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Bioactive zein/chitosan systems loaded with essential oils for food-packaging applications

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Abstract

BACKGROUND: There has recently been increased interest in biodegradable and sustainable packaging within the food industry. Biopolymer materials based on renewable biomass can be used as alternatives to conventional plastic packaging. A corn protein, zein, possesses excellent film-forming properties because of its hydrophobic nature. It can be used for making edible films and for producing nanofibrous layers. Combination with polysaccharides like chitosan offers promising prospects for the production of delivery systems for the controlled release of active substances. The current trend is to minimize the content of chemical additives; thus essential oils are suitable alternatives to synthetic antimicrobials.

RESULTS: This study aimed to develop various zein/chitosan-based film-forming solutions, films, and coatings with antimicrobial substances to prepare active food packaging. Thymol and three essential oils (thyme, cinnamon, oregano) were applied as bioactive ingredients against bacteria, yeasts, and fungi. The incorporation of these natural active compounds led to a decrease in particle size in most film-forming solutions and a reduction of zeta potential compared to controls. Release of the bioactive compound into an aqueous environment was proved by antimicrobial test. A zein/chitosan-based coating with thymol was applied on fresh strawberries. Microbiological analysis over 10 days confirmed the efficient control of bacterial and fungal growth.

CONCLUSION: Zein/chitosan (7:1) systems are suitable as bioactive compound carriers to make barriers and to prevent moisture loss, ensuring microbial food quality and prolonging the shelf life of fruits. These systems can serve as sustainable active food packaging.

Keywords: Zein, chitosan, essential oil, antimicrobial aktivity, food packaging

INTRODUCTION

In recent decades there has been increased interest, within the food industry, in the development of innovative biomaterials with antioxidant and antimicrobial properties to improve food safety, prolong shelf life, and satisfy growing consumer demand.[1,2] The incorporation into films of essential oils or other natural active substances such as enzymes, peptides, bacteriocins, bacteriophages, and fermented additives can slow the surface growth of bacteria, yeasts, and fungi, and thus serve as an alternative to chemical antimicrobials.[3] Edible or biodegradable films can also substitute or fortify natural layers to prevent moisture loss while selectively enabling the controlled exchange of important gases.[4]

Chitosan, produced by deacetylation from chitin, represents a suitable polysaccharide for preparing non-toxic, biocompatible, and degradable films.[5,6] It is obtained from crustacean bodies or fungal hyphae cells.[7] Chitosan and its derivatives have been shown to have antioxidant, antihypertensive, anticoagulant, antidiabetic, anti-obesity, antiallergic, anti-inflammatory, antimicrobial, anticancer, neuroprotective, and matrix metalloproteinase-inhibitory effects.[8,9] On the other hand, chitosan films are known for their worse mechanical and barrier properties. Generally, to overcome the weaknesses of individual polymers and optimize the properties of final systems, combinations with other materials can be used. The addition of protein into casting polysaccharide solution is a technique to improve the characteristics of chitosan films. Zein, obtained from the endosperm of the corn kernel, is a biodegradable and biocompatible protein-based material suitable for preparing edible films. Zein-based films are known for their smoothness, good barrier properties, and high thermal sta-bility.[10-12] In addition to the enhanced passive function of these blended composite systems, active compounds such as a variety of essential oils including cinnamon, arise, orange and oregano, α-tocopherol, and nanoparticles, have been added to chitosan/ zein blend films to broaden their functionality in active packaging.[6,11,13]

The objective of this study is to prepare zein/chitosan-based blend systems enriched with thymol, cinnamon, thyme, and oregano essential oils and investigate their physico-chemical properties and antimicrobial efficiency with potential for active packaging applications.

MATERIALS AND METHODS

Materials, chemicals and microorganisms

Low-molecular-weight chitosan was purchased by Sigma-Aldrich (St. Louis, Missouri, USA). Zein was obtained from TCI (Tokyo, Japan). Three types of essential oils (Thymus vulgaris Spain; Cinna-momum zeylanicum Germany; Origanum vulgare Romania) were supplied by Nobilis Tilia s.r.o. (Krásná Lípa, Czech Republic). Thymol and emulsifier Tween 20 were provided by Sigma-Aldrich. The following microorganisms were obtained from the Czech Collection of Microorganisms: Gram-negative rods Escherichia coli (ATCC 25922), gram-positive cocci Staphylococcus aureus (ATCC 25923), Candida parapsilosis (ATCC 22019), Aspergillus brasiliensis (ATCC 16404). Mueller-Hinton (MH) Agar and Nutrient Agar both supplied by Himedia Laboratories (Mumbai, India) were used for the growth of bacteria (37 °C/24 h). Sabouraud Agar provided by Himedia Laboratories was used for the cultivation of yeasts and molds (20 °C/5 days). Plate Count Agar (PCA), Chloramphenicol Yeast Glucose Agar (CYGA) and Violet Red Bile Agar (VRBA) were obtained from Himedia Laboratories.

Preparation of film-forming solutions and films

A stock solution of chitosan (0.5% w/v) in 1.0% v/v acetic acid was prepared by dissolving chitosan polymer in an ultrasonic bath at 40 °C for 30 min, followed by stirring for about 4 h with a magnetic stirrer until complete dissolution occurred. A stock solution of zein (0.5% and 2.0% w/v) in 75% ethanol was prepared by dissolving an appropriate amount of zein under stirring at 25 °C for 20 min.

