of unpleasant flavors and aromas, as well as pose health threats. Therefore, cheese makers pursue ways to increase shelf life as well as the quality and safety of cheese products [1].

Antimicrobial agents can be incorporated in packaging films to extend food storage periods. Controlled release of these agents inhibits the growth of microorganisms and consequently prolongs the shelf life of packaged products [2]. The main antimicrobials tested in edible films are the bacteriocins nisin [3] and natamycin [4], the enzyme lysozyme [5] and various essential oils [6].

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria [3]. Nisin is a bacteriocin produced by *Lactococcus lactis* [7] and has been considered generally recognized as safe (GRAS) by the Food and Agriculture Organization (FAO) since 1988. It is widely used in several countries in products such as milk, cheese, other dairy products, canned tomatoes and other vegetables, canned soups, mayonnaise and baby foods [8]. In Brazil, nisin is approved for use as a biopreservative in all types of cheese up to a maximum of 12.5 mg kg^{-1} [9]. Moreover, it has the advantage of not changing the taste of food while inhibiting *Listeria monocytogenes*, a bacterium that contaminates milk and dairy products [10].

The gram-positive bacterium *Listeria monocytogenes* causes listeriosis, disease whose clinical manifestations include miscarriage, sepsis, meningoencephalitis, gastroenteritis and fatal foodborne infection. Pregnant women, neonates, aged and debilitated patients are predominantly affected. *Listeria monocytogenes* is the only species of the genus *Listeria* that is a human pathogen [11].

Some studies have examined the use of nisin in milk and cheese, but nisin can also inhibit the multiplication of lactic acid bacteria, depending on the dose used in cheese manufacture, and can consequently affect the desired sensory characteristics. The use of nisin in cheese packaging is effective because it does not interfere in cheese production [12].

Conventional packaging materials are mainly petroleum based, but due to environmental and sustainability issues, the use of edible films and coatings has been increasingly investigated [13]. Among the packaging materials available, cellulose products have attracted growing interest due to their edibility, biodegradability and potential as good carriers of a wide range of antimicrobial agents [14].

In the early 2000s, the incorporation of nisin in cellulose-based packaging films was reported [15, 16]. Besides its plant source, cellulose can also be produced by bacteria *Gluconacetobacter xylinus* [17]. In static culture, bacterial cellulose is synthesized as a film on the surface of the growth medium. The utility of bacterial cellulose to the producing microorganism is not clear. There are several theories, such as: retaining moisture to prevent bacteria from dehydrating; helping bacteria to become floatable in an aerobic environment; reducing the opportunity for organisms other than cellulose-producing bacteria; and protecting bacteria from the hazardous effects of UV radiation because of its opaque nature [18].

Bacterial cellulose membranes have unique characteristics compared to other cellulose sources, such as high purity, crystallinity, tensile strength and water retention capacity, so they have good potential for a variety of applications [19].

The use of adsorbed nisin in bacterial cellulose films has potential applicability in smart packaging for control of *Listeria monocytogenes* in cheese. In this work, bacterial cellulose films were produced and nisin was incorporated as antimicrobial. The film was evaluated as primary packaging for Minas Frescal cheese (uncured cheese made in the Brazilian state of Minas Gerais) aiming at controlling *Listeria monocytogenes*. To the best of our knowledge, this is the first study using bacterial cellulose incorporated with nisin for cheese packaging.

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MATERIALS AND METHODS

Production of bacterial cellulose

HS medium [20] was inoculated (3% v/v) with *Gluconacetobacter hansenii* ATCC 23769 strain (pre-cultivated in HS broth at 28 ºC for 48 hours) and incubated (Infors HT Ecotron shaker, Bottmingen, Switzerland) statically at 28 ºC for 72 hours. Then the films were washed with distilled water, subjected to alkaline treatment with 0.5 mol L^{-1} of NaOH for 1 h at 90 ºC and then washed with distilled water to neutral pH.

Bacterial cellulose films for nisin absorption assays were produced in sterile centrifuge tubes with 5 cm diameter containing 5 mL of HS medium. Bacterial cellulose (BC) for packaging cheeses was produced in Petri dishes (15 cm) containing 35 mL of HS medium.

