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Antimicrobial Films Based on Chitosan and Methylcellulose Containing Natamycin for Active Packaging Applications

Serena Santonicola ^{1,2} , Verónica García Ibarra ¹, Raquel Sendón ¹ , Raffaolina Mercogliano ² and Ana Rodríguez-Bernaldo de Quirós ^{1,*}

¹ Department of Analytical Chemistry, Nutrition, and Food Science, Faculty of Pharmacy, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain; serena.santonicola@unina.it (S.S.); veronica.garcia.ibarra@usc.es (V.G.I.); raquel.sendon@usc.es (R.S.)

² Department of Veterinary Medicine and Animal Production, University of Naples, 80137 Naples, Italy; raffaella.mercogliano@unina.it

* Correspondence: ana.rodriguez.bernaldo@usc.es; Tel.: +34-881-814-965

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Abstract: Biodegradable polymers are gaining interest as antimicrobial carriers in active packaging. In the present study, two active films based on chitosan (1.5% *w/v*) and methylcellulose (3% *w/v*) enriched with natamycin were prepared by casting. The antimicrobial's release behavior was evaluated by immersion of the films in 95% ethanol (*v/v*) at different temperatures. The natamycin content in the food simulant was determined by reversed-high performance liquid chromatography with diode-array detection (HPLC-DAD). The apparent diffusion (D_p) and partition ($K_{p/s}$) coefficients were calculated using a mathematical model based on Fick's Second Law. Results showed that the release of natamycin from chitosan based film ($D_p = 3.61 \times 10^{-13} \text{ cm}^2/\text{s}$) was slower, when compared with methylcellulose film ($D_p = 3.20 \times 10^{-8} \text{ cm}^2/\text{s}$) at the same temperature ($p < 0.05$). To evaluate the antimicrobial efficiency of active films, cheese samples were completely covered with the films, stored at 20 °C for 7 days, and then analyzed for moulds and yeasts. Microbiological analyses showed a significant reduction in yeasts and moulds (7.91 log CFU/g) in samples treated with chitosan active films ($p < 0.05$). The good compatibility of natamycin with chitosan, the low D_p , and antimicrobial properties suggested that the film could be favorably used in antimicrobial packagings.

Keywords: active packaging; chitosan; methylcellulose; natamycin

1. Introduction

Antimicrobial food packaging is one of the most promising applications of active packaging, and acts to reduce, inhibit or retard microorganism growth that could contaminate the packaged food [1–3].

During recent decades, due to the environmental and economic implications of the use of materials made from petrochemical derivatives, various research groups have been looking toward green polymers as alternatives to film and coating manufacturing for plastic packages [4–9]. Bio-based packaging materials can be created on the basis of polymers directly extracted/removed from natural materials [10]. Elaboration of these films and coatings has been possible thanks to the filmogenic capacity of natural biopolymers, which have a good aptitude for forming a continuous and cohesive matrix with adequate mechanical properties [10,11]. Chitosan is a natural weak cationic polysaccharide, derived from deacetylation of chitin, which is the major component of the shells of crustaceans such as crab, shrimp, and crawfish [2,12]. It is non-toxic, biodegradable, biofunctional, and biocompatible [13,14]. Chitosan-based films have good mechanical properties, and has some

advantages over other biomolecule-based polymers used as packaging materials due to its antibacterial behavior [13–16]. Methylcellulose application as a film and coating component is also very attractive. It is one of the most important commercial cellulose ethers, and it has been used in many industrial applications [17]. Methylcellulose films have a flexible and transparent character. They also possess low oxygen and moisture vapor transmission rates when compared to other hydrophilic edible films [18].

