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# Development and Characterization of Sustainable Antimicrobial Films Incorporated with Natamycin and Cellulose Nanocrystals for Cheese Preservation

**Miriane Maria de Sousa [1], Vânia Miria C. Clemente [1], Rosilene Maria de S. Santos [1], Mariane Oliveira [1], José Osvaldo Ramos Silva [1], Laís Fernanda Batista [1], Clara Suprani Marques [1,2,*], Amanda Lélis de Souza [1], Éber Antônio Alves Medeiros [1] and Nilda de Fátima Ferreira Soares [1]**

[1] Food Technology Department, Federal University of Viçosa, Viçosa 36570-900, Brazil
[2] Department of Agrarian Sciences, Federal Institute of Minas Gerais, Campus Bambuí, Bambuí 38900-000, Brazil
* Correspondence: supraniclara@gmail.com

**Abstract:** Environmental pollution and food safety are both issues of global concern. In this sense, sustainable and antimicrobial nanocomposites based on cellulose/poly (vinyl alcohol) blend incorporated with natamycin and cellulose nanocrystals (CNC) were manufactured and characterized. The developed films were evaluated according to their mechanical and optical properties, and their barrier to oxygen and water vapor permeation. The antimycotic activity was evaluated in vitro against fungi and yeasts. The film's potential to act as an active packaging for Minas cheese preservation was also assessed. The incorporation of CNC increased the films' tensile strength; however, it did not influence the barrier properties to water vapor ($4.12 \times 10^{-7}$ g·cm.m$^{-1}$·h$^{-1}$·Pa$^{-1}$) and oxygen ($3.64 \times 10^{-13}$ g·cm·m$^{-1}$·h$^{-1}$·Pa$^{-1}$). The incorporation of natamycin, on the other hand, resulted in films that were more opaque (around 24%) and of a yellowish color. The active nanocomposites developed showed antimicrobial effects against all analyzed fungi and yeasts (approximately 35 mm of inhibition zone) and were able to control the growth of *S. cerevisiae* in cheese, reducing a log cycle until the 12th day of storage. Since they performed well in vitro and on food, it was concluded that the films showed potential to be applied in Minas cheese preservation.

**Keywords:** active packaging; cellulose nanocrystal; food packaging; food spoilage microorganisms; methyl cellulose; nanotechnology



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## 1. Introduction

Cheese production is one of the most important activities of the dairy industry in Brazil [1]. In order to guarantee a final product with microbiological quality, improve the product shelf life, minimize risks to consumers' health, as well as to reduce economic losses due to food waste, certain precautions are required throughout the production chain, such as the implementation of good hygiene practices and food safety management systems [2,3]. Besides that, several innovative technologies have been developed and used to preserve perishable goods and to ensure food safety.

Among these new technologies, it is worth noting the active packaging with the incorporation of antimicrobial substances [4,5]. An important advantage of this kind of technology is that the addition of the preservative agents into the polymer matrix of the packaging enables reducing the addition of preservatives directly into the food formulation, which is in accordance with emerging consumer trends such as lower additive content in foods [6,7]. Moreover, since most microbial growth takes place on the food surface, the presence of antimicrobial agents in the packaging may improve their preservative action [8].

Antimicrobial packaging has been tested on different perishable foodstuffs, such as meat, fruits, vegetables, bakery goods, and dairy products, with promising results [9–13].

Regarding dairy products, preservative agents such as natamycin, lysozyme, and nisin are interesting antimicrobials to consider as active packaging additives since they are natural compounds already used in cheese production [4,13,14]. Oliveira et al. [4], for example, manufactured an active packaging incorporated with nisin, which showed relevant activity against *Staphylococcus aureus*, a pathogen occasionally linked to foodborne outbreaks [15].

Natamycin, also known as piramicin, is a natural antimycotic produced by the Gram-positive bacteria *Streptomyces natalensis*. It is considered generally recognized as safe (GRAS) by Food and Drug Administration (FDA, Silver Spring, MD, USA) and used in cheese as an antimicrobial agent, preventing product contamination by yeasts and molds [14]. Since fungal spoilage occurs mainly on the cheese surface and the current methods used to cover the food with natamycin, such as spraying and coating, have low efficiency, its incorporation into active food packaging has been considered and studied in recent years [16,17]. The active packaging developed by Fayed et al. [17] with natamycin nanoparticles was able to control the growth of *Aspergillus flavus* on Romy cheese, as well as significantly reduce toxin production. Similarly, the natamycin-based low-density polyethylene film elaborated by Anari et al. [18] exhibited activity against spoilage yeasts common in yogurt and could contribute to increasing the shelf life of this kind of product.

Another important topic that attracts the attention of researchers on a worldwide scale is the hazards regarding the accumulation of non-degradable plastics and their negative impact on the environment. In this context, there is a growing interest in the research of biodegradable polymers aiming at the production of sustainable packaging [19]. Bio-renewable sources, such as cellulose and its derivatives, have been studied as possible materials for green packaging; however, despite their desirable sustainable nature, these kinds of polymers originate films that present many limitations when compared to petroleum-based films since their barrier, mechanical and thermal properties leave something to be desired [5,20]. Low impact resistance, brittleness, and higher permeation to water vapor and other gases are characteristics usually related to bio-based packaging [5,20,21]. Furthermore, the higher hydrophilicity exhibited by several biopolymers restricts their range of applications in the food industry [21].

