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# Hyperbaric Storage of Vacuum-Packaged Fresh Atlantic Salmon (*Salmo salar*) Loins by Evaluation of Spoilage Microbiota and Inoculated Surrogate-Pathogenic Microorganisms

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**Abstract**
Hyperbaric storage at low and room temperature (HS/LT, 60 MPa/10°C; HS/RT, 75 MPa/25°C; 30 days) of vacuum-packaged Atlantic salmon (*Salmo salar*) was studied and compared with storage under atmospheric pressure (AP, at 5, 10, and 25°C). Spoilage and inoculated surrogate-pathogenic (*Bacillus subtilis* endospores, *Escherichia coli*, and *Listeria innocua*) microorganisms were monitored during storage. Under AP, the spoilage microorganisms increased during storage time, reaching total aerobic mesophiles values higher than the acceptable limit after 15 and 5 days at AP/5°C and AP/10–25°C, respectively. Contrarily, both HS conditions inhibited and inactivated the spoilage microorganisms, reaching total aerobic mesophiles to values below the detection limit after 30 days under HS/RT. Under AP, surrogate-pathogenic microorganism counts increased, while for both HS samples *B. subtilis* endospores reached counts below the detection limit after 30 days while *E. coli* and *L. innocua* counts were also reduced. In conclusion, HS decreased initial population of spoilage and inoculated surrogate-pathogenic microorganisms, showing that besides the shelf-life extension (due to microbial growth inhibition), it also increased microbial safety (by microbial inactivation) of vacuum-packaged Atlantic salmon.

**Keywords** Hyperbaric storage · *Salmo salar* · Spoilage microbiota · Inoculated surrogate-pathogens · *Bacillus subtilis* endospores

## Introduction

Fresh and minimally processed fish is one of the most perishable food products due to chemical and enzymatic reactions and its fast-microbial deterioration. Furthermore, the main cause of this rapid deterioration is the activity under refrigeration of specific spoilage microorganisms, including psychrotolerant Gram-negative bacteria activity, such as *Pseudomonas*, *Shewanella*, and *Photobacterium* (family: *Vibrionaceae*) [1]. Some pathogens are either frequently present the aquatic environment, e.g., *Listeria monocytogenes* and *Bacillus* spp., or originated from animal/human reservoirs (habitat in which the infectious agent normally lives, grows, and multiplies, as example: *Escherichia coli*) [2]. Because of the growing demand for fresh and minimally processed fish, research on new preservation methods is required not only to increase the shelf-life of foods but also to ensure microbiological safety.

Use of low temperature during storage and chemical techniques, for controlling water activity, enzymatic, oxidative, and microbial spoilage, are among the most common in the fish industry today. Preservation by refrigeration and freezing is widely accepted by consumers, even though other methods for preservation such as salting, drying, canning, and smoking are also applied [3]. During refrigerated storage, several physicochemical changes occur in fish muscle that can lead to important losses on sensory and nutritional qualities with an important impact on the commercial value. However, storage using low temperatures is one of the most energy-intensive technologies applied in food supply chain, involving several sustainability-related challenges. Controlling refrigeration temperatures is therefore important for all stages of the food supply chain, but maintaining refrigeration is also

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energy-intensive, accounting for about 50% of the electrical consumption by food industry [4].

Hyperbaric storage (HS) has attracted great interest due to the possibility to store food products under moderate pressures, increasing their shelf-life and quality as compared with refrigeration. HS at room temperatures (HS/RT) or at reduced temperatures (HS/LT) has been applied on fish products, proving its efficiency in extending shelf-life [5–10]**.** Cape hake (*Merluccius* spp.) loins [8] and Atlantic mackerel (*Scomber scombrus*, L.) fillets [7] were stored at HS/LT (defined by the authors as hyperbaric cold storage at 50 MPa and 5°C), controlling microbial load at 7 and 12 days, respectively. In previous work, Atlantic salmon (*Salmo salar*) was packaged in the presence of air and preserved at HS/RT [5] and HS/LT [9]. These both conditions showed an increase of the microbial shelf-life to at least 25 and 50 days, respectively, compared with refrigeration (5ºC, 3 days). As of manuscript submission time, no published studies regarding the effect of HS in pathogenic microorganisms inoculated in fresh or processed fish were found. Some results are available concerning the effect of HS/RT during 10 days on inoculated pathogenic microorganisms (*E. coli* and *L. innocua*), but in watermelon juice [11]. These authors verified that watermelon juice stored at 50–100 MPa (RT 18–21°C) caused microbial load reductions to below the detection limit for both microorganisms [11]. Although there was an increase of *L. innocua* load above acceptable limits after 10 days at 50 MPa, reaching similar values to conventional refrigeration at atmospheric pressure [11]. Concerning spores, results of the HS effect are also very scarce. However, a recent study showed that HS at 50 and 100 MPa (RT: 18–21°C) for 60 days lowered counts of *Bacillus subtilis* endospores in different matrices, with this decrement increasing in the following order: McIlvaine buffer > carrot juice > Brain Heart Infusion-broth. This order was interpreted as the effect of the increment of the nutritional conditions for *B. subtilis* germination [12]. Additionally, endospores of *Alicyclobacillus acidoterrestris* inoculated in pasteurized apple juice were stored for 30 days under HS/RT (18 to 23°C) under atmospheric pressure [13]. This study demonstrated that in the case of highly acid foods such as apple juice, HS/RT pressures below to 100 MPa and ambient temperature were effective in inactivating these spores of *A. acidoterrestris* for up to 30 days [13].

