Antimicrobial properties of chitosan solutions, chitosan films and gelatin-chitosan films

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Abstract: The antimicrobial properties of chitosan solutions and films against selected bacteria and the effect of chitosan incorporation into gelatin films were studied. The bactericidal effect of chitosan solutions increased with time and temperature of sample incubation. Two psychrotrophic strains *Pseudomonas fluorescens* and *Listeria innocua* were more sensitive to chitosan than mesophilic strains *Escherichia coli* and *Staphylococcus aureus*. The growth of bacteria under chitosan discs was inhibited. In the case of two component gelatin-chitosan films strong antimicrobial effect was also observed.

Keywords: chitosan, chitosan film, gelatin-chitosan film, antimicrobial films.

Przeciwdrobnoustrojowe właściwości roztworów chitozanowych, folii chitozanowych i żelatynowo-chitozanowych

Streszczenie: Zbadano właściwości przeciwdrobnoustrojowe roztworów i folii chitozanowych oraz dwuskładnikowych folii żelatynowo-chitozanowych, wobec wybranych bakterii. Stwierdzono zwiększenie bakteriobójczego działania roztworów chitozanu wraz ze wzrostem temperatury i wydłużeniem czasu inkubacji. Dwa psychrotrofowe szczepy *Pseudomonas fluorescens* i *Listeria innocua* wykazały większą wrażliwość na działanie chitozanu niż mezofilne szczepy *Escherichia coli* i *Staphylococcus aureus*. Wzrost badanych drobnoustrojów został całkowicie zahamowany pod krążkami z folii chitozanowych. Silne właściwości przeciwdrobnoustrojowe zaobserwowano również w przypadku dwuskładnikowych folii żelatynowo-chitozanowych.

Słowa kluczowe: chitozan, folia chitozanowa, folia żelatynowo-chitozanowa, folie przeciwdrobnoustrojowe.

Packaging is an important and integral part of food production. The main role of packaging is to protect food products from external influences and damage. Nowadays, however, food package is expected to be not only a physical barrier against undesirable factors of external environment, but also to perform other, additional functions. So called active packages extend shelf life and enhance the quality and safety of food. They can carry additives such as antioxidants, antimicrobials, colorants, flavors, some nutrients, and spices. One of the most promising solutions of active package system is antimicrobial packaging, as microorganisms constitute serious hazard in food. In this area chitosan seems to be the most suitable polymer, as it demonstrates a strong antimicrobial activity against different groups of microorganisms, such as bacteria, both gram-positive and gram-negative, as well as fungi [1—4], and simultaneously it has good film forming properties [5, 6]. Furthermore, this bioactive polysaccharide has been affirmed as generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) [7].

At present, there are many literature data available on the antibacterial activity of chitosan in solutions. This activity depends on many factors, such as deacetylation degree (*DD*), molecular weight of chitosan, its concentration in a solution, or pH and the ionic strength [8]. Chitosan below pH 6 is positively charged because of the protonation of amino groups in glucosamine residues [9] and the interaction of positively charged molecules of chitosan with anionic components of microorganisms — lipopolysaccharides (Gram-negative bacteria) and teichoic acid (Gram-positive bacteria) are considered the main reason for antimicrobial activity of this polymer. Such activity was not shown by some of the authors at pH = 7, and it was explained as a result of predomination of uncharged amino groups and poor solubility of the polymer under these conditions [10, 11]. However, in spite of chitosan solutions, the results obtained on anti-

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microbial activity of chitosan films are less numerous and very often inconsistent. Some authors even state that chitosan films do not reveal antimicrobial activity [12]. Therefore, for practical use of chitosan films as an antimicrobial food packaging material there is still a need for studies that will help to answer the question whether chitosan in the form of films is able or not to sufficiently inactivate bacteria. Although, there can be many reasons for discrepancies in reported data, it seems that the main one is the method applied for determination of antimicrobial activity of chitosan films. The agar diffusion method is the most widely used method for this purpose that at the first look can show the lack of antibacterial properties of chitosan films because the inhibition zones do not appear [12—16]. Therefore, the first aim of our work was to determine the activity of chitosan films against selected bacteria, using the method proposed by Ko *et al.* [17] in the case of protein films containing nisin. This method enables the quantitative determination of bacteria inactivation and so far was not applied to chitosan films. For comparison, the agar diffusion technique was also applied. The other method reported [18—23], as well as advantages or disadvantages of particular techniques will be discussed later. The ability of chitosan in the form of solutions (with different *DD* and similar molecular weight) to inactivate two Gram-negative (*Escherichia coli*, *Pseudomonas fluorescens*) and two Gram-positive bacteria species (*Staphylococcus aureus*, *Listeria innocua*) was examined before the study of the antimicrobial activity of chitosan films.

