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Chitosan/Thyme Oil Systems as Affected by Stabilizing Agent: Physical and Antimicrobial Properties

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Abstract: Antimicrobial biopolymer films and coatings are of great interest for many applications. Different chitosan systems were prepared and characterized to evaluate the effect of their composition on the physical and antimicrobial properties. Three types of emulsifiers (Tween 20, 80, and 85) were used as stabilizing agents, combined with thyme essential oil (from two producers) applied as an active substance. A predominant role of the applied stabilizer and its hydrophilic–lipophilic balance value was proven. The incorporation of thyme essential oil and surfactant into the chitosan matrix led to a significant decrease of particle size in film-forming solutions, as well as a thickness increase and the enhancement of the barrier properties in chitosan films. Antimicrobial effects were provided even at the lowest tested concentration of thyme essential oil. Hence, the prepared chitosan films represent promising candidates in antimicrobial packaging applications.

Keywords: antimicrobial activity; biopolymer film; chitosan; surfactant; thyme oil

1. Introduction

Food and pharmaceutical packaging plays an important role in keeping the quality and freshness of the products and prolonging their shelf life. The selection of suitable materials with optimum environmental and utilization properties belongs among the key factors in developing an effective coating fulfilling the industrial and consumer's requirements [1–3]. Polysaccharides represent an important category of polymers with good biodegradability and biocompatibility, which are essential properties required from biomaterials. In this group, chitosan, comprising glucosamine and *N*-acetylglucosamine, ranks in a high position. This natural, nontoxic, biodegradable cationic polysaccharide is usually obtained through deacetylation of chitin and is available in various forms—solution, flakes, fibers, films, etc. [4,5]. Chitosan is applied in a range of industrial areas, e.g., as a chelating agent, flocculant for wastewater treatment, and a fungicidal agent for crop protection in agriculture. Other potential applications include cosmetics and skin care, and antimicrobial or anticoagulant agents. Chitosan has the ability to promote wound-healing processes because of its ability to support tissue regeneration and stimulate hemostasis. In addition, chitosan itself has proven

antimicrobial effects, which can also be effectively used in biomedical research [6]. The film-forming properties of chitosan can be exploited in tissue engineering, drug delivery systems, and the production of active food packaging [3,7].

These films and coatings can also serve as carriers of different active substances that include antimicrobials, antioxidants, or flavors. Among antimicrobial agents, bacteriocins, enzymes, organic acids, and/or essential oils can be mentioned [2]. It is known that essential oils represent attractive natural-based substances possessing antimicrobial properties that are strongly affected by the composition and functional groups of these biologically active compounds [8,9]. Ecological and geographical aspects, as well as harvest conditions, are the predominant factors here. The highest efficiency was shown in oils based on thyme, cinnamon, oregano, and clove [10,11]. Wang et al. [7] investigated effects of different essential oils in combination with chitosan film. The study revealed that antimicrobial properties were enhanced when cinnamon and clove bud oils were incorporated, and other physical properties were modified strongly depending on the specific oil type. Thyme essential oil is characteristic for a high content of biologically active compounds, such as thymol, carvacrol, p-cymene, and γ-terpinene. It was proven that carvacrol and thymol exhibit not only significant bacteriostatic and bactericidal properties, but also high antioxidant capacity [8,12,13]. Altiok et al. [8] prepared chitosan films enriched with thyme oil for potential application in healing wounds. The minimum inhibition concentration of thyme oil was set to 1.2% (v/v). Moreover, hydrophobic essential oils can improve not only the antimicrobial properties, but also the water-barrier properties of polymers [14]. In a study by Perdones et al. [15], chitosan systems with basil and thyme essential oils in combination with oleic acid were investigated. Water vapor permeability was improved in all samples. Barrier properties were also enhanced in chitosan films containing carvacrol and grape-seed extract [16].

Since the incorporation of essential oils may lower the system stability and cause phase separation, other substances are usually required to control the stability, homogeneity, and surface properties of polymer films. Among these, various amphiphilic compounds with both a hydrophilic and a hydrophobic part are worth mentioning. Hydrophilic–lipophilic balance (HLB) is an important factor determining the final functional properties and applications of a surface-active molecule [17]. The class of polysorbate non-ionic surfactants (commercially known as Tweens) comprise ethoxylated sorbitan attached to a hydrophobic chain based on a fatty-acid residue [18]. Tween 80 was applied as an emulsifier into chitosan solutions enriched with carvacrol and grape-seed extract [16]. The effects of Tween 20 and Tween 80 were compared in the study of Tongnuanchan et al. [14], who evaluated their impact on the structural and thermal properties of films based on fish skin gelatin incorporated with basil and citronella oils. A strong effect of surfactant type on microstructural and thermal properties of prepared systems was proven in Reference [17]. Tween 80 and Span 80 surfactants were added to the chitosan film-forming solution with lemon essential oil to evaluate their effects on structural, optical, and physical properties. While a more hydrophilic Tween 80 enhanced the compatibility and stability of essential oil in the chitosan matrix, Span 80 supported the oil transport to the film surface.

The objective of this work was to evaluate the physical and antimicrobial properties of chitosan systems prepared with various emulsifiers and enriched with thyme essential oil, with regard to their potential application as active packaging to enhance the quality and shelf life of food products, such as meat or vegetables.

2. Materials and Methods

2.1. Materials

Low-molecular-weight chitosan was provided by Sigma-Aldrich (Prague, Czech Republic). Two types of thyme essential oils were used, supplied by Nobilis Tilia s.r.o. (Krásná Lípa, Czech Republic) and Cosmetics Atok International s.r.o. (Trmice, Czech Republic). Emulsifiers Tween 20, 80, and 85 (T20, T80, T85) were supplied by Sigma-Aldrich. Microorganisms (*Escherichia coli* CCM 3954, *Salmonella*

enterica subsp. *Enterica ser. Typhimurium* CCM 7205, *Staphylococcus aureus* CCM 3953, *Bacillus cereus* CCM 2010, *Candida albicans* CCM 8215, and *Aspergillus niger* CCM 8155) were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic). Mueller–Hinton Agar (Himedia Laboratories, Telangana, India) at 37 °C (24 h) and Sabouraud Agar (Himedia Laboratories) at 20 °C (72 h) were used for the growth of bacterial and fungal cultures, respectively.

2.2. Analysis of Essential Oils by Gas Chromatography

Gas chromatography (GC) of essential oils was carried out on a DANI GC Master Fast Gas Chromatograph (Cologno Monzese, Italy) equipped with a Zebron™ ZB-5MS (Aschaffenburg, Germany) capillary column (30 m × 0.25 mm × 0.5 µm) and flame ionization detector (FID). The initial column temperature was set to 50 °C and then gradually increased to 120 °C (4 °C·min^{-1}), after which it was raised to 230 °C at 15 °C·min^{-1} (left for 15 min). The nitrogen was used as the carrier gas (flow rate 1 mL/min). The injection port was heated and maintained at 200 °C, and the detector temperature was 270 °C. The volume of diluted samples of the essential oils in 1 mL of methanol was 1 µL (split ratio of 1:25)

2.3. Preparation of Chitosan Dispersions and Films

The dispersions were prepared by dissolving 1 g of chitosan in 100 mL of acetic acid (1% *v*/*v*) under continuous stirring at ambient temperature for 24 h. After chitosan solution filtration, a mixture of emulsifier (20%) and thyme oil (80%), marked as TEO, was added in concentrations ranging from 0.5 wt.% to 5 wt.%. The dispersions were subjected to premixing on a Vortex V-1 Plus device (Biosan, Riga, Latvia) for 1 min and then homogenization with Ultra Turrax IKA® T-25 (IKA, Staufen, Germany) for 5 min at 15,600 rpm. After homogenization, 25 mL of the solutions were cast on sterile Petri dishes (9 cm in diameter) and dried at 35 °C in an air-circulated oven. Prior to further testing, the dried films were stored at 25 °C and 60% relative humidity. The description of samples is specified in Table 1.

Table 1. Designation of samples.

Sample	Essential Oil Supplier	Emulsifier Type
A20-*x*[1]	Cosmetics Atok	Tween 20
A80-*x*	Cosmetics Atok	Tween 80
A85-*x*	Cosmetics Atok	Tween 85
N80-*x*	Nobilis Tilia	Tween 80

[1]: *x* indicates the concentration of emulsifier and thyme oil (TEO), which ranged from 0.5 wt.% to 2 wt.%.