The aim of this work was to study the behaviour of spoilage microbiota and surrogate-pathogenic microorganisms in fresh vacuum-packaged Atlantic salmon stored under HS/RT (60 MPa/10°C) and HS/LT (75 MPa/25°C). Spoilage microbiota as total aerobic mesophiles, anaerobic bacteria, Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp. and hydrogen sulphide-producing bacteria, and inoculated *E. coli*, *L. innocua* and *B. subtilis* endospores (as surrogates for pathogens of *L. monocytogenes*, *E. coli* O157:H7, and *B. cereus* endospores, respectively) were evaluated.

## Materials and Methods

### Spoilage Microbiota Assay

In a first round, Atlantic salmon (*Salmo salar*) was acquired from a local market before each storage experiment and the skin from the salmon loins was removed in aseptic conditions, using a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain). Salmon loins with ca. 60 g and similar dimensions (10 cm × 4 cm × 1.5 cm) were selected from two individuals with similar weight (ca. 9 kg) and length (ca. 120 cm). Salmon loins were vacuum-packaged in low-oxygen permeable barrier bags (PA/PE-90; Plásticos Macar – Indústria de Plásticos Lda., Palmeira, Portugal) and sealed under vacuum at − 1 bar of pressure (Vacuum Packaging Machine Culinary, Albipack, Águeda, Portugal). Then, samples were stored under HS/RT (60 MPa/10°C) and HS/LT (75 MPa/25°C), and also under atmospheric conditions (5, 10, and 25°C), and the behaviour of spoilage microorganisms during storage was analysed.

For spoilage microbiota analyses, 5 g of fish muscle were transferred aseptically into a stomacher bag and 45 mL of Ringer's solution 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 mesophiles, anaerobic bacteria, Enterobacteriaceae, lactic acid bacteria, and *Pseudomonas* spp. were determined by the method described previously by Fidalgo et al. [5]. Hydrogen sulphide-producing bacteria, including *Shewanella putrefaciens*, were determined by the same method used by Teixeira et al. [14]. Total aerobic mesophiles were determined in pour plate of 1 mL of diluted sample using plate count agar (PCA, Merck), incubated at 30°C for 3 days. Anaerobic bacteria counts were determined in the same media (PCA, Merck) for 72 h at 30°C in anaerobiosis conditions (using an anaerobic system, Anaerocult®, Merck). Enterobacteriaceae counts were quantified in pour plate of 1 mL of diluted sample using violet red bile dextrose agar (VRBDA, Merck), incubated at 37°C for 24 h, and large colonies with purple haloes were quantified. Lactic acid bacteria counts were determined by pour plating 1 mL of diluted sample using De Man, Rogosa, and Sharpe (MRS, Merck) agar, followed by incubation during 4 days at 25°C. *Pseudomonas* spp.

were determined by the spread plating 0.1 mL of diluted sample in Pseudomonas selective agar base (Merck) supplemented with Pseudomonas CFC selective supplement (Merck), and plates were incubated at 25°C for 2 days. $H_2S$-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.

## Inoculated Microorganisms' Assay

In a second set of experiments, samples (10 ± 0.5 g) of dorsal muscle from Atlantic salmon obtained and prepared as previously described were divided in three lots inoculating each with for *B. subtilis* endospores, *E. coli*, or *L. innocua* inoculations. Control samples without inoculated microorganisms were similarly prepared and stored under the same conditions used for the inoculated samples. We have performed previous microbiological analyses on the endospore stock solution and then heat-treated aliquots at 80°C for 20 min to eliminate possible vegetative microorganisms. The results of these microbiological analyses revealed practically no differences between unheated and heat-treated spores, suggesting that the inoculum was basically 100% spores.

### Inoculated Microorganism Preparation

The *B. subtilis* endospores were prepared as described by Reineke et al. [15] with minor modifications. *B. subtilis* ATCC 6633 (DSM-347, Deutsche Sammlung von Mikroorganismen und Zellkulturen—DSMZ, Braunschweig, Germany) was grown in BHI-agar at 30°C for 24 h. Then, a single colony was isolated to obtain an overnight liquid culture (in BHI-broth, kept at 30°C with shaking at 150 rpm). Hereafter, the liquid culture (0.1 mL) was aseptically spread-plated onto BHI-agar plates and incubated at 30°C for 24 h. The sporulation process was verified by phase-contrast microscopy, and it took 15 days to achieve more than 95% of bright-phase endospores. Afterwards, the endospores were harvested by flooding the cultures with cold (4°C), sterile distilled water, and by scratching the agar plates with a bend glass rod, followed by threefold centrifugation for 15 min at 5,000×*g* at 4°C. The washed endospores were stored in distilled water and kept at 4°C until use.

