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Evaluation of the Antimicrobial Potential of Alginate and Alginate/Chitosan Films Containing Potassium Sorbate and Natamycin

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The antimicrobial potential of alginate films (AFs) and alginate/chitosan composite films with two different mass proportions of the biopolymers, 82.5:17.5 (CF1) and 65:35 (CF2), containing potassium sorbate (KS) or natamycin was evaluated. At the practical limit of KS addition (0.17 g KS per gram of alginate) for pure AFs, no inhibition zones were observed against *Debaromyces hansenii*, *Penicillium commune* and *Penicillium roqueforti* by the agar diffusion test. Above this concentration, films became opaque, brittle and showed a whitish precipitate over their surface, making them not suitable for use. However, alginate and alginate/chitosan composite films containing natamycin were able to inhibit the growth of the three microorganisms listed above. Natamycin was effective at concentrations as low as 0.005 g per gram of biopolymer for AF and 0.01 g per gram of biopolymer for both composite films, alginate/chitosan 65:35 (CF1) and alginate/chitosan 82.5:17.5 (CF2) against all microorganisms tested. The inhibitory zone diameter increased as concentration of natamycin increased. Active films containing 0.04 g natamycin per gram of biopolymer obtained in the present study exhibited suitable functional attributes and showed excellent perspectives as active antimicrobial films intended for food protection applications.

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KEY WORDS: antimicrobial films; active packaging; alginate; chitosan; natamycin

INTRODUCTION

There is a growing interest in the development of new formulations of antimicrobial agents in various practices, including food coating and packaging and surface treatments in biomedical devices. However, the direct application of antimicrobial agents onto food surfaces, by dipping or spraying, could be inefficient, owing to the rapid diffusion of the active substances within the bulk of the product. To design proper antimicrobial films to be used in food preservation, the rate of the release of the active agents from the film to the food surface should be the basic concern, thus helping to maintain high concentrations where most deterioration processes take place.[1,2]

Antimicrobial active films represent an innovative packaging concept designed to meet today's consumer expectation for high quality and minimal processed food products. Besides, the use of natural polymers as packaging materials may relieve the environmental impact caused by excessive use of conventional polymeric material. Biofilms can be prepared from renewable sources such as polysaccharides, proteins and/or lipids.[3] The blending of polymers, which results in preparation of new materials with improved physicochemical and mechanical properties, has received considerable

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attention from researchers in the past several decades. The final properties of the blends are determined by polymer characteristics and their compatibility.[4–6]

Alginates are the main biomacromolecules extracted from brown seaweed. In molecular terms they are anionic linear copolymers of (1,4) D-mannuronic acid and L-guloronic acid residues arranged in a nonregular blockwise pattern. Alginates form strong and quite brittle films with poor water resistance. However, alginates have a unique ability to react irreversibly with polyvalent metal cations, in particular calcium ions, to produce low-solubility materials.[7] Chitosan is a weak cationic polysaccharide, formed mainly by (1,4)2-amino-2-deoxy-β-D-glucan. It is a natural carbohydrate polymer derived from deacetylation of chitin, which is a major component of the shells of crustaceans such as crab, shrimp and crawfish. Because of the good film-forming property, biocompatibility and a certain degree of antimicrobial activity,[8,9] chitosan has often been chosen as a raw material to prepare composite films.[10] Chitosan-based films have been proven to have adequate gas barrier properties, moderate mechanical properties but high water vapor permeability (WVP) and water solubility because of their hydrophilic nature.[11] To overcome these disadvantages, many researchers have investigated the polyelectrolyte complex (PEC) of chitosan with other polymers like sodium alginate,[12] pectin,[13] carrageenan and xanthan gum.[14] These oppositely charged polysaccharides will form PECs that have interesting characteristics for controlled release applications.[12,15]

The antimicrobial compounds and their incorporation into packaging materials have been well described and reviewed.[1,16] Recent studies have investigated the antimicrobial potential of several active substances against a vast number of microorganisms.[17–21] Commonly used antimicrobial molecules are organic acids (sorbic acid and benzoic acid), enzymes (lactoperoxidase, lactoferrin, avidin and lysozyme), microbial preservatives produced from starter cultures (nisin, lactacin/lactococin/lacticin, natamycin [NA] and variacin) and plant sources (herbs, spices, extracts, essential oils and their isolated components).[22,23] NA, also known as pimaricin, is a natural antimycotic agent produced by *Streptomyces natelensis*. Because of its low solubility, NA is used as a surface treatment on foods to control fungal growth but is not effective against bacteria or viruses.[24,25] It inhibits the fungi by binding to cell membrane sterols, especially ergosterol, causing cell lyses. NA was approved as generally recognised as safe (GRAS) by the Food and Drug Administration (FDA) in the United States and as a natural preservative in the European Union (E235).[26]

The incorporation of antimicrobial agents into film matrix adds new functionality to the packaging material. However, the addition of these agents may cause changes in the structure and physical properties of films. Changes in film structure and consequently in the release pattern of the active substance could arise from interactions between the active solutes and the film constituents.[27,28]

In the present study, the antimicrobial potential of alginate films (AFs) containing potassium sorbate (KS) and of alginate and alginate/chitosan composite films containing NA was evaluated.

MATERIALS AND METHODS

Materials

Medium viscosity sodium alginate, obtained from *Macrocystis pyrifera* seaweed, purchased from Sigma-Aldrich (St. Louis, USA), with an average molecular mass (M_w) of 1.61×10^6 Da, and chitosan with M_w of 3.70×10^5 Da and deacetylation degree of 96.7% (Polymar, Fortaleza, Brazil) were used as biopolymer matrices in the composite and pure films. Calcium chloride dihydrate (Merck, Darmstadt, Germany) was used as cross-linking agent and glycerol (Synth, Diadema, Brazil) as plasticizer. KS (Sigma-Aldrich, St. Louis, USA) and NA (Natamax®, Danisco, São Paulo, Brazil), were used as antimicrobial agents.