2.4. Particle Size and Zeta Potential of Film-Forming Solutions

The measurement was carried out on a Zetasizer Nano ZS device (Malvern Instruments, Ltd., Malvern, UK) after diluting the samples using filtrated distilled water (VWR® syringe filter 0.2 µm, Stříbrná Skalice, Czech Republic). The particle size was gauged via laser diffractometry (90° scattering angle, 1.33 refractive index, 0.001 absorption (Malvern Instruments, Ltd.). Zeta potential was analyzed using the Smoluchowski model. All measurements were carried out at 25 ± 1 °C.

2.5. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectra were recorded on an Alpha-T FTIR spectrometer (Bruker, Billerica, MA, USA). The essential oils were measured using the KBr disc method (1 mg of thyme oil was ground with 150 mg of KBr (FTIR purity Acros Organics)). The films were measured without any previous treatment. The spectrum was recorded in the spectral range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} (16 scans). Evaluation of the spectra was performed using the OPUS program (version 7.5).

2.6. Morphological Characterization by Scanning Electron Microscopy

Morphology of the surfaces and cross-sections of the chitosan films was visualized using a Vega 3 high-resolution scanning electron microscope (Tescan, Brno, Czech Republic). The samples were sputtered with a conductive coating layer prior to visualization.

2.7. Film Thickness

The film thickness was gauged with a digital micrometer (Schut, Trossingen, Germany) as an average of ten measurements from each film at an accuracy of ± 0.006 mm.

2.8. Contact Angle

The wettability of the chitosan films was analyzed via measuring the contact angles using the sessile drop method on a Surface Energy Evaluation System by Advex Instruments (Brno, Czech Republic) at ambient temperature. The final values were calculated as an average from three measurements. Demineralized water was used as the reference liquid; the volume of each deposited droplet was 5 µL.

2.9. Mechanical Properties

Prior to the measurements, the samples were conditioned at 25 °C and 50% relative humidity for 48 h. Mechanical properties were determined on a texture analysis machine TA1 Series (AMETEK Test & Calibration Instruments, Largo, FL, USA) using a NEXYGENPlus texture analysis software (version 4.0.1.184). For the puncture test, the films were cut to square-shaped samples (40×40 mm^2), which were placed between two plates with a circular hole (20 mm in diameter) and secured with a clamping device. A cylindrical probe (2 mm in diameter) was pressed through the center of the sample at a speed of 1 mm/s. The puncture strength (PS) in N·mm^{-1} was then calculated from four measurements according to Equation (1).

$$\mathrm{PS} = \frac{F_{\max}}{T} \tag{1}$$

where $F_{\max}$ is the maximum puncture strength (N), and T is the average thickness of the film sample (mm). Puncture deformation (PD) in mm was evaluated from the distance when the probe contacted the specimen and the break point.

Tensile strength (TS) of the films was measured on samples of 10 mm × 60 mm size. The ends of the strips were mounted into the tensile grips so that the exposed specimen area was 10 mm × 40 mm. The crosshead speed was set to 1 mm·s^{-1}. Tensile strength (TS) was then evaluated by the NEXYGENPlus software (version 4.0.1.184) from the maximum stretching strength (N), thickness, and width of the specimen (mm), according to Equation (2).

$$\mathrm{TS} = \frac{F_{\mathrm{t}}}{TW} \tag{2}$$

where F_{t} is the maximum tensile strength (N), T is the average thickness of the film sample (mm), and W is the width of the film sample (mm).

Elongation at break (E) was determined as a percentage by dividing the elongation at the break point by the initial specimen length multiplied by 100. The resultant values were calculated from four measurements.

2.10. Moisture Content and Water Solubility of Films

Chitosan samples (2×2 cm^2) were weighed (initial weight M_1) and then dried at 105 °C until constant weight (dry sample weight M_2). A moisture content was calculated as a percentage according to Equation (3).

$$\text{MC}\ (\%) = \left(\frac{M_1 - M_2}{M_1}\right) \times 100 \quad (3)$$

The dried film pieces were then immersed in distilled water and, after agitation (for 24 h at ambient temperature), they were carefully rinsed with water and transferred into an oven to dry at 105 °C until constant weight (M_3). The solubility in water was calculated according to Equation (4). All experiments were carried out in triplicate.

$$S(\%) = \left(\frac{M_2 - M_3}{M_2}\right) \times 100 \quad (4)$$

2.11. Water Vapour Barrier Properties of Films

Barrier properties were studied through to the measurement of water vapor permeability (WVP) according to ASTM E96–95 [19]. The films were sealed at the mouth of a test dish filled with distilled water (100% relative humidity) that was placed in a desiccator containing silica gel (0% relative humidity). The periodic weighing of dishes was carried out as a function of time every 2 h until changes in values did not differ in more than 0.001 g. The obtained values were used for the calculation of the water vapor transmission rate (WVT) in $g{\cdot}h^{-1}{\cdot}m^{-2}$ and the water vapor permeability (WVP) in $g{\cdot}Pa^{-1}{\cdot}h^{-1}{\cdot}m^{-2}$ according to Equations (5) and (6).

$$\text{WVT} = \frac{\left(\frac{m}{t}\right)}{A} \quad (5)$$

$$\text{WVP} = \frac{\text{WVT}}{\Delta p} = \frac{\text{WVT}}{S(R_1 - R_2)} \quad (6)$$

where $\frac{m}{t}$ is weight loss vs. time ($g{\cdot}h^{-1}$), A is the test area in m^2 (the mouth area of the test dish), Δp is the difference in water vapor pressure (Pa), S is the saturation pressure of vapor at the tested temperature, R_1 is relative humidity in the test dish expressed as a fraction, and R_2 is relative humidity at the vapor sink expressed as a fraction.

2.12. Antimicrobial Effectiveness of Essential Oils and Films

The agar diffusion method was used for testing the antimicrobial activity of chitosan films incorporated with thyme essential oil. The discs of chitosan films (9 mm in diameter) were put on agar plates previously inoculated with 1 mL of 0.5 McF turbid microbial suspension prepared in sterile saline solution. After incubation at given conditions, the diameters of inhibition zones around the discs were recorded. Antimicrobial testing was carried out in triplicate.

2.13. Statistical Analysis

The statistical evaluation of the experimental data was carried through one-way analysis of variance (ANOVA), using Statistica software (version 10, StatSoft, Inc., Tulsa, OK, USA), at the significance level of $p < 0.05$.

3. Results and Discussion

3.1. Analysis of Essential Oils by Gas Chromatography

It is well known that the composition of essential oils may differ substantially depending on a locality, conditions, and time of a plant collection. Results of gas chromatography (Table 2) confirm a similar composition in both thyme oils with predominant thymol and p-cymene, in addition to linalool, carvacrol, and β-caryophyllene. However, in Atok essential oil (EO) a higher thymol (51.7%) and carvacrol (2.7%) amount was found. On the other hand, a higher amount of p-cymene (25.3%) was detected in Nobilis EO compared to Atok (16.6%). Anyway, both oil samples were classified as a thymol

chemotype [20,21]. Regarding other components, differences between these two oils were negligible. Carvacrol and thymol are known as very effective biocides; thus, a significant effect on antimicrobial properties can be expected. This fact is in accordance with the microbiology testing (Section 3.10), where samples with Atok EO proved to be efficient even at the lowest tested concentration (0.5 wt.%).

Table 2. Constituents of Atok and Nobilis thyme oil.

Component	Atok		Nobilis	
	R_t (min)	Area (%)	R_t (min)	Area (%)
α-Pinene	8.78	0.7	8.78	2.9
β-Pinene	9.93	0.2	9.93	0.7
β-Myrcene	10.01	0.9	10.01	2.7
p-Cymene	11.03	16.6	11.04	25.3
Eucalyptol	11.28	0.9	11.29	2.3
Linalool	12.68	5.4	12.68	4.8
Camphor	13.79	0.2	13.80	1.6
Menthol	14.19	0.7	14.19	1.0
Terpinen-4-ol	14.28	1.0	14.28	1.2
Terpineol	14.48	0.3	14.48	0.2
Citronellol	14.89	0.5	14.89	0.5
Neral	15.03	0.5	15.04	0.4
Cinnamaldehyde	15.55	0.2	15.55	0.1
Thymol	15.68	51.7	15.70	34.5
Carvacrol	15.80	2.7	15.81	1.8
Eugenol	–	–	16.48	0.2
β-Caryophyllene	17.36	2.8	17.37	3.4
Bisabololoxid-A	17.55	0.1	17.55	0.1
α-Humulene	17.72	0.1	17.72	0.1
β-Farnesene	17.83	0.2	17.83	0.1

