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EFFECT OF LYSOZYME OR NISIN ON SURVIVAL OF SOME BACTERIA TREATED WITH HIGH PRESSURE AT SUBZERO TEMPERATURE

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ABSTRACT

The aim of this work was to examine the inactivation of some Gram-positive and Gram-negative bacteria exposed to the pressure of 193 MPa at –20 °C in the presence of lysozyme or nisin at concentration of 400 μg/ml. The highest effect of pressure at subzero temperature and lysozyme was found with pressure sensitive *Pseudomonas fluorescens*; viable cells of this strain were not detected in 1 ml of sample after combined treatment. The action of pressure at subzero temperature and lysozyme or nisin against *Escherichia coli* led to synergistic reduction by 0.7 or 1.6 log cycles, respectively, while it was practically insignificant for two *Staphylococcus aureus* strains. Viability loss of *E. coli* and *S. aureus* occurred during storage for 20 h of the samples at 37 and 5 °C, which were previously pressurized with lysozyme or nisin. The synergistic effect of pressure and nisin at pH 5 against *E. coli* cells just after the pressure treatment was lower than that at pH 7, however, the extent of the lethal effect after storage was higher.

Key words: Inactivation of microorganisms, High pressure, Subzero temperature, Lysozyme, Nisin

INTRODUCTION

High-pressure technique is commercially used for preservation of, mainly, acid food products. However, food with pH close to neutral is a better medium for growth of microorganisms that survived the pressure treatment. Moreover, frequently in the pressure treated population of vegetative bacteria survivor tails can occur (35). It is known that Gram-negative bacteria and cells in the exponential growth phase are more sensitive to pressure treatment than Gram-positive bacteria and cells being in the stationary phase of growth (3, 35). Moreover, species very resistant to high pressure exist even among Gram-negative bacteria. Variations in pressure resistance were also observed among strains belonging to the same species of food-born pathogens and spoilage bacteria (1, 21, 28, 29). It should be also taken

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into consideration that barotolerant mutants can be formed in the pressure process (14). Furthermore, microorganisms are usually more resistant to pressure treatment in food systems than in a buffer (4, 8, 29, 34). For these reasons pressure above the moderate range should be used in cold pasteurization of food. This could limit the practical applications of this technique due to changes in some components of food, especially proteins and because of the higher operating costs with the increase of pressure dose. One of the possibilities for achieving the increase in inactivation of bacteria in low-acidity foods is using pressure in combination with antimicrobial factors. Lysozyme, lactoperoxidase, lactoferrin, lactoferricin, nisin and pediocin are known to enhance the effectiveness of high pressure in inactivation of some bacteria (8, 9, 10, 17, 18, 19, 20, 22, 24, 27). The synergistic effect is a result of sublethal injury of cells that makes them more sensitive to antimicrobial substances (14, 17, 18, 20). Moreover, the high pressure induces outer membrane permeabilization in Gram-negative bacteria. It sensitizes the cells to action of bacteriocins and lysozyme (14, 22, 23, 27). The temperatures used in experiments conducted by the cited authors were in the range of 20-50 °C. However, Steeg *et al*. (36) observed the enhanced synergistic inactivation of some bacteria when high pressure in the presence of nisin was used at temperature 10 °C. This effect of nisin can probably deepen during further reduction of temperature. Moreover, the limited available data showed higher level of inactivation of microorganisms under high pressure at subzero temperature than in a certain range of temperature above 0 °C (12, 15, 26, 31, 32, 33).

Therefore, it is not excluded that such conditions may enhance the synergistic effect of combined action of high pressure and nisin against microorganisms. Furthermore, it would be worth also to check if lysozyme exerts any antimicrobial activity under pressure at such low temperature as –20 °C. It is known that this polypeptide can act on bacteria according to lytic and non-lytic mechanisms, depending on their type and the environmental conditions (6, 25). Therefore, the aim of this work was to examine the inactivation of some Gram-positive and Gram-negative bacteria exposed to pressure of about 193 MPa at –20 °C in unfrozen system in the presence of lysozyme and nisin followed by prolonged storage at 5 and 37 °C. Such data were not found in the available literature.

