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The effect of high pressure on formation of volatile amines in minced meat of cod (*Gadus morhua*)

Edyta Malinowska-Pańczyk, Ilona Kołodziejska

Department of Food Chemistry, Technology and Biotechnology, Chemical Faculty, Gdansk University of Technology, G. Narutowicza 11/12, 80-233 Gdańsk, Poland

*Department of Food Chemistry, Technology and Biotechnology

Chemical Faculty

Gdansk University of Technology

G. Narutowicza 11/12, 80-233 Gdańsk, Poland

Tel. +48-58-3472656

Fax: +48-58-3472694

E-mail: edyta.malinowska-panczyk@pg.gda.pl

Abstract

The effects of high-pressure at subzero temperature (193 MPa, at -20°C) on inactivation of natural microflora of cod meat and degradation of trimethylamine oxide (TMAO) to trimethylamine (TMA) during refrigerated storage and to dimethylamine (DMA) and formaldehyde (FA) during frozen storage were investigated. The content of TMA, DMA and FA in cod meat did not change immediately after pressure treatment. During 40 days of frozen storage of pressurized meat at -5°C concentration of DMA-N and FA was, respectively, about 10 and 7 times lower than in the stored unpressurized meat. It is the result of pressure-induced inactivation of cod meat TMAOase. There was no correlation between the total numbers of bacteria and TMA-N content. The total bacterial count has increased during refrigerated storage of pressurized meat, although for a short period of time more slowly than in the untreated samples. During refrigerated storage the accumulation of TMA-N in pressurized samples was much lower than in unpressurized meat. The pressure treatment of fish meat leads to considerable reduction of enzymatic and bacterial decomposition of TMAO to DMA and TMA, respectively. Therefore, such processing can improve sensory quality of meat and allow to extend its shelf-life.

Keywords: high pressure at subzero temperature; trimethylamine oxide; trimethylamine; dimethylamine; formaldehyde; *Gadus morhua*

1. Introduction

The methods of food preservation are of fundamental importance for quality and safety of food. Among them, high pressure technique still arouses interest not only as a way of inactivation of non thermal microorganisms but also as a possibility of controlling enzyme activity [1]. Pressure acts as a physical factor that disrupts intramolecular interactions balance between the protein and solvent. Hydrophobic interactions which play an important role in maintaining the quaternary structure of proteins are especially sensitive to high pressures (<150MPa). It is known that pressure usually decreases enzymes activity by affecting their structure[2]. Pressure induced inactivation of enzymes can be useful in the case of endogenous enzymes present in food which lead to deterioration of quality during storage or processing of raw material, among others - fish. It is well documented that trimethylamine oxide (TMAO) is one of the compounds that exert indirectly a strong impact on the sensory properties of fish and squid muscles during storage and processing. It may be microbiologically reduced to trimethylamine (TMA), which is co-responsible for the fishy smell, at refrigerated storage. The rate of TMA accumulation during storage of meat depends on the storage temperature and initial microbial contamination of meat. The content of TMA is one of the important criteria for quality evaluation of fresh fish or fish products. It has been proposed that TMA-N value between 12 and 24 mg kg^{-1} tissue should be considered the maximum allowable level in international trading (FAO Fisheries Technical Paper). According to the International Commission on Microbiological Specifications for Foods (ICMSF, 1986), the maximum initial total microbial count should not exceed 5 log cfu g^{-1}.