An appropriate amount of 20% w/v thymol solution was pipetted into the zein solution. In the case of essential oils, the procedure was reversed, with the appropriate amount of oil weighed first and then a given volume of zein solution was added. After 30 min, an appropriate amount of chitosan solution was added dropwise from the burette, with stirring, and after another 30 min, 2 mLof 5% w/v Tween 20 solution was added. The stirring was then stopped after another 30 min. Zein and chitosan solutions (ratio 7:1,5:1) with active substances - thymol, thyme, cinnamon, oregano (2%, 3% w/v) - were prepared. Analogously, as a control, a sample without active substances was prepared.

The prepared polymer solutions (25 mL) with and without active substances were poured into plastic Petri dishes (diameter 9 cm) in a laminar box and then dried in an oven at 35 °C overnight. The dried films were kept at room temperature. Nevertheless, cinnamon films were not homogeneous and could not be used for further analyses.

Particle size and zeta potential

The measurements of polymer solutions with active compounds were carried out on a Zetasizer Nano ZS device (Malvern Instruments, Ltd, Malvern, UK) after diluting the samples with the filtrated distilled water (VWR syringe filter, 0.2 pm, Stříbrná Skalice, Czech Republic). The particle size was gauged by laser diffractom-etry (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 in triplicate and then evaluated.

pH measurement

The pH values were measured using a battery-powered CPH 51 pH meter with a combined 301 TMS electrode (Malá Skála, Czech Republic) at room temperature.

Surface tension

The surface tension of solutions was measured on an EasyDyne tensiometer K20 Kruss GmbH (Hamburg, Germany) by the Wil-helmy plate method. All measurements were carried out at 25 ± 5 °C in triplicate.

Viscosity measurement

The viscosity of solutions was measured at a shear rate of 200 rpm by use of a Brookfield DV - III Ultra Rheometer Ametek, Brookfield (Middleboro, MA, USA) with a spindle SC4-18. The results were expressed as relative viscosity normalized by the viscosity of water. All measurements were carried out at 25 $\pm$ 5 °C in triplicate.

Antimicrobial activity - disk diffusion method

The antimicrobial effects of zein/chitosan film-forming solutions with active substances were determined by the disk diffusion method. The film-forming solutions (10 μL) were pipetted on sterile circular paper disks (diameter 6 mm), which were placed on agar plates previously inoculated with 1 mL of 0.5 McF turbidity scale suspension (Escherichia coli, Staphylococcus aureus, Candida parapsilosis, Aspergillus brasiliensis) in sterile saline solution. The MH plates with bacteria were incubated at 37 °C/24 h; Sabouraud plates for yeasts and molds were cultivated at room temperature for 5 days. After incubation, the diameters of inhibition zones, including the area under the disk, were measured. Six replicates were performed for each sample and data was calculated using MS Office software Excel (Microsoft, 2020).

The antimicrobial effects of zein/chitosan films with active substances were determined by the disk-diffusion method using circular samples (diameter 9 mm) placed directly on agar plates inoculated by bacteria, yeast, and mold, cultivated and evaluated as described previously.

The release conditions of antimicrobial compounds from zein/ chitosan film (incorporated with thymol, thyme, and oregano) were determined by the release test. Film samples were cut into disks (diameter 9 mm), placed separately in plastic tubes with 20 mL of phosphate buffer (pH 7), and stored in an incubator at 37 °C. The samples of zein/chitosan disks were withdrawn at time intervals of 3 and 24 h, and 5 and 12 days) and transferred to agars (MH, Sabouraud) pre-inoculated with 1 mL of 0.5 McF turbidity scale suspension of microorganisms (bacteria, yeasts, and molds). Agars with zein/chitosan disks were incubated, measurements, and data processing were performed according to the protocol described previously.

Coating method

Film-forming solutions (2% w/v Z:CH 7:1 without bioactive compound - control, and 2% w/v Z:CH 7:1 with 3% w/v thymol) were used in the strawberry dipping experiment. The strawberries ($Fragaria\ x\ ananassa$) were purchased from a local market, where they were freshly picked. They were chosen carefully for their uniformity in size, appearance, and color, and were without visible traces of decay. After transport into the laboratory, each strawberry was weighed. The mean weight of all tested strawberries at the beginning of the experiment was 8.63 $\pm$ 1.12 g (n = 90). The strawberries were washed with distilled water for 3 min and dried individually for 30 min at room temperature. Coatings were applied by dipping for 1 min in the control and sample solution incorporated with thymol. For negative control, the same process was performed dipping in distilled water. The strawberries were dried by hanging them up in a laminar box for 1 h at room temperature. They were kept separately in sterile plastic boxes, ten pieces for each batch - negative control, dipped control, dipped sample - and stored at 4 °C and 7580% relative humidity for 10 days. The whole experiment was conducted in triplicate.

Weight loss

Weight loss was measured by weighing the strawberries before and during the storage period and weight loss percentage14 was calculated by the following equation (Eqn (**1**)):

$$weight\ loss\,(\%) = \frac{W_i - W_f}{W_i} \times 100 \qquad (1)$$

where W_i is the initial weight and W_f is the final weight of the tested strawberry during the storage period at 0,3,6, and 10 days.