MATERIALS AND METHODS

Preparation of lysozyme and nisin solutions

Hen egg white lysozyme (69 480 U/mg proteins, Fluka, Switzerland) dissolved in potassium phosphate buffer (10 mM, pH 7.0) at concentration of 2 mg/ml and nisin (Fluka, Switzerland) dissolved in 0.01 M HCl at concentration of 2 mg/ml, prepared directly before experiments, were used.

Cultures and growth conditions

The following bacterial strains were used: *Pseudomonas fluorescens* WSRO121 from Collection of Dairy Cultures of Department of Microbiology, University of Warmia and Mazury, Olsztyn, Poland; *Escherichia coli* K-12 PCM2560 (NCTC10538) and *Staphylococcus aureus* PCM2054 (ATCC25923) from Polish Collection of Microorganisms, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wrocław, Poland; *Staphylococcus aureus* DSM2569 from German Collection of Microorganisms and Cell Cultures and *Listeria innocua* III_1 (isolated from fish) kindly provided by Department of Food Microbiology, University of Agriculture, Szczecin, Poland.

Inocula of strains were prepared by inoculating 100 ml

of tripticase soy broth supplemented with 0.6% yeast extract (TSBYE; purchased from BTL Sp. z o.o., Łódź, Poland) with 100 µl of liquid culture (at stationary phase of growth) and incubating at 37 °C (*E. coli, S. aureus, L. innocua*) or 28 °C (*P. fluorescens*) for 24 h with shaking. Under these conditions the cells were in the stationary phase.

In order to obtain an appropriate growth phase of cells, 100 µl of the stationary-phase culture were inoculated into 100 ml of fresh TSBYE and incubated with shaking at optimal temperature of growth for particular microorganisms. The bacteria growth was measured by determination of optical density at 660 nm for 48 h. The middle exponential and stationary phase were determined from the obtained curves of growth.

Preparation of cell suspensions

The cells in a stationary or exponential phase of growth were centrifuged at 1300xg for 20 min at 5 °C, and the pellets were resuspended in phosphate-buffered saline at pH 7.0 or in some experiments in Mc Ilvaine's buffer at pH 5.0 to give viable counts of about $10^8/10^9$ cfu/ml. Antimicrobials were added to the final concentration of 400 µg/ml (lysozyme) or 100 and 400 µg/ml (nisin).

Pressure treatment

The pressure was generated similarly as described by Hayakawa *et al*. (15). The procedure was based on the phenomenon that in a sealed vessel filled with water and kept at subzero temperatures, increasing volume of forming ice I generates internal pressure. According to Bridgman (2) high pressure reduces the freezing and melting points of water to a minimum of –22 °C at 207.5 MPa. Therefore, above this temperature, the sample placed in a sealed vessel is affected y the pressure in unfrozen state.

The equipment used to generate pressure during the xperiments was designed and constructed by Edward Dunajski in our Department (Fig. 1).

Cell suspensions (3 ml of each) were placed in sterile glass test tubes (with volume of 3 ml), and sealed with a stopper. At this moment a small excess of liquid was pushed out from the tubes without leaving any bubbles of air inside. Due to the special design the stopper holds the tightness of the tube and it is able to move. This allows to expose the sample to the generated pressure.

The tube containing cell suspension and then a metal spring were placed in a cylindrical metal vessel filled with distilled water. The vessel was closed without leaving any bubbles of air inside. It was immersed in four stages during 40 min (4x10 min) with the closed side down, in a temperature-controlled bath containing a mixture of ethanol, propylene glycol and distilled water (1:1:1, v/v) as a coolant (Fig. 1). So initially, only the bottom part of the vessel was immersed in the cooling bath. The role of the spring was to keep the tube, at this moment and later, when the whole pressure vessel was in the cooling mixture, at upper - nonfrozen zone - of the pressure vessel. The time during which the assumed temperature was achieved has been determined in preliminary experiments using a thermocouple. As high pressure lowers the freezing point of water, thus the material should be in the unfrozen state up to about –20 °C. This fact confirmed the results of experiments in which hydrogels were kept at –20 °C in the pressure vessel and simultaneously at the same temperature under atmospheric pressure. The damage of structure of the latter hydrogel caused by water crystallization was visible. Such changes did not appear when hydrogel was cooled in the sealed vessel.