At frozen conditions TMAO is converted to dimethylamine (DMA) and formaldehyde (FA) in the reaction catalyzed by endogenous TMAO demethylase [3]-[4]. The interaction of FA with reactive protein groups leading to the formation of stable

protein aggregates is one of the reasons for undesirable changes in texture of many fish species (in gadoid species) during frozen storage. FA and DMA do not accumulate as a result of biochemical processes of life but are formed *post mortem*. TMAO demethylase activity has been identified in 30 species of 10 families of marine fish and 8 species of invertebrates [5]. The enzyme has been found in the water soluble fraction of several tissues and differs in molecular weight and cofactor requirements. Dark muscle and other tissues, such as kidney or spleen, have a higher rate of activity (several fold or more than 1000 times higher, respectively) than ordinary muscle. The pH optimum for enzymatic system varies from 5.0 to 7.5. This enzyme is denatured by heat. At cold temperature denaturation this protein seems to be rather low since the system is active at frozen storage temperatures. It has been shown that TMAO demethylase is active down to -29°C [6]. However, the activity of this enzyme decreases with decreasing frozen storage temperatures (Rehbein, 1988; Sotelo et al., 1995b). The inactivation of the enzyme in fish tissue could extend the freshness of frozen fish.

Available literature data on high pressure decomposition of TMAO are limited. Only Gou et al. [4, 5] found that TMA and DMA contents were reduced during refrigerated storage of pressure treated squid and the decrease in DMA was accompanied by the decrease in TMAO demethylase activity. There is no published information on changes in TMAO of pressurized fish stored at refrigerated and freezing temperatures.

The objective of this study was to investigate the effect of high pressure at subzero temperature on inactivation of natural microflora of cod meat and degradation of TMAO to TMA during refrigerated storage as well as TMAO decomposition to DMA and FA during frozen storage. The treatment was conducted at subzero temperature to enhance the high pressure effect. Such conditions were reported to be more effective in

inactivation of microorganisms than the use of pressure at ambient temperature[9], [10]. Furthermore, high pressure reduces the freezing and melting points of water to -22°C at 207 MPa allowing to keep the samples in unfrozen state [11].

2. Materials and methods

2.1. Materials and samples preparation

Cod (*Gadus morhua)* fillets were purchased from a local market and stored on ice before use. Samples of meat were minced in a mechanical grinder model 986.86 Zelmer, vacuum packed in polyethylene bags (50 g) and pressurized.

2.2. Pressure treatment

The pressure was generated as described by Malinowska-Pańczyk et al. [12]. The method is based on the phenomenon of generating pressure in response to the increasing volume of forming ice I in a sealed vessel filled with water and kept at subzero temperatures. Moreover, according to Bridgman [13] high pressure reduces the freezing and melting points of water to a minimum of -22°C at 207 MPa. Therefore, above this temperature, the sample placed in a sealed vessel is affected by the pressure in unfrozen state. Samples of meat were pressurized at 193 MPa and -20°C. Total pressurization/decompression steps lasted about 50 min. After processing, the samples were stored at 4°C or at -5 and -20°C.

2.3. Preparation of TMAOase extract and determination of its activity

In order to prepare crude extract of TMAOase, the 10 g of minced meat samples were homogenized (Heidolph SilentCrusher M) with 20 mL 20 mM Tris-acetate buffer (pH 7.0) containing 0.1 M NaCl and 0.1% Triton X-100 (12 000 rpm for 1 min). The homogenate was centrifuged at 12000×*g* for 30 min at 4°C. The supernatant was used as the crude TMAOase extract.

TMAOase activity was assayed using TMAO as a substrate in the presence of cofactors[14]. Enzyme solution in the amount of 0.5 mL was added to 2.5 mL of buffer (24 mM Tris-acetate, containing 24 mM TMAO, 2.4 mM cysteine, 2.4 mM ascorbate, 0.24 mM $FeCl_2$, pH 7.0). The mixtures were incubated at 25°C for 20 min and 1 mL of 10% trichloroacetic acid was added to terminate the reaction. The reaction mixture was centrifuged at 8000×*g* for 15 min and the supernatant was subjected to DMA determination. One unit of TMAOase was defined as activity, which released 1 μmol min^{-1}.