In order to improve the bioplastic properties, the incorporation of nanomaterials into a compatible polymeric matrix is suggested since this allows the obtainment of an improved bio-nanocomposite that might meet the mechanical and barrier requirements for food packaging [22]. Among the several nanomaterials studied, we highlight nanocrystalline cellulose (CNC): nanostructures obtained from a renewable source (cellulose) that can be added to a polymeric matrix, acting as a mechanical and barrier reinforcement without altering its biodegradable nature. In this context, this study aimed at the production of antimicrobial and sustainable packaging, which consisted of a methylcellulose (MC) and poly(vinyl alcohol) (PVOH) blend incorporated with CNC and natamycin. The mechanical, optical, and barrier properties of the films were evaluated, as well as the film's capability to preserve Minas cheese.

## 2. Materials and Methods

### *2.1. Materials*

The following materials were used in the present research: natamicyn 50% (Natamax natural antimicrobial, Danisco Brazil LTDA, Brazil); CNC was extracted from softwoodpulp supplied by Klabin (São Paulo, SP, Brazil); glycerol (Sigma-Aldrich, St. Louis, MO, USA); poly(vinyl alcohol) (PVOH, PM = 85,000–124,000 $g \cdot mol^{-1}$, Sigma-Aldrich, St. Louis, MO, USA); methylcellulose (MC, Sigma-Aldrich, St. Louis, MO, USA); lithium chloride; sodium chloride; potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO, USA; Sabouraud dextrose broth (Kasvi, Padova, Italy). Minas cheese was purchased from a local market in Viçosa, Minas Gerais, transported to the laboratory, and kept under refrigeration until analyses. The yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, and the food spoilage molds *Alternaria alternata*, *Rhizopus stolonifer*, *Fusarium semitectum*, and *Aspergillus niger* were

obtained from the culture collection of the Food Packaging Laboratory, Federal University of Viçosa.

### *2.2. Methods*

#### 2.2.1. Experimental Design

The experimental design was completely randomized, and a two-factor five-level rotatable central composite design (RCCD) with five repetitions at the central point, totaling thirteen trials, was implemented to study the effect of the incorporation of CNC and natamycin in the optical, mechanical, barrier, and antimycotic properties of the MC/PVOH blend. The coded levels and their respective real values of concentrations of natamycin and CNC are displayed in Table 1.

**Table 1.** Composition of the two-factor five-level RCCD of the elaborated films incorporated with cellulose nanocrystals (CNC) and natamycin. Coded levels and their respective real values.

| Treatments | Coded Levels | | Real Values | |
|---|---|---|---|---|
| | X1 | X2 | CNC (% wt./wt.) | Natamycin (% wt./wt.) |
| 1 | −1 | −1 | 0.70 | 0.70 |
| 2 | +1 | −1 | 4.30 | 0.70 |
| 3 | −1 | +1 | 0.70 | 4.30 |
| 4 | +1 | +1 | 4.30 | 4.30 |
| 5 | −1.41 | 0 | 0 | 2.50 |
| 6 | +1.41 | 0 | 5.00 | 2.50 |
| 7 | 0 | −1.41 | 2.50 | 0 |
| 8 | 0 | +1.41 | 2.50 | 5.00 |
| 9 | 0 | 0 | 2.50 | 2.50 |
| 10 | 0 | 0 | 2.50 | 2.50 |
| 11 | 0 | 0 | 2.50 | 2.50 |
| 12 | 0 | 0 | 2.50 | 2.50 |
| 13 | 0 | 0 | 2.50 | 2.50 |

The % was based on the final polymers' mass.

#### 2.2.2. Elaboration of the Polymeric Blend Incorporated with CNC and Natamycin

Different concentrations of CNCs (Table 1, Section 2.2.1) were dispersed in 200 mL of distilled water, homogenized in ultraturrax (5 min, 20,000 rpm, model T25, IKA), and ultrasonicated (450 W, Unique Group, Rio de Janeiro, RJ, Brazil) for 5 min. After, 2.60 g of PVOH and 6.25 g of MC were added to the CNC dispersion and heated at 70 °C for 4 h under continuous stirring until solubilization, followed by gelification. Different concentrations of natamycin were added subsequently (Table 1, Section 2.2.1), followed by the incorporation of the plasticizer glycerol (30% wt./wt.). All concentrations were calculated based on the total polymer mass. The obtained dispersions were continuously stirred for 20 min and poured on glass plates (18 cm × 34 cm) until solvent evaporation.

