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Selected Useful Properties of Polylactide Films Containing Nisaplin and Natamax

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Abstract: In this article, we present polymer materials consisting of polylactide (PLA) and nisaplin (N), as well as PLA and natamax (X). These materials were obtained using the solvent method and tested by various test methods, i.e., functional properties—water vapor permeability, light transmission, gloss, and bactericidal activity against strains *E. coli* (ATCC 8739P), *S. aureus* (ATCC 65388), and *P. aeruginosa* (ATCC 8739). Furthermore, analyses were conducted to evaluate their efficacy against pathogenic fungi, including *A. niger*, *A. flavus*, *A. glaucus*, and *A. versicolor*. Mutagenicity analyses were performed using the standard Ames Test with *Salmonella typhimurium*. The main test methods used were ISO 22196, ISO 846. The results obtained confirm the potential suitability of the films of PLA with nisaplin and natamax for applications in the food packaging industry.

Keywords: nisaplin; natamax; polylactide

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1. Introduction

Polylactide (PLA) is one of the most highly regarded biodegradable polymers in industry and one of the most widely described in science [1,2]. Its beneficial properties allow it to be used as a material for various applications, mainly in two important areas, including consumer products and small medical equipment [3,4]. Common-use products are mainly containers and packaging films, as well as medical accessories including bioresorbable implants, surgical sutures, stents, tissue engineering scaffolds, and hygiene and dressings materials [5,6]. One of the earliest areas of PLA application was the production of capsules for slow drug release [7,8]. PLA is very often subjected to modifications [9–13].

Bactericidal substances can be introduced into packaging in several ways. One of them involves introducing them into the polymer matrix during production, the other allows biocides to be applied to the surface of previously produced materials by coating and immobilization. Bactericidal compounds introduced into the polymer matrix during processing must be resistant to high temperature and have low volatility [14]. Bactericidal substances can also be applied to packaging by coating the finished product (packaging). Thanks to this method, bactericidal substances that are not resistant to high temperature and shear forces can be used (due to the elimination of the process of extrusion of composites and mixtures) [14].

Coating involves the physical adsorption of a bactericidal substance on the polymer surface and is the simplest way to obtain bactericidal packaging. Bactericidal substances can be directly applied by typical coating, impregnation, sprinkling, spraying, or printing [14]. Some bactericidal substances migrate from the surface of the material too quickly; in such cases, a carrier is used together with the biocide. The carriers are methylcellulose (MC), hydroxypropyl methylcellulose (HPMC), polyamide resin, glucomannan, alginate, chitosan,

and vinyl acetate-ethylene copolymer [14]. Organic acids, bacteriocins, lysozyme, and essential oils are most often used to coat the packaging surface [15]. Packaging materials with antibacterial properties, obtained by physical adsorption of a biocide to the surface of the material, can be used for packaging food products. The condition for the effective operation of such packaging is its direct adhesion to the protected (fixed) product [16].

Another way to obtain bactericidal packaging is immobilization. It involves immobilizing a bactericidal substance on the surface of polymeric materials through covalent or ionic bonds. When using this method, the presence of functional groups in polymers and bactericidal substances is necessary. Immobilization of bactericidal substances can occur in two ways. The first one involves direct immobilization of the biocide on the polymer surface, the second one requires the presence of intermediate compounds. Binding agents may be dextrans, glutaraldehyde, ethylenediamine, or polyethyleneimine [17]. Positive bactericidal results (mainly against Gram-positive bacteria) are obtained in the case of immobilization of lysozyme, which becomes strongly bound to polyvinyl alcohol via glutaldehyde. The use of packaging containing bactericidal substances, regardless of the method of introducing them or applying them to the polymer, leads to protection of food against the development of undesirable microorganisms, extending the shelf life of products, increasing health safety, reducing the possibility of microbiological infection, minimizing the development of microorganisms during transport and storage, and increasing effectiveness of preservation methods (i.e., packaging in a modified atmosphere, pressure preservation) of food products [16].

The bactericidal compounds introduced into the polymer matrix during processing must be resistant to high temperatures and have low volatility. There are also methods by which less resistant substances, including bacteriocins, can also be incorporated into polymers [14–17].

Bacteriocins are ribosomally synthesized antimicrobial proteins (AMPs), most often used directly for food preservation [18]. Some of the best known bacteriocins are nisin (trade name Nisaplin®, Danisco, Denmark) and natamycin (Natamax, Danisco, Denmark), which have antimicrobial properties against pathogens of concern in the food industry [19,20]. Nisin acts against vegetative forms of bacteria and can also effectively inactivate bacterial spores by altering their structure [21]. There have already been attempts to incorporate these substances into various matrices to produce food packaging materials. Cé et al. [22] introduced nisin and natamycin into a chitosan matrix and obtained antimicrobial activity against seven species of bacteria and two species of fungi that are a problem in food production and storage.