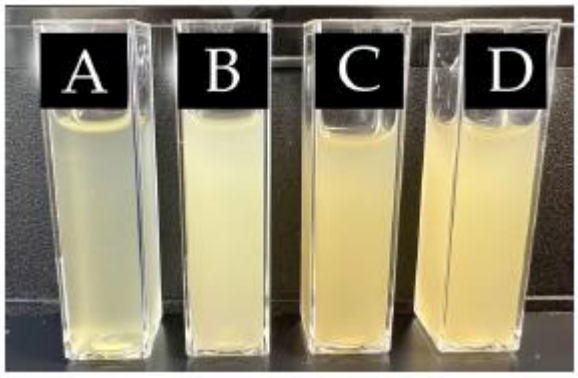

Figure 1. Zein (2% w/v)/chitosan solutions (ratio 7:1) without (A), and with 2% w/v addition of antimicrobial compound - thymol (B), thyme (C), or oregano (D).

Microbiological analysis of strawberries

Microbiological analysis was performed on days 0, 3, 6 and 10 of cold storage. Five grams was weighed into a sterile bag and 45 mL of sterile saline solution was added than it was homogenized and decimal dilutions of homogenate were prepared. Three different agar types were inoculated according to the determined parameters - PCA for total count of microorganisms - TCM, 30 ° C/48 h, CYGA for yeasts and molds, 20 °C/7 days and VRBA for coliform bacteria, 37 °C/24 h. Results were expressed as Log CFU g^{-1}.

Statistical analysis

The values obtained were calculated as means $\pm$ standard deviations using Excel. The experimental data were evaluated using a one-way ANOVA followed by a Tukey test using Statistica software version 10, StatSoft, Inc. (Tulsa, OK, USA) with a significance level of $P < 0.05$.

RESULTS

Physico-chemical properties of film-forming solutions

The appearance of all the prepared solutions were observed. The visual characteristics can indicate the stability of the solutions (**Fig. 1**).The samples were homogeneous with no obvious aggregation or sedimentation. Solutions were nontransparent with a yellowish shade, which was slightly darkened after adding essential oils. No significant changes were observed in the solutions, even after 5 weeks of storage at room temperature, evidencing the stability of the film-forming solutions that were prepared.

Zeta potential is another important factor affecting the physical stability of dispersion systems. As shown in **Fig. 2**, the zeta potentials of samples containing 0.5% w/v zein/chitosan with thymol, thyme,

and oregano rose to positive values in comparison with the control sample without the active substance.

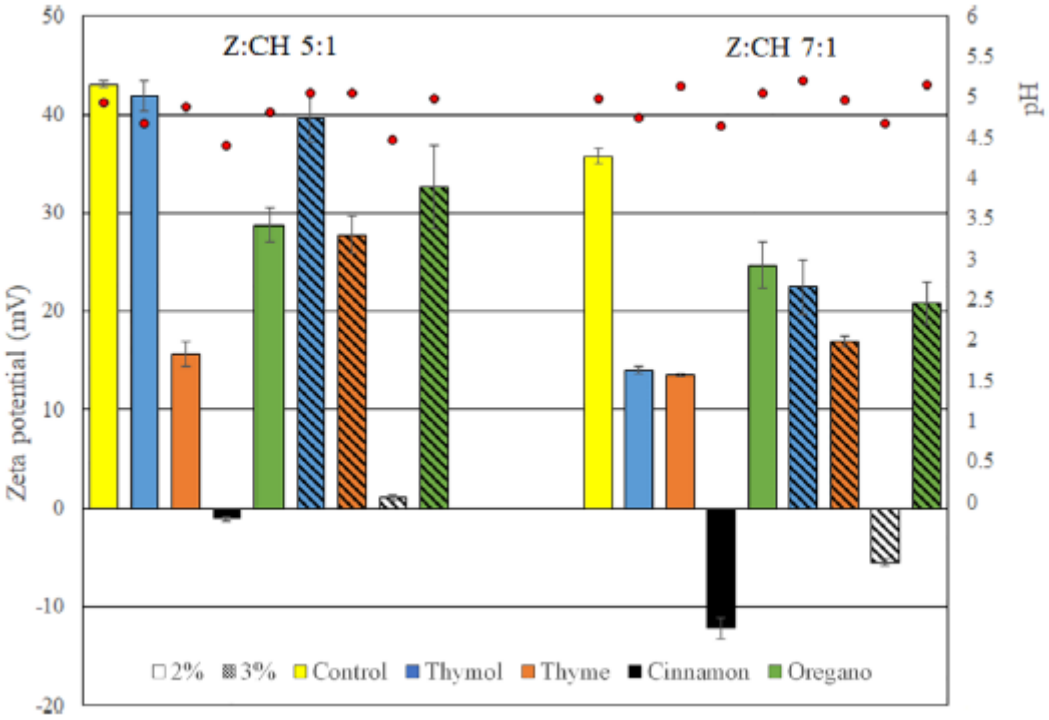

Figure 2. Zeta potential and pH values for 0.5% w/v zein/chitosan solutions (ratio 5:1, 7:1) with 2% and 3% w/v addition of antimicrobial compounds (thymol, thyme, cinnamon, and oregano).