#### 2.2.3. Thickness and Mechanical Properties

Film thickness was measured, in μm, with a digital micrometer (model 547–401, Mitutoyo, Kawasaki, Japan). Ten specimens of each treatment were analyzed, and the measurement was realized in ten random points of each sample [9]. The films were also evaluated according to their mechanical properties of ultimate tensile strength (UTS, in MPa), elongation at break (EB, in %), and modulus of elasticity (Young's modulus, YM, in MPa) using a Universal Testing Machine (model 3367, Instron Corporation, Norwood, MA, USA) equipped with a 1 kN load cell. Five specimens (25 mm × 150 mm) of each treatment were evaluated. The initial distance of grids separation was 100 mm, and the standardized rate of separation was 50 mm·min$^{-1}$ [23].

2.2.4. Color and Opacity Measurement

The color of the films was assessed by a Color Quest XE spectrophotometer (Hunter Lab, Reston, VA, USA), working with D65, 10° angle, and using the CIELAB scale for the L*, a* and b* coordinates. Opacity (OP), yellowness index (YI), and b* (tendency to yellow) were the parameters investigated. Two samples were analyzed for each treatment, and five measurements were performed at random points of each sample [24].

2.2.5. Water Vapor Permeability (WVP)

WVP was investigated by the gravimetric method according to ASTM E96/E96M-10 [25] with modifications [9]. The obtained films were cut into circles (Ø = 83 mm) and sealed with paraffin in poly(methyl methacrylate) cups containing a saturated solution of lithium chloride (RH = 12% ± 5% at 25 °C ± 2 °C). After, the cups were placed in desiccators containing a saturated solution of sodium chloride (RH = 75% ± 5% at 25 °C ± 2 °C). The cups were weighted periodically, enough to provide ten data points after the steady state was reached. The results were expressed as $g \cdot cm \cdot m^{-1} \cdot h^{-1} \cdot Pa^{-1}$.

2.2.6. Oxygen Permeability ($O_2P$)

The permeability to oxygen was verified in a VAC-V1 gas permeability tester (Labthink Instruments, Jinan, China). The experiment was carried out at 23 °C ± 2 °C and 50% RH. The exposed area of the films was 38.48 $mm^2$, and the analysis was conducted for 8 h. The transmission rate of oxygen through film was expressed as $g \cdot cm \cdot m^{-1} \cdot h^{-1} \cdot Pa^{-1}$.

2.2.7. In Vitro Assessment of the Antimycotic Activity of the Films

The yeasts *S. cerevisiae* and *K. lactis* were grown on Sabouraud broth, incubated for 24 h at 32 °C, plated on acidified PDA, and incubated once more for 7 days at 25 °C ± 2 °C. After, isolated colonies were selected, suspended in a 5 mL 0.85% (wt./wt.) saline solution, and adjusted to 0.5 McFarland standard (around $1.0 \times 10^6$ cells$\cdot mL^{-1}$) [26]. Regarding the molds, the conidia were grown on Sabouroud broth for 7 days at 25 °C ± 2 °C, plated on acidified PDA, and incubated again at the same conditions. Subsequently, the conidia were collected and transferred to 5 mL 0.85% (wt./wt.) saline solution and adjusted to optical density ($OD_{530}$) of 0.10 (GBC UV/Vis 918), which represented around $5 \times 10^4$ conidia$\cdot mL^{-1}$ [26].

After, aliquots of 100 µL of the suspensions prepared were spread on acidified PDA, and samples of the films (Ø = 10 mm) were placed at the center of each plate. The plates inoculated with molds or yeasts were incubated for 7 days at 25 °C ± 2 °C, and the inhibition zone verified was measured in mm.

2.2.8. Assessment of the Active Films on Minas Cheese Preservation

The surface of the food sample (25 g) was decontaminated with alcohol 70% (*v*/*v*). Subsequently, aliquots of 100 µL of *S. cerevisiae* ($10^4$ CFU$\cdot mL^{-1}$) (Section 2.2.7) were spread on cheese surface, and, right after, the samples were left to dry for 15 min [27]. The inoculated samples were packaged with the following elaborated films: film A (5% of CNC and 2% natamycin) and film B (5% of CNC and 0% of natamycin, taken as the control). The samples were stored at 4 °C for 15 days. Every three days, for a 15-day period, the samples (25 g) were homogenized with 225 mL of peptone water (0.1% *v*/*v*), diluted accordingly, and spread plated on PDA agar. The plates were incubated for 7 days at 25 °C ± 2 °C, and the results were expressed as log of colony forming unit of *S. cerevisiae per* gram of cheese (Log CFU$\cdot g^{-1}$) [9].

2.2.9. Statistical Analysis

The models representing the parameter's behavior as a function of CNC and/or natamycin concentrations were obtained using analysis of variance (ANOVA) with a 5% level of significance followed by regression analysis. The software Minitab 17 was used.

## 3. Results and Discussion

### *3.1. Films' Appearance*

The MC and PVOH blends incorporated with CNC and natamycin were successfully achieved, as can be seen in Figure 1. Macroscopically, they presented a smooth and homogeneous aspect. Natamycin incorporation, especially, resulted in films with a yellowish color and less transparent than films incorporated with CNC only. Complementarily, Table 2 shows the results obtained for opacity, yellow index, and tendency to yellow.