Currently there are many data in the literature on functional, mechanical and barrier properties of gelatin films as food packaging materials. However, this polymer is susceptible to microbiological degradation. The contact of films with moist products can lead to their fast destruction and then to shortening of the shelf life of the product instead of its extending. The presence of chitosan in gelatin films could probably prevent them from undesirable degradation as well as induce antimicrobial properties. The published data on antimicrobial properties of gelatin-chitosan films are very limited [24]. Therefore, the second aim of this study was to determine the activity of such two-component films against some bacterial species. Furthermore, proteins containing films are often crosslinked, among others by transglutaminase (TGase), in order to reduce their excessive solubility [25—29]. This enzyme catalyses formation of the covalent linkages between γ-carboxyamide groups of peptide-bound glutamine residues and ε-amino groups of lysine or primary amino groups of a number of components [30]. Thus, it is not excluded that amino groups of chitosan participate in these reactions. The involvement of chitosan amino groups in crosslinking reactions can cause a decrease in its antimicrobial activity. So the third aim of this work was the comparison of antimicrobial properties of gelatin-chitosan films crosslinked and not crosslinked with TGase.

EXPERIMENTAL PART

Materials

Chitosan samples with different *DD* and molecular weight were obtained from krill chitin in the Sea Fisheries Institute in Gdynia according to the method described in [31]. *DD* of chitosans was determined by potentiometric titration according to Broussignac method [32] and was calculated using the equation:

$$DD\ (\%) = 2.03\ (v_2 - v_1) / [m + 0.0042\ (v_2 - v_1)] \qquad (1)$$

where: v_1, v_2 — volumes (in cm^3) of 0.1 M of NaOH solution corresponding to the two inflection points on the titration curve, m — mass (in g) of the sample.

The viscosity-average molecular weight ($\overline{M}_{r,v}$) was estimated from the limiting viscosity number determined in the solvent 0.5 M of acetic acid and 0.2 M of sodium acetate using the Mark-Houwink parameters $\alpha = 0.76$, $K_\eta = 3.5 \cdot 10^{-2}$ cm^3/g at 25 °C and using the coefficients set for this solvent by Terbojevich i Cosani [33]. Values of *DD* and $\overline{M}_{r,v}$ of investigated chitosan are collected in Table 1.

The gelatin was obtained from the skins of Baltic cod as described by Kolodziejska *et al.* [34].

Transglutaminase (TGase) with trade name Activa® GS was received from Ajinomoto North America, Inc.

Trypticase soy broth, trypticase soy agar (TSA) and buffered saline (PBS) were delivered by Merck KGaA.

Film preparation

To prepare one-component solutions, fish gelatin was dissolved in deionized water, and chitosan in deionized water adjusted to pH ≈ 6 using 0.5 M of HCl solution, to achieve the final concentration of 5 and 2 wt %, respectively. The pH of fish gelatin solution was about 6.6. Gelatin-chitosan films (4:1 by weight) were obtained by mixing the appropriate volume of 2 wt % solution of chitosan with 25 wt % solution of gelatin. The resulting mixture was stirred during 2 h of incubation at 50 °C and centrifuged at 2000 × g for 15 min at 20 °C. TGase was added to the film forming solutions, cooled to the room temperature, to reach the final concentration of 0.2 mg/cm^3. The enzymatic reaction was running during that process.

In all experiments 22 g of solutions were cast on Petri plates 120 × 120 mm, spread manually to the outside borders. The films were dried at the room temperature for 24—48 h at 35—45 % relative humidity (*RH*).