After pressure treatment at –20 °C the vessel was stuck out from cooling bath in 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. The samples were stored in an ice bath prior to

determination of viable counts. Unpressurized samples were used as controls. In some experiments the samples were incubated for 2 or 20 h with or without lysozyme/nisin added after the pressure treatment.

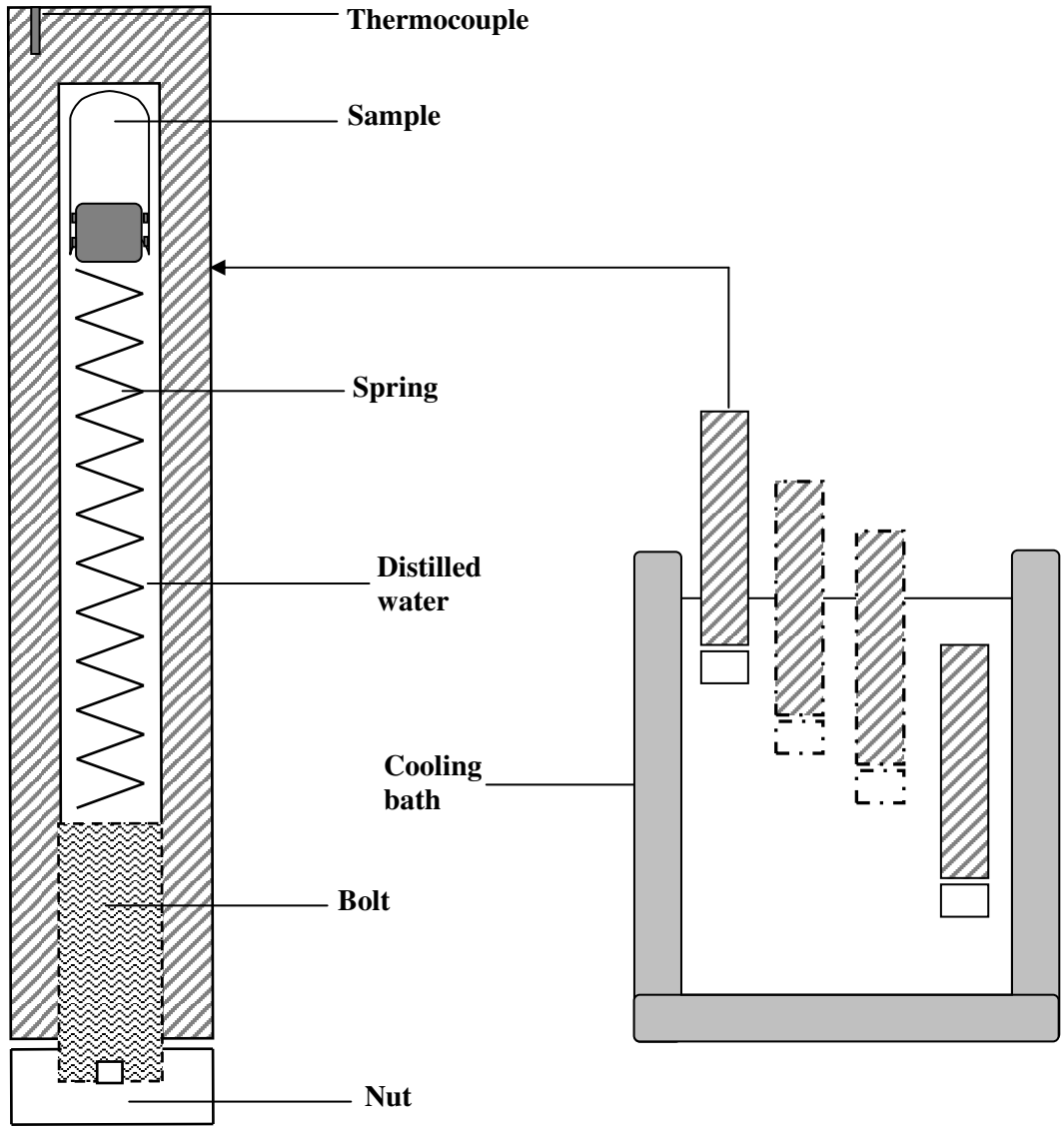

Figure 1. Scheme of the high pressure vessel and sample installation

Enumeration of viable cells

Pressure-treated samples and untreated control cell suspensions were serially diluted with buffered saline (pH 7.0). Dilutions were plated on tripticase soy agar supplemented with 0.6 % yeast extract (TSAYE; purchased from BTL Sp. z o.o., Łódź, Poland)) and plates were incubated for 48 h at 37 °C (*E. coli*, *S. aureus*, *L. innocua*) or 28 °C (*P. fluorescens*).