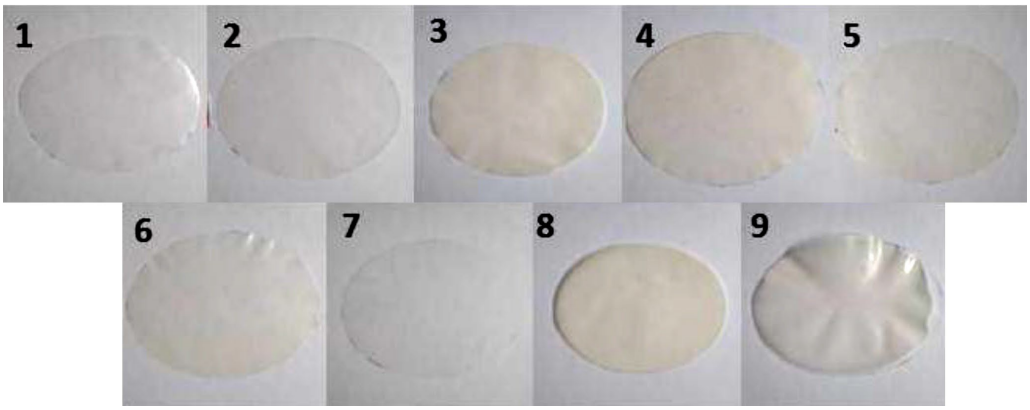


**Figure 1.** Photographs of the methyl cellulose and poly(vinyl alcohol) films elaborated with nanocrystalline cellulose (CNC) and natamycin: 0.7% of CNC and 0.7% of natamycin (1), 4.3% of CNC and 0.7% of natamycin (2), 0.7% of CNC and 4.3% of natamycin (3), 4.3% of CNC and 4.3% of natamycin (4), 0% of CNC and 2.5% of natamycin (5), 5% of CNC and 2.5% of natamycin (6), 2.5% of CNC and 0% of natamycin (7), 2.5% of CNC and 5% of natamycin (8), and 2.5% of CNC and 2.5% of natamycin (9).

**Table 2.** Results obtained for optical parameters (tendency to yellow, b*, opacity, OP, and yellow index, YI), thickness, mechanical properties (ultimate tensile strength, UTS, elongation at break, EB, Young's modulus, YM), water vapor permeability (WVP), and oxygen permeability ($O_2P$) of films elaborated with methyl cellulose, poly(vinyl alcohol), cellulose nanocrystals, and natamycin.

| Treatment | b* | OP (%) | YI | Thickness (μm) | UTS (MPa) | EB (%) | YM (MPa) | WVP ($10^{-7}$ g·cm·m$^{-1}$·h$^{-1}$·Pa$^{-1}$) | $O_2P$ ($10^{-13}$ g·cm·m$^{-1}$·h$^{-1}$·Pa$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2.78 | 16.85 | 4.67 | 163 | 19.74 | 64.21 | 6.59 | 3.14 | 1.03 |
| 2 | 2.83 | 16.39 | 4.69 | 177 | 29.11 | 72.78 | 9.53 | 4.45 | 1.33 |
| 3 | 5.19 | 24.18 | 9.18 | 191 | 22.64 | 73.97 | 7.39 | 3.25 | 1.26 |
| 4 | 5.45 | 21.35 | 9.38 | 182 | 20.64 | 65.66 | 7.63 | 4.61 | 9.40 |
| 5 | 3.86 | 19.97 | 6.51 | 181 | 21.43 | 79.54 | 6.28 | 4.27 | 1.37 |
| 6 | 4.04 | 18.57 | 7.49 | 164 | 29.37 | 71.86 | 10.36 | 3.29 | 7.15 |
| 7 | 1.80 | 16.62 | 3.05 | 146 | 26.79 | 78.83 | 6.53 | 3.97 | 9.34 |
| 8 | 6.40 | 23.39 | 10.80 | 193 | 22.85 | 71.72 | 7.52 | 3.27 | 1.37 |
| 9 | 4.64 | 21.29 | 8.12 | 161 | 26.63 | 81.93 | 5.93 | 4.73 | 2.05 |
| 10 | 4.06 | 18.34 | 6.8 | 172 | 27.73 | 76.78 | 9.28 | 4.52 | 1.41 |
| 11 | 4.68 | 19.59 | 7.98 | 194 | 27.48 | 74.19 | 7.82 | 5.15 | 8.89 |
| 12 | 4.46 | 18.65 | 7.18 | 149 | 28.65 | 75.38 | 9.69 | 4.47 | 1.39 |
| 13 | 4.28 | 19.04 | 7.72 | 163 | 26.65 | 77.07 | 8.05 | 4.49 | 1.43 |

From the results for optical properties, it was possible to adjust models (non-significant lack-of-fit) that explained the behavior of the three parameters analyzed as a function of the concentration of natamycin only, as displayed in Figure 2. CNC concentration, in turn, did not impact these parameters, suggesting a good dispersion of the nanomaterial in the polymer matrix. The yellowish color of films containing natamycin is possibly due to the slightly yellow color of the powder. In addition, the increased opacity verified when higher amounts of natamycin were added into films was probably the result of the hydrophobic

portion of the peptide and its poor solubility in water, which affected the antimicrobial dispersion in the hydrophilic matrix and resulted in opaquer films [14,28].

