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THE EFFECT OF COLD CHAIN DISRUPTION ON THE MICROBIOLOGICAL PROFILE OF CHILLED FISH

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ABSTRACT

Cold chain interruption during transport of samples to the laboratory may become a confounder in food hygiene practice. This study evaluates the influence of inadequate transport conditions on the microbiological quality of fish fillets from common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), and Atlantic cod (*Gadus morhua*) packaged in plain, vacuum, or modified atmosphere packaging (MAP). The experiments simulated the temperature increase during sample transport to 3, 5, 8, 11, 14, 17, 20, and 25 °C with exposure times of 1, 2, 3, and 4 h. Aerobic plate count (APC), psychrotrophic microorganisms count (PMC), *β*-D-glucuronidase-positive *Escherichia coli*, and *Salmonella* spp. were evaluated immediately after the exposure to the elevated temperature (0 h), 3 h, and 24 h after the return to the normal temperature. Variability was observed even in the initial microbial contamination of fish fillets. Storage at higher temperatures led to an increase in microbial contamination of fish fillets Different types of packaging also played a role. In non-fatty fish (cod), CO_2-containing MAP led to a decrease in pH and, therefore, to lower microbial growth. In fatty fish (salmon), the presence of CO_2 did not lead to the reduction in pH and the degree of microbial contamination was, therefore, not affected by this intrinsic factor. If cold chain interruption occurs during the transport of samples to the laboratory, it is necessary to perform the analysis immediately as even storage in cold conditions until the next day can lead to increased levels of microbial contamination of such samples.

Keywords: plain packaging, modified atmosphere, vacuum packaging, aerobic plate count, psychrotrophic microorganisms

INTRODUCTION

Fish consumption is nowadays increasing, both due to their positive effects on health caused by the abundant presence of unsaturated fatty acids and their use in many fashionable foods (e.g. raw fillet used for uncooked sushi and sashimi dishes) **(Castrica *et al.*, 2021)**. This has increased the need for long-term storage preserving the safety of the food. However, fresh fish are a highly perishable product with a very short storage life, which limits their distribution and marketing **(Gómez-Estaca *et al.*, 2018).**

According to the so-called Hurdle Effect Theory, the conditions of food storage, i.e., external factors (temperature, relative air humidity, composition of the packaging atmosphere, duration of storage), combined with the internal factors (food composition, water activity, pH, redox potential and texture) are the principal factors influencing shelf life and safety **(Leistner, 2000)**. The growth of Gram-negative microorganisms, such as *Pseudomonas* spp., *Shewanella putrefaciens*, and *Aeromonas* spp., under aerobic conditions, is one of the principal reasons for the limited shelf life of chilled fish **(Masniyom, 2011; Cyprian *et al.*, 2013)**. The microorganisms associated with fish and seafood can be directly related to the fishing area, type of fish, environmental factors, harvesting methods, storage, and transportation **(Masniyom, 2011; Terentjeva *et al.*, 2015; Olalemi and Oulyemi, 2018; Merlo *et al.*, 2019)**. Temperature affects not only the shelf-life of fish meat but also increases the spoilage microbiota population, thus altering the quality of meat **(Durrani *et al.*, 2021; Giarratana *et al.*, 2022)**. **Liu *et al.* (2022)** confirmed the effect of increasing temperature during cold chain interruption on the aerobic plate count, *Enterobacteriaceae*, *Pseudomonas* spp. and *Vibrio* spp. Microbial spoilage and biochemical reactions were also shown to increase the pH of meat and seafood products in sealed packaging **(Ezati *et al.*, 2021; Mu *et al.*, 2022)**.

Other reasons for fish meat perishability include its high water activity (a_w), neutral pH, and presence of autolytic enzymes. These factors, combined with the lower content of connective tissue compared to other flesh foods, lead to the more rapid spoilage of fish compared to the mammalian muscles **(Pereira de Abreu *et al.*, 2010)**. The rate of deterioration is highly temperature-dependent; the spoilage can, therefore, be inhibited by the use of low storage temperature (i.e. at a temperature approaching that of melting ice) **(Regulation (EC) No 853/2004; Cyprian *et al.*, 2013)**.

Microbial contamination, together with lipid and protein oxidation, are major concerns for fish meat and products thereof in terms of food safety. Modified atmosphere packaging (MAP) and various types of active packaging are widely applied in the packaging of fish. Oxygen is often extracted from the package to delay oxidative rancidity and inhibit the growth of aerobic microorganisms **(Zouharová *et al.*, 2023)**. Safety is a major concern for all foods of animal origin, as these provide a suitable environment for many microorganisms, including pathogenic ones, and are a common source of food-borne diseases. Therefore, it is crucial to maintain the high microbiological quality of animal origin foods as well as the sensory and nutritional values.

