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Starch edible film supporting natamycin and nisin for improving microbiological stability of refrigerated argentinian Port Salut cheese

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ABSTRACT

The effectiveness of natamycin and nisin supported in edible films (NANI) was evaluated, at 7 ± 1 °C, in relation to the improvement of the microbiological stability of Port Salut cheese. This film inhibited the growth of yeasts and moulds and controlled the growth of psychrotrophic bacteria originally present in the Port Salut cheese stored at refrigeration temperature. It also inhibited the development of a mixed culture (*Saccharomyces cerevisiae* and *Listeria innocua*) present in the cheese due to a superficial contamination, throughout the entire storage. With respect to a postprocessing contamination affecting the film present in a covered cheese, control film, commercial film and film containing nisin and natamycin (NANI), acted as barriers precluding the mixed culture growth on the cheese surface. But only NANI inhibited the growth of that culture on the film. These results are demonstrating that the film NANI is an extremely effective method to control the population of microorganisms present in both the cheese and the film, enabling to offer the consumer a safer product.

1. Introduction

Food safety is a global priority and one of the major objectives of the current food legislation (Quintavalla & Vicini, 2002). Cheeses offer a suitable environment for the survival and growth of microorganisms, specially *Listeria monocytogenes* and yeasts (Melo, Andrew, & Faleiro, 2015). Cheese is a ready to eat food, susceptible to physical, chemical and microbiological deterioration during storage and distribution (Cha & Chinnan, 2004). One of the frequently isolated spoilage yeast in this type of food is *Saccharomyces cerevisiae*. Its presence causes an undesirable flavour, affects visual appearance and reduces the shelf life of food (Corsetti, Rossi, & Gobbetti, 2001; Welthagen & Viljoen, 1998). Among the high-risk pathogens, *L. monocytogenes* is particularly troublesome to cheese industry; postprocessing contamination of food with this bacteria is a critical problem of public health, since outbreaks of listeriosis are responsible for high mortality rates and this pathogen represents a major cause of product recalls worldwide (López-Pedemonte, Roig-Sagués, De Lamo, Hernández-Herrero, & Guamis, 2007). In fact, surfaces of cheeses may be contaminated at any stage during process, storage and distribution, since *L. monocytogenes* shows ability to grow at chilling temperatures and to tolerate salt and low pH (Chambel et al., 2007; Gameiro, Ferreira-Dias, Ferreira, & Brito, 2007). *Listeria innocua* is used as a surrogate of *L. monocytogenes*, because it is physiologically close to it and non-pathogenic (Soares Pinto et al., 2009). Additionally, both microorganisms can be isolated from cheeses.

Among natural antimicrobials, nisin is the first antimicrobial peptide receiving GRAS (generally recognized as safe) status for food applications by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives, and its use in various food products is allowed in several countries (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996). It exhibits antimicrobial activity towards a wide range of Gram positive bacteria, including *L. monocytogenes* (Martins, Cerqueira, Souza, Carmo Avides, & Vicente, 2010). Nisin is produced by strains of *Lactococcus lactis* subsp. *Lactis* and is widely used as a preservative in food, including dairy products (Al-Holy,

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Al-Nabulsi, Osaili, Ayyash, & Shaker, 2012; Fernández, Jagus, & Mugliaroli, 2014).

Natamycin is a natural antimycotic polyene produced by *Streptomyces natalensis* and is currently employed in dairy-based food products to prevent yeasts and moulds contamination (Dervisoglu, Gul, Aydemir, Yazici, & Kahyaoglu, 2014; Dzigbordi, Adubofuor, & Faustina Dufie, 2013; Kallinteri, Kostoula, & Savvaidis, 2013). Natamycin has been considered as a GRAS product by the FDA (Koontz, Marcy, Barbeau, & Duncan, 2003) and is also indicated as a natural preservative by the European Union (EEC N° 235). It has been approved as a food additive in over 40 countries. Natamycin is active against yeasts and moulds but not against bacteria, viruses and protozoa (Te Welscher et al., 2008).