A significant difference occurs in solutions containing cinnamon that exhibit neutral or negative values with higher cinnamon concentrations. An increased zeta potential was proved in the samples containing higher chitosan concentration, which was in accordance with the theoretical assumptions due to the positive charge in the acetic acid solution. After adding the active substances to the zein/chitosan solution, the zeta potential decreased in most samples in comparison with the control. This phenomenon can probably be attributed to the chemical interactions between the active substances and chitosan. Sufficient electrostatic stabilization was proven in the prepared 0.5% (w/v) zein/chitosan film-forming solutions (5:1) with and without thymol due to the positive zeta potential values exceeding +30 mV at laboratory temperature.

The pH values of zein/chitosan solutions were in the range from 4.4 to 5.4 (**Fig. 2**). Regarding the samples with active substances, the highest pH value (5.4) was measured in the 2% w/v zein/chitosan film-forming solution (ratio 7:1) with 2% w/v thymol incorporated. The lowest value was found to be 4.4 in the 0.5% w/v zein/chitosan solution (ratio 5:1) with 2% w/v cinnamon.

The size of the particles can significantly influence the structural and sensory properties of the samples. **Table 1** summarizes the average particle size of film loaded with bioactive compounds forming solutions relative to controls. These measurements showed a significant decrease in almost all cases. The lowest decrease (96%) was observed in the case of 2% w/v zein/chitosan 7:1 with 3% w/v of cinnamon oil. The particle size decreased with increasing zein concentration. A decreasing trend in the average particle size of solutions was also observed with increasing concentration of essential oils. However, this trend was not observed for the samples with thymol incorporated.

The surface tension and viscosity were measured as the important parameters for further processing of film-forming solutions. The average measured surface tension for the sample Z:CH 7: 1 without active substance was 27.3 mN m^{-1}. Addition of active substances (3% thymol) to the solution reduced the value to 27.0 mN m^{-1}. In other samples, the results showed no significant changes in surface tension values, which fluctuated around 27 mN m^{-1}, regardless of the control or modified film-forming solution.

Table 1. Particle size (nm) for film-forming solutions with different concentrations of zein, ratio zein/chitosan, and active substance Particle size (nm) for zein/chitosan film-forming solutions

			Particle size (nm) for zein/chitosan film-forming solutions				
Zein (w/v)	Z:CH	Active compound (w/v)	Control	Thymol	Thyme	Cinnamon	Oregano
0.5%	5:1	2%	$513.3 \pm 1.3^{\dagger a}$	20.5 ± 0.1^{b}	62.2 ± 1.0^{c}	179.8 ± 16.7^{d}	86.1 ± 2.4^{e}
	7:1		365.1 ± 5.4^{a}	111.2 ± 0.3^{b}	114.2 ± 2.3^{c}	61.5 ± 0.8^{d}	217.3 ± 0.6^{a}
	5:1	3%	513.3 ± 1.3^{a}	258.1 ± 7.7^{b}	55.2 ± 2.2^{c}	26.9 ± 0.3^{d}	77.1 ± 1.8^{e}
	7:1		365.1 ± 5.4^{a}	66.3 ± 0.6^{b}	47.6 ± 0.5^{c}	14.5 ± 0.3^{d}	31.4 ± 0.1^{e}
2.0%	5:1	2%	268.8 ± 7.2^{a}	114.2 ± 0.6^{b}	146.7 ± 4.5^{c}	182.0 ± 3.3^{d}	209.4 ± 11.6^{a}
	7:1		223.6 ± 4.3^{a}	164.9 ± 4.4^{b}	97.6 ± 2.2^{c}	161.3 ± 1.4^{d}	195.8 ± 1.8^{e}

†Different lowercase superscripts in the row mean statistical difference ($P < 0.05$) from control.

Table 2. Antimicrobial activity of zein/chitosan film-forming solutions (0.5% w/v zein) incorporated with antimicrobial compound (2% w/v, 3% w/v) performed by disk diffusion method (disk diameter 6 mm)