The bactericidal activity of bacteriocins may deteriorate (destabilize) during their incorporation into the polymer, under the influence of factors such as high temperature, pH, pressure, or shear forces, so in order to prevent this, among other things, the know-how of the technology and production process of a particular product is used [23]. The introduction of antibacterial compounds into PLA in the process of extrusion of granules and then films results in the compounds (even in low concentrations) being evenly distributed in the polymer matrix [24]. For this reason, among others, obtaining bactericidal polymeric materials is one of the most preferred methods in processing and industry [24–26].

The aim of the article was to produce a polylactide (PLA) film with the addition of nisaplin and natamax in three different concentrations, 0.2, 0.6, and 1.0% wt., in a different way than what is commonly described in the literature, namely obtaining composites by extrusion. An attempt was made to prepare polymeric materials with bacteriocins in three different concentrations using the solvent method, for which we have a patent application. In addition, the prepared films were subjected to functional analyses, such as water vapor permeability, transmittance, gloss, and biological assessments, such as determining bactericidal, fungicidal, and antimutagenic properties. This was performed to check the appropriate application and utilitarian potential of the produced films and to determine whether the obtained materials are suitable for use in the broadly understood packaging industry.

2. Materials and Methods

2.1. Materials

- Polylactide PLA, biodegradable polymer (L) (2003D type, Ingeo Biopolymer 2003D, Nature Works LLC, Minnetonka, MN, USA).
- Chloroform, solvent (Chempur, Piekary Śląskie, Poland).
- Nisaplin (N), bacteriocin, biocidal substance based on nisin (Danisco, Denmark). Nisaplin® is composed of Nisin (E234) min. 1000 IU per mg.
- Natamax (X), bacteriocin, biocidal substance based on natamycin (Danisco, Denmark). Natamax® is composed of Natamycin preparation, min. 1000 IU per mg.

2.2. Preparation of Materials

Separate liquefied solutions of PLA (L) (2003D type, Ingeo Biopolymer 2003D, Nature Works LLC, Minnetonka, MN, USA) were prepared using chloroform (Chempur, Piekary Śląskie, Poland) and then combined with nisaplin (N) and natamax (X) solutions to obtain homogeneous mixtures [27,28]. Subsequently, these mixtures were allowed to solidify at 23 °C for 48 h, resulting in thin films. The thickness of the films ranged from 0.080 to 0.090 mm. The resulting films were measured at 20 different locations with an accuracy of ±0.001 mm.

Bacteriocins in powder form, nisaplin and natamax (Danisco, Denmark), were added as a biocidal substance in quantities of 0.2%, 0.6%, and 1.0% wt. The detailed composition and symbolism of individual samples are included in Table 1.

Table 1. Composition and designations of individual samples.

Samples	Polylactide—(L) [%]	Nisaplin (N) [%]	Natamax (X) [%]
L	100	-	-
LN0.2	99.8	0.2	-
LN0.6	99.4	0.6	-
LN1.0	99.0	1.0	-
LX0.2	99.8	-	0.2
LX0.6	99.4	-	0.6
LX1.0	99.0	-	1.0

2.3. Water Vapor Permeability

According to the standard (PN-EN ISO 15106-1:2007), water vapor permeability (Pv) was determined using a laboratory device type L80-5000 (PBI Dansensor). This study involves determining the amount of water vapor that can penetrate a given material in the form of films sample, for a given unit of time and at a constant temperature of 38 °C. Five repetitions were performed for each sample, and the result was averaged [2,29].

2.4. Gloss of Materials

Gloss measurements were performed in accordance with ASTM D2457: "Standard method for testing the specular gloss of plastic films and solid materials", using Micro-Gloss 45° (spectro-quide sphere gloss CD-6834, BYK-Gardner, GmbH, Geretsried, Germany). Tests were carried out under the following conditions: temperature 23 ± 2 °C, humidity 50% and optical geometry of 45°. Gloss [gU] was determined according to the following scale: below 15—matte, 15–30—semi-matte, 31–50—semi-gloss, 52–80—gloss, above 80—high gloss [30]. Gloss is created as a result of reflection and scattering of light directly over or on the surface of solids and liquids. It depends on several factors, namely: refractive index, absorption, transparency and the nature/type of the surface itself. The color and shape of the surface and the lighting also have some influence on the visual impression of gloss. The intensity of gloss depends primarily on the light reflectance coefficient, expressed as the ratio of reflected light to incident light.

2.5. Light Transmittance of Materials

The light transmittance of transparent materials was determined according to the PN-EN ISO 13468-1:2003 standard Plastics Determination of the total light transmittance of transparent materials. Part 1: Single beam camera [31]. To conduct the study, the Hazemeter M 57 Diffusion Systems test stand was used.

2.6. SEM Analysis

The morphology of the PLA films with and without BT was studied using a HITACHI SU8010 scanning electron microscope (SEM Hitachi High-Technologies Co., Tokyo, Japan). Photographs of the topography of the samples were taken using an SEM detector at 1000× magnification. Prior to each analysis, the surfaces of the studied materials were sprayed with a layer of gold.