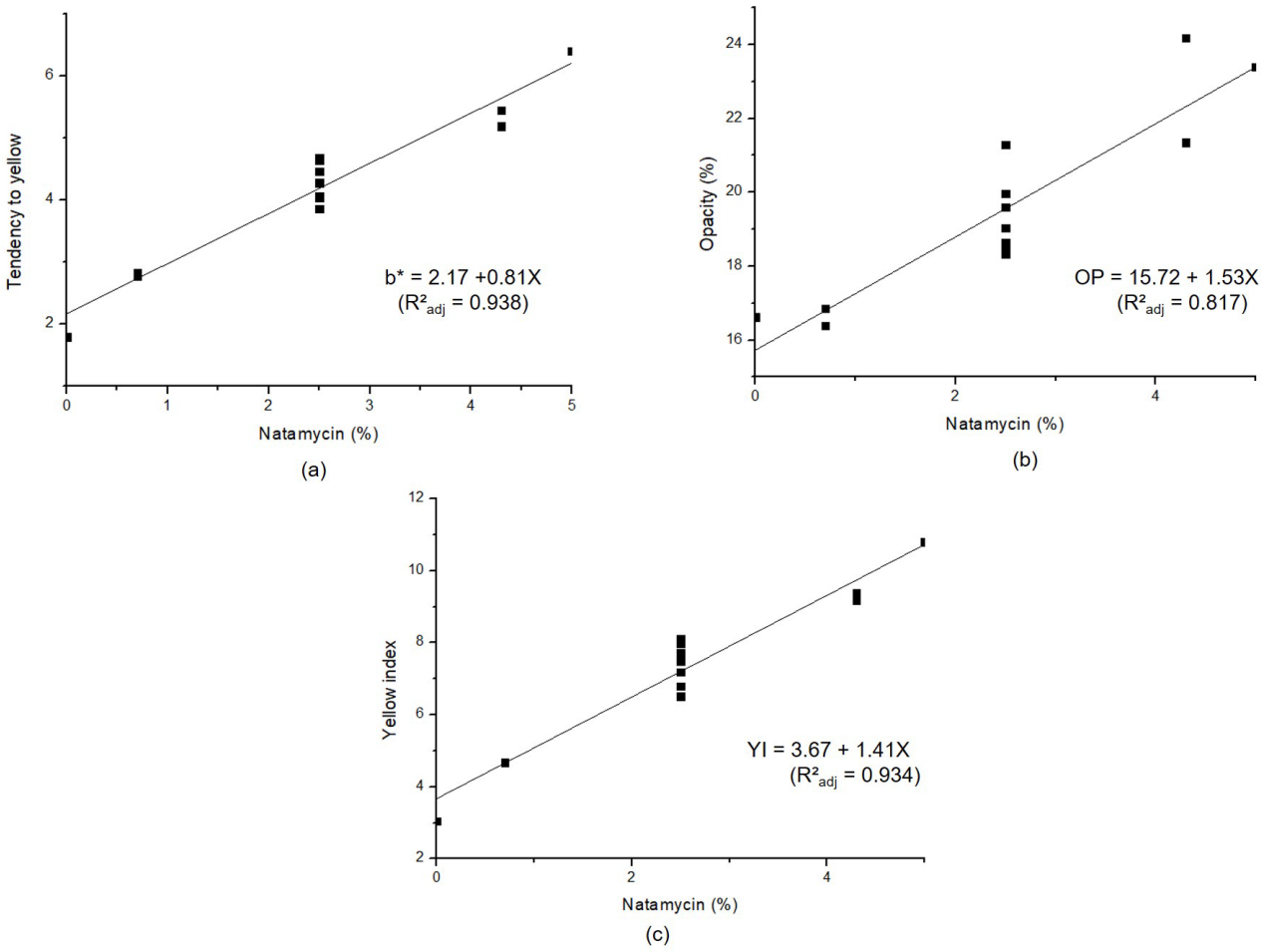


**Figure 2.** Plots of optical parameters as a function of natamycin concentration (% wt./wt., represented by X), their regression model, and adjusted $R^2$: tendency to yellow (b*, **a**), opacity (OP, **b**), and yellow index (YI, **c**).

### *3.2. Mechanical Properties*

When developing new materials for food packaging, it is crucial to evaluate their mechanical properties since these features will allow us to infer about the packaging behavior when under certain conditions, such as mechanical stress. The results for thickness, UTS, EB, and YM are also displayed in Table 2. It was possible to adjust a regression model for only UTS, and the behavior was a function of both CNC (X1) and natamycin (X2) concentrations, as demonstrated in Equation (1). The other parameters (thickness, EB, and YM) were not explained by any models, therefore, we considered only the global mean value: thickness of 172 μm; 68.74% of EB; YM of 7.89 MPa.

$$UTS = 14.01 + 5.92X_1 + 4.30X_2 - 0.48{X_1}^2 - 0.58{X_2}^2 - 0.88X_1X_2 \rightarrow ({R^2}_{adj} = 0.910) \quad (1)$$

Complementarily to the adjusted model, Figure 3 shows the surface response obtained for UTS.