2.4. Determination of TMA, DMA and FA

Extraction of TMA, DMA and FA from cod samples (unpressurized and pressurized meat at 193 MPa) were performed immediately after processing and during storage at 4°C, -5°C and -20°C. A sample of meat (10 g) was homogenized with 20 mL of 7.5% trichloroacetic acid (TCA) at a speed of 12 000 rpm for 1 min. The homogenate was centrifuged at 3000×*g* for 15 min. The supernatant was placed in a volumetric flask and TCA at 7.5% concentration was added to make up to 25 mL. In the case of free formaldehyde determination, the supernatant was neutralised to pH 6.0 and the final volume was made up to 25 mL using distilled water. The supernatants were used for analysis.

The TMA and DMA were determined according to Dyer [15] and Dyer and Mounsey [16], respectively, with slight modification, that is toluene was used instead of benzene. In order to determine the TMA-N content, 0.5 ml of formaldehyde, 5 ml of toluene and 1.5 ml K_2CO_3 (50%) were added to 2 ml of the extract. The solution was mixed and the toluene layer (2.5 ml) was collected. The toluene layer was dried by adding anhydrous Na_2SO_4. Subsequently, 1.25 ml of picric acid (0.02% in toluene) was added to the dried toluene layer and the absorbance was measured at 410 nm.

The determination of DMA was conducted by adding 8 ml of 5% CS_2 in toluene and 0.8 ml copper-ammonia reagent to 8 ml of the extract. Then the mixture was heated in water bath at 50ºC for 5 min. Subsequently 0.4 ml 30% acetic acid was added and the mixture was stirred vigorously. The clear toluene layer (5 ml) was dried by adding anhydrous Na_2SO_4 and the absorbance was measured at 435 nm.

The standard curves for TMA and DMA were prepared in the same manner as described for the sample. The amounts of TMA and DMA were expressed in mg TMA-N or DMA-N per kg of meat.

Free FA was determined according to Nash [17].

2.5. Microbiological analysis

Microbiological enumerations were performed directly after the pressure treatments and after appropriate time of storage at 4ºC. Samples of meat (10 g) were transferred to a stomacher bag and homogenized in the stomacher (Masticatorbasic Panoramic) with 0.1 % peptone water (1:9, w/v) for 1 min. Successive decimal dilutions were also prepared in 0.1 % peptone water. The appropriate serial dilutions were then plated onto plate count agar (two replications) and incubated for 48 h at 30°C (Total Bacterial Count - TBC). The number of coliforms was estimated using Violet Red Bile Dextrose Agar (VRBD Agar). The media were purchased from Merck KGaA.

The results presented in the tables and figures are averages from three pressure treatments in which experiments were performed in three replications ± standard deviation. The differences between treatments were evaluated statistically by analysis of variance (one-way procedure) using the program Statgraphics, Statistical Graphic Corporation.

3. Results and discussion

3.1. The influence of high pressure on production of DMA and FA during frozen storage of cod meat

The raw cod meat contained low concentration of DMA (4.5 mg DMA-N kg^{-1}) and FA (0.46 mg FA kg^{-1}). The DMA and FA formation during frozen storage depended on the temperature and treatment. As has been expected the process of DMA and FA accumulation was faster when the unpressurized samples were stored at -5°C than at -20°C. After 40 days at -5°C the 1 kg of meat contained 133 mg of DMA-N and 166 mg of FA, whereas at -20°C only 18 mg and 27 mg, respectively. Similar dependence during frozen storage of meat of some fish species, including cod meat was shown previously by other authors [18] [19].