The data presented in the tables and figures are mean values obtained from three independent experiments. The bars on the figures indicate the mean standard deviations for the data points. The results were evaluated statistically by analysis of variance (one-way procedure) or Students t-test using the program Statgraphics, Statistical Graphic Corporation.

RESULTS AND DISCUSSION

Antibacterial effect of high pressure and lysozyme

The pressure that can be achieved at –20 °C in the closed vessel amounts to about 193 MPa. The tested bacteria differed in sensitivity to pressure mainly in the exponential phase of growth and the most pressure resistant were *S. aureus* strains (Fig. 2). The number of viable cells from the stationary phase of growth decreased under these conditions no more than by one log cycle, with exception of *P. fluorescens*. The pressure reduction in the viability of this strain was about 4 log cycles. The large pressure sensitivity of *P. fluorescens* was also evidenced by Gervilla *et al.* (11). Therefore, in the next experiments only bacterial cells in the stationary phase of growth were used.

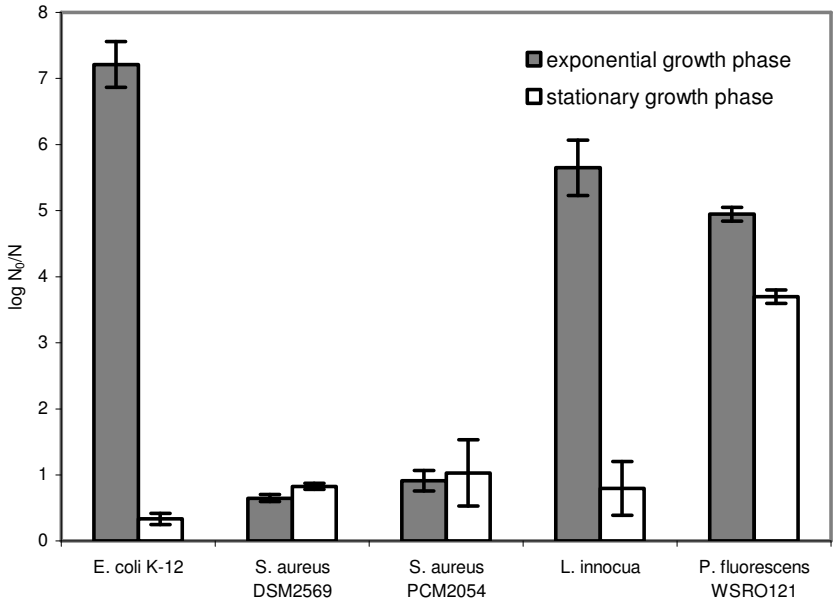

Figure 2. Viability loss of some Gram-negative and Gram-positive bacteria after pressure treatment at 193 MPa and –20 °C
N_0 – the number of cells in the control (about 10^8 – 10^9 cfu/ml)
N – the number of cells detected after pressurization

As expected, lysozyme at ambient pressure was not effective against Gram-negative bacteria: *E. coli* and *P. fluorescens* (Table 1). Moreover, the viability loss in the presence of lysozyme in the case of tested Gram-positive *L. innocua* and two strains of *S. aureus* did not exceed 0.5 log cycle. As reported by Pellegrini *et al.* (30) lysozyme at atmospheric pressure did not attack *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 1228 cells. It is known that modification of the peptidoglycan of some Gram-positive bacteria results in resistance to its degradation by lysozyme (5).

When combined pressure 193 MPa at –20 °C and lysozyme were used the extra reduction of *E. coli* of 0.7 log cycle was obtained (Fig. 3). In samples pressurized in the

presence of lysozyme the viability of *E. coli* cells did not decrease during incubation for 2 h at 37 °C (Fig. 3 B and C). Moreover, there were no significant differences in the number of viable cells in the pressurized samples without lysozyme and in the samples incubated for 2 h at 37 °C with lysozyme added after pressure treatment (Fig. 3 A and D). These data suggest that some of the observed antimicrobial impact of lysozyme did not take place after decompression but during pressurization at subzero temperature. Lysozyme probably does not show enzyme activity at –20 °C; thus a non-lytic mechanism of lysozyme action under these conditions could not be excluded. The most likely non-lytic mechanism of antimicrobial activity of lysozyme is based on disruption of bacterial membrane (6).

