COMBINATIONAL APPROACHES FOR ANTIMICROBIAL PACKAGING: NATAMYCIN AND NISIN

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ABSTRACT

Food safety is a global priority and one of the major objectives of the current food legislation. The right combination of strategies for food industrialization including the packaging step, ensures the achievement of that objective. New food products and new industrialization processes impose the need for the development of new packaging materials that assure food protection and that address the changing demands of the food industry and of the consumers. The general perception of the importance of minimizing the environmental damage has catalyzed the exploration of new bio-based packaging materials such as biodegradable and edible films because they are environmental friendly. Additionally, consumer demand for more natural foods has promoted the research about natural antimicrobials like natamycin and nisin.

In this chapter, it is reviewed the available information on antimicrobial packaging containing the natural antimicrobials natamycin and nisin simultaneously and, in particular, its antimicrobial effectiveness. According to published and new results evaluated, packaging containing natamycin and nisin is a very efficient strategy to control food contamination. Additionally, the use of biodegradable materials to produce the packaging contributes to environment protection.

Key Words (5-10): food safety, antimicrobial food packaging, natamycin, nisin, bacteria, moulds and yeasts.

INTRODUCTION

Food safety is a global priority and one of the major objectives of the current food legislation. However, food microbiological risks are even today one of the main sources of foodborne illnesses. Additionally, the greatest losses in food are attributed to microbiological alterations, which decrease their shelf life (Quintavalla and Vicini, 2002).

The growing consumer demand for minimally processed, more natural, fresh and convenient food products, as well as the continuous changes at industrial distribution levels associated with globalization, carriage major challenges for food safety and quality (Realini and Marcos, 2014).

Packaging

The adequate application of industrialization processes is essential in order to obtain safer products and, packaging is a key step for its achievement.

New food products and industrialization processes impose the need for the development of new packaging materials that assure food protection while addressing the changing demands of the industry and consumers.

The four basic functions of traditional food packaging are protection, communication, convenience and containment (Yam et al., 2005). Food packaging innovations should be discussed on the basis of their scientific and technological contributions to the basic functions of traditional food packaging, and also on their general contributions towards a more sustainable world, considering the harmful impact of packaging waste (Han, 2014; Vanderroost et al., 2014). As a consequence, packaging innovations should take into account a broad range of sustainability issues such as waste prevention, efficient use of resources, process optimization and recycle, among others. Accordingly, there is an increasing tendency to employ environmental friendly materials with the intention of substituting non-degradable materials, thus reducing the environmental pollution resulting from waste accumulation (Imran et al., 2010). To address the environmental issues, and concurrently optimized

54 the shelf-life and quality of foods, it has begun the exploration of new bio-based packaging materials
55 such as biodegradable and edible films (Burke, 2006; Tharanathan, 2003).

56 Biodegradable polymers, are defined as polymers created from raw materials of agricultural or
57 marine sources and broken down through biological or chemical reactions. They are gaining
58 popularity over plastics materials (Amass et al., 1998; Cha and Chinnan, 2004).

59 Some renewable biopolymers such as polysaccharides, proteins, gums, lipids derived from
60 animal and plant origin (Ray and Bousmina, 2005) and their combination, contribute to the
61 environmental protection while reducing residues from the agro-industry.

62 However, some limitations arise in the use of these biopolymeric films, such as their poor water
63 barrier and weak mechanical properties. Many efforts have been made by the scientist to overcome
64 this shortcoming. In this sense, modification of polymeric structure, blends of protein and
65 polysaccharide, lipid and polysaccharide or lipid and protein, among others, have been evaluated
66 (Delville et al., 2003; Sorrentino et al., 2007). If these biopolymers are edible, packaging produced
67 with them is named as edible films.

68 Active packaging is the most relevant innovative idea applied for consumer satisfaction
69 (Ahvenainen, 2003; Lee et al., 2009). It has been defined as a system in which the product, the
70 package and the environment interact in a positive way to extend the shelf life of product or to achieve
71 some characteristics that cannot be obtained otherwise (Cutter, 2002; Miltz et al., 1995). This type of
72 packaging contributes to the protection function of traditional food packaging. Active packaging sales
73 were nearly $8.8 billon in 2011 and are expected to grow up to $11.9 billon in 2017 (BBC, 2013).

74 Addition of antimicrobial agents reduces or prevents the growth of spoilage and pathogenic
75 microorganisms in food (Franssen et al., 2004). They can be either incorporated in foods during their
76 preparation, or applied on their surface (Kim et al., 2002). However, these operations present limited
77 benefit as they result in a rapid loss of antimicrobial activity due to a rapid decrease of active
78 concentration, resulting from interactions with food components and dilution (Ture et al., 2011).

Antimicrobial active packaging allows greater efficiency in food protection, offering better stability of antimicrobial agents, and ensuring control of its release over a period of time to maintain the product's quality and safety (Balasubramanian et al., 2009; Guiga et al., 2010). In particular, edible packaging can serve as carrier of antimicrobials compounds, constituting an additional stress factor to be applied for food preservation and contributing to food safety (Reppas et al., 2009). Furthermore, they can also decrease the interaction of antimicrobials with other additives and food components due to its support in a polymeric matrix (Campos et al., 2011). Morover, the antimicrobial active edible packaging can be a good barrier to post-processing contamination during storage period (Ollé Resa et al., 2013).