The content of DMA and FA in cod meat determined immediately after pressure treatment (193 MPa at -20°C) was similar as in the untreated sample. However, during frozen storage at -5°C of pressurized meat, the production rate of these two compounds was decreased in comparison to unpressurized stored meat. After 40 days of storage, the concentration of DMA-N and FA amounted to 14 and 24 mg kg^{-1} of meat, respectively and was similar as in the samples stored at -20°C. It is the result of pressure-induced inactivation of cod meat endogenous demethylase. The activity of TMAOase was lowered from 4.8 units mg^{-1} of proteins to 0.1 units mg^{-1} of proteins immediately after pressurization (Table 1). There is no data in available literature on the effect of pressure on the rate of DMA and FA accumulation during frozen storage of pressurized fish meat. However, Gou et al.[20] showed that the formation of DMA was considerably reduced in meat of pressure treated squid (*Todarodes pacificus*) stored under refrigerated conditions and it was also correlated with the decrease in TMAO-ase activity. The data presented in Figure 1 show that in the case of pressurized cod meat stored at -20°C the

content of DMA and FA was slightly lower than in unpressurized meat but these differences were not statistically significant.

3.2. The influence of high pressure on natural microflora and production of TMA during refrigerated storage of cod meat

The content of TMA-N in cod meat amounted to 2 mg kg^{-1} of meat (Figure 2) regardless of initial total bacterial count. It was shown that samples coming from different batches of meat varied in levels of total bacterial count (Tables 2 and 3). The TBC from batch I amounted to 6.4, while from batches II and III 4.2 and 4.4 log cfu g^{-1}, respectively (Tables 2 and 3). These differences in TBC were most likely caused by various hygienic conditions maintained after catching the fish.

During storage of meat at refrigerated conditions, the rate of TMA formation depended on the temperature and initial microbial contamination. The sample with relative high initial TBC (batch I - 6.4 log cfu g^{-1}) after 7 days of storage at 4ºC, contained 422 mg of TMA-N kg^{-1} of meat, and this from batch II (initial TBC - 4.2 log cfu g^{-1}) contained only 223 mg of TMA-N kg^{-1} of meat (Figure 2). The samples could not be stored for longer time due to a very intensive fish odour. The accumulation of TMA in the meat stored at 2ºC (batch III) was much slower than in the samples stored at 4ºC (batch II). The TBC in batch II after 7 days amounted to 8.8 log cfu g^{-1} while in batch III 6.7 log cfu g^{-1} (the initial TBC in both batches was similar) and simultaneously TMA-N content was 223 and 28 mg TMA-N/kg, respectively.

The level of TMA in meat immediately after pressurization at 193 MPa was the same as in the unpressurized sample (Figure 2). During refrigerated storage the accumulation of TMA in pressurized samples was much lower than in unpressurized meat. The content of TMA-N did not exceed 10 mg TMA-N kg^{-1} of meat after 1 week independently of storage temperature. TMA-N content in pressurized meat increased

only to 22 mg kg^{-1} of meat and 45-65 mg kg^{-1} of meat stored for 2 weeks at 2 and 4°C, respectively. As TMA is formed from reduction of TMAO by microbial enzymes, the decrease in the rate of this reaction during refrigerated storage of pressurized cod meat samples should result from inactivation of natural microflora of cod meat. The data presented in Tables 2 and 3 show that just after pressure treatment at 193 MPa and -20ºC the total bacterial count was reduced by about 1.5 to 2.0 log cycles. During storage of meat for 7 days at 2 and 4°C coliform bacteria were below detection level (<1 log cfu g^{-1}) in the pressurized sample while in the control sample their number increased by about 0.5 to 2.5 log cycles depending on the initial contamination and temperature of storage.

On the other hand, the TBC increased during refrigerated storage of pressurized meat, although for a short period of time more slowly than in the untreated control samples. Nevertheless, the number of bacteria in all pressurized samples was high. In meat stored at 4ºC (batch I), after 7 days, the number of bacteria increased from 4.0 log cfu g^{-1} to 7.9 log cfu g^{-1}, and in the samples from batch II increased from 2.2 log cfu g^{-1} to 6.2 log cfu g^{-1} in spite of very low content of TMA (Table 2). These results show that there is little correlation between the total number of bacteria and TMA content. It can be also concluded that bacteria responsible for degradation of TMAO were inactivated. Already in the 1950s Castell et al. [21] and in the 1980s Huss et al. [22] Gram and Huss [23] reported that only a part of the total microflora participates in the spoilage. Among the spoilage bacteria able to decompose TMAO to TMA are *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Vibrionaceae* sp. [2[4]. Also some species belonging to *Enterobacteriaceae,* e.g. *E. coli*, produce TMAO reductase [24]. All above-mentioned species of bacteria are gram-negative. It is well known that this group of bacteria is sensitive to high pressure treatment [25] 23].