Incorporation of nisin by adsorption method

A nisin solution of 50000 IU/mL was prepared by dissolving 0.5 g of nisin (Sigma-Aldrich - Gillingham, Dorset, UK) in 10 mL of 0.01M HCl. The solution was centrifuged at 3000 g for 15 min in a sterile centrifuge tube and the supernatant was filtered through a 0.45 µm membrane [21]. This solution was diluted with 0.01M HCl to obtain other nisin concentrations: 10000 IU mL^{-1}, 5000 IU mL^{-1}, 2500 IU mL^{-1} and 1000 IU mL^{-1}. In a laminar flow cabinet (Nuaire Class 2, Plymouth, Minnesota, United States), the produced BC was immersed in nisin solutions during 0, 2, 4 and 6 hours, followed by immersion in sterile 15% glycerol solution. All BC samples were oven dried at 60 °C for 1 hour (Bunker NI1705, Piracicaba, São Paulo, Brazil).

Antimicrobial activity assay

Listeria monocytogenes ATCC 19117 strain was inoculated in brain-heart infusion broth (BHI) (Oxoid - Basingstoke, Hampishire, England), incubated for 18 hours at 30 °C. Then the culture was diluted in 0.9% NaCl at a concentration of 10^{-2} CFU mL^{-1} and 1 mL of the diluted culture was used to inoculate Tryptone Soy Agar (TSA) (Difco – Detroit, Michigan, United States) by the pour plate method. After the medium solidified, BC membranes with different nisin concentrations were positioned over the agar surface, in the middle of the plate. The agar plates were then incubated at 30 °C for 48 h (Bunker NI1705, Piracicaba, São Paulo, Brazil) and the antimicrobial activity of the cellulose films was observed.

Inoculation of cheeses and packaging

The bacterial suspensions used in this procedure were prepared from cultures of *Listeria monocytogenes* ATCC 19117. To obtain the suspensions, the microorganism was activated in BHI broth at 37 °C for 24 h. Subsequently, they were subjected to decimal dilutions in 0.1% peptone water and the inoculum concentration was adjusted with a Densimat densometer (Biomerieux, Craponne, France) to 10^{-2} and 10^{-4} CFU mL^{-1}. The number of inoculum colonies was determined by plating on TSA agar plates after incubation at 37 °C for 24 h (Bunker NI1705, Piracicaba, São Paulo, Brazil).

Minas Frescal cheese (25 g) was produced in the laboratory of Embrapa Agroindústria de Alimentos. Samples of cheese without *Listeria* inoculum were considered as controls. Inoculated cheese samples were submerged in 0.85% saline containing 10^3 CFU mL^{-1} *Listeria monocytogenes* for 20 minutes. After this, they were packaged in bacterial cellulose with or without nisin. Figure 1 shows cheese with and without bacterial cellulose. The samples were stored at 10 ºC for 1 day or 7 days, as presented in Table 1. After the incubation period, all the cheese samples were submitted to *Listeria* microbiological analysis.

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Table 1: Storage conditions of cheese with or without nisin incorporated in bacterial cellulose

Cheese	Bacterial Cellulose	Inoculum	Storage days
Control	NA*	NA*	24 h
	with nisin		
	without nisin		
Inoculated	with nisin	10^2	7 dias
	without nisin		
Inoculated	with nisin	10^4	7 dias
	without nisin		

*NA: Not applicable. Chesse samples were not packaged

A B

Figure 1: Minas Frescal cheese without bacterial cellulose (A) and with bacterial cellulose packaging (B).

Microbiological analysis

BCs of incubated cheeses were removed and transferred to 225 mL of sterile 0.1% peptone water in sterile Stomacher® bags. The cheese was manually compressed and homogenized in the solution followed by successive dilutions. An aliquot of 0.1 mL of each dilution was spread on the surface of Oxford agar (Oxoid - Basingstoke, Hampishire, England) in Petri dishes. The dishes were then incubated at 30 °C for 48 hours (Bunker NI1705, Piracicaba, São Paulo, Brazil).

Statistical analysis

The statistical analysis was carried out using the GraphPad Prism version 5.01 software. The statistical significance of the evaluated data was determined by one-way analysis of variance (ANOVA) and the Tukey test with significance level α= 0.05.

RESULTS AND DISCUSSION

Antimicrobial activity assay

The antibacterial activity of the bacterial cellulose films incorporated with nisin against *L. monocytogenes* on TSA plates can be seen in Figure 2. The antimicrobial activity of bacterial cellulose films was proportional to the concentration of nisin solution. These concentrations were studied considering that below 625 IU mL^{-1}, nisin, despite presenting antimicrobial activity in synthetic media [22, 23,24], was found to be

inefficient in food [25], and above 10000 IU mL^{-1} becomes economically prohibitive. The diffusion assay showed that the lowest dilution combined with the shortest period caused inhibition of *L. monocytogenes* of 2500 IU mL^{-1} of nisin after 4 hours. There was no inhibition zone on cellulose films containing nisin at 1000 IU mL^{-1}. Based on these results, active bacterial cellulose films for cheese packaging were prepared with exposure to 2500 IU mL^{-1} nisin for 4 h.