*E. coli* ATCC 25922 and *L. innocua* ATCC 33090 (DSMZ) were grown in tryptic soy agar (TSA) at 37°C for 24 and 48 h, respectively. Then, a single colony was isolated to obtain an overnight liquid culture (in tryptic soy broth, kept at 37°C with shaking at 150 rpm). Afterwards, the liquid culture was streaked in TSA plates that were incubated for 24 and 48 h for *E. coli* ATCC 25,922 and *L. innocua* ATCC 33,090, respectively. The pure cultures were then kept at 4°C until use.

### Microorganism Inoculation on Salmon Samples

Portions of 10 ± 0.5 g of dorsal muscle from Atlantic salmon were aseptically placed in previously UV-light sterilized, low permeability polyamide–polyethylene bags, to avoid contamination. Then, 0.5 mL of *B. subtilis* endospore suspension were inoculated on the surface of salmon loins at a concentration of about 6.62 ± 0.01 log CFU/g. The endospores used in this work were not heat-treated to avoid changes on their pressure resistance in order to simulate the worst-case scenario on food preservation; according to Vercammen et al. [16], non-heat-treated endospores in low acidic and elevated water activity food products showed to be more pressure-resistant than those heat-treated.

In order to evaluate the effect of HS in a gram-negative and a gram-positive microorganism, and also surrogates of pathogenic strains, non-pathogenic *E. coli* ATCC 25922 and *L. innocua* ATCC 33090 were inoculated in fresh Atlantic salmon. The inoculations were carried out by suspending plated pure cultures of each microorganism in Ringer's solution, being the obtained suspension adjusted to 0.5 McFarland (Microplate Spectrophotometer Multiskan Go, Thermo Scientific, USA). Then, 0.5 mL of the suspension were inoculated on the surface of dorsal muscle from Atlantic salmon (10 ± 0.5 g), at a final concentration of 4.40 ± 0.19 and 5.91 ± 0.11 log CFU/g for *E. coli* and *L. innocua*, respectively. After inoculating each microorganism, the PA/PE bags were heat-sealed as close as possible to the salmon loins to avoid spread of the inoculum, as well as to maximize the contact between the inoculum and the loins.

### Inoculated Microorganisms' Enumeration

For *B. subtilis*, 10 g of salmon were transferred aseptically into a stomacher bag and 90 mL of physiological solution (0.9% NaCl) solution were added, and homogenizing the mixture for 60 s (STOMACHER 400, Seward Laboratory Systems Inc., FL, USA), which corresponded to the dilution $10^{-1}$. To assess both germinated and ungerminated endospores after each storage condition, an aliquot of each salmon sample was heated at 80°C for 20 min to inactivate the vegetative bacteria [12]. Then, decimal dilutions were performed (1.0 mL of each sample for 9.0 mL of physiological solution) in all prepared samples (heated and non-heated samples) that were pour plated (0.5 mL) in HiCromeTM Bacillus Agar (HiMedia, Mumbai, India) and incubated at 30°C for 24 h, being only enumerated the green colonies, as instructed by the culture media manufacturer.

For *E. coli* ATCC 25922 and *L. innocua* ATCC 33090, 10 g of salmon were transferred aseptically into a stomacher bag and 90 mL of Ringer's solution were added, being the mixture 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. *E. coli* ATCC 25922 was spread plated (1 mL) and counted in Coliform Count Agar after incubation at 37 ± 1°C for 24 h [17], while *L. innocua* ATCC 33090 was spread plated (0.1 mL) in PALCAM Listeria agar base with the selective supplement PALCAM (FD061), and incubated at 37 ± 1°C for 48 h [18].

Microbial counts were expressed as logarithm of colony-forming units per gram (log CFU/g), being quantified from triplicate of samples and for each sample with triplicate plates.

### Storage Conditions

To avoid deterioration, samples from both assays were kept on ice, and storage experiments were initiated as soon as all samples were prepared (within a maximum of 2 h).

The pressure conditions were decided took into account previous works [5, 9]. For 60 MPa/10°C, the unit used was a SFP FPG13900 Model (Stansted Fluid Power, Stansted, UK), with a pressure vessel of 35 mm inner diameter and 250 mm height. For 75 MPa/25 °C, the unit used Model 2-L high-pressure equipment (FPG7100, Stansted Fluid Power) with a 100-mm diameter and 250-mm height. A mixture of propylene glycol and water (40:60, v/v) was used as pressurization fluid, taking 10 to 12 s to reach 75 MPa and 7 to 8 s to reach 60 MPa, while decompression time took 2 to 3 s. The experiments were carried out between December 2018 and February 2019.

### Statistical Analysis

Data were tested with one-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 $p < 0.05$.