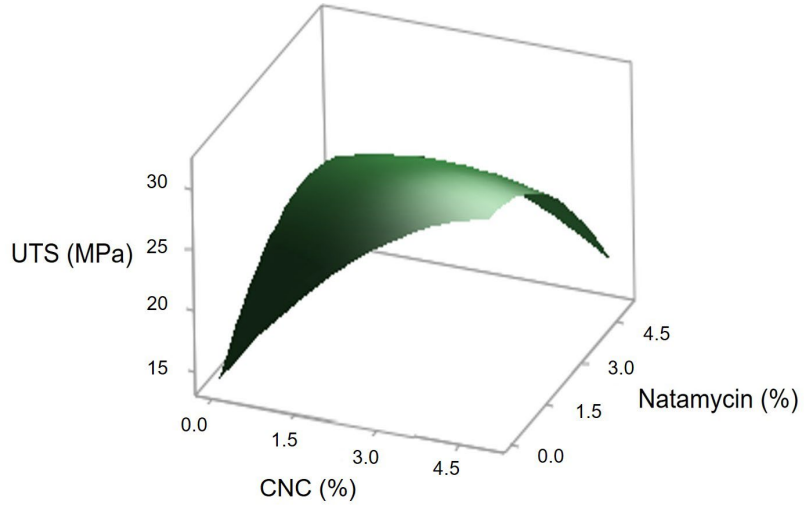


**Figure 3.** Three-dimensional response surface plot for ultimate tensile strength (UTS) as a function of cellulose nanocrystals (CNC, $X_1$) and natamycin ($X_2$) concentrations.

The findings indicated that both CNC and natamycin impacted the films' UTS, which assumed a parabolic shape. This suggested that, beyond certain concentrations, the additive's incorporation had a negative effect on the UTS parameter. Furthermore, the term $X_1X_2$ in Equation (1) suggested that, for this parameter, there was an interaction between CNC and natamycin: the UTS was likely to increase when higher concentrations of CNC and lower concentrations of natamycin were used. Other research also verified an increase in the tensile strength of sustainable films of PVOH incorporated with CNC [29,30]. This behavior may be explained by the high amount of –OH groups on the CNC structure, which are able to strongly interact with the hydrophilic polymeric matrices (MC and PVOH), increasing the compaction of the chains. This more homogenous structure may play an important role in stress redistribution all over the chains. Besides that, the high specific surface area and aspect ratio are CNCs' features that allow their application as potential reinforcing materials [31]. Natamycin, on the other hand, contributed less to the mechanical parameters, which may be due to its hydrophobic region and lower compatibility with both polymers.

### *3.3. Water Vapor and Oxygen Permeability*

The results obtained for WVP and $O_2P$ are presented in Table 2. In the food packaging field, it is of utmost importance to ensure that gas exchanges between the food and the external environment are minimal. While water vapor permeation can adversely affect both the product's shelf life and its sensorial quality, oxygen permeation triggers oxidation, which in turn leads to undesirable changes in flavor, texture, and appearance.

It was not possible to adjust a regression model for either WVP or $O_2P$ as a function of CNC and natamycin concentrations (significant lack of fit, indicating not suitable models). Due to this, we considered only the global mean values: $4.12 \times 10^{-7}$ g·cm·m$^{-1}$·h$^{-1}$·Pa$^{-1}$ for WPV and $3.64 \times 10^{-13}$ g·cm·m$^{-1}$·h$^{-1}$·Pa$^{-1}$ for $O_2P$. Although it is said in the literature that CNC is able to improve the barrier properties of biodegradable packaging, hindering the flow of molecules through tortuous paths, contrary to our expectations, neither CNC nor natamycin influenced the gas permeability of the elaborated films [32,33].

### *3.4. In Vitro Antimycotic Activity*

At first, the films' antimycotic activity was assessed in vitro against four molds of importance in food preservation, as well as two yeasts. Photographs of the inoculated plates after the incubation period of seven days are displayed in Figure 4.