4. Conclusions

The pressure treatment of fish meat at 193 MPa leads to considerable reduction of enzymatic and bacterial decomposition of TMAO to DMA (during frozen storage) and TMA (during refrigerated storage), respectively. It results from inhibition of demethylase and probably TMAO reductase activity. Pressurization of meat under the applied conditions inactivates coliform bacteria, but the decrease in TMA accumulation is accompanied by retarding bacterial growth expressed as the total bacterial number during 7 days of refrigerated storage of pressurized cod meat. However, even this moderate pressure treatment of cod with relatively high bacterial contamination allows to extend the shelf-life of meat for about two days and through limitation of TMA improves sensory quality of meat.

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Figure captions

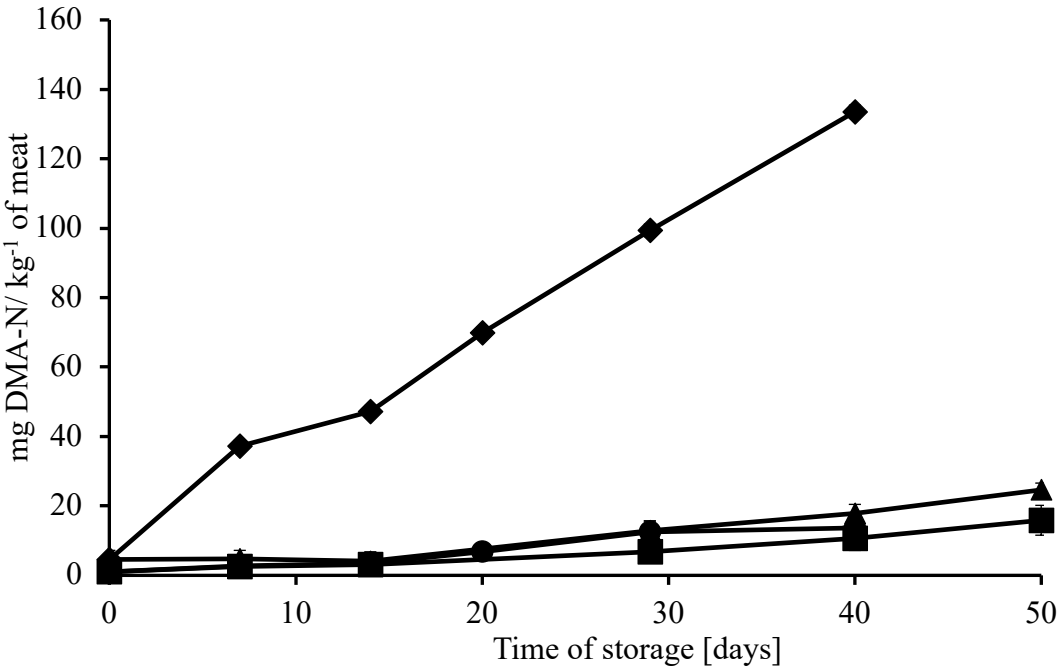
Figure 1.

Formation of DMA (A) and FA (B) in cod meat stored at -5°C: unpressurized (◆), pressurized at 193 MPa (•) and at -20°C: unpressurized (▲), pressurized at 193 MPa (■)

Figure 2.

