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Natamycin efficiency for controlling yeast growth in models systems and on cheese surfaces

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A B S T R A C T

Consumers demand for more natural ingredients in processed foods is a result of a requirement for more healthy and safe food with also a need for a balanced and adequate diet.

Natamycin is a polyene macrolide antibiotic which is active against yeasts and moulds but not against bacteria, viruses and protozoa.

In this study the effectiveness of natamycin delivered by different methods against *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii* and *Yarrowia lipolytica* using both food models and cheese with natural antimicrobials.

It was observed that natamycin concentration and yeast type influenced whether the natamycin effect in tapioca starch films was cidal or inhibitory. This was also observed when the antimicrobial was applied directly to a liquid system for comparison purposes. Bioavailability was not compromised by the polymeric supporting matrix and natamycin efficiency against a *S. cerevisiae* contamination that preceded antimicrobial application was superior when film action was compared with spraying.

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1. Introduction

Foods are prone to microbiological deterioration throughout storage and distribution. Addition of antimicrobial agents may enable extension of the shelf-life and safety of fresh and packaged foods, by reducing or even preventing the growth of pathogenic and spoilage microorganisms (Franssen, Rumsey, & Krochta, 2004).

Consumers demand for more natural ingredients in processed foods is a result of a requirement for more healthy and safe foods with also a need for a balanced and adequate diet. As a consequence, the demand for natural food ingredients has increased and the use of natural antimicrobial compounds from a wide variety of sources has been widely investigated (Gould, 1997).

Natamycin is a natural antimycotic polyene characterized by the presence of a large macrocyclic lactone ring containing a series of conjugated double bonds and one or more sugar residues (Hammond & Lambert, 1978). It has a molecular weight of 665.7Da, is produced by *Streptomyces natalensis* and is commonly employed in dairy-based food products to prevent yeasts and moulds contamination (El-Diasty, El-Kaseh, & Salem, 2008; Gallo & Jagus, 2006; Reps, Jedrychowski, Tomasik, & Wisniewska, 2002). It has been approved as a food additive in over 40 countries and has been considered as a GRAS (generally recognized as safe) product by the FDA (Koontz, Marcy, Barbeau, & Duncan, 2003) and also indicated as a natural preservative by the European Union (EEC N° 235).

Natamycin kills yeasts by specifically binding to ergosterol but without permeabilizing the plasma membrane. It inhibits vacuolar fusion through the specific interaction with ergosterol (te Welscher et al., 2008, 2010). Therefore, it is active against yeasts and moulds but not against bacteria, viruses and protozoa. Sterols are known to have an ordering effect on the membrane, it is thought that they reside in specific sterol-rich domains in membranes and they are also known to be involved in endocytosis, exocytosis, vacuolar fusion (Wachtler & Balasubramanian, 2006), pheromone signalling (Jin, McCaffery, & Grote, 2008), membrane compartmentalization (Klose et al., 2010), and the proper functioning of membrane proteins (Zhang et al., 2010). According to Athar and Winner (1971), structural modification and/or decreased expression of ergosterol via mutations in the sterol biosynthesis pathway substantially diminish fungal pathogenicity *in vivo*.

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Natamycin can be applied directly by incorporation in liquid systems (Gallo & Jagus, 2006). In the case of food solid surfaces, it can be applied using different techniques like spraying, dipping or brushing, for the purpose of controlling microbial growth. According to Ture, Eroglu, Ozen and Soyer (2011), direct application of additives can have limited benefits. The antimicrobials can exhibit a loss of activity due to the reduction of their active concentration resulting from interaction or reaction with other additives or components present in the food matrix. Incorporation of antimicrobials in food interfaces by means of the use of edible films where they are entrapped helps to decrease the rate of diffusion from the surface to the bulk of the product, thus assisting in the maintenance of high concentrations of the active ingredient where it is required (Kristo, Koutsoumanis, & Biliaderis, 2008). It can also diminish the interaction with other additives and food components due to its presence being restricted mainly to the surface (Campos, Gerschenson, & Flores, 2011). As a consequence edible matrices with antimicrobial activity constitute a promising form of antimicrobial delivery in the frame of food preservation (Dos Santos Pires et al., 2008; Fajardo et al., 2010; Ollé Resa, Gerschenson, & Jagus, 2012; Ture et al., 2011).