Recently, the food industry and the research community showed an increasing interest in active edible films supporting natural antimicrobials, with the objective of enhancing food safety. There are several advantages for considering this strategy for antimicrobial applications: the incorporation of antimicrobials entrapped in a biopolymeric matrix helps to decrease the rate of diffusion from the surface to the bulk of the food product, thus assisting in the maintenance of high concentrations of the active ingredient in the surface, trend that is positive if the surface is the place where it is required. Its support in a matrix can also diminish the interaction with other food additives and components of the food and with oxygen and moisture of the environment. According to different researchers, edible matrices with antimicrobial activity constitute a promising form of antimicrobial delivery in the frame of food preservation (Fajardo et al., 2010; Ollé Resa et al., 2014a; Pires et al., 2008; Ture, Eroglu, Ozen, & Soyer, 2011).

Since food contamination is produced by mixed populations, it is necessary to use effective antimicrobials for bacteria and also moulds and yeasts. Several authors developed biopolymeric matrices containing nisin or natamycin (Basch, Jagus, & Flores, 2013; Cao-Hoang, Chaine, Grégoire, & Waché, 2010; Fajardo et al., 2010; Ollé Resa et al., 2013; Ramos et al., 2012; Ture et al., 2011). However, scarce data (Ollé Resa et al., 2014a; Ollé Resa et al., 2014b) exists in relation to the activity of these natural antimicrobials incorporated together in these matrices and used in real foods against a mixed culture.

To the best of our knowledge, there has been no research reported on the activity of nisin and natamycin, incorporated together in tapioca starch films, against a mixed culture present in refrigerated argentinian Port Salut cheese. Therefore, the aim of this study was to evaluate the effectiveness of natamycin and nisin supported in edible films to improve microbiological stability of refrigerated Port Salut cheese in relation to: a) the native microbiota, b) an external postprocessing contamination by a mixed culture (*S. cerevisiae* and *L. innocua)* on the surface of Port Salut cheese or on the surface of the covering film.

2. Materials and methods

2.1. Materials

Tapioca starch was provided by Industrias del Maíz S.A. (Argentina). Glycerol was provided by Mallinckrodt (Argentina). The antimicrobials, commercial natamycin (Delvocid® Salt) containing 50%w/w NaCl and 50%w/w natamycin, and commercial nisin (Nisin®) containing 97.5%w/w NaCl and 2.5%w/w nisin, were provided by DSM (The Netherlands) Argentina branch.

A commercial film constituted by the copolymer of polyvinyl/polyvinylidene chlorides (Cryovac®, Sealed Air Argentina SA) named as "CF" was also tested in this work. Port Salut cheese (La Serenisima®, Argentina) was purchased in a local supermarket.

2.2. Film preparation

Mixtures of starch, glycerol and water (2.5:1:46.5, in weight) were mixed to constitute the control film, named CNANI. For preparing the film containing natamycin and nisin (NANI), 300 g of slurry was prepared with the mixture previously stated, but 20 g of water was replaced by: i) 10 g of a solution of natamycin of adequate concentration for obtaining a final concentration of 0.027 g natamycin/100 g slurry (or 9.25 mg natamycin/dm^2 of film) and ii) 10 g of a solution (pH 2) of nisin of adequate concentration for obtaining a final concentration of 0.0068 g nisin/100 g slurry (or 2.31 mg nisin/dm^2 of film).

In both cases, starch gelatinization was performed at a constant rate of ~1.5 °C/min attaining a final temperature of 82 °C. Vacuum was applied to remove air from the gel when necessary. The slurry was dispensed in aliquots of 12 g in plates of 7 cm diameter. The drying of the films was performed at 37 °C during 48 h in a convection chamber. Once constituted, films were peeled off from plates and, before evaluating film properties, samples were conditioned at 28 °C, in a desiccator over saturated solution of NaBr (water activity, $a_w \cong 0.575$) for 7days.

2.3. Direct application of antimicrobials

In order to compare the antimicrobial effectiveness of the film NANI with natamycin and nisin direct application on the surface of the cheese (treatment named as "DA"), solutions containing antimicrobials were used. For this assay, a stock solution of natamycin and nisin was prepared with 3125 ppm and 12500 ppm, respectively. An aliquot (20 μl) of this solution was extended with a glass rod on the surface of the cheeses. This allowed a concentration of antimicrobials in the cheese surface comparable to the one contained in NANI (9.25 mg natamycin and 2.31 mg nisin/dm^2 of cheese).

2.4. Microbiological assay

2.4.1. Strains and growth conditions

S. cerevisiae (CBS 1171, strain collection SC) was grown in 150 ml Sabouraud broth (Biokar Diagnostic, France) with continuous agitation using a shaker (Cetin, Argentina) in a controlled camera at 28 °C, until early stationary phase was achieved.