Cultures and growth conditions

The following bacterial strains were used: *Escherichia coli* K-12 PCM 2560 (NCTC 10538) and *Staphylococcus aureus* PCM 2054 (ATCC 25923) from Polish Collection of Microorganisms, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wrocław, Poland, *Pseudomonas fluorescens*

WSRO 121 from Collection of Dairy Cultures of Department of Microbiology, University of Warmia and Mazury, Olsztyn, Poland, and *Listeria innocua* DSM 20649 from German Collection of Microorganisms and Cell Cultures.

Cultures in stationary phase were prepared by inoculating 100 cm^3 of SB with 0.1 cm^3 of liquid culture (at stationary phase of growth) and incubating at 37 °C (*Escherichia coli*, *Staphylococcus aureus*) or at 28 °C (*Listeria innocua*, *Pseudomonas fluorescens*) for 24 h with shaking.

Preparation of cell suspensions

The cells in the stationary phase of growth were centrifuged at 1300 × g for 20 min at 5 °C, and the pellets were resuspended PBS (pH = 6.0) to give viable bacteria number of about 10^8— 10^9 CFU/cm^3 in the final sample (CFU — colony forming unit). Chitosans were dissolved in PBS (pH = 5.8) at a final concentration of 4 mg/cm^3. Chitosans were added to reach the final concentration of 0.2 wt %. Samples were stored at 4 or 20 °C for 24 h and then the viable cells were determined.

The cell suspensions were serially diluted with PBS (pH = 7.0). Dilutions were plated on TSA and plates were incubated for 48 h at 37 °C (*Escherichia coli*, *Staphylococcus aureus*) or at 28 °C (*Listeria innocua*, *Pseudomonas fluorescens*).

Determination of antimicrobial activity of the films

Agar diffusion method

The culture of each microorganism was diluted with PBS to 10^5—10^6 CFU/cm^3 and spread (0.1 cm^3/plate) on TSA. Uniform 22 mm diameter discs were cut with a whole-puncher from the prepared gelatin, chitosan and gelatin-chitosan films, and each film disc was placed in the center of the inoculated petri dish. The plates were incubated at 37 °C (*Escherichia coli*, *Staphylococcus aureus*) and 28 °C (*Listeria innocua*, *Pseudomonas fluorescens*). After the incubation, the number of colony was counted under the contact area of discs. The tests were performed in triplicate.

The method proposed by Ko *et al.* [17]

0.015 cm^3 of bacterial suspension (10^5 CFU/g) was placed on the prepared film discs (22 mm in diameter and 0.02 g in weight). The films were incubated for 1 h or 24 h at the ambient temperature. After the incubation, the film discs were placed into sterile plastic tubes and diluted with PBS, and then homogenized for 1 min at 10 000 rpm. The solution was decimally diluted with PBS and plated in duplicate on plate count agar. The plates were incubated at 37 °C (*Escherichia coli*, *Staphylococcus aureus*) and at 28 °C (*Pseudomonas fluorescens*, *Listeria innocua*) for 24 h, and the number of cells (given in CFU/cm^3) was then determined.

The results in the tables are the average of three replications. The differences between treatments were evaluated statistically by the analysis of variance (one-way procedure) using the program Statgraphics, Statistical Graphic Corporation.

RESULTS AND DISCUSSION

Antimicrobial activity of chitosan in solutions

Values of logarithm of number of cells in the samples containing various types of chitosan before and after 24 h incubation at 20 or 4 °C are listed in Tables 1 and 2. These results show antibacterial activity of chitosan with different *DD* and high $\overline{M}_{r,v}$ (above 700 000) against *Escherichia coli*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Listeria innocua* cells. Immediately after the addition, chitosan with *DD* of 73 % did not affect the number of all tested bacteria, while chitosan with higher *DD*, *i.e.* 90 and 96 %, reduced it by about 0.6—1.9 log cycles, depending on the bacteria species. As this relationship was found also in the case of chitosan with low $\overline{M}_{r,v}$ [35—37], it can be concluded that the content of free amine groups in the polymer is the main factor deciding about its antimicrobial activity.