			Inhibition zones (mm)			
			S. aureus	*E. coli*	*C. parapsilosis*	*A. brasiliensis*
,		Control	NI†^{a}	NI^{a}	NI^{a}	NI^{a}
Z:CH 5:1	2%	Thymol	$8.0 \pm 0.1^{c,d}$	$10.0 \pm 0.1^{f,g}$	$7.0 \pm 0.7^{b,c}$	NI^{a}
		Thyme	7.0 ± 0.5^{c}	$8.0 \pm 0.1^{c,d}$	7.0 ± 0.1^{b}	NI^{a}
		Cinnamon	NI^{a}	$8.5 \pm 0.6^{c,d}$	NI^{a}	7.0 ± 0.2^{b}
		Oregano	8.0 ± 0.1^{c}	$8.5 \pm 0.6^{c,d}$	7.0 ± 0.1^{b}	NI^{a}
	3%	Thymol	9.0 ± 0.1^{d}	12.5 ± 0.6^{h}	$9.3 \pm 0.3^{d,e}$	NI^{a}
		Thyme	$8.5 \pm 0.6^{c,d}$	$10.0 \pm 0.1^{f,g}$	$8.0 \pm 0.1^{c,d}$	NI^{a}
		Cinnamon	14.5 ± 0.6^{i}	$10.5 \pm 0.6^{f,g}$	9.0 ± 0.1^{d}	7.0 ± 0.3^{b}
		Oregano	$8.0 \pm 0.1^{c,d}$	$10.0 \pm 0.1^{f,g}$	$8.5 \pm 0.6^{c,d}$	NI^{a}
Z:CH 7:1	2%	Thymol	$10.3 \pm 0.3^{f,g}$	10.0 ± 0.4^{f}	$8.0 \pm 0.1^{b,c}$	NI^{a}
		Thyme	$7.0 \pm 0.1^{b,c}$	$8.0 \pm 1.1^{c,d}$	$7.0 \pm 0.1^{b,c}$	NI^{a}
		Cinnamon	NI^{a}	9.0 ± 0.7^{d}	$7.5 \pm 0.6^{b,c}$	$7.4 \pm 0.4^{b,c}$
		Oregano	$7.0 \pm 0.1^{b,c}$	9.0 ± 0.3^{d}	$7.0 \pm 0.1^{b,c}$	NI^{a}
	3%	Thymol	$9.0 \pm 0.1^{d,f}$	$12.0 \pm 0.3^{g,h}$	10.0 ± 0.1^{f}	NI^{a}
		Thyme	$7.5 \pm 0.6^{b,c}$	$10.0 \pm 0.1^{f,g}$	$8.5 \pm 0.6^{c,d}$	NI^{a}
		Cinnamon	15.0 ± 0.1^{i}	$10.5 \pm 0.6^{f,g}$	$9.5 \pm 0.2^{e,f}$	$7.5 \pm 0.6^{b,c}$
		Oregano	$8.5 \pm 0.6^{b,c}$	$9.5 \pm 0.6^{d,f}$	7.0 ± 0.5^{b}	NI^{a}

†NI, no inhibition zone; different lowercase superscripts ir the column mean statistical difference ($P < 0.05$) from control.

The viscosity measurements revealed the reduction in values after the incorporation of active substances. The zein/chitosan 7:1 solution without active compound was chosen as the representative sample. Its average relative viscosity was 3.89 ± 0.03, while the decrease to 3.49 ± 0.02 was obtained after the bioactive substance addition (3% w/v thymol).

Antimicrobial activity of film-forming solutions

The antimicrobial properties of zein/chitosan film-forming solutions were investigated by the agar diffusion method against *S. aureus, E. coli, C. parapsilosis, and A. brasiliensis*. The inhibition zones of the tested solutions are summarized in **Table 2**. It can be observed that control zein/chitosan film-forming solutions (both ratios 5:1 and 7:1) without active compounds showed neitherantibacterial nor antifungal activity. The addition ofcinna-mon exhibited the most significant antibacterial activity against gram-positive S. aureus, at a concentration of 3% w/v; on the other hand, 3% w/v thymol solution was the most active against gramnegative E. coli. Antifungal activity was observed against yeast C. parapsilosis in the case of the thymol solution (3% w/v). However, only the cinnamon solution (3% w/v) proved effective against the mold A. brasiliensis. The other active ingredients showed very low (C. parapsilosis) or no (A. brasiliensis) antifungal activity.

Antimicrobial activity of prepared films

The film-forming solutions with a minimum zein concentration of 2% w/v were applied successfully to prepare films as a type of carrier system for active substances, using a solvent-casting technique. All films were homogenous except for cinnamon oil samples, which could not be used for further testing due to their significant lack of homogeneity. It is evident from the results that the films with thymol and oregano provided stronger antimicrobial activity, especially against the fibrous micromycete Aspergillus brasiliensis, than thyme oil films. The antibacterial effect was not shown to be selective among different bacteria species (**Table 3**).

Table 3. Antimicrobial activity of zein/chitosan films (2% w/v zein) with the addition of 3% w/v antimicrobial compound (film disk diameter 9 mm)

	Inhibition zone (mm)			
	S. aureus	*E. coli*	*C. parapsilosis*	*A. brasiliensis*
Z:CH 5:1 Control	NI[†a]	NI[a]	NI[a]	NI[a]
Thymol	19.5 ± 0.6^{b}	21.0 ± 0.9^{b}	40.0 ± 1.1^{b}	62.5 ± 2.5^{b}
Thyme	17.3 ± 0.5^{b}	17.3 ± 0.5^{c}	16.5 ± 1.6^{c}	38.5 ± 1.5^{c}
Oregano	17.7 ± 0.5^{b}	22.3 ± 0.5^{b}	23.0 ± 1.1^{d}	49.0 ± 4.0^{d}
Z:CH 7:1 Control	NI[a]	NI[a]	NI[a]	NI[a]
Thymol	22.0 ± 0.1^{c}	32.3 ± 1.3^{d}	38.5 ± 0.6^{b}	$58.0 \pm 2.0^{b,e}$
Thyme	18.3 ± 0.5^{b}	22.7 ± 0.5^{b}	16.5 ± 1.6^{c}	$50.5 \pm 6.5^{d,e}$
Oregano	15.0 ± 0.9^{d}	18.3 ± 0.5^{c}	25.5 ± 2.5^{d}	34.0 ± 1.0^{f}

† NI, no inhibition zone; different lowercase superscripts in the column mean statistical difference ($P < 0.05$) from control.

Finally, a focused study was performed of the release of active substances over time (**Table 4**). Samples of film disks were taken at 0, 3, 24 h, and 5 and 12 days. After 24 h, thyme and thymol (zein/chitosan ratio 7:1) exhibited antibacterial activity against S. aureus and E. coli. Antimicrobial activity against C. parapsilosis was shown, except for thyme (both ratios) after 24 h.