The present study assessed the effect of elevated transport temperature on the microbiological quality of common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), and Atlantic cod (*Gadus Morhua*) fillets.

MATERIAL AND METHODS

Fish fillets samples

Three types of fish were used in the presented study: fatty fish (Atlantic salmon, *Salmo salar*), medium-fat fish (common carp, *Cyprinus carpio*), and low-fat fish (Atlantic cod, *Gadus morhua*). The fish fillets were purchased in the retail and transported to the laboratory at melting ice temperature (1 ± 1 °C). Immediately upon receipt at the laboratory, the fillets were aseptically cut into portions. Individual portions of fish (approx. 150 g each) were placed in plastic bowls made of polypropylene and covered with polyethylene stretch film (plain packaging). For modified atmosphere packaging (MAP), the fish and the bowls were placed in plastic bags, a gas mixture of 70% N_2 and 30% CO_2 was applied to the packages and the packages were sealed. For vacuum packaging, fish samples were placed into individual bags made of polyamide + polyethylene film and subsequently evacuated and sealed using a TekVac 411 vacuum packer (Distform, Spain). Before starting the experiment, all prepared samples were cooled down to a temperature of 1 ± 1 °C (temperature of melting ice) overnight.

Simulation of the cold chain interruption

Before cold chain interruption, packages from each packaging type and fish were randomly selected (24 for vacuum and MAP, 27 for plain packaging) and used for determination of initial microbiological parameters. Subsequently, a simulation of the violation (increase) of the external temperature during the transport of the samples was carried out using the following temperatures: 3, 5, 8, 11, 14, 17, 20, and 25 °C. The duration of the samples' exposure to the elevated temperature was 1, 2, 3, and 4 hours. Twelve packages were used for each combination of temperature, packaging and fish. Immediately after that, the samples were returned to the melting ice temperature (plain packaging) or 3°C (MAP and vacuum packaging), where they were left until sample collection. Analytical samples were collected immediately after exposure to the elevated temperature (0 h, samples labeled V0), after 3 h (V3), and 24 h (V24) after returning to the low (storage) temperature, respectively (i.e., four packages per time point of sample collection). From each package, three parallel samples were taken for analyses. Fillets stored at the temperature of melting ice without interruption were used as control samples for plain packaging. For MAP and vacuum packaging, the controls were stored at the temperature of 3 °C (temperature recommended by the manufacturer and required by legislation).

Microbiological and chemical parameters

The aerobic plate count (APC; **ČSN EN ISO 4833-1/2014**), the psychrotrophic microorganisms count (PMC; **ČSN ISO 17410/2020**), count of *β*-D-glucuronidase-positive *Escherichia coli* (**ČSN ISO 16649-2/2003**) and the presence of *Salmonella* spp. (**ČSN EN ISO 6579-1/2020**) were evaluated. APC, PMC, and *E. coli* were determined for each collected analytical sample. The presence of *Salmonella* spp. was evaluated before the start of the increased storage temperature simulation and then in all samples taken 24 hours after exposure to the increased temperature (V24 samples). Basic processing of the collected samples was carried out in accordance with **ČSN EN ISO 6887-1/2018** using sterile saline enriched with peptone (0.85% NaCl + 0.1% peptone, pH 7) as a dilution medium. The upper acceptable limits were set for each combination of fish species and packaging type, taking into account also the initial bacterial condition, in accordance with generally appplicable rules for calculating of statistical discrimination limits for quality evaluation of analytical methods performance (**Suchánek *et al.*, 1997**).

Immediately after the microbiological examination, pH value and water activity were determined. A microprocessor pH meter 211 (Hanna Instruments, USA) was used to determine the pH value. The pH was measured in each individual sample with an injection electrode at a temperature of 25 ± 1 °C. Water activity was determined using a LabMaster a_w-meter (Novasina AG, Switzerland) at a temperature of 25 ± 1 °C. Considering the time needed to measure one sample, the water activity was always measured for a pooled sample characterizing an individual package.

Statistical analysis

The obtained values of CFU/g counts were logarithmically transformed using the decimal logarithm (log CFU/g) and expressed using the mean and standard error of the mean. For each type of fish (carp, salmon, cod), ANOVA followed by Tuckey's post-hoc test was used to compare the initial values of aerobic plate count (APC), psychrotrophic microorganisms count (PMC), pH, and water activity (a_w) among the three types of packaging (plain, vacuum, MAP). For the purposes of further statistical analysis, the APC (and PMC) values were adjusted as the ratio of the measured value and the average initial value for the given temperature. A general linear model (GLM) – ANCOVA was used to evaluate the effect of temperature, cold chain interruption length, sample examination time, and type of packaging on adjusted APC and PMC. Sample examination time and type of packaging were included as categorical factors and temperature and cold chain interruption length as continuous predictors. All performed tests were evaluated at a significance level of $\alpha = 0.05$. The Statistica software, version 13.5, was used for data processing.

