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Biodegradable Film Development by Nisin Z Addition into Hydroxypropylmethylcellulose Matrix for Mozzarella Cheese Preservation

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Abstract

Currently, improvement of food preservation has been a substantial challenge for industries to increase shelf-life of products and to maintain food quality during storage. These goals are often tied to the sustainable tendency for use of eco-friendly packaging to store these products without loss of the packaging features. Therefore, the aim of this study was to produce biodegradable antimicrobial films by the incorporation of nisin Z peptide under different concentrations (0 %, 5 %, 10 %, 15 % and 20 % wt.) into hydroxypropylmethylcellulose (HPMC) matrices. The active film properties were evaluated in terms of their antimicrobial capacity in vitro, mechanical performance and microscopic characteristics. Hence, active films containing 10 % (wt.) of nisin Z and control films were placed in contact with sliced mozzarella cheese for eight days, and microbiological growth was monitored during storage. Nisin Z's antimicrobial effects were observed against the Gram-positive microorganisms such as *Staphylococcus aureus* and *Listeria innocua*, regardless if the compound was free as a suspension or incorporated into HPMC matrices. However, the expected low action of nisin Z against Gram-negative bacteria, as reported in literature, was not observed since *Salmonella enterica* Choleraesuis's growth was inhibited. Moreover, active films with added nisin Z (10 % wt.) were more effective than the control film to inhibit mesophilic microorganisms in mozzarella cheese during 8 days of storage. The mechanical properties of the films were not influenced by nisin Z incorporation, since the addition of the compound enhanced the active function without the loss of mechanical properties required for a good food packaging. These results suggest that biodegradable films produced by nisin Z addition into HPMC matrix are an excellent biomaterial for mozzarella cheese preservation.

Keywords: HPMC; Antimicrobial packaging; Mozzarella cheese; Active packaging; Food preservation

1 Introduction

Food packaging is a great strategy to differentiate products in the market under consumers' attention. The use of a suitable packaging for food quality maintenance, coupled with the demands of consumers, is one of the most important factors for success in a competitive market. The packaging material must meet the criteria for preserving the food and its nutrients, and protect against environmental factors such as light, moisture, oxygen and microorganisms, in order to prevent or hinder contact between the external environment and the contents inside the pack-

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Nomenclature

HPMC	hydroxypropymethylcellulose	SS	solution stock
MIC	minimum inhibitory concentration	EB	elongation at break
CFU	colony-forming unit	ME	modulus of elasticity
HCl	hydrochloric acid	ML	maximum load
SEM	scanning electron microscopy		

aging (Bradley, Castle & Chaudhry, 2011; Jorge, 2013).

Although traditional food packaging has many properties to ensure the integrity of foods during the marketing, distribution and storage, they are not sufficient to satisfy new consumers' requirements about food safety, which is, packaging that promotes food quality as well as fulfilling its basic packaging function (Dainelli, Gontard, Spyropoulos, Zondervan-van den Beuken & Tobback, 2008; Yam, Takhistov & Miltz, 2005). Active packaging is defined as systems that interact and change food conditions in order to extend product shelf life, as well as improve its safety or alter its sensory properties, thus maintaining or improving the food quality (Azeredo, 2012). How active packaging functions can be classified according to O2-scavenging, antioxidants, flavourings, absorbers and antimicrobials (Soares et al., 2009; Vermeiren, Devlieghere, van Beest, de Kruijf & Debevere, 1999). Antimicrobial packaging is a promising type of active packaging that confers antimicrobial compounds incorporated into the polymer matrices to eliminate or inhibit microorganisms present in the food (Cowan, 1999; Cox et al., 2000; Holley & Patel, 2005; Lambert, Skandamis, Coote & Nychas, 2001). In recent years, there has been a noticeable increase in research on antimicrobial packaging to assure or improve the food quality. These researchers have tended to study natural antimicrobials, such as bioactive compounds of vegetables and fruits; essential oils; and bacteriocins that are not adverse to consumers' health (Mlalila, Hilonga, Swai, Devlieghere & Ragaert, 2018; Mulla et al., 2017; Niu, Liu, Song, Han & Pan, 2018; Pola et al., 2016; Sarwar, Niazi, Jahan, Ahmad & Hussain, 2018; Woraprayote et al., 2018).