The most commonly known spoilage yeasts are facultative anaerobic fermentative organisms, producing ethanol and carbon dioxide from simple sugars. Some fermentative yeasts are the most osmophilic organisms known, capable of slow growth at water activity (a_w) as low as 0.6 (Martorell, Fernández-Espinar, & Querol, 2005). Representative genera include *Saccharomyces* and *Zygosaccharomyces*. In particular, *Saccharomyces cerevisiae* is especially interesting because it is a yeast for which there is a good understanding of its physiology, biochemistry and genetic responses (Betts, Linton, & Betteridge, 2000; Praphailong & Fleet, 1997).

Zygosaccharomyces rouxii has unusual physiological characteristics which are largely responsible for their ability to cause spoilage, including resistance to weak-acid preservatives, extreme osmotolerance and ability to ferment hexose sugars (James & Stratford, 2003). *Z. rouxii* is characterized by its ability to tolerate low a_w environments, being one of the most xerophilic organisms known. It possesses two plasma-membrane antiporters with different substrate specificities which confer this property (Martorell, Stratford, Steels, Fernández-Espinar, & Querol, 2007; Pribylova, Papouskova, & Sychrova, 2008).

Yarrowia lipolytica strains are readily isolated from dairy products, and also from salads containing meat or shrimps. This yeast, frequently found in cheeses, was also reported to be associated with browning phenomenon (Carreira, Paloma, & Loureiro, 1998). The inability of this yeast to survive under anaerobic conditions permits its easy elimination from dairy products. *Y. lipolytica* does not require nitrogen limitation for induction of sporulation in contrast to *S. cerevisiae* and some other yeasts. Diploid strains sporulate on solid or in liquid complete medium between 20 °C and 30 °C, when glucose is exhausted (Barth & Gaillardin, 1997).

All these microorganisms comprise strains that are capable of growth during cold storage (Sorhaug & Stepaniak, 1997).

According to Ollé Resa et al. (2012) and Krause Bierhalz, da Silva and Kieckbusch (2012) the incorporation of natamycin on edible films constituted by tapioca starch and alginate and pectin, affect the physical properties of the films.

Although extensive information on the antimicrobial properties of natamycin is available in the literature, scarce data exist about the activity of natamycin against different yeasts when incorporated alone or supported in edible films.

Therefore, the objective of this study was to evaluate the effectiveness of natamycin against *S. cerevisiae*, *Z. rouxii* and *Y. lipolytica*, when different application techniques are assayed in model systems and in a food matrix.

2. Materials and methods

2.1. Materials

Tapioca starch was provided by Industrias del Maíz S.A. (Argentina) and glycerol by Mallickrodt (Argentina). Commercial natamycin (Delvocid® Salt) containing 50% NaCl and 50% natamycin was kindly supplied by DSM (Argentine). Whey protein concentrate (WPC35) with 35% w/w protein content was kindly supplied by Arla S.A. (Argentina). Its composition was: lactose 48.8% w/w; protein 38.3% w/w; ash 7.5% w/w; moisture 3.2% w/w and fat 2.2% w/w. Port Salut cheese (La Serenisima, Argentina), purchased in a local supermarket was used for the evaluation of the antimycotic activity of the films in a real food.

2.1.1. Film preparation

Mixtures of starch, glycerol and water (2:1:37, in weight) with and without commercial natamycin added were prepared. In this last case, 10 ml of the water were replaced with a solution of natamycin of adequate concentration for obtaining different final concentrations in the films, such as: film I with 1.85 mg natamycin/dm^2, film II with 3.70 mg natamycin/dm^2, or film III with 9.25 mg natamycin/dm^2. 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 bubbles from the gel. The slurry was dispensed in aliquots of 12 g in silicone plates of 7 cm diameter. The drying of the films was performed at 37 °C during 48 h in a convection chamber. Films without natamycin are named as films C. Once constituted, films were peeled off from plates and, before evaluating their properties, were conditioned at 28 °C, over saturated solution of NaBr (water activity, $a_w \cong 0.575$) during 7 d to assure equilibration.

2.1.2. Liquid WPC preparation

WPC35 was dissolved in sterile distilled water at the desired concentration (16% w/v) and the pH was adjusted to 5.5 by 0.5 N HCl addition (von Staszewski & Jagus, 2008).

2.2. Microbiological assay

2.2.1. Strains and growth conditions

S. cerevisiae (CBS 1171, strain collection SC), *Z. rouxii* (ATCC 28.253) and *Y. lipolytica* (National Institute of Infectious Diseases "Dr. Carlos G. Malbrán", CABA, Argentina) were grown in 150 ml Sabouraud broth (Biokar, France) at 28 °C in a continuously agitated temperature-controlled shaker until early stationary phase was achieved.