RESULTS AND DISCUSSION

Aerobic plate count (APC)

The total amount of microorganisms expressed as aerobic plate count (APC) recorded before the cold chain interruption was 7.21 ± 0.07 log CFU/g for common carp fillets in plain packaging, 6.69 ± 0.14 log CFU/g in vacuum packaging and 7.23 ± 0.08 log CFU/g in MAP, respectively (Tab. 1).

Table 1 Values measured before the exposure to a higher temperature and range of values measured after exposure to a higher temperature for fish samples. APC – aerobic plate count (log CFU.g^{-1}), PMC – psychrotrophic microorganisms count (log CFU.g^{-1}), a_w – water activity, SE – standard error of the mean

	Fish species	Packaging	Values before exposure to a higher temperature		Values after exposure to a higher temperature	
			N	mean ± SE	N	mean ± SE
APC	Common carp	Plain	27	7.21 ± 0.07	288	7.50 ± 0.04
		Vacuum	24	6.69 ± 0.14	270	6.81 ± 0.05
		MAP	24	7.23 ± 0.08	270	7.34 ± 0.03
	Atlantic salmon	Plain	27	3.91 ± 0.12	288	3.78 ± 0.03
		Vacuum	24	3.35 ± 0.08	270	3.42 ± 0.03
		MAP	24	4.55 ± 0.14	270	4.48 ± 0.04
	Atlantic cod	Plain	27	5.30 ± 0.14	288	5.29 ± 0.04
		Vacuum	24	5.11 ± 0.13	270	5.22 ± 0.04
		MAP	24	4.49 ± 0.11	270	4.48 ± 0.03
PMC	Common carp	Plain	27	7.20 ± 0.08	288	7.51 ± 0.04
		Vacuum	24	6.78 ± 0.14	270	6.84 ± 0.04
		MAP	24	7.26 ± 0.07	266	7.31 ± 0.03
	Atlantic salmon	Plain	27	3.38 ± 0.09	288	3.25 ± 0.03
		Vacuum	24	3.24 ± 0.08	270	3.24 ± 0.03
		MAP	24	4.24 ± 0.17	270	4.24 ± 0.04
	Atlantic cod	Plain	27	5.47 ± 0.15	288	5.55 ± 0.04
		Vacuum	24	5.42 ± 0.14	270	5.53 ± 0.04
		MAP	24	4.44 ± 0.11	270	4.43 ± 0.03
pH	Common carp	Plain	27	6.39 ± 0.04	288	6.43 ± 0.01
		Vacuum	24	6.42 ± 0.03	270	6.42 ± 0.01
		MAP	24	6.39 ± 0.03	270	6.27 ± 0.01
	Atlantic salmon	Plain	27	6.15 ± 0.01	288	6.12 ± 0.01
		Vacuum	24	6.19 ± 0.02	270	6.20 ± 0.01
		MAP	24	6.29 ± 0.01	270	6.24 ± 0.01
	Atlantic cod	Plain	27	7.04 ± 0.04	288	7.08 ± 0.01
		Vacuum	24	6.88 ± 0.03	270	6.86 ± 0.01
		MAP	24	6.68 ± 0.03	270	6.67 ± 0.01
a_w	Common carp	Plain	3	0.9817 ± 0.0007	96	0.9809 ± 0.0002
		Vacuum	3	0.9840 ± 0.0015	90	0.9809 ± 0.0003
		MAP	3	0.9863 ± 0.0009	90	0.9844 ± 0.0002
	Atlantic salmon	Plain	4	0.9828 ± 0.0008	96	0.9807 ± 0.0002
		Vacuum	4	0.9840 ± 0.0013	90	0.9794 ± 0.0003
		MAP	3	0.9857 ± 0.0015	90	0.9823 ± 0.0004
	Atlantic cod	Plain	3	0.9827 ± 0.0007	96	0.9794 ± 0.0003
		Vacuum	3	0.9813 ± 0.0013	90	0.9807 ± 0.0004
		MAP	3	0.9780 ± 0.0000	90	0.9784 ± 0.0003

These values were statistically significantly different (ANOVA: F (2,72) = 9.312, P < 0.001) with the vacuum-packed samples having a significantly lower APC value than the other two. After the cold chain interruption, APC values ranged from 5.40 ± 0.08 to 9.07 ± 0.45 log CFU/g depending on the packaging type, high temperature the sample was exposed to, duration of the cold chain interruption, and the time until examination after the cold chain interruption (ANCOVA: the influence of all predictors was significant, with P < 0.001; Tab 2). The higher temperature and longer cold chain interruption were associated with higher APC. APCs were generally notably higher in vacuum packaging samples and in samples examined after 24 hours (Tab 2, S1–S3).