Biodegradable packaging materials can act as supporters of antimicrobials [19]. Much research has been devoted to the design of antimicrobial packaging containing natural antimicrobial agents for specific or broad microbial inhibition [20]. Bacteriocins are an attractive option, as they constitute natural preservatives, avoiding the addition of synthetic compounds to food [3,21]. These antimicrobial proteins/peptides, produced by bacteria, are nontoxic and nonantigenic to humans, and have GRAS (generally recognized as safe) status [3,22]. Natamycin, produced during fermentation by the bacterium *Streptomyces natalensis*, is a naturally occurring antifungal agent classified as a macrolide polyene, which acts through the specific interaction with ergosterol of yeast membranes [23,24]. According to Directive 95/2/EC, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry cured sausage at a maximum level of 1 mg/dm² in the outer 5 mm of the surface [25,26]. Natamycin has no adverse effect on the rind or the flavor of the cheese. The depth of penetration of this antimicrobial compound depends on of the initial concentration, cheese type and storage time [27]. Incorporation of antimicrobials in food interfaces by the use of films helps to decrease the rate of diffusion from the surface to the bulk of the product assuring the maintenance of high concentrations of the active agent where it is required [28]. In addition, due to the low water solubility of natamycin, the incorporation into a coating improves distribution in the cheese and, therefore, the surface protection from mould growth [4]. This antimicrobial agent has been successfully used in different active systems [29–31]. Chitosan coating containing natamycin decreased mold/ yeast population on Saloio cheese after 27 days of storage [4]. Moreover, the application of chitosan films determined an increase of the shelf life of different cheese types, such as Mozzarella [32], Emmental [33], Regional Saloio [34], and Apulia spreadable cheese [35]. Furthermore, natamycin-impregnated cellulose-based films showed inhibitory effects against *Penicillium roquefortii* on the surface of Gorgonzola cheese [36] and, in combination with nisin, prolonged the shelf life of sliced mozzarella cheese by 6 days compared to the control [37]. Methylcellulose and wheat gluten films containing natamycin showed ability in the prevention and control of toxigenic moulds on dairy products [18].

For the selection of an antimicrobial, the possible interactions among the antimicrobial, the film-forming biopolymer, and other food components, which can modify the antimicrobial activity and the characteristics of the film, must be considered [15]. Diffusion (D) and partition (K) coefficients for antimicrobials in packaging films can help to design efficient active packaging and to predict the shelf-life of food products [19,38]. Therefore, the aim of this study was to develop two active films, based on chitosan and methylcellulose, incorporating natamycin as antimicrobial agent, and to study the release behavior of antimicrobial agents from the active films. The antimicrobial effectiveness of chitosan and methylcellulose films containing natamycin to prevent yeast and mould growth on cheese surface was also investigated.

2. Materials and Methods

2.1. Materials

The materials used to prepare the active films were: Chitosan with a degree of deacetylation of approximately 75–85% (medium molecular weight, 200–800 mPa·s viscosity, soluble in 1% Acetic acid aqueous solution) (Sigma chemicals, St-Louis, MO, USA); Glycerol (molecular biology grade, Calbiochem); Methylcellulose (3500–6000 mPa viscosity) (Sigma-Aldrich, Darmstadt, Germany). Natamycin was provided by Sigma (Steinheim, Germany). Ethanol (analytical grade), Methanol (HPLC grade), Acetonitrile (HPLC grade) were provided by Merck (Darmstadt, Germany). Acetic acid solution

(HPLC grade) was provided by Sigma-Aldrich (Germany). The water used to prepare all solutions was purified by a Milli-Q water purification system (Millipore) (Bedford, MA, USA).

2.2. Preparation of Films

Chitosan films (1.5% *w/v*) were prepared by dissolving chitosan in acetic acid aqueous solution 1% (*v/v*). Subsequently, the solution, containing glycerol as plasticizer (0.2 g/g biopolymer), was kept under stirring for 2 h at a constant temperature at 80 °C and then 12 h at room temperature until the chitosan was fully dissolved.

Methylcellulose (3% *w/v*) was mixed with a water-ethanol solution (50:50 *v/v*) and then homogenized for 5 min. After the addition of glycerol (0.4 g/g biopolymer), the solution was kept under stirring, and heated to 80 °C for 2 h. Natamycin was incorporated at room temperature into the film solutions to reach the final concentration of 0.01% (*w/v*). The solutions were then cast in 8.5 cm polyacrylic plates and dried at 30 °C for 12 h. A saturated solution of magnesium nitrate was put in the oven to achieve a relative humidity of 53%.