Natural antimicrobials

Over the last few years, consumer demand for foodstuff of natural origin (termed as "Bio"), high quality and elevated safety, minimally processed with longer shelf-life and ease-to-eat with a fresh taste and appearance, have turned out to be of cardinal importance (Sobrino-Lòpez and Martín-Belloso, 2008). Accordingly, the application of chemical preservatives has been considered as a disadvantage by consumers looking for high quality and more natural foods, and the use of natural antimicrobial compounds from a wide variety of natural sources has been the object of abundant research (Gould, 1997; Tiwari et al., 2009).

The applications of natural antimicrobial for food preservation relies heavily on the use of multiple barriers known as"hurdle technology" to minimize the risk of microbial activity and therefore, ensure food safety and improved economic benefit (Gupta et al., 2012; Leistner, 2000).

Among natural antimicrobials, nisin is the first antimicrobial peptide with a generally recognized as safe (GRAS) category 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 et al., 1996). It exhibits antimicrobial

104 activity towards a wide range of Gram positive bacteria, including *L. monocytogenes* (Martins et al.,
105 2010).

106 Nisin is produced by strains of *Lactococcus lactis* subsp. *Lactis* and is widely used as a
107 preservative in food, including dairy products (Al-Holy et al., 2012; Fernández et al., 2014). It displays
108 inhibitory activity towards a broad range of Gram-positive organisms, including *L. monocytogenes*
109 (Martins et al., 2010). Nisin binds electrostatically to the negatively charged phospholipids and
110 increases the permeability of the membrane by pore formation, resulting in rapid efflux of essential
111 intracellular small molecules (Breukink et al., 1997). It also interferes with cell wall biosynthesis.
112 These phenomena are mediated by the ability of nisin to bind lipid II, a peptidoglycan precursor of the
113 bacterial cell wall (Bauer and Dicks, 2005).

114 Natamycin is a natural antimycotic polyene, characterized by the presence of a large
115 macrocyclic lactone ring containing a series of conjugated double bonds and one or more sugar
116 residues (Hammond and Lambert, 1978), with a molecular weight of 665.7Da. It is produced by
117 *Streptomyces natalensis* and, is currently employed in dairy-based food products to prevent yeasts
118 and moulds contamination (El-Diasty et al., 2008; Gallo and Jagus, 2006; Reps et al., 2002).
119 Natamycin has been considered as a GRAS product by the FDA (Koontz et al., 2003) and is also
120 indicated as a natural preservative by the European Union (EEC N° 235). It has been approved as a
121 food additive in over 40 countries.

122 Natamycin kills yeasts by specifically binding to ergosterol but without permeabilizing the plasma
123 membrane. It inhibits vacuolar fusion through the specific interaction with ergosterol (te Welscher et
124 al., 2008, 2010). Therefore, it is active against yeasts and moulds but not against bacteria, viruses
125 and protozoa.

126

127 ***Active antimicrobial films for the control of mixed populations***

128 Recently, the food industry and the research community showed an increasing interest in active
129 packaging supporting natural antimicrobials, with the objective of enhancing food safety. There are

several advantages for considering this strategy for antimicrobials application: the incorporation of antimicrobials entrapped in packaging materials 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. It can also diminish the interaction with other additives and food components present in the food bulk. In particular, edible matrices with antimicrobial activity constitute a promising form of antimicrobial delivery in the frame of food preservation (Fajardo et al., 2010; Olle Resa et al., 2014a; Pires et al., 2008; Ture et al., 2011).

Since food contamination is produced by mixed populations, it is necessary to use effective antimicrobials for bacteria and also mould and yeasts. Several authors developed packaging containing nisin or natamycin (Basch et al., 2013; Cao-Hoang et al., 2010; Fajardo et al., 2010; Ollé Resa et al., 2013; Ramos et al., 2012; Ture et al., 2011). However, scarce data exist in relation to the activity of these natural antimicrobials incorporated together in food packaging.

Therefore, the aim of this chapter is: a) to review the available information on antimicrobial packaging containing the natural antimicrobials natamycin and nisin simultaneously concerning physico-chemical and antimicrobial properties; b) To report recent research about the use of edible film containing natamycin and nisin simultaneously as a barrier against a mixed culture in a preservation process for Port Salut cheese.

PACKAGING FORMULATION: PHYSICO-CHEMICAL PROPERTIES

The first step in the production of antimicrobial films is the development of the matrix and its characterization. It is also necessary to evaluate the effect produced by the incorporation of antimicrobials on the properties of the film.

Hanušová et al. (2010) developed a coextruded polyamide/polyethylene film, coated by polyvinyldichloride lacquer, containing natamycin (Delvocid®, DSM, The Netherlands) and nisin

(Nisaplin®, Danisco A/S, Denmark). This film named INVOS, was prepared with a flexography printing machine under industrial scale conditions. The final thickness of the film was 5.0±0.9 µm, with a concentration of 16.7% w/w of each antimicrobial. The authors studied the natamycin migration from the film to distilled water at 23ºC during 48 h, and informed a maximal level of migration of about 0.83±0.04 mg/dm^2. The migration of nisin was evaluated from the packaging film into acidified physiological solution in the same conditions as mentioned for the natamycin migration test. The maximal level observed was in this case, about 800±7 IU/dm^2, where IU means international units. The results indicated that both antimicrobials could be released from the synthetic lacquer coating on the polymer packaging film.