2.2.2. Antimicrobial activity in a model liquid food (liquid WPC)

This test was used to determine the antimicrobial effectiveness of natamycin applied directly to a liquid system. The systems evaluated were: WPC (system containing WPC and without natamycin), WPC/N1 (system containing WPC and with 20 ppm of natamycin or 0.02 mg natamycin/g system) and WPC/N2 (system with WPC and containing 50 ppm of natamycin or 0.05 mg natamycin/g system). These systems were inoculated with the different microorganisms and incubated at 25 °C. To determine the viable population of microorganisms, samples were serially diluted with peptone water. Microbial counts were then made on plates of chloramphenicol glucose agar (YGC, Biokar Diagnostics, France). The number of CFU/ml was determined after incubation at 28 °C for 48 h. Determinations were carried out in duplicate.

2.2.3. Barrier to microbial contamination

Petri dishes containing YGC agar with pH adjusted to a value of 5.2 (citric acid 50% w/w), were used to resemble a food product.

Disks of 1.0 cm diameter were cut from films C, I, II and III, and placed on the surface of the agar. Immediately, 10 μl of each culture containing $1 \times 10^{3-4}$ CFU/ml, were dispensed on the disks. Samples were incubated at 25 °C during 168 h and periodically sampled, to test yeast viability.

In order to evaluate the antimicrobial effectiveness of the films in a food matrix, Petri dishes containing cubes of cheese with a weight of 5 ± 0.3 g of the food, were used. Squares of 5.0 cm of side were cut from the films C and III, and brought in contact with the upper surface of the cheese. Immediately, 20 μl of each culture containing $1 \times 10^{3-4}$ CFU/ml, were dispensed on the films. Samples were incubated at 25 °C during 168 h and periodically sampled, to test yeast viability.

In both cases (food model and cheese), the initial number of viable cells and the counts at different storage times were evaluated in the films. For this, dilution drops (20 μl) were spotted in duplicate onto agar YGC and the number of CFU/ml was determined after incubation at 28 °C for 72 h. In the cheese test, there were also evaluated the counts for the different storage times in the cheese after removal of the film. Determinations were made in duplicate in two separate experimental runs.

2.2.4. Agar diffusion test

The agar diffusion test was used to determine the antimicrobial effect of the films on the test microorganisms when contamination precedes edible films application. Briefly, 100 μl of inoculum containing 1×10^{6} CFU/ml of each yeast, were spread on the surface of Petri dishes containing agar YGC.

Film discs (7 mm diameter) without natamycin (control, C) and with 9.25 mg natamycin/dm^2 of film of natamycin (film III), were placed on the plates previously inoculated which were pre-incubated at 4 °C for 48 h and, afterwards, incubated at 28 °C for 72 h (Hanušová et al., 2010). The inhibitory activity was quantified by measuring the total diameter of inhibition (disc plus inhibition zone). Determinations were made in duplicate.

2.2.5. Cheese diffusion test

The cheese diffusion test was used to determine the antimicrobial effect of films in a real system food. Briefly, 20 μl of inoculum containing $1 \times 10^{2-4}$ CFU/ml of each yeast were spread on the surface of cubes of cheese (5 ± 0.3 g), contained in Petri dishes. Squares (5 cm side) cut from the different films were used to cover the cheese with and without inoculum. The plates were incubated at 25 °C for 216 h.

This assay was performed with film III. For comparison purposes a spraying treatment of 50ppm natamycin was also applied. Samples of cheese without film covering (WF) were also assayed.

The initial and surviving number of viable cells at different storage times were evaluated. Dilution drops (20 μl) were spotted in duplicate onto agar YGC and the number of CFU/ml was determined after incubation at 28 °C for 72 h. Inhibition of microorganisms' growth was expressed as log CFU/ml. Determinations were made in duplicate in two separate experimental runs.

2.2.6. Transmission electron microscopy (TEM)

Cells of *S. cereviseae* in stationary phase were evaluated through transmission electron microscopy (TEM Philips EM 301, USA) when grown in Sabouraud broth (untreated) or in Sabouraud broth supplemented with 50 ppm of natamycin and stored for 24 h at 28 °C (treated), for the purpose of characterizing the effect of the antimycotic on the yeast.

2.2.6.1. Negative staining test. a drop of the suspension of the yeast in Sabouraud broth was mounted on a carbon-coated 200 mesh copper grid and stained on a drop of 1% uranyl acetate for 90 s. Samples were examined using a transmission electron microscope (Philips EM 301).

