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The Combined Effect of Moderate Pressure and Chitosan on *Escherichia coli* and *Staphylococcus aureus* Cells Suspended in a Buffer and on Natural Microflora of Apple Juice and Minced Pork

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Summary

The effect of chitosan and pressure of 193 MPa at –20 °C on *Escherichia coli* and *Staphylococcus aureus* cells suspended in a buffer at pH=5.8 as well as on the natural microflora of minced pork and apple juice has been evaluated. Immediately after pressure treatment of the tested bacteria in the presence of chitosan, the synergistic antimicrobial effect was higher against *E. coli* than against *S. aureus*, which amounted to additional 3.6 and 0.7 log cycles, respectively, compared to either treatment acting alone. However, incubation of *S. aureus* cells for 20 h at 37 °C after pressure treatment in the presence of chitosan led to complete inactivation of these bacteria. The combined effect of moderate pressure and chitosan did not decrease the total bacterial or psychrophilic and psychrotrophic count in minced pork in comparison with meat treated only with one factor, but the growth of psychrophilic and psychrotrophic bacteria was inhibited during storage up to 8 days at 5 °C. In apple juice, the combined effect of moderate pressure and chitosan only slightly increased the inactivation of bacterial population. However, during storage of samples at 5 °C for 15 days, the total bacterial count was about 1 log cycle lower than after the pressure treatment, while psychrophiles and psychrotrophs were not detected in 1 mL of the samples. The yeasts in apple juice were inactivated after pressure treatment alone. Synergistic reduction of moulds amounted to 2 log cycles and increased during storage at 5 °C. After 5 days, moulds were not detected in 1 mL of the samples.

Key words: inactivation of microorganisms, moderate pressure, subzero temperature, chitosan

Introduction

High pressure is one of the innovative techniques of food preservation that meets consumers' expectations regarding high quality of food products. To minimize changes of some food components such as proteins or lipids and for economical reasons, high pressure at moderate doses and at mild temperature should be used. However, under such conditions, with the use of mild pressure alone, a substantial inactivation of microorganisms, including vegetative cells of some bacteria is not achieved. Therefore, natural antimicrobial substances such as lysozyme, lactoperoxidase, lactoferrin, lactoferricin, nisin and pediocin are included into the process to increase the effectiveness of high pressure inactivation (*1–11*). Although the combined activity of these anti-

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microbials and high pressure increases the level of inactivation of microorganisms, other substances are still searched for in order to make the process more efficient.

Among the antimicrobial substances, chitosan, a deacetylated product of chitin, is of interest. This bioactive polysaccharide has been affirmed as Generally recognized as safe (GRAS) by the US FDA (*12*). Chitosan and its derivatives show strong antimicrobial activity against different groups of microorganisms, such as bacteria and fungi. In contrast to many other antimicrobials, chitosan displays antibacterial activity against both Gram-positive and Gram-negative bacteria. However, there are differences in sensitivity to chitosan within both Gram--types of bacteria (*13–17*) and therefore, it is difficult to determine whether chitosan as antimicrobial agent is more effective against Gram-positive or against Gram--negative bacteria.

The antimicrobial activity of chitosan depends on the degree of polymer deacetylation and molecular mass, its concentration in a solution or pH and the composition of the medium. These have been reviewed by Shahidi *et al.* (*18*) and Rabea *et al.* (*19*).

Chitosan below pH=6 is positively charged because of the protonation of amino groups in glucosamine residues. Therefore, the most probable mechanism of antimicrobial activity of chitosan results from the interaction of positively charged molecules of chitosan with the negatively charged surface of microbial cells. This leads to an increase in cell permeability and, as a consequence, to the leakage of many intracellular compounds (*20–24*). Moreover, chitosan as a chelating agent is able to selectively bind metals and essential nutrients, thus inhibiting the growth of microorganisms (*25,26*). According to Zheng and Zhu (*27*), the antimicrobial mechanism depends on the molecular mass of chitosans. The authors suggest that chitosans with high molecular mass form a film on the surface of the cell that does not allow nutritious compounds to enter the bacterial cell. These authors also suggest that chitosans with low molecular mass, below 5 kDa, can get into the cell and, through interactions with negatively charged cellular constituents, interfere with metabolic reactions. However, this mechanism seems to be unlikely because of the chitosan polarity.