L. innocua (CIP 80.11) was grown in 150 ml tryptona soya broth with yeast extract (TSBYE, Biokar Diagnostics, France) overnight, with continuous agitation using a shaker in a 28 °C controlled temperature chamber. Finally, 2 ml of inoculated broth was added onto fresh TSBYE and the system was agitated till desired cell concentration was achieved.

For preparation of mixed culture, each of the microorganisms was grown in its corresponding broth to achieve desired density (specified for each assay). Thereafter, 30 ml aliquots of each culture were centrifuged at 10,000 rpm, each cell pellet was resuspended in 15 ml of TSBYE and both suspensions were mixed together.

2.4.2. Microbiological stability of refrigerated Port Salut cheese

Changes in psychrotrophic bacteria, *Listeria* spp. and total yeasts and moulds, microbial groups that commonly cause spoilage in Port Salut cheese, were measured during storage at refrigerated temperature. Briefly, pieces of cheese (2.5 × 2.5 × 0.5 cm; 5 ± 0.3 g) were cut with a sterile knife and placed on sterile petri dishes. Squares (5 cm side) from the different films (CF, CNANI and NANI) were cut and used to cover the cheese and also a cheese without film (CH) was evaluated. The samples were incubated at 7 ± 1 °C for 22 days.

At selected times, cheese samples were homogenized in peptone water (1:10) and dilution drops (20 μl) were spotted in duplicate onto the adequate media to determine the number of

CFU/ml of each microorganism. Yeasts and moulds were enumerated on plates containing yeast selective agar YGC (Biokar Diagnostic, France) incubated at 28 °C for 48–72 h. Psychrotrophic bacteria were determined using plate count agar (PCA, Biokar Diagnostics, France) after incubation for 5 days at 7 ± 1 °C. *Listeria* spp. was enumerated on plates containing the *L. innocua* selective agar Oxford (Biokar Diagnostics, France) incubated at 37 °C for 48 h. Enumeration of colonies was performed, and microorganism growth was expressed as log CFU/ml. Determinations were performed in duplicate in two separate experimental runs.

2.4.3. Diffusion method

Using the same methodology as applied by Ollé Resa et al. (2014a), the cheese diffusion test was used to determine the antimicrobial effect of films in Port Salut cheese. Briefly, pieces of cheese (2.5 × 2.5 × 0.5 cm; 5 ± 0.3 g) were cut with a sterile knife and placed on sterile Petri dishes. Afterwards, 20 μl of the mixed culture of *S. cereviseae* and *L. innocua* containing 1×10^4 CFU/ml each, were spread on the surface of the cheeses. Film squares (5 cm by side) of the different films (CF, CNANI and NANI) were placed on the cheeses previously inoculated. Also, a direct application (DA) was tested. The samples were incubated at 7 ± 1 °C for 192 h.

The initial and surviving numbers of viable cells in the cheeses were evaluated at different times. For this, cheese samples were homogenized in peptone water (1:10) and dilution drops (20 μl) were spotted in duplicate onto agar YGC and Oxford, and the number of CFU/ml was determined after incubation at 28 °C for 72 h and 37° for 48 h, respectively. Enumeration of colonies was performed, and microorganism growth was expressed as log CFU/ml. Determinations were performed in duplicate in two separate experimental runs.

2.4.4. Barrier to mixed culture contamination

Barrier efficacy of the film was tested evaluating the capacity of the film to prevent the contamination of the cheese, and also its ability to control and eliminate the microorganisms present over the film. The assay performed by Ollé Resa et al. (2014a) was applied. Briefly, 5.0 × 5.0 cm pieces of all films (CF, CNANI and NANI) were cut and brought in contact with the surface of the cheese. Then, 10 μl of mixed culture of *S. cereviseae* and *L. innocua* containing 1×10^6 CFU/ml each, were dispensed on the films. Samples were incubated at 7 ± 1 °C during 192 h and periodically sampled, to test microorganism viability.

In order to investigate if the film prevented the contamination of the cheese during the assay, after removal of the film, cheese samples were homogenized in peptone water (1:10) and dilution drops (20 μl) were spotted in duplicate onto agar YGC and Oxford, and the number of CFU/ml was determined after incubation at 28 °C for 72 h and 37° for 48 h, respectively.