2.2.6.2. Glutaraldehyde fixation. treated and untreated cells were fixed with glutaraldehyde (2.5g/100 ml phosphate buffer) for 18 h, rinsed three times for 10min with phosphate buffer (0.1 mol/l pH 7.4) and post-fixed with osmium tetraoxide (1.0g/100 ml) for 2 h at 4 °C. After fixation, the cells were rinsed three times for 10min with phosphate buffer (0.1 mol/l pH 7.4) and then dehydrated using 30, 50, 70 and 95ml/100 ml acetone sequentially for 15min each. Next, the cells were dehydrated three times for 30min with pure acetone. After dehydration, the cells were treated with propylene oxide twice for 10min at 4 °C. The cells were sequentially infiltrated with a mixture of propylene oxide: Durcupan's ACM epoxy resin (3:1, 1:1 and 1:3) for 45min. Polymerization of the resin to form specimen blocks was performed in an oven at 60 °C for 72 h. The specimen blocks were hand trimmed with a razor blade and sectioned with a diamond knife in an ultramicrotome Sorvall MT2 Porter-Blum (DuPont Instruments, Sorvall Operations, Newton, Conn.). Thin sections (70–80 nm) were placed on 300-mesh copper grids. The sections were stained for 15–20min in uranyl:ethyl alcohol (1:1), washed three times for 2min and then incubated in a drop of Reynold's lead citrate and examined using a transmission electron microscope.

2.3. Statistical analysis of data

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

3. Results and discussion

3.1. Antimicrobial activity in a model liquid food (liquid WPC)

Fig. 1 shows the effect of different concentrations of natamycin on *S. cereviseae*, *Z. rouxii* and *Y. lipolytica* present in liquid WPC.

In the absence of natamycin an increase in counts for all the yeasts occurred. In the presence of natamycin, its activity did not show an immediate reduction of the initial microbial population and the inhibition of all yeasts evaluated was exerted throughout storage. Also amphotericin B exerts its fungicidal activity against *Candida* species throughout storage (Cantón, Pemán, Gobernado, Espinel-ingroff, & Pema, 2004).

The inactivation of yeasts increased with the natamycin concentration for all the yeasts. In the case of *S. cerevisiae* a dose of 20 ppm of natamycin (WPC/N1) showed that cell number was reduced to 1.25 log CFU/ml after 96 h and had a fungistatic effect for the additional 4 d evaluated. The treatment with 50 ppm of natamycin (WPC/N2) on the same initial population, produced a greater reduction of cells, and after 96 h resulted in less than 10 CFU/ml (Fig. 1a). Gallo & Jagus, (2006) studied the inactivation of *S. cerevisiae* in liquid cheese whey treated with different levels of natamycin (12,5 mg/l to 100 mg/l) and with different inoculum sizes showing that the inactivation of *S. cerevisiae* increased with storage time and concentration of natamycin.

Z. rouxii was less sensitive to natamycin (Fig. 1b). The presence of 20 ppm and 50 ppm reduced the counts 0.5 log cycle and 1.3 log cycle, respectively, and maintained this level until the end of the storage. To our knowledge, no other authors studied the activity of natamycin against *Z. rouxii*. Cerrutti & Alzamora, (1996) showed that it is possible to inhibit growth of *S. cerevisiae*, *Z. rouxii*, and other yeasts in apple puree (pH 3.5) containing 2000 ppm of

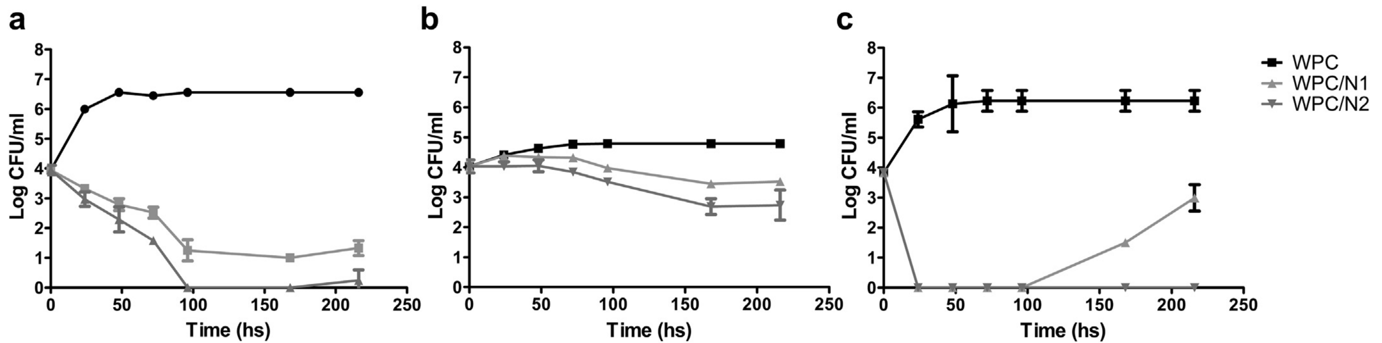

Fig. 1. Antimicrobial activity in a model liquid food (liquid WPC). a = *S. cerevisiae*, b = *Z. rouxii* and c = *Y. lipolytica*.

vanillin for 40 d storage at 27°, but the addition of these antimicrobial to the banana puree (pH 4) at the same concentration was not effective to inhibit the growth of *Z. rouxii* and *S. cerevisiae*.

