



Quality of Fresh Atlantic Salmon (*Salmo salar*) Under Hyperbaric Storage at Low Temperature by Evaluation of Microbial and Physicochemical Quality Indicators

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Abstract

Atlantic salmon (*Salmo salar*) quality was evaluated by hyperbaric storage at low temperature (HS/LT; 40–60 MPa, 5–15 °C) and compared with control samples stored at the same storage temperatures (5–15 °C) and atmospheric pressure (AP). Results showed that HS/LT was efficient to slowdown spoilage microbial growth, with additional inactivation at 60 MPa/10 °C after 50 days. Contrarily, AP-5/10 °C samples exceeded the established limit after 15 days. Furthermore, the established limit of total volatile base-nitrogen was surpassed at 60 MPa/10 °C only after 30 days (contrarily to 6 days at AP/10 °C), but with stable trimethylamine-nitrogen content in the former. Formaldehyde and dimethylamine-nitrogen contents increased after 6 days of HS/LT, but only the former progressively increased until the 10th day, indicating a possible formation by the action of enzymatic activity, but also by other chemical reactions. Additionally, HS/LT slightly increased secondary product content from the lipid oxidation, although to a lower extent compared with AP (at the different storage temperatures). Concluding, HS/LT may represent an interesting methodology to control microbial activity and important physicochemical parameters (such as volatile amines, colour, and lipid oxidation) of Atlantic salmon muscle portions for 15–30 days, compared with 6 days for AP/5 °C (conventional refrigeration).

Keywords Hyperbaric storage · Refrigeration · *Salmo salar* · Microbial stability · Volatile amines · Physicochemical properties

Introduction

Several spoilage mechanisms are responsible for fish deterioration: metabolic activity of microorganisms, endogenous enzymatic activity (autolysis), and chemical lipid oxidation, considering fish products, mainly fresh fish, a highly perishable product with short shelf-life, being this very limiting for commercialization and leading to excessive losses (Ghaly et al. 2010). Lipid oxidation causes the formation of negative odours and lipid peroxides, resulting in taste, texture, and consistency losses, and reduced nutritional value. Transition metals are primary activators of molecular oxygen, which reacts with double bonds of fatty acids, particularly

polyunsaturated fatty acids (PUFAs) that are highly susceptible to oxidation (Hultin 1994).

Microbial spoilage in fish produces amines, organic acids, sulphides, alcohols, aldehydes, and ketones with unpleasant and unacceptable off-flavours (Ólafsdóttir et al. 1997). The occurrence of volatile basic compounds, such as ammonia, trimethylamine-nitrogen (TMA-N), and dimethylamine-nitrogen (DMA-N), is one of the characteristic features attributed to chemical changes occurring in marine fish muscle during spoilage. The analytical parameter, which includes these volatile compounds, is the total volatile basic nitrogen (TVB-N), and this is widely considered to be a useful index of fish freshness (Ozogul 2009). The main volatile compound accumulated during chilled fish is TMA-N, originating from the bacterial reduction of TMA-N-oxide (TMAO), via the enzyme TMAO reductase (TMAOase), and this is considered to be the main reason for the off-odours of fish products (Ólafsdóttir et al. 1997). When the bacterial growth is slowed down/inhibited, as is the case of refrigeration/frozen storage, conversion of TMAO to DMA and formaldehyde (FA) by the enzyme TMAO aldolase occurs, also with negative effects on fish quality (Benjakul et al. 2004).

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Hyperbaric storage (HS) has attracted great interest lately due to the possibility to preserve food products above atmospheric pressure at room (RT) or low temperatures (LT), increasing shelf-life and quality comparatively to the conventional method of refrigeration. The effect of HS/LT for fish preservation was already studied by Ko et al. (2006) and Fidalgo et al. (2018) using tilapia fillets and Atlantic salmon muscle portions, respectively, and at HS/LT by Otero et al. (2017) and Otero et al. (2019), using Cape hake (*Merluccius capensis*) loins and Atlantic mackerel (*Scomber scombrus*, L.) fillets, respectively, with good results for fresh fish shelf-life extension. In such studies, HS/LT (50 MPa/5 °C) of both hake loins and Atlantic mackerel fillets resulted in a maintenance of microbial load and TVB-N content after 7 days and 12 days, respectively. However, drip losses and an increase of the shear resistance and whiteness of the raw fish were verified, but after cooking, these effects were imperceptible. These two studies clearly show the potential of HS/LT to increase fresh fish shelf-life, being of great interest further and deeper studies of the HS effect on fresh fish, as for instance the study of longer storage periods. For instance, HS/LT was recently studied for watermelon juice, being observed a microbial shelf-life of at least 58 days at 62.5/75 MPa (15 °C), due to a reduction of initial loads, by at least 2.5 log CFU/mL, while pH and colour values did not change (Lemos et al. 2017).

Therefore, the aim of this work was to define the best condition of HS/LT for shelf-life extension of Atlantic salmon, and evaluation of spoilage microbial activity and physicochemical parameters related to quality up to 50 days. For that, an initial screening was carried out, using different pressure/temperature storage conditions (40–60 MPa/5–15 °C) and storage experiments over 10 days. Control samples stored at atmospheric pressure (AP, 0.1 MPa) and at the same temperature range (5–15 °C) during the same time were also performed. The samples were microbiology evaluated (total aerobic psychrophiles, anaerobic bacteria, Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp., and hydrogen sulphide-reducing bacteria). Afterwards, the results obtained led to carry out a further storage assay using the condition for which the best results were obtained in the screening assay, 60 MPa/10 °C, during a longer storage time (50 days). These results were compared with control samples stored at AP (5 or 10 °C) for 6 days, by studying spoilage microorganisms, physicochemical indicators of quality, instrumental colour parameters, and lipid oxidation mechanism.

Materials and Methods

Sample Preparation and Storage Experiments

Atlantic salmon was acquired from a local market before each experiment storage and portions of dorsal muscle (5–10 g)

were cut in aseptic conditions, removing the skin. Salmon portions were packaged in low-oxygen permeable barrier bags (PA/PE-90; Plásticos Macar–Indústria de Plásticos Lda., Palmeira, Portugal). To avoid deterioration, samples were kept on ice, and storage assays were initiated as soon as possible that all samples were prepared (within maximum of 2 h). Storage experiments were carried out between January and June 2017.