Also, the initial and surviving numbers of viable cells were evaluated in the films, at different storage times. Removed films were homogenized in peptone water (1:10) and dilution drops (20 μl) were spotted in duplicate onto agar YGC and onto agar Oxford for the evaluation of the number of CFU/ml after incubation at 28 °C for 72 h and at 37 °C for 48 h, respectively. Enumeration of colonies was performed, and microorganism growth was expressed as log CFU/ml. Determinations were made in duplicate in two separate experimental runs.

2.5. Statistical analysis of data

Data were analysed through two-way ANOVA with an α: 0.05 and Tukey was the post-hoc test applied. Results are reported based on their mean and standard deviation (Sokal & Rohlf, 2000). The software GraphPad Prism®, version 5.01(Graph Pad Software, Inc., California) was used for the treatment and analysis of data.

3. Results and discussion

3.1. Microbiological stability of refrigerated Port Salut cheese

Cheese is usually refrigerated at 7 ± 1 °C, so it is interesting to evaluate the antimicrobial activity of the film containing both antimicrobials at this temperature against the native microbiota. The study was performed with cheese without film (CH) and covered with edible control film (CNANI), commercial film (CF), and edible film containing antimicrobials (NANI). Since CH, CNANI and CF presented the same behaviour, only CNANI is shown in Fig. 1a. The counts of yeasts and moulds and psychotropic bacteria in the cheese were below 10 CFU/ml initially but resumed growth after the fifth day, reaching a value of 7.5 log CFU/ml at the end of the storage. No growth of *L. innocua* spp. was observed, demonstrating the quality of the studied cheese.

When the microbiological stability of Port Salut cheese covered with film NANI was studied (Fig. 1b), the total count of psychrotrophic bacteria showed no growth until 15 days of storage, and then grew to 3 log CFU/ml, being the regrowth 10 days later than the one observed for cheese covered with film CNANI. The greatest effect of the film NANI was observed in relation to the yeasts and moulds, because no growth was observed throughout the storage, revealing antimicrobial diffusion to cheese surface. *L. innocua* spp. growth was not observed in the cheese.

El-Diasty, El-Kaseh, and Salem (2008) evaluated total yeast counts during storage at 4 °C of both treated (with 10 mg of natamycin/Kg of yoghurt) and untreated yoghurt. They detected in the latter, a yeast growth from the third day, while in the treated yoghurt no growth of yeasts was detected till 35 days of storage.

3.2. Effectiveness of film NANI against an external contamination with a mixed culture prior to the application of edible film. Diffusion method

The release of antimicrobials from film NANI was studied for the semi-soft cheese Port Salut. For comparison purposes, the direct application (DA) of the antimicrobials was also evaluated during storage at 7 ± 1 °C.

The behaviour of *S. cerevisiae* present in a mixed culture inoculated on the surface of the cheese covered with different films or treated with direct application of antimicrobials is shown in Fig. 2a. The systems containing natamycin (NANI and DA) showed a fungistatic effect for the first 72 h of storage. However, *S. cerevisiae* resumed its growth, reaching at the end of the storage a count 1.5 log cycles lower than systems without antimicrobials (CNANI and CF). The cheese covered with film NANI or submitted to direct application of natamycin and nisin (DA), presented the same behaviour, indicating that the bioavailability of the antimicrobials present in the film is the same for both treatments.

The behaviour of *L. innocua* present in the mixed culture, inoculated on the surface of Port Salut cheese is shown in Fig. 2b. The film NANI caused a bactericidal effect on *L. innocua* until the first 144 h and then, the bacteria resumed its growth and reached a final count of approximately 4 log cycles lower than those observed for cheeses covered with CNANI and CF films at the end of the storage. Direct application of antimicrobials (DA) had a bacteriostatic effect along the whole test, maintaining the initial count of 4 log cycles. It is interesting to compare the nisin activity for the different forms of application (NANI vs DA). For DA, proteins and lipids present in cheese might have interacted with nisin decreasing its effectiveness (Sanjurjo, Flores, Gerschenson, & Jagus, 2006). Conversely, the