Bacteriocins are peptides, produced by Gram-positive and Gram-negative bacteria, which exhibit antimicrobial activity against other bacteria in medium (Arena et al., 2016; Karpinski & Szkaradkiewicz, 2013). One of the most studied and applied bacteriocins in the food industry is nisin, which is considered non-toxic and safe for human health (Karpinski & Szkaradkiewicz, 2013). Nisin is a peptide composed by 34 amino acid units, presenting a cationic and hydrophobic character, and it is considered a lantibiotic. This bacteriocin is produced by species of *Lactococcus lactis* subsp. *Lactis* and has been studied since its discovery in 1928 (Nascimento, Moreno & Kuaye, 2008; Siamansouri, Mozaffari & Alikhani, 2013). There are five natural variants of nisin: nisin A, nisin Z, nisin Q, nisin U and nisin F (De Kwaadsteniet, Fraser, Van Reenen & Dicks, 2006). The bacteriocins most used in industry and food research are nisin A and nisin Z, which structurally differ in only one amino acid at position 27, and both are produced by strains of *Lactococcus lactis*. The differences between them are few, however, nisin Z is slightly more diffusible in agar and more soluble at neutral pH than nisin A (Mulders, Boerrigter, Rollema, Siezen & Devos, 1991).

According to the active packaging concept, this work sets out to develop active films, incorporated with peptide nisin Z into HPMC matrix, to come up with a novel material that provides anti-

microbial activity, in order to improve mozzarella cheese preservation during storage.

2 Materials and Methods

2.1 Material

Nisin Z was purchased from Handary (Belgium). Hydroxypropylmethylcellulose (HPMC) was obtained from Sigma-Aldrich (United States). Glycerol was purchased from Labsynth (Brazil). Broth TSB, Mueller-Hinton agar and PCA agar were purchased from DIFCO® (USA). Hydrochloric acid (HCl) was obtained from SPLabor (Brazil). Strains of *Salmonella enterica* serotype Choleraesuis (ATCC 10708), *Staphylococcus aureus* (ATCC 6538), and *Listeria innocua* (ATCC 33090) were acquired from Food Packaging Laboratory (UFV, Brazil). Deionized water (Millipore Inc.) was used at electrical resistivity of 18.2 MΩ.cm.

2.2 Determination of Minimum Inhibitory Concentration of nisin Z (MIC)

The minimum inhibitory concentration (MIC) of nisin Z was performed in accordance with the broth macro dilution method standardized by the Clinical and Laboratory Standards Institute (CLSI, 2012), with modifications. The microorganisms evaluated were *S. aureus*, *L. innocua* and *S. enterica* Choleraesuis. Initially, a stock solution (SS) containing deionized water pH 4.6 (adjusted with 0.1 mol.L^{-1} HCl solution) and nisin Z (concentration determined according previous test) was produced. SS solution was diluted in Muller-Hinton broth according to Table 1. Then 2.0 mL of each dilution (Table 1) and 2.0 μL of the microorganism suspension (turbidity based on McFarland 0.5 standard solution which corresponds to a concentration of 10^8 CFU.mL−1) were added to tubes, which were incubated at 35 ^{o}C for 18 h. To confirm the result, after the incubation time, Mueller-Hinton agar plates were inoculated, in duplicate, with 0.1 mL of each dilution tube, and incubated at 35 ^{o}C for 18 h. The nisin's MIC was the lowest concentration of the antimicrobial agent capable to inhibit the visible microorganism growth. The experiment was performed in three repetitions, in duplicate, for each microorganism.