Pires et al. (2008) designed an active film containing antimicrobials with a good biodegradability. The authors employed cellulose derivative polymer as the matrix material, and nisin (Christin®, Crh. Hansen A/S, Denmark) and natamycin (Natamax®, Danisco A/S, Denmark) as antimicrobials. They applied the casting technique to produce the films, and analysed the film thickness, the mechanical properties and the microscopic characteristics by scanning electron microscopy. Additionally, the authors studied the diffusion of the antimicrobials contained in the film into mozzarella cheese.

The results indicated that the films containing nisin or its combination with natamycin, presented a lower tensile strength and elongation to break than the control film (without antimicrobials). While this last film showed a homogeneous structure, films containing antimicrobials presented a non-uniform distribution of the antimicrobial crystals.

The quantification of natamycin in the cheese slices that were in contact with film containing only this antimicrobial, indicated that natamycin diffused from the film within 3 days of contact with the cheese, mantaining its concentration in the cheese during the storage period. Additionally, cheese samples in contact with film containing both antimicrobials, showed higher concentration of natamycin in the mozzarella cheese along the storage. The authors hypothesized that the nisin included in the film matrix, could be embedded into the polymer chains, increasing the space between them and as a consequence, facilitating the diffusion of the natamycin molecules to the cheese. On the contrary,

181 based on chromatographic studies, nisin was not detected in sliced cheese in contact with films
182 containing one or two antimicrobials, indicating a strong interaction between nisin and the film matrix.
183 The most abundant bibliographic information about packaging containing natamycin and nisin
184 involves edible films. Pintado et al. (2010) developed a whey protein isolate based edible film,
185 containing glycerol or sorbitol as plasticizer. Nisin (Nisaplin®, Danisco A/S, Denmark) and natamycin
186 (Natamax® Salt, Danisco Beaminstewr Ltd., UK) were incorporated as antimicrobials with a final
187 concentration in the film solution of 50 IU nisin/ml and 0.002 or 0.005 g of natamycin/ml. They used
188 hydrochloric acid or malic acid to decrease the solution pH. Film solution was homogenized and
189 aseptically spread on 90 mm diameter disposable plates. The plates with film solution were dried at
190 23℃ and 50% relative humidity. Afterwards, the films were peeled out from the plates and stored
191 under these conditions. Film thickness, water vapor permeability (WVP), mechanical and rheological
192 properties were evaluated. The authors informed that the designed edible films could be a carrier of
193 the two antimicrobials without compromising the mechanical properties of the films.

194 Ollé Resa et al. (2014a) prepared control edible films with a mixture of starch, glycerol and
195 water (2.5:1:46.5 in weight). For preparing the film with antimicrobials, part of the water was replaced
196 by natamycin (Delvocid® Salt, DSM, The Netherlands) and nisin (Nisin®, DSM, The Netherlands)
197 solutions for obtaining a final concentration of 9.25 mg natamycin/dm^2 of film and 2.31 mg nisin/dm^2 of
198 film respectively. In all cases, after starch gelatinization, the slurry was dispensed in plates and dried
199 at 37 ℃ during 48 h in a convection chamber. Afterwards, films were peeled off from plates, and
200 equilibrated to a water activity of 0.575 before characterization.

201 The authors compared the control film with the film containing antimicrobials in relation to their
202 physicochemical properties, roughness and hydrophobicity. They observed that the addition of
203 antimicrobials lowered the stress at break and increased the strain at break. This phenomenon can be
204 attributed to the plasticizing action of the antimicrobials (Ramos et al., 2012). Also the Young modulus
205 diminished in the presence of these antimicrobials and the authors attributed this trend to nisin action.
206 Basch et al. (2013) observed that nisin addition in a film of tapioca starch and

hydroxylpropylmethylcellulose, produced a decrease in the Young modulus. Ollé Resa et al. (2013) informed that the presence of natamycin in tapioca starch films did not significantly change this parameter.

Non significant differences between water vapor permeability values of films with or without antimicrobials were reported in this study.

Since the contact angle is the most common measure of wettability or surface hydrophobicity (Muscat et al., 2013), the authors studied this parameter and observed that the presence of the antimicrobials in the designed films increased the contact angle, indicating an increase in the surface hydrophobicity and also a reduced wettability. The roughness value, which is related to the surface irregularities, presented significant differences for control and antimicrobial films and this behavior was attributed to the presence of nisin. Also La Storia et al. (2008) informed an important increase of the surface roughness in a polyethylene film with the presence of nisin.

PACKAGING FORMULATION: ANTIMICROBIAL ACTIVITY

Hanušová et al. (2010) tested the antimicrobial capacity of non-biodegradable INVOS film containing natamycin and nisin, against selected microorganisms, on agar media and on two traditional Czech cheeses. They observed that the tested film was able to inhibit *Penicillum expansum*, *Fusarium culmorum* and also *Lactobacillus helveticus* in agar media. When the authors tested the Blatácké zlato cheese packaged in the INVOS film, they observed that the aerobic sporforming bacteria *Bacillus cereus*, present in the surface of the cheese, were inhibited during 28 days of storage at 23ºC. Also the INVOS film prevented the growth of *Penicillum expansum* in the surface of the cheese.These results proved that the antimicrobials natamycin and nisin could be released from the tested film, and could inhibit sensible microorganisms. On the contrary, the total bacteria count increased on the surface of the cheese packaged with the film without antimicrobials and stored in the same condition.

232 However, the INVOS film was inefficient in Olomoucké tvaruzky, a surface ripened cheese, to
233 inhibit *Listeria monocytogenes* AW2007, originally isolated from this type of cheese. Morover, the
234 authors observed that the antimicrobial nisin released from the film inhibited the culture
235 microorganisms responsible for the ripening process. As a consequence, the cheese did not ripe and
236 remained hard at the end of the storage.