T a b l e 1. Deacetylation degree (*DD*) and viscosity-average molecular weight ($\overline{M}_{r,v}$) of chitosan used in experiments

Symbol of sample	*DD*, %	$\overline{M}_{r,v}$
Chitosan-73	73	1 086 000
Chitosan-90	90	766 000
Chitosan-96	96	759 000

T a b l e 2. The antimicrobial properties of chitosan with different deacetylation degree against Gram-negative bacteria

Conditions of sample incubation	The number of cells (given in log CFU/cm^3)			
	without chitosan	with chitosan-73	with chitosan-90	with chitosan-96
Escherichia coli				
Control	$9.2^{a,A}$	$9.0^{a,A}$	$8.1^{a,B}$	$8.1^{a,B}$
24 h at 20 °C	$9.0^{a,A}$	$7.4^{b,B}$	$3.3^{b,C}$	$1.7^{b,D}$
24 h at 4 °C	$9.1^{a,B}$	$7.6^{b,B}$	$6.4^{c,C}$	$7.7^{c,B}$
Pseudomonas fluorescens				
Control	$9.2^{a,A}$	$9.2^{a,A}$	$8.6^{a,B}$	$8.0^{a,C}$
24 h at 20 °C	$8.9a^{,A}$	$8.3^{b,B}$	$3.5^{b,C}$	$0.6^{b,D}$
24 h at 4 °C	$9.1^{a,A}$	$8.9^{a,A}$	$4.9^{c,B}$	$3.3^{c,C}$

a—c — values for a particular column followed by different letters differ significantly ($p < 0.05$).
A—D — values for a particular row followed by different letters differ significantly ($p < 0.05$).

The bactericidal effect of chitosan increased during subsequent incubation of samples and depended on the

temperature. At 20 °C bacteria were more susceptible to chitosan than at 4 °C (Tables 2 and 3). These results confirmed our earlier data obtained with a limited number of bacteria species [38]. Also Tsai and Su [39] showed that within the range of 4 to 37 °C, chitosan antibacterial activity increased with the increasing temperature. According to [39], the low temperature stress may lead to the decrease in the number of binding sites at the cell surface (or electronegativity) available for chitosan.

The increase in antimicrobial activity along with increasing chitosan *DD* was clearly evidenced during incubation of the samples. It is also in agreement with the data obtained by Chung and Chen [40], for chitosan with relatively low $\overline{M}_{r,v}$. Chitosan with *DD* of 73 % exerted the least effect on all bacterial strains. After incubation for 24 h at 20 °C, the number of *Escherichia coli* and *Pseudomonas fluorescens* decreased only by 1.6 and 0.9 log cycle, whereas in the presence of chitosan with *DD* of 90 % by 4.8 and 5.1 log cycles, respectively. The highest antibacterial activity was observed for chitosan with *DD* of 96 % — the number of *Escherichia coli* and *Pseudomonas fluorescens* after incubation at 20 °C was reduced by 6.4 and 7.4 log cycles, respectively (Table 2). In the case of Gram-positive *Staphylococcus aureus* and *Listeria innocua*, inactivation also increased with increasing chitosan *DD* (Table 3).

T a b l e 3. The antimicrobial properties of chitosan with different deacetylation degree against Gram-positive bacteria

Conditions of sample incubation	The number of cells (given in log CFU/cm^3)			
	without chitosan	with chitosan-73	with chitosan-90	with chitosan-96
Staphylococcus aureus				
Control	$9.0^{a,A}$	$8.8^{a,A}$	$8.3^{a,B}$	$8.4^{a,B}$
24 h at 20 °C	$9.0^{a,A}$	$7.8^{a,B}$	$6.0^{b,C}$	$3.7^{b,D}$
24 h at 4 °C	$8.9^{a,A}$	$8.7^{a,A}$	$7.1^{c,B}$	$6.4^{c,C}$
Listeria innocua				
Control	$9.3^{a,A}$	$8.8^{a,B}$	$8.6^{a,B}$	$7.1^{a,C}$
24 h at 20 °C	$8.8^{b,A}$	$7.0^{b,B}$	$1.2^{b,C}$	not detected
24 h at 4 °C	$9.3^{a,A}$	$7.9^{c,B}$	$7.5^{c,C}$	$3.6^{b,D}$

a—c — values for a particular column followed by different letters differ significantly ($p < 0.05$).

A—D — values for a particular row followed by different letters differ significantly ($p < 0.05$).