2.3. Film Thickness Measurement

The thickness of the samples was determined using a manual digital micrometer (0.001 mm, Mitutoyo, Mizonokuchi, Japan). Measurements were repeated in 5 different regions of each sample and then an average value was calculated.

2.4. Experimental Procedure for Kinetics of Natamycin Release

During migration tests, the films were fixed in glass tubes so that both sides of the tested films were in contact with food simulant. According to EU Commission Regulation No 10/2011 [39], ethanol 95% (*v/v*) was used as a substitute food simulant for fatty food. Chitosan and methylcellulose films were cut into pieces of 14.7 cm² area and immersed into 20 mL of ethanol 95% (*v/v*). The migration kinetics of the antimicrobial agent from methylcellulose film were studied at different temperatures: 10, 20 and 40 °C (± 0.2 °C). To compare the release behavior between the different active films, natamycin migration test from chitosan film was performed at 40 °C (± 0.2 °C).

To determine the amount of natamycin released, aliquots (500 μ L) of the food simulant were taken out from the tubes at preset times, filtered and injected by HPLC-DAD (high-performance liquid chromatographic with diode-array detection). The analytical conditions were obtained by modification of a previously reported method [40]. The preserving agent released from the film into the simulant was determined as follows: the calculation of the final migration level included the correction for changing simulant volume, as well as for the amount of natamycin taken during the previous sampling. Then, to calculate the remaining amount in the film, a piece of film was introduced in a glass tube containing 20 mL of ethanol 80% (*v/v*) solution at 40 °C for 24 h. The experiment was performed in duplicate.

HPLC-DAD Analysis

An HPLC HP1100 system (Hewlett Packard, Waldbronn, Germany) equipped with a quaternary pump, a degassing device, an autosampler, a column thermostating system, a diode-array detector (DAD), and Agilent Chem-Station for LC and LC/MS systems software (Agilent, Santa Clara, CA, USA), was used. Separation was performed on a Kromasil ODS (C18) (150 \times 3.20 mm² i.d., 5 mm particle size) column thermostatted at 25 °C. Acetonitrile (A) and Milli-Q water (B) were used as mobile phase. The injection volume was 20 μ L. Samples were eluted in gradient mode under the following conditions: 0 min (20% A–80% B); 10 min (60% A–40% B); 15 min (60% A–40% B); 20 min (20% A–80% B). The flow rate was 0.6 mL/min. Three selected wavelengths were set in DAD detector, 291, 304 and 319 nm, corresponding to the three absorption peaks of the characteristic natamycin spectrum [40]. The wavelength used to quantify the antifungal was 304 nm.

2.5. Diffusion Coefficient (D) and Partition Coefficient (K) Measurement

The diffusion coefficients of natamycin from the active films into the substitute food simulant ethanol 95% (v/v) were calculated using a mathematical model based on Fick's Second Law (1):

$$\frac{\partial C_p}{\partial t} = D \frac{\partial^2 C_p}{\partial x^2} \quad (1)$$

where C_p is the concentration of the migrant in the film at time t and position x .

An analytical solution of this differential equation, that describes the diffusion kinetics, was proposed by Crank [41]. After a slight modification, this can be expressed by the following Equations (2) and (3) [42]:

$$\frac{m_{F,t}}{A} = c_{P,0} \rho_P d_P \left(\frac{\alpha}{1 + \alpha} \right) \times \left[1 - \sum_{n=1}^{\infty} \frac{2\alpha(1 + \alpha)}{1 + \alpha + \alpha^2 q_n^2} \exp \left(-D_P t \frac{q_n^2}{d_P^2} \right) \right] \quad (2)$$