2.7. Antibacterial Effect

The antibacterial properties of the films were determined according to the ISO standard (ISO 22196:2011) using the following bacterial strains that are pathogens: *E. coli* (ATCC 8739P), *S. aureus* (ATCC 65388), and *P. aeruginosa* (ATCC 8739). Bacterial strains were inoculated in nutrient broth with the following composition [g/L]: peptone—5.0, meat extract—3.0, distilled water—1 L, pH 7.4. After inoculation of the medium, incubation was carried out at 37 °C for 24 h. From the obtained cultures, 1 mL of the suspension was sterilely collected and transferred to Eppendorf type tubes. The cultures were centrifuged at 8.000 rpm using a MiniSpin® centrifuge (Eppendorf). The supernatant was removed, and the sediment was resuspended in 1 mL of diluent, which was nutrient broth (1/500 NB) with the composition [g/L]: meat extract—0.006, peptone—0.02, NaCl—0.01, distilled water—1L, pH 6.8–7.2. The obtained cell suspension was transferred to a densitometer (Densi-La-Meter®II, Lachema) to adjust its optical density to 0.5 which, according to the McFarland scale, corresponds to 1.5×10^8 bacterial cells in 1 mL. Then, using a diluent (1/500 NB), the obtained suspension was diluted to a number of 7.5×10^5 bacterial cells in 1 mL.

The 0.1 mL of the final suspensions of the tested strains prepared in this way were placed on a 5 × 5 cm film. The films were covered with a sterilized 4 × 4 cm slide in order to evenly distribute the microorganisms on the surface of the test film (Figure 1).

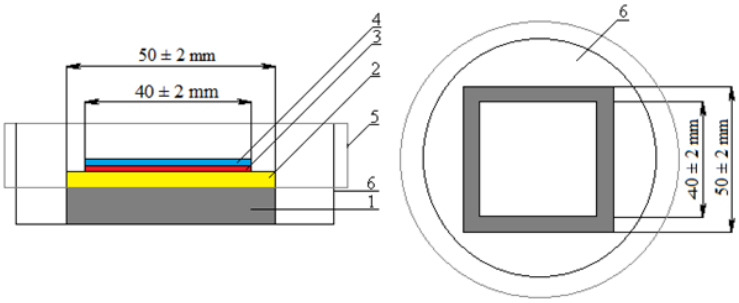

Figure 1. Scheme of the experimental system: (1) stabilizing stand, (2) film sample, (3) test bacterial inoculum (0.4 cm^3), (4) slide, (5) dish lid, (6) Petri dish.

Samples prepared in this way were incubated for 24 h at 35 °C. After this time, the number of living and growing bacterial cells on the surface of the test and control films (PLA without nisaplin or natamax) was determined. Before determining the number of cells, they were recovered from the tested films. For this purpose, the films were rinsed with 10 mL of SCDLP medium with the composition [g/L]: casein peptone—17.0, soy peptone—3.0, NaCl—5.0, NaH_2PO_4—2.5, glucose—1.0, water distilled—1 L, pH 6.8–7.2. The obtained suspension was diluted 10 times and inoculated by the pour plate method on PCA medium with the following composition [g/L]: yeast agar—2.5, tryptone—5.0, glucose—1.0, agar—15, distilled water—1 L, and pH 7.0–7.2. After 48 h of incubation at

35 °C, the grown colonies were counted and their number was converted to the number of cells. According to the ISO 22196:2011 standard, films are considered to have bactericidal properties if the reduction of bacterial cells capable of growing is at least two orders of magnitude greater than in control samples. Antibacterial activity, or reduction in microbial counts (R) was determined according to the guidelines specified in the standard [32].

2.8. Study of Fungistatic Properties

Testing of fungistatic properties was carried out in accordance with the methodology outlined in [33] Strains used: *A. niger*, *A. flavus*, *A. glaucus*, and *A. versicolor*. The samples were treated with a suspension of a mixture of fungal spores in the presence of a complete medium with the following composition: $NaNO_3$—2 g/L, KH_2PO_4—0.7 g/L, K_2HPO_4—0.3 g/L, KCl—0.5 g/L, $MgSO_4 \cdot 7H_2O$—0.5 g/L, agar—20 g/L, glucose—30 g/L, water—1 L [21].

Any inhibition of growth, both on the tested material (film) and on the medium (zone of growth inhibition), indicates the fungistatic or fungicidal activity of the film. Samples measuring 4 × 4 cm were placed on dishes containing the medium. The films were covered with 0.1 mL of a suspension of fungal spores with a concentration of 10^6 spores/mL [33].

Any inhibition of fungal growth, both on the surface of the polymeric material sample and around it (inhibition zone), indicates the fungistatic activity of the polymeric material.

In accordance with the standard, the division into 3 lots of samples was applied: (a) lot 0—control samples, stored at standardized temperature and relative humidity; (b) batch I—samples inoculated with microorganisms and incubated at 24 ± 1 °C; and (c) lot S—uninoculated samples, stored under the same conditions as lot I.