Storage experiments are divided in two parts: (1) A screening assay using different combinations of pressure/low temperatures over 10 days of storage was carried out (40 MPa/5 °C, 50 MPa/5 °C, 50 MPa/10 °C, 50 MPa/15 °C, 60 MPa/10 °C, 60 MPa/15 °C); (2) The best condition, 60 MPa/10 °C, in a microbial stability point of view, was further studied during a longer storage time of 50 days. Control samples were also kept at the same storage temperatures (5–15 °C) under AP conditions, in exactly the same conditions (in the dark and immersed in the same fluid used for compression). Samples from first assay (1) were evaluated through microbial analyses, while in the second assay (2) were also performed pH measurements, TVB-N, TMA-N, DMA-N, and FA determinations, colour, and lipid oxidation (primary, secondary and tertiary lipid oxidation). For each storage condition, a different batch of fresh salmon samples was used.

The first set of HS experiments (1) was performed using a 100-mL high-pressure equipment (high-pressure system U33, Institute of High Pressure Physics, Warsaw, Poland) equipped with a pressure vessel of 35-mm inner diameter and 100-mm height. The second set (2) was carried out using a different 200-mL high-pressure equipment (SFP FPG13900, Stansted Fluid Power, Stansted, UK), which has a pressure vessel of 35-mm inner diameter and 250-mm height. Both high pressure equipment use a mixture of propylene glycol and water (40:60, v/v) as pressurization fluid.

Microbial Analyses and pH Determination

For microbial analyses, salmon muscle (5 g) was transferred aseptically into a stomacher bag and Ringer's solution (1:9, w/v) were added. The mixture was homogenized for 60 s (STOMACHER 400, Seward Laboratory Systems Inc., FL, USA) which corresponded to the dilution 10^{-1} . Then, decimal dilutions were made using Ringer's solution. Total aerobic psychrophiles, anaerobic bacteria, Enterobacteriaceae, lactic acid bacteria, and *Pseudomonas* spp., hydrogen sulphide-producing bacteria (H_2S -producing bacteria including *Shewanella putrefaciens*), were determined by the method described by ISO 4833 (2003), Teixeira et al. (2014), García-Soto et al. (2013), ISO 8523 (1991), and Teixeira et al. (2014), respectively. H_2S -producing bacteria were enumerated in pour plate using Lyngby iron agar (Merck), by inoculation of 1 mL of diluted samples and incubation at 20 °C for 4 days, counting the black colonies. In all cases, petri

dishes containing 15–300 colony-forming units (CFU) were selected for counting, and microbial loads were calculated as logarithm of colony-forming units per gramme (log CFU/g).

The pH of salmon samples was measured with a properly calibrated glass electrode at 25 °C (Crison, Barcelona, Spain).

Total Volatile Base-Nitrogen Assessment

TVB-N values were measured using the method of Antonacopoulos (1960), which was modified by Vázquez et al. (2018). Briefly, 10 g fish muscle were extracted with 60 g/L perchloric acid in 30 mL water and brought up to 50 mL. An aliquot of the acid extracts was rendered alkaline to pH 13 with 200 g/L aqueous NaOH and then steam distilled. Finally, the TVB-N content was determined by titration of the distillate with 10 mM HCl. Results were expressed as mg TVB-N/100 g muscle.

Trimethylamine-Nitrogen Assessment

Trimethylamine-nitrogen (TMA-N) values were determined by the picrate method, as previously described by Tozawa et al. (1971). This involves the preparation of a 5% trichloroacetic acid extract of fish muscle (10 g muscle/25 mL trichloroacetic acid). Results were expressed as milligrammes TMA-N/100 g muscle.

Dimethylamine-Nitrogen and Formaldehyde Determination

Before DMA-N and FA quantification, extraction using trichloroacetic acid was performed, according to Benjakul et al. (2004). Fish muscle (2 g) and 8 mL of 5% trichloroacetic acid were homogenized using a MICCRA D-9 Homogenizer (MICCRA GmbH, Müllheim, Deutschland). The homogenate was centrifuged at 3000 ×g for 15 min (Heraeus Biofuge Stratos, Thermo, Electron Corporation, Massachusetts, EUA). To the pellet, 4 mL of 5% trichloroacetic acid were added and homogenized as previously mentioned. The supernatants were combined and neutralized to pH 6.0–6.5 and the final volume was made up to 20 mL using distilled water. The supernatant was then used for DMA-N and FA determinations.

DMA-N was determined by the copper-dithiocarbamate method, as described by Dyer and Mounsey (1945), with slight modifications. To 2 mL of neutralized supernatant, 1 mL of copper-ammonia reagent was added and mixed thoroughly, followed by the addition of 4 mL of 5% carbon disulphide-toluene solution. The tubes were then closed tightly, and incubation at 50 °C for 2 min. After mixing for 20 s, 400 mL of 30% acetic acid were added, followed by mixing again for 15 s. After leaving for 10 min at room temperature, the toluene layer was transferred to a tube containing 0.5–

1.0 g of anhydrous sodium sulphate to remove the water. The absorbance was measured at 440 nm (LAMBDA 35 UV/Vis spectrometer, PerkinElmer Instruments, Inc.). Results were expressed as mg DMA-N/100 g muscle.

FA was determined using the acetylacetone reagent according to the method of Nash (1953) with slight modifications. To the neutralized supernatant (3 mL), 3 mL of acetylacetone reagent was added and mixed thoroughly. The reaction mixture was incubated at 60 °C for 15 min and cooled in running water. The absorbance was measured at 412 nm (LAMBDA 35 UV/Vis spectrometer, PerkinElmer Instruments, Inc.) and the results expressed as microgrammes FA/100 g muscle.