Film preparation

AFs and alginate and chitosan composite films with two different mass proportions of alginate and chitosan, 82.5:17.5 (CF1) and 65:35 (CF2), were obtained by casting in a two-stage cross-linking procedure.

The AF-forming solution (1.5% w/w) was prepared in distilled water already containing 0.6 g glycerol per gram of alginate at room temperature. The solution was mechanically stirred at 1000 rpm

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(Fisatom, model 713, São Paulo, Brazil) for approximately 1 h at room temperature to ensure homogeneity. Afterwards, the temperature of the system was raised to 70°C, and a dilute aqueous calcium chloride solution was slowly added to the alginate solution at a flow rate of 1.0 ml/min delivered by a peristaltic pump (Masterflex C/L, model 77120–70, Vernon Hills, USA), until a total amount of 0.04 g $CaCl_2 \cdot 2H_2O$ per gram of alginate was transferred (approximately 30 min). The increase in temperature, the low flow rate and the strong agitation were necessary to avoid local gelation, and as a consequence, film heterogeneities. KS (0.17, 0.33, 0.67, 1.0 and 1.33 g per gram of biopolymer) or NA (0, 0.005, 0.01, 0.02, 0.04 and 0.08 g per gram of biopolymer) were then added to the polymeric solution and the system was further stirred for another 10 min.

For the preparation of composite films, the film forming solutions of the two biopolymers (1.5% w/w) containing glycerol as plasticizer (0.6 g per gram of biopolymer) were prepared separately. Alginate solution was prepared as described above for pure AFs. Chitosan was dissolved in an acetic acid aqueous solution (1%) and was mechanically stirred at 1,000 rpm (Fisatom, model 713, São Paulo, Brazil) for approximately 1 h at room temperature. The solution was then vacuum filtered to remove impurities and was kept still until use. The alginate solution was stirred at 14,000 rpm using an ultra-homogenizer (Ultra Turrax T18 basic, IKA, Staufen, Germany). Then the appropriate amount of chitosan solution was fed to the alginate solution at a flow rate of 1.0 ml/ min by a peristaltic pump (Masterflex C/L, model 77120–70, Vernon Hills, USA) through the bottom of the reactor. As for pure AFs, NA was then added to the composite polymeric solution and the system was further stirred for another 10 min. This solution was then transferred to an ultrasound bath (Thornton, model T14, Vinhedo, Brazil) for 10 min to remove air bubbles.

Aliquots (50 g) of the partially cross-linked film forming solution (pure alginate and mixture of alginate and chitosan) were poured into polystyrene Petri dishes ($d = 14$ cm) and dried in a convection oven (Nova Ética, model 420D, Vargem Grande Paulista, Brazil) at 40°C for 20 h. After detaching the resulting film from the support, the cross-linking was complemented in a second stage, by total immersion of the films in 50 ml of an aqueous calcium chloride solution (5% w/v) containing glycerol (5% v/v) for 30 min. The film was then immersed in 50 ml of distilled water containing glycerol (5% v/v) for 2 min. The excess surface liquid was removed, and the films were placed over inverted Petri dishes and dried in a ventilated ambient for approximately 6 h, at room temperature and RH > 60% with the film borders fixed by Teflon® rings to avoid wrinkling of the edges. All films were conditioned at room temperature and 52% RH inside desiccators for 3 days before use and characterisation.

Antimicrobial activity (Agar disc diffusion test)

Three microorganism species from ITAL collection (Food Technology Institute, Campinas, São Paulo State, Brazil) were chosen for antimicrobial activity evaluation of the films: *Penicillium roqueforti*, *Penicillium commune* and *Debaromyces hansenii*. All these microorganisms comprise strains with psychrotrophic characteristics which represent examples of cold storage-resistant species most frequently isolated from naturally contaminated cheese rind samples.[29,30] Cultures were grown at 25°C for 5 days on standard 90-mm Petri dishes containing approximately 20 ml of malt extract agar freshly prepared according to the formulation and directions in Pitt and Hocking.[31] Spores were collected and transferred to a flask containing 50 ml of sterile 0.1 % peptone water, 0.01 % Tween 80® and glass beads. The suspension was stirred and then filtered over sterile gauze. The stock solution of each microorganism was submitted to decimal dilutions in sterile peptone water 0.1% to obtain a culture of approximately 10^4–10^3 CFU/ml.

An agar disc diffusion test was performed to evaluate the antimicrobial activity of the films. Circular film samples ($d = 2.5$ cm) were exposed to UV light (110 V, 254 nm) for 30 min each side before the test. After that, film discs were aseptically transferred to the center of standard 90-mm Petri dishes containing approximately 20 ml of malt extract agar previously inoculated with 0.1 ml of spore suspension. After incubation at 25°C for 5 days, inhibition zones were measured with a micrometer considering the distance from the film center to the nearest CFU.[32] Films without antimicrobial addition and Petri dishes without film samples were used as controls. Each treatment was performed in triplicate and the average diameter of the inhibition zone was reported.

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Film thickness, δ

The film thickness was controlled by pouring a constant mass (50 g) of the film forming solution over the support. The thickness of the conditioned films was measured using a digital micrometer (Mitutoyo, model MDC-25S, Kawasaki, Japan). Measurements were taken at ten different positions of the film surface and the mean value was reported.