A new possibility for processing and preservation of food has been created by using high pressure below 0 °C. Such low temperature and high pressure conditions allow a more effective inactivation of microorganisms than the pressure treatment in the range of 0–30 °C (*28–30*).

At present there is no literature data available on the combined effect of moderate or high pressure at subzero temperature and chitosan on the microorganisms in model system and on natural microflora of food. The objective of this study is to investigate antimicrobial effect of chitosans with different deacetylation degrees and pressure of 193 MPa at –20 °C in a sealed vessel on Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria as well as on the natural microflora of some food (minced pork and apple juice).

Materials and Methods

Chitosan preparation

Chitosan with deacetylation degree of 75 % and molecular mass of 1408 kDa (chitosan-75) and chitosan with deacetylation degree of 96 % and molecular mass of 1674 kDa (chitosan-96) were obtained from krill chitin in the Sea Fisheries Institute in Gdynia, Poland (*31*). Chitosans were dissolved in phosphate buffered saline (pH=5.8) at a final concentration of 4 mg/mL.

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 the Polish Collection of Microorganisms, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wrocław, Poland.

Cultures in stationary phase were prepared by inoculating 100 mL of TSBYE (tryptic soy broth supplemented with 0.6 % yeast extract) with 100 μL of liquid culture (at stationary phase of growth) and incubating it at 37 °C 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 in phosphate buffered saline (pH=6.0) to give viable counts of about 10^8–10^9 CFU/mL of the final concentration. Chitosans were added to the final concentration of 2 mg/mL.

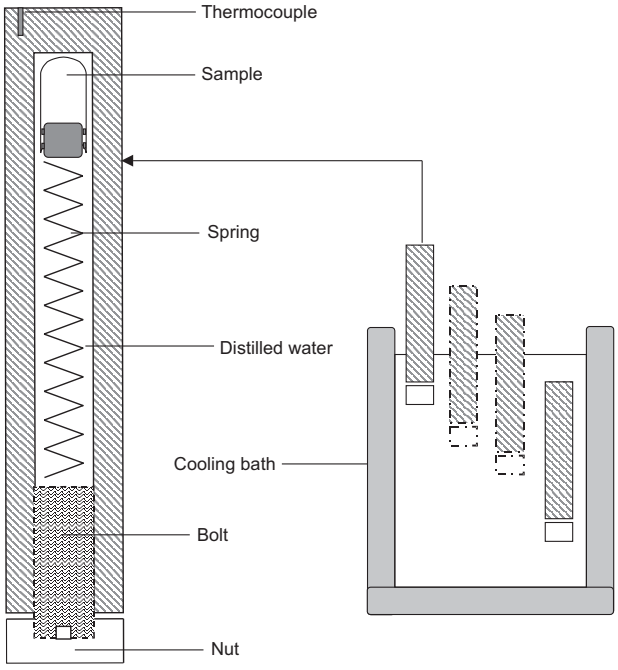
Preparation of food samples

Raw apple juice (pH=3.7) and minced pork were purchased from a local market and stored at 4 °C before use. A solution of 4 mg/mL of chitosan-96 was thoroughly mixed with minced pork or apple juice to obtain the final concentrations of chitosan of 2 mg/g or 2 mg/mL, respectively.

Pressure treatment

The pressure was generated in a natural way as proposed by Hayakawa *et al.* (*29*), without using an oil pressure pump. The method is based on the process of generating pressure as a result of the increasing volume during forming of ice I in a sealed vessel filled with water and kept at subzero temperatures. Moreover, according to Bridgman (*32*), high pressure reduces the freezing point of water to –22 °C at 207.5 MPa. Therefore, above this temperature, the sample placed in a sealed vessel is affected by the pressure in unfrozen state. The equipment used to generate pressure during the experiments was designed and constructed at the Department of Food Chemistry, Technology and Biotechnology, Chemical Faculty, Gdańsk University of Technology, Poland (Scheme 1).

Cell suspensions, apple juice (3 mL of each) or minced pork (about 3 g) were placed in sterile glass test tubes sealed with a stopper without leaving any bubbles of air inside and kept at 0 °C before pressurization. The tube was sealed with a specially designed tightly-fitting



Scheme 1. High pressure vessel and sample installation

stopper that is able to move within the tube. This allows pressure to be transmitted to the sample.