Formation of TMA in unpressurized (■) – batch I, (▲) – batch II, (●) – batch III and pressurized at 193 MPa cod meat stored at 4°C (□)- batch I, (△) – batch II, and at 2°C (○) – batch III

A



B

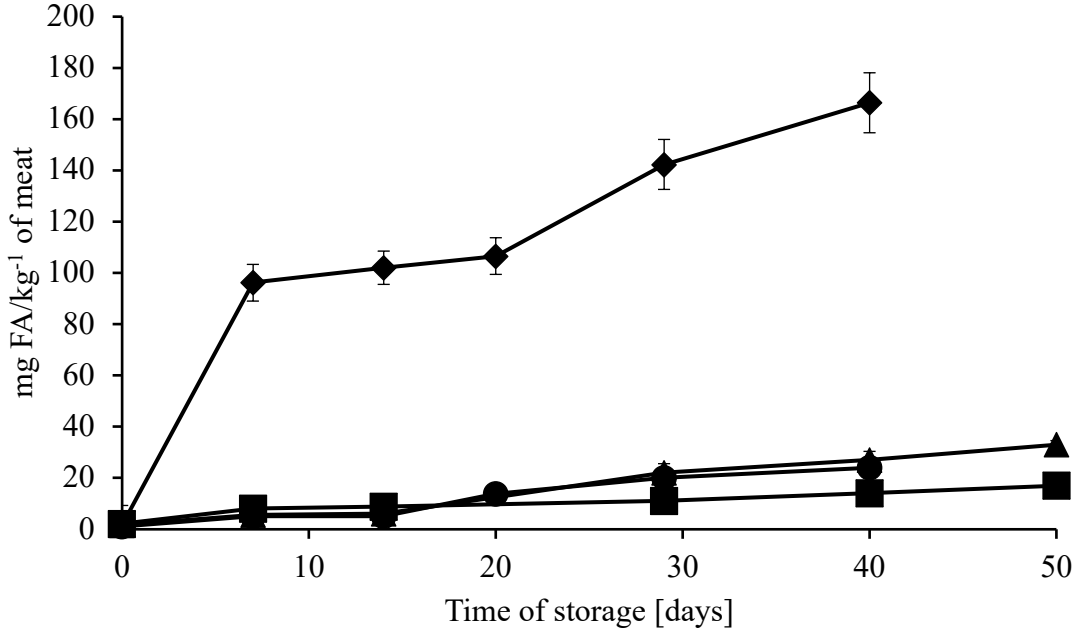


Figure 1.