Colour Analysis

Colour was measured at 25 °C and using a Konica Minolta CM-2300d spectrophotometer (Osaka, Japan). The colour parameters were recorded according to the CIElab system and directly computed through the original SpectraMagic™ NX software (Konica Minolta, Osaka, Japan), obtaining the following parameters: L^* value (0, dark; 100, light), a^* value (+, red; –, green) and b^* value (+, yellow; –, blue). These colour parameters were used to obtain the total colour difference (ΔE^*):

$$\Delta E^* = \left[(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2 \right]^{1/2}$$

Lipid Oxidation Assessment

Lipid content was quantified according to the method of Bligh and Dyer (1959), being the extraction done with a chloroform:methanol:distilled water mixture (1/2/1). The total lipid content ranged from 15.4 to 21.9%. For peroxide value, determination (primary lipid oxidation) was carried out following the ferric thiocyanate method (Chapman and Mackay 1949). The peroxide value was expressed as mg Fe (III)/kg lipids. Secondary lipid oxidation was evaluated by quantification of secondary lipid oxidation products using the thiobarbituric acid-reactive substance (TBARS) method, which was performed as described by Vyncke (1970). TBARS results were expressed as microgrammes malondialdehyde (MDA)/g fish. Tertiary lipid oxidation compounds resulting from the interaction between oxidized lipids and nucleophilic compounds (namely protein-like molecules) were measured by fluorescence spectroscopy (Hitachi F2000 fluorescence spectrophotometer (Tokyo, Japan). In agreement with previous research (Aubourg 1999), fluorescence measurements were carried out at 393/463 nm and 327/415 nm in the aqueous phase (methanol-water layer) resulting from the lipid extraction of fish muscle (Bligh and Dyer 1959). The relative

fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission wavelength pair and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 $\mu\text{g}/\text{mL}$ in 0.05 M H_2SO_4) at the corresponding wavelength pair. The fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463 \text{ nm}}/RF_{327/415 \text{ nm}}$.

Statistical Analysis

For the first set of experiments (10-days of storage), microbiology results are shown in relative microbial variation ($\log N - \log N_0$), calculated by difference between the logarithmic load at the end of storage (N) and the initial logarithmic load at day 0 (N_0). This way, positive values denote microbial growth, while negative values indicate microbial reduction compared with initial counts. Fish samples corresponding to each storage condition were tested with a two-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey's honestly significant difference, HSD) to identify differences between conditions and during storage period. The level of significance was established at $p < 0.05$.

For the second set of experiment (50 days of storage), microbial, pH, TVB-N, TMA-N, DMA-N, FA, colour, and lipid oxidation results are shown in absolute values. Fish samples (initial samples/fresh fish, and samples stored under AP and HS) were tested with a one-way ANOVA, followed by a multiple comparisons test (Tukey's HSD) to identify differences between samples. The level of significance was also established at $p < 0.05$.

Results and Discussion

Storage of Atlantic Salmon for 10 days: Pressure/Temperature Storage Effect

Table 1 showed the initial microbial counts for raw salmon muscle used in this study. Initial total aerobic psychrophiles counts were between 4.19 and 5.40 log CFU/g, which were very similar to those obtained in previous work (Fidalgo et al. 2018). According to the limit of 7.0 log CFU/g of aerobic plate counts established by ICMSF (1986), the salmon samples used in this study were in good conditions for human consumption before the storage experiments.

Evolution of the microbial counts in relative values of Atlantic salmon muscle portions stored under the different conditions of pressure/temperature is shown in Fig. 1 (the statistical analysis is presented in Table 2). Control samples, stored at AP at 5, 10, and 15 °C, showed a progressive increase ($p < 0.05$) of total aerobic psychrophiles counts during the 10 days of storage, with increments of 1.49 log, 2.05 log, and 1.93 log, respectively, at the 3rd day of storage, which

Table 1 Initial microbial load (minimum – maximum) of the different samples of Atlantic salmon used in the storage experiments

Microbial parameters (Log CFU/g)	Results	RSD
Total aerobic psychrophiles	4.19–5.40	9%
Anaerobic bacteria	1.72–4.39	28%
Enterobacteriaceae	1.90–2.91	16%
Lactic acid bacteria	1.95–3.15	34%
<i>Pseudomonas</i> spp.	4.16–6.06	13%
H_2S -producing bacteria	3.65–5.47	22%

RSD relative standard deviation

correspond to values next or above the acceptable limit of 7.0 log CFU/g (ICMSF 1986) (Fig. 1(a)). Consequently, these samples were already unacceptable for consumption at the 6th day, according to the same acceptability limit, and showed evident visual signs of spoilage, mainly samples stored at AP/15 °C. For the other microorganisms analysed, these samples showed a similar behaviour to total aerobic psychrophiles, with samples stored at AP/15 °C presenting counts above the acceptable limit after 10 days of storage.

Increasing pressure level, microbial evolution was progressively affected during storage time, similarly to what was verified in our previous work using storage at a room temperature of 25 °C at 75 MPa (Fidalgo et al. 2018). However, the present results showed that decreasing the temperature storage under a lower pressure allowed obtaining an improvement of the microbial stability. For instance, for the pressure level of 50 MPa, at 5 °C, a microbial load reduction ($p < 0.05$) was verified, of about 1.45 log, 1.00 log, 0.56 log, and 1.67 log for total aerobic psychrophiles (Fig. 1(a)), Enterobacteriaceae (Fig. 1(c)), *Pseudomonas* spp. (Fig. 1(e)), and H_2S -producing bacteria (Fig. 1(f)), respectively. For anaerobic bacteria (Fig. 1(b)) and lactic acid bacteria (Fig. 1(d)), the initial microbial load was maintained during the whole storage time. On the other hand, increasing the temperature storage to 10 °C and maintaining the same pressure storage (50 MPa) only allowed obtaining a microbial growth slowdown. In this latter condition (50 MPa/10 °C), there is only an exception for lactic acid bacteria counts, which showed a reduction at the 3rd day of about 1.61 log, further increasing to values similar to AP/5 °C, at the 10th day of storage. However, using a higher pressure of 60 MPa (at same 10 °C of storage temperature), this condition was enough to reduce the initial microbial counts for all the microorganisms analysed after 10 days of storage (1.29 log, 1.22 log, 1.99 log, 1.58 log, and 2.12 log for total aerobic psychrophiles, Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp., and H_2S -producing bacteria, respectively), except for anaerobic bacteria, for which a maintenance of the initial microbial load was observed.