237 Pires et al. (2008) evaluated the antimicrobial capacity of a biodegradable cellulose derivative
238 polymer based film, containing natamycin and nisin. The authors studied the *in vitro* efficacy against
239 *Staphylococcus aureus*, *Listeria monocytogenes*, *Penicillum* sp. and *Geotrichum* sp. They spred 0.1
240 ml of suspension of each microorganism tested onto the adequate media. Circular samples of the
241 antimicrobial film and of the control film were placed over the culture media. Afterwards, the plates
242 were incubated at 35±2ºC, 24-48 h or 23±2ºC and 3-5 days for bacteria and moulds, respectively.
243 The diameter of the halos or its absence indicated the antimicrobial efficacy of the films.

244 The films containing nisin or both antimicrobials presented a zone of inhibition of 2.7 cm in the
245 case of *S. aureus*. These films also inhibited *L. monocytogenes*, but the inhibition was only visible in
246 the contact area between the film and the culture media. Since the formation and the size of the halo
247 depends on the diffusion of the antimicrobial into the culture media and the microorganism growth
248 rate, the authors hypothesize that the difference observed between the two microorganisms tested,
249 could be attributed to the unequal diffusion of the antimicrobial (nisin and natamycin) and sensitivity of
250 each microorganism. The antimicrobial film produced halos of 4.8 and 2.3 cm in diameter when tested
251 the effectiveness against *Penicillum* sp. and *Geotrichum* sp. respectively.

252 Additionally, the authors also investigated the film antimicrobial activity in refrigerated sliced
253 mozzarella cheese, considering that the product could be contaminated as a consequence of a poor
254 hygienic control of the equipment during the slicing process. They placed the films (control and
255 antimicrobial film) between two slices of cheese, sealed the system in bags of polyethylene/polyamide
256 laminate, and stored at 12ºC. The samples were analysed at different times between 0 and 15 days,
257 for *Staphylococcus sp.,* moulds and yeasts, and psychrotrophic bacteria. The authors informed that

the antimicrobial film was able to inhibit moulds and yeasts growth through 9 days of storage, improving the shelf life of the cheese in 6 days compared with the control film. However, after 12 days of storage no difference between this film and the control film was observed. Also, the antimicrobial film extended the lag phase of psychrotrophic bacteria for 6 days, but did not produce a decrease in count along the storage. Since nisin is effective against gram-positive bacteria, and the group of psychotropic bacteria include also gram-negative ones, it is possible to expect a low efficiency. Moreover, the antimicrobial film was not able to inhibit *Staphylococcus sp.* growth. In this case, the authors attributed the inefficacy of the film to the insufficient diffusion of nisin from the film into the cheese.

As previously mentioned, bibliographic information about antimicrobial packaging containing natamycin and nisin, is fundamentally constituted by edible films. Pintado et al. (2010) evaluated through a diffusion type assay, the antimicrobial activity of whey protein film containing natamycin and nisin, against spoilage and pathogenic microorganisms isolated from cheese surface. They informed that *Yarrowia lipolytica* and *Penicillum* spp. were inhibited by the film containing both antimicrobials, being the zones of inhibition independent of the acid used to lower the pH of the film solution. On the contrary, *Listeria monocytogenes* presented a higher inhibition zone when the film contained malic acid (3.3mm) instead of hydrochloric acid (0.6mm). No inhibitory effect was observed against *Pseudomonas aeruginosa*, which is nisin resistant (Thomas et al., 2000). The authors compared the film containing the two antimicrobial with films containing each antimicrobial alone. They observed that there was a differentiated target and an independent action of each antimicrobial, and concluded that the designed films presented a positive effect against *L. monocytogenes, Y. lipolytica* and *Penicillum* spp., and proposed to evaluate the effectiveness of these films for wrap cheese.

Ollé Resa et al. (2014b) studied the effectiveness of natamycin and nisin supported toghether in tapioca starch films (namely NANI), against *Saccharomyces cerevisiae* and *Listeria innocua***,** present in a model system and in a Port Salut cheese. Moreover, they compared the performance of the tested microorganisms as simple and mixed culture.

The authors applied the agar diffusion method to determine the antimicrobial effect of the film with and without antimicrobials (NANI and CONTROL), against the tested microorganisms in an agar food model. They observed that the film CONTROL could not inhibit the microorganism growth at the interface of the film and the agar, indicating that starch and glycerol did not exert an antimicrobial effect. On the contrary, when the antimicrobial film was tested in TSYE agar (tryptone soya with yeast extract, unrestricted agar), two halos were formed. The authors informed that the smallest diameter corresponded to the inhibition of bacteria growth and the largest to the one of yeast, being both diameters similar to that observed for simple cultures of each microorganism. Therefore, they concluded that the bioavailability of one antimicrobial is not affect by the presence of other antimicrobial.

Also, the authors evaluated the effectiveness of the designed films in a commercial Port Salut cheese. The cheese was inoculated with the mixed culture of *S. cerevisiae* and *L. innocua* by spreading on its surface. Afterwards, the films CONTROL and NANI were placed on the inoculated cheese and stored at 25℃. Additionally, a cheese with a direct application of a solution containing the same antimicrobial concentrations as the film was evaluated. The film without antimicrobial could not inhibit the microorganisms present in the cheese surface. However, for *L. innocua*, the film NANI and the direct application, showed counts lower than 10 CFU/ml till the end of the storage (196 h). Furthermore, these treatments decreased the initial *S. cerevisiae* count reasuming afterwards the growth, but at the end of the storage, the film NANI was the most effective one.