Zein/chitosan 5:1 films with thymol, and Z/CH films with oregano (both ratios) showed antifungal activity against A. brasiliensis, even after 24 h (**Fig. 3**). No inhibitory effect was observed in all samples after either 5 or 12 days. Release kinetics is the crucial parameter for the active packaging applied to food products.

Table 4. Release test - antimicrobial activity of zein/chitosan (2% w/v zein) films with 3% w/v active compound for S. aureus, E. coli, C. parapsilosis, and A. brasiliensis (disk diameter 9 mm)

	Z:CH 7:1			Z:CH 5:1		
Time (h)	Thymol	Thyme	Oregano	Thymol	Thyme	Oregano
S. aureus						
0	$19.0 \pm 3.1^{e,f,g}$	16.5 ± 0.5^{e}	19.0 ± 0.3^{f}	23.0 ± 1.1^{g}	18.5 ± 0.6^{f}	19.0 ± 0.4^{f}
3	$15.0 \pm 6.2^{b,c,d,e,f}$	$13.5 \pm 0.5^{c,d}$	$16.5 \pm 2.6^{d,e,f}$	14.0 ± 0.1^{d}	$10.5 \pm 1.6^{b,c}$	$14.0 \pm 5.1^{b,c,d,e,f}$
24	9.5 ± 0.6^{b}	10.0 ± 1.1^{b}	NI[†a]	NI[a]	NI[a]	NI[a]
E. coli						
0	20.5 ± 0.6^{g}	16.0 ± 0.1^{e}	$18.0 \pm 1.1^{e,f}$	$19.5 \pm 0.6^{f,g}$	$15.0 \pm 0.1^{c,d}$	$18.0 \pm 1.1^{e,f}$
3	$11.5 \pm 3.6^{b,c,d}$	$11.5 \pm 2.6^{b,c}$	$16.5 \pm 0.6^{d,e}$	17.0 ± 0.1^{e}	13.0 ± 1.1^{c}	$15.5 \pm 2.6^{c,d,e}$
24	NI[a]	10.0 ± 1.1^{b}	9.5 ± 0.6^{b}	NI[a]	NI[a]	13.0 ± 1.1^{c}
C. parapsilosis						
0	27.5 ± 0.6^{i}	$14.5 \pm 0.6^{d,e}$	$17.5 \pm 0.6^{f,g}$	21.0 ± 1.1^{h}	$13.0 \pm 0.1^{c,d}$	18.5 ± 0.6^{g}
3	$12.5 \pm 3.6^{b,c,d,e}$	9.5 ± 0.6^{b}	18.5 ± 0.6^{g}	$12.5 \pm 1.6^{b,c,d}$	10.0 ± 1.1^{b}	$15.0 \pm 2.1^{c,d,e,f}$
24	10.5 ± 1.6^{b}	NI[a]	11.5 ± 0.6^{b}	11.0 ± 1.1^{b}	NI[a]	$12.0 \pm 1.1^{b,c}$
A. brasiliensis						
0	35.5 ± 0.6^{e}	42.5 ± 0.6^{g}	39.0 ± 1.1^{f}	45.0 ± 1.1^{h}	29.0 ± 1.1^{d}	45.5 ± 0.6^{h}
3	44.0 ± 0.1^{c}	13.0 ± 1.1^{b}	$39.5 \pm 2.6^{e,f,g}$	52.0 ± 0.1^{i}	14.5 ± 0.6^{b}	32.0 ± 2.1^{d}
24	NI[a]	NI[a]	24.0 ± 1.1^{c}	13.0 ± 0.1^{b}	NI[a]	14.0 ± 2.1^{b}

[†]NI, no inhibition zone; different lowercase superscripts in the row for the same microorganism mean statistical difference ($P < 0.05$) from no inhibition zone.

Coating method

The film-forming solutions were used for coating strawberries to evaluate their suitability for food applications. The batch negative control was prepared by dipping in water; the dipped control was dipped into zein/chitosan solution without a bioactive compound; and the dipped sample was prepared as zein/chitosan coating with 3% w/v thymol. Weight loss was calculated as a percentage during 10 days of cold storage. The weight of negative control strawberries decreased to 2.49% after 3 days, to 20.17% after 6 days and to 21.63% after 10 days. Weight loss in dipped control and dipped sample after 3 days were not significantly different from negative control. On the other hand, significantly ($P < 0.05$) lower loss was observed in dipped control (12.77/19.73%) and in a dipped sample (16.22/18.30%) after 6/10 days. These results suggest that dipping in zein/chitosan solution prevents drying.

Microbiological analysis was performed on days 0, 3, 6, and 10 during cold storage. The results showed that strawberries' aerobic microbiota were significantly affected by coating (**Table 5**). No coliform bacteria were found in any group of strawberries during the whole experiment. Negative control strawberries had about 4 Log orders of microbial counts (bacteria and fungi) after 10 days. On the other hand, Z/CH 7:1 dip without bioactive compound caused a decrease in counts. Dip with 3% w/v thymol caused a reduction in all tested types of microorganisms. It was shown that antimicrobial zein/chitosan-based food packaging with thymol could slow the growth of bacteria, yeasts, and molds and prolong the shelf life of strawberries as easily perishable fruit.