Our data also showed that in the presence of chitosan there were no clear differences in inactivation of cells between Gram-negative and Gram-positive bacteria. It was shown that the sensitivity to chitosan differed between psychrotrophs and mesophiles. The psychrotrophic Gram-positive *Listeria innocua* and Gram-negative *Pseudomonas fluorescens* cells were most sensitive to chitosan. To our knowledge, there is no data in the available literature on this subject.

Antimicrobial activity of chitosan and gelatin-chitosan films

Some authors have shown that inhibition zones do not appear when the agar diffusion method for determination of chitosan films activity is used [12—16]. However, there is insufficient evidence to state that chitosan films do not exert antimicrobial activity. The lack of this zone results from the fact that chitosan in the form of film is not able to diffuse through the agar medium [14, 16, 18]. Moreover, Coma *et al*. [18] and Abdollahi *et al*. [41] did not observe such zones even when a chitosan solution was tested, what was also explained by the limitation of chitosan diffusion. Subsequently, our results, presented in Table 4 showed that although inhibition zones were not present, the growth of bacteria under chitosan discs was inhibited. Previously, some authors observed as well the reduction of bacterial growth when chitosan was incorporated into the agar medium or under chitosan coating and films [18—21]. Additionally we have found that the level of inhibition of bacterial growth depended on the *DD* value of chitosan. The films from chitosan with *DD* of 90 % caused complete inhibition of growth of Gram-negative *Escherichia coli*, *Pseudomonas fluorescens* and Gram-positive *Staphylococcus aureus* and *Listeria innocua* under film discs, while a few of Gram-positive bacterial cells were able to grow under films from chitosan with *DD* of 73 %. For comparison, under the gelatin film about 50 CFU was observed (Table 4). Coma *et al*. [19] showed 100 % inhibition of *Staphylococcus aureus* and *Listeria monocytogenes* and about 80 % inhibition of *Pseudomonas aeruginosa* cells on agar medium coated with chitosan (*DD* = 98 %). Complete inhibition of *Listeria innocua* under chitosan film (*DD* = 85 %) was reported by Portes *et al*. [20]. The literature data also show that the antimicrobial effect of chitosan films depends on their molecular weight as well as on the kind of microorganism and the level of *inoculum*, and the phase of bacteria growth [21, 22]. Furthermore, chitosan films placed between 2 slices of bologna inoculated with *L. monocytogenes*, reduced the number of bacteria by 1 to 3 log cycles (depending on the initial bacterial population) and *Escherichia coli* O157:H7 by about 1 log cycle [16].

The antimicrobial properties of chitosan films were also confirmed using the technique of Ko *et al*. [17] based on inoculation of the known population of microorganisms on a film surface, and then the determination of the viability of bacteria on agar medium. The results are collected in Table 5. The level of bacterial viability depended on the time during which the cells were in contact with the films surfaces. One hour of incubation of the cells on the surface of films caused low inactivation, even in the case of films prepared from chitosan-90. The number of *Escherichia coli* and *Pseudomonas fluorescens* decreased by 2.1 and 1.9 log cycles, whereas *Staphylococcus aureus* and *Listeria innocua* cells were not reduced even by 1 log cycle. However, the increase in inactivation of the tested bacte-

T a b l e 4. The antimicrobial activity of chitosan and gelatin-chitosan films against Gram-negative and Gram-positive bacteria determined using disc diffusion method

Type of films	The number of cells (given in CFU under disc)			
	Gram-negative bacteria		Gram-positive bacteria	
	Escherichia coli	*Pseudomonas fluorescens*	*Staphylococcus aureus*	*Listeria innocua*
Gelatin	58[a]	42[a]	55[a]	57[a]
Chitosan-73	No growth[a]	No growth[b]	2[b]	5[b]
Gelatin-chitosan-73	58[a]	No growth[b]	56[a]	55[a]
Gelatin-chitosan-73 crosslinked with TGase	57[a]	No growth[b]	54[a]	36[c]
Chitosan-90	no growth[b]	no growth[b]	no growth[c]	no growth[d]
Gelatin-chitosan-90	no growth[b]	no growth[b]	no growth[c]	no growth[d]
Gelatin-chitosan-90 crosslinked with TGase	no growth[b]	no growth[b]	7[d]	no growth[d]

a—d — values for a particular column followed by different letters differ significantly ($p < 0.05$).