2.3 Preparation of antimicrobial films

The antimicrobial films were prepared according to the method of Sanchez-Gonzalez, Vargas, Gonzalez-Martinez, Chiralt and Chafer (2009), with modifications (Figure 1). 150 mL of deionized water pH 4.6 (adjusted with 0.1 mol.L^{-1} HCl solution) was heated to 80 ^{o}C. Afterwards, 2 % (w/v) of hydroxypropylmethylcellulose (HPMC) and 20 % (wt.) of glycerol were added to the water and then, the polymer dispersion was mixed on a magnetic stirrer at 700 rpm for 5 min. This was followed by nisin Z addition at 0 %, 5 %, 10 %, 15 % and 20 % (wt.) concentrations, wherein the percentage added was relative to HPMC mass. The polymeric material was poured into bordered glass plate and left for 72 h at 25 ± 2.0 ^{o}C.

2.4 Characterization of films

In vitro antimicrobial activity of films

The *in vitro* antimicrobial activity of films was measured according to the agar diffusion method. Films discs (ϕ = 0.8 cm), containing nisin Z (0 %, 5 %, 10 %, 15 % and 20 % (wt.)), were placed in contact with inoculated Mueller-Hinton agar, that was obtained by the friction of a swab soaked in the microorganism suspension. The microorganism suspension had the same turbidity as the standard 0.5 McFarland solution (corresponding to a bacterial concentration of 10^8 CFU.mL^{-1}). The antimicrobial effectiveness of the films was determined by comparing the inhibition zone formed after the Petri dishes were incubated for 24 h at 35 ^{o}C. The microorganisms evaluated were *S. aureus*, *L. innocua* and *S. enterica* Choleraesuis.

Table 1: Dilutions of nisin Z dispersion to determinate nisin Z's MIC

Solution	Aliquot (mL)	Volume of Muller-Hinton broth (mL)
S1	2.0 mL of SS	0.0
S2	1.0 mL of SS	1.0
S3	1.0 mL of SS	3.0
S4	1.0 mL of SS	7.0
S5	1.0 mL of S4	1.0
S6	1.0 mL of S4	3.0
S7	1.0 mL of S4	7.0
S8	1.0 mL of S7	1.0
S9	1.0 mL of S7	3.0
S10	1.0 mL of S7	7.0

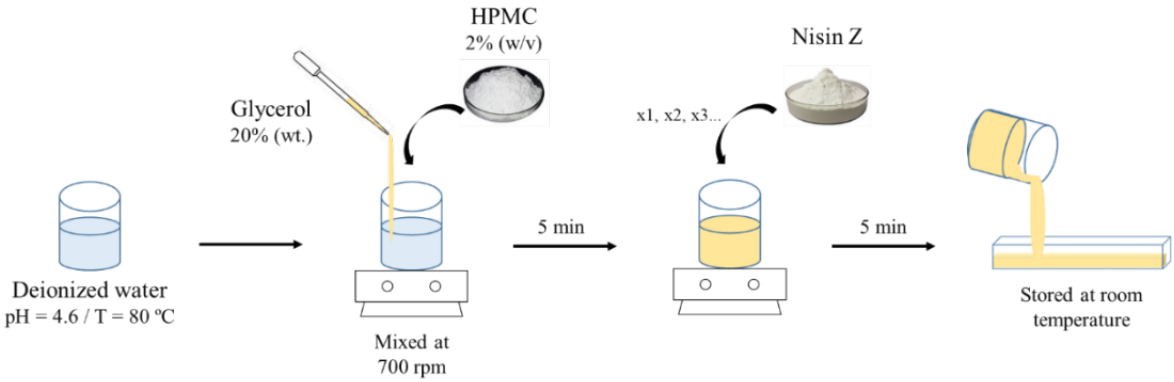

Figure 1: Schematic illustration of antimicrobial and control films' preparation

Mechanical properties of antimicrobial films

Maximum load (N), elongation at break (%) and modulus of elasticity (MPa) of films with added nisin Z or not (control film) were measured using the Universal Machine of Mechanical Testing (Instron Corporation, Norwood, MA, USA), according to ASTM (2012) method. The specimens (17.5 x 2.5 cm) were grabbed by two grips initially separated by 125 mm, and stretched at a crosshead speed of 12.5 mm.min^{-1}. Each treatment was performed in three repetitions, and each repetition in quintuplicate.