Table 2 Results of GLM – ANCOVA. The effect of temperature, exposure duration, examination time, and type of packaging on APC (aerobic plate count) and PMC (psychrotrophic microorganisms count) in fish

Fish species		Effect of variables			
		Temperature	Duration of exposure	Examination time	Packaging
Common carp	APC	F (1,821) = 31.559 P < 0.001	F (1,821) = 16.602 P < 0.001	F (2,821) = 90.989 P < 0.001	F (2,821) = 20.763 P < 0.001
	PMC	F (1,817) = 39.160 P < 0.001	F (1,817) = 23.753 P < 0.001	F (2,817) = 75.719 P < 0.001	F (2,817) = 48.139 P < 0.001
Atlantic salmon	APC	F (1,821) = 10.656 P = 0.001	F (1,821) = 2.693 P = 0.101	F (2,821) = 1.303 P = 0.272	F (2,821) = 13.873 P < 0.001
	PMC	F (1,821) = 12.284 P < 0.001	F (1,821) = 32.902 P < 0.001	F (2,821) = 57.351 P < 0.001	F (2,821) = 8.072 P < 0.001
Atlantic cod	APC	F (1,821) = 4.701 P = 0.030	F (1,821) = 0.042 P = 0.838	F (2,821) = 5.011 P = 0.007	F (2,821) = 1.915 P = 0.148
	PMC	F (1,821) = 4.587 P = 0.033	F (1,821) = 0.165 P = 0.685	F (2,821) = 19.706 P < 0.001	F (2,821) = 4.618 P = 0.010

Carp fillets in plain packaging exceeded the limits fairly often. APCs were below limits only when investigated immediately after exposure to the higher temperature, particularly after shorter exposures and at lower temperatures. In samples examined 24 h after the cold chain interruption, the limits were violated in most of cases. Vacuum and, particularly, modified atmosphere packaging showed better results – in samples exposed to lower degrees of cold chain interruption (lower temperatures and shorter exposure) limits were not exceeded even after 24 hours after exposure (Tab 3, S1–S3).

Table 3 Violation of limits for aerobic plate count for individual temperatures in common carp, Atlantic salmon, and Atlantic cod samples in plain (P), vacuum (V), and modified atmosphere (M) packaging

Examination time	Temperature	Time of exposure of samples to higher storage temperature			
		1 hour	2 hours	3 hours	4 hours
E0	0 °C				
	3 °C			Salmon-V, Cod-V	
	5 °C		Cod-V		Carp-M, Salmon-V
	8 °C	Salmon-V	Salmon-M, Cod-M		Carp-V
	11 °C	Cod-V	Salmon-P		Salmon-V
	14 °C	Carp-M, Cod-V			Carp-V, Salmon-V,M
	17 °C	Carp-M, Cod-V	Carp-M		Carp-P,V
	20 °C	Salmon-M, Cod-M	Cod-P,M		Carp-M, Salmon-M, Cod-P
	25 °C	Cod-V	Salmon-P	Carp-P, Salmon-P, Cod-V	Carp-P,M
E3	0 °C				
	3 °C			Cod-V	
	5 °C	Carp-M, Cod-V	Carp-P,M, Cod-V	Carp-P,M, Salmon-V, Cod-V	Salmon-V
	8 °C	Salmon-M			Cod-V
	11 °C	Salmon-P, Cod-V			Carp-P,M, Salmon-P
	14 °C	Cod-V	Carp-M	Carp-P	Carp-P,M
	17 °C		Cod-V	Salmon-V	Carp-V
	20 °C	Salmon-M	Carp-V	Carp-P, Cod-P,M	Carp-V, Salmon-M
	25 °C	Salmon-P,M	Cod-V,M	Salmon-P, Cod-P	Carp-P,V, Salmon-P, Cod-V,M
E24	0 °C	Carp-P			Carp-P, Cod-P
	3 °C	Cod-P	Carp-M, Cod-V,M		Carp-P,M
	5 °C	Carp-P, Cod-V	Carp-P,Cod-V,M	Carp-M	Carp-P,M
	8 °C	Carp-P,V	Carp-P	Carp-P,V, Cod-V	Carp-P, Cod-P
	11 °C	Carp-P	Carp-P,M, Salmon-P	Carp-P,M	Carp-P, Salmon-V, Cod-V,
	14 °C	Carp-P	Carp-P	Carp-P,M	Carp-P,V, Salmon-V, Cod-V
	17 °C	Carp-V, Cod-V	Carp-P,V,M, Salmon-M	Carp-P,V, Salmon-P,V	Carp-P, Salmon-P,V, Cod-P
	20 °C	Carp-P,V, Cod-P	Carp-P,V, Salmon-M	Carp-V, Cod-V	Carp-P,V, Cod-P,V,M
	25 °C	Salmon-P, Cod-V	Carp-P,M, Salmon-P, Cod-P,V,M	Carp-P,V,M, Salmon-P, Cod-V	Carp-P,M, Cod-P,V,M