$$\alpha = \frac{1}{K_{P/F}} \frac{V_F}{V_P} \quad (3)$$

where $m_{F,t}$ is the mass of the migrant transferred from P into F after time t , (μg); A is the area of P in contact with F (cm^2); $C_{P,0}$ is the initial concentration of the migrant in P (mg/kg); ρ_P is the density of P (g/cm^3); t is the migration time (s); d_P is the thickness of P (cm); V_P is the volume of P (cm^3); V_F is the volume of F (cm^3); q_n is the positive root of the equation $\tan q_n = -\alpha \cdot q_n$; D_P is the diffusion coefficient for the migrant in the polymer (cm^2/s); $K_{P/F}$ is the partition coefficient for the migrant between P and F .

Partition coefficient between the film and food simulant ($K_{P/S}$) was calculated according to the following Equation (4):

$$K_{P/S} = \frac{C_P}{C_S} \quad (4)$$

where C_P is the concentration of a substance in the film at equilibrium, in $\mu\text{g}/\text{g}$; C_S is the concentration of a substance in the simulant at equilibrium, in $\mu\text{g}/\text{g}$.

Experimental data were fitted to the proposed model using Solver function of the commercial software Microsoft Excel 2007[®] (Redmond, WA, USA).

To measure the fit between the experimental and estimated data the root of mean-square error % (RMSE (%)) was calculated according to the following Equation (5):

$$\text{RMSE}(\%) = \frac{1}{M_{P,0}} \sqrt{\frac{1}{n} \sum_{i=1}^n ((m_{F,t})_{\text{exp},i} - (m_{F,t})_{\text{pred},i})^2} \times 100 \quad (5)$$

where n is the number of experimental points per migration/release curve; i is the number of observations; $M_{P,0}$ is the initial amount of the migrant in the polymer (μg).

2.6. Microbiological Analysis

A commercial semi-hard cheese was purchased from a local supermarket and stored in a refrigerator (4 °C) until use. Fifteen pieces of cheese were randomly assigned to five treatments: samples coated with Polyethylene (used as blank), samples coated with chitosan and with methylcellulose films, and finally samples coated with chitosan and with methylcellulose films containing natamycin.

Cheese samples (10 g) were completely covered with the films (28.26 cm^2), sealed in polyethylene bags, and finally stored at 20 °C for 7 days.

At the times of bacterial enumeration, cheese samples were aseptically removed from their packaging and the films were separated from cheese slices with sterile forceps. An aliquot (10 g) of

cheese was aseptically collected and placed in 400 mL homogenizing bag along with 90 mL of 0.1% (*w/v*) of peptone water and massaged for 60 s at high speed in a Stomacher (AES Chemunex, Coburg, FR, Germany). Decimal dilutions were prepared from the initial homogenate. After that, 0.1 mL were spread onto potato dextrose agar (PDA) plates and incubated at 25 °C for 3 days, before counting colonies. Three samples of each treatment were analyzed.

2.7. Statistical Analysis

Statistical analysis of data was performed with package SPSS 15.0 (SPSS Inc., Chicago, IL, USA). ANOVA test was applied to determine significant differences ($p < 0.05$) according to the release rate of antimicrobial agent from methylcellulose films at different temperatures, and from chitosan and methylcellulose film at the same temperature, according to mould/yeast counts.

3. Results and Discussion

3.1. Film Appearance Characterization

All of the films were transparent. The average thickness ranged between 38 and 51 μm for chitosan, and from 56 to 76 μm for methylcellulose films.

Natamycin was added to the film solutions at concentrations of 6.6 and 3.3 mg per gram of chitosan and methylcellulose, respectively. The increase in natamycin concentration (from 0.01% to 0.1% and 1%), affected the appearance of both types of films, making them opaque and unsuitable for use. This behavior has also been observed by other authors [24]. For the migration kinetics, it is essential to determine the initial concentration of the active compound in the developed films. The concentrations of natamycin in the chitosan and methylcellulose films prior to the migration assays were 0.6% (*w/w*) and 0.3% (*w/w*), respectively.