Table 1. The viability of some Gram-negative and Gram-positive bacteria of stationary phase after 2 h incubation at 37 °C in the presence of lysozyme (400 μg/ml)[1].

Strain	Log CFU/ml	
	Without lysozyme	**With lysozyme**
S. aureus PCM2054	8.9±0.1[a]	8.5±0.1[b]
S. aureus DSM2569	8.8±0.1[a]	8.7±0.0[a]
E. coli K-12	8.8±0.1[a]	8.8±0.1[a]
L. innocua III_1	8.6±0.1[a]	8.1±0.1[b]
P. fluorescens WSRO121	8.8±0.1[a]	8.6±0.1[a]

[1]The values for a particular row followed by different letters differ significantly ($p<0.05$)

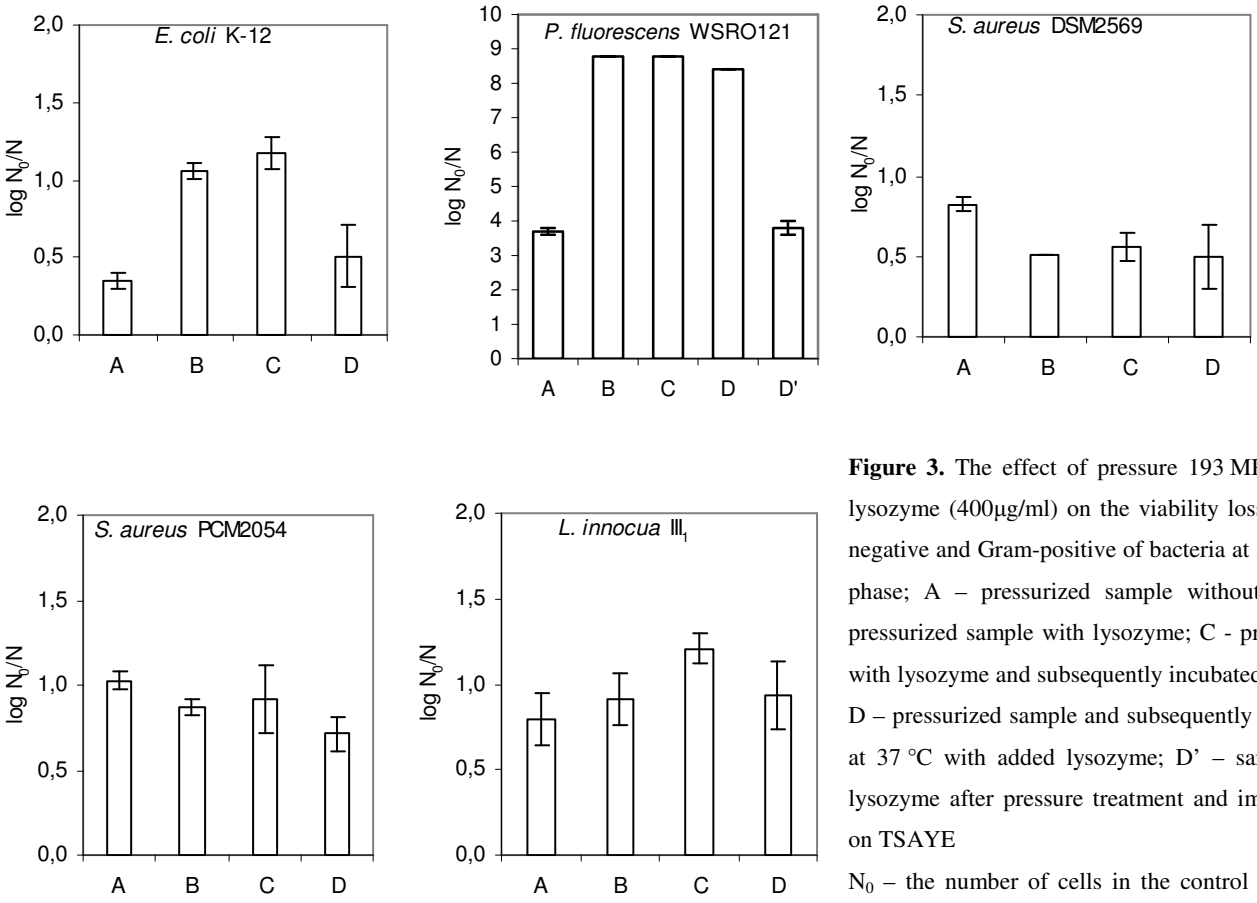

Figure 3. The effect of pressure 193 MPa at –20 °C and lysozyme (400μg/ml) on the viability loss of some Gram-negative and Gram-positive of bacteria at stationary growth phase; A – pressurized sample without lysozyme; B - pressurized sample with lysozyme; C - pressurized sample with lysozyme and subsequently incubated for 2 h at 37 °C; D – pressurized sample and subsequently incubated for 2 h at 37 °C with added lysozyme; D' – sample with added lysozyme after pressure treatment and immediately plated on TSAYE

N_0 – the number of cells in the control (about 10^8 – 10^9 cfu/ml)

N – the number of cells detected after pressurization

According to some authors (13, 22, 23, 24) the sensitization of Gram-negative bacteria to lysozyme or nisin under high pressure is a result of a transient permeabilization of the outer membrane. It means that bacteria show sensitivity to the antimicrobial substances only during pressurization. The results of the present study show also such an effect when the samples after pressure treatment were incubated for a short time, up to 2 h at 37 °C. During that time the level of bacteria inactivation did not increase (Fig 3). In the experiments of other authors with lysozyme added after pressure treatment the time of incubation did not exceed 180 min at 25 ºC (13, 25). However, data presented in Table 2 indicate that the reduction of *E. coli* cells took place when storage time of samples pressurized in the presence of lysozyme was elongated to 20 h. The number of viable cells of *E. coli* K-12 