Legend: E0 – examination of the sample immediately after exposure, E3 – examination of the sample 3 hours after exposure, E24 – examination of the sample 24 hours after exposure

APC before the cold chain interruption statistically significantly differed depending on the packaging also in the Atlantic salmon. In plain packaging, APC was 3.91 ± 0.12 log CFU/g; in vacuum packaging, it was lower at 3.35 ± 0.08 log CFU/g but in MAP, the highest value of 4.55 ± 0.14 log CFU/g (Tab. 1) was

observed (ANOVA: F(2,72) = 26.841, P < 0.001; all three types of packaging significantly differed). After cold chain interruption, the APC values in salmon ranged from 2.62 ± 0.14 to 5.79 ± 0.52 log CFU/g and were statistically significantly affected by the high temperature and type of packaging (ANCOVA: P < 0.001 for both predictors), with higher APC values recorded in samples exposed to higher temperatures and in those from vacuum packaging (Tab 2, S4–S6).

In Atlantic salmon samples, APC limits were exceeded in a lower number of cases than in the carp. More occurrences of violation of the limits were recorded in plain packaging than in vacuum or modified atmosphere packaging. Generally, the limits were not exceeded at lower-intensity cold chain interruptions (i.e., shorter, lower temperatures) (Tab 3, S4–S6).

In Atlantic cod, APC before cold chain interruption was 5.30 ± 0.14 log CFU/g in plain packaging, 5.11 ± 0.13 log CFU/g in vacuum packaging, and 4.49 ± 0.11 log CFU/g in MAP, respectively (Tab. 1). These values were statistically significantly different (ANOVA: F (2,72) = 10.816, P < 0.001), with MAP samples being different from the other packaging types. After cold chain interruption, the values ranged from 3.36 ± 0.06 to 7.09 ± 0.11 log CFU/g and were affected by the temperature (ANCOVA: P = 0.030) and time from the exposure to the higher temperature (P = 0.007). Higher APCs were recorded after exposures to higher temperatures and longer time to sampling and at (Tab 2, S7– S9).

APC limits were exceeded more often than in salmon, and vacuum packaging yielded worse results than plain packaging or MAP. In all, limits were not exceeded at lower exposure temperatures and shorter exposure times (Tab 3, S7– S9).

Psychrotrophic microorganisms count (PMC)

Similar to APC, PMC recorded in common carp samples before cold chain interruption also differed depending on the packaging type (ANOVA: F (2,72) = 6.207, P = 0.003). The difference was caused by the vacuum-packaged samples, the PMC of which was significantly lower (6.78 ± 0.14 log CFU/g) than in the other two packaging types (plain 7.20 ± 0.08 log CFU/g, MAP 7.26 ± 0.07 log CFU/g) (Tab. 1). After cold chain interruption, PMC values ranged from 5.31 ± 0.08 to 8.55 ± 0.10 log CFU/g and were affected by the type of packaging, exposure temperature, and duration, and the time from exposure to evaluation (ANCOVA: influence of all predictors P < 0.001; Tab 2). PMC values were higher with the higher temperature and longer cold chain interruption; significantly higher PMC values were found in plain packaging and in samples examined after 24 hours (Tab 2, S10–S12).

PMC limits were violated in most carp samples in plain packaging, the values were not exceeded only in samples after exposure, especially after shorter cold chain interruption or those exposed to lower temperatures. In samples evaluated 24 hours after the interruption, limits were exceeded in most cases. Where carp samples in MAP or vacuum packaging are concerned, limits were not exceeded at lower exposure temperatures and shorter exposure times – after short exposures to higher temperatures, the limits were not violated even 24 hours after exposure (Tab 4, S10–S12).

Table 4 Violation of limits for psychrotrophic microorganism count for individual temperatures in common carp, Atlantic salmon, and Atlantic cod in plain (P), vacuum (V), and modified atmosphere (M) packaging