3.2. Natamycin Release Kinetics

Figures 1 and 2 illustrate the release profiles of natamycin from chitosan and methylcellulose films in the substitute food simulant (Ethanol 95% (*v/v*)) at different storage temperatures as a function of time.

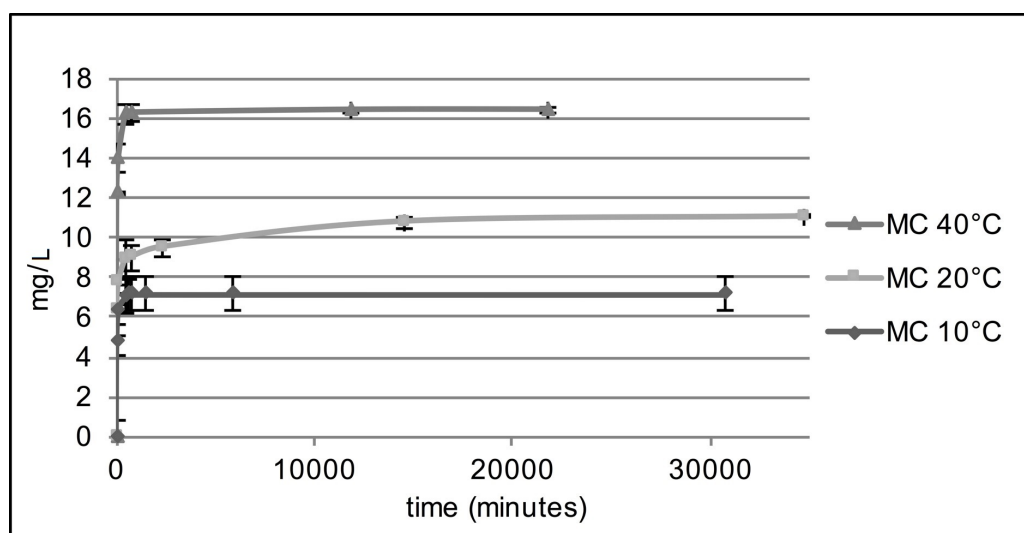


Figure 1. Release profiles of natamycin from methylcellulose films (MC) at different temperatures. Each dot represents the mean of the experimental data with an error bar of 2 replications.

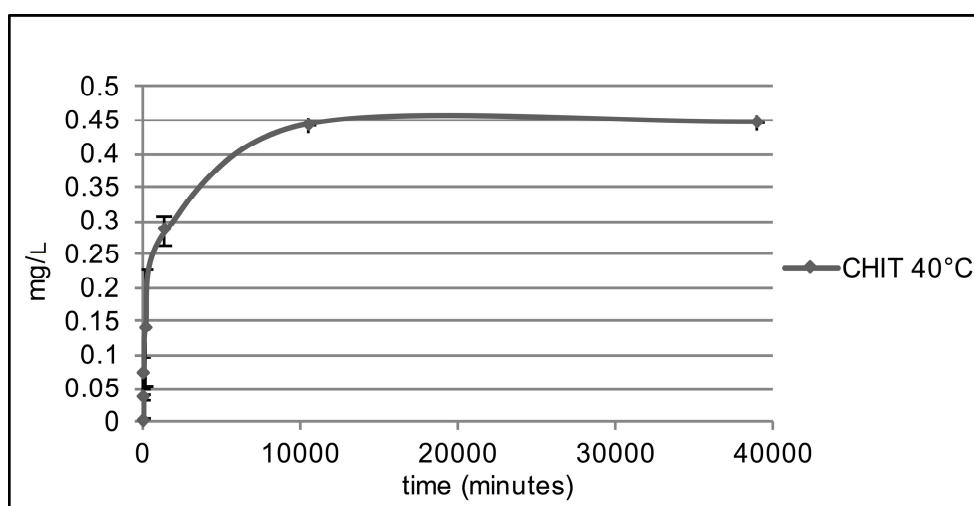


Figure 2. Release profiles of natamycin from chitosan film (CHIT) at 40 °C. Each dot represents the mean of the experimental data with an error bar of 2 replications.