Solubility in water, SW

The solubility in water of the films was measured as proposed by Irissin-Mangata *et al.*[33]. The moisture weight fraction, ω, of the film was gravimetrically determined in a vacuum oven (Lab-Line, Squaroid, USA) at 105°C for 24 h. Disks cut from the same film were weighed (total mass m_o) and immersed in 50 ml of distilled water using a 250-ml beaker maintained under mild agitation (175 rpm) at 25°C for 24 h (Shaker Bath Orbit, Lab-Line, USA). The final dry matter (m_f) of the sample was determined in the same vacuum oven (105°C/24 h). The fractional solubilised matter (*SW*) was calculated as a function of the initial dry matter using Equation 1.

$$SW = \frac{m_o(1-\omega) - m_f}{m_o(1-\omega)} \tag{1}$$

Water vapor permeability, WVP

WVP coefficients were determined according to method E96–95.[34] Film samples were sealed over a circular opening of a Plexiglas® permeation cell containing anhydrous calcium chloride. These cells were individually kept in hermetically closed chambers (≈500ml) containing a saturated solution of sodium chloride at 25°C± 0.5°C to maintain an RH difference of 75%. After the system attained steady-state conditions (≈2h), the cell weight was measured every 12 h for 3 days using an analytical balance (Ohaus, model Analytical Plus, AP210, Switzerland). The WVP was calculated by Equation 2.

$$\mathrm{WVP} = \frac{G\delta}{A \times \Delta\mathrm{RH} \times P_w} \tag{2}$$

where δ is the film average thickness (mm), G is the permeation rate (g/day) calculated by linear regression of weight gain versus time, A is the permeation area ($1.521 \times 10^{-3}\mathrm{m}^2$), ΔRH is the difference in relative humidity (0.75) and P_w is the partial water vapor pressure at test temperature (25°C) (3.167 kPa).

Statistical analysis

Analysis of variance and Tukey test were used to determine statistically significant differences ($P < 0.05$) among averages, using the Software Statistica V.1.1.5.

RESULTS AND DISCUSSION

Incorporation of antimicrobial agents and film characteristics

AFs produced by the two-stage cross-linking method were homogeneous, transparent, flexible and visually attractive. Alginate and chitosan composite films were also homogenous and flexible, but a yellowish and hazier appearance was observed when the chitosan content in the film was increased. Composite film surfaces were slightly striated, typical of PECs.[12,13,35] All films showed low solubility in water after the final cross-linking procedure (second stage), with values varying from 0.14 to 0.27 g H_2O per gram of dry mass for AF and CF2, respectively. The addition of the antimicrobials did not significantly affect solubility in water of films.

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The incorporation of KS at different concentrations (0.17, 0.33, 0.67, 1.00 and 1.33 g per gram of alginate) was initially tested in the pure AFs. Films containing 0.17 g KS per gram of alginate kept the same visual appearance of KS free AFs. However, above this concentration, films became opaque, brittle and showed a white powder precipitate over the film surface, making them not suitable for use. The most commonly used antimycotics by the food industry (KS and sodium benzoate) tend to form insoluble salts with calcium. As the film formation procedure involves contact with calcium chloride solutions in the first and second reticulation stages, the calcium salts tend to accumulate over the film surface after film drying. Hence, the practical critical limit of KS added to the films was established at 0.17 g KS per gram of biopolymer. The same white precipitate was reported by Zactiti and Kieckbusch [36] in AFs when the concentration of sorbate exceeded 0.13 g per gram of alginate. Turbiani *et al*.,[37] studying the reticulation of AFs with calcium benzoate, observed that the maximum concentration of the additive that could be added to the film forming solution, without calcium precipitation on the film surface, was 0.22 g per gram of alginate.

NA has been considered effective against several species of fungi at relatively low concentrations.[18,20,24] Hence, to avoid the drawbacks observed for AFs with the addition of KS, the use of this natural antimicrobial was investigated. The addition of NA in pure AFs and alginate and chitosan composite films up to a concentration of 0.08 g per gram of biopolymer did not greatly affect the overall appearance of both types of films, but all films lost their transparency with the increase in NA concentration. Türe *et al*.[38] did not observe any alteration in the visual appearance of wheat gluten and methyl cellulose films with NA addition up to a concentration of 0.013 and 0.067 g NA per gram of biopolymer, respectively.

The thickness and WVP coefficients of alginate and composite films with and without the addition of NA are shown in Tables 1 and 2, respectively. Composite films tended to be thicker than pure AFs, probably due to the formation of a less tightly packed molecular structure when chitosan was added to

Table 1. Thickness of alginate and composite films with different NA concentrations.

NA concentration (g/100 g biopolymer)	Thickness, δ (μm)		
	AF	CF1	CF2
0	47 (2.9)^{a,A}	54 (3.7)^{a,B}	62 (3.1)^{a,C}
0.5	47 (1.8)^{a,A}	56 (3.1)^{a,b,B}	65 (4.9)^{a,C}
1	59 (4.7)^{b,c,A}	56 (1.9)^{a,b,A}	66 (2.7)^{a,B}
2	55 (5.9)^{a,b,A}	59 (3.2)^{b,c,A}	62 (2.1)^{a,B}
4	58 (2.8)^{b,c,A}	60 (2.7)^{c,d,A}	65 (2.6)^{a,B}
8	65 (7.6)^{c,A}	63 (1.8)^{d,A}	68 (3.8)^{b,A}

Data are presented as the mean (SD) of 10 experimental determinations.
AF, alginate film; CF1, alginate and chitosan composite film 82.5:17.5; CF2, alginate and chitosan composite film 65:35.
Averages with the same lower case letter in the same column indicate no significant difference ($P < 0.05$).
Averages with the same capital letter in the same row indicate no significant difference ($P < 0.05$).

Table 2. WVP coefficients of alginate and composite films with different NA concentrations.