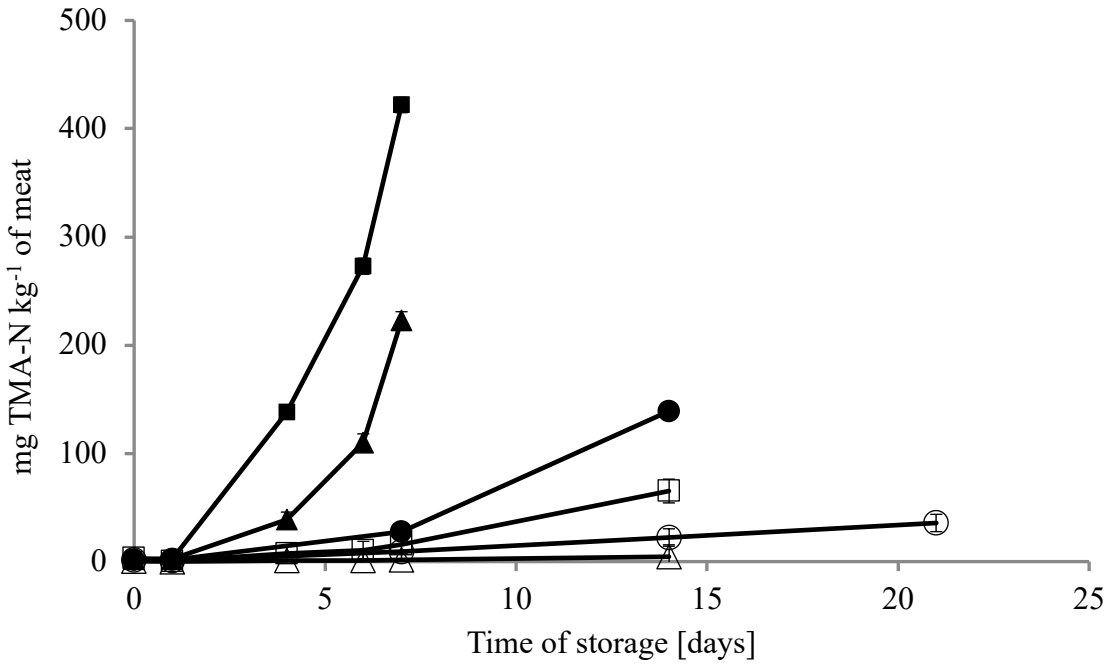


Figure 2.

61 Table 1. The influence of high pressure on activity of endogenous TMAO-demethylase of cod

62 minced meat

Minced cod meat	TMAOase activity [units mg^{-1} proteins]
Unpressurized	4.8 ± 0.3
Pressurized at 193 MPa	0.1 ± 0.02

63

64
65

66 Table 2. The influence of high pressure of 193 MPa on natural microflora of cod minced meat

67 immediately after treatment and after 2, 4 and 7 days of storage at 4°C; A) batch I, B) batch II

68 A

Cod minced meat	Time of storage [days]			
	0	2	4	7
	Log of total bacterial count g^{-1}			
Control sample (nonpressurized)	6.4±0.03[a]	7.8±0.08[a]	8.8±0.02[a]	8.4±0.02[a]
Treated at 193 MPa	4.0±0.01[b]	5.4±0.11[b]	7.5±0.16[b]	7.9±0.15[b]
	Log of total coliform bacterial count g^{-1}			
Control sample (nonpressurized)	3.0±0.19[a]	3.9±0.01[a]	4.15±0.13[a]	4.8±0.05[a]
Treated at 193 MPa	<1[b]	<1[b]	<1[b]	<1[b]

69 B)

Control sample (nonpressurized)	4.2±0.04[a]	4.6±0.03[a]	5.8±0.10[a]	8.8±0.02[a]
Treated at 193 MPa	2.2±0.02[b]	2.3±0.03[b]	3.5±0.62[b]	6.2±0.10[b]
	Log of total coliform bacterial count g^{-1}			
Control sample (nonpressurized)	1.3±0.1[a]	1.9±0.01[a]	2.8±0.11[a]	3.8±0.03[a]
Treated at 193 MPa	<1[b]	<1[b]	<1[b]	<1[b]

70 [a-b] For each bacterium the values for a particular column followed by different letters differ

71 significantly ($p<0.05$)

72
73

74 Table 3. The influence of high pressure of 193 MPa on natural microflora of cod minced meat

75 immediately after treatment and after 1, 7 and 14 days of storage at 2°C

Cod minced meat	Time of storage [days]			
	0	1	7	14
	Log of total bacterial count g^{-1}			
Control sample (nonpressurized)	4.4±0.01[a]	4.9±0.03[a]	6.7±0.01[a]	9.4±0.03[a]
Treated at 193 MPa	2.9±0.01[b]	2.9±0.05[b]	4.6±0.06[b]	7.5±0.02[b]
	Log of total coliform bacterial count g^{-1}			
Control sample (nonpressurized)	1.0±0.1[a]	1.1±0.01[a]	1.5±0.05[a]	2.1±0.07[a]
Treated at 193 MPa	<1[b]	<1[b]	<1[b]	<1[b]

76 [a-b] For each bacterium the values for a particular column followed by different letters differ

77 significantly ($p<0.05$)

78