ria took place when the time was extended to 24 h. Similarly to the data obtained by using the agar diffusion method, the live cells of all bacterial strains were not detected after 24 h contact with both chitosan films (*DD* of 73 and 90 %). The inactivation effect was also shown when chitosan films were placed in liquid medium inoculated with *Staphylococcus aureus* and incubated at 37 °C [22]. On the other hand, Foster and Butt [12], using a similar technique as Fernandez-Saiz *et al*. [22] did not observe any antibacterial activity of chitosan films obtained from chitosan solution with pH about 5. Their results, contrary to those obtained by other authors (including our data), can result from the fact that they tested γ-ray sterilized films. As reported by Fernandez-Saiz *et al*. [22] heat sterilized films (with or without steam contact) lost their antimicrobial activity. The cited authors showed using ATR-FT-IR technique that COO^- groups were significantly reduced in such films in comparison with unsterilized samples. According to the authors, only gelled chitosan or films prepared from acidic solutions are active against microorganisms, as in these conditions the polymer amine groups are protonated or „activated" [22]. Furthermore, the cited authors reported that only a soluble fraction from chitosan films revealed the antimicrobial activity. However, it seems that the solubility of films is not necessary for their antimicrobial activity. We showed previously that films from chitosan-73 *DD* and 96 % were soluble only in about 3 % after incubation for 24 h in buffer with pH = 6 [42]. Moreover, in the methods used in the present work for the determination of bactericidal activity of chitosan, samples were not introduced to suspension of bacteria, but an appropriate amount of *inoculum* was placed on the film surface. Similarly, Vartiainen *et al*. [43] also observed the reduction of bacteria inoculated on the chitosan film immobilized in a BOPP matrix. Nevertheless, there is still discussable antimicrobial mode of action of chitosan in the form of films. Next, as reviewed by Kong *et al*. [8] chitosan in the form of nanoparticles or macrospheres exhibited antimicrobial activity over a broader range of pH than did the solubilized chitosan. It was also found that chitosan in the form of microspheres in a solid dispersing system demonstrated antimicrobial activity against *Escherichia coli* suspended in a medium at pH = 7.5 [44]. According to these authors, positively charged amino groups in chitosan are not necessary for its activity — chitosan acts as a chelating agent of divalent cations, which leads to the destabilization of the outer membrane of *Escherichia coli*. In the next stage, changes of permeability of cytoplasmic membrane take place by binding microspheres with membrane phospholipids by hydrophobic interactions. The antibacterial activity of chitosan in the form of films was also shown in PBS with pH = 7.2 [23]. On the other hand, it was reported that neutralized films or an as-received polymer in the form of flakes [22] or powder [24] do not exhibit bactericidal activity. The analysis of the above presented results shows that further studies are needed to explain in detail the relationship between the form of chitosan and its antimicrobial ability.

In the next step, we checked whether incorporating of chitosan into gelatin films would be effective in inhibition of the bacterial growth. In such two-component gelatin-chitosan films antimicrobial effect was also observed, especially in the presence of highly deacetylated chitosan. None of the tested bacteria were detected under gelatin-chitosan-90 films. For gelatin films with chitosan-73, the stage of inhibition of bacteria growth depended on sensitivity of the tested strains. Only *Pseudomonas fluorescens* growth was totally inhibited under film discs (Table 4). Generally, similar results were obtained with the second method used for determination of antimicrobial activity of the films. All strains of bacteria were completely inactivated after 24 h incubation with films from gelatin and chitosan-90, while in the case of one-component gelatin films the increase in the number of bacteria by about 2—4 log cycle was observed (Table 5). Films from gelatin containing chitosan-73 after 24 h completely inactivated *Pseudomonas fluorescens* and *Listeria innocua*

T a b l e 5. The antimicrobial activity of chitosan and gelatin-chitosan films against Gram-negative and Gram-positive bacteria determined with the method proposed by Ko *et al.* [17]