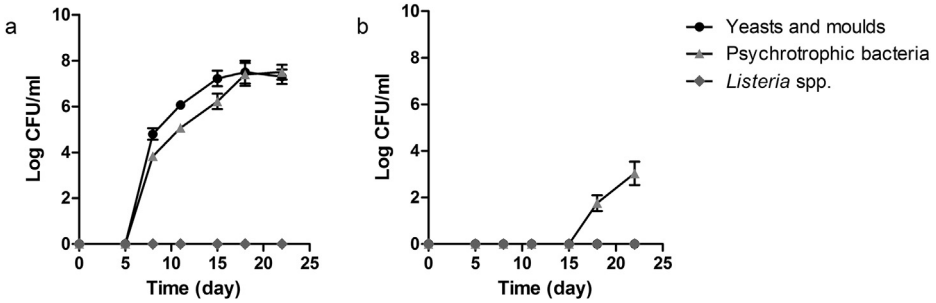

Fig. 1. Microbiological stability of refrigerated Port Salut cheese. Panel a: cheese covered with film without antimicrobials (CNANI), Panel b: cheese covered with film containing natamycin and nisin (NANI).

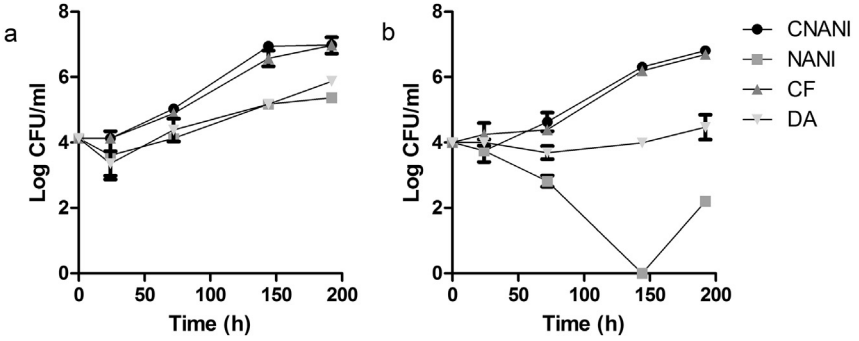

Fig. 2. Cheese diffusion method. Growth of mixed culture during storage at 7 ± 1 °C. The cheese samples were covered with: control film (CNANI), film containing natamycin and nisin (NANI), commercial film (CF). Also direct application of natamycin and nisin (DA) was assayed. Panel a: *Saccharomyces cereviseae*. Panel b: *Listeria innocua*.

support of antimicrobials in the film, allowed a slow release of nisin, reducing the interactions while preserving antimicrobial availability over the whole time of the assay.

3.3. *Effectiveness of film NANI acting as barrier to mixed culture contamination*

The suitability of formulated films to act as a barrier against an external microbial contamination, at refrigerated temperature, was evaluated. Results obtained during storage at 7 ± 1 °C are shown in Figs. 3 and 4. To evaluate the barrier activity of the films it is necessary to know whether or not the films allowed the passage of contamination into the cheese and if, simultaneously, these films could reduce or eliminate the contamination that occurred on their surface during storage.

Survival of *S. cerevisiae* present in a mixed culture, inoculated on the surface of various films and on the cheese surface in the case of DA and stored at 7 ± 1 °C, is shown in Fig. 3. Microbial analysis for Port Salut cheese (with and without film) is presented in Fig. 3a. A cheese without inoculum and without film (CH) and an inoculated cheese without film (ICH), were also evaluated for comparison purposes. The inoculated cheese without film (ICH) showed an initial yeast count of 4 log CFU/ml, increasing 3 log cycles in 196 h. The CH showed yeast counts below 10 CFU/ml until 144 h of storage; from that time and on, the yeast reassumed the growth achieving at the end of storage a value of 4 log CFU/ml, trend that revealed the presence of native flora. Covered cheeses with films CF and CNANI showed a yeast count similar to cheese CH, indicating that these films did not allow the passage of contaminant yeast. However they did not exert any antimicrobial effect on native yeast present in cheese evaluated. The cheese covered with film NANI (containing both antimicrobials) presented a count lower than 10 CFU/ml throughout the trial, without allowing the growth of native yeasts. Instead, the cheese with the direct application of antimicrobials (DA) produced a 1 log cycle reduction of yeast counts the first 24 h but immediately reassumed growth reaching a value of 5 log CFU/ml at the end of the storage.