DISCUSSION

To confirm any effect from the incorporation of natural active components into zein/chitosan film-forming solutions and resulting films, the physico-chemical and antimicrobial properties were

evaluated. Zeta potential measurement, reflecting the potential electrostatic stability of dispersion systems, depends mainly on the chemical properties of the polymer, on the stabilizing agents, and on the pH of the medium.[15]

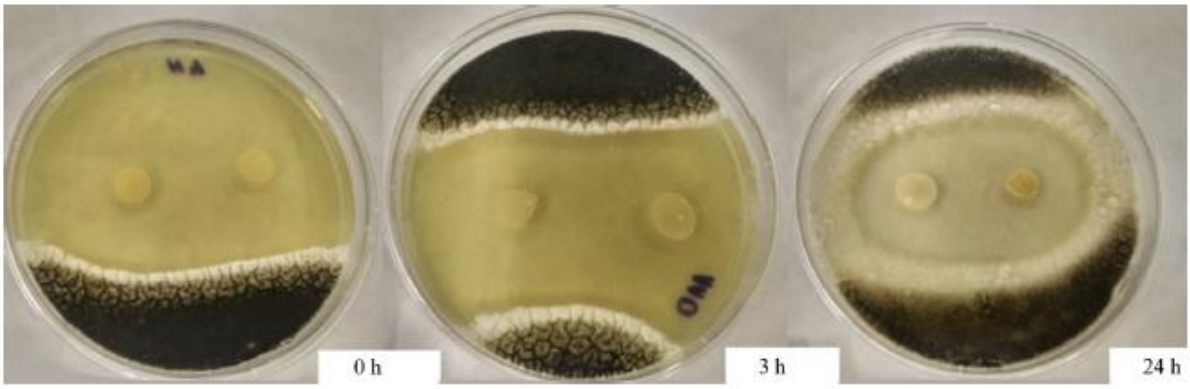

Figure 3. Release test - inhibition zones of zein (2% w/v)/chitosan films (7:1) incorporated with 3% w/v oregano against A. brasiliensis during time spent in the phosphate buffer at 37 °C.

Table 5. Microbiological analysis (Log CFU/g) of strawberries - negative control (dipped pound), dipped sample (Z:CH 7:1 with 3% w/v thymol) during 10 days of cold storage

Days	0	3	6	10
TCM†				
Negative control	$5.84^{a,A}$	$5.63^{a,b,A}$	$4.99^{b,A}$	$4.38^{c,A}$
Dipped control	$5.83^{a,A}$	$2.83^{b,B}$	$2.69^{b,B}$	$2.56^{b,B}$
Dipped sample	$5.87^{a,A}$	$2.74^{b,B}$	$2.49^{b,B}$	$<2.30^{b,B}$
Yeasts and moulds				
Negative control	$2.64^{a,A}$	$2.86^{a,A}$	$3.76^{b,A}$	$3.97^{c,A}$
Dipped control	$2.32^{a,A}$	$2.81^{b,A}$	$<2.30^{c,B}$	$<2.30^{c,B}$
Dipped sample	$2.42^{a,A}$	$<2.30^{b,B}$	$<2.30^{b,B}$	$<2.30^{b,B}$

†TCM, total count of microorganisms; different lower case superscripts mean statistically significant differences ($P < 0.05$) among values in a line for TCM and yeast and molds separately; different upper case superscripts mean statistically significant difference among values in a column for TCM and yeast and molds separately.

The measurement showed higher absolute values in the samples with the lower ratio of zein (5:1 systems). Except for the solutions containing 2% w/v thymol and 2 or 3% w/v cinnamon, all the systems exhibited the zeta potential values over +30.0 mV or almost reaching this value, which is considered as sufficient for the electrostatic repulsion between the individual particles. On the other hand, particles with a zeta potential between −10.0 and + 10.0 mV were evaluated as neutral with low electrostatic stability.[15]

High absolute values of zeta potential not only prevent aggregation of colloidal systems but can also predict bacterial adhesion, which is known to be dependent on van der Waals and electrostatic interactions. The mutual interactions between the polymer surface and the bacteria then play a role in the overall energy balance. Bacteria provide negatively charged surfaces resulting in binding to positively charged polymer materials.[16] This could explain the higher effectivity of cinnamon towards the tested microorganisms.

Instability of dispersion systems often results from the processes of coalescence, or flocculation, i.e. the particle size increase. As a consequence, gravitational separation, such as creaming or

sedimentation, can occur. Thus, particle size plays an important role in the appearance, structure, stability, and taste of colloidal systems.[17] Zhang etal. found that the average particle size of chito-san film-forming solutions was 393.6 nm, which increased to 423.7 nm after the addition of zein to the system, presumably caused by its high surface hydrophobicity.[18] This increase corresponds with our results for both ratios of polymers with incorporated active components (**Table 1**). When the control and modified samples are compared, statistically significant results ($P < 0.05$) were obtained in almost all cases.