Examination time	Temperature	Time of exposure of samples to higher storage temperature			
		1 hour	2 hours	3 hours	4 hours
E0	0 °C				
	3 °C			Cod-V	
	5 °C		Cod-V		Carp-M
	8 °C		Cod-V		Cod-P,M
	11 °C	Cod-V			
	14 °C	Carp-M, Cod-V		Carp-P	Carp-M, Salmon-V,M
	17 °C	Cod-V	Carp-M		Carp-P,V, Cod-P
	20 °C		Carp-V, Cod-M	Cod-P	Carp-M, Cod-P
	25 °C	Carp-V, Cod-V		Carp-P, Cod-V	Carp-P,V,M, Cod-V
E3	0 °C				
	3 °C			Carp-M, Cod-V	
	5 °C	Cod-V	Carp-P, Cod-V	Carp-P, Carp-M	Carp-P
	8 °C		Carp-P		Cod-V
	11 °C		Cod-P		Carp-P
	14 °C	Cod-V		Carp-P	Carp-M, Cod-V
	17 °C	Cod-M	Cod-V	Carp-V	
	20 °C		Carp-V	Carp-P, Cod-P,M	Salmon-M
	25 °C	Carp-V	Carp-P, Cod-M	Carp-P, Salmon-M, Cod-P	Carp-P,V, Cod-V
E24	0 °C	Carp-P	Carp-P		Carp-P, Cod-P
	3 °C	Salmon-P,M, Cod-P	Cod-V,M		Carp-P, Salmon-P,M
	5 °C	Carp-P, Cod-V	Carp-P, Cod-P,V	Carp-M, Salmon-P	Carp-P,M, Salmon-P,V, Cod-P
	8 °C	Carp-P	Carp-P, Cod-P,M	Carp-P, Cod-V	Carp-P, Cod-P,V
	11 °C	Carp-P	Carp-P	Carp-P,M, Salmon-M	Carp-P, Salmon-M
	14 °C	Carp-P	Carp-P	Carp-P,M	Carp-M, Salmon-V, Cod-V
	17 °C	Carp-P, Cod-V	Carp-P,V,M, Salmon-P	Carp-P, Salmon-P,V	Carp-P, Salmon-P,V, Cod-P,M
	20 °C	Carp-P, Salmon-V, Cod-P	Carp-P, Salmon-M	Carp-P, Salmon-M, Cod-P	Carp-P,M, Cod-P,M
	25 °C	Cod-V	Carp-P,M, Cod-P,V,M	Carp-P,V,M, Cod-V	Carp-P,M, Salmon-M, Cod-P,V

Legend: E0 – examination of the sample immediately after exposure, E3 – examination of the sample 3 hours after exposure, E24 – examination of the sample 24 hours after exposure

PMC values before cold chain interruption differed also in Atlantic salmon (ANOVA: F (2,72) = 21.180, P < 0.001). In plain packaging, PMC was 3.38 ± 0.09 log CFU/g, compared to vacuum packaging with PMC of 3.24 ± 0.08 log CFU/g and MAP with 4.24 ± 0.17 log CFU/g (Tab. 1); MAP differed from the other two types of packaging. After cold chain interruption, the PMC values in salmon ranged from 2.32 ± 0.32 to 5.65 ± 0.21 log CFU/g and were statistically significantly influenced by the packaging type, temperature, cold chain interruption, and time from exposure to the interruption (ANCOVA: effect of all predictors P < 0.001; Tab 2). As in carp, PMC values were higher after more intensive cold chain interruption (higher temperature, longer exposure), and higher values were detected in samples evaluated after 24 hours, and in the vacuum and MAP packaging (Tab 2, S13–S15).

Similar to APC, PMC limits were violated less commonly in Atlantic salmon than in the carp. Limits were rarely exceeded in samples taken immediately after cold chain interruption or three hours later; in samples examined 24 hours after cold chain interrruption, limits were not exceeded only after less intensive cold chain interruption (i.e., at shorter exposures and exposures to a lower temperature; Tab 4, S13–S15).

In Atlantic cod, the initial PMC values differed between the plain (5.47 ± 0.15 log CFU/g), vacuum (5.42 ± 0.14 log CFU/g), and modified atmosphere packaging

(4.44 ± 0.11 log CFU/g) (Tab. 1) (ANOVA: F(2,72) = 18.566, P < 0.001). The lowest PMCs were observed in MAP. After cold chain interruption, the PMC values ranged from 3.16 ± 0.16 to 7.38 ± 0.11 log CFU/g. PMC was affected by the packaging type (ANCOVA: P = 0.010), temperature (P = 0.033), and time to sample examination (P < 0.001). Higher values were associated with higher exposure temperature, longer time to evaluation, and in plain and vacuum packaging (Tab 2, S16–S18).

PMC limits were violated in more instances in cod than in salmon. In plain or vacuum packaging samples, limit values were violated more often than in MAP samples, including samples examined immediately after exposure (Tab 4, S16–S18).

***E. coli* a *Salmonella* spp.**

A vast majority of samples contained less than 1.70 log CFU/g of *E. coli* (i.e., values below the limits of detection of the used platemethod; *E. coli* examination was positive only in carp samples – out of the 363 carp samples in vacuum packaging, 6 samples were positive for *E. coli* at 1.70–2.48 log CFU/g, without association with exposure temperature or length of time until examination. The presence of *Salmonella* spp. was not detected in any of the studied samples.

The variability in the amount of microorganisms contaminating fish muscles is high, as corroborated by our results. This is caused by many factors, including fishing area, type of fish, environmental factors, harvesting methods, storage, and transportation **(Masniyom, 2011; Terentjeva *et al.*, 2015).** Although we have done our best to limit this variability (more than 900 samples per fish were used in the described experiment), we were not able to completely eliminate this variability. The fact that various batches of fish had to be used in such a huge number of samples is the most likely reason for this observation.