3.2. *Particle Size and Zeta Potential of Film-Forming Solutions*

Particle size and charge of film-forming solutions belong among the important factors that give evidence of the physical stability of the systems. It is generally known that if all the particles possess a sufficiently positive (>+30 mV) or negative (<−30 mV) zeta potential value, they repel each other and, thus, the stability of given systems increases. On the other hand, the stability dramatically decreases when the zeta potential value is in the range from 0 to ±10 mV, because of a higher tendency to aggregate [22]. Zeta potential expresses an electrostatic charge depending on physicochemical properties of liquids, and affects the processes such as aggregation, adsorption, and dispergation. The effect of rising TEO concentration on ζ-potential value is obvious in Table 3. As shown below, the value for A85-0.5 (57.3 mV) is almost coincident with the ζ-potential of chitosan that was 58.7 mV. As far as other systems are concerned, the lowest TEO concentration induced a ζ-potential value drop. The results also revealed a different trend depending on the emulsifier used. The ζ-potential of samples A20 and A85 increased with higher TEO content up to ca. 60 mV, which exceeds the value of pure chitosan solution (58.7 mV). This value corresponds to the fact that, in an acid environment, the amino groups in the chitosan molecule are positively charged. It is expected that, at TEO concentrations used (0–2 wt.%), applied emulsifiers (Tween 20 and 85) support chitosan adsorption at particle boundaries. A similar phenomenon was shown in Reference [15], who focused on films based on chitosan with thyme and/or basil oil containing oleic acid as a potential stabilizing agent. The presence of oleic acid led to an increase in ζ-potential, probably due to its adsorption onto EO droplets followed by interaction with chitosan molecules. On the other hand, in samples with Tween 80, increasing TEO content resulted in a rather decreasing potential, regardless of the type of essential oil. A primary effect of essential oil is presumed here, since both EO types had a negative ζ-potential, specifically −23.1 and −25.6 mV for Atok and Nobilis Tilia oil, respectively. Similarly, Bonilla et al. [23] recorded negative values of thyme essential oil that was then combined with chitosan polymer. Several factors

clearly influence the final ζ-potential values, in addition to the charge of individual components, whereby the HLB value of applied emulsifiers is also important [24,25]. Even the potential impurities affecting the resultant charge of the system have to be considered. However, a sufficient electrostatic stabilization was proven in the prepared chitosan film-forming solutions due to the obtained positive zeta potential values exceeding +30 mV in most cases. Following the fact that a direct relationship between the stability of film-forming solutions and films can be presumed, even the physical and stability properties of prepared chitosan/TEO films could be easily tailored to specific applications.

Table 3. Zeta potential and particle sizes of chitosan systems with different combinations of essential oil (EO) and emulsifiers. TEO—thyme essential oil and emulsifier.

TEO Concentration (wt.%)	Sample			
	A20	**A80**	**A85**	**N80**
Zeta Potential (mV ± S.D.)				
0	58.7 ± 1.2^{a}			
0.5	$40.5 \pm 5.7^{b,A}$	$43.8 \pm 1.7^{b,A}$	$57.3 \pm 0.8^{a,B}$	$34.1 \pm 1.7^{b,C}$
1	$37.4 \pm 1.2^{b,A}$	$28.3 \pm 1.4^{c,B,C}$	$30 \pm 3.0^{b,c,A}$	$27.9 \pm 1.2^{c,B,C}$
2	$50.6 \pm 1.2^{c,A}$	$30.1 \pm 1.2^{c,B}$	$59.6 \pm 0.7^{a,C}$	$34.2 \pm 0.9^{b,D}$
Particle Size (nm ± S.D.)				
0	4050 ± 79^{a}			
0.5	$5700 \pm 491^{b,A}$	$5200 \pm 157^{a,A}$	$6900 \pm 596^{b,B}$	$5600 \pm 273^{b,A}$
1	$4100 \pm 251^{a,B}$	$4600 \pm 89^{a,A}$	$5400 \pm 220^{c,B}$	$3030 \pm 77^{c,C}$
2	$1640 \pm 56^{c,A}$	$2220 \pm 64^{b,B}$	$2650 \pm 51^{d,C}$	$1910 \pm 70^{d,D}$

$^{a–d}$ and $^{A–D}$: different lower-case/upper-case letters in the same column/line indicate significant differences, respectively ($p < 0.05$).

Size of particles is an important factor influencing the final physical and sensory properties, such as appearance, structure, stability, and taste. In all samples, a monomodal particle size distribution was obtained, which indicates an optimum interfacial coverage of the oil particles. Table 3 summarizes the particle size of individual film-forming solutions with different concentrations of EO and emulsifier. It is obvious that, at the lowest tested concentrations (0.5 wt.% and 1 wt.% TEO), the particles were quite large, exceeding the measurability limit of the Zetasizer instrument and also the average particle size of unmodified chitosan solution in acetic acid. However, in the case of all samples, the increasing TEO content led to a substantial decrease in the size of particles. The most significant drop (91%) between the lowest and highest TEO concentration was visible in Atok oil with Tween 20 (HLB 16.7). Certain differences can be seen in the sample with Atok and Tween 85 (HLB 11), where the average particle size for the lowest concentration was about 1000 nm higher when compared to other emulsifiers. The bigger particles were also seen in other samples containing Tween 85 with the lowest HLB value. This result corresponds with the SEM analysis (Section 3.4). Evaluating the film forming solutions (FFS) with the same emulsifier (Tween 80, HLB 15) and different thyme oils (Atok and Nobilis), comparable values of particle size were obtained. This fact gives evidence of the significant effect of emulsifier type and its HLB value. In some other works [7,14,23], the addition of essential oils caused aggregation, flocculation, and/or even phase separation. No similar phenomena were observed in our samples, and a high emulsification activity of all surfactants was shown.

3.3. Fourier-Transform Infrared Spectroscopy (FTIR)

The mutual interactions between chitosan polymer and active/stabilizing agents were studied by Fourier-transform infrared spectroscopy; obtained results for chitosan film and chitosan film enriched with 2 wt.% TEO are shown in Table 4 and Figure 1. As can be seen from the main absorption bands (Table 4), thyme oil exhibited some characteristic peaks, e.g., at 3390 cm^{-1} (O–H), 2961 cm^{-1} (C–H stretching of methyl and isopropyl groups on thymol phenolic structure), 1619 cm^{-1} (conjugated

double bond of ring), 1457 cm^{-1} (CH deformation), and 1289 cm^{-1} (C–O–C) [26,27]. The FTIR spectrum of chitosan showed characteristic signals between 3500 and 3000 cm^{-1} related O–H and N–H bonds in the amino group. Due to the addition of TEO, this band was weakened, which suggests the fact that the most reactive functional amino and hydroxyl chitosan groups were weakened too, probably due to the interactions with thyme oil functional groups [28]. Bands located in the wavelength range from 2950 to 2800 cm^{-1} correspond to C–H stretching from the $–CH_2$ chitosan group. Following chitosan modification with thyme EO, a peak at 2959 cm^{-1} was indicated. Newly emerged peaks in the wavelength range from 900 to 600 cm^{-1} refer to the potential interaction between chitosan and EO, i.e., incorporation of EO into the chitosan film [26]. A comparison of spectra in the selected interval (1800–400 cm^{-1}) of the chitosan film and the chitosan film enriched with 2 wt.% TEO is shown in Figure 1.

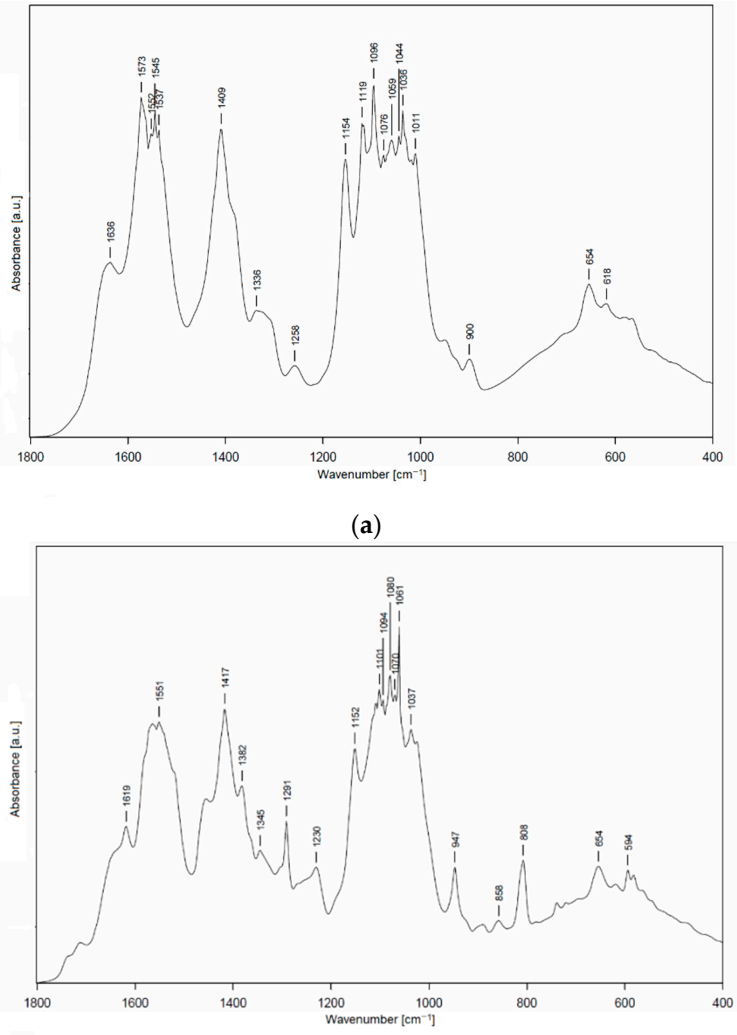

Figure 1. Fourier-transform infrared (FTIR) spectra of the chitosan film (**a**) and the chitosan film modified with 2 wt.% TEO (**b**) in the interval 1800–400 cm^{-1}.