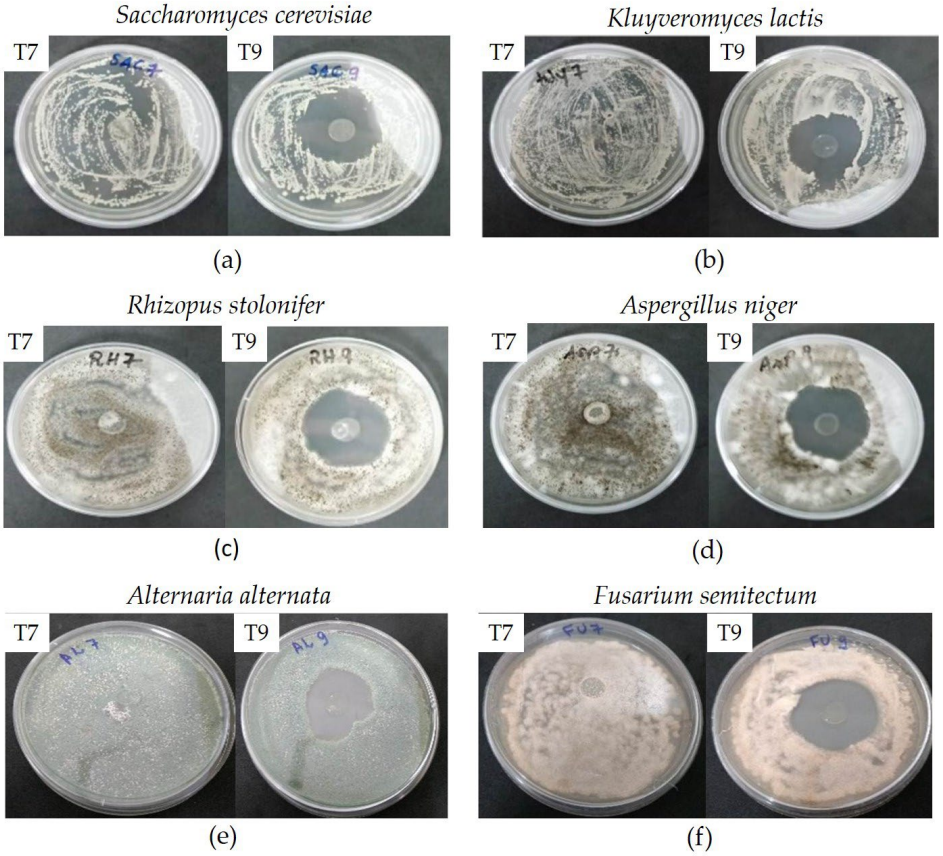


**Figure 4.** Photographs of plates after incubation of different yeasts (*S. cerevisiae* (**a**) and *K. lactis* (**b**)) and molds (*R. stolonifer* (**c**), *A. niger* (**d**), *A. alternate* (**e**), and *F. semitectum* (**f**)) at 25 °C for 7 days. T7: films with 2.5% of CNC and 0% of natamycin. T9: films with 2.5% of CNC and 2.5% of natamycin.

Films elaborated without natamycin (T7) did not show antimicrobial activity, as can be seen in Figure 4. When incorporated with natamycin, all the films displayed antimicrobial properties, suggesting that they could be used as active films for food preservation against these microorganisms. Regardless of the natamycin concentration, the inhibition zones verified for each mold or yeast investigated were similar, and the mean values obtained are presented in Table 3. The only exception was when the films were tested against *A. alternata*, in which it was possible to adjust a polynomial model of the inhibition zone as a function of natamycin concentration (Table 3).

**Table 3.** Inhibition zones obtained when the elaborated films with different concentrations of natamycin (0.7%, 2.5%, 4.3%, and 5.0%) were assessed against molds and yeasts of importance in food preservation.

| Microorganism | Inhibition Zone (mm) | $R^2_{adj}$ |
|---|---|---|
| *Alternaria alternata* | $IZ = 3.12 + 17.13X - 2.37X^2$ | 0.92 |
| *Aspergillus niger* | $34.7 \pm 4.4$ | - |
| *Rhizopus stolonifer* | $35.4 \pm 3.0$ | - |
| *Fusarium semitectum* | $34.2 \pm 4.0$ | - |
| *Saccharomyces cerevisiae* | $36.7 \pm 3.5$ | - |
| *Kluyveromyces lactis* | $35.0 \pm 2.7$ | - |

IZ = inhibition zone; X = natamycin concentration (wt./wt.).

Natamycin is a preservative allowed for dairy products, and it has been the object of study of several pieces of research regarding active food packaging. Similar to the present study, Chakravartula et al. (2020) [34] verified that blend films elaborated with cassava

starch, chitosan, and natamycin presented potential to be used as active packaging due to the antifungal activity displayed by the films. Films incorporated with natamycin were also manufactured by Grafia et al. (2018) [16]. The authors tested the active films in vitro against *A. niger* previously isolated from onions and achieved interesting outcomes.

Literature shows that the mechanism of action of natamycin against fungi involves interference in the cells' vacuole and a preferred binding to ergosterol, a sterol molecule found in fungi cells membrane responsible for cell integrity [35–37]. Besides that, natamycin is also referred to as an efficient inhibitor of the transport of nutrients, compromising cell function [37].

### *3.5. Application of the Active Packaging in Minas Cheese*

The central composite design allowed us to optimize the packaging system and define the film formulation that should be tested on food based on the outcomes found for the investigated properties. Minas cheese, a traditional-Brazilian product, was chosen as the food sample in this step since preliminary tests indicated that the film remained well preserved after contact with the food. The active films tested on the Minas cheese samples were elaborated with 5% of CNC and 2% of natamycin (film A), and 5% of CNC (film B, control). In this sense, cheese samples previously inoculated with *S. cerevisiae* were packaged with the films and stored for up to 15 days at low temperatures (4 °C). Regression models were adjusted for each case, and they are presented below (Equations (2) and (3)):

$$Y_A = 3.62 + 0.20T \rightarrow (R^2adj = 0.878) \quad (2)$$

$$Y_B = 3.79 + 0.44T - 0.014T^2 \rightarrow (R^2adj = 0.925) \quad (3)$$

in which $Y_A$ is the yeast growth, in log CFU·g$^{-1}$, when the film A was used, $Y_B$ is the yeast growth, also in CFU·g$^{-1}$, when the film B was used. For both equations, T represents the time, in days.