A microbial inhibition was obtained using the other pressure/temperature conditions (50 and 60 MPa, at 15 °C),

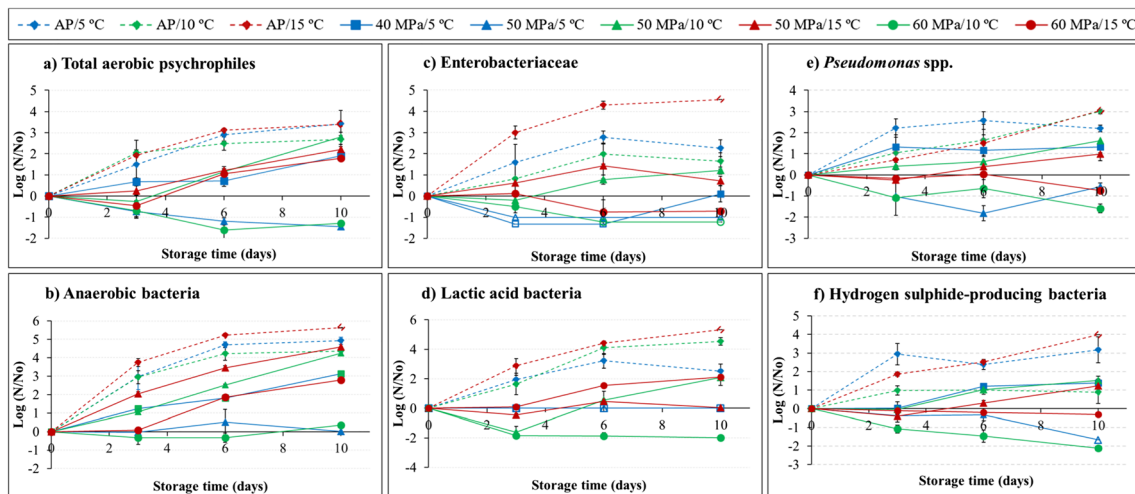


Fig. 1 Relative values of total aerobic psychrophiles (a), Enterobacteriaceae (b), anaerobic bacteria (c), lactic acid bacteria (d), *Pseudomonas* spp. (e), and H₂S-producing bacteria (f) evolution on Atlantic salmon during 10 days of storage under: hyperbaric storage at low temperature (40 and 50 MPa at 5 °C; 50 and 60 MPa at 10 °C; and 50 and 60 MPa at 15 °C), and under atmospheric pressure (AP, 0.1 MPa) at

the same temperatures (5, 10, and 15 °C). Results are shown in relative microbial variation ($\log(N/N_0)$), calculated by difference between the logarithmic load at the end of storage (N) and the initial logarithmic load range at day 0 (N_0) is presented in Table 1, while the statistical analysis is shown in Table 2. Unfilled symbols mean that the microbial values are below the detection limit (≤ 1.00 log CFU/mL)

showing total aerobic psychrophiles counts higher than 7 log CFU/g (established limit) at the 10th day, except for 40 MPa/5 °C, which showed a very close value to the acceptable limit, of about 6.47 log CFU/g (an increase of 1.91 log), at the end of storage. Accordingly, these samples resulted in an increase of microbial shelf-life to at least 6 days, unlike AP/5 °C that showed microbial load above the acceptable limit at the same sampling day. Anaerobic bacteria presented a similar behaviour to total aerobic psychrophiles counts, while the other microorganisms, as the case of Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp. and H₂S-producing bacteria, were more pressure sensible.

Recently, for hake loins (Otero et al. 2017) and Atlantic mackerel fillets (Otero et al. 2019) stored at 50 MPa/5 °C during 7 days and 12 days, respectively, it was reported that microbial growth was inhibited at the beginning of the storage period, while microbial inactivation was verified in the samples stored under pressure during all storage time. In the current work, similar results were obtained for the same storage condition (50 MPa/5 °C), but for a longer storage time.

As a result, the two pressure/temperature conditions of 50 MPa/5 °C and 60 MPa/10 °C were the most efficient to preserve fresh Atlantic salmon muscle portions, since in addition to microbial growth inhibition, it was observed an additionally microbial inactivation during the 10 days of storage. Since 5 °C is a conventional refrigeration temperature, it was decided to pursue this work, studying a longer storage time of 50 days, in order to verify the possible achievable microbial shelf-life increase of fresh salmon fish at 60 MPa/10 °C, since this allows for energetic costs reduction (10 °C versus 5 °C).

Storage of Atlantic Salmon for 50 Days

Microbial Evaluation

Microbial evolution of the longer time experiment (50 days) using the pressure/temperature condition of 60 MPa/10 °C is shown in Table 3. The initial microbial loads (presented in fresh salmon) were very close to those obtained in the first experiment (10 days), and a microbial load decrease ($p < 0.05$) was also verified at day 6 in samples stored at 60 MPa/10 °C, as was verified in the 10-day experiment. On the other hand, and similarly to the previously observed results, storage at AP at 5 and 10 °C caused an increase of the initial microbial load for all the microorganisms, except for Enterobacteriaceae on samples stored at AP/5 °C (initial value was maintained after 6 days). Differently, during the 50 days of storage at 60 MPa/10 °C, a progressive reduction ($p < 0.05$) was observed for all the microorganisms analysed, with values below the detection limit for Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp., and H₂S-producing bacteria being verified. In the case of total aerobic psychrophiles and anaerobic bacteria, a reduction of 1.25 log and 0.41 log, respectively, was obtained. These results clearly show the great potential of HS/LT to obtain microbial stability of fresh Atlantic salmon during 50 days of storage.

Physicochemical Evaluation

pH Samples stored at 60 MPa/10 °C and at AP (5 and 10 °C) were also evaluated by pH measurements (Table 4). The

Table 2 Statistical analyses of microbial evolution (in relative values) on Atlantic salmon stored during 10 days under: hyperbaric storage at low temperature (40 and 50 MPa at 5 °C; 50 and 60 MPa at 10 °C; and 50 and 60 MPa at 15 °C), and under atmospheric pressure (AP, 0.1 MPa) at the same temperatures (5, 10, and 15 °C). Different letters denote significant

differences ($p < 0.05$) between storage days for each storage condition (a and b) and between storage conditions for each storage day (A–F). Asterisk and number sign mean that microbial counts were ≥ 300 CFU on the 10^{-6} dilution of samples or below the detection limit (≤ 1 CFU/g), respectively