Fungal growth was assessed based on the below scale:

0. No visible growth under the microscope.
1. Growth invisible to the naked eye, but clearly visible under a microscope
2. Growth noticeable by the unaided eye covering up to 25% of the test area.
3. Growth noticeable by the unaided eye covering up to 50% of the test area.
4. Significant increase covering more than 50% of the test area.
5. Intense growth covering the entire test surface.

Incubation was carried out at 29 ± 1 °C and 90% (±5%) relative humidity for 28 days. Visual assessment was performed using photos taken with an automatic aCOLyte3 Automatic colony counter (Synbiosis, Pegasus Court, Frederick, TX, USA). A Leica stereoscopic microscope (Leica, Wetzlar, Germany, UE) was used for microscopic observations at 40× magnification of the sample image using a Leica camera (Leica, Wetzlar, Germany, UE) [33].

2.9. Ames Test

The potential mutagenicity of samples containing nisaplin and natamax was tested in vitro using the Ames test. In this analysis, M9 minimal medium with the composition [g/L]: ($Na_2HPO_4 \times 12H_2O$—6.78, KH_2PO_4—3, NaCl—0.5, NH_4Cl—1, agar—3, $MgSO_4$—$CaCl_2$—0.1, glucose—20, ampicillin—0.001, biotin—0.48, histidine—0.042) was inoculated with the *Salmonella typhimurium* strain. Samples were then placed on the medium, except for the plates, which were to serve as a control, i.e., to confirm bacterial growth. The plates were incubated at 37 °C for 48 h. The lack of growth of a significant number of bacterial cells around the sample indicated that the film had no mutagenic properties [34].

3. Results and Discussion

3.1. Film Thickness

The resulting films were measured at 20 different locations with an accuracy of ±0.001 mm. Table 2 summarizes the obtained results, showing means and standard deviations.

Table 2. Thickness of the obtained materials based on plasticized PLA (L) without and with the addition of nisaplin (LN0.2, LN0.6, LN1.0) and natamax (LX0.2, LX0.6, LX1.0).

Samples	L	LN0.2	LN0.6	LN1.0	LX0.2	LX0.6	LX1.0
Thickness [mm]	0.090 ± 0.009	0.087 ± 0.015	0.080 ± 0.014	0.087 ± 0.019	0.086 ± 0.021	0.078 ± 0.009	0.084 ± 0.012

The thickness did not depend on the added substance, and the changes in the thickness of the film were small. The results obtained show similarity to those obtained previously for the same type of film [9].

3.2. Permeability of Water Vapor

Water vapor permeability is a very important parameter considered in the packaging industry. The relationship between these properties is that the higher the water vapor permeability values, the lower the barrier, while the lower the water vapor permeability values, the higher the barrier. Depending on what is in the packages, either low or high water vapor permeability is desired. Water vapor permeability is a very important functional property. The higher the water vapor permeability values, the lower its barrier properties.

Test results regarding the effect of nisaplin and natamax on the permeability (P_V) of water vapor through films PLA are shown in Figure 2.

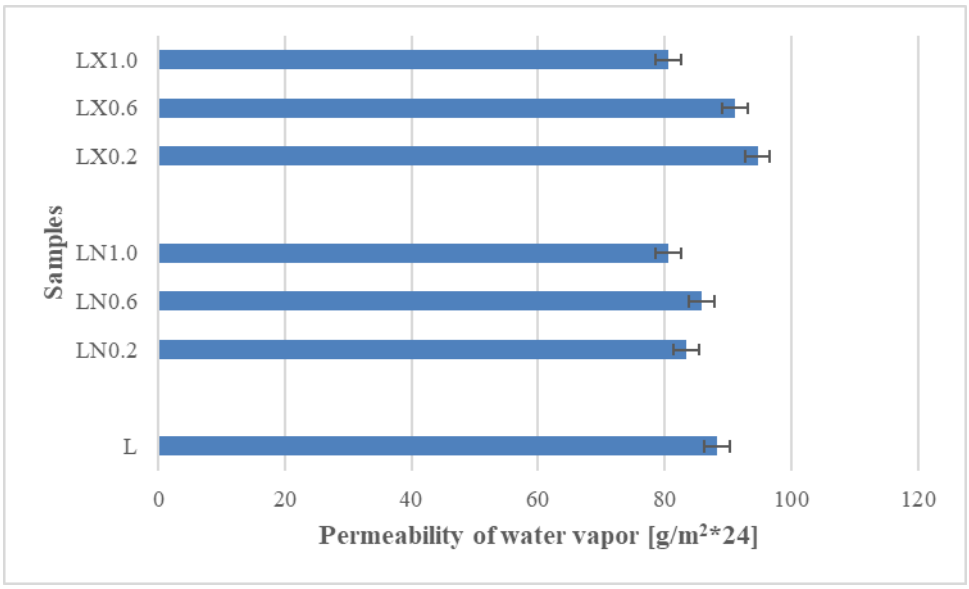

Figure 2. The permeability of water vapor through the films of PLA with nisaplin and natamax.