The tube containing a sample and a metal spring was placed in a cylindrical metal vessel filled with distilled water. The vessel was closed without leaving any bubbles of air inside. It was immersed for 40 min (4×10 min) with the closed side down in a temperature-controlled bath containing a mixture of ethanol, propylene glycol and distilled water (1:1:1, by volume) as a coolant (Scheme 1). Initially, only the bottom part of the vessel was immersed in the cooling bath. The role of the spring was to keep the tube in the upper, nonfrozen zone of the pressure vessel at all times. The temperature was measured inside the upper part of the pressure vessel with a thermocouple (Fig. 1). The visible plateau at –2 °C and the less observable one at lower temperatures are a consequence of heat released during the water–ice phase transitions that take place in the bottom part of the pressure vessel gradually immersed in the cooling bath. As high pressure lowers the freezing point of water, the sample should be in the unfrozen state up to about –20 °C, which was achieved after 33 min. The level of pressure was calculated on the basis of the solid-liquid equilibrium phase diagram of water (*32*). At the temperature of –20 °C the pressure in the sealed vessel reaches the level of 193 MPa (*33*). The fact that the sample was in the unfrozen state has been confirmed by the results of experiments in which polysaccharide hydrogels were kept simultaneously in the pressure vessel and under atmospheric pressure both at –20 °C. The damage of the structure of the latter hydrogel caused by water crystallization was visible. Such changes did not appear when hydrogel was cooled in the sealed vessel.

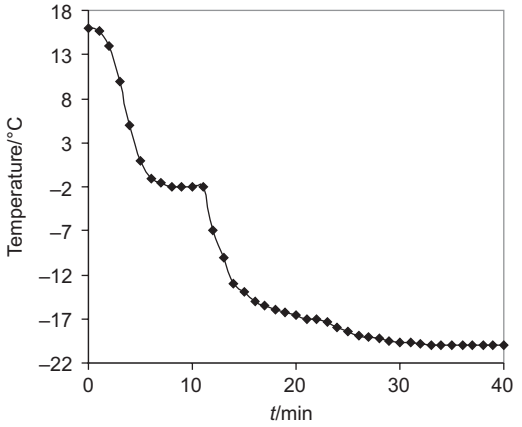


Fig. 1. The temperature history recorded in the upper segment of the pressure vessel, in the unfrozen water

After pressure treatment at –20 °C, the vessel was lifted from the cooling bath to half of its length and warmed in the upper part to 15 °C, measured with a thermocouple. Then the vessel was taken out and placed for a few minutes in a water bath at 20 °C. The total time of decompression did not exceed 10 min (after that time there were not any ice crystals in the pressure vessel). After pressure treatment the samples were stored in an ice bath prior to determination of viable counts. Unpressurized samples were used as controls.

Enumeration of viable cells

Pressure-treated cell suspensions and controls were serially diluted with phosphate buffered saline (pH=7.0). Dilutions were plated on tryptic soy agar supplemented with 0.6 % yeast extract (TSAYE) and plates were incubated for 48 h at 37 °C.

Samples of apple juice were serially diluted with 0.1 % peptone water. In the case of minced pork, the initial 10-fold dilution was prepared by homogenizing 1 g of meat with 9 mL of 0.1 % peptone water before further dilutions. Appropriate serial dilutions of apple juice and pork were then plated onto plate count agar and incubated for 48 h at 30 °C (total bacterial count) or for 10 days at 7 °C (psychrophiles and psychrotrophs). Additionally, Sabouraud agar was used to enumerate total counts of yeast and mould in apple juice and the plates were incubated at 20 °C for 72 h. The media were purchased from BTL Sp. z o.o., Łódź, Poland.

Statistical analyses

Results of the effects of pressure and chitosan are average values of three replicates with standard deviation. The differences between treatments were evaluated statistically by the one-way analysis of variance (ANOVA) using the program Statgraphics, Statistical Graphic Corporation, version 2.1.