When the researchers studied the effectiveness of the antimicrobial film to prevent the post-processing contamination of the model system (barrier properties of the film) by a mixed culture**, t**hey observed that both films (CONTROL and NANI), prevented the contamination of the agar along the entire storage period. However, only the film NANI inhibits the growth of *S. cerevisiae* and controls the growth of *L. innocua* on the film, providing a safer product for the consumer. Additionaly, in order to simulate a contamination along different stages of food storage, the authors developed an assay in which the inoculum was dispensed on the agar at 0 d and 5 d of film-agar contact. The initial and

surviving number of viable cells at different storage times after inoculation was evaluated in the films. The results showed that natamycin present in the film NANI was effective in preventing an external contamination of *S. cerevisiae* even after 5 d of contact at 25°C. On the other hand, the efficacy of the nisin present in film NANI, changed with the contact period, being extremely effective when the contamination was produced initially. However, when contamination occurred after 5 d of contact, the bacteria presented an initial reduction of counts followed by a regrowth. Also, all the films prevented the contamination of the agar.

RECENT DEVELOPMENTS CONCERNING ANTIMICROBIAL EDIBLE FOOD PACKAGING CONTAINING NATAMYCIN AND NISIN

Although there is information about barrier properties of edible films containing antimicrobials in model systems, scarce data exist in relation to the behavior of the films containing natamycin and nisin in real foods and against a mixed culture.

Jagus, Gerschenson and Ollé Resa evaluated these properties for an edible tapioca starch film, containing natamycin and nisin, in a commercial cheese. The usefulness of films proposed as barriers was tested against a mixed culture.

The films were prepared with and without antimicrobials. Starch (Industrias de Maiz S.A., Argentina), glycerol (Mallinkrodt) and water (2.5:1:46.5 w/w) were mixed to obtain the control film (C). For antimicrobial film (FNANI) preparation, part of the water was replaced by the antimicrobial solution to obtain a final concentration of 9.25 mg natamycin/dm^2 of film (Delvocid® Salt, DSM, The Netherlands), and 2.31 mg nisin/dm^2 of film (Nisin®, DSM, The Netherlands). The assay was performed using Port Salut (La Serenísima©, Argentina) cheese samples.

The application technique was adapted from the one designed by Ollé et al. (2014a). Briefly, disks of 1.0 cm diameter were cut from films C and FNANI, and brought in contact with the surface of the cheese. Then, 10 µl of the mixed culture (*Sacharomyces cerevisiae* CBS 1171, strain collection SC and *Listeria innocua*, CIP 80.11), containing 10^6 CFU/ml each, were dispensed on the film disk. A

commercial film (Cryovac ®, Sealed Air Argentina S.A.) named CF and a direct application (DA) with a solution containing the same antimicrobial concentrations as film FNANI were also assayed.

To evaluate the barrier activity of the films it is necessary to know whether or not the films allow the passage of pollution into the cheese, and if simultaneously, this film can reduce or eliminate contamination that occurred on its surface during storage. With this objective, samples (cheese with and without treatments) were incubated at 25 ºC for 168 h and, the viability of the microorganisms in the film and in the cheese were periodically evaluated. Enumeration of colonies was performed in agar YGC (Biokar Diagnostics, France) and agar Oxford (Biokar Diagnostics, France), and microorganism's growth was expressed as log CFU/ml. Determinations were made in duplicate in two separate experimental runs.

Survival of *S. cerevisiae* present in a mixed culture, inoculated on the surface of various films and stored at 25 ºC, is shown in **Figure 1.** Microbial analysis for the films is presented in **Figure 1a.** The films without antimicrobials (C and CF) allowed the growth of yeast on its surface. On the other hand, the film containing natamycin and nisin (FNANI) had fungicidal effect from the first 24 hours and until the end of storage (216 hours). 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 ºC.

Microbial analysis for Port Salut cheese (with and without film) is presented in **Figure 1b**. Only for comparison, a cheese without inoculum and without film (CH), and an inoculated cheese without film (ICH), were evaluated. The inoculated cheese without film (ICH) showed an initial yeast count of 4 log cycles, increasing 3 log cycles in 48 hs. The CH shows yeast counts below 10 log CFU/ml until

72 hours of storage; from that time and on, the yeast resumed the growth achieving at the end of storage a value of 6 log cycles, a level similar to ICH trend that revealed the presence of native flora. Covered cheeses with films C and CF 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 FNANI presented a count lower than 10 log CFU / ml throughout the trial, without allowing the growth of native flora. Instead, the cheese with the direct application of antimicrobials (DA) produced the reduction of yeast counts immediately to less than 10 log CFU/ml, maintaining this value until 168 hours. Finally, yeast resumed growth reaching a value of 2.5 log cycles at 216 hours.

Figure 1

Survival of *L. innocua* present in a mixed culture, inoculated on the surface of various films and stored at 25 ℃, is shown in **Figure 2,** and microbial analysis for the films is presented in **Figure 2a.** The films without antimicrobials (C and CF) allowed the growth of the bacteria on its surface, presenting a bacteria count of 7 log cycles at the end of the storage. Conversely, film containing natamycin and nisin (FNANI) presented a bactericidal effect throughout the experiment. Basch et al. (2011) have previously reported the antimicrobial activity of edible films based on tapioca starch and HPMC and containing nisin. They reported that this film produced a rapid decrease of the inoculated *L. innocua*, reaching at the end of the storage a population 5 log cycle lower than the films without antimicrobials.