at 37 and 5 °C was, respectively, 1.9 or 2.7 log cycles lower than that for samples pressurized and stored in the absence of lysozyme. It was also found that although there was no significant reduction in the number viable cells after 24 h of storage at 5 °C of pressurized sample without lysozyme, sublethal injury of the cells was observed. The difference between the number of *E. coli* cells isolated from TSAYE and MacConkey selective medium increased from 0.1 log cycle (unstored pressurized sample) to about 3 log cycles after storage at 5 °C (unpublished data). Furthermore, analysis of the data presented in Table 2 suggests that all sublethal injured cells remain sensitive to lysozyme.

The synergistic effect of high pressure and lysozyme was not observed in experiments with *L. innocua* and two strains of *S. aureus.* During storage for 24 h at 5 and 37 °C the number of viable cells of *S. aureus* PCM2054 pressurized in the presence of lysozyme decreased but only additive effect was evidenced (Table 2).

The highest effect of pressure 193 MPa at –20 °C and lysozyme was found with pressure sensitive *P. fluorescens*; viable cells of this strain were not detected in 1 ml of sample after combined treatment. Moreover, the results of the experiment with lysozyme added after pressure treatment of bacteria and immediately plated on agar medium showed that the level of inactivation of the tested bacteria was the same as that for the sample pressurized without lysozyme (Fig 3D' and A). These results again confirmed that lysozyme exerted antimicrobial activity during pressure treatment at low temperature. In addition, during storage of such samples for 2 h at 37 °C further inactivation of bacteria was observed reaching the same level as in the case of bacteria pressurized in the presence of lysozyme. It indicates a large extent of sublethal damage of *P. fluorescens* cells.

Table 2. The effect of pressure 193 MPa at –20 °C and lysozyme (400 μg/ml) on the viability of *S. aureus* and *E. coli* cells at stationary growth phase, suspended in the buffer with pH 7.0[1]