NA concentration (g/100 g biopolymer)	WVP (g.mm/m^2.day.kPa)		
	AF	CF1	CF2
0	4.06 (0.26)^{a,A}	4.91 (0.09)^{a,B}	7.41 (0.15)^{a,C}
0.5	3.95 (0.03)^{a,A}	4.87 (0.03)^{a,B}	7.81 (0.57)^{a,C}
1	4.05 (0.33)^{a,A}	5.04 (0.15)^{a,B}	7.79 (0.23)^{a,C}
2	4.01 (0.18)^{a,A}	5.00 (0.18)^{a,B}	7.61 (0.07)^{a,C}
4	4.13 (0.05)^{a,A}	6.00 (0.42)^{b,B}	7.65 (0.15)^{a,C}
8	5.63 (0.47)^{b,A}	6.39 (0.24)^{b,A}	7.72 (0.15)^{a,B}

Data are presented as mean (SD) of three experimental determinations.
AF, alginate film; CF1, alginate and chitosan composite film 82.5:17.5; CF2, alginate and chitosan composite film 65:35.
Averages with the same lower case letter in the same column indicate no significant difference ($P < 0.05$).
Averages with the same capital letter in the same row indicate no significant difference ($P < 0.05$).

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the film matrix. NA addition also slightly increased film thickness. Santiago-Silva *et al.*[39] obtained cellulose films two to three times thicker, when 25% and 50% of pediocin, in relation to biopolymer mass, were added. In another study, also dealing with cellulose films, Pires *et al.*[18] reported significant increases in film thickness with NA addition (8%, in relation to biopolymer mass).

A significant increase in WVP was only observed with the addition of 4% and 8% of NA, in relation to biopolymer mass, for CF1 and for pure AFs, respectively (Table 2). WVP coefficients of CF2 were not affected by the antimicrobial agent in the concentration range tested. The increase in this attribute, with the addition of higher levels of NA, may be associated with a looser packing of the film macromolecules increasing the free volume of the polymeric structure.[28] Recently, da Silva *et al.*[40] observed, using scanning electron microscopy, the presence of NA crystals at concentrations higher than 4%. The recrystallisation of NA in the alginate and alginate/chitosan film matrix probably affected the WVP of these films. The increase in chitosan content in the film formulations greatly increased the WVP. As a combined dissolution and diffusion controlled process, WVP is governed not only by the concentration and the chemical structure of the components but also by chain mobility, which is dependent on formation kinetics, intermolecular forces, degree of cross-linking and crystallinity.

Antimicrobial effect of active films

Films containing KS. The antimicrobial activities of pure AFs containing KS (0.17 g per gram of alginate) were tested against *D. hansenii*, *P. commune* and *P. roqueforti*, which are prevalent spoilage organisms in dairy products. No inhibition zones were detected for the three microorganisms tested as can be visually observed in Figure 1. Microbial growth was observed even under and over the film

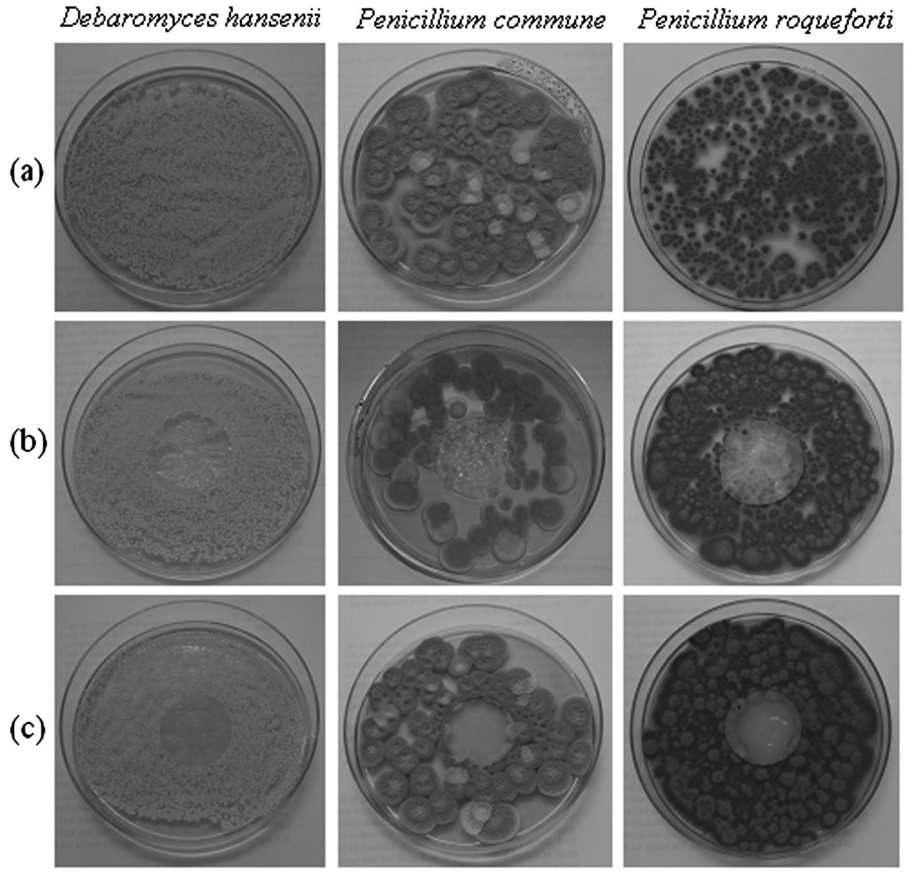

Figure 1. Antimicrobial effect of AF with KS (0.17 g per gram of alginate) against *Debaromyces hansenii*, *Penicillium commune* and *Penicillium roqueforti* (after 5 days of incubation at 25°C): (a) controls (no film); (b) films not submitted to final cross-linking stage; (c) films submitted to final cross-linking stage.

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surface. To validate the hypothesis that the high level of cross-linking with calcium obtained in the second reticulation stage could be hindering the release of the active agent to the agar medium, AFs not submitted to this final reticulation stage (second stage) were also tested. These films had expressive swelling and an increase in diameter of the film after contact with the agar but no inhibitory effect was observed (Figure 1b).