With respect to the migration kinetics, results showed that the release of natamycin from the chitosan-based films was slower, when compared with the methylcellulose films at the same temperature ($p < 0.05$). In addition, after 12 h at 40 °C, methylcellulose film released about 90% of the natamycin, while less than 15% of the initial amount of natamycin in the chitosan film was released when equilibrium conditions were reached.

The diffusion and the transport mechanism of the active agents from the film matrix to the food surfaces are the most important factors in developing an antimicrobial food packaging. The diffusion coefficient indicates the rate at which the release of the active agent takes place. Diffusivities can be used to quantify the release behavior of the antimicrobials, and also to obtain information about polymeric networks [11]. The partition and apparent diffusion coefficients of natamycin from chitosan (at 40 °C) and methylcellulose films (at 10, 20, 40 °C) are included in Table 1. For methylcellulose films, in order to test the linearity between the D_p and the temperature, an Arrhenius-type equation was applied, and $R^2 = 0.9384$ was obtained.

Table 1. Partition ($K_{P/S}$) and apparent diffusion (D_p) coefficients (mean values) of natamycin from the active films in food simulant at different temperatures (T).

Films	T	D_p (cm ² /s)	$K_{P/S}$	RMSE (%)
chitosan + natamycin	40 °C	3.61×10^{-13}	1054.11	0.63
methylcellulose + natamycin	10 °C	7.30×10^{-10}	271.64	5.38
methylcellulose + natamycin	20 °C	1.14×10^{-9}	127.85	4.59
methylcellulose + natamycin	40 °C	3.20×10^{-8}	3.74	10.93

According to the literature [19], for each tested temperature, and for the same temperature (40 °C), the diffusion coefficients of natamycin from methylcellulose films were higher, when compared to chitosan films. As expected, the diffusion process of natamycin from methylcellulose films generally occurred faster at higher temperatures ($p < 0.05$). However, literature data showed that the temperature variations could result in a negligible change in the diffusion coefficient of antimicrobial compound from chitosan film [19].

The low release of natamycin from chitosan film could be related to different factors. Apparent diffusion coefficients for natamycin from alginate/chitosan film into water at an order of magnitude in the range of 10^{-11} – 10^{-12} have been reported [11]. These values are considered very low when compared with diffusivities of other antimicrobials incorporated in polymeric matrices,

suggesting a chemical interaction between natamycin and chitosan [11]. Chen et al. [43] attributed this fact to a possible electrostatic interaction between NH_3^+ groups of chitosan and COO^- groups of antimicrobials. Methylcellulose carries no electrical charge [44], and the possibility of interactions between the polymer and the antimicrobial is lower.

Diffusion coefficients at an order of magnitude of 10^{-10} and 10^{-12} cm^2/s have been reported for natamycin from chitosan films to phosphate-buffered saline solution and cheese, respectively [4]. The diffusion coefficient was higher for the phosphate-buffered saline solution because of the swelling effect in the release phenomenon [4]. Similar results were obtained by Hanušova et al. [45] with polyvinyl dichloride lacquer coatings also used as a natamycin carrier in cheese packaging. Water-ethanol solution 95% (*v/v*), used as substitute food simulant in our study, exhibits lower water activity, which subsequently results in lower degradation, swelling and solubilization phenomena in biopolymers, making the antimicrobial diffusion through the film matrix difficult [38]. Moreover, chitosan maintains its structure in a neutral environment, but is solubilized and degraded in an acidic medium [46]. As the water-ethanol solution used in the present study had a neutral pH, chitosan film demonstrated higher stability with a compact structure and, therefore, lower diffusion coefficient values [19]. A major advantage of slow release over direct addition of the antimicrobial into the food is continuous microbial inhibition obtained over an extended period [47]; in contrast, a rapid release may cause migration of the active agent to internal parts of the food, reducing the protection at the surface [1].