Table 4. The main signals of Fourier-transform infrared (FTIR) analysis.

Wavelength (cm^{-1})	Chitosan (cm^{-1})	EO Atok (cm^{-1})	Film with TEO (cm^{-1})
3000–3500 (N–H, O–H)	3510, 3466, 3435, 3417, 3393, 3358, 3333, 3315, 3290, 3266, 3245, 3224, 3186, 3174, 3156, 3125, 3102	3390	3372
2500–3000 (C–H)	2923, 2891	2961, 2927, 2870	2959
1500–2000 (amides with C=O, N–H)	1636, 1573, 1545	1619, 1584, 1517	1618, 1560
1000–1500 (C–O, C–C)	1409, 1336, 1258, 1154, 1119, 1096, 1036, 1011	1457, 1420, 1381, 1289, 1227, 1153, 1112, 1088, 1060	1417, 1382, 1291, 1230, 1152, 1089, 1061
500–1000 (C–H aromatic)	900, 656	945, 858, 809	947, 858, 808, 654, 594

3.4. Morphological Characterization by Scanning Electron Microscopy

After the film preparation, a visual evaluation of the physical appearance was carried out. Control chitosan films were smooth and transparent, with a slightly yellow tinge. They became darker yellow and more opaque when TEO was added. However, even at 2 wt.% TEO concentration, the surface of the films was continuous with no deformation or greasy feeling (Figure 2).

(**a**) (**b**) (**c**)

Figure 2. Appearance of chitosan films: (**a**) control; (**b**) A80-0.5; (**c**) A80-2.

Microstructural changes in polymer films caused by the modification with TEO were observed with the help of scanning electron microscopy. Figure 3 shows the surfaces and cross-sections of chitosan films without and with Atok thyme EO and various emulsifiers (TEO content was 2 wt.%). As shown below, the chitosan film with the absence of any active substances and/or emulsifiers had a compact homogeneous structure without pores or fractures. On the other hand, films modified with TEO showed a structure with droplets of various sizes, depending on the type of surfactant used. The sample containing Tween 20 revealed no uniform particle distribution, ranging from 2 to 5 μm both on the surface and cross-section (Figure 3b). In the case of Tween 80 (Figure 3c), an increase in the number of droplets was evident on the film surface; in the cross-section, a relatively even distribution of particles with lower polydispersity degree (on average from 2 to 3 μm) was seen. Figure 3d (left), i.e., the film containing Tween 85, reveals the bigger markedly spherical-shaped formations, whereas the cross-section of the same sample shows rather oval particles (Figure 3d (right)), which could be the consequence of the drying process of the film and the solvent evaporation. Amongst the emulsifiers tested, Tween 80 provided the most uniformly distributed particles, which were maintained within the polymer structure without the tendency to show creaming or other instability behavior. A profound role of surfactant was found by Tongnuanchan et al. [14], who tested gelatin films with essential oils and three types of emulsifiers. While soy lecithin ensured a smooth and continuous structure, Tween emulsifiers (20 and 80) resulted in discontinuity and general instability.

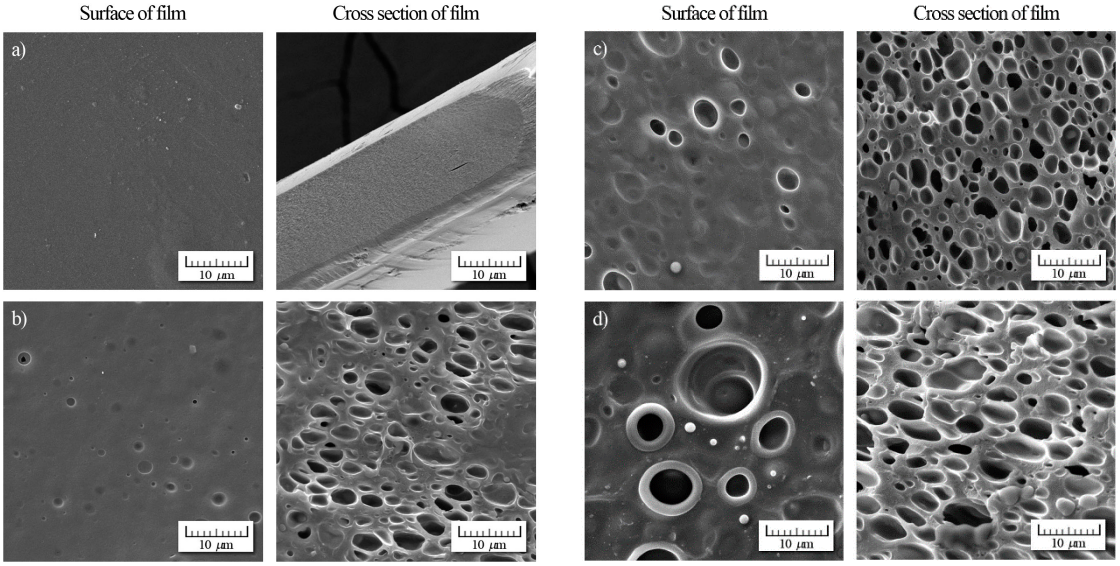

Figure 3. SEM of surfaces (**left**) and cross-sections (**right**) of chitosan films with 2 wt.% TEO (Atok): (**a**) chitosan; (**b**) Tween 20 (HLB 16.7); (**c**) Tween 80 (HLB 15); and (**d**) Tween 85 (HLB 11).

Structural changes significantly depend on components included in the original film-forming solution; thus, this refers to the presence of hydrophobic essential oils, as well as emulsifiers with different HLB values, in our case. The most noticeable particles were revealed in films with Tween 85, which has the lowest HLB (11) from all tested surfactants. It is supposed that this emulsifier has a tendency to shift the oil droplets close to the film surface, which is evident from Figure 3d (left). The effect of surfactant on the morphology of chitosan films containing EO was studied in the paper of Peng et al. [17]. It was proven that the emulsifier with higher HLB (Tween 80) ensured the formation of a more homogeneous and smoother structure. An emulsifier Span 80 with significantly lower HLB was applied here for comparison purposes. Nonetheless, a substantial effect of emulsifier type on the morphological characteristics was confirmed. The type of essential oil affects the final film structure as well. As stated in the study of Valderrama et al. [29], the incorporation of thyme oil into chitosan led, as with our samples, to the formation of oil droplets anchored in the polymer matrix. The addition of cinnamon EO resulted in a rather sheet-like, multilayered, and more compact structure. On the other hand, clove EO caused a more noticeable deformation in the form of cracks [30,31].

3.5. Film Thickness

It is well known that the thickness of polymer films can be considered another important characteristic that may influence the barrier properties. The thickness of chitosan film without TEO was 0.04 ± 0.01 mm. A higher TEO concentration led to increasing thickness, due to the increased interactions between applied active substance, emulsifier, and chitosan. The values of thickness in the samples containing various combinations of EOs and emulsifiers did not differ significantly, and ranged from 0.04 to 0.14 mm (Table 5). The increasing thickness of chitosan films with essential oil addition was proven in the study of Sun et al. [25], in which the value ranged from 0.09 to 0.13 mm. Similarly, in the work of Ojagh [30], the measured thickness increased with a higher active substance concentration; the value was about 0.11 mm for the samples containing 2 wt.% cinnamon EO.

Table 5. Thickness (unit: mm) of chitosan films.