Previous studies have shown inhibitory activity of antimicrobial films containing KS against microorganisms with an agar diffusion method.[41–43] Sayanjali *et al*.[43] observed that the minimal inhibitory concentration of KS added to carboxymethyl cellulose films that showed inhibition against *Aspergillus* species was 1 g per gram of biopolymer. In a recent study, Türe *et al*.[44] reported an inhibitory effect of wheat gluten films containing KS against *Aspergillus niger* and *Fusarium incarnatum*. However, in their studies, the minimum effective concentration of KS was approximately 10 times higher than the concentration used in this study.

The selection of the antimicrobial is generally limited by the incompatibility of the active substance with the biopolymer or other component of the film, or its degradation during film fabrication.[16] The pH influences ionisation (association/dissociation) and therefore can alter antimicrobial activity of organic acids and their salts.[16,45] Sorbic acid has a *p*Ka of 4.8 and shows greater activity in pH lower than 6.0, being practically ineffective in pH higher than 6.0. The pH of the AF forming solution was approximately 6.5, which could have contributed to the noninhibitory effect of KS added AFs. Besides, previous published results on the release pattern of KS from a different film matrix indicate a quick desorption of the KS from the film, which could inhibit the microbial growth at the early stage of storage. However, this release could also induce a great concentration gradient between the agar surface and the bulk, increasing its diffusion rate into the agar and consequently the concentration at the surface could have become insufficient to ensure microbial growth inhibition.[46]

Films containing NA. Antimicrobial activities of alginate and alginate/chitosan composite (FC1 and FC2) films containing NA were also tested against *D. hansenii*, *P. commune* and *P. roqueforti*. Examples of inhibitory zones formed can be visually observed in Figures 2, 3 and 4. NA-added films were very effective in inhibiting the growth of all three species of fungi tested. It can be noticed that for the three types of film (AF, CF1 and CF2) and for all microorganisms tested, the diameter of the inhibition zone increased with the increase in NA concentration. The clear zones formed were concentric to the disc films, suggesting a uniform diffusion of the active substance through the agar media. As for the control Petri dishes (plates containing film without NA addition), fungal growth under and over the film sample was observed. All films kept their integrity and original size (no radial swelling) during the entire experiment.

The effect of NA concentration added to alginate and composite films on growth inhibition of *D. hansenii*, *P. commune* and *P. roqueforti* are shown in Tables 3–5, respectively. Inhibitory zone diameters varied from 2.64 cm, for CF2 containing 0.01 g NA per gram of biopolymer against *D. hansenii*, and 5.80 cm for AF containing 0.08 g NA per gram of biopolymer against *P. roqueforti. P. commune* and *P. roqueforti* were more susceptible to the antimicrobial action, showing larger inhibitory zone diameters than *D. hansenii* over the entire NA concentration range studied. Minimum inhibitory concentration (MIC) values were chosen as the boundary between no effect and minimum inhibitory effect against tested fungi. The MIC value of NA added to pure AFs was 0.005 g NA per gram of biopolymer against the three species of fungi, whereas for alginate and chitosan composite films (CF1 and CF2), the MIC value was 0.01 g NA per gram of biopolymer. De Oliveira *et al*.,[24] tested NA concentrations from 0% to 4% (in relation to biopolymer dry mass) in cellulosic films against *P. roqueforti*. These authors reported an inhibitory effect in concentrations as low as 1% (for 95 μm thickness films) and 2% (for 33 μm thickness films).

As visually observed in Figure 2, the data in Tables 3–5 confirm that an increase in NA concentration tended to increase the inhibition zone diameter for all films. Doubling the concentration of NA from 0.04 to 0.08 g per gram of biopolymer, however, did not lead to an expressive increase in the inhibition zones, meaning that adding more than 4% of NA would be pointless. The same trend was observed by Türe *et al*.,[20] evaluating the antimicrobial efficiency of NA against *Aspergillus niger* and *P. roqueforti*. Their studies revealed that inhibition zone diameters leveled out around NA concentrations of 0.007 and 0.033 g per gram of biopolymer for wheat gluten and methyl cellulose films,