For PLA/nisaplin mixtures (labeled LN) water vapor permeability decreased, reaching the level 8.5% lower (at a concentration of 1% by weight) than the control (PLA) film. PLA/natamax films (labeled LX) (0.2, 0.6 and 1.0% wt.) showed a similar decrease in water vapor as the concentration of this bacteriocin increased. At the highest concentration (1% wt.) of natamax, water vapor permeability was reduced by 6.9% compared to pure PLA. Films containing nisaplin and natamax at the lowest concentration used (0.2% wt.) were the least susceptible to changes in water vapor permeability. This shows that the least changes in the barrier properties of these films have occurred [2,26]. Even a small percentage of added nisaplin and natamax changes the water vapor permeability. These two molecules behave differently because they are two different compounds and have different chemical structures, even though they belong to the bacteriocin group.

3.3. Transmittance and Gloss Determination

The results of tests determining transmittance, haze, and gloss of individual composite film samples are presented in Table 3.

Table 3. Results of transmittance and gloss determination.

Name of Samples	Light Transmission [%]	Hazing [%]	Gloss [gU]
L	86.34 ± 0.40	42.56 ± 1.21	86.90 ± 0.78
LN0.2	87.30 ± 1.09	56.52 ± 1.58	85.70 ± 0.57
LN0.6	85.98 ± 1.10	63.89 ± 0.61	85.00 ± 0.64
LN1.0	85.04 ± 0.90	65.34 ± 0.73	84.02 ± 0.87
LX0.2	85.32 ± 1.20	44.52 ± 1.23	86.10 ± 1.13
LX0.6	85.89 ± 0.98	49.50 ± 1.22	85.50 ± 0.43
LX1.0	86.50 ± 0.73	55.20 ± 1.11	83.42 ± 0.55

The obtained results of transmittance and haze tests show that the films had the highest transmittance 87.3% (LN1) and 86.5% (LX10). The same samples had the lowest haze value of 56.52% and 55.2%. These results indicate that the films containing both nisaplin and natamax have very good transparency. The transmittance and haze results for the LN1.0 sample were 87.3% and 56.5%, respectively, while for the LX1.0 sample, the same parameters reached values of 86.5% and 55.2%.

The highest gloss value was characteristic of the film marked with the symbol L (86.9 gU). In turn, the greatest decrease in the value of the tested parameter occurred for the LN1.0 and LX1.0 samples and amounted to 3.3 and 4%, respectively, compared to the reference sample (L). All analyzed film samples were characterized by high gloss, which is the highest level according to the applicable international scale.

3.4. Films Analysis by SEM

Images of the film surface are shown in Figure 3, which were carried out using SEM analysis. The first image, Figure 3a, shows a control sample of polylactide (L). The second (Figure 3b) and third (Figure 3c) photos show the surface of the foil (LN1.0, LX1.0) containing the highest concentration of the additive, i.e., 1% by weight, nisaplin and natamax, respectively. The similarity of Figure 3b,c may result from the same nature of the biocidal substances added to the polymer, i.e., bacteriocins (nisaplin, natamax). Figure 3a has a different surface from the others, the difference may be due to the fact that it is a control sample containing polylactide, without other additives, so another surface is possible.

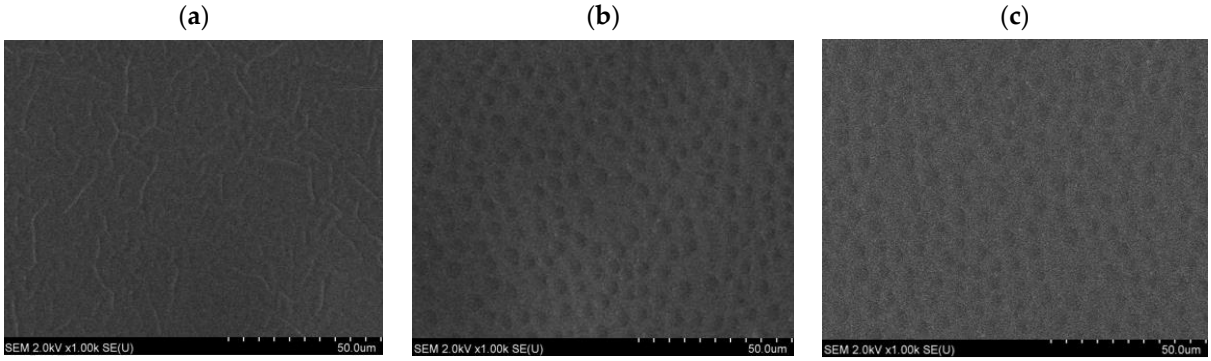

Figure 3. SEM images of the studied materials: (**a**) L, (**b**) LN1.0, (**c**) LX1.0).

Thanks to the results obtained through these analyses, it is possible to critically look at other results, such as the biocidal effect or the homogeneity of the obtained films. The biocidal substance affects the morphology and surface of the polymer. If the compound is uniformly distributed and dispersed in the polymer matrix, a smooth surface is obtained.