Microbial analysis of the films is presented in Fig. 3b. The films without antimicrobials (CF and CNANI) allowed the growth of yeast on its surface; therefore, these films are not suitable to act as a barrier against an external contamination of a mixed culture of *S. cerevisiae* and *L. innocua*. On the other hand, the film containing natamycin and nisin (NANI) had fungicidal effect from the first 24 h and until the end of storage. Other authors have previously reported the ability of films based on different hydrocolloids and containing natamycin for acting as a barrier to external mycotic contaminations. Ollé Resa et al. (2013), studied a tapioca starch based film containing natamycin and observed that the preservative was available to prevent an external contamination of *S. cerevisiae* and that the antimycotic effect exerted by the films depended on the natamycin content. Also Ramos et al. (2012) studied the efficacy of films produced from whey protein isolate containing natamycin as antimicrobial agent and observed that the natamycin incorporated in the film led *Y. lipolytica* to depletion within 3 h of storage at 30 °C.

Survival of *L. innocua* present in a mixed culture, inoculated on the surface of various films and stored at 7 ± 1 °C, is shown in Fig. 4. Microbial analysis for Port Salut cheese (with and without film) is presented in Fig. 4a. Again, a cheese without inoculum and without film (CH) and an inoculated cheese without film (ICH), were tested. The inoculated cheese without film showed an initial bacterial count of 4 log CFU/ml which increased its value acquiring at the end of storage a value of 7 log CFU/ml. The CH shows bacterial counts below 10 CFU/ml over the entire storage; this result demonstrated the good quality of the cheese, showing absence of *Listeria* spp. Covered cheeses with films CNANI, CF and NANI (all inoculated)

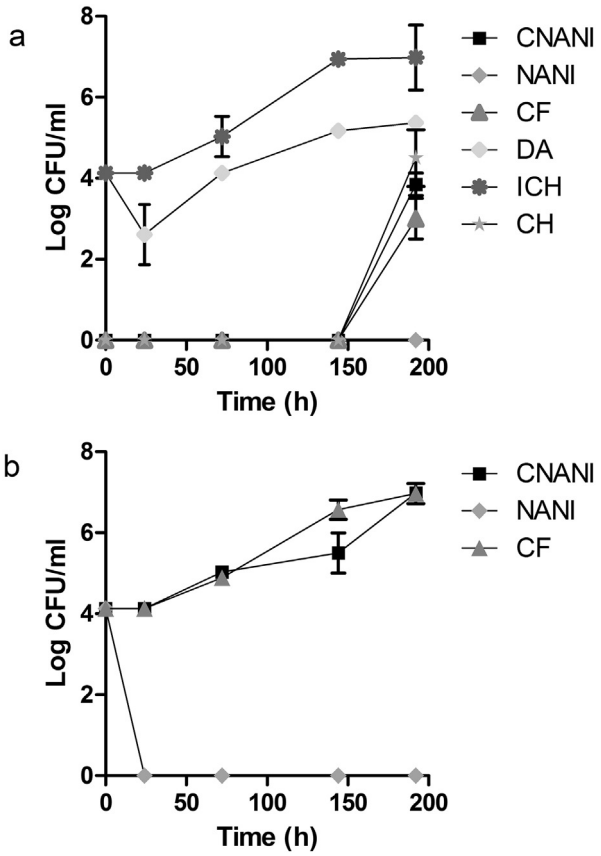

Fig. 3. Effectiveness of different treatments applied on refrigerated Port Salut cheese against an external contamination by a mixed culture containing *Saccharomyces cerevisiae*. The cheese samples were covered with: control film (CNANI), film containing natamycin and nisin (NANI), commercial film (CF). Cheese with direct application of natamycin and nisin (DA), without inoculum and without film (CH), and inoculated cheese without film (ICH) were also studied. Panel a: microbiological analysis of Port Salut cheese, Panel b: microbiological analysis of the films evaluated.