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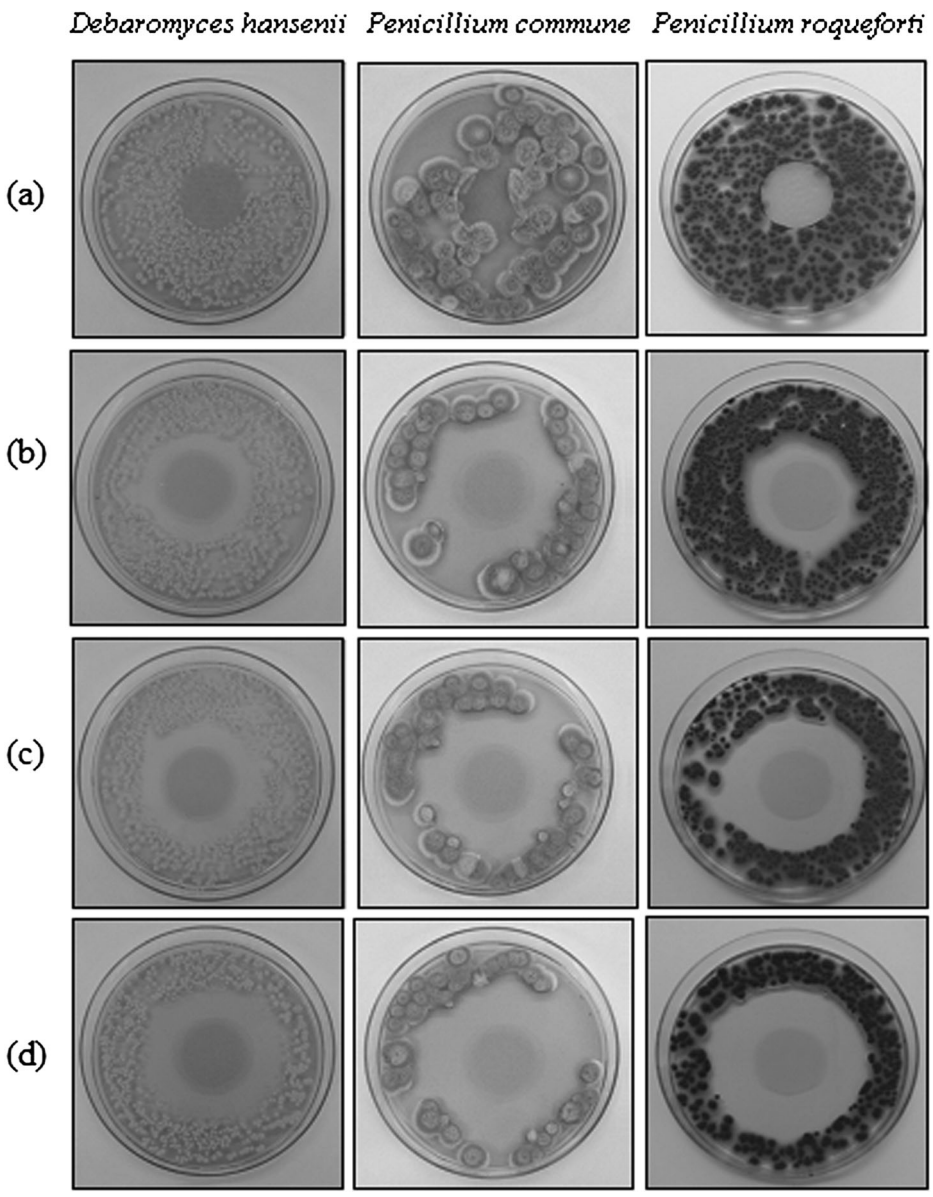

Figure 2. Antimicrobial effect of active alginate (A) and composite films 1 (B) and 2 (C) with different NA concentrations against *Debaromyces hansenii*, *Penicillium commune* and *Penicillium roqueforti* (after 5 days of incubation at 25°C): (a) 0, (b) 0.005, (c) 0.01, (d) 0.04 g NA per gram of biopolymer.

respectively. In the same study, the authors demonstrated that NA was more effective against *P. roqueforti* than *Aspergillus niger*.

A comparison between the performance of pure AFs and the composite films indicates that films containing chitosan (CF1 and CF2) showed smaller inhibition zone diameters. In general, for all species of fungi tested, inhibition zone diameters decreased by increasing the chitosan concentration in the film formulations. These results can indicate a possible electrostatic interaction between NA molecules and chitosan polymeric chains, which could be hindering the release of the active substance to agar. In fact, diffusion experiments in water indicated slower release kinetics, and therefore lower diffusion coefficients of NA added to alginate and chitosan composite films compared with pure AFs.[47] On the other hand, Stark and Tan [26] observed that the chemical stability of NA is favored in neutral pH and that possible degradation could take place at low pH values. The pH of the film forming solution of pure AF was close to 7, whereas the pH for composite films was approximately 5.5, which

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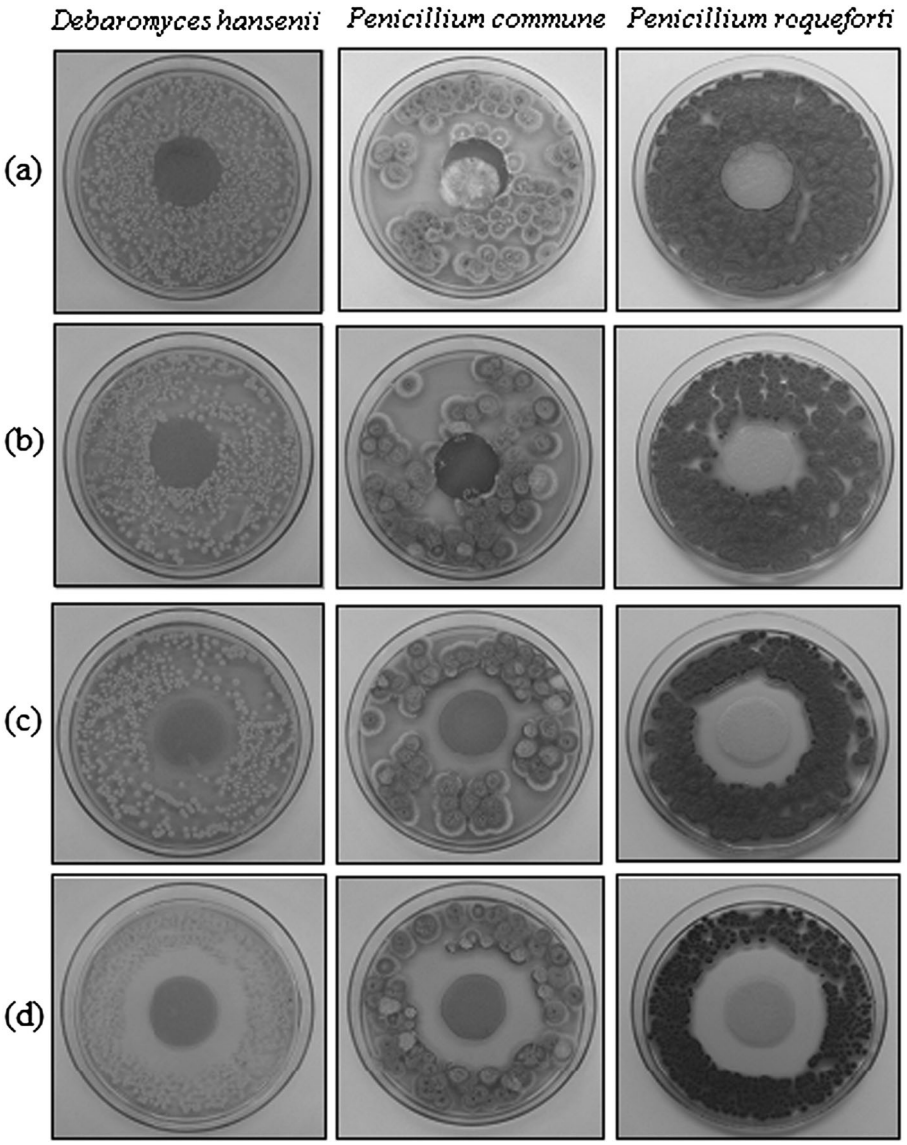