Type of films	The number of cells (given in log CFU/cm^3)							
	Gram-negative bacteria				Gram-positive bacteria			
	Escherichia coli		*Pseudomonas fluorescens*		*Staphylococcus aureus*		*Listeria innocua*	
	1 h	24 h	1 h	24 h	1 h	24 h	1 h	24 h
Gelatin	4.8 ± 0.1^{a}	6.7 ± 0.37^{a}	5.2 ± 0.4^{a}	7.7 ± 0.1^{a}	3.4 ± 0.13^{a}	7.3 ± 0.2^{a}	4.2 ± 0.23^{a}	6.8 ± 0.1^{a}
Chitosan-73	3.8 ± 0.16^{b}	Nd^{b}	5.9 ± 0.4^{a}	Nd^{b}	2.8 ± 0.12^{b}	Nd	$4.5 \pm 0.06^{a,b}$	Nd
Gelatin-chitosan-73	3.5 ± 0.06^{c}	3.3 ± 0.15^{b}	5.5 ± 0.5^{a}	Nd^{b}	2.5 ± 0.05^{c}	5.0 ± 0.17^{b}	4.6 ± 0.20^{b}	Nd
Gelatin-chitosan-73 crosslinked with TGase	3.6 ± 0.28^{b}	3.1 ± 0.31^{c}	5.8 ± 0.2^{a}	Nd^{b}	$2.7 \pm 0.02^{b,d}$	4.8 ± 0.54^{b}	4.7 ± 0.15^{b}	Nd
Chitosan-90	2.7 ± 0.05^{d}	Nd^{d}	3.3 ± 0.05^{b}	Nd^{b}	2.8 ± 0.13^{b}	Nd	$4.4 \pm 0.28^{a,b}$	Nd
Gelatin-chitosan-90	3.9 ± 0.19^{b}	Nd^{d}	3.7 ± 0.08^{c}	Nd^{b}	2.6 ± 0.15^{d}	Nd	4.7 ± 0.19^{b}	Nd
Gelatin-chitosan-90 crosslinked with TGase	3.8 ± 0.39^{b}	Nd^{d}	3.2 ± 0.11^{b}	Nd^{b}	2.8 ± 0.15^{b}	2.6 ± 0.65^{c}	4.3 ± 0.05^{a}	Nd

Nd — not detected.

a—d — values for a particular column followed by different letters differ significantly ($p < 0.05$).

but only partially *Escherichia coli* and *Staphylococcus aureus* cells. The antimicrobial activity of these two-component films was lower than the activity of the one-component chitosan films. The decrease in antimicrobial properties of chitosan films in the presence of guar gum at the concentration above 15 % was reported by Rao *et al*. [23] and explained as the formation of intermolecular hydrogen bonds between amine groups of chitosan and hydroxyl groups of the guar gum. Results obtained from the film analysis by using FT-IR [45] showed that interactions of chitosan and gelatin include not only formation of hydrogen bonds within and between chains of both polymers, but also electrostatic interactions between COO^- groups of gelatin and NH_3^+ groups of chitosan. These interactions and lower concentration of chitosan in comparison with one-component films are probably the reason for decreasing of positively charged groups in chitosan and lead to the reduction in antibacterial activity of the films. As we mentioned in the introduction, it could be expected that crosslinking of film components with TGase could also decrease antibacterial properties of the films. However, as a rule, crosslinking of chitosan-gelatin films with TGase did not affect their antimicrobial properties (Tables 4 and 5). The reduction of antimicrobial activity was observed only in the case of modified TGase gelatin-chitosan-90 films against *Staphylococcus aureus* cells (Table 5).

CONCLUSIONS

The studies clearly demonstrate that chitosan in the form of films exerts antimicrobial effect on Gram-negative and Gram-positive bacteria. Therefore, such films can be used as antimicrobial food packaging material, as they not only protect food from undesirable environmental factors but can also reduce microbial contamination on the surface of the packed products. Moreover, incorporation of chitosan into gelatin films makes them active packages. The presence of chitosan in gelatin packages should also prevent them from too fast degradation during storage of the packed products.

The stage of bacteria inactivation rises with the increase in *DD* of the polymer, similarly as for chitosan in solution. However, the mechanism of inhibition of bacteria growth or inactivation in contact with chitosan films has not been fully recognized yet, and therefore further studies are needed to explain the relationship between the form of chitosan and its antimicrobial capacity.

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