Results and Discussion

The effect of chitosan on E. coli and S. aureus cells

Chitosan-96 with average molecular mass of 1674 kDa, at a concentration of 2 mg/mL, reduced the number of viable *S. aureus* and *E. coli* cells by about 1.6 and 0.8 log cycles, respectively, during incubation of samples

for 1 h at 37 °C (Table 1). When incubation was extended up to 20 h, further decrease of the *S. aureus* count reached 3.4 log cycles and *E. coli* cells were not detected in 1 mL of the sample (Table 1). Since chitosan-96 with high molecular mass was used in our experiments, the lethal effect probably resulted from interactions of protonated amine groups of polymer with negatively charged surface of bacterial cells. Furthermore, the antimicrobial effectiveness of chitosan was greater against *E. coli* than against *S. aureus*. Similar results were obtained by Wang (*34*), Uchida *et al.* (*13*) and Simpson *et al.* (*35*). According to Chung *et al.* (*36*), it results from higher negative charge on the surface of Gram-negative bacteria than that on the surface of Gram-positive bacteria. However, some other authors showed that chitosans had higher antimicrobial efficiency against *S. aureus* than against *E. coli* (*16,37,38*) or displayed the same effect against both species of bacteria (*14,15,39,40*). It seems that all these discrepancies among the data can result not only from different tests and conditions of experiments that were used to determine antimicrobial properties of chitosan, but also because different strains of *S. aureus* or *E. coli* were tested in particular experiments.

It has been shown that temperature is an important factor that influences the level of bacteria inactivation by chitosan. The reduction in *S. aureus* and *E. coli* counts after incubation for 20 h at 5 °C was 3 and 5 log cycles, respectively, lower than at 37 °C (Table 1). Tsai and Su (*24*) also found that increasing temperature in the range of 4 to 37 °C enhanced bactericidal activity of chitosan against *E. coli* suspended in the buffer medium.

Antimicrobial activity of chitosan-75 was much lower in comparison with more deacetylated chitosan-96 (Table 1). Such relationship had previously been found by other authors (*17,40,41*). According to them, enhancing antimicrobial activity with the increase of deacetylation degree of chitosan results in the increase of positive charge of polymer molecules. Moreover, Chung *et al.* (*36*) showed that more chitosan was adsorbed by bacterial cells as the deacetylation degree increased and there was a positive correlation between the inhibition efficiency of chitosan and the amounts of adsorbed polymer.

The effect of pressure and chitosan on E. coli and S. aureus cells

Immediately after pressure treatment of the tested bacteria in the presence of chitosan-96, the synergistic antimicrobial effect was higher against *E. coli* than against *S. aureus*. The additional reduction of these bacteria amounted to 3.6 and 0.7 log cycles, respectively (Table 1). However, incubation of *S. aureus* cells in the presence of chitosan-96 for 20 h at 37 °C after pressure treatment led to complete inactivation of these bacteria and calculated synergistic reduction was 3.4 log cycles. Although the storage of samples for 20 h at 5 °C after pressurization in the presence of chitosan was less effective than at 37 °C and did not cause a complete inactivation of both tested bacterial strains, the antimicrobial effect of combined pressure and chitosan was still strong. The *S. aureus* and *E. coli* counts decreased by 6 log cycles (synergistic reduction 3.6 log cycles) and 5.5 log cycles (syn-

Table 1. Effect of pressure[1] and chitosan[2] on *E. coli* K-12 and *S. aureus* PCM2054 cells[3]