## Results and Discussion

### Effects of Hyperbaric Storage on Spoilage Microbiota and Muscle pH

Table 1 presents the initial microbial load of Atlantic salmon loins used in the storage experiment, and also the evolution of pH muscle during storage. Total aerobic mesophiles, anaerobic bacteria, Enterobacteriaceae, *Pseudomonas* spp., and $H_2S$-producing bacteria counts were 3.37 ± 0.34, 2.05 ± 0.08, 2.12 ± 0.13, 3.54 ± 0.28, and 2.15 ± 0.26 log CFU/g, respectively, while for lactic acid bacteria counts were below the quantification limit (≤ 2.00 log CFU/g). These results are in agreement with those reported in previous works [5, 9]

In previous works [5, 9], microbial load in AP samples increased ($p < 0.05$) during storage, reaching total aerobic mesophiles values to above the established limit (7.0 log CFU/g; [19]) after 3–10 days of storage at AP (25, 10, and 5°C). In the current work, the established limit was surpassed between the 5th and 15th days for AP/5°C, and after

**Table 1** Spoilage microbiota evolution and pH of Atlantic salmon loins stored at 60 MPa/10°C, 75 MPa/25°C, and atmospheric pressure (AP) at 5°C during 30 days; at AP/10°C during 15 days; and at AP/25°C during 5 days. For total aerobic mesophiles, anaerobic bacteria, Enterobacteriaceae, lactic acid bacteria, and $H_2S$-producing bacteria, values of 2.00 and 1.00 log CFU/mL correspond to the limit de quantification and detection, respectively (for *Pseudomonas* spp., correspond to 3.00 and 2.00 log CFU/mL, respectively). Different letters denote significant differences ($p < 0.05$) between storage conditions and storage time (a–c)

| Storage conditions | Storage time | Total aerobic mesophiles | Anaerobic bacteria | Enterobacteriaceae | Lactic acid bacteria | *Pseudomonas* spp. | H2S-producing bacteria | pH |
|---|---|---|---|---|---|---|---|---|
| Fresh fish | 0 days | 3.37 ± 0.34d | 2.05 ± 0.08e | 2.12 ± 0.13 cd | 2.00d | 3.54 ± 0.28d | 2.15 ± 0.26d | 6.13 ± 0.06abc |
| 60 MPa/10°C | 5 days | 2.49 ± 0.05e | 2.00e | 2.00 cd | 2.00d | 2.00e | 1.00e | 6.13 ± 0.02abc |
| | 15 days | 2.35 ± 0.20e | 2.18 ± 0.17e | 2.00d | 1.00e | 2.00e | 2.00d | 6.18 ± 0.02abc |
| | 30 days | 2.15 ± 0.14e | 1.00f | 1.00d | 1.00e | 2.00e | 1.00e | 6.19 ± 0.01abc |
| 75 MPa/25°C | 5 days | 2.16 ± 0.28e | 2.00e | 1.00d | 1.00e | 3.00d | 1.00e | 6.19 ± 0.01abc |
| | 15 days | 2.03 ± 0.05e | 1.00f | 1.00d | 1.00e | 2.00e | 1.00e | 6.27 ± 0.06a |
| | 30 days | 1.00f | 1.00f | 1.00d | 1.00e | 2.00e | 1.00e | 6.24 ± 0.02ab |
| AP/5°C | 5 days | 5.18 ± 0.31c | 3.94 ± 0.18d | 2.46 ± 0.33c | 2.00d | 4.88 ± 0.31c | 4.25 ± 0.07c | 6.15 ± 0.03abc |
| | 15 days | 7.06 ± 0.20b | 4.69 ± 0.41c | 2.08 ± 0.13 cd | 4.19 ± 0.50c | 5.00 ± 0.01c | 4.18 ± 0.32c | 5.91 ± 0.02ad |
| | 30 days | 7.11 ± 0.12b | 6.69 ± 0.41b | 4.08 ± 0.13b | 6.18 ± 0.47b | 6.00 ± 0.01b | 6.17 ± 0.33b | 6.12 ± 0.08bc |
| AP/10°C | 5 days | 7.20 ± 0.05b | 7.20 ± 0.28b | 3.99 ± 0.52b | 4.07 ± 0.08c | 5.00 ± 0.01c | 4.00 ± 0.01c | 5.86 ± 0.02e |
| | 15 days | 8.04 ± 0.28a | 8.26 ± 0.12a | 5.00 ± 1.40b | 7.88 ± 0.38a | 5.49 ± 0.11bc | 7.00 ± 0.01a | 6.18 ± 0.12abc |
| AP/25°C | 5 days | 8.48a | 8.48a | 8.16 ± 0.25a | 7.54 ± 0.30a | 7.61 ± 0.63a | 6.48 ± 0.36ab | 6.05 ± 0.04 cd |

5 days for AP/10 and 25°C, with total aerobic mesophiles counts of 7.06 ± 0.20, 7.20 ± 0.05 and ≥ 8.48 log CFU/g, respectively.

Both HS samples showed during storage microbial inhibition and inactivation during storage. HS/LT at 60 MPa/10°C reduced counts ($p < 0.05$) to 2.49 ± 0.05 log CFU/g of total aerobic mesophiles counts after 5 days (which corresponds to a reduction of about 0.9 log units) and retained in similar values ($p > 0.05$) after 30 days (2.15 ± 0.14 log CFU/g), while for HS/RT condition of 75 MPa/25°C, a more pronounced total aerobic mesophiles load reduction ($p < 0.05$) was observed to a value of 2.16 ± 0.28 log CFU/g after 5 days (a reduction of about 1.2 log units), decreasing thereafter to below the detection limit (≤ 1.00 log CFU/g) after 30 days of storage (a reduction of about 2.4 log units). For other microorganisms (anaerobic bacteria, Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp., and $H_2S$-producing bacteria) count values below detection limit after the 30 days of storage. The behaviour of the spoilage microbiota was thus similar to that observed in previous studies [5, 9], but in previous work, salmon samples were packaged in the presence of air, contrary to the current work, where samples were vacuum-packaged.