Minor changes or even defects may also appear due to sample drying or solvent evaporation. The films were produced using the solvent method, so some changes may result from the evaporation of the chloroform that was used to produce the films. A certain hypothesis can be made regarding the type of interaction between the additive and the polymer. Namely, regardless of the additive used, it may affect the formation of hydrogen bonds. Depending on the nature of the additive used, additional chemical bonds may also be formed, e.g., ionic bonds or cross-linking bonds, ester bonds. Analyzing the SEM results, it can be concluded that the type of additive, its concentration, the nature of the polymer matrix, and the method of obtaining the foil have a direct impact on the surface structure [24].

3.5. Antibacterial Properties

When analyzing polymer materials with altered properties, it is important to distinguish between two concepts, namely effectiveness and antibacterial activity. Antibacterial activity is a difference in the logarithm of the number of cells found on an antibacterial-treated product and an untreated product after inoculation and incubation of bacteria. Antibacterial effectiveness is the ability of an antibacterial agent to inhibit the growth of bacteria on the surface of materials treated with an antibacterial agent, as determined by the value of the antibacterial activity [32]. The biocidal properties of PLA films containing nisaplin and natamax are presented in Table 4.

Table 4. Antimicrobial activity of samples with nisaplin (LN0.2, LN0.6, LN1.0) and natamax (LX0.2, LX0.6, LX1.0) against pathogenic strains in relation to the control (L) [33].

Sample Description	Sample Description	R	% Reduction	Antimicrobial Efficacy
E. coli (ATCC 8739P)	L	-	-	-
	LN0.2	1.2	>90.0	satisfactory
	LN0.6	1.4	>90.0	satisfactory
	LN1.0	1.9	>99.0	very good
	LX0.2	1.5	>90.0	satisfactory
	LX0.6	1.6	>90.0	satisfactory
	LX1.0	1.9	>99.0	very good
S. aureus (ATCC 65388)	L	-	-	-
	LN0.2	1.9	>99.9	very good
	LN0.6	2.0	>99.9	very good
	LN1.0	2.4	>99.9	very good
	LX0.2	2.1	>99.9	very good
	LX0.6	2.5	>99.9	very good
	LX1.0	2.7	>99.9	very good
P. aeruginosa (ATCC 8739)	L	-	-	-
	LN0.2	1.6	>90.0	satisfactory
	LN0.6	1.6	>90.0	satisfactory
	LN1.0	1.9	>90.0	very googd
	LX0.2	1.8	>90.0	satisfactory
	LX0.6	2.1	>99.9	very good
	LX1.0	2.3	>99.9	very good

Bacteria are divided into two groups: Gram-negative and Gram-positive. This division is caused by differences in the structure of the cell wall; therefore, these bacteria have different effects, e.g., antibiotics, but also bacteriocins or other bactericidal substances. Gram (+)-bacteria do not have an outer cell membrane, and their wall is thicker and composed of a layer of peptidoglycans, which is murein. In Gram (−)-bacteria, an outer cell membrane is present, and their cell wall is thinner because it contains fewer layers of murein. Both *Escherichia coli* and *Pseudomonas aeruginosa* are Gram-negative bacteria, while *Staphylococcus aureus* is a Gram-positive bacterium. All bacterial strains used in the tests have recommendations from international standards that set the limits of the bactericidal

properties of materials. Each strain is a human pathogen, and the most common strains occur in nosocomial infections. The differences obtained in the results of the antibacterial properties of the film may depend on the strain's membership in a given group, but also on the type of a given microorganism, as well as on the difference in the mechanism of action of bacteriocins, which may consist in attacking the bacterial cell wall or the membrane surrounding the bacteria, interfering with bacterial reproduction or blocking protein production by bacteria.

The best antibacterial effect against *E. coli*, *S. aureus*, and *P. aeruginosa* was observed for PLA films containing the highest concentration of nisaplin, i.e., for samples (LN1.0) and natamax (LX1.0). Interestingly, biocidal properties against *S. aureus* were recorded for all samples with nisaplin (LN0.2, LN0.6, LN1.0) and for all samples with natamax (LX0.2, LX0.6, LX1.0). In addition to sample LX10, sample LX0.6 showed very strong bactericidal properties against *P. aeruginosa*. The remaining samples obtained satisfactory results in terms of bactericidal properties against *E. coli*, *S. aureus*, and *P. aeruginosa*.

It can be concluded that the higher (1.0%) the content of a given bacteriocin (nisaplin or natamax) in the PLA films, the better antibacterial effect was obtained. Work by other scientists also contains information regarding biocidal properties. The authors of these works used, among others bacteriocins, tar, oils. The similarity of these articles, including ours, lies in similar concentrations (e.g., 0.2, 1.0%) of the substances used and the type of microorganisms (e.g., *Staphylococcus aureus*, *Pseudomonas aeruginosa*) against which these properties were analyzed [35–39].