Sample	log CFU/mL		
	without chitosans	with chitosan-96	with chitosan-75
	Staphylococcus aureus		
unpressurized and non-stored	(8.2±0.1)^{a}	(8.4±0.3)^{a}	(7.8±0.1)^{i}
stored for 1 h at 37 °C	(8.3±0.1)^{a}	(6.8±0.1)^{d}	(7.3±0.1)^{j}
stored for 20 h at 37 °C	(8.1±0.3)^{a,b}	(3.4±0.2)^{e}	(6.8±0.1)^{k}
stored for 20 h at 5 °C	(8.2±0.1)^{a}	(6.4±0.1)^{f}	(7.7±0.1)^{i}
stored for 1 h at 37 °C and then pressurized	(7.9±0.1)^{b}	(5.8±0.1)^{g}	(6.2±0.2)^{l}
pressurized and subsequently stored for 20 h at 37 °C	(8.0±0.1)^{a,b}	nd	(5.6±0.2)^{m}
pressurized and subsequently stored for 20 h at 5 °C	(7.5±0.1)^{c}	(2.2±0.3)^{h}	(5.9±0.1)^{m}
	Escherichia coli		
unpressurized and non-stored	(8.1±0.1)^{a}	(7.6±0.1)^{d}	(8.0±0.1)^{a}
stored for 1 h at 37 °C	(8.0±0.1)^{a}	(6.8±0.1)^{e}	(7.7±0.1)^{i}
stored for 20 h at 37 °C	(8.4±0.1)^{b}	nd	(4.7±0.1)^{j}
stored for 20 h at 5 °C	(8.1±0.1)^{a}	(4.8 ±0.1)^{f}	(6.4±0.2)^{k}
stored for 1 h at 37 °C and then pressurized	(8.0±0.1)^{a,c}	(3.6 ±0.0)^{g}	(3.8±0.2)^{g}
pressurized and subsequently stored for 20 h at 37 °C	(7.8±0.1)^{c}	nd	(4.4±0.2)^{j}
pressurized and subsequently stored for 20 h at 5 °C	(7.8±0.0)^{c}	(2.5±0.1)^{h}	(3.2±0.1)^{l}

nd – not detected
$^{a–m}$values for a particular row or column followed by different letters differ significantly ($p<0.05$)
[1]193 MPa, –20 °C
[2]c=2 mg/mL
[3]suspended in buffer with pH=5.8

ergistic reduction 2 log cycles), respectively (Table 1). According to Papineau *et al.* (*37*) there was no synergy between the activity of chitosan and high hydrostatic pressure of 238 MPa against *S. aureus* MF-31 and *E. coli* V517. However, it seems that some results obtained by these authors show that such effect took place in the case of *S. aureus* cells.

The combined effect of moderate pressure and chitosan-75 with lower deacetylation degree was less effective in the inactivation of bacteria than that with chitosan-96 (Table 1). However, similarly to the experiments carried out with pressure and chitosan-96, *E. coli* cells were more sensitive to the treatment with both of these factors than *S. aureus*, although there was not such a big difference in the viability of cells depending on the temperature during storage of the samples after pressurization.

Considering the activity of chitosan under high pressure with different deacetylation degree, it can be concluded that, like in the case of chitosan alone, the most important factor is the number of protonated amine groups. The positive charge of chitosan depends on the deacetylation degree of the polymer as well as on the pH of the medium. In such case it is very important to take into account the possibility of pH change of the buffer under high pressure. In the work of Quinlan *et al.* (*42*) the pH of the phosphate buffer decreased about 0.4 units at 250 MPa and 25 °C. Such changes of pH possibly occurred in our experiments. Therefore, one reason for synergistic effect of chitosan and high pressure against bacteria could be the increase of chitosan charge due to decrease of the buffer pH. Finally, it seems that both chitosan and pressure treatment increase the cell permeability, causing enhanced destructive activity against bacteria.

Combined effect of moderate pressure and chitosan-96 on inactivation of natural microflora of minced pork and apple juice

The total bacterial, and psychrophilic and psychrotrophic count determined after the treatment of meat at 193 MPa and –20 °C was decreased by about 1 log cycle as compared to that from the untreated samples (Table 2). For example, at a temperature above 0 °C this level of inactivation was achieved with minced chicken treated with the pressure of 500 MPa at 40 °C (*43*). On the other hand, higher inactivation, especially of psychrotrophic bacteria, was observed in pork loin and minced beef after the pressure treatment with 400 MPa at 22–25 °C (*44,45*), and mechanically recovered poultry meat pressurized in three cycles with 450 MPa at 2 °C (*46*). The higher sensitivity to pressure of psychrotrophic bacteria than mesophiles is a result of the loss of ability to grow at low temperature (*46*). Therefore, it is difficult to compare the results obtained by different authors because the microflora of the tested meat samples can be diverse as well as the conditions of pressure treatment.