Y. lipolytica presented a different response to the treatment with natamycin. A concentration of 20 ppm of natamycin exerted a fungicidal effect, but the yeast reassumed the growth after 100 h of storage (Fig. 1c). A higher concentration of the antimycotic (50 ppm) maintained the level of *Y. lipolytica* in less than 10 CFU/ml until the end of the storage. Ramos et al. (2012) who studied the MIC for natamycin against *Y. lipolytica* reported a value of 50 ppm.

3.2. Barrier to microbial contamination

Several authors have evaluated the performance of edible matrices as barriers to microbial contamination and reservoirs for controlled diffusion of antimicrobials (Flores, Haedo, Campos, & Gerschenson, 2007; Franssen, 2002; Ozdemir & Floros, 2001; Sebti, Blanc, Carnet-Ripoche, Saurel, & Coma, 2004). Accordingly, a test was performed to evaluate the usefulness of films proposed as barriers to microbial contamination.

The three yeasts studied grew when the film C was assayed.

It was observed that the films containing natamycin, showed a different behaviour depending on the microorganism and the concentration of the preservative. Films II and III showed fungicidal action on *S. cerevisiae*, while the film I exerted fungistatic action (Fig. 2a). *Z. rouxii* showed a concentration dependent sensitivity to natamycin up to 48 h of storage but, in all cases, the action was fungicidal. Afterwards, all the concentrations evaluated exerted the same effect (Fig. 2b). *Y. lipolytica* was shown to be very sensitive to the presence to natamycin and a fungicidal effect was observed, irrespective of the antimicrobial concentration (Fig. 2c).

Other authors studied the ability of films to act as barrier to external contamination with different matrices and antimicrobials. Basch, Carpenco, Jagus & Flores (2011) studied the performance of nisin (Nis), potassium sorbate (KS) and their combination (Nis/KS), when incorporated in films made with tapioca starch and its mixtures with hydroxypropyl methylcellulose. In the case of an external contamination by *Zygosaccharomyces bailii*, films with KS or Nis/KS decreased the rate of yeast growth, compared with the film without antimicrobials (control film). However, combined test films showed a better performance than the films containing only KS, presenting after 24 h, counts that were 1.4 log cycles lower than control films. Ramos et al. (2012) studied the efficacy of edible films produced from whey protein isolate, glycerol and natamycin as antimicrobial agent. The authors showed through the viable cell counts assay that natamycin incorporated in the film displayed a cidal effect against *Y. lipolytica*. This antimicrobial led the yeast to depletion within 3 h of storage at 30 °C.

The barrier test performed in cheese with films C and III produced similar results to the ones previously reported for the food model, which means that only the films with natamycin exerted a fungicidal effect against the three yeasts (data not shown). In this assay, additionally, all the films with and without natamycin prevented the contamination of the cheese by the microorganisms inoculated in the surface.

3.3. Agar diffusion test

In the agar diffusion test, it was observed that film C allowed the growth of all the yeasts (data not shown), while the presence of the film III showed clear zones of different diameters dependent on the contaminating microorganism (Table 1). *Y. lipolytica* showed the smallest diameter of inhibition.

It is important to state that the diameter of the inhibition zone is the result of the balance between the rate of antimicrobial diffusion to the agar and the rate of microorganism growth. Considering that diffusion of natamycin is the same for the three yeasts evaluated because the same media was used for their growth and, taking into