Storage time	Storage conditions	Total aerobic psychrophiles	Anaerobic bacteria	Enterobacteriaceae	Lactic acid bacteria	<i>Pseudomonas</i> spp.	Hydrogen sulphide-producing bacteria		
3 days	0.1 MPa	5 °C	AB b	A b	B b	BC b	A a	A a	
		10 °C	A a	A b	BC b	B b	AB b	B a	
		15 °C	A b	A b	A c	A b	ABC b	B b	
	40 MPa	5 °C	BC b	BC b	E b#	C a#	AB a	C b	
		50 MPa	5 °C	D a	D a	E a#	C a#	D ab	CD a
			10 °C	CD c	C c	CD b	DE c	BCD b	C b
	60 MPa	15 °C	CD b	B c	BC a	CD a	CD b	CD b	
		10 °C	D a	D a	DE a	E a	D a	D a	
		15 °C	D b	D c	C a	C b	CD a	C a	
6 days	0.1 MPa	5 °C	A a	AB a	B ab	B a	A a	A a	
		10 °C	A a	B a	BC a	A a	AB b	C a	
		15 °C	A a	A a	A b	A a	ABC b	A b	
	40 MPa	5 °C	B b	C b	E b#	D a#	BCD a	C a	
		50 MPa	5 °C	C a	D a	E a#	D a#	E b	D a
			10 °C	B b	C b	D ab	CD b	BCD ab	C a
	60 MPa	15 °C	B b	B b	CD a	CD a	CD ab	CD a	
		10 °C	C a	D a	E b#	E a	E a	E ab	
		15 °C	B a	C b	E A	BC a	D a	D a	
10 days	0.1 MPa	5 °C	A a	AB a	B a	BC b	AB a	A a	
		10 °C	AB a	B a	BC ab	A a	A a	B a	
		15 °C	A a*	A a*	A a*	A a*	A a*	A a*	
	40 MPa	5 °C	B a	C a	DE a	C a#	BC a	B a	
		50 MPa	5 °C	C a	D a	F a#	C a#	D a	D b#
			10 °C	A a	B a	BC a	B a	BC a	B a
	60 MPa	15 °C	B a	B a	CD a	C a	C a	B a	
		10 °C	C a	D a	F b#	D a	D a	D b	
		15 °C	B a	C a	EF a	B a	D a	A a	

initial pH obtained in raw salmon samples was 6.02 ± 0.02 , very similar to values obtained in previous work (pH 5.95–6.07) (Fidalgo et al. 2018). Storage at AP/10 °C caused a decrease ($p < 0.05$) of pH, from 6.02 ± 0.02 to 5.86 ± 0.03 and 5.80 ± 0.04 after 6 days and 15 days, respectively, which is probably caused by the accumulation of lactic acid produced by microbial glycolysis, as it was referred in previous work (Fidalgo et al. 2018), due to the contribution of acid lactic bacteria to the decrease of pH muscle. For storage at AP/5 °C, there was also a similar decrease of the initial pH, being verified a reduction to 5.88 ± 0.04 after 15 days. Differently, at 60 MPa/10 °C, no changes in pH was observed. These results are similar to those obtained by Otero et al. (2017) and Otero et al. (2019), which reported that after 7 days and 12 days of storage at 5 °C, respectively, no variations were detected in the pH values of Cape hake loins and

Atlantic mackerel fillets, respectively, both in AP and HS samples.

Total Volatile Basic Nitrogen The level of total volatile basic nitrogen (TVB-N, Table 4) was initially 26.13 ± 1.62 mg N/100 g, similarly to values reported by other authors (Calanche et al. 2013) and did not exceed the legal limit (35 mg/100 g muscle) stipulated for Atlantic salmon muscle in accordance to the Regulation (EC) No 1022/2008.

Storage at AP/10 °C caused an increase ($p < 0.05$) of TVB-N content in about 1.69-fold, showing salmon deterioration throughout the storage, which could be correlated with the higher microbial load of these samples. However, 6 days at AP/5 °C did not affect ($p > 0.05$) the formation of TVB-N, showing similar values to the initial one. Baixas-Nogueras et al. (2002) observed an increase on TVB-N values for

Table 3 Microbial evolution of Atlantic salmon stored at 60 MPa/10 °C during 50 days, and AP (5 and 10 °C) during 15 days. Different letters denote significant differences ($p < 0.05$) between storage conditions (a–e)

Microbial analyses (1)	Fresh fish	60 MPa/10 °C				AP/5 °C		AP/10 °C	
	0 days	6 days	15 days	30 days	50 days	6 days	15 days	6 days	15 days
Total aerobic psychrophiles	4.31 ± 0.08 ^c	3.82 ± 0.15 ^{cd}	3.44 ± 0.11 ^{de}	3.31 ± 0.05 ^{de}	3.06 ± 0.13 ^c	5.57 ± 0.51 ^b	≥ 7.00 ^{a(3)}	6.86 ± 0.44 ^a	≥ 7.00 ^{a(3)}
Anaerobic bacteria	2.40 ± 0.25 ^c	1.93 ± 0.17 ^c	≤ 1.00 ^{d(2)}	≤ 1.00 ^{d(2)}	1.99 ± 0.60 ^c	4.12 ± 0.10 ^b	na ⁽⁴⁾	6.66 ± 0.50 ^a	na ⁽⁴⁾
Enterobacteriaceae	2.47 ± 0.06 ^b	≤ 1.00 ^{c(2)}	≤ 1.00 ^{c(2)}	≤ 1.00 ^{d(2)}	≤ 1.00 ^{C(2)}	2.49 ± 0.54 ^b	na ⁽⁴⁾	5.50 ± 0.73 ^a	na ⁽⁴⁾
Lactic acid bacteria	2.00 ± 0.17 ^c	2.25 ± 0.27 ^c	≤ 1.00 ^{d(2)}	≤ 1.00 ^{d(2)}	≤ 1.00 ^{D(2)}	3.23 ± 0.05 ^b	na ⁽⁴⁾	5.93 ± 0.21 ^a	na ⁽⁴⁾
<i>Pseudomonas</i> spp.	4.04 ± 0.23 ^c	2.90 ± 0.82 ^d	≤ 2.00 ^{d(2)}	≤ 2.00 ^{d(2)}	≤ 2.00 ^{D(2)}	6.02 ± 0.41 ^b	na ⁽⁴⁾	7.09 ± 0.18 ^a	na ⁽⁴⁾
H ₂ S-producing bacteria	2.23 ± 0.31 ^c	≤ 1.00 ^{d(2)}	≤ 1.00 ^{d(2)}	≤ 1.00 ^{d(2)}	≤ 1.00 ^{D(2)}	4.65 ± 0.03 ^b	na ⁽⁴⁾	5.75 ± 0.69 ^a	na ⁽⁴⁾

⁽¹⁾ Microbial loads are in Log CFU/g muscle

⁽²⁾ Counts were below the detection limit (≤ 1 log CFU/g or ≤ 2 log CFU/g in case of *Pseudomonas* spp.)

⁽³⁾ Counts were above the acceptable limit (≥ 7 log CFU/g) according to ICMSF (1986)

⁽⁴⁾ Not analysed

Mediterranean hake (*Merluccius merluccius*) only after 10 days at 6–8 °C (under AP conditions), and only a slight increase after 20 days in samples stored in ice. Samples stored at AP/10 °C exceeded the legal limit (Regulation (EC) No 1022/2008) after 6 days of storage, showing an average value of 44.24 ± 4.59 mg N/100 g.