Figure 3. Antimicrobial effect of composite films 1 (CF1) with different NA concentrations against *Debaromyces hansenii*, *Penicillium commune* and *Penicillium roqueforti* (after 5 days of incubation at 25°C): (a) 0, (b) 0.005, (c) 0.01, (d) 0.04 g NA per gram of biopolymer.

could suggest that the lower inhibition effect is due to NA chemical degradation. Several studies in the literature point out that the antimicrobial effect of active films and coatings is related to antimicrobial substance action mode and spectra but also to the interaction between the film matrix and the active substance.[1,27,48–50] Chen *et al*.,[51] investigated the effect of adding 2% KS and sodium benzoate to methyl cellulose and to chitosan films against *Rhodotorula rubra* and *Penicillium notatum*. They observed that only the methyl cellulose films showed an inhibitory effect and accredited the different efficiencies to chemical interactions between chitosan and antimicrobials.

Many researchers have pointed out an inherent antimicrobial effect of chitosan molecules.[2,52,53] However, no additional inhibitory effect of chitosan (present in CF1 and CF2 formulations) was observed for the three microorganisms tested. To verify if chitosan alone would show an inhibitory effect against the mentioned species of fungi, a pure chitosan film was prepared and submitted to the diffusion agar disc assay. No inhibition zone was developed, and microbial growth under and over the film surface was observed (data not shown). The same lack of inhibitory action of chitosan films

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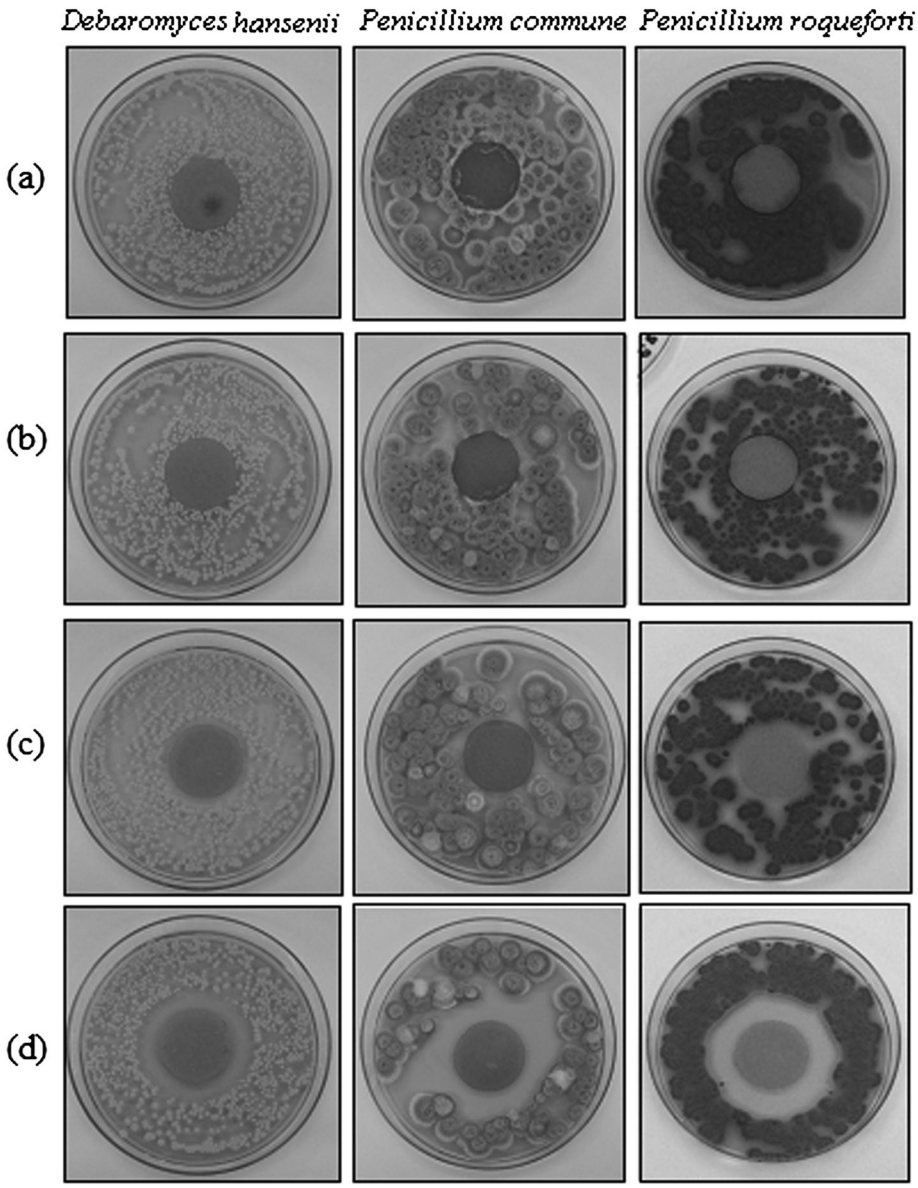