Scanning electron microscopy (SEM)

The microscopic structure of films was recorded by scanning electron microscope (Hitachi, model TM3000, Japan). Film samples (0.5 cm^2) were fixed in stubs covered with a carbon layer (Camilloto, 2009). The acceleration voltage was in automatic mode. The images were taken at 7000x magnification.

Antimicrobial activity of the HPMC-based active film in cheese

The potential *in situ* antimicrobial ability of the films containing nisin Z was evaluated in mozzarella cheese slices. The active film with the best antimicrobial properties *in vitro*, 10 % (wt.)

of nisin Z, was chosen to conduct the application test according to the methodology of Soares and Hotchkiss (1998), with modifications. Cheese slices were intercalated with active films, containing enough area to cover the sample surface. Then, they were placed in expanded polystyrene trays, wrapped with polyethylene-nylon and stored at 5 ± 2 ^{o}C during 8 days (Figure 4a). 25 g of cheese samples were taken, aseptically, on 0, 2, 4, 6 and 8 days of storage and then, they were homogenized with 225 mL of 0.1 % (w/v) peptone water in sterile bags by a shake stomacher. 1 mL of this suspension was transferred to a tube containing 9 mL of sterile 0.1 % (w/v) peptone water, obtaining a dilution of 10^{-2}. From this, subsequent dilutions were obtained and for each dilution, 0.1 mL was spread on the non-selective PCA agar, in duplicate, and incubated at 37 ^{o}C for 24 h. Plates containing 25 to 250 colonies were selected to determine the amounts of microorganism capable of growth (Log CFU.mL^{-1}). Equations describing the growth behavior of mesophilic microorganisms in cheese over the time were adjusted with 95 % significance level.

2.5 Statistical analysis

The data were analyzed by Analysis of Variance (ANOVA), and when it was possible, a regression equation (at 5 % probability level) was adjusted, for each response variable, considering as a factor the nisin Z concentrations incorporated in the films. All statistical analysis was performed with Minitab statistical program, version 17.

3 Results and Discussion

3.1 Minimum inhibitory concentration (MIC) of nisin Z

The MIC for *S. aureus*, *L. innocua* and *S. enterica* Choleraesuis were determined to verify the antimicrobial potential of the nisin Z. According to the Table 2, nisin Z was more effective in inhibiting microbial growth of Gram-positive *S. aureus* and *L. innocua* than Gram-negative bacteria *S. enterica* Choleraesuis. Unlike Gram-positive bacteria, which have a peptidoglycan layer, Gram-negative bacteria have an outer lipopolysaccharide layer that acts as a barrier and prevents the diffusion of bacteriocins into the cell (Gyawali & Ibrahim, 2014). One of the most accepted mechanisms of nisin activity, as an antimicrobial compound, is its interaction with the anionic lipids present in the cytoplasmic membrane that promote a disturbance, forming pores that cause alteration in the vital ions gradient, leading to cell death (Breukink & de Kruijff, 2006; Tong, Ni & Ling, 2014).

The MIC for the bacteria *L. innocua* and *S. aureus* were in agreement with inhibition concentrations reported by other authors (Niaz et al., 2018; Ramos et al., 2012; Sadiq et al., 2016; Yoneyama, Fukao, Zendo, Nakayama & Sonomoto, 2008). In addition, the MIC determined for *S. enterica* Choleraesuis was above the values found in other studies, however, with slight changes of microbial species and types of nisin (Field et al., 2012; Kim, Jung, Kim & Shin, 2006). The divergent responses are justified by the variation in the fluidity of the bacteria cytoplasmic membrane as a function of several factors such as temperature, pH and the presence of chelators (Prudencio, Mantovani, Cecon, Prieto & Dantas Vanetti, 2016).