HS at 60 MPa/10 °C showed a maintenance ($p > 0.05$) of the TVB-N formation to at least the 15th day of storage, increasing thereafter. These results are in agreement with those obtained by Otero et al. (2017), who verified that HS at 50 MPa/5 °C seemed not to affect TVB-N content of hake loins during 7 days, contrarily to a threefold increase obtained at AP/5 °C at the same sampling day. On the other hand, Otero et al. (2019) observed that HS at the same conditions (50 MPa/5 °C), after 12 days, resulted in an increase of TVB-N content on Atlantic mackerel samples. In our work, after 30 days and 50 days of storage of salmon samples, TVB-N values increased in about 1.48- and 1.62-fold, respectively, reaching values similar to AP/10 °C after 6 days, which were higher than the legal limit (Regulation (EC) No 1022/2008). High pressure processing (250 and 400 MPa, 5 min) of sea bass did not cause changes on TVB-N values up to at least 18 days (Teixeira et al. 2014). However, results from high pressure processing cannot be easily extrapolated to HS, since in the latter pressure is much lower, while time under HS is much longer.

Özyurt et al. (2009) stated that the rise in TVB-N is related to the activity of spoilage bacteria and endogenous enzymes. This parameter includes TMA-N (produced by spoilage bacteria), ammonia (produced by deamination of amino acids and nucleotides catabolites), and DMA-N (produced by autolytic enzymes mainly during frozen storage). As in HS samples, there was no microbial growth after 30 days of storage, probably the TVB-N formation was caused by enzymatic activity.

Trimethylamine-Nitrogen Initial salmon samples showed a value of 0.03 ± 0.01 mg TMA-N/100 g, values close to that obtained by other authors (0.05 ± 0.01 mg TMA-N/100 g) (Rodríguez et al. 2008) also in Atlantic salmon (Table 4). According to Hansen et al. (2009), salmon contains naturally low levels of TMAO and the TMA-N formation is low, which is in agreement with the low levels found in this study and in other salmon species (Dondero et al. 2004). After 6 days of storage, TMA-N formation was clearly affected by storage under AP/10 °C, presenting an increase ($p < 0.05$) of the initial values of about 570-fold. In addition, although not as pronounced and without significant statistical differences ($p < 0.05$), there was an increase of TMA-N values on samples stored at AP/5 °C of about 7.80-fold of the initial value, and a progressive increase ($p < 0.05$) on HS samples over storage time, showing an increase of about 57-fold the initial value at the 50th day of storage. The level of TMA-N found in fresh fish rejected by sensory panels varies between species, but it is typically around 10–15 mg TMA-N/100 g in aerobically stored fish (Dalgaard et al. 1993). Consequently, only samples stored at AP/10 °C could be rejected, since these fish samples showed a value of 15.88 ± 1.52 mg TMA-N/100 g.

TMA-N value is one of the most commonly employed quality methods to assess microbial activity in marine species kept under refrigerated conditions. According to Baixas-Nogueras et al. (2002), hake samples stored in ice showed values around 4 mg/100 g only after 20 days, but earlier and higher TMA-N values (> 15 mg/100 g after 10 days) were observed for samples stored at 6–8 °C (at AP), which agree to what was obtained in the current study.

On the other hand, according to the microbial inhibition (and inactivation) under HS (60 MPa/10 °C) during the 50 days of storage, an inhibition of the TMA-N formation was expected to occur. However, the conversion of TMAO into TMA-N observed in this study may be caused by non-

Table 4 Physicochemical parameters evolution of Atlantic salmon stored at 60 MPa/10 °C during 50 days, and AP (5 and 10 °C) during 15 days. Different letters denote significant differences ($p < 0.05$) between storage conditions (a–e)

Physicochemical analyses	60 MPa/10 °C					AP/5 °C			AP/10 °C				
	Fresh fish					6 days	15 days	30 days	50 days	6 days	15 days	6 days	15 days
	0 days	6 days	15 days	30 days	50 days								
pH	6.02 ± 0.02 ^b	6.08 ± 0.01 ^a	6.09 ± 0.02 ^a	6.10 ± 0.01 ^a	6.08 ± 0.02 ^a	6.01 ± 0.02 ^b	5.88 ± 0.04 ^c	5.86 ± 0.03 ^c	5.86 ± 0.04 ^c	5.86 ± 0.03 ^c	5.80 ± 0.04 ^c	na(6)	na(6)
TVB-N(1)	26.13 ± 1.62 ^b	27.32 ± 1.29 ^b	29.01 ± 1.25 ^b	38.65 ± 3.49 ^a	42.22 ± 6.11 ^a	25.87 ± 1.16 ^b	na(6)	44.24 ± 4.59 ^a	44.24 ± 4.59 ^a	44.24 ± 4.59 ^a	na(6)	na(6)	na(6)
TMA-N(2)	0.03 ± 0.01 ^b	0.16 ± 0.07 ^b	0.58 ± 0.22 ^b	0.71 ± 0.29 ^b	1.58 ± 0.45 ^b	0.22 ± 0.17 ^b	na(6)	15.88 ± 1.52 ^a	15.88 ± 1.52 ^a	15.88 ± 1.52 ^a	na(6)	na(6)	na(6)
DMA-N(3)	0.134 ± 0.004 ^c	0.156 ± 0.005 ^b	0.158 ± 0.005 ^b	0.158 ± 0.012 ^b	0.151 ± 0.008 ^{bc}	0.186 ± 0.003 ^b	na(6)	0.143 ± 0.007 ^{bc}	0.143 ± 0.007 ^{bc}	0.143 ± 0.007 ^{bc}	na(6)	na(6)	na(6)
FA(4)	75.73 ± 17.84 ^c	206.89 ± 5.57 ^c	258.19 ± 14.89 ^b	292.43 ± 5.19 ^{ab}	302.68 ± 13.42 ^a	208.27 ± 17.33 ^c	na(6)	163.28 ± 3.39 ^d	163.28 ± 3.39 ^d	163.28 ± 3.39 ^d	na(6)	na(6)	na(6)
Colour													
<i>L</i> *	56.54 ± 1.39 ^{ab}	54.71 ± 3.05 ^b	59.98 ± 0.57 ^a	59.32 ± 0.53 ^{ab}	60.60 ± 1.72 ^a	56.77 ± 2.17 ^{ab}	na(6)	57.63 ± 2.26 ^{ab}	57.63 ± 2.26 ^{ab}	57.63 ± 2.26 ^{ab}	na(6)	na(6)	na(6)
<i>a</i> *	16.61 ± 3.19 ^a	19.11 ± 2.88 ^a	20.00 ± 1.16 ^a	18.87 ± 1.38 ^a	19.53 ± 3.62 ^a	16.59 ± 2.32 ^a	na(6)	19.54 ± 2.23 ^a	19.54 ± 2.23 ^a	19.54 ± 2.23 ^a	na(6)	na(6)	na(6)
<i>b</i> *	18.06 ± 4.09 ^a	19.68 ± 2.70 ^a	21.15 ± 1.05 ^a	19.36 ± 0.57 ^a	20.60 ± 3.24 ^a	18.00 ± 3.55 ^a	na(6)	20.00 ± 4.04 ^a	20.00 ± 4.04 ^a	20.00 ± 4.04 ^a	na(6)	na(6)	na(6)
ΔE^*		5.12 ± 1.96 ^a	5.80 ± 1.28 ^a	3.93 ± 1.09 ^a	6.96 ± 1.07 ^a	3.75 ± 1.29 ^a	na(6)	5.17 ± 2.59 ^a	5.17 ± 2.59 ^a	5.17 ± 2.59 ^a	na(6)	na(6)	na(6)
Lipid oxidation(5)													
Primary	3.80 ± 1.28 ^a	3.35 ± 0.51 ^a	3.67 ± 0.85 ^a	3.74 ± 0.15 ^a	3.91 ± 0.97 ^a	3.30 ± 0.37 ^a	na(6)	4.32 ± 1.01 ^a	4.32 ± 1.01 ^a	4.32 ± 1.01 ^a	na(6)	na(6)	na(6)
Secondary	0.36 ± 0.04 ^b	1.89 ± 0.15 ^a	2.66 ± 0.36 ^a	2.38 ± 0.12 ^a	2.65 ± 0.68 ^a	0.35 ± 0.03 ^b	na(6)	0.47 ± 0.01 ^b	0.47 ± 0.01 ^b	0.47 ± 0.01 ^b	na(6)	na(6)	na(6)
Tertiary	0.05 ± 0.01 ^d	0.10 ± 0.01 ^{cd}	0.06 ± 0.01 ^{cd}	0.12 ± 0.01 ^{bcd}	0.15 ± 0.01 ^{bc}	0.21 ± 0.03 ^b	na(6)	0.42 ± 0.05 ^a	0.42 ± 0.05 ^a	0.42 ± 0.05 ^a	na(6)	na(6)	na(6)