TEO Concentration (wt.%)	Sample			
	A20	**A80**	**A85**	**N80**
0	0.04 ± 0.01^{a}			
0.5	$0.07 \pm 0.01^{b,A}$	$0.05 \pm 0.01^{a,B}$	$0.04 \pm 0.02^{a,B}$	$0.06 \pm 0.01^{b,A}$
1	$0.08 \pm 0.01^{c,A}$	$0.12 \pm 0.02^{b,B}$	$0.07 \pm 0.01^{b,A}$	$0.07 \pm 0.01^{b,A,C}$
2	$0.11 \pm 0.03^{d,A}$	$0.14 \pm 0.03^{c,B}$	$0.10 \pm 0.02^{c,A}$	$0.11 \pm 0.01^{c,A}$

$^{a–d}$ and $^{A–D}$: different lower-case/upper-case letters in the same column/line indicate significant differences, respectively ($p < 0.05$).

3.6. Contact Angle

Contact angle is defined as the angle between a film surface and the tangent leading from the contact position of a liquid drop on the surface. It is assumed that contact-angle value will increase with sample hydrophobicity [30].

The pure chitosan film revealed a relatively high contact angle of 60.7°. However, it was proven that chitosan hydrophilicity is also significantly dependent on the deacetylation degree (DA), which was 87% in our chitosan sample; the contact angle increases with higher DA [32]. The contact angles of modified chitosan films with TEO (concentration range from 0.5 wt.% to 2 wt.%) are summarized in Table 6. It is clear that a drop in contact angle occurred in all cases when compared to the control sample. This fact reveals the predominant effect of hydrophilic emulsifier Tween. On the other hand, the samples containing the same emulsifier Tween 80, but diverse types of thyme essential oil exhibited significantly different results. The contact angle measured with Nobilis EO was higher, approaching the values of unmodified chitosan film. The reason could lie in the different composition of these essential oils. For example, a higher amount of polyphenols with their hydrophilic groups can lead to increased interactions of the film surface with water, followed by a decrease in contact angle. This phenomenon was also discussed by other authors [1,25]. However, it is important to state that the values are strongly dependent on the location of a droplet's placement on the film surface. In the case where the sample is not ideally homogeneous, the results may be substantially affected.

Table 6. Contact angles (°) of chitosan films.

TEO Concentration (wt.%)	Samples			
	A20	**A80**	**A85**	**N80**
0	60.7 ± 1.0^{a}			
0.5	$30.7 \pm 0.5^{b,A}$	$46.0 \pm 0.8^{b,B}$	$42.1 \pm 1.8^{b,B}$	$58.3 \pm 1.2^{a,b,C}$
1	$39.0 \pm 2.0^{c,A}$	$37.0 \pm 4.0^{b,c,A}$	$43.1 \pm 0.8^{b,A}$	$56.0 \pm 1.0^{b,c,B}$
2	$47.7 \pm 0.7^{d,A}$	$27.5 \pm 1.6^{c,B}$	$42.0 \pm 3.0^{b,A}$	$56.4 \pm 1.2^{c,C}$

$^{a–d}$ and $^{A–D}$: different lower-case/upper-case letters in the same column/line indicate significant differences, respectively ($p < 0.05$).

3.7. Mechanical Properties

Mechanical properties are among the most important characteristics for final applications of polymer films. Tensile strength, elongation at break, puncture strength, and puncture deformation were measured for chitosan samples selected on the basis of previously ascertained physical and antimicrobial properties. Tensile strength is defined as the maximum stress exerted on a specimen during a tensile test [8]. As may be seen in Table 7, the tensile strength of the chitosan control sample was 35 MPa, which is comparable with the value attained in the work of Pranoto et al. [33] (37 MPa). The incorporation of thyme oil and Tween surfactants led to a significant decrease (average 50%) in tensile strength compared to the control chitosan sample. The loss of tensile strength after the incorporation of active substances, which can be a result of the film structure's disruption, was also noticed in some of other studies [8,31,33]. This result was previously supported by Cagri et al. [34]

who confirmed that all other additives except cross-linking agents caused a decrease in tensile strength. Elongation at break, which gives information on the specimen elasticity, was 5.4% for the control sample, and the value increased (from 8% to 9%) with TEO addition except for the film containing Nobilis thyme oil, in which the value of elongation decreased when compared to the control sample. Similarly, samples with a higher degree of elasticity were prepared by Hosseini et al. [31] by incorporating thyme and clove oil into chitosan films. On the other hand, the addition of cinnamon oil resulted in a significant drop in this parameter. A loss of elasticity was observed in chitosan samples containing different ratios of carvacrol and grape-seed extract, or containing lemon oil [1,17]. Tensile properties definitely depend on the preparation conditions, as well as composition, especially the presence of a plasticizer.

Table 7. Mechanical properties of chitosan films (TS: tensile strength, *E*: elongation at break, PS: puncture strength, PD: puncture deformation).

Sample	TS (MPa)	*E* (%)	PS (N/mm)	PD (mm)
Control	35.2 ± 1.2^{a}	5.4 ± 0.4 a	238.2 ± 37.8^{a}	3.9 ± 0.4^{a}
A20-2	13.6 ± 1.1^{b}	9.0 ± 0.9^{b}	56.3 ± 1.8^{b}	$3.1 \pm 0.6^{b,c}$
A80-2	16.7 ± 0.7^{c}	8.8 ± 0.8^{b}	67.5 ± 2.4^{c}	$3.4 \pm 0.5^{a,b}$
A85-2	15.8 ± 1.3^{c}	8.3 ± 1.1^{b}	53.6 ± 1.0^{b}	2.6 ± 0.2^{c}
N80-2	11.7 ± 0.9^{b}	4.8 ± 1.0^{a}	73.3 ± 1.9^{c}	$3.2 \pm 1.1^{a,b}$

[a–d]: different lower-case/upper-case letters in the same column/line indicate significant differences, respectively ($p < 0.05$).

Puncture strength (PS) and puncture deformation (PD) report on the specimen integrity and behavior during penetration. The values of PS in Table 7 point to the significant drop (ca. 75%) in puncture strength in films modified by TEO in comparison to the control chitosan sample. With regard to the effect of individual emulsifiers, similar values ($p > 0.05$) were obtained for the pairs A20-2 and A-85-2, and A80-2 and N80-2. The values of puncture deformation were also lower when compared to the chitosan sample and ranged from 2.6 to 3.2 mm in modified samples.

When mechanical properties of the samples containing various emulsifiers were compared, the film with Tween 80 could be emphasized as having higher tensile and puncture strength values. This result is in accordance with the SEM analysis that proved the most homogeneous structure with uniformly distributed particles in this sample (Section 3.4).

3.8. Moisture Content and Water Solubility of Films

Increasing concentration of essential oil mostly contributes to formation of covalent links among functional groups of chitosan chains. This process is then followed by lower availability of hydroxyl and amino groups, which limits the polysaccharide–water interactions [30]. The values in Table 8 show a slightly decreasing trend of moisture content (MC) when TEO concentration rose from 0.5 to 2 wt.% TEO. The decrease was also reported when the values were compared to pure chitosan film (20.93%). A similar trend, i.e., a decrease in MC of chitosan films with EO content, was reported in the study of Ojagh [30], who modified chitosan with various concentrations of cinnamon essential oil (up to 2 vol.%) and a constant concentration of Tween 80. A significant drop in water content was also observed in chitosan samples enriched with young apple polyphenols [25].

Table 8. Moisture content and solubility of chitosan films.

TEO Concentration (wt.%)	Sample			
	A20	**A80**	**A85**	**N80**
Moisture Content (wt.%)				
0	20.93 ± 0.6^{a}			
0.5	$19.6 \pm 0.4^{a,A}$	$18.5 \pm 0.8^{a,b,A}$	$24.0 \pm 2.5^{a,A}$	$17.47 \pm 1.0^{a,b,A}$
1	$17.5 \pm 0.4^{b,c,A}$	$16.6 \pm 0.3^{b,A}$	$19.0 \pm 1.0^{a,A}$	$14.8 \pm 0.6^{b,A}$
2	$17.31 \pm 0.1^{c,A}$	$16.7 \pm 0.1^{b,B}$	$21.0 \pm 1.0^{a,A}$	$13.8 \pm 0.3^{b,BC}$
Solubility (wt.%)				
0	22.5 ± 0.3^{a}			
0.5	$23.2 \pm 0.1^{a,A}$	$19.6 \pm 1.3^{a,A}$	$20.0 \pm 0.5^{a,A}$	$18.8 \pm 2.5^{a,A}$
1	$32.7 \pm 0.5^{b,A}$	$26.5 \pm 0.2^{b,B}$	$24.0 \pm 3.0^{a,B}$	$32.5 \pm 6.1^{b,A}$
2	$38.8 \pm 0.1^{c,A}$	$31.6 \pm 0.0^{c,B}$	$27.0 \pm 1.0^{a,C}$	$33.1 \pm 0.1^{c,D}$

$^{a–d}$ and $^{A–D}$: different lower-case/upper-case letters in the same column/line indicate significant differences, respectively ($p < 0.05$).