3.2 Antimicrobial activity of films *in vitro*

Equations ($P<0.05$) and behaviors that described the antimicrobial activity of the films against *L. innocua* and *S. aureus* are shown in Table 3 and Figure 2, respectively. There was not inhibitory action of the films in *S. enterica* Choleraesuis presence, regardless of the nisin Z concentration added into the HPMC film, so no equation was adjusted ($P>0.05$).

The treatments evaluated were not able to inhibit *S. enterica* Choleraesuis due to the low action of nisin Z against Gram-negative microorganisms, corroborating with MIC values (item 3.1). Furthermore, the nisin concentrations added in the films may not have been enough to diffuse in an amount capable of *S. enterica* Choleraesuis inhibition. The antimicrobial activity against *S.*

Table 2: MIC values for the microorganisms evaluated.

Microorganism	MIC ($mg.mL^{-1}$)
S. aureus	0.19
L. innocua	0.19
S. enterica Choleraesuis	2.00

aureus and *L. innocua* (Figure 2c) showed a maximum inhibition zone at 10 % (wt.) of nisin Z addition into HPCM. Above 10 % wt. of nisin Z concentration into the polymer matrices, the halos formed maintained almost the same size, and the differences observed were not so prominent. Therefore, from an economic point of view, the same antimicrobial effect can be obtained using less nisin Z, reducing costs, and from the health point of view, the intake of this additive by the consumer is decreased. Similar behavior was observed in other polymer matrices, such as studies carried out by Cao-Hoang, Chaine, Gregoire and Wache (2010) that produced films based on sodium caseinate, with added nisin. More studies on antimicrobial activity *in vitro* of active packaging are required because these packages may better inhibit microorganisms than their direct addition in the food or in the culture medium. The contact of nisin with the food surface, promoted by the direct contact between the food and the packaging, can facilitate its diffusion whilst diminishing the complexation with food components or headspace (de Barros, Kunigk & Jurkiewicz, 2010). In addition, the peptide nisin Z added into the polymer matrix can be used to control release and to promote antimicrobial activity over time, from the production until the food consumption (Sadiq et al., 2016; Salmieri et al., 2014; Shahbazi, Shavisi & Mohebi, 2016).

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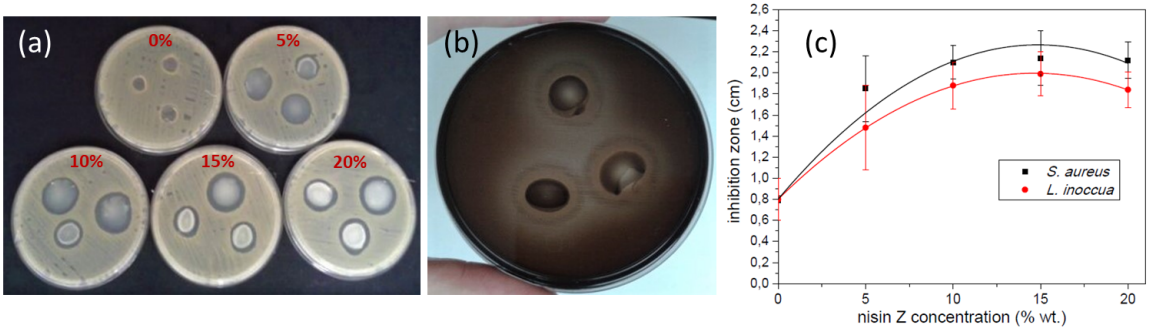

Figure 2: Inhibition images of films containing different concentrations of nisin Z against the microorganisms *S. aureus* (a) and *L. innocua* (b) and their inhibition behaviors for each microorganism (c).

Table 3: Equations obtained by regression analysis for the "halo test" in *S. aureus*, *L. innocua* and *S. enterica* Choleraesuis presence

Microorganism	**Equation**[a]	**R^2**	**Faj**[b]
S. aureus	$Y_1 = 0.086 + 3.099*x - 0.140*x^2$	0.789	0.543
L. innocua	$Y_2 = -0.150 + 3.180*x - 0.157*x^2$	0.794	0.273
S. enterica Choleraesuis	$Y_3 = 0.800$	-	-

[a] Was used the Quadratic model (α =0.05).
[*] Significant by t-Test (P<0.05).
[b] Faj: P-value for the lack of fit (not significant for P>0.05).

bazi et al., 2016).