(1) TVB-N: total volatile basic-nitrogen (mg N/100 g muscle)

(2) TMA-N: trimethylamine-nitrogen (mg TMA-N/100 g muscle)

(3) DMA-N: dimethylamine-nitrogen (mg DMA-N/100 g muscle)

(4) FA: formaldehyde ($\mu\text{g FA}/100\text{ g muscle}$)(5) Primary lipid oxidation by peroxides values (mg Fe (III)/kg lipids), secondary lipid oxidation by TBARS ($\mu\text{g malondialdehyde (MDA)}/\text{g muscle}$); and tertiary lipid oxidation by fluorescence ratio

(6) Not analysed

enzymatic processes, endogenous tissue enzymes, or enzymes produced by microorganisms before the storage process. Besides that, Méndez et al. (2017) stated that increase of TMA-N can be related to protein breakdown caused by high pressure processing (125–200 MPa, 0 min of holding time). Nevertheless, a good correlation between microbial inactivation by high pressure processing and the TMA-N formation was observed in many fish muscles (Karim et al. 2011).

Dimethylamine-Nitrogen and Formaldehyde DMA-N content on the initial samples (fresh salmon) was 0.134 ± 0.004 mg DMA-N/100 g (Table 4), which agrees with the values obtained by other authors using Coho salmon (1.0–1.9 mg DMA-N/kg) (Ortiz et al. 2012) and hake (1.83 mg DMA-N/kg) (Vázquez et al. 2018). After 6 days under refrigeration (AP/5 °C), an increase ($p < 0.05$) of about 1.38-fold was observed. However, for samples stored at AP/10 °C (6 days), there was no effect ($p > 0.05$) on DMA-N formation. For HS samples, a small increase was verified (to a maximum of about 0.158 mg DMA-N/100 g after 15 days and 30 days) and decreasing to 0.151 ± 0.008 mg DMA-N/100 g after 50 days.

Initial salmon sample showed an FA content of about 75.73 ± 17.84 µg/100 g (Table 4), which is in agreement with values reported before by Chung and Chan (2009) using Atlantic salmon (< 1 mg/kg) and Vázquez et al. (2018) using hake samples (~ 0.83 mg FA/kg). All stored samples showed an increase ($p < 0.05$) of the initial FA content. After 6 days of storage, there were no differences ($p > 0.05$) between samples stored at AP/5 °C and HS/LT (60 MPa/10 °C), presenting an increase of FA values to 208.27 ± 17.33 µg FA/100 g and 206.89 ± 5.57 µg FA/100 g, respectively, while at AP/10 °C, a less intense increase was verified (163.28 ± 3.39 µg FA/100 g) at the same storage day. For HS samples, FA content increased progressively ($p < 0.05$) until the 50th day of storage to a value of 302.68 ± 13.42 µg FA/100 g, which corresponds to a rise in about fourfold of the initial value.

As it was explained previously, TMA-N formation is mainly caused by microbial activity, while concerning DMA-N, its formation can be explained based on the TMAO breakdown catalysed by an endogenous enzyme widely present in fish species (i.e. TMAOase) and be produced equimolecularly with FA (Benjakul et al. 2004). A marked inhibitory effect of storage pressure on DMA-N formation was observed in the current study and this effect was verified in other studies when high pressure treatments (150–450 MPa for 2 min) were applied on hake before freezing (Vázquez et al. 2018). It is important to mention that there was a low DMA-N and FA formation on AP/10 °C-stored samples, which might be due to the high TMA-N content in the same samples, indicating that practically all TMAO was converted to TMA-N. However, FA content increased about fourfold on HS/LT samples along the storage time, which indicates that possibly TMAOase activity is not totally inhibited and occurs enzymatic reduction

of TMAO to DMA-N and FA. However, DMA-N content increased only between the 6th and 30th days (*ca* 1.2-fold), resulting in similar values to the initial one after 50 days of storage. These results could indicate that, besides natural formation of FA in fish and seafood by enzymatic reaction, other biochemical reactions can also occur (Noordiana et al. 2011).