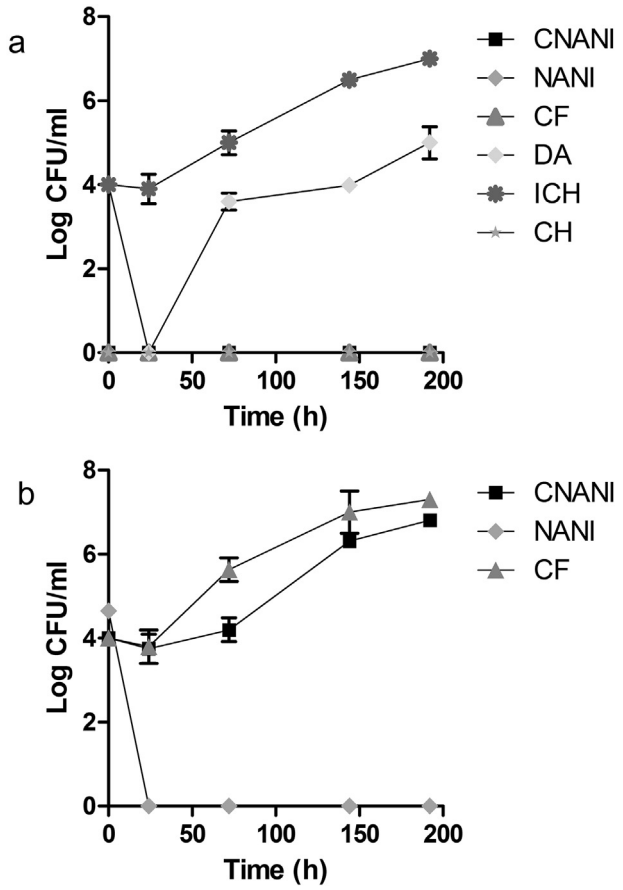

Fig. 4. Effectiveness of different treatments applied on refrigerated Port Salut cheese against an external contamination by a mixed culture containing *Listeria innocua*. The cheese samples were covered with: edible control film (CNANI), film containing natamycin and nisin (NANI), commercial film (CF). Cheese with direct application of natamycin and nisin (DA), without inoculum and without film (CH), and inoculated cheese without film (ICH) were also studied. Panel a: microbiological analysis of the Port Salut cheese, Panel b: microbiological analysis of the films evaluated.

showed similar bacterial count to that obtained for the cheese CH without inoculum and without film (less than 10 CFU/ml throughout all storage), indicating that these films did not allow the passage of contaminant bacteria. The cheese with the direct application of antimicrobials (DA) reduced the count of *L. innocua* to a value lower than 10 CFU/ml immediately. Then, the bacteria reassumed its growth reaching a value of 5 log CFU/ml, being 2 log cycles lower than cheese CH.

Microbial analysis of the films is presented in Fig. 4b. The films without antimicrobials (CF and CNANI) allowed the growth of the bacteria on its surface, presenting a bacteria count of 7 log CFU/ml at the end of the storage. Conversely, film containing natamycin and nisin (NANI) presented a bactericidal effect throughout the experiment. Basch, Carpenco, Jagus, and Flores (2011) have previously reported the antimicrobial activity of edible films based on tapioca starch and HPMC and containing nisin. They informed that this film produced a rapid decrease of the inoculated *L. innocua*, reaching a population 5 log cycles lower than the films without antimicrobials at the end of the storage.

Also, Ollé Resa et al. (2014a) evaluated the performance as barrier of films containing natamycin and nisin (NANI) against a mixed culture on a model system stored at 25 °C. They observed that this film prevented the contamination of the agar and inhibited the growth of the mixed culture over the film.

Commercial film showed a good barrier capability but allowed the growth of the mixed culture on its surface. The microorganisms present over the commercial film might produce the contamination of the food during film withdrawal prior to consumption, showing that the good barrier property of the film is not enough to ensure the microbiological safety of the cheese.

Since edible films are consumed with food, it is important that they are free of microorganisms; this fact highlights the film containing natamycin and nisin as the most appropriate treatment for cheese preservation.

4. Conclusion

The results obtained in this study indicate that the edible film containing natamycin and nisin (NANI) inhibited the growth of yeasts and moulds and controlled the growth of psychrotrophic bacteria originally present in the Port Salut cheese stored at refrigeration temperature.

Film NANI controlled *S. cerevisiae* growth and inhibited the growth of *L. innocua*, present together in the surface of refrigerated Port Salut cheese. In the case of nisin, the support in the film determined a greater effectiveness than its direct application. Additionally, it inhibited the development of a postprocessing mixed culture (*S. cerevisiae* and *L. innocua*) contamination of a covered Port Salut cheese along a storage of 8 days at 7 ± 1 °C.

These results are demonstrating that the film NANI is an extremely effective method to control the contamination of Port Salut cheese, improving the microbial stability in relation to native microbiota and postprocessing contamination. In this way it will be possible to offer the consumer a safer food product.

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