In meat stored up to 8 days at 5 °C in the presence of chitosan at the concentration of 2 mg/g, the total bacterial, and psychrophilic and psychrotrophic count was about 1 log cycle lower than in meat samples treated without chitosan (Table 2). Similar results were obtained by Sagoo *et al.* (*47*) in the case of pork sausages containing 0.6 % of chitosan glutamate. Such level of inactivation was also observed in minced beef after 10 days of storage at 4 °C, but in the presence of chitosan at concentration of 1 % (*48*). Below this concentration bacterial growth was not retarded more than in the control meat.

The combined effect of moderate pressure and chitosan did not decrease the total bacterial or psychrophilic and psychrotrophic counts in comparison with

Table 2. Effect of pressure[1] and chitosan-96[2] on total bacterial count, and psychrophilic and psychrotrophic count in minced pork stored at 5 °C

Sample	*t*(storage)/day				
	0	4	6	8	13
	Total bacterial count/(log CFU/g)				
without chitosan and unpressurized	$(4.8\pm0.1)^{a}$	$(5.9\pm0.0)^{c}$	$(8.0\pm0.1)^{f}$	$(9.4\pm0.1)^{h}$	$(10.4\pm0.1)^{l}$
with chitosan and unpressurized	$(3.8\pm0.1)^{b}$	$(4.7\pm0.2)^{d}$	$(6.8\pm0.2)^{g}$	$(8.2\pm0.1)^{i}$	$(10.1\pm0.1)^{m}$
pressurized	$(3.9\pm0.3)^{b,e}$	$(4.1\pm0.1)^{e}$	$(4.1\pm0.1)^{e}$	$(6.0\pm0.1)^{j}$	$(9.0\pm0.1)^{n}$
pressurized with chitosan	$(3.9\pm0.1)^{b}$	$(3.8\pm0.1)^{b}$	$(4.0\pm0.1)^{e}$	$(4.5\pm0.2)^{k}$	$(6.8\pm0.1)^{o}$
Sample	*t*(storage)/day				
	0	4	6	8	13
	Psychrophilic and psychrotrophic count/(log CFU/g)				
without chitosan and unpressurized	$(4.3\pm0.1)^{a}$	$(6.1\pm0.1)^{c}$	$(8.1\pm0.1)^{f}$	$(9.2\pm0.2)^{i}$	$(10.4\pm0.1)^{m}$
with chitosan and unpressurized	$(3.6\pm0.1)^{b}$	$(4.6\pm0.1)^{d}$	$(6.8\pm0.1)^{g}$	$(8.2\pm0.1)^{j}$	$(10.1\pm0.1)^{n}$
pressurized	$(3.4\pm0.1)^{b}$	$(3.5\pm0.1)^{b}$	$(3.8\pm0.1)^{h}$	$(6.1\pm0.1)^{k}$	$(9.0\pm0.1)^{o}$
pressurized with chitosan	$(3.5\pm0.1)^{b}$	$(3.1\pm0.2)^{e}$	$(3.7\pm0.1)^{b,h}$	$(4.1\pm0.1)^{l}$	$(7.9\pm0.2)^{p}$

[a–p]values for a particular row or column followed by different letters differ significantly ($p<0.05$)
[1]193 MPa at –20 °C
[2]c=2 mg/g

meat treated only with one factor (Table 2). During storage at 5 °C of meat samples pressurized in the presence of chitosan, there was no reduction in the total bacterial count in contrast to the results obtained in the model system with pure cultures of mesophilic bacteria in the buffer solution. However, the growth of bacteria was inhibited during storage up to 8 days since the total bacterial, and psychrophilic and psychrotrophic count only slightly increased during that time (Table 2). The counts were about 5 log cycles lower than those in the corresponding control samples. However, after 13 days of storage, the total bacterial, and psychrophilic and psychrotrophic count increased up to 10^7 and 10^8 CFU/mL, respectively, but the counts were still lower than in the control samples (unpressurized and without chitosan).

Although chitosan exerts bactericidal effect against bacteria in model system, natural microflora of apple juice, including yeasts and moulds, was not inactivated by this polymer at the concentration of 2 mg/mL (Tables 3 and 4). Pressurization of apple juice without chitosan at 193 MPa and –20 °C reduced the total bacterial and mould counts only by 0.5 and 0.7 log cycles, respectively. The most sensitive to pressure were yeasts. They were not detected in 1 mL of the sample after pressurization (Table 4). The literature data also show that yeasts are more sensitive to pressure than bacteria and moulds (*49*,*50*).