In Figure 5, it is possible to observe the yeast counts in Minas cheese during the storage time.

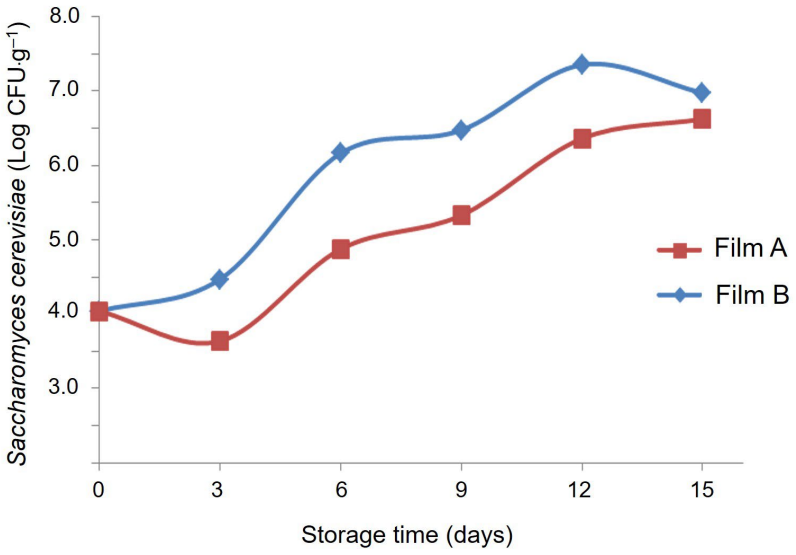


**Figure 5.** *Saccharomyces cerevisiae* count, in log CFU·g$^{-1}$, as a function of time, in Minas cheese stored at 4 °C for 15 days and packaged with methyl cellulose/poly (vinyl alcohol) blend films incorporated with 2% of natamycin and 5% of cellulose nanocrystals (Film A) and 5% of CNC (Film B).

It can be observed that the film incorporated with natamycin impacted the growth of *S. cerevisiae* in cheese in approximately one log cycle until the 12th day of refrigerated storage when compared to the film incorporated with only CNC. This means that a reduction of approximately 90% of the yeast population was achieved. Although *S. cerevisiae* is an

important fermenting agent for the food industry, mostly involved in the manufacturing of bakery products and alcoholic beverages, it is considered a contaminant microorganism in cheese, usually related to product spoilage [38,39]

To date, fungal spoilage in cheese is one of the major challenges faced by the dairy industry [40]. In a study with 61 cheesemakers from the United States, 71% reported that the growth of undesirable mold on cheese surfaces was one of the most common issues in the area [41]. The occurrence of strange color and pigment development, probably caused by microbial growth, was also mentioned by around 54% of the respondents [39]. In addition, 28% of the interviewed cheesemakers mentioned that around 5 to 10% of their production was lost due to quality issues, indicating an economic loss that can be significant.

In order to minimize contaminants in dairy, it is essential to ensure the quality of the milk, as well as invest in and enforce good hygiene practices throughout the entire production chain. The adoption of novel technologies, such as active packaging, could be a complementary strategy to enhance quality and contribute to prolonging the product's shelf life. In this sense, these findings suggest that the application of a sustainable packaging MC/PVOH-based incorporated with CNC and natamycin in Minas cheese could play an important role in product preservation. Although CNC does not present antimicrobial activity, the material can be added as a nanofiller to enhance the mechanical properties of the packaging, while natamycin functions as the active component effective against molds and yeasts.

## 4. Conclusions

In the present study, an MC/PVOH/CNC/natamycin blend film was successfully manufactured and showed potential to be used as an antimicrobial film in Minas cheese preservation. Initially, it was hypothesized that the presence of CNC would improve the films' mechanical and barrier properties; however, only the UTS parameter was influenced by the CNC concentration. Nevertheless, this was an important finding since CNC did act as a mechanical reinforcing material. Future studies should be more focused on enhancing the barrier properties of the film since it is a significant parameter of food preservation. Furthermore, the presence of CNC did not impact the optical properties of the elaborated packaging, which was also a positive result. Natamycin, on the other hand, had a negative effect on the optical parameters and on the mechanical properties of the films, probably due to lower compatibility among the antimicrobial and the polymers. Although it enabled the film to act as an antimicrobial packaging against yeasts and molds, as well as displayed a good performance when tested on Minas cheese, its compatibility and interaction with the polymers should be studied in greater depth to allow the obtainment of active films with not only antimicrobial properties, but also better mechanical, optical, and barrier properties.

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