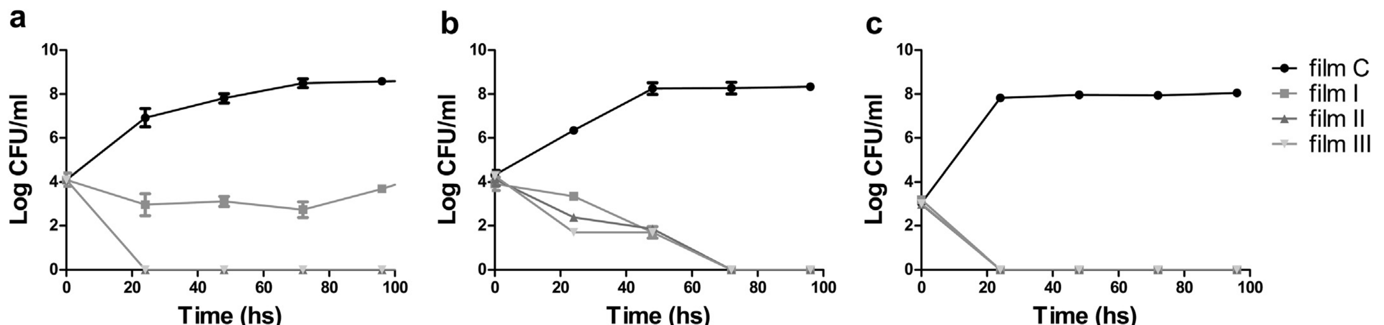

Fig. 2. Films as barriers to external contamination by (a) *S. cerevisiae*, (b) *Z. rouxii* and (c) *Y. lipolytica*.

Table 1
Agar diffusion test.

Yeast	Diameter of inhibition zone (cm)
S. cerevisiae	2.95 ± 0.07[a]
Y. lipolytica	2.25 ± 0.21[b]
Z. rouxii	3.10 ± 0.14[a]

Values with different letters showed significant differences (α: 0.05).

account that it is being analyzed the performance of the same edible film containing natamycin (film III), it can be said that the different diameters observed for the inhibition zone, were associated with different growth rate and sensitivity to natamycin for the different yeasts evaluated. It is noteworthy that in the barrier to microbial contamination test it was observed that when testing the performance of the film III, *Y. lipolytica* was the fastest growing yeast, showing in the present assay the smaller inhibition diameter. In contrast, *Z. rouxii* and *S. cerevisiae* were the yeasts that grew more slowly and showed herein a larger inhibition diameter (Fig. 2 and Table 1).

Pintado, Ferreira & Sousa (2010) studied the effect of nisin, natamycin and malic acid incorporated into a film of whey protein concentrate against *Y. lipolytica*. These authors observed that films containing natamycin and malic acid produced a zone of inhibition of 8.0 mm and 11.9 mm respectively. These values are similar to those obtained in the present work since the diameter of inhibition of *Y. lipolytica* was 2.25 cm which corresponded to an inhibition zone of 6.7 mm. It is important to remark that the films probably grew in size along the experiment due to swelling of the hydrophilic matrices used, which may have influenced the results observed (Flores et al., 2007).

Fajardo et al. (2010) studied the release of natamycin from chitosan films to a liquid medium (phosphate buffer solution) obtaining values of diffusion coefficients of ($3.60 \times 10^{-10} \pm 0.26 \times 10^{-10}$) $cm^2 s^{-1}$ at 4 °C which are similar to those obtained by Hanušová et al. (2010) at 23 °C for natamycin supported in polyvinyl chloride. Krause Bierhalz et al. (2012) informed that the release of natamycin with time when edible films were immersed in water, fitted well to Fick's second law diffusional model, with effective diffusivity values ranging from 3.2×10^{-9} (pectin films) to 9.2×10^{-12} cm^2/s (alginate films). In the work of Fajardo et al. (2010), the release of natamycin to cheese as a solid food model allowed to observe lower values of diffusion coefficient ($1.29 \times 10^{-12} \pm 0.35 \times 10^{-12}$) $cm^2 s^{-1}$ at 4 °C and the authors attributed this difference in the diffusion coefficient to the characteristics of the cheese: solid system and with smaller water activity (a_w) than in the case of the liquid system. Although the release was slower, 80% of the natamycin present in the films was released after 23 d of storage. In the present research, it was observed a diffusion of natamycin from the film to the medium (Table 1) favored by its high water activity (a_w 0.99). As previously reported (Fig. 2), edible matrices were still effective as a barrier to external contamination at 100 h of storage indicating the potential efficiency of these films to be used as a vehicle for natamycin.

3.4. Cheese diffusion test

For the purpose of evaluating the response of the wide range of microorganisms, including mould and yeasts, present in cheese, the performance of the film and the spray system in a cheese without inoculation was evaluated.

Fig. 3a shows that the yeasts present in the cheese were inhibited by film III. With application of spray or without film, the yeasts reassumed the growth after 48 h and 24 h of storage respectively.

In the inoculated cheese, results obtained showed that the three yeasts grew without restriction in the cheese without film, WF (Fig. 3b–d). On the contrary, in the presence of film III or when the sample had been sprayed with natamycin solution, different rates of growth were observed for each yeast and treatment.