The solubility of polymer films refers to their resistance to water. The results from solubility measurements of the chitosan films are shown in Table 8. The solubility reached at 0.5 wt.% TEO approached the values of pure chitosan (22.5%). This value was comparable with other studies [2,16]. When a higher amount of TEO was added, a significant ($p < 0.05$) solubility increase could be seen in most of samples. This increase can be related to the hydrophilic groups included in phenolic compounds that dispose to interactions with water molecules. These results were confirmed even by the contact-angle measurements (see Section 3.6). The highest solubility was achieved in the samples with Tween 20 (HLB 16.7), unlike the samples with Tween 85 emulsifier (HLB 11) that showed values ranging from 20% to 27%. With regard to TEO concentration, a higher solubility was measured with the content of 2 wt.% (up to 38% in case of Tween 20). A similar trend was shown in a study [25] investigating chitosan films with polyphenols, which are common components of thymol oil. The solubility of samples containing 0.25% active substance was 19.93%, which was then increased to 40.56% at 1% active substance concentration. On the contrary, the moisture content of these samples decreased from 25.9% to 16.94%. In the work of Peng et al. [17], the incorporation of lemon essential oil promoted a reduction in solubility, whereas the combination with Span 80 surfactant induced a significant increase in solubility, although this type of surfactant is characteristic for its lower HLB value. This phenomenon could be explained by the cross-linking effects between chitosan and lemon oil in the absence of surfactant. On the other hand, the resultant system did not exhibit high stability, which led to migration of essential oil from the film surface, an increase in the contact area with water, and a consequent solubility increase. It is clear that the aforementioned physical properties are, similarly to particle size and zeta potential, affected by multiple factors. In addition to the applied essential oil, the emulsifier type and its HLB value are important parameters that can determine both the interactions between individual components and the potential solubilization of thyme oil into the amphiphilic structure of emulsifiers.

3.9. Water Vapor Barrier Properties of Films

The water vapor permeability (WVP) of polymer films should be low for optimum practical applications in order to prevent or at least reduce the transfer of moisture between ambient atmosphere and a protective film. The water vapor transfer itself depends on the ratio of hydrophobic and hydrophilic parts of the film, as well as thickness or polymer crystallinity. It is known that amorphous structures transmit a higher proportion of water vapor than crystalline polymers, in which the crystalline structure impedes the transfer of water molecules [35].

The permeability is generally expressed as time of water vapor transfer per material area unit caused by the pressure difference between two surfaces under give conditions of temperature and

moisture. The main purpose is to lower the oxygen presence, which significantly shortens a shelf life of products [36]. The effect of chitosan modification by thyme essential oil and different surfactants (TEO concentration 2 wt.%) on barrier properties of the films is graphed in Figure 4. Apparently, a significant drop of water vapor permeability was achieved in all modified samples when compared to the WVP of pure chitosan film, which was higher by one order of magnitude (16.5×10^{-3} g·Pa^{-1}·h^{-1}·m^{-2}). The lowest permeability (WVP = 9.6×10^{-3} g·Pa^{-1}·h^{-1}·m^{-2}) was obtained with the sample A85-2, i.e., the film containing Atok essential oil and Tween 85. The WVP values of A20-2 and A80-2 samples were almost identical (ca. 10×10^{-3} g·Pa^{-1}·h^{-1}·m^{-2}).

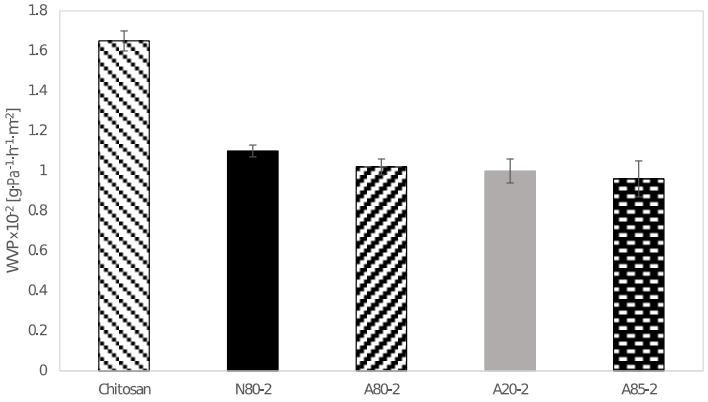

Figure 4. Water vapor permeability (WVP) of chitosan films without and with 2 wt.% thyme essential oil and emulsifier (TEO).

The effect of incorporation of essential oils into the chitosan matrix on WVP decrease was also observed by other authors. Ojagh et al. [30], who studied the effects of various concentrations of cinnamon oil for potential applications in active packaging, proved that not only did WVP decrease, but the film thickness was also affected by this modification. Observing the physical, antioxidant, and antimicrobial properties of chitosan films enriched with carvacrol resulted in a similar trend of water vapor drop, which was explained by the modification of hydrophobic film parts caused by a change in ratio of chitosan and carvacrol [25,37].

3.10. Antimicrobial Effectiveness of Films

It is known that chitosan itself has some antimicrobial properties, arising from its molecular structure, which contains free amino groups that can bind to bacterial cell surfaces, leading to disruption of the membranes and leakage of intracellular components [38]. However, chitosan films prepared by the solvent casting technique from acetic acid solution did not show an inhibition zone around the testing disc, while microbial growth was only suppressed directly under the sample (in Figures 5–7, this is indicated by the straight line related to the diameter of the testing disc). This was probably due to the limited activity and diffusion of chitosan molecules entrapped in the film [39].

The results of antimicrobial testing of the chitosan samples enriched with thyme essential oil measured by the agar diffusion method against six different pathogens are given in Figures 5–7. Generally, it was shown that increasing TEO concentration led to higher inhibition zones.

In the case of Tween 20, already the lowest TEO concentration (0.5 wt.%) was efficient against Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*) and Gram-negative (*Escherichia coli*, *Salmonella typhimurium*) bacteria (Figures 5 and 6). As can be seen in Figure 7, there was no antifungal activity at this TEO content. On the other hand, at 2 wt.% TEO, the inhibition zones were almost twice as big as in the case of bacteria (almost 50 mm against *Aspergillus niger*). A similar trend was observed with the samples containing Atok thyme oil and Tween 80 or Tween 85. Antibacterial activity was shown already at 0.5 wt.%, while the inhibition of *Candida albicans* and *Aspergillus niger* was obtained at higher TEO concentrations.

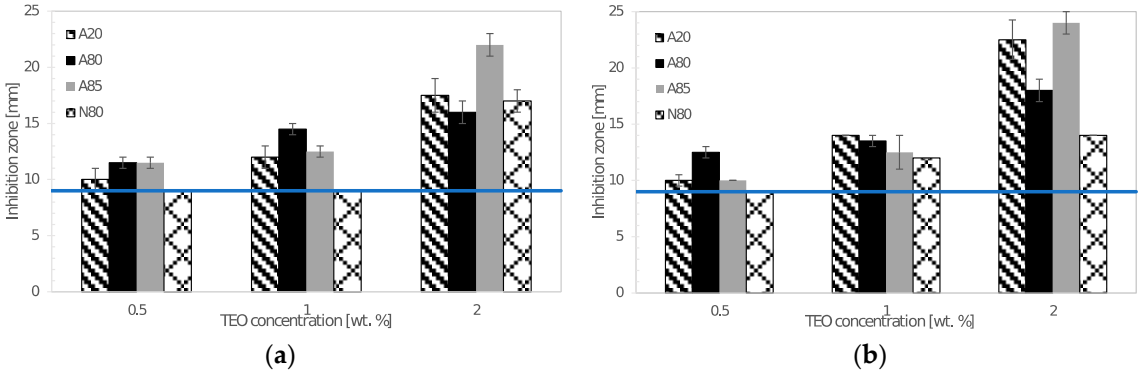

Figure 5. Inhibition zones of chitosan films against (**a**) *Escherichia coli* and (**b**) *Salmonella typhimurium*. The straight line indicates the control chitosan sample.