The partition coefficient ($K_{P/S}$) indicates the ratio between the concentration of the active compound in the film and the concentration in the food simulant at equilibrium [48,49]. The partition coefficients, shown in Table 1, correspond to the values predicted by the mathematical model used. Higher $K_{P/S}$ values are achieved with higher concentrations of the active agent in the film; in contrast, a lower $K_{P/S}$ indicates that more migrant is absorbed into food from the polymer. However, various parameters, such as temperature, pH, the chemical structure of the migrant, molecular size and structure, and the fat content of foods, can influence the partition coefficient [49].

In order to measure the fit between the experimental and estimated data, the root of mean-square error % (RMSE (%)) was calculated. Generally, acceptable values were obtained. In particular, the best fit between the experimental and estimated data was found for chitosan films [8].

3.3. Antimicrobial Activity of Films

The microbial assays were performed under accelerated conditions at 20 °C. The mean values of the counts obtained for moulds and yeasts were 7.91 log (CFU/g) and 8.25 log (CFU/g), respectively, for chitosan and methylcellulose films containing natamycin. Values slightly higher for mould/yeast counts were observed in cheese coated with chitosan (8.30 log CFU/g), methylcellulose (8.95 log CFU/g) and polyethylene (8.27 log CFU/g) films, when compared to the active films. It is important to note that results were obtained under no-inoculation conditions. Therefore, large differences in the counts were not to be expected for the whole cheese, and not only on the cheese surface [4].

Antimicrobial agents can be applied by dipping, spraying, or brushing to food surfaces for controlling microbial growth [18]. However, these techniques are laborious, and have limited benefits, because of a rapid loss of activity resulting from the interaction between the antimicrobial compound and food components, and from a dilution phenomenon occurring when the additive diffuses to the bulk of the food [24,29]. The use of packaging films containing antimicrobial agents could better control the migration of the agents away from the surface [50]. In earlier studies, antimycotic activity of natamycin-incorporating films has been shown against several moulds [30–32]. The low antimicrobial activity of methylcellulose films could be explained by the rapid release, which may cause migration of the active agent to internal parts of the food, reducing the protection at the surface [1]. On the contrary, the slow release of natamycin from chitosan film into the food surface determined a significant ($p < 0.05$) reduction of mould/yeast counts with respect to polyethylene films, which were used as

a blank. Chitosan has been tested as a carrier of other natural antimicrobials, e.g., lysozyme [51], lysozyme and EDTA [52], essential oils [13,38], and nisin [19]. In particular, several studies have reported that chitosan antimicrobial films containing natamycin possess the potential ability to inhibit microorganisms on food products [4,11,24]. The incorporation of natamycin in chitosan-based film could act as an additional post-processing safety measure, once the inhibitory effect on microbial growth of both components [4].

Antimicrobial films or coatings resulted in more effective microorganism inhibition when applied to nutrient media than when applied to real systems, owing to the complex structure of foods [18]. In fact, the minimum inhibitory concentration of natamycin against *A. niger* and *P. roquefortii* was found to be two times higher in cheese application with respect to in vitro studies [18].

4. Conclusions

Briefly, the kinetics results showed that, at the same temperature, the release of natamycin from chitosan-based film ($D_p = 3.61 \times 10^{-13} \text{ cm}^2/\text{s}$) was slower, when compared with methylcellulose film ($D_p = 3.20 \times 10^{-8} \text{ cm}^2/\text{s}$) ($p < 0.05$). Moreover, a significant reduction in yeast and mould was observed in cheese samples treated with chitosan films containing natamycin ($p < 0.05$). The controlled release of natamycin from chitosan film would address the limitation of rapid loss of natamycin applied directly to the cheese surface. However, further studies are needed to measure their antimicrobial activities on selected microorganisms and on other real food surfaces.

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Author Contributions: Ana Rodríguez Bernaldo de Quirós, Raquel Sendón and Raffaelina Mercogliano conceived and designed the experiments and analyzed the data; Serena Santonicola performed the experiments and wrote the paper; and Veronica García Ibarra performed the microbiological experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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