Figure 4. Antimicrobial effect of composite films 2 (CF2) with different NA concentrations against *Debaromyces hansenii*, *Penicillium commune* and *Penicillium roqueforti* (after 5 days of incubation at 25°C): (a) 0, (b) 0.005, (c) 0.01, (d) 0.04 g NA per gram of biopolymer.

against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium, Listeria monocytogenes* and *Bacillus cerues* was found by Pranoto *et al*.[19]. These authors hypothesised that the antimicrobial effect of chitosan depends on the direct contact of microorganisms on the active sites of chitosan molecules. Coma *et al*.,[52] on the other hand, failed to find an inhibitory zone against *L. monocytogenes* by testing chitosan films on culture media but obtained complete inhibition of this microorganism by adding the biopolymer directly to the culture media, at a concentration of 8% (v/v). The same tendency was found by Foster and Butt,[54] who observed no inhibitory effects of chitosan films against *E. coli*, *S. aureus* or *S. epidermidis* species but almost complete inhibition when solutions used to prepare films were tested on bacterial lawns and in liquid cultures. These authors also pointed out that increased acidity of chitosan solutions (pH 5) was responsible for the bactericidal effects of this biopolymer.

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Table 3. Inhibition zone diameters of *Debaromyces hansenii* (after 5 days of incubation at 25°C) exposed to alginate and composite film discs ($d = 2.5$ cm) containing NA in agar diffusion test.

NA concentration (g/100 g biopolymer)	Inhibition zone diameter (cm)		
	AF	CF1	CF2
0	+	+	+
0.5	3.92 (0.01)[a]	+	+
1	4.12 (0.01)[b]	3.44 (0.08)[a]	2.64 (0.02)[a]
2	4.30 (0.00)[c]	4.14 (0.12)[b]	3.44 (0.06)[b]
4	4.76 (0.01)[d]	4.52 (0.01)[b]	3.70 (0.04)[b]
8	5.14 (0.01)[e]	4.48 (0.01)[b]	4.32 (0.01)[c]

Data are presented as mean (SD) of three experimental determinations.
Averages with the same letter in the same column indicate no significant difference ($P < 0.05$).
AF, alginate film; CF1, alginate and chitosan composite film 82.5:17.5; CF2, alginate and chitosan composite film 65:35; +, no inhibition.

Table 4. Inhibition zone diameters of *Penicillium commune* (after 5 days of incubation at 25°C) exposed to alginate and composite film discs ($d = 2.5$ cm) containing NA in agar diffusion test.

NA concentration (g/100 g biopolymer)	Inhibition zone diameter (cm)		
	AF	CF1	CF2
0	+	+	+
0.5	4.40 (0.07)[b]	+	+
1	4.74 (0.02)[b]	3.82 (0.08)[b]	3.00 (0.06)[b]
2	4.92 (0.01)[bc]	4.46 (0.04)[c]	3.90 (0.01)[c]
4	5.52 (0.01)[d]	4.48 (0.06)[c]	4.10 (0.08)[cd]
8	5.60 (0.01)[d]	4.90 (0.03)[d]	4.60 (0.06)[e]

Data are presented as mean (SD) of three experimental determinations.
Averages with the same letter in the same column indicate no significant difference ($P < 0.05$).
AF, alginate film; CF1, alginate and chitosan composite film 82.5:17.5; CF2, alginate and chitosan composite film 65:35; +, no inhibition.

Table 5. Inhibition zone diameters of *Penicillium roqueforti* (after 5 days of incubation at 25°C) exposed to alginate and composite film discs ($d = 2.5$ cm) containing NA in agar diffusion test.

NA concentration (g/100 g biopolymer)	Inhibition zone diameter (cm)		
	AF	CF1	CF2
0	+	+	+
0.5	3.20 (0.07)[a]	+	+
1	4.60 (0.07)[b]	3.76 (0.01)[a]	2.90 (0.11)[a]
2	4.92 (0.01)[b]	4.06 (0.05)[a]	4.00 (0.08)[b]
4	5.48 (0.01)[c]	4.48 (0.04)[b]	4.50 (0.06)[b]
8	5.80 (0.07)[c]	5.28 (0.01)[c]	4.56 (0.15)[b]

Data are presented as mean (SD) of three experimental determinations.
Averages with the same letter in the same column indicate no significant difference ($P < 0.05$).
AF, alginate film; CF1, alginate and chitosan composite film 82.5:17.5; CF2, alginate and chitosan composite film 65:35; +, no inhibition.

CONCLUSIONS

This study showed that the practical limit of KS addition into AF cross-linked with calcium is 0.17 g KS per gram of alginate. At this concentration, active films were not able to inhibit the growth of *D. hansenii*, *P. commune* and *P. roqueforti* by the agar diffusion test. However, above this concentration, films became opaque and brittle with a white powder precipitated over the film surface, making them unsuitable for use. On the other hand, alginate and alginate/chitosan composite films containing NA

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were able to inhibit the growth of the three microorganisms tested. NA proved to be a very efficient antimycotic agent when incorporated in alginate and alginate/chitosan composite films. It was effective at concentrations as low as 0.005 g per gram of biopolymer for AF and 0.01 g per gram of biopolymer for both composite films (CF1 and CF2) against all microorganisms tested. The inhibitory zone diameter increased as concentration of NA increased. In general, the visual appearance of all films was not markedly affected by NA addition up to a concentration of 0.08 g NA per gram of biopolymer, but films were slightly thicker and less transparent by increasing the antimicrobial concentration. Except for CF2, the WPV of the films tended to increase at higher concentrations of NA. Compared with pure AFs, the addition of chitosan in composite film formulations produced more flexible films; however, films showed an increase in solubility in water and WVP and became yellowish and hazier. Active films containing 0.04 g NA per gram of biopolymer obtained in the present study showed excellent perspectives for acting as antimicrobial films intended for dairy food protection applications. However, further tests are needed to confirm the antimicrobial efficacy of these films on dairy food systems.

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