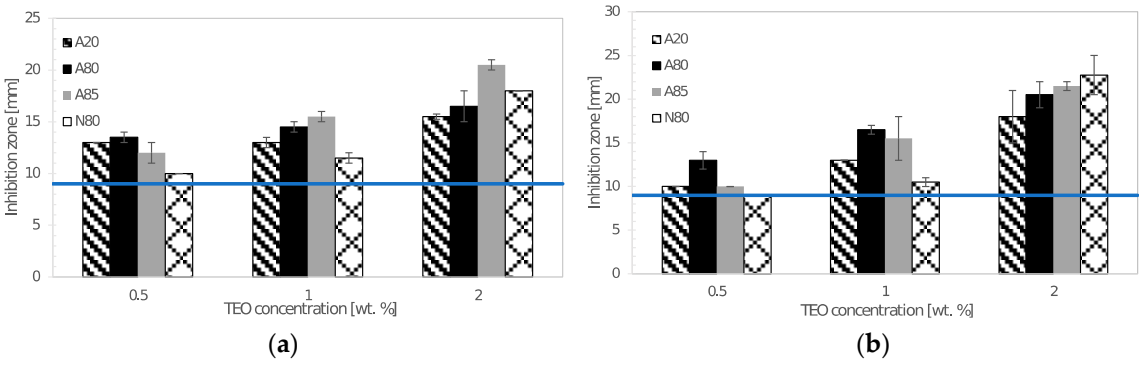

Figure 6. Inhibition zones of chitosan films against (**a**) *Staphylococcus aureus* and (**b**) *Bacillus cereus*. The straight line indicates the control chitosan sample.

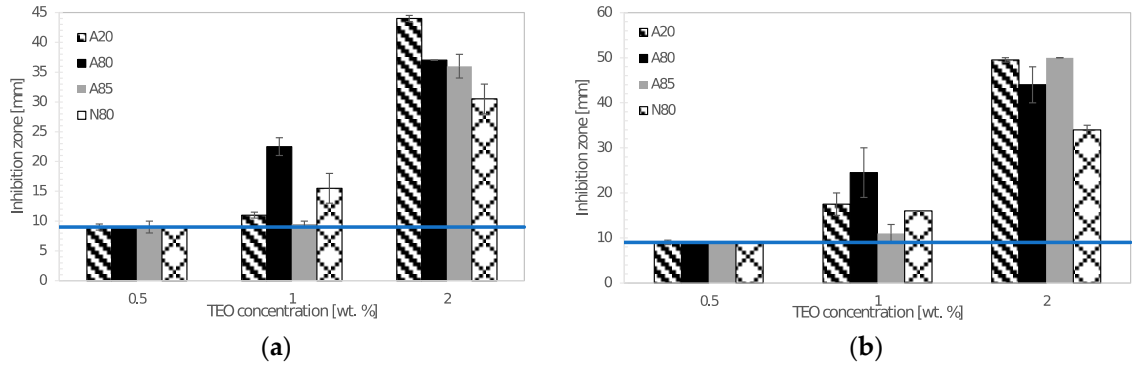

Figure 7. Inhibition zones of chitosan films against (**a**) *Candida albicans*, and (**b**) *Aspergillus niger*. The straight line indicates the control chitosan sample.

Slightly different results were obtained with the samples containing thyme essential oil from Nobilis Tilia and Tween emulsifier 80 (N80). Inhibition effect at the lowest TEO concentration (0.5 wt.%) was observed only against bacteria *Staphylococcus aureus* (Figure 6a). *Escherichia coli* proved a higher resistance against chitosan films (even at 1 wt.% TEO concentration, no inhibition zone around the sample was shown). Generally, the samples containing Nobilis EO exhibited rather lower activity against tested microorganisms when compared with Atok essential oil. This can be explained by a different amount of active substances in both oils, especially thymol and carvacrol. Maximum inhibition zone (50 mm) was shown against *Aspergillus niger* with the sample containing Tween 85 and Atok EO.

Antimicrobial effects of thyme oil were also confirmed in other works [40,41]. Hosseini et al. [10] investigated chitosan-based films enriched with thyme and clove essential oil, from which the thyme

EO exhibited the stronger antimicrobial activity against Gram-positive *Listeria monocytogenes* and *S. aureus*, and Gram-negative *Salmonella enteridis*. The tested concentrations were 0.5%, 1%, and 1.5% *v*/*v*. In the work of Ballester-Costa et al. [41], four *Thymus* species were applied into chitosan films to study the antibacterial and antioxidant properties. An agar disc diffusion method showed the higher efficiency of *Thymus mastichina* and *Thymus capitatus*. The chitosan film itself was not effective against the tested bacteria.

It is assumed that a predominant effect consists in the type and amount of components present in the essential oil. As shown by GC analysis (Section 3.1), the thyme essential oil includes a significant ratio of thymol, a hydrophobic phenolic constituent, which is believed to be responsible for antimicrobial action.

4. Conclusions

Low-molecular-weight chitosan was enriched with stabilizing agents of different HLB values (Tween 20, 80, and 85) and thyme essential oil to evaluate the final physical and antimicrobial properties. A predominant role of stabilizer was shown during the measurement of zeta potential of polymer dispersions; systems with Tween 20 and 80 revealed values reaching the zeta potential of unmodified chitosan solution (+58.7 mV), while, on the other hand, the sample with Tween 80 showed a significantly lower value (about +30 mV), regardless of thyme oil used.

Prepared films exhibited a sufficient antimicrobial activity against tested Gram-negative and Gram-positive bacteria, yeasts, and molds. Atok thyme oil proved to be more effective at the lowest tested concentrations, which was probably due to the higher thymol and carvacrol content that was observed by GC analysis. FTIR spectra of modified chitosan films revealed the presence of new peaks that could signal the interaction between polymer and active substance, which was successfully incorporated into the chitosan matrix. The results of mechanical testing of modified samples are satisfying, even though a decrease in tensile and puncture strength was ascertained when compared to the control sample. The microstructural study revealed relatively homogeneously distributed TEO droplets of various size depending on the type of emulsifier used, whereby the most noticeable particles were observed in the sample containing Tween 85 with the lowest HLB value. With regard to a comprehensive evaluation of the final properties, Tween 80 and Atok thyme essential oil seems to be the optimum choice for stabilizing and active agent, respectively. The modification of chitosan with TEO mixture provided stable polymer films with improved water-barrier characteristics and efficient antimicrobial properties, which could serve as a cost-effective and environmentally favorable active coating for food preservation.

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References

1. Marsh, K.; Bugusu, B. Food packaging—Roles, materials and environmental issue. *J. Food Sci.* **2007**, *72*, 39–55. [CrossRef] [PubMed]
2. Kaya, M.; Khadem, S.; Cakmak, Y.S.; Mujtaba, M.; Ilk, S.; Akyuz, L.; Salaberria, A.M.; Labidi, J.; Abdulqadira, A.H.; Deligözf, E. Antioxidative and antimicrobial edible chitosan films blended with stem, leaf and seed extracts of *Pistacia terebinthus* for active food packaging. *RSC Adv.* **2018**, *8*, 3941–3950. [CrossRef]
3. Lan, W.; He, L.; Liu, Y. Preparation and properties of sodium carboxymethyl cellulose/sodium alginate/chitosan composite film. *Coatings* **2018**, *8*, 291. [CrossRef]