Microbial analysis for Port Salut cheese (with and without film) is presented in **Figure 2b**. Again, a cheese without inoculum and without film (CH), and an inoculated cheese without film (ICH), were tested. The inoculated cheese without film shows an initial bacterial count of 4.5 log cycles which increased its value acquiring at the end of storage a value of 7 log cycles. The CH shows bacterial counts below 10 log CFU/ml over the entire storage. This result demonstrates the good quality of the cheese, showing absence of *Listeria* spp. Covered cheeses with films CF, C and FNANI showed similar bacterial count to that obtained for the cheese CH without inoculum and without film (less than

10log 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* immediately and kept it lower than 10log CFU/ml up to 48 hours of storage. Then, the bacteria reasumed its growth reaching a value of 3 cycles log and maintaining that value until the end of storage.

Figure 2

These results indicate that all films (CF, C and FNANI) acted as a barrier to post-processing contamination. This means that the presence of these films prevented the access of the contaminating microorganism to the food. However, it is noteworthy that only the film containing both antimicrobials (FNANI) inhibited the development of mixed culture throughout the entire storage. Additionally, this film inhibited the growth of yeasts originally present in the cheese studied. These results are demonstrating that the film FNANI is an extremely effective method to control the population of microorganisms present on both sides of the film that is, between the cheese surface and the film and on the surface of the film, enabling to offer the consumer a safer product.

Since edible films are consumed with food, it is important that they are free of microorganisms, which highlights the film containing natamycin and nisin as the most appropriate edible film. In the case of commercial film, it is removed at the time of cheese consumption, but this process can result in contamination of the food if the packaging film does not have adequate microbiological status, fact that allow to conclude that the barriers capability of the film is not enough to ensure the microbiological safety of the cheese.

CONCLUSION

The overall results presented in this chapter indicate that the inclusion of natamycin and nisin in the packaging modifies the physico-chemical properties of the materials, trend that must be considered because it can compromise the packaging performance. According to bibliography, the inclusion of these antimicrobials gives origin to an efficient hurdle to control microbial contamination

414 during process or post-process. New results reported showed that an edible packaging based on
415 tapioca starch is a very interesting and efficient method to control microbial food contamination
416 produced by mixed cultures of *Saccharomyces cerevisiae* and *Listeria innocua* in cheese during post-
417 processing.

418

419 **REFERENCES**

420 Ahvenainen, R., 2003. Novel food packaging techniques. Elsevier, Cambridge, England.

421 Al-Holy, M., Al-Nabulsi, A., Osaili, T., Ayyash, M., Shaker, R., 2012. Inactivation of Listeria innocua in
422 brined white cheese by a combination of nisin and heat. Food Control 23, 48–53.

423 Amass, W., Amass, A., Tighe, B., 1998. A review of biodegradable polymers: uses, current
424 developments in the synthesis and characterization of biodegradable polyesters, blends of
425 biodegradable polymers and recent advances in biodegradation studies. Polym. Int. 47, 89–144.

426 Balasubramanian, A., Rosenberg, L., Yam, K., Chikindas, M., 2009. Antimicrobial packaging: potential
427 vs. reality—a review. J. Appl. Packag. Res. 3, 193–221.

428 Basch, C., Carpenco, J., Jagus, R., Flores, S., 2011. Individual and combined performance of nisin
429 and potassium sorbate supported in tapioca strach edible fims. Proc. 11th. Int. Congr. Eng. Food
430 (ICEF 11) 2, 979–980.

431 Basch, C., Jagus, R., Flores, S., 2013. Physical and antimicrobial properties of tapioca Starch-HPMC
432 edible films incorporated with nisin and/or potassium sorbate. Food Bioprocess Technol. 6,
433 2419–2428.

434 Bauer, R., Dicks, L., 2005. Mode of action of lipid II-targeting lantibiotics. Int. J. Food Microbiol. 101,
435 201–216.

436 BBC, R., 2013. Active, Controlled, and Intelligent Packaging for Foods and Beverages.
437 http://www.bccresearch.com/pressroom/fod/advanced-packaging-solutions-market-value-
438 projected-nearly-$44.3-billion-2017. (Last accesed: 30/01/2015).

439 Breukink, E., van Kraaij, C., Demel, R., Siezen, R., Kuipers, O., de Kruijff, B., 1997. The C-terminal
440 region of nisin is responsible for the initial interaction of nisin with the target membrane.
441 Biochemistry 36, 6968–6976.

442 Burke, J., 2006. Biodegradeable-compostable packaging: The promise and the problems. Pap.
443 Packag. 47.

444 Campos, C., Gerschenson, L., Silvia, S., 2011. Development of Edible Films and Coatings with
445 Antimicrobial Activity. J. Food Bioprocess Technol. 4, 849–875.

446 Cao-Hoang, L., Chaine, A., Grégoire, L., Waché, Y., 2010. Potential of nisin-incorporated sodium
447 caseinate films to control Listeria in artificially contaminated cheese. Food Microbiol. 27, 940–4.

448 Cha, D., Chinnan, M., 2004. Biopolymer-based antimicrobial packaging: A review. Crit. Rev. Food Sci.
449 Nutr. 44, 223–237.