Prepared film-forming solutions were also characterized by the surface tension measurement using the Wilhelmy plate method that showed the values around 27 mN m^{-1} regardless of the control or modified sample. The same technique was used for chitosan-based film forming solutions in an earlier study,[19] where the chitosan samples with and without the presence of oregano oil gave a value about 41 mN m^{-1}. In another recent study,[20] the surface tension of zein solutions in acetic acid was measured by the same method and similar values as in our case (from 27 to 30 mN m^{-1}) were obtained. Zein polymer plays a dominant role here.[21] The decrease in average relative viscosity after adding bioactive compound can be the consequence of weakened inter- or intramolecular forces of zein and chitosan polymer after the active molecule incorporation into their matrix. In this way, the previously ordered network structure can be disturbed while new hydrogen bonds arise.[22]

Due to the increasing requirements for material with reduced environmental impacts, essential oils - oregano, thyme, and cinnamon - were selected for the zein/chitosan-based film preparation.[3] Zein and chitosan polymers were chosen for their non-toxicity, biodegradability, biocompatibility, and excellent film-forming properties.[23] Chitosan also possesses antimicrobial activity caused by the interaction of positively charged amino groups of glucosamine units in chitosan with negatively charged components in microbial cell membranes, possibly leading to cell membrane rupture, intercellular leakage, and eventually cell death.[24] However, control zein/ chitosan samples of solutions and films without active components did not show any inhibitory effect in this study. Factors such as temperature, pH, molecular weight of chitosan or its source have to be considered, as well as potential limited diffusion of chitosan molecules captured in the polymer film matrix.[25,26]

The inhibitory activity of prepared zein/chitosan-based systems with bioactive compounds against tested microorganisms was observed. Gram-positive *S. aureus* and gram-negative *E. coli* bacteria were the most sensitive strains. On the other hand, *C. parapsilosis* and *A. brasiliensis* showed considerable resistance. The antimicrobial activity of essential oils depends on their specific composition. Antimicrobial effects of cinnamon essential oil can be ascribed to the main constituent, cinnamaldehyde, which is able to disrupt bacterial membranes resulting in their cytoplasmatic leakage and cell lysis. Gofii et al. also found antibacterial activity from cinnamon essential oil in combination with clove essential oil against Escherichia coli, Yersinia enterocolitica, Pseudomonas aeruginosa, Salmonella choleraesuis, Staphylococcus aureus, Listeria monocytogenes, Enterococcus and Bacillus.[27] The main components responsible for antimicrobial activity of thyme and oregano essential oils include carvacrol, thymol,p-cymene, ϒ-terpinene.[28] The activity of these essential oils incorporated in soy-based edible coatings was studied against *E. coli* O157: H7, L. monocytogenes, and *S. aureus* earlier[29] and, as in our study, the highest inhibition was provided against *S. aureus*,fol-lowed by *L. monocytogenes* and *E. coli* O157. Generally, grampositive bacteria have been reported to be more sensitive to plant essential oils than their gram-negative counterparts due to impaired diffusion of hydrophobic compounds across the outer cell wall of gram-negative bacteria.[30]

The results of antimicrobial activity of prepared films correlate with the release test, where the highest values were reported for the tested mold, A. brasiliensis, even after 24 h. To the best of our knowledge, this is the first report of a relatively undemanding method to evaluate the residual concentration of

bioactive compound in the film matrix. The release conditions of the incorporated antimicrobials through the packaging/food interface must be controlled. Factors such as chemical compatibility with the polymer, the swelling and plasticization of the polymer matrix by the food, environmental relative humidity, the release temperature and pH have to be taken into account.[31,32] If the diffusion rate into the environment occurs in a short period, the minimum inhibitory concentration cannot be reached, resulting in a poor inhibitory effect. On the other hand, too slow diffusion of antimicrobials may also lead to low inhibition of microbial growth.[33] Demonstration of the practical film application as antimicrobial packaging should be proceed after in vitro tests. Real food is a mixture of many chemical substances, which can unpredictably react with compounds contained in packaging.[34] An in vivo study conducted on cherry tomatoes indicated greater efficacy of starch/chitosan nanoparticle-based film in comparison with neat starch film.[35] Incorporation of microcapsules with grape seed extract and carvacrol to chitosan films extended the shelf life of salmon for 4-7 days.[36] Shelf life was also extended for pork patties wrapped into chitosan/clove/nisin packaging.[37] Chitosan nanoparticles were even applied successfully to harvested strawberries in the study by Salha,[14] where the efficient control of coated fruit decay was ensured. The significant inhibition of aerobic microorganisms was proved by coating strawberries in our study, no matter if the bioactive compound is present. On the other hand, thymol coating was able to completely reduce fungal microbiota after 3 days of cold storage. The results proved that prepared zein/chitosan-based systems with bioactive compounds forms a barrier that can significantly inhibit microbial growth on fruits and thus serve as an efficient coating, preserving the quality and extending the shelf life.

CONCLUSION

Bioactive compounds (thymol and thyme, cinnamon, and oregano essential oils) were applied as antimicrobial ingredients against bacteria, yeasts, and fungi in zein/chitosan systems. These can serve as suitable carriers for bioactive compounds in the form of films or coatings. Zein/chitosan (7:1) with 3% w/v thymol was successfully applied on fresh strawberries to prolong their shelf-life and quality. The optimization of conditions for releasing bioactive compounds to prolong antimicrobial activity, e.g. encapsulation in micellar particle prior to addition into polymer film or coating, is planned during further experiments. Prepared zein/chitosan systems enriched with natural active substances can serve as an efficient alternative to chemical antimicrobials. Edible or biodegradable films can also substitute for natural layers to prevent moisture loss while selectively enabling the exchange of essential gases.

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