Moreover, microbial growth during storage depended on the preservation conditions. The rapid decay of fish is particularly caused by psychrophilic microorganisms **(Golian *et al.*, 2021)** and its rate is, in particular, temperature-dependent; that's why the fish are chilled or frozen immediately after being caught. Fresh fish are brought to the market at melting ice temperature (-1 to +2 °C), fresh chilled fish in vacuum or protective atmosphere packaging at temperatures between -1 and +5 °C **(Regulation (EC) No 853/2004; Fernandes, 2009; Golian *et al.*, 2021)**. The temperatures of storage of control samples in our experiment were chosen to meet these legislative requirements for individual types of packaging, i.e., 1 ± 1 °C for plain packaging, and 3 °C for MAP and vacuum packaging. A critical value of 6 log CFU/g of spoilage bacteria (mainly psychrotrophic) associated with a significant decay of the sensorial characteristics was exceeded after 9 days of storage at 1 ± 0.5 °C and 3 days for fluctuating temperature ranging between 1 and 7 °C (**Giarratana *et al.*, 2022**).

Nitrogen, oxygen, and carbon dioxide at various concentrations are the most common gases used in modified atmosphere packaging (**Macé *et al.*, 2013; Zouharová *et al*., 2023)**. In our study, we used a mixture of 70% N_2 and 30% CO_2, which is a commercially widely available gas mixture. For packaging fresh chilled fish in a protective atmosphere, manufacturers typically use oxygen-free gas mixtures, with the mixture used in our experiment being widely employed for this purpose.

In the case of common carp and Atlantic cod samples, MAP and vacuum packaging performed better (more samples within the limit) in both APC and PMC than in plain packaging. This corresponds to the results by many authors reporting the inhibition of microorganism growth caused by the lack of oxygen in the packaging environment. Most oxygen reactions in foods contribute to spoilage, especially lipid oxidation **(Lee *at al.*, 2015**). In MAP, the absence of oxygen leads to the inhibition of lipid oxidation, which contributes to the extension of the shelf life of packaged fish**.** CO_2 inhibits the autolytic degradation of fish muscle tissue during storage and inhibits microbial growth, thus usually extending the shelf life of fish by 30–60 % **(Arvanitoyannis and Stratakos, 2012**). It is highly soluble in water and lipids and this solubility greatly increases with decreasing temperature**.** Therefore, pH of the muscle tissue further decreases with the duration of the contact of CO_2 with the food and its concentration at low storage temperatures **(Lee *et al.*, 2015)**, which further increases the antimicrobial capacity of CO_2. On the other hand, the high CO_2 content negatively affects the sensory quality of fish muscles, particularly the change in structure **(Masniyom, 2011)**.

Based on all the above, storage of fresh fish in an oxygen-free modified atmosphere can greatly reduce the lipid oxidation and growth of aerobic bacteria **(Macé *et al.*, 2013; Merlo *et al*., 2019)**. Lactic acid bacteria, in particular *Lactococcus piscium* and *Hafnia alvei*, count among the bacteria that can cause spoilage of salmon stored in oxygen-free MAP **(Merlo *et al.*, 2019)**. **Macé *et al.* (2013)** identified *Lactococcus piscium* and gram-negative fermentation bacteria *Photobacterium phosphoreum* and *Enterobacteriaceae* (*Serratia* spp.) to be the dominant bacterial groups/species on salmon steaks stored in vacuum or MAP (50 % CO_2: 50 % N_2) at the time of spoilage.

The extension of shelf-life and slowing down of spoilage by vacuum packaging is another well-known fact. **Pantazi *et al.* (2008)** reported that the shelf-life of vacuum-packaged Mediterranean swordfish improved to nine days, which was seven days more than in aerobic packaging.

Water activity and pH

The initial pH recorded before the cold chain interruption in common carp samples was 6.39 ± 0.04 in plain packaging, 6.42 ± 0.03 in vacuum packaging, and 6.39 ± 0.03 in MAP, respectively (Tab 1). These values did not statistically significantly differ (ANOVA: F (2,72) = 0.274, P = 0.761). After cold chain interruption, pH in neither plain packaging (6.43 ± 0.01), nor vacuum packaging (6.42 ± 0.01) changed significantly, while a decrease in pH to 6.27 ± 0.01 was found in MAP. These results are in accordance with **Ježek and Buchtová (2012)**, who determined the pH in common carp fillets stored for 11 days to range between 6.28 ± 0.23 and 6.62 ± 0.19. Similarly, **Kachele *et al.* (2017)** measured initial pH in common carp filets at the beginning of storage to be 6.41.