Initial pH of salmon loins was 6.13 ± 0.06, in agreement with values reported in previous work [5, 9]. After 15 days of storage at AP/5°C, pH decreased ($p < 0.05$) to a value of 5.91 ± 0.02, increasing ($p < 0.05$) thereafter to 6.12 ± 0.08; at AP/10°C, with a similar decrement being observed ($p < 0.05$) after 5 days of storage. In previous works [5, 9], a similar behaviour was obtained during long storage at AP/5°C, which was attributed to the accumulation of acid lactic from the glycolysis process [20]. Our results indicated an increase of fermentative acid lactic bacteria along storage, being this a possible reason for the pH reduction. However, after 30 and 15 days at AP/5°C and AP/10°C, respectively, the pH increased ($p < 0.05$) to similar values to those obtained in the fresh salmon. The increase of pH could be assigned to the production of alkaline bacterial metabolites spoilage process during storage [20]. The spoilage rate verified by the alkaline metabolites production increases with the increase of storage temperature, being observed that at AP/25°C, no pH variations were found after 5 days of storage. Under both HS conditions, pH remained stable ($p > 0.05$) along storage, ranging between 6.13–6.19 and 6.19–6.24 for 60 MPa/10°C and 75 MPa/25°C, respectively.

Recently, a study focusing on physical properties, lipid stability, and volatiles profile of vacuum-packaged fresh Atlantic salmon stored under the same condition of 60 MPa/10°C was also performed [10]. No variations in drip loss and water holding capacity were observed for HS/LT samples, as well as lower changes on muscle fibres, visible by scanning electron micrographs, and a decrease of resilience property (only after 30 days), when salmon samples were compared with those stored at AP [10]. In addition, HS/LT showed a polyene index similar to fresh samples during the 30 days of storage, confirmed by the lower lipid oxidation state of these samples, compared with AP storage [10]. Moreover, volatile profile of HS/LT samples showed to be more similar to the fresh ones, retaining fresh-like alcohols and aldehydes, generally not detected in AP samples after 15 days, with the latter presenting spoilage-related compounds probably derived from microbial activity [10], which are strongly correlated to the microbial results obtained in the current work.

## Effect of Hyperbaric Storage on *Bacillus subtilis* Endospores

Figure 1 shows the behaviour of *B. subtilis* endospores during storage of salmon samples inoculated with *B. subtilis* endospores under different pressure/temperature conditions. The endogenous microbial load of bacteria and endospores (named endogenous total load) was also determined for control uninoculated salmon samples. No endogenous *B. subtilis* endospores were detected during all storage experiments.

In addition, endogenous total load decreased ($p < 0.05$) for HS samples, 60 MPa/10°C and 75 MPa/25°C, from an initial value of 3.65 ± 0.09 to 2.74 ± 0.19 and ≤ 1.30 log CFU/g after 30 days of storage.

After inoculation with *B. subtilis* endospores, initial salmon samples showed an initial load of 5.02 ± 0.18 log CFU/g, which correspond to the sum of endogenous and inoculated *B. subtilis* endospores (EI_total load). Endospores load were obtained by heating salmon samples (at 80°C for 20 min) to kill the vegetative forms of *B. subtilis* (obtaining only endospores—I_endospores), resulting in an initial value of 4.37 ± 0.22 log CFU/g.

For both HS samples, EI_total load decreased ($p < 0.05$) during storage time. HS/LT at 60 MPa/10°C showed a slower decrease than HS/RT at 75 MPa/25°C. After 5 days, a reduction of about 1.1 and 2.5 log units was obtained for 60 MPa/10 °C and 75 MPa/25°C, respectively, yielding values of 2.72 ± 0.27 and ≤ 2.30 log CFU/g, respectively, after 30 days of storage. Contrarily, AP samples showed an increase along storage time, showing values of 8.43 ± 0.17 log CFU/g after 15 days at AP/25°C, and 7.31 ± 0.09 and 6.47 ± 0.15 log CFU/g after 30 days at AP/10 and 5°C, respectively.

Regarding I_endospores, HS conditions caused an inactivation of *B. subtilis* endospores to values below the detection limit (≤ 1.30 log CFU/g) after 30 days of storage. This effect may be attributed to a combined effect of hydrostatic pressure and the food matrix nutrients that trigger the endospore germination pathways but, due to the pressure hurdle, the endospores could not undergo the outgrowth process, thus being inactivated [12]. On the other hand, I_endospore counts