3.4 Scanning electron microscopy (SEM)

According to the micrographs taken of the film surfaces (Figure 3), it was possible to observe the presence of HPMC clusters in all films, regardless of nisin Z concentrations, represented by white agglomerates in different formats and sizes. The clusters are originated by hydrophobic interactions between the methyl replacement groups present on the HPMC structure. The presence of these aggregates showed the heterogeneity of the film at the microscope level, which were also found by other authors such as Sanchez-Gonzalez et al. (2009).
Several unformed and agglomerated points were visualized in the micrographs of the films containing nisin Z. These points increased with increasing nisin Z concentration into the polymer matrix (Figure 3b, c, d, e), which was also reported by other authors (Meister Meira et al., 2014; Scaffaro, Botta, Marineo & Puglia, 2011). This result agrees with the antimicrobial behavior *in vitro* of the films (item 3.2). There were no prominent differences in the film surfaces when nisin concentration varied from 10 %, 15 % and 20 % wt., which may have promoted similar rates of nisin diffusion into the medium among these treatments. The presence of pores in all films was due to the formation of bubbles from the film-forming process (Bastarrachea et al., 2010). Changes observed in the film's microstructure such as aggregates, bubbles, degree of compaction and cohesion can influence several characteristics of materials like intermolecular, thermal and barrier properties.

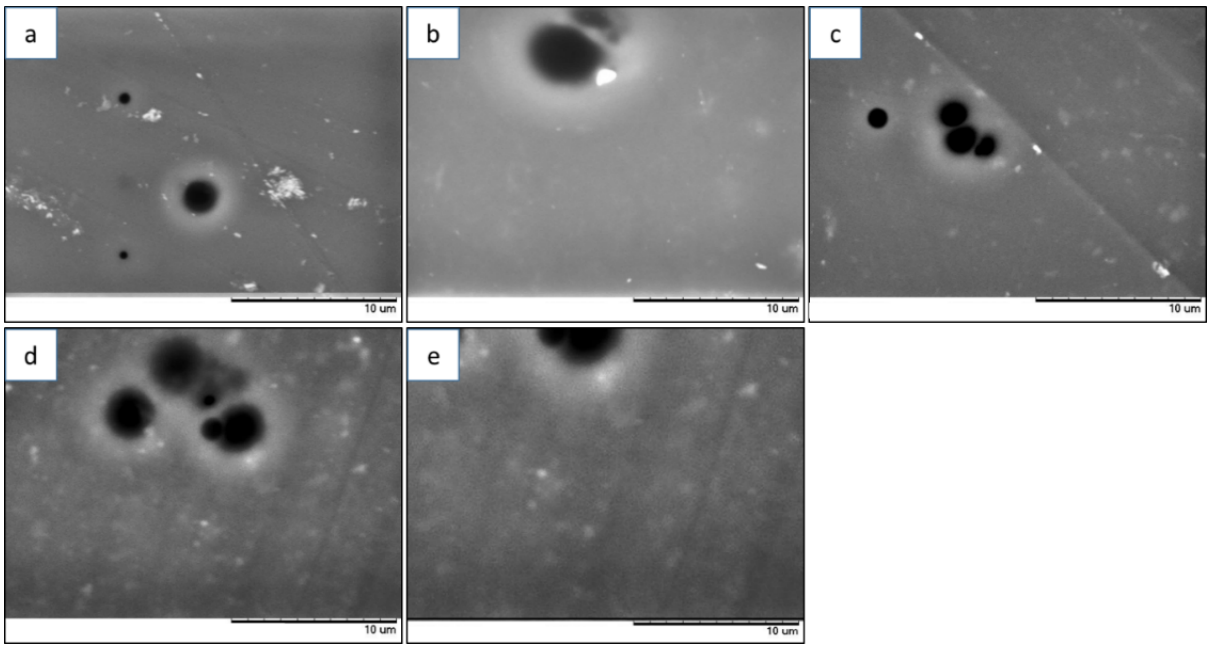

Figure 3: SEM micrographs of the antimicrobial films added by nisn Z at 0 % (a), 5 % (b), 10 % (c), 15 % (d) and 20 % (wt.) (e) (magnification of 7000x and the acceleration voltage in automatic mode)