As a result of their activity, bacteriocins cause cell death (bactericidal effect) or inhibit the development of microorganisms and limit their reproduction (bacteriostatic effect). Bacteriocins cause the formation of pores in the cytoplasmic membrane or interfere with the biosynthesis of the cell wall (Figure 4).

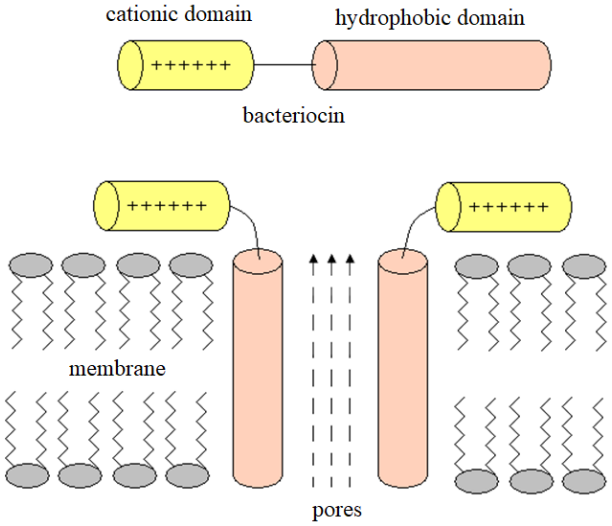

Figure 4. Mechanism of action of bacteriocins on the cytoplasmic membrane.

Through the created pores, ions, amino acids, and ATP molecules flow out and the membrane potential and the pH gradient are disturbed. The synthesis of macromolecules such as proteins, polysaccharides, DNA and RNA is inhibited as a result of low ATP levels and ion deficiency. However, due to the lack of nutrient transport, the growth and development of microbial cells is inhibited [8].

3.6. Fungistatic Properties

The fungicidal effect of the film is shown in Table 5.

Table 5. Assessment of microbial growth.

Sample	Mixture of Fungi (*A. niger*, *A. flavus*, *A. glaucus*, and *A. versicolor*)
Control mixture of fungi	5
L	4
LN0.2	3
LN0.6	2
LN1.0	0
LX0.2	4
LX0.6	3
LX1.0	0

By analyzing and determining the growth of fungi on the surface of the samples according to the scale in Table 6, it can be concluded that samples LN1.0 and LX1.0 are fungicidal, as the fungal growth on their surface was determined with the value "0". The LN0.2 samples turned out to be slightly worse, with fungal growth on the surface marked as "2". However, on the LN0.2 and LX0.6 films, fungal growth covered more than 25% of the surface (value "3"). The control sample, which was pure polylactide, was covered with fungi on more than 50% of its surface (value "4"). Microscopic images were taken for selected control samples and those with the strongest fungicidal activity and are presented in Figure 5. They show the results of the growth of four fungal strains after a period of four weeks, for the samples with the highest content of nisaplin (LN1.0) and natamax (LX1.0).