Initial pH values in Atlantic salmon were 6.15 ± 0.01 (plain), 6.19 ± 0.02 (vacuum), and 6.29 ± 0.01 (MAP), respectively, which was statistically significantly different between samples (ANOVA: F (2,72) = 25.377, P < 0.001). Surprisingly, pH in salmon samples in MAP were significantly higher than in the samples with plain and vacuum packaging. After cold chain interruption, the pH values in MAP decreased to 6.24 ± 0.01, but still remained higher than in plain (6.12 ± 0.01) and vaccum packaging (6.20 ± 0.01) (Tab 1). The values are consistent with pH 6.0 – 6.4 reported for fresh Atlantic salmon fillets by **Chan *et al.* (2020). Rode *et al.* (2016)** reported a similar range for pH in salmon, namely 6.16-6.27. **Chan *et al.* (2021)** also determined pH in salmon meat to be approx. 6.2.

In Atlantic cod, initial pH values were also statistically significantly different among packaging types (ANOVA: F (2,72) = 32.131, P < 0.001), being the lowest in the MAP (6.68 ± 0.03), and the highest in plain packaging (7.04 ± 0.04). After cold chain interruption, this order remained unchanged, with lowest pH value in MAP (6.67 ± 0.01) and highest in plain packaging (7.08 ± 0.01) (Tab 1). These values are consistent with the pH value of 6.7 ± 0.4 determined for atlantic cod in MAP reported by **Sørensen *et al.* (2020). Bisenius *et al.* (2019)** studied pH values in Atlantic cod in association with detection of food additives. In untreated fillets, they detected a mean pH of 6.5. **Ezati et al. (2021)** reported an increase in pH of fish samples after 36 h at 25 °C from 5.6 to 6.9.

As stated by both **Lee *et al.* (2015) and Babic *et al.* (2015)**, CO_2 in MAP reduces the pH of the food. **Babic** *et al.* (2015) found that during storage of common carp fillets in two types of CO_2-containing MAP (40 % CO_2+60 % N_2 and 100 % CO_2, respectively) for 15 days, the pH of of fillets gradually declined in both types of MAP. This decrease was more pronouced (down to the pH of 6.2) in the CO_2-richer atmosphere. This may be one of the reasons why in common carp and Atlantic cod, the MAP pH values are lower than those in plain packaging and vacuum packaging (Tab 1). In Atlantic salmon fillets, however, this trend was not confirmed – on the contrary, pH values were the highest for MAP samples. This can be caused by the fact that salmon has high fat content and CO_2 is absorbed in the fat, thus not forming carbonic acid (especially as we used the modified atmosphere with a relatively low CO_2 content).

Initial water activity values in common carp ranged between 0.981 and 0.988, not statistically different between packaging types (ANOVA: F (2,6) = 4.594, P = 0.062). After cold chain interruption, a_w values in the carp ranged between 0.976 and 0.992 (Tab 1).

Similar to the carp, a_w showed no significant differences between packaging types in Atlantic salmon, either (ANOVA: F (2,8) = 1.480, P = 0.284). Before cold chain interruption, it ranged from 0.981 to 0.988, and after it, from 0.973 to 0.990 (Tab 1).

In Atlantic cod, the initial a_w were 0.982–0.984 (plain packaging), 0.980–0.984 (vacuum packaging), and 0.978 (MAP). A statistically significant difference was observed between samples in plain and modified atmosphere packaging (ANOVA: F (2,6) = 7.800, P = 0.021). After cold chain interruption, the a_w values in cod ranged from 0.974 to 0.992 (Tab 1).

Water activity is an important factor affecting fish spoilage. All determined values were above a_w = 0.97, which is consistent with the character of fresh foods susceptible to rapid spoilage. **Abbas *et al.* (2009)** reported a_w values to range typically 0.971–0.995 in fresh fish, which was consistent with all values determined in the presented study as well. **Da Silva *et al.* (2020**) reported a_w = 0.98 for salmon-containing sushi, and **Iacumin *et al.* (2021)** a_w > 0.97 in cold-smoked fish products (trout, salmon, and sea bass).

CONCLUSION

In all, we can say that the initial microbial contamination of fish fillets is highly variable, even within the same batch of fish. The level of common carp contamination is generally higher than in sea fish (Atlantic cod, Atlantic salmon). Cold chain interruption, which may occur, for example, during the transport of samples into the laboratory, can affect the results of the microbiological examination. This is particularly the case if the samples are exposed to the higher temperature for a prolonged period of time. For this reason, if such a cold chain interruption is found, samples should be analyzed immediately upon arrival at the laboratory.

The results of this study can be used to establish guidelines for supervisory authorities, specifying the methods for sample collection and transport of chilled samples for microbiological testing. This may reduce the number of samples rejected by the laboratories due to improper transport. The results of the study can

also be used as a defense against defendants' challenges of the results of analyses of samples taken during routine inspections. Finally, the results can be also valuable for producers of chilled fish.

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