Strain	Sample	Log CFU/ml of sample	
		Without lysozyme	With lysozyme
S. aureus PCM2054	Control (unpressurized and unstored)	8.8±0.1[a]	8.7±0.2[a]
	Control stored for 20 h at 5 °C	8.7±0.1[a]	8.2±0.2[c]
	Control stored for 20 h at 37 °C	8.6±0.3[a]	8.3±0.2[a,c]
	Pressurized	7.7±0.2[b]	7.8±0.1[b]
	Pressurized and subsequently stored for 20 h at 5 °C	7.6±0.1[b]	7.3.±0.1[d]
	Pressurized and subsequently stored for 20 h at 37 °C	7.8±0.2[b]	7.4±0.1[d]
E. coli K -12	Control (unpressurized and unstored)	8.6±0.1[a]	8.6±0.1[a]
	Control stored for 20 h at 5 °C	8.5±0.1[a]	8.0±0.0[c]
	Control stored for 20 h at 37 °C	8.4±0.1[a,b]	8.0±0.1[c]
	Pressurized	8.2±0.1[b]	7.5±0.3[d]
	Pressurized and subsequently stored for 20 h at 5 °C	8.1±0.2[b]	5.4±0.e[d]
	Pressurized and subsequently stored for 20 h at 37 °C	8.3±0.2[a,b]	6.4±0.1[f]

[1] For each bacterium, the values for a particular row or column followed by different letters differ significantly ($p<0.05$)

Antibacterial effect of high pressure and nisin

Nisin at concentration of 100 μg/ml and 400 μg/ml, pressure 193 MPa at –20 °C used separately or combined did not exert any bactericidal effect on *S. aureus* PCM2054 cells just after pressure treatment. Otherwise, *E. coli* was more sensitive to combined action of high pressure and nisin (Table 3). Synergistic reduction of these bacteria after pressure treatment in samples containing nisin at concentration of 400 μg/ml amounted to 1.7 log cycles and was higher than in the case of lysozyme. According to Masschalck *et al*. (23) various compounds characterize different bactericidal spectra under pressure. However, in the present study less significant antimicrobial effect of high pressure at subzero temperature and lysozyme than pressure and nisin can result also from low enzymatic activity of lysozyme. Nisin working in different way than lysozyme can be more effective in inactivation of bacteria under these conditions.

Table 3. The effect of pressure 193 MPa at –20 °C and nisin on the viability of *S. aureus* and *E. coli* cells at stationary growth phase, suspended in the buffer with pH 7.0[1]

Strain	Sample	Log CFU/ml of sample		
		Without nisin	With nisin (100 μg/ml)	With nisin (400 μg/ml)
S. aureus PCM2054	Control (unpressurized and unstored)	8.8±0.1[a]	8.7±0.2[a]	8.7±0.2[a]
	Control stored for 20 h at 5 °C	8.5±0.1[b]	8.3±0.2[a,b]	8.5±0.3[a,b]
	Pressurized	8.1±0.1[c]	7.9±0.1[c]	8.1±0.3[b,c]
	Pressurized and subsequently stored for 20 h at 5 °C	7.8±0.1[d]	8.2±0.1[b]	6.9±0.1[d]
E. coli K -12	Control (unpressurized and unstored)	8.4±0,3[a]	8.4±0.2[a]	8.4±0.2[a]
	Control stored for 20 h at 5 °C	8.2±0.1[a]	8.5±0.3[a]	8.5±0.1[a]
	Pressurized	8.1±0.1[a]	7.8±0.1[b]	6.5±0.1[c]
	Pressurized and subsequently stored for 20 h at 5 °C	8.2±0.1[a]	7.7±0.1[b]	6.1±0.1[d]

[1] For each bacterium, the values for a particular row or column followed by different letters differ significantly ($p<0.05$)

The results obtained by Steeg *et al*. (36) showed that bactericidal effect of combined pressure and nisin increased when treatment was conducted at temperature below 15 °C. The synergistic reduction of *E. coli* NCTC 9001 treated with pressure of 200 MPa in the presence of nisin was above 3 log ycles at 10 °C while at 40 °C only 0.3 log cycle. The results f the present study show lower synergistic effect (Table 3), lthough temperature was lower than in the experiments arried out by Steeg *et al*. (36). These differences can result from higher sensitivity to high pressure of *E. coli* NCTC 9001 than of the strain *E. coli* NCTC 10538 used in our study. Masschalck *et al*. (23) reported that the sensitization of bacteria by high pressure to antimicrobial compounds varies even among strains belonging to the same species. Subsequently, the cell density in the present study was $10^8/10^9$ cfu/ml while in that conducted by Stegg *et al*. (36) amounted to 10^5 cfu/ml. As found Garcia-Graells *et al* (9) *E. coli* was more sensitive to antimicrobial compounds like

lactoperoxidase system and high pressure at low cell density (10^6 cfu/ml) than at high cell density (10^9 cfu/ml).