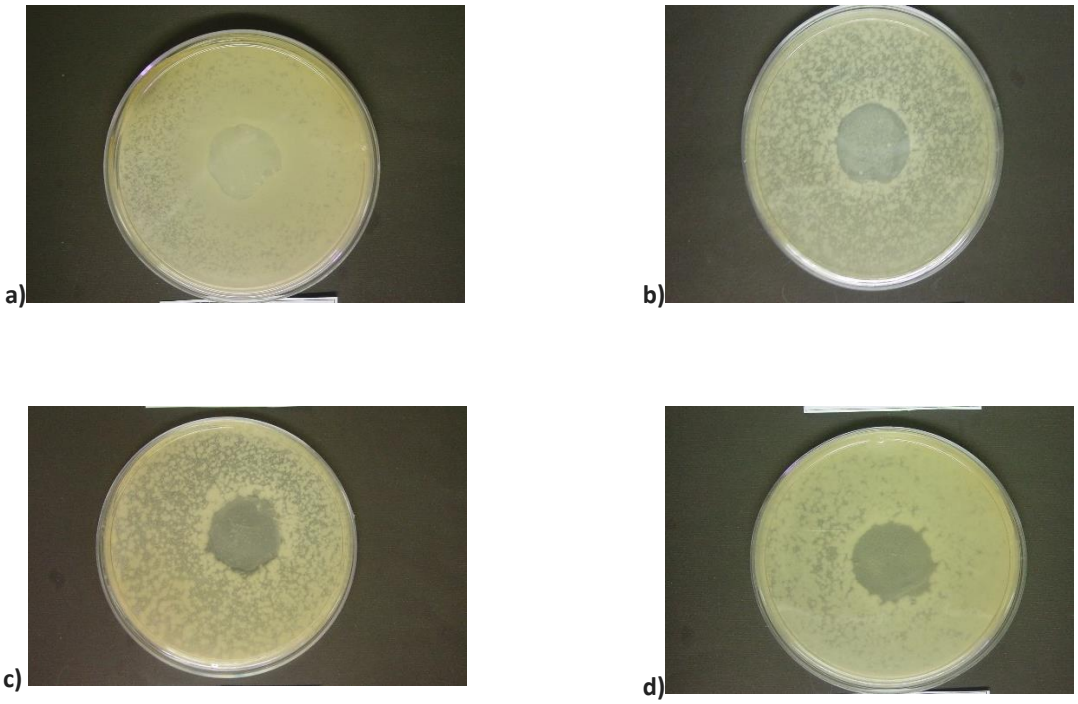

Figure 2: Antimicrobial activity of cellulose films against *L. monocytogenes* on TSA plates. Cellulose film exposed to different nisin solutions for 4 hours a) 1000 IU mL^{-1} b) 2500 IU mL^{-1} c) 5000 IU mL^{-1} d) 10000 IU mL^{-1}

Microbiological analysis

In this study, the antimicrobial activity in synthetic media was enough to control *Listeria* growth in cheese, even though some previous studies have shown that nisin has stronger antimicrobial activity against *L. monocytogenes* in synthetic media than in foods [26, 27]

In all conditions, the use of BC with nisin at 2500 IU mL^{-1} as primary packaging of Minas Frescal cheese reduced the presence of *Listeria monocytogenes* when the cheese was stored under refrigeration (10 ºC). After 24 hours, the control sample with nisin presented the lowest count among the controls (5.4 log CFU g^{-1}). The control with cellulose film not containing nisin presented a higher count compared to the control without cellulose film. A similar result was reported by Nguyen et al. [25] and the authors attributed this to the effect of the hydrated cellulose film, providing a better microenvironment for bacterial growth than the surface without cellulose.

After 7 days, cheese inoculated with 10^2 presented 6.1 – 7.1 log CFU g^{-1} and cheese inoculated with 10^4 presented 7.1 – 8.0 log CFU g^{-1}. In both cases, samples packaged in BC with nisin presented a reduction of 1 log CFU g^{-1} in comparison with BC without nisin.

Few studies have reported the use of BC as active packaging, but all of them have demonstrated satisfactory results. These studies have focused on control of contamination in sausage. Using BC films, Zhu et al. [28] tested ε–polylysine, Padrão et al. [29] investigated lactoferrin, and Nguyen et al. [25] evaluated nisin. Cellulose food packaging incorporated with nisin has been investigated with cellulose from plants [15, 16) and

more recently with nanocellulose composites [30, 31]. The use of nanocellulose, including nanocellulose from BC, is a trend in food packaging [32, 33].