The combined effect of moderate pressure and chitosan slightly increased the inactivation of bacterial population. However, during storage of apple juice at 5 °C for 15 days, the total bacterial count was about 1 log cycle lower than that immediately after the pressure treatment, and psychrophiles and psychrotrophs were not detected in 1 mL of the sample (Table 3). These experiments, in contrast to that with minced pork, clearly showed that psychrophiles and psychrotrophs are more sensitive to pressure than mesophiles. In the case of moulds, synergistic effect of pressure and chitosan was observed after the pressure treatment. It amounted to 1 log cycle and increased during storage at 5 °C; after 5 days moulds were not detected in 1 mL of the sample (Table 4).

Unlike at low temperature, the growth of bacteria and yeasts was not inhibited in apple juice with the addition of chitosan or in pressurized juice without chitosan during storage at 20 °C (Tables 3 and 4). Moreover, the yeasts outnumbered the moulds as the time of storage prolonged (Table 4). Also, Roller and Covill (*51*) found that chitosan glutamate at 25 °C, in contrast to 5 °C, was ineffective as a preservative in mayonnaise-based shrimp salads. Furthermore, Tsai *et al.* (*38*) reported that antimicrobial effect of chitosanoligosaccharides against some pathogenic bacteria inoculated into sterilized milk was much higher during storage at 4 than at 37 °C. However, the results of our experiments showed that the combined effect of moderate pressure and chitosan effectively inhibited bacterial and fungal growth during storage of apple juice for 15 days not only at 5 but also at 20 °C (Tables 3 and 4).

Conclusions

Chitosan, contrary to other antimicrobial substances such as lysozyme and nisin, is effective in inactivation of Gram-negative bacteria in model buffer system. The combined effect of moderate pressure at a temperature below 0 °C and chitosan allows achieving high synergistic bactericidal effect against pure culture of bacteria including pressure resistant Gram-positive *Staphylococcus aureus*.

Table 3. Effect of pressure[1] and chitosan-96[2] on the total bacterial count, and psychrophilic and psychrotrophic count in apple juice stored at 5 and 20 °C

Sample	*t*(storage)/day							
	0	5	10	15	0	5	10	15
	Psychrophilic and psychrotrophic count				Total bacterial count			
	log CFU/mL							
Storage at 5 °C								
without chitosan and unpressurized	$(4.8\pm0.1)^{a}$	$(4.9\pm0.2)^{a}$	$(5.7\pm0.0)^{e}$	$(6.7\pm0.1)^{g}$	$(4.1\pm0.1)^{a}$	$(4.3\pm0.1)^{a}$	$(3.8\pm0.2)^{e}$	$(3.7\pm0.1)^{e}$
with chitosan and unpressurized	$(4.1\pm0.0)^{b}$	$(4.5\pm0.1)^{d}$	$(4.2\pm0.0)^{b}$	$(5.4\pm0.2)^{h}$	$(3.9\pm0.1)^{a}$	$(3.3\pm0.0)^{d}$	$(3.2\pm0.1)^{d}$	$(3.2\pm0.1)^{d}$
pressurized	$(2.8\pm0.2)^{c,f}$	$(2.9\pm0.1)^{c}$	$(2.6\pm0.1)^{f}$	$(2.8\pm0.2)^{c,f}$	$(3.6\pm0.1)^{b}$	$(3.4\pm0.2)^{d}$	$(3.3\pm0.1)^{d}$	$(3.4\pm0.1)^{d}$
pressurized with chitosan	nd	nd	nd	nd	$(3.0\pm0.1)^{c}$	$(3.0\pm0.1)^{c}$	$(3.1\pm0.1)^{c,d}$	$(2.0\pm0.2)^{f}$
Storage at 20 °C								
without chitosan and unpressurized	$(4.8\pm0.0)^{a}$	$(6.1\pm0.1)^{d}$	$(7.1\pm0.1)^{e}$	$(8.7\pm0.0)^{h}$	$(4.1\pm0.1)^{a}$	$(6.2\pm0.3)^{d}$	$(6.1\pm0.1)^{d}$	$(6.9\pm0.2)^{j}$
with chitosan and unpressurized	$(4.1\pm0.1)^{b}$	$(5.8\pm0.2)^{d}$	$(7.4\pm0.1)^{f}$	$(7.7\pm0.2)^{f}$	$(3.9\pm0.1)^{a}$	$(6.8\pm0.2)^{e}$	$(7.1\pm0.1)^{e,h}$	$(7.5\pm0.1)^{k}$
pressurized	$(2.8\pm0.1)^{c}$	$(2.8\pm0.1)^{c}$	$(6.3\pm0.1)^{g}$	$(7.4\pm0.1)^{f}$	$(3.6\pm0.1)^{b}$	$(7.2\pm0.1)^{f}$	$(7.2\pm0.1)^{f,h}$	$(7.6\pm0.1)^{k}$
pressurized with chitosan	nd	nd	nd	nd	$(3.0\pm0.0)^{c}$	$(2.3\pm0.0)^{g}$	$(2.7\pm0.1)^{i}$	$(3.5\pm0.0)^{l}$