Table 4. The effect of pressure 193 MPa at –20 °C and nisin (400 μg/ml) on the viability of *S. aureus* and *E. coli* cells at stationary growth phase, suspended in the buffer with pH 5.0[1]

Strain	Sample	Log CFU/ml	
		Without nisin	With nisin
S. aureus PCM2054	Control (unpressurized and unstored)	8.3±0.3[a]	7.6±0.1[b]
	Control stored for 20 h at 5 °C	7.6±0.1[b]	6.6±0.1[c]
	Pressurized	8.4±0.1[a]	7.5±0.2[b]
	Pressurized and subsequently stored for 20 h at 5 °C	7.4±0.1[b]	4.0±0.3[d]
E. coli K-12	Control (unpressurized and unstored)	8.3±0.1[a]	8.0±0.2[a]
	Control stored for 20 h at 5 °C	8.2±0.1[a,b]	8.0±0.1[a]
	Pressurized	8.0±0.2[a,b]	8.1±0.1[a]
	Pressurized and subsequently stored for 20 h at 5 °C	7.9±0.2[b]	7.1±0.1[c]

[1] For each bacterium, the values for a particular row or column followed by different letters differ significantly ($p<0.05$)

Then, similarly as in experiments with lysozyme, there was the viability loss of *E. coli* and *S. aureus* during storage of the samples at 5 °C, which were previously pressurized with nisin; the numbers of viable cells were decreased by 0.4 (*E. coli*) and 1.2 log cycles (*S. aureus*) as compared with those just after pressure treatment. However, the inactivation of *E. coli* pressurized with nisin after 24 h storage was lower than that obtained in the case of lysozyme. On the other hand, taking into account higher antimicrobial effectiveness of nisin during pressurization than of lysozyme, the total reduction of *E. coli* was similar for both antimicrobial substances.

Gänzle *et al*. (7), reported that under ambient pressure ıe sensitivity of *E. coli* to nisin increased in acidic ɔnditions. However, Steeg *et al*. (36) showed that ntimicrobial effect of nisin and high pressure on *E. coli* NCTC 9001 cells was much lower in samples at reduced pH. According to the authors, the culture obtained at pH 7 was treated by pressure at pH 4.5 and the shock induced by pH drop probably reduced the transmembrane potential and in consequence the antimicrobial efficiency of nisin. In the present work the antimicrobial effect of pressure and nisin at pH 5 against *E. coli* cells just after pressure treatment was also much lower than that at pH 7 (Table 3 and 4). However, the extent of the lethal effect during incubation of the sample after pressure treatment was higher. It can result from higher stability of nisin at acidic pH than at neutral pH (16, 25). In the case of *S. aureus* the higher synergistic effect of pressure and nisin was obtained at pH 5 as compared with that of pH 7 and differences deepened during storage of the sample after pressure treatment at 5 °C (Table 3 and 4). *S. aureus*, in comparison to *E. coli*, was also more sensitive to acidic conditions and nisin during storage under ambient pressure. It is known that enteric bacteria are tolerant towards adverse environmental conditions such as low pH or other factors (7) and Gram-negative bacteria are more resistant to nisin than Gram-positive ones (3).

CONCLUSIONS

The data suggest that lysozyme exerts antimicrobial effect during pressurization at subzero temperature what points to non-lytic mechanisms of its action under these conditions. Further studies are needed to explain this phenomenon.

Pressurization of bacteria at subzero temperature in buffer solution leads to sublethal injury of bacterial cells during subsequent prolonged storage of the samples. In the consequence, these cells become sensitive to antimicrobial substance such as nisin and lysozyme.

Furthermore, the maximal pressure of about 200 MPa that is possible to achieve in the sealed vessel at subzero

temperature combined with nisin or lysozyme and refrigerated storage increases synergistically reduction of bacteria. However, such conditions can be insufficient to complete inactivation of vegetative microorganisms, especially in food system. Therefore, taking into account also the economical aspect of using high pressure, it would be worth searching for some other, more effective antimicrobials to extend the shelf-life and enhance the safety of food.

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