450 Cutter, C., 2002. Microbial Control by Packaging: A Review. Crit. Rev. Food Sci. Nutr. 42, 151–161.

451 Delves-Broughton, J., Blackburn, P., Evans, R., Hugenholtz, J., 1996. Applications of the bacteriocin,
452 nisin. Antonie Van Leeuwenhoek 69, 193–202.

453 Delville, J., Joly, C., Dole, P., Bliard, C., 2003. Influence of photocrosslinking on the retrogradation of
454 wheat starch based films. Carbohydr. Polym. 53, 373–381.

455 El-Diasty, E., El-Kaseh, R., Salem, R., 2008. The effect of natamycin on keeping quality and
456 organoleptic characters of yoghurt. Arab J. Biotechnol. 12, 41–48.

457 Fajardo, P., Martins, J., Fuciños, C., Pastrana, L., Teixeira, J., Vicente, A., 2010. Evaluation of a
458 chitosan-based edible film as carrier of natamycin to improve the storability of Saloio cheese. J.
459 Food Eng. 101, 349–356.

460 Fernández, M., Jagus, R., Mugliaroli, S., 2014. Effect of combined natural antimicrobials on spoilage
461 microorganisms and Listeria innocua in a whey cheese “ricotta.” Food Bioprocess Technol. 1–10.

462 Franssen, L., Rumsey, T., Krochta, J., 2004. Whey protein film composition effects on potassium
463 sorbate and natamycin diffusion. J. Food Sci. 69, 347–350.

464 Gallo, L., Jagus, R., 2006. Modelling Saccharomyces cerevisiae inactivation by natamycin in liquid
465 cheese whey. Brazilian J. Food Technol. 9, 311–316.

466 Gould, G., 1997. Methods of preservation and extension of shelf life. Int. J. Food Microbiol. 33, 51–64.

467 Guiga, W., Swesi, Y., Galland, S., Peyrol, E., Degraeve, P., Sebti, I., 2010. Innovative multilayer
468 antimicrobial films made with Nisaplin?? or nisin and cellulosic ethers: Physico-chemical
469 characterization, bioactivity and nisin desorption kinetics. Innov. Food Sci. Emerg. Technol. 11,
470 352–360.

471 Gupta, K., Chatterjee, C., Gupta, B., 2012. Isolation and characterization of heavy metal tolerant
472 Gram-positive bacteria with bioremedial properties from municipal waste rich soil of Kestopur
473 canal (Kolkata), West Bengal, India. Biologia (Bratisl). 67, 827–836.

474 Hammond, S., Lambert, P., 1978. Membrane-active antimicrobial agents, in: Antibiotics and
475 Antimicrobial Action. Edward Arnold Publishers Limited, London, pp. 34–36.

476 Han, J., 2014. A review of food packaging technologies and innovations, in: Han, J. (Ed.), Innovations
477 in Food Packaging. San Diego, USA, pp. 3–12.

478 Hanušová, K., Šťastná, M., Votavová, L., Klaudisová, K., Dobiáš, J., Voldřich, M., Marek, M., 2010.
479 Polymer films releasing nisin and/or natamycin from polyvinyldichloride lacquer coating: Nisin
480 and natamycin migration, efficiency in cheese packaging. J. Food Eng. 99, 491–496.

481 Imran, M., El-Fahmy, S., Revol-Junelles, A., Desobry, S., 2010. Cellulose derivative based active
482 coatings: Effects of nisin and plasticizer on physico-chemical and antimicrobial properties of
483 hydroxypropyl methylcellulose films. Carbohydr. Polym. 81, 219–225.

484 Kim, K., Ko, C., Park, H., 2002. Mechanical Properties, Water Vapor Permeabilities and Solubilities of
485 Highly Carboxymethylated Starch-Based Edible Films. J. Food Sci. 67, 218–222.

486 Koontz, J., Marcy, J., Barbeau, W., Duncan, S., 2003. Stability of natamycin and its cyclodextrin
487 inclusion complexes in aqueous solution. J. Agric. Food Chem. 51, 7111–7114.

488 La Storia, A., Ercolini, D., Marinello, F., Mauriello, G., 2008. Characterization of bacteriocin-coated
489 antimicrobial polyethylene films by atomic force microscopy. J. Food Sci. 73, 48–54.

490 Lee, H., Zhou, B., Liang, W., Feng, H., Scott, E., 2009. Inactivation of Escherichia coli cells with
491 sonication , manosonication, thermosonication, and manothermosonication: Microbial responses
492 and kinetics modeling. J. Food Eng. 93, 354–364.

493 Leistner, L., 2000. Basic aspects of food preservation by hurdle technology. Int. J. Food Microbiol. 55,
494 181– 186.

495 Martins, J., Cerqueira, M., Souza, B., Carmo Avides, M., Vicente, A., 2010. Shelf life extension of
496 ricotta cheese using coatings of galactomannans from nonconventional sources incorporating
497 nisin against Listeria monocytogenes. J. Agric. Food Chem. 58, 1884–1891.

498 Miltz, J., Passy, N., Mannheim, C., 1995. Trends and applications of active packaging systems. Spec.
499 Publ. R. Soc. Chem. 162, 201–210.

500 Muscat, D., Adhikari, R., McKnight, S., Guo, Q., Adhikari, B., 2013. The physicochemical
501 characteristics and hydrophobicity of high amylose starch–glycerol films in the presence of three
502 natural waxes. J. Food Eng. 119, 205–219.

503 Ollé Resa, C., Gerschenson, L., Jagus, R., 2013. Effect of natamycin on physical properties of starch
504 edible films and their effect on Saccharomyces cerevisiae activity. Food Bioprocess Technol. 6,
505 3124–3133.