nd – not detected
[a–l]values for a particular row or column followed by different letters differ significantly ($p<0.05$)
[1]193 MPa at –20 °C
[2]*c*=2 mg/mL

Table 4. Effect of pressure[1] and chitosan-96[2] on the total yeast and mould counts in apple juice stored at 5 and 20 °C

Sample	*t*(storage)/day							
	0	5	10	15	0	5	10	15
	Yeast count				Mould count			
	log CFU/mL							
Storage at 5 °C								
without chitosan and unpressurized	(3.2±0.1)^{a}	(4.0±0.1)^{b}	(4.7±0.1)^{d}	(5.5±0.1)^{e}	(3.5±0.2)^{a}	(2.7±0.0)^{d}	(3.1±0.1)^{e}	(3.1±0.0)^{e}
with chitosan and unpressurized	(3.1±0.1)^{a}	(1.9±0.2)^{c}	(2.0±0.0)^{c}	(1.9±0.1)^{c}	(3.3±0.1)^{a}	(2.6±0.2)^{d}	(2.6±0.2)^{d}	(2.2±0.1)^{f}
pressurized	nd	nd	nd	nd	(2.7±0.0)^{b}	(2.7±0.1)^{b,d}	(2.9±0.1)^{d}	(2.7±0.1)^{b,d}
pressurized with chitosan	nd	nd	nd	nd	(1.4±0.1)^{c}	nd	nd	nd
Storage at 20 °C								
without chitosan and unpressurized	(3.2±0.1)^{a}	(6.7±0.1)^{b}	(6.7±0.2)^{b}	(6.3±0.1)^{d}	(3.5±0.1)^{a}	(8.0±0.0)^{d}	(8.0±0.1)^{d}	(8.0±0.1)^{d}
with chitosan and unpressurized	(3.1±0.1)^{a}	(6.0±0.1)^{c}	(6.7±0.1)^{b}	(7.0±0.1)^{e}	(3.3±0.1)^{a}	nd^{e}	nd^{e}	nd^{e}
pressurized	nd	nd	nd	nd	(2.7±0.2)^{b}	(6.2±0.1)^{e}	(5.5±0.2)^{f,g}	(5.8±0.1)^{g}
pressurized with chitosan	nd	nd	nd	nd	(1.5±0.1)^{c}	nd	nd	nd

nd – not detected
$^{a–g}$values for a particular row or column followed by different letters differ significantly ($p<0.05$)
[1]193 MPa at –20 °C
[2]*c*=2 mg/mL

Among natural microflora of a fruit juice, the most sensitive to pressure alone were yeasts. The living cells in pressurized apple juice at pH=3.8 were not detected during storage at 5 °C for 15 days. Such effect was also observed against moulds in the case of combined effect of pressure and chitosan. The bacterial population of food was reduced to a much lesser extent even when pressure together with chitosan was used. This can result from a protective effect that components of food such as saccharides and proteins exert on microorganism cells and from large species diversity of microflora in food products. Additionally, especially in meat systems, interactions of chitosan with negatively charged groups of proteins or other components can lead to the reduction of antimicrobial effectiveness of a polymer. However, although the moderate pressure treatment alone or combined with antimicrobial substances such as chitosan does not allow cold pasteurisation of food, it enables significantly extended shelf life of refrigerated food.

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