3.5 Mechanical properties of antimicrobial films

In order to evaluate the film's mechanical properties caused by addition of nisin Z into the polymer matrix, the samples were submitted to stress tests and the maximum load (ML), the elongation at break (EB) and the modulus of elasticity (ME) were determined (Table 4).

None of the mechanical properties had significant equation adjusts ($P>0.05$) as nisin Z concentration increased (Table 4). The mechanical properties were constant regardless of antimicrobial peptide addition into the matrices and this behavior can be related to the lower interaction between nisin Z and the polymer chains. According to Ko, Janes, Hettiarachchy and Johnson (2001), no changes in the mechanical characteristics of the films occurred due to the hydrophilicity features of the HPMC polymer matrix, which was responsible for the lower interaction with hydrophobic nisin. In addition, the mechanical performance of polymer films involves other factors such as matrix interface filling and irregular dispersion of additives in matrices that can be visualized by microscopic analysis (item 3.3). These results are similar to those reported by other authors (Basch, Jagus & Karina Flores, 2013; Meister Meira et al., 2014). Therefore, the mechanical studies suggest no difference between films with added nisin Z and the control films, indicating its mechanical potential to be applied as food packaging.

3.6 Antimicrobial activity of HPMC-based film applied in cheese

A significant effect ($P<0.05$) in microbial growth, caused by time, can be observed during storage for both treatments evaluated (Figure 4b). This behavior can be explained by the refrigerator temperature used (5 ^{o}C), which caused initial inhibition due thermal shock of mesophilic bacteria in the mozzarella cheese. Only after the fourth day of storage, it was observed that the resistant microbial population returned to growth. Al-

Table 4: Mechanical properties of films with added nisin Z or not

Nisin Z (% wt.)	ML* (N)	EB* (%)	ME* (MPa)
0	$153.6^{a} \pm 29.9$	$14.1^{b} \pm 0.8$	$2841.0^{c} \pm 317.9$
5	$143.2^{a} \pm 26.7$	$14.0^{b} \pm 5.3$	$2409.6^{c} \pm 400.0$
10	$162.6^{a} \pm 26.5$	$16.8^{b} \pm 3.7$	$2565.8^{c} \pm 770.0$
15	$145.3^{a} \pm 24.7$	$17.5^{b} \pm 4.0$	$1862.0^{c} \pm 674.3$
20	$150.2^{a} \pm 23.2$	$16.2^{b} \pm 2.7$	$1956.3^{c} \pm 752.0$

* Equal letters in the same column indicate not significant by the Tukey test.