506 Ollé Resa, C., Gerschenson, L., Jagus, R., 2014b. Natamycin and nisin supported on starch edible
507 films for controlling mixed culture growth on model systems and Port Salut cheese. Food Control
508 44, 146–151.

509 Ollé Resa, C., Jagus, R., Gerschenson, L., 2014a. Effect of natamycin, nisin and glycerol on the
510 physicochemical properties, roughness and hydrophobicity of tapioca starch edible films. Mater.
511 Sci. Eng. C 40, 281–287.

512 Pintado, C., Ferreira, M., Sousa, I., 2010. Control of pathogenic and spoilage microorganisms from
513 cheese surface by whey protein films containing malic acid, nisin and natamycin. Food Control
514 21, 240–246.

515 Pires, A., Soares, N., Andrade, N., Silva, L., Camilloto, G., Bernardes, P., Dos Santos Pires, A., De
516 Ferreira Soares, N., De Andrade, N., Mendes Da Silva, L., Peruch Camilloto, G., Campos
517 Bernardes, P., 2008. Development and evaluation of active packaging for sliced mozzarella
518 preservation. Packag. Technol. Sci. 21, 375–383.

519 Quintavalla, S., Vicini, L., 2002. Antimicrobial food packaging in meat industry. Meat Sci. 62, 373–
520 380.

521 Ramos, O., Silva, S., Soaresa, J., Fernandesa, J., Poçasa, M., Pintadoa, M., Malcata, F., 2012.
522 Features and performance of edible films, obtained from whey protein isolate formulated with
523 antimicrobial compounds. Food Res. Int. 45, 351–361.

524 Ray, S., Bousmina, M., 2005. Biodegradable polymers and their layered silicate nanocomposites: In
525 greening the 21st century materials world. Prog. Mater. Sci. 50, 962–1079.

526 Realini, C., Marcos, B., 2014. Active and intelligent packaging systems for a modern society. Meat
527 Sci. 98, 404–419.

528 Reppas, C., Swidan, S., Tobey, S., Turowski, M., Dressman, J., 2009. Hydroxypropylmethylcellulose
529 significantly lowers blood cholesterol in mildly hypercholesterolemic human subjects. Eur. J. Clin.
530 Nutr. 63, 71–7.

531 Reps, A., Jedrychowski, L., Tomasik, J., Wisniewska, K., 2002. Natamycin in ripening cheeses.
532 Pakistan J. Nutr. 1, 243–247.

533 Sobrino-Lòpez, A., Martín-Belloso, O., 2008. Use of nisin and other bacteriocins for preservation of
534 dairy products. Int. Dairy J. 18, 329–343.

535 Sorrentino, A., Gorrasi, G., Vittoria, V., 2007. Potential perspectives of bio-nanocomposites for food
536 packaging applications. Trends Food Sci. Technol. 18, 84–95.

537 Te Welscher, Y., Jones, L., van Leeuwen, M., Dijksterhuis, J., De Kruijff, B., Eitzen, G., Breukink, E.,
538 2010. Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with
539 ergosterol. Antimicrob. Agents Chemother. 54, 2618–2625.

540 Te Welscher, Y., Ten Napel, H., Balagué, M., Souza, C., Riezman, H., De Kruijff, B., Breukink, E.,
541 2008. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing
542 the membrane. J. Biol. Chem. 283, 6393–6401.

543 Tharanathan, R., 2003. Biodegradable films and composite coatings: past, present and future. Crit.
544 Rev. Food Sci. Technol. 14, 71–78.

545 Thomas, L., Clarkson, M., Delves-Broughton, J., 2000. Nisin. Natural Food Antimicrobial Systems.,
546 Boca Raton.

Tiwari, B., Valdramidis, V., O'Donnell, C., Muthukumarappan, K., Bourke, P., Cullen, P., 2009. Application of natural antimicrobials for food preservation. J. Agric. Food Chem. 57, 5987–6000.

Ture, H., Eroglu, E., Ozen, B., Soyer, F., 2011. Effect of biopolymers containing natamycin against Aspergillus niger and Penicillium roquefortii on fresh kashar cheese. Int. J. Food Sci. Technol. 46, 154–160.

Vanderroost, M., Ragaert, P., Devlieghere, F., De Meulenaer, B., 2014. Intelligent food packaging: The next generation. Trends Food Sci. Technol. 39, 47–62.

Yam, K., Takhistov, P., Miltz, J., 2005. Intelligent Packaging: Concepts and Applications. J. Food Sci. 70, 1–10.

Figure 1: Effectiveness of different treatments applied on Port Salut cheese against an external contamination by a mixed culture containing *Saccharomyces cerevisiae.* Commercial film (CF), edible Control (C), film containing natamycin and nisin (FNANI), solution containing natamycin and nisin (direct application, DA), cheese without inoculum and without film (CH), and inoculated cheese without film (ICH). a: microbiological analysis of the films evaluated, b: microbiological analysis of the Port Salut cheeses.

Figure 2: Effectiveness of different treatments applied on Port Salut cheese against an external contamination by a mixed culture containing *Listeria innocua.* Commercial film (CF), edible Control (C), film containing natamycin and nisin (FNANI), solution containing natamycin and nisin (direct application, DA), cheese without inoculum and without film (CH), and inoculated cheese without film (ICH). a: microbiological analysis of the films evaluated, b: microbiological analysis of the Port Salut cheese.