Colour The colour parameters values obtained in the initial raw salmon were as follows: 56.54 ± 1.39 for L^* (lightness), 16.61 ± 3.19 for a^* (redness), and 18.06 ± 4.09 for b^* (yellowness) (Table 4), similar to values obtained in a previous work (Fidalgo et al. 2018). Nevertheless, according to the literature, there are several reasons for colour differences of salmon muscle, which could be caused by different contents in astaxanthin carotenoid, as well as to the haem pigments (Yagiz et al. 2010). Accordingly, to minimize these effects, several measurements in different locations of the muscle salmon were carried out in the current study.

Salmon samples stored at atmospheric pressure (5 and 10 °C) after 6 days presented a maintenance ($p > 0.05$) of the colour parameters (lightness, redness, and yellowness). Furthermore, compared with fresh fish, colour parameters were not statistically ($p > 0.05$) affected by pressure storage, showing values between 54.71–60.60, 19.11–20.00, and 19.36–21.15 for lightness, redness, and yellowness parameters, respectively, as can be seen on Table 4. Since these parameters did not change in any sample, this is reflected on the value of ΔE^* , resulting in no differences ($p > 0.05$) between samples. The ΔE^* parameter shows the comparison between samples stored under the different conditions and the respective initial value obtained in raw fresh salmon samples.

The presence of carotenoids (astaxanthin and canthaxanthin), and somewhat by haem proteins, on the salmon muscle contributes to the characteristic red colour (Yagiz et al. 2010), being a very important organoleptic property for the customer acceptance (Alfnes et al. 2006). Generally, according to the literature, a reduction of salmon redness was obtained by increasing pressure and treatment time, due to a pressure-induced myofibrillar denaturation, including the astaxanthin-binding proteins or structural changes of astaxanthin-actinin (Ojagh et al. 2011), as it was observed in salmon muscle treated from 100 to 200 MPa and 5 °C for 10–60 min (Amanatidou 2000). In the current study, redness of salmon muscle stored under pressure was not affected, as also the other colour parameters, revealing that under these storage conditions astaxanthin and/or myoglobin are probably unaffected. These results are in agreement with those obtained in a previous work (Fidalgo et al. 2018) using higher pressure level/storage temperature (75 MPa/25 °C, during 10 days or 25 days). On the other hand, Otero et al. (2017) and Otero et al. (2019) verified that storage under pressure (50 MPa/5 °C, during 7 days) increased the whiteness of the raw hake

loins and Atlantic mackerel fillet, respectively, but after cooking the differences disappeared.

Lipid Oxidation Salmon muscle is very susceptible to oxidation, due to the high content in unsaturated fatty acids and the oxidation state of salmon muscle samples. The present study was evaluated by primary, secondary, and tertiary lipid oxidations, by the quantification of the peroxide value, TBARS, and fluorescence ratio, respectively.

Initially, the peroxide value and TBARS were 3.80 ± 1.28 mg Fe (III)/kg lipids and 0.36 ± 0.04 μ g MDA/g muscle, which are in agreement to those obtained in previous work (Fidalgo et al. 2018). Concerning fluorescent compounds, the initial value was 0.05 ± 0.01 , being lower to that obtained in a previous work (0.85 ± 0.06) (Fidalgo et al. 2018).

Primary lipid oxidation did not change ($p > 0.05$) on HS/LT and AP samples, with values ranged between 3.30 and 4.32 mg Fe (III)/kg lipids (Table 4). No variations on values ($p > 0.05$) of TBARS were obtained for both storage conditions at AP (5 and 10 °C). However, HS/LT caused an increment on TBARS values, being observed an accelerated secondary lipid oxidation immediately after 6 days to 1.89 ± 0.15 μ g MDA/g muscle, remaining thereafter statistically unchanged ($p > 0.05$) during all storage time (50 days: 2.65 ± 0.68 μ g MDA/g muscle). Otherwise, an increase of fluorescent compounds formation ($p < 0.05$) to a value of 0.47 ± 0.01 and 0.21 ± 0.03 for samples was stored at AP (10 and 5 °C samples, respectively), which correspond to an increase of about 9.5-fold and fourfold of the initial value, respectively. On the other hand, no variations ($p > 0.05$) were observed for HS/LT samples during storage time, with the tertiary lipid oxidation products being kept in values similar to fresh salmon samples.

According to Aubourg (1999), the mechanism of lipid oxidation is a process that resulted from interactions between hydroperoxides (primary lipid oxidation) and compounds formed on the secondary oxidation (mainly, electrophilic compounds) and protein-type molecules (mainly, nucleophilic compounds) present in the salmon muscle. This mechanism could be the explanation for the obtained results in the AP conditions (lower values of TBARS and the higher values of fluorescence ratio). Contrarily, an increase of the TBARS values was observed in the HS samples, indicating a possible pro-oxidant effect of pressure, but not as intense as for AP samples. Furthermore, in a previous work (Fidalgo et al. 2018), higher TBARS values were observed for HS samples stored at a higher storage pressure/temperature (75 MPa/25 °C), with values reaching 7.35 ± 0.30 μ g MDA/g fish after 25 days. These results indicate that a lower storage temperature (10 °C) probably retards lipid oxidation, which is in agreement to the results obtained by Otero et al. (2019), verifying that HS, at 50 MPa/5 °C, did not enhance lipid oxidation in mackerel fillets. Additionally, the higher values of

TBARS and lower values of fluorescence ratio observed for HS in the present work indicate that probably HS reduced the reactions involved in the mechanism from secondary to tertiary lipid oxidations. Additionally, pressure storage possibly can result in a higher availability of astaxanthin in salmon muscle and exert a protective effect on the fish lipids (Yagiz et al. 2007).

Conclusions

This work showed that with the condition of 60 MPa/10 °C, it was possible to increase the microbial and physicochemical stability of Atlantic salmon to a minimum of 15 days and a maximum of 30 days, by inhibition of microbial growth (and additional microbial inactivation) and slowdown of chemical indicators formation (volatile amines), as the case of TVB-N, TMA-N, and DMA-N. Furthermore, these samples did not show colour parameter differences along storage time. Nevertheless, much more research is required, namely on the oxidation mechanisms (enzymatic and non-enzymatic) that occur during HS of fresh fish and how to avoid or reduce them, or at the level of other quality analyses (for example using inoculated pathogenic microorganisms, enzymatic activity, textural, and sensorial parameters).

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