In the case of *S. cerevisiae*, film III exerted initially a fungicidal effect (Fig. 3b). Even thought, growth was thereafter restored, reaching at the end of the storage a population of about 5 log cycles lower than cheese WF. On the contrary, the spray of natamycin only produced a slight reduction in the first 24 h, and afterwards reassumed the growth, reaching a population similar to WF at the end of the evaluated period. It is interesting to note that in a preliminary

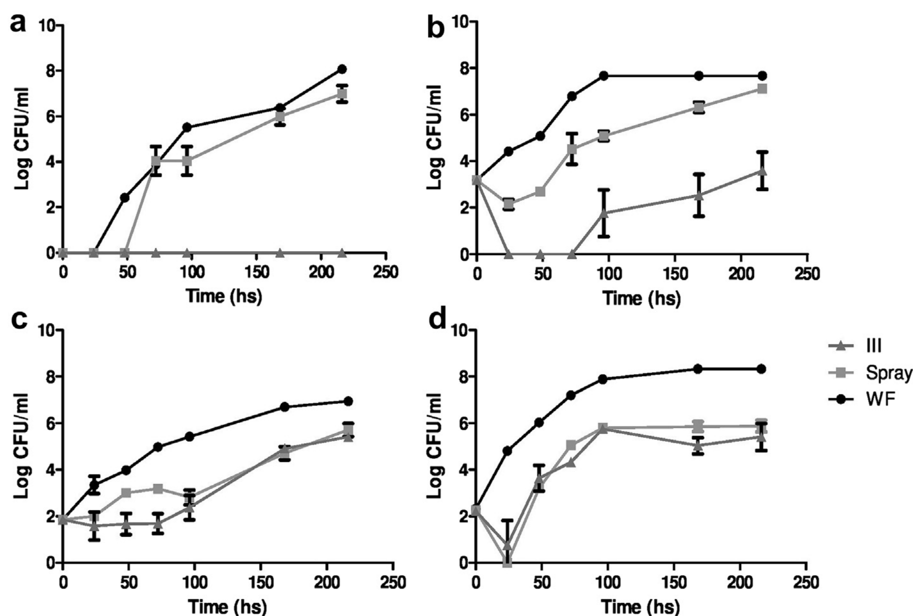

Fig. 3. Cheese diffusion test: comparison of (a) without inoculum; (b) inhibition of *S. cerevisiae*; (c) inhibition of *Z. rouxii*; (d) inhibition of *Y. lipolytica* in the presence of an edible film containing natamycin (III), with a spray of 50ppm of natamycin (Spray) and without film (WF).