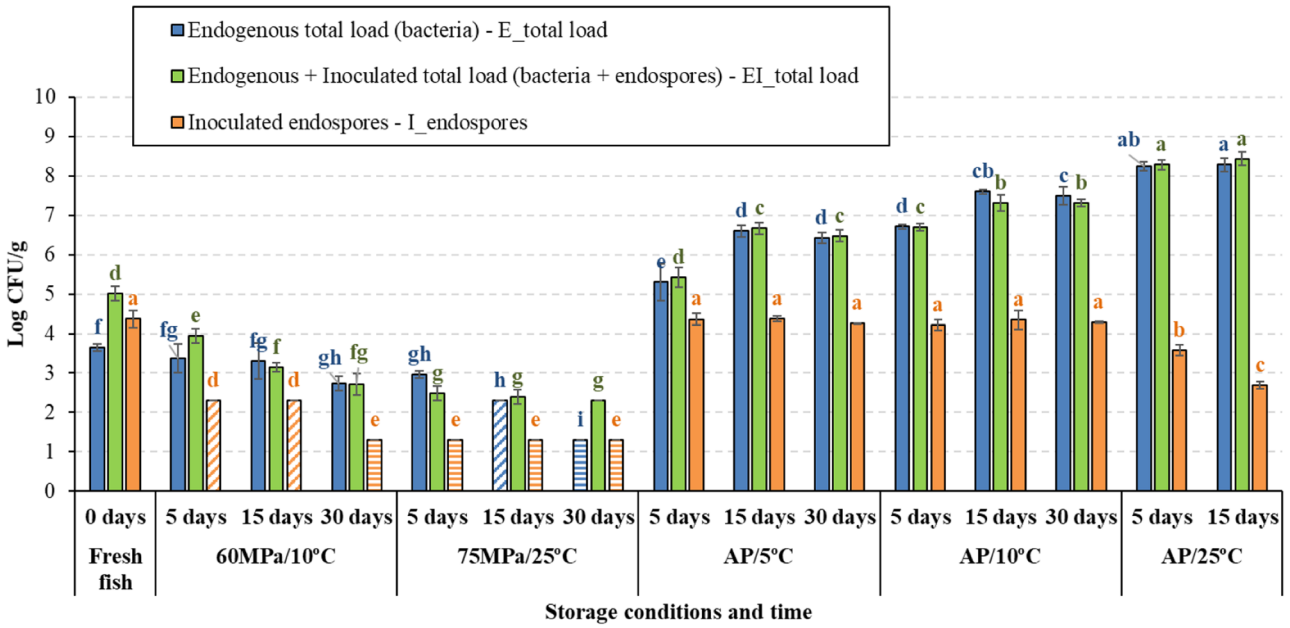


**Fig. 1** *Bacillus subtilis* count evolution in Atlantic salmon kept under different storage conditions: hyperbaric storage at low temperature (HS/LT, 60 MPa/10°C); hyperbaric storage at room temperature (HS/RT, 75 MPa/25°C); atmospheric pressure and same temperatures (AP/10°C and AP/25°C) and refrigeration (AP/5°C). There were no detected *B. subtilis* endospores in non-inoculated samples, so endogenous total load (E_total load) only corresponds to vegetative forms (bacteria). Empty bars with diagonal and horizontal dashes mean that the values are below the quantification limit (≤ 2.30 log CFU/mL) and the detection limit (≤ 1.30 log CFU/mL), respectively. Different letters (a–f) indicate significant differences ($p < 0.05$) between different storage conditions/storage times

were maintained in similar values ($p > 0.05$) for samples stored at AP/5 and 10°C, while for AP/25°C, there was a decrease ($p < 0.05$) of I_endospore count to 2.69 ± 0.08 log CFU/g. According to Pinto et al. [12], inoculated *B. subtilis* endospores in carrot juice and BHI-broth (nutrient-rich matrices) showed similar behaviour to those obtained in the present work for salmon samples, attributing this behaviour to germination and outgrowth of the endospores to vegetative forms, increasing total microbial load (vegetative and endospores forms), and decreasing endospores counts (after heat-treatment of 80°C for 20 min).

Sterilization, which involves high temperatures combined with long processing times, is used for inactivation of spore-forming bacteria and bacterial endospores [21]. However, this process results in reduction of food product quality. High-pressure processing (1200 MPa for 14 h) showed to be not enough to inactivate *B. subtilis* endospores [22], being the combination of pressure (≥ 500 MPa during short holding times) and temperature (60–90°C) efficient [21]. Moreover, application of temperature in muscle food products, as the case of fish, caused changes in sensorial and quality properties, becoming less appreciated by consumers. So, HS seemed to be an efficient method to improve fresh fish safety. In the current work, HS performed at 60 MPa/10°C and 75 MPa/25°C for 30 days inactivated *B. subtilis* endospores and thus eliminating a potential microbial safety risk fresh Atlantic salmon. These results agree to those obtained previously by Pinto et al. [12], who observed that HS/RT (50 and 100 MPa at RT: 18–21°C) for 60 days also caused *B. subtilis* endospores reductions (inoculated in three different matrices: McIlvaine buffer, carrot juice and BHI-broth) along storage.

## Effect of Hyperbaric Storage on *E. coli* and *L. innocua*

In order to evaluate the effect of HS in gram-negative and gram-positive surrogate-pathogenic microorganisms, non-pathogenic *E. coli* ATCC 25,922 and *L. innocua* ATCC 33,090 were inoculated in fresh Atlantic salmon. Figure 2 shows the behaviour of *E. coli* and *L. innocua* counts in inoculated salmon samples stored under the different conditions. Control microbial analyses of these microorganisms were also performed in non-inoculated salmon samples not only to infer possible endogenous *E. coli* or *L. innocua* in fresh Atlantic salmon but also their behaviour along the storage experiments.