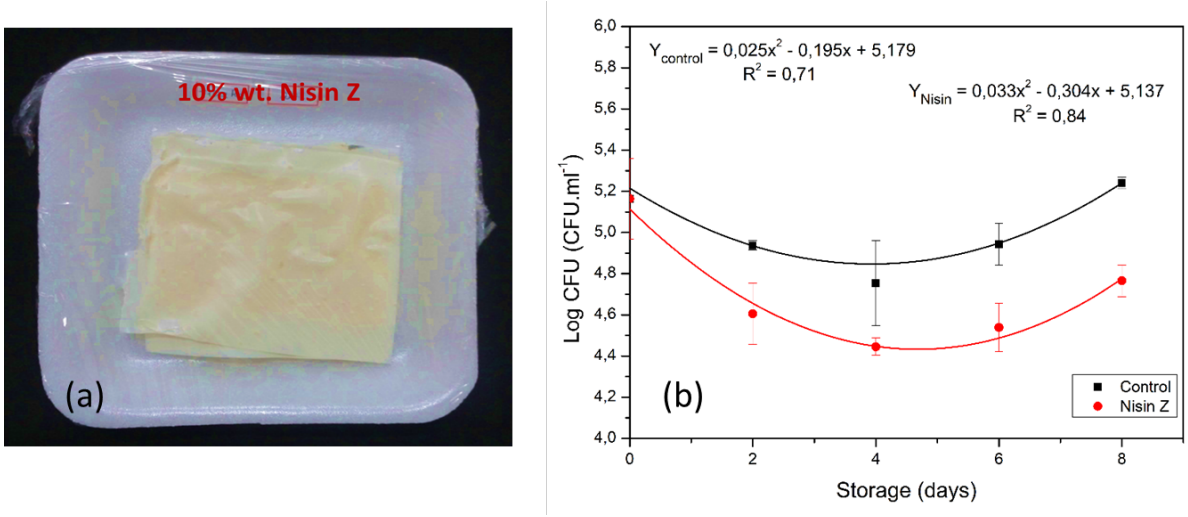

Figure 4: Image of cheese slices intercalated with active film (10 % wt. of nisin Z) placed in expanded polystyrene trays (a); behavior and adjusted equations (P<0.05) for the growth of aerobic mesophilic in the mozzarella packed with the control film and the antimicrobial film (y) during storage (x) (b). Each treatment was performed in three repetitions, and each repetition in triplicate.

though low temperatures inhibit some mesophilic microorganisms, cold-adapted bacteria can survive and proliferate under low temperature conditions (Remenant, Jaffres, Dousset, Pilet & Zagorec, 2015). Other authors, even at higher storage temperatures (Conte, Scrocco, Sinigaglia & Del Nobile, 2007; Gorrasi et al., 2016), found similar growth behavior in packed mozzarella cheese.

The incorporation of nisin Z into the HPMC film influenced (P<0.05) the microbial growth when compared with the control film, gives lower microbial counts at all times, as can be observed by regression analysis (Figure 4b). This difference was highest on the eighth day of storage, where the film with added nisin Z was approximately 0.5 logarithmic cycle lower than the control film. This result indicates that there was diffusion over time of the nisin Z added into the polymer matrix to the cheese, influencing the cheese microbiota and inhibiting microorganisms. The inhibition behavior agrees with other studies that applied different polymer matrices and different antimicrobial agents to control microorganisms in mozzarella cheese (Dannenberg et al., 2017; Gorrasi et al., 2016; Lucera et al., 2014).

Nisin activity is attributed to its interaction with the anionic lipids in the cytoplasmic membrane of

bacterial cells, resulting in a plasma membrane disturbance. This peptide increases the membrane permeability by formation of pores, resulting in an efflux of intracellular material, essential components, adenosine triphosphate (ATP), amino acids, potassium ions, and promotes several changes that end in cell death (Breukink et al., 2003). The effectiveness of HPMC-based films presented in this work agrees with many studies where nisin has been incorporated in packaging to inhibit microorganisms for application in dairy products (Cui, Wu, Li & Lin, 2016; Martins, Cerqueira & Vicente, 2012).

4 Conclusion

Compared to petroleum-based plastics, biopolymers can be a less environmentally aggressive solution. HPMC-based film can be developed for several applications, where one of them is to act as an antimicrobial packaging by nisin Z incorporation. Nisin Z is a bioactive peptide that presents antimicrobial action mainly for *L. innocua* and *S. aureus*. The mechanical properties of the HMPC films are not altered by the nisin Z incorporation which is an interesting result when it comes to the mechanical strength of the food packaging. From the SEM images, it was possible to observe the presence of nisin Z on the film surfaces, which indicates direct contact with the medium and explains the antimicrobial activity *in vitro* of films in contact with mozzarella cheese. The results found in this work showed a great potential to apply these films to increase the shelf life of dairy foods, ensuring the quality and safety of the products.

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