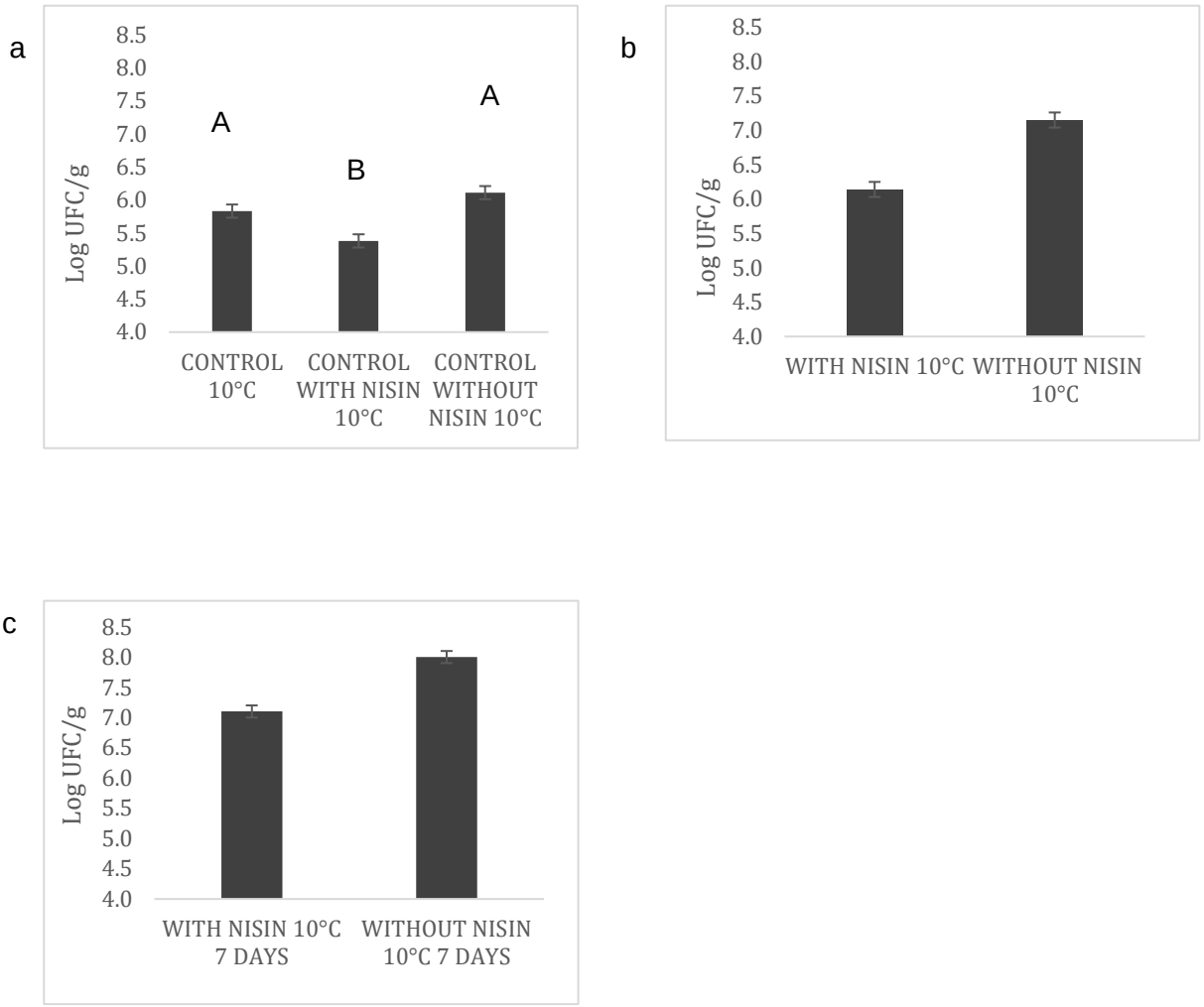

Figure 3: Numbers of *L. monocytogenes* in cheese (a) without packaging, packaging with nisin and without nisin for 24 hours (b) inoculated cheese (10^2) with nisin and without nisin after 7 days (c) inoculated cheese (10^4) with nisin and without nisin after 7 days.

CONCLUSIONS

Bacterial cellulose (BC) films were produced and incorporated with nisin at 2500 IU ml $^{-1}$ after 4 hours exposure. These films were efficient to control *Listeria monocytogenes* growth on TSA agar and cheese. The use of these films reduced *Listeria* growth by 1 log CFU g^{-1} in Minas Frescal cheese after storage for 7 days. The use of BC in active food packaging is a promising and sustainable alternative.

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