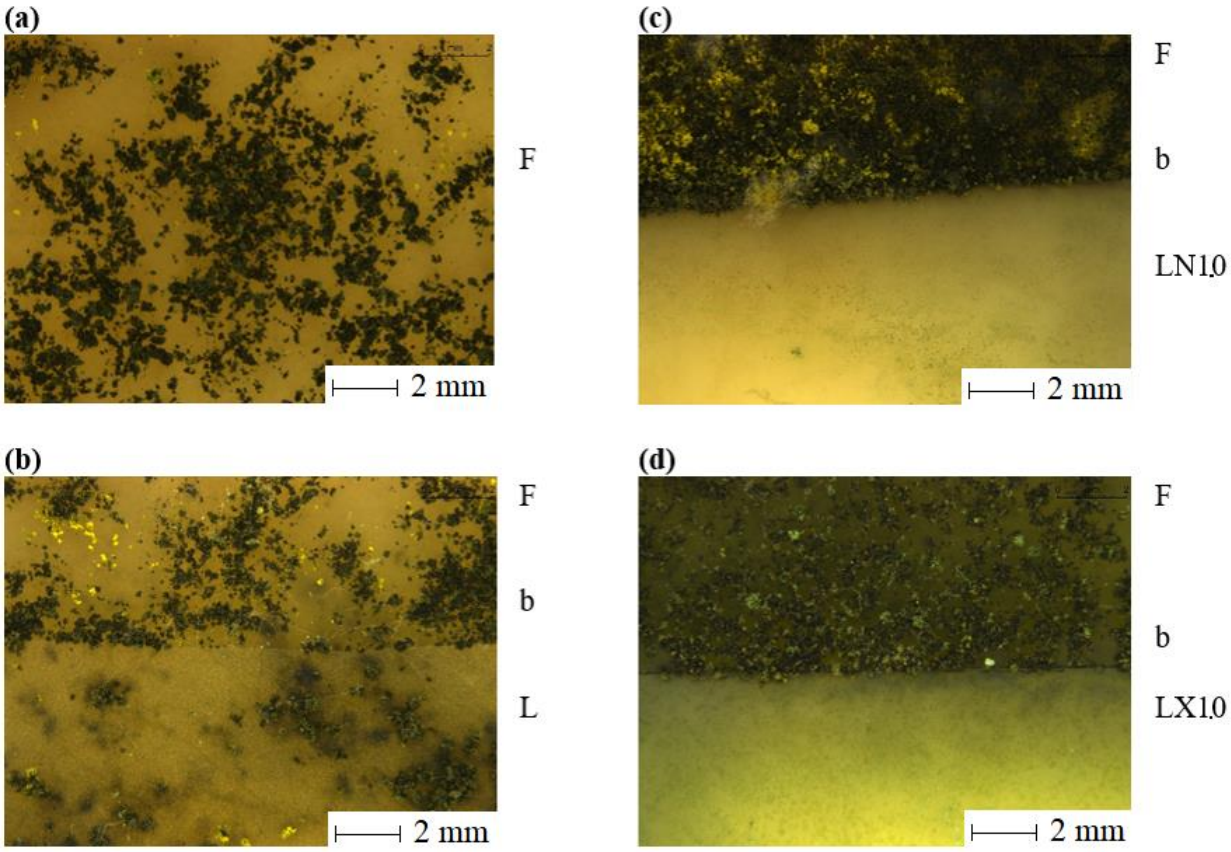

Figure 5. (**a**) Growth of fungi mix (*A. niger*, *A. flavus*, *A. glaucus*, and *A. versicolor*) on the surface films without (L), (**b**) on the surface of PLA (L), and (**c**) with 10% wt. nisaplin (LN1.0), (**d**) 10% wt. natamax (LX1.0). Abbreviations: F—agar with fungi, b—border line (between agar with fungi and the film).

Table 6. Growth of the *Salmonella typhimurium* strain around the sample indicating a mutagenic effect or its absence.

Symbol of Samples	Mutagenicity Effect
L	-
LN0.2	-
LN0.6	-
LN1.0	-
LX0.2	-
LX0.6	-
LX1.0	-

The results show that the analyzed films containing bacteriocins show bactericidal and antifungal properties. Compared with the results for films with the same content of substances, but obtained by the solvent method, the data are similar [40]. The strongest effect of the film on microorganisms was noted for the bacterial strain *A. versicolor* and the fungi *A. glaucus*, *A. niger*, and *A. flavus*. The paper [41] shows how important it is to know antifungal properties and what are the ways and possibilities of dealing with the problem of infections caused by fungi.

3.7. Ames Test—Determination of Mutagenicity

Table 6 presents the results of tests providing information on the mutagenicity of polymer films. The results obtained as a result of these analyses clearly indicate the lack of mutagenicity of all tested samples. Mutagenicity is determined on a scale of "+" or "−", respectively.

Many studies indicate that mutagenicity tests are extremely important in the analysis of chemical or biological substances. Particularly noteworthy is the work [42], which presents all important issues regarding the bacterial strains to be used in the study, the conditions for conducting the study, and the interpretation of the results. We conducted our work in a similar way, as described in this work.

4. Conclusions

PLA/nisaplin and PLA/natamax materials have a bactericidal effect against the following strains: *E. coli* (ATCC 8739P), *S. aureus* (ATCC 65388), and *P. aeruginosa* (ATCC 8739). Moreover, the effectiveness of the film against a mixture of *A. niger*, *A. flavus*, *A. glaucus*, and *A. versicolor* fungi was demonstrated. An additional advantage of the film is the confirmed lack of mutagenicity demonstrated using the Ames Test. In addition, water vapor permeability results at the level of 80 [g/m^2*24 h] and very good parameters regarding light transmittance and gloss of the film 80 [gU], prove its aesthetic values.

The obtained operational results confirm the potential utilitarian usefulness of PLA film with nisaplin and natamax in the concentration range of 0.2–1.0% wt. for use in the food packaging industry. Due to their biocidal and fungicidal properties and lack of mutagenicity, PLA films containing nisaplin or natamax are suitable for packaging food products such as cheese, cold cuts, pasta, and other flour products. In addition, they are also suitable for packing fruit (apples, pears, plums, bananas, grapes) or vegetables (carrots, leeks, chives, beetroots). Additionally, such foils can be used to pack gardening products such as plants, e.g., cut flowers (roses, tulips, carnations) and potted flowers (cacti, succulents, house flowers).

An undoubtedly desirable feature of our foils is aesthetics, which is extremely important in terms of marketing and advertising of a given product, which has been confirmed by very good gloss and transparency results.

5. Patents

Richert, A., Dąbrowska, G.B., Dąbrowski, H.P., 2020. Bactericidal polylactide film and the method of its preparation. Patent Application P.433979 (in Polish).

Richert, A., Dąbrowska, G.B., 2022. A Method of Obtaining a Biodegradable Film From Biodegradable Polymers and a Biodegradable Film Containing Biodegradable Polymers. Patent Application P.442284 (in Polish).

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