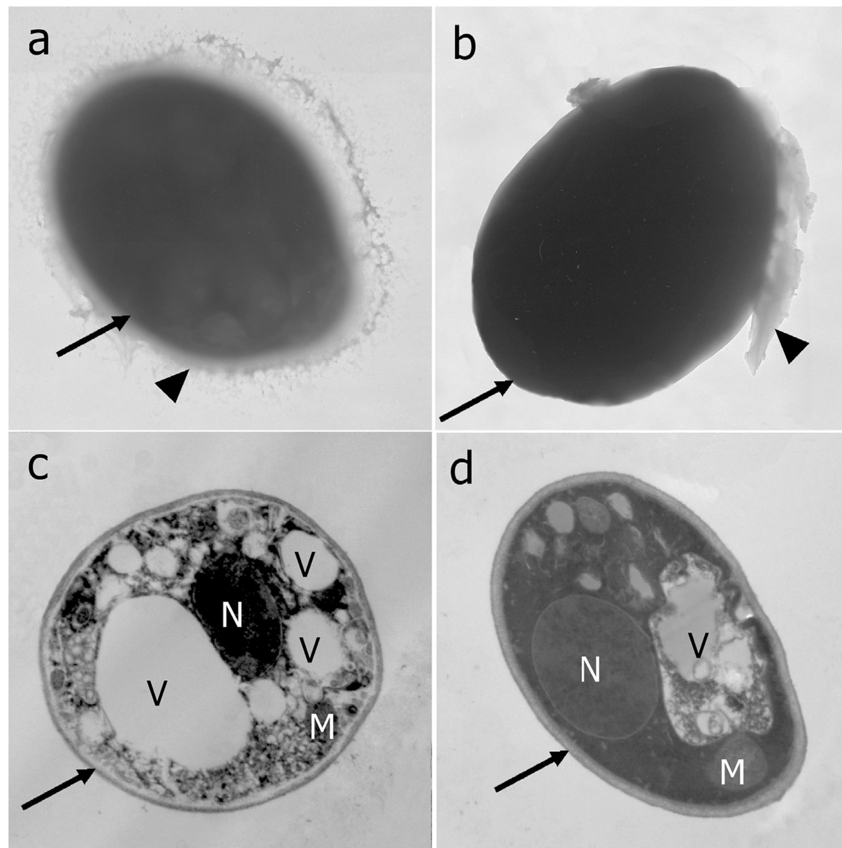

Fig. 4. Transmission electron microscopy micrographs of *S. cerevisiae*: untreated (panels a and c) and treated with 50ppm of natamycin (panels b and d). Negative staining (panels a and b), glutaraldehyde fixation (panels c and d). Nucleus (N), vacuole (V), mitochondria (M), arrowhead (outer layer) and arrows (cell wall). Bars: 1 μm.

assay performed with *S. cerevisiae*, that cheese covered with control film (C) or a commercial film (Cryovac BK50,Sealed Air Argentina) showed similar growth pattern to cheese without film (WF).

For the other two yeasts both treatments (film III and spray) showed a similar performance, and at the end of storage a population of 1.5 log resulted which was 2.8 log cycles lower than the one observed for cheese WF for *Z. rouxii* and *Y. lipolytica*, respectively (Fig. 3c and d).

These results indicate the effectiveness of tapioca starch based films containing natamycin for acting as reservoir and for inhibiting the *S. cerevisiae*, *Z. rouxii* and *Y. lipolytica* growth in a food matrix, due to the liberation of the antimycotic from the film. Fajardo et al. (2010) stated that the controlled release of natamycin from chitosan films to a cheese matrix would minimise or prevent the rapid loss of the antimicrobial when applied directly on cheese surface.

3.5. Transmission electron microscopy (TEM)

The cell wall of *S. cerevisiae* is a sturdy structure providing physical protection and osmotic support. The mechanical strength of the wall is mainly attributed to the inner layer, which consists of β1,3-glucan and chitin, and represents about 50–60% of the wall dry weight. An outer layer, which consists of heavily glycosylated mannoproteins emanating from the surface (Klis, Mol, Hellingwerf, & Brul, 2002), is involved in many vital functions (De Nobel, Klis, Priem, Munnik, & van den Ende, 1990; Jigami & Odani, 1999; Orlean, 1997; Reynolds & Fink, 2001). Cell wall proteins account for about one third of the wall dry weight and they are covalently linked to the β1,3-glucan-chitin network (Moukadiri, Jaafar, & Zueco, 1999).

Through the negative staining test, it was possible to observe the outer layer of the yeast cell detecting that untreated cells maintained an intact outer layer (Fig. 4a) while the treated cells (Fig. 4b) showed a damaged outer layer. It is known that natamycin interacts with proteins (Geijp, Stark, & Van Rijn, 1998) and it is probable that the antimycotic interacts with the mannoproteins of the outer layer producing the changes observed in Fig. 4b.

After glutaraldehyde fixation the cell organelles could be observed. The TEM micrographs of untreated yeasts showed cells with a normal ultrastructure, with nucleus, continuous cytoplasmatic membrane, compact and defined cell wall (Fig. 4c). It is remarkable that treated yeasts revealed dramatic changes in cell wall architecture and abnormalities in the sub-cellular structures (Fig. 4d), such as an increase in cell wall thickness, an electron-dense cytoplasm, accumulation of substrates in vacuoles and mitochondrial swelling. These findings are similar to those reported by Belanger, Nast, Fratti, Sanati & Ghannoum (1997), Koul, Vitullo, Reyes & Ghannoum (1999) and Ishida et al. (2009) who showed that the treatment with different azoles produced similar abnormalities in the cells of *Candida* spp.

4. Conclusion

The present work has demonstrated that natamycin was effective against *S. cerevisiae*, *Z. rouxii* and *Y. lipolytica* delivered by different techniques. In liquid systems natamycin exhibited different degrees of effectiveness depending on its concentration and the type of yeast.

The tapioca starch based films containing natamycin showed an enhanced inhibition of *S. cerevisiae* growth on cheese surfaces

contaminated previous to antimicrobial application, when compared with spray application. Additionally the preservative incorporated in the film was available to prevent an external contamination of yeasts during storage. Natamycin exerted an important effect, acting as a fungicide or a fungistatic depending on its concentration and the yeast evaluated, but showed always to be bioavailable when supported in tapioca starch films. The inactivation of yeasts increased with the natamycin concentration for all the yeasts evaluated. Hence, these results indicate that this natural antimicrobial can be potentially used for controlling yeasts in food systems being an adequate application technique its support in tapioca starch matrices.

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