4. Dumitriu, S. Polysaccharides as biomaterials. In *Polymeric Biomaterials*, 2nd ed.; Dumitriu, S., Ed.; CRC Press: Boca Raton, FL, USA, 2001; pp. 1–61.
5. Santonicola, S.; Ibarra, V.G.; Sendón, R.; Mercogliano, R.; de Quirós, A.R. Antimicrobial films based on chitosan and methylcellulose containing natamycin for active packaging applications. *Coatings* **2017**, *7*, 177. [CrossRef]
6. Ahmed, S.; Ikram, S. Chitosan based scaffolds and their applications in wound healing. *Achiev. Life Sci.* **2016**, *10*, 27–37. [CrossRef]
7. Wang, L.; Liu, F.; Jiang, Y.; Chai, Z.; Li, P.; Cheng, Y.; Jing, H.; Leng, X. Synergistic antimicrobial activities of natural essential oils with chitosan films. *J. Agric. Food Chem.* **2011**, *59*, 12411–12419. [CrossRef] [PubMed]
8. Altiok, D.; Altiok, E.; Tihminlioglu, F. Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications. *J. Mater. Sci. Mater. Med.* **2010**, *21*, 2227–2236. [CrossRef] [PubMed]
9. Galluci, M.N.; Oliva, M.; Casero, C.; Dambolena, J.; Luna, A.; Zygadlo, J.; Demo, M. Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. *Flavour Frag. J.* **2009**, *24*, 348–354. [CrossRef]
10. Hosseini, M.H.; Razavi, S.H.; Mousavi, S.M.A.; Yasaghi, S.A.S.; Hasansaraei, A.G. Improving antibacterial activity of edible films based on chitosan by incorporating thyme and clove essential oils and EDTA. *J. Appl. Sci.* **2008**, *8*, 2895–2900. [CrossRef]
11. Guitiérrez, L.; Batlle, R.; Sánchez, C.; Nerín, C. New approach to study the mechanism of antimicrobial protection of an active packaging. *Foodborne Pathog. Dis.* **2010**, *7*, 1063–1069. [CrossRef] [PubMed]
12. Jiang, T.; Feng, L.; Zheng, X. Effect of chitosan coating enriched with thyme oil on postharvest quality and shelf life of Shitake mushroom (*Lentinus edodes*). *J. Agric. Food Chem.* **2012**, 188–196. [CrossRef] [PubMed]
13. Perricone, M.; Arace, E.; Corbo, M.R.; Singaglia, M.; Bevilacqua, A. Bioactivity of essential oils: A review on their interaction with food components. *Front. Microbiol.* **2016**, *6*, 76. [CrossRef] [PubMed]
14. Tongnuanchan, P.; Benjakul, S.; Prodpran, T. Structural, morphological and thermal behaviour characterizations of fish gelatin film incorporated with basil and citronella essential oils as affected by surfactants. *Food Hydrocoll.* **2014**, *41*, 33–43. [CrossRef]
15. Perdones, Á.; Chiralt, A.; Vargas, M. Properties of film-forming dispersions and films based on chitosan containing basil or thyme essential oil. *Food Hydrocoll.* **2016**, *57*, 271–279. [CrossRef]
16. Rubilar, J.F.; Cruz, R.M.S.; Silva, H.D.; Vicente, A.A.; Khmelinskii, I.; Vieira, M.C. Physico-mechanical properties of chitosan films with carvacrol and grape seed extract. *J. Food Eng.* **2013**, *115*, 466–474. [CrossRef]
17. Peng, Y.; Yin, L.; Li, Y. Combined effects of lemon essential oil and surfactants on physical and structural properties of chitosan films. *Int. J. Food Sci. Technol.* **2013**, *48*, 44–50. [CrossRef]
18. Balakrishnan, B.; James, N.R.; Jayakrishnan, A. Tween 20-modified poly(vinyl chloride) exhibits enhanced blood-compatibility. *Polym. Int.* **2005**, *54*, 1304–1309. [CrossRef]
19. *ASTM E96–95 Standard Test Methods for Water Vapor Transmission of Materials*; ASTM International: West Conshohocken, PA, USA, 1995.
20. Grigore, A.; Paraschiv, I.; Colceru-Mihul, S.; Bubueanu, C.; Draghici, E.; Ichim, M. Chemical composition and antioxidant activity of *Thymus vulgaris* L. volatile oil obtained by two different methods. *Rom. Biol. Lett.* **2010**, *15*, 5436–5443.
21. Stahl-Biskup, E.; Venskutonis, R.P. Thyme. In *Handbook of Herbs and Spices*, 2nd ed.; Peter, K.V., Ed.; Woodhead Publishing: Cambridge, UK, 2012; Volume 1, pp. 499–525.
22. Sabbah, M.; Di Pierro, P.; Esposito, M.; Giosafatto, C.V.L.G.; Mariniello, L.; Porta, R. Stabilization of charged polysaccharide film forming solution by sodium chloride: Nanoparticle *Z*-average and zeta potential monitoring. *J. Biotechnol. Biomater.* **2016**, *6*, e128. [CrossRef]
23. Bonilla, J.; Vargas, M.; Atarés, L.; Chiralt, A. Physical properties of chitosan-basil essential oil edible films as affected by oil content and homogenization conditions. *Procedia Food Sci.* **2011**, *1*, 50–56. [CrossRef]
24. Ćirin, D.M.; Poša, M.M.; Krstonošić, V.S.; Milanović, M.L. Conductometric study of sodium dodecyl sulfate-nonionic surfactant (Triton X-100, Tween 20, Tween 60, Tween 80 or Tween 85) mixed micelles in aqueous solutions. *Hem. Ind.* **2012**, *66*, 21–28. [CrossRef]
25. Sun, L.; Sun, J.; Chen, L.; Niu, P.; Yang, X.; Guo, Y. Preparation and characterization of chitosan film inforporated with thinned young apple polyphenols as an active packaging material. *Carbohydr. Polym.* **2017**, *163*, 81–91. [CrossRef] [PubMed]

26. Raphaël, K.J.; Meimandipoour, A. Antimicrobial activity of chitosan film forming solution enriched with essential oils; an in Vitro Assay. *Iran J. Biotechnol.* **2017**, *15*, 111–119. [CrossRef] [PubMed]
27. Ghahrarokhi, M.G.; Barzegar, M.; Sahari, M.A.; Azizi, M.H. Enhancement of thermal stability and antioxidant activity of thyme essential oil by encapsulation in chitosan nanoparticles. *J. Agric. Sci. Technol.* **2016**, *18*, 1781–1792.
28. Hromiš, N.M.; Bulut, S.N.; Lazic, V.L.; Popović, S.Z.; Šuput, D.Z.; Markov, S.L.; Vaštag, Ž.G.; Dzinić, N.R. Effect of caraway essential oil on the antioxidant and antimicrobial activity of chitosan film. *Food Feed Res.* **2015**, *42*, 31–42. [CrossRef]
29. Valderrama, N.; Albarracín, W.; Algecira, N. Physical and microbiological evaluation of chitosan films: Effect of essential oils and storage. *World Acad. Sci. Eng. Technol. Int. J. Agric. Biosyst. Eng.* **2015**, *9*, 262–269. [CrossRef]
30. Ojagh, S.M.; Rezaei, M.; Razavi, S.H.; Hoseini, S.H.S. Development and evaluation of a novel biodegradable film made from chitosan and cinnamon essential oil with low affinity toward water. *Food Chem.* **2010**, *122*, 161–166. [CrossRef]
31. Hosseini, M.H.; Razavi, S.H.; Mousavi, M.A. Antimicrobial, physical and mechanical properties of chitosan-based films incorporated with thyme, clove and cinnamon essential oils. *J. Food Process Preserv.* **2009**, *33*, 727–743. [CrossRef]
32. Foster, L.J.R.; Ho, S.; Hook, J.; Basuki, M.; Marcal, H. Chitosan as a biomaterial: Influence of degree of deacetylation on its physiochemical, material and biological properties. *PLoS ONE* **2015**, *10*, e0135153. [CrossRef] [PubMed]
33. Pranoto, Y.; Rakshit, S.K.; Salokhe, V.M. Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. *LWT—Food Sci. Technol.* **2005**, *38*, 859–865. [CrossRef]
34. Cagri, A.; Ustunol, Z.; Ryser, E.T. Antimicrobial, mechanical, and moisture barrier properties of low pH whey protein-based edible films containing p-aminobenzoic or sorbic acids. *J. Food Sci.* **2001**, *66*, 865–870. [CrossRef]
35. Alves, V.L.C.D.; Bruna, P.M.R.; Cruz, R.M.S.; Vicente, A.A.; Khmelinskii, I.; Vieria, M. Preparation and characterization of a chitosan film with grape seed extract-carvacrol microcapsules and its effect on the shelf-life of refrigerated Salmon (*Salmo salar*). *LWT* **2017**, *89*, 525–534. [CrossRef]
36. Dutta, P.K.; Dutta, J.; Tripathi, V.S. Chitin and chitosan: Chemistry, properties and applications. *J. Sci. Ind. Res.* **2004**, *24*, 20–31.
37. Yuan, G.; Lv, H.; Yang, B.; Chen, X.; Sun, H. Physical properties, antioxidant and antimicrobial activity of chitosan films containing carvacrol and pomegranate peel extract. *Molecules* **2015**, *20*, 11034–11045. [CrossRef] [PubMed]
38. Coma, V.; Martial-Gros, A.; Garreau, S.; Copinet, A.; Salin, F.; Deschamps, A. Edible antimicrobial films based on chitosan matrix. *J. Food Sci.* **2002**, *67*, 1162–1169. [CrossRef]
39. Sedlaříková, J.; Doležalová, M.; Egner, P.; Pavlačková, J.; Krejčí, J.; Rudolf, O.; Peer, P. Effect of oregano and marjoram essential oils on the physical and antimicrobial properties of chitosan based systems. *Int. J. Polym. Sci.* **2017**, *2017*, 2593863. [CrossRef]
40. Rota, M.C.; Herrera, A.; Martínez, R.M.; Sotomayor, J.A.; Jordán, M.J. Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. *Food Control.* **2008**, *19*, 681–687. [CrossRef]
41. Ballester-Costa, C.; Sendra, E.; Fernández-López, J.; Viuda-Martos, V. Evaluation of the antibacterial and antioxidant activities of chitosan edible films incorporated with organic essential oils obtained from four Thymus species. *J. Food Sci. Technol.* **2016**, *53*, 3374–3379. [CrossRef] [PubMed]