In inoculated salmon samples, the initial *E. coli* counts were 4.40 ± 0.19 log CFU/g (Fig. 2a). After 5 days at AP/25°C, microbial load increased ($p < 0.05$) for a value of 8.78 ± 0.05 log CFU/g, keeping ($p > 0.05$) in similar values after 15 days (8.69 ± 0.01 log CFU/g). According to Adoga et al. [23], *E. coli* is one of principal spoilage organisms

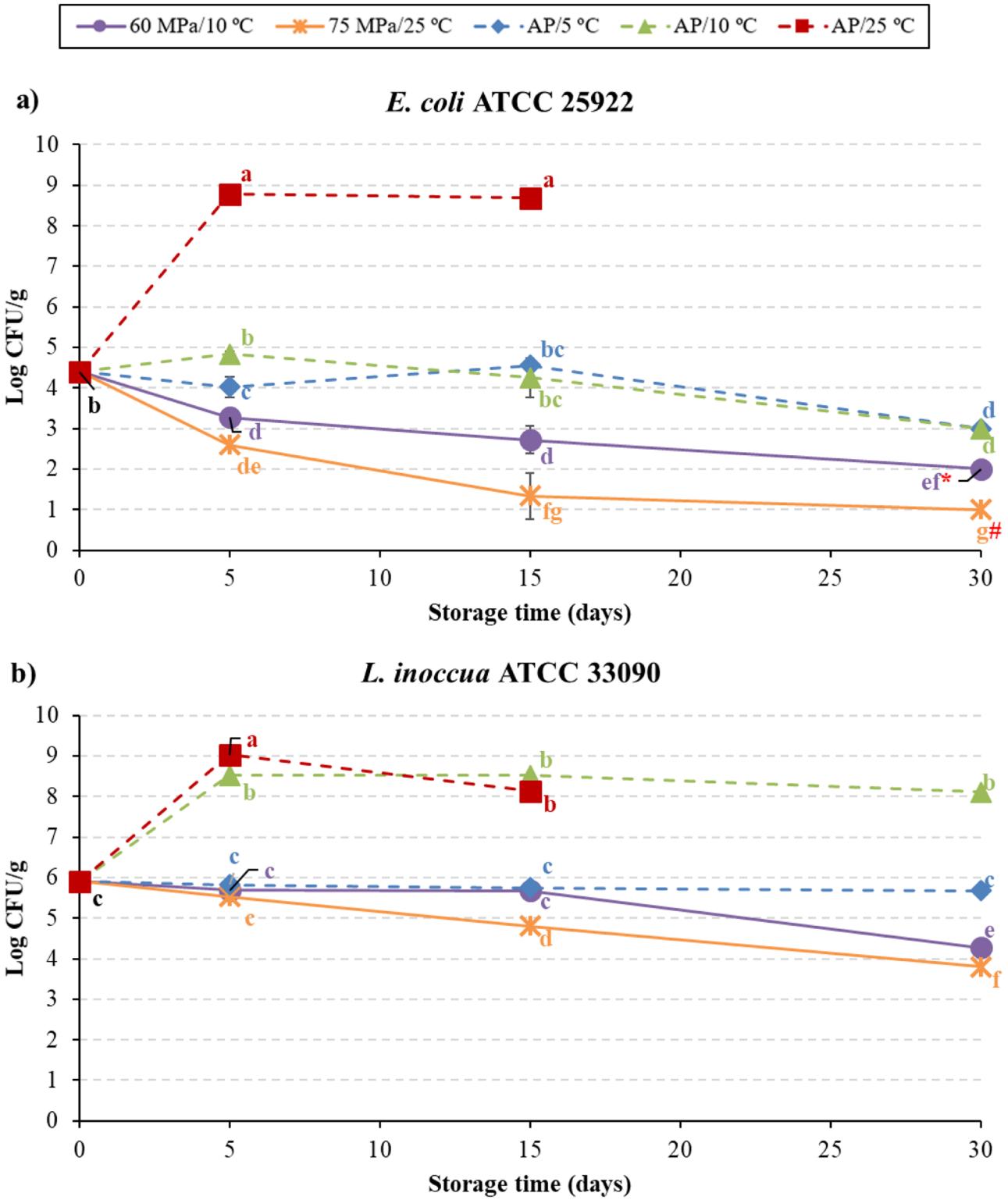


**Fig. 2** *Escherichia coli* (**a**) and *Listeria innocua* (**b**) count evolution in Atlantic salmon kept under different storage conditions: hyperbaric storage at low temperature (HS/LT, 60 MPa/10°C); hyperbaric storage at room temperature (HS/RT, 75 MPa/25°C); atmospheric pressure and same temperatures (AP/10°C and AP/25°C) and refrigeration (AP/5°C). Red symbols of * and # mean that the values are below the quantification limit (≤ 2.00 log CFU/mL) and the detection limit (≤ 1.00 log CFU/mL), respectively. Different letters (a–f) indicate significant differences ($p < 0.05$) between different storage conditions/storage times

during a short storage at 30–31°C during 12 h, which agrees with results obtained in the present work at AP/25°C. At AP/10 and 5°C, *E. coli* seemed to be unaffected, maintaining its loads in similar values ($p > 0.05$) during 15 days (4.25 ± 0.49 and 4.55 ± 0.05 log CFU/g, respectively), and decreasing ($p < 0.05$) to a value of 3.00 ± 0.01 log CFU/g in both conditions after 30 days. Samples stored under these both conditions revealed an increase of lactic acid bacteria counts and, according to Aymerich et al. [24], some strains of lactic acid bacteria are antagonistic against many microorganisms, including spoilage and pathogenic bacteria, as it is the case of *E. coli*, because they produce bacteriocins, which may explain the observed *E. coli* reduction in the same samples. Contrarily, under HS at 60 MPa/10°C and 75 MPa/25°C, *E. coli* counts decreased during storage time, resulting in values below limits of quantification (≤ 2.00 log CFU/g) and detection (≤ 1.00 log CFU/g), respectively.

Regarding *L. innocua*, the initial counts were 5.91 ± 0.11 log CFU/g (Fig. 2b). For inoculated samples stored at AP/25 and 10°C, there was a pronounced increase ($p < 0.05$) of microbial load along storage, resulting in counts of 8.13 ± 0.20 and 8.11 ± 0.13 log CFU/g, after 15 and 30 days, respectively. Additionally, at AP/25°C, there was a slight decrease of about 10% from the 5th to the 15th day, which can be associated to a possible lower availability of nutrients, when bacteria increased to high population densities, which might have led to microbial competitions [25]. Storage at AP/5°C showed no changes ($p > 0.05$) during the 30 days of storage (with counts between 5.75 and 5.83 log CFU/g). Bacteriocin-producing lactic acid bacteria has the ability to grow and produce bacteriocins at low temperatures [26], being probably the reason to the inhibition of *L. innocua* growth in samples stored at AP/5ºC. HS/LT and HS/RT showed a slow decrease of *L. innocua* during storage, which was only verified after 30 days at 60 MPa/10°C and after 15 days at 75 MPa/25°C (4.27 ± 0.05 and 4.80 ± 0.01 log CFU/g, respectively). After 30 days, these samples presented *L. innocua* counts of 4.27 ± 0.05 and 3.80 ± 0.34 log CFU/g (60 MPa/10 °C and 75 MPa/25°C, respectively).

In the present study, *E. coli* did not grow in vacuum packaged salmon samples stored at AP/10 and 5°C, as well as, in samples stored under both HS conditions. In addition, HS/LT and HS/RT showed reductions in *E. coli* counts. For *L. innocua*, a similar behaviour was observed, HS allowed to decrease initial microbial population, showing that besides the shelf-life extension, it also ensured microbiological safety. *L innocua* seemed to be more pressure-resistant than *E. coli* (reductions of about 2.4–3.4 and 1.6–2.1 log units, respectively, at 60 MPa/10 °C -75 MPa/25°C, respectively). This behaviour might be related with the fact that gram-positive bacteria (as *L. innocua*) are more pressure-resistant than gram-negative bacteria (as *E. coli*) due to the presence of a peptidoglycan layer on gram-positive bacteria [27]. These results are in agreement with those reported by Pinto et al. [11], in inoculated watermelon juice with *E. coli* and *L. innocua* and stored at 75 and 100 MPa (at RT: 18–23°C) during 10 days.

## Conclusions

Both HS conditions at LT (60 MPa/10°C) and at RT (75 MPa/25°C) allowed a shelf-life extension of vacuum-packaged Atlantic salmon up to 30 days, the maximum storage time used in this study. These two conditions showed an inhibition and additional inactivation of initial spoilage microbial load after 30 days, being the effect more pronounced at HS/RT than at HS/LT, resulting in a decrease of the total aerobic mesophiles to values below the detection limit (≤ 1.00 log CFU/g) at HS/RT. For the other microorganisms, a reduction to values below the detection limit after the 30 days of storage for both HS conditions was also observed.

HS at 60 MPa/10°C and 75 MPa/25°C showed to be effective methods to control spoilage and some surrogate-pathogenic microorganisms in Atlantic salmon during 30 days of storage. Both HS conditions inhibited and reduced initial spoilage microbial load, being observed a more pronounced effect at HS/RT as compared with HS/LT. Additionally, both HS conditions also caused an inactivation of *B. subtilis* endospores to values below detection limit after 30 days of storage. Furthermore, HS/LT and HS/RT allowed to obtain a reduction of initial *E. coli* and *L. innocua* counts, being observed that the later seemed to be more pressure-resistant than the former. This behaviour might be related with the fact that gram-positive bacteria (as *L. innocua*) are more pressure-resistant than gram-negative bacteria (as *E. coli*) due to the presence of a peptidoglycan layer on gram-positive bacteria.

These results showed the great potential of HS for preservation of low acidity/high $a_W$ foods, as the case of fresh fish, whose shelf-life is very limited. This opens the possibility for considerable shelf-life extensions of fresh fish with also an enhancement in microbial safety. Additionally, HS at RT has shown an important advantage of a possible energy savings, when compared with conventional refrigeration, since energy is only required for compression and decompression of the pressure vessel, and not during storage.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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