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Abstract

High hydrostatic pressure (HHP; 200 MPa for 15 min), in combination with liquid smoke and freezing was used to eliminate *Listeria monocytogenes* in BHI broth, raw and smoked trout. The bactericidal effect of liquid smoke (L9 and G6) solutions, HHP and their combinations were evaluated against *L. monocytogenes* LO28, EGD-e and 10403S and further continued with the most resistant strain (10403S) to the combined treatment. For first time, a synergistic effect of liquid smoke and HHP was observed, further enhanced by freezing at −80 °C prior to HHP. The effect of HHP and liquid smoke on *L. monocytogenes* 10403S, prior to freezing was highest in BHI compared to raw and smoked trout. However, major synergistic effect of HHP, liquid smoke and freezing was observed, reaching a 5.48 log CFU/g reduction when smoked trout was used. High levels of injury also occurred, among the treatments on *L. monocytogenes* 10403S.

Industrial relevance

HHP is a novel, non-thermal technique that emerged in food protection within the last three decades and further knowledge needs to be generated. This paper illustrates for first time, the possibility of using a very low pressure in combination with liquid smoke and freezing to eliminate *L. monocytogenes*. It was demonstrated that treatment of trout samples with liquid smoke followed by freezing prior to pressurization at 200 MPa for 15 min reduced the number of *L. monocytogenes* by more than 5 log CFU/g. Such a remarkable bacterial inactivation at a very low pressure (compared to common industrial practices) is a significant achievement that could allow production of safer and novel products by HHP at an affordable price, as the cost of equipment manufacture

as well as the maintenance and running costs could be reduced substantially at lower operation pressures.

Keywords: High Hydrostatic Pressure, *Listeria monocytogenes*, freezing, liquid smoke, sub-lethal injury, rainbow trout

1. Introduction

Fish and seafood are much appreciated because of their high nutritional value but are perishable and represent a high microbiological risk for the consumer (Novotny et al., 2004). According to the Food and Agriculture Organization (FAO, 2018), live, fresh or chilled seafood represent 45 % of the world's human consumption, while cured (dried, salted, in brine, fermented smoked) seafood represent 12 %.

To extend the shelf life and maintain the quality of smoked fish, vacuum packaging is normally used, but to ensure food safety and to guarantee high product quality, strict temperature control of refrigeration storage is required. *Listeria monocytogenes* is a serious concern for consumer safety in ready-to-eat (RTE) products (Beaufort, 2011), because of the capability to survive under various conditions and even grow under refrigeration temperatures. The direct contamination of food processing equipment and subsequently foods with *L. monocytogenes* is easy as it is ubiquitous in nature and can result in outbreaks of foodborne disease called listeriosis, a rare but severe illness with a high mortality rate (>30%; EFSA, 2015).

Although a broad range of preservation techniques are used to preserve seafood and control the growth of foodborne pathogens (Neil, 2012; Boziaris, 2014), microbial spoilage and food contamination remains a problem that needs to be controlled effectively (Khan et al., 2017). Furthermore, novel food processing methods, such as

High Hydrostatic Pressure (HHP) offer solutions that can implement food safety and prolong the shelf life of fish (Erkan et al., 2010a) and RTE seafood products (Georget et al., 2015; Syed et al., 2016; Possas et al., 2017).

Nowadays, consumers desire minimally processed food products with less additives, but with improved safety and shelf-life (Spence, 2006; Sun-Waterhouse et al., 2014; Moses et al., 2014). HHP can provide food products with high retention of nutritional quality and minimal effects on the organoleptic characteristics (Rastogi & Knorr, 2013). During the last decades the use of this technique has been extended to various types of foodstuff, including fish and seafood (Elamin et al., 2015). Furthermore, several products processed with HHP are commercially available in regions of North America, Europe, Oceania and Asia. In seafood products, HHP is being used as a non-thermal technology for inactivation of bacterial pathogens and for reducing the spoilage microorganisms (Koutchma, 2014). One of the major disadvantages of HHP is the high cost of the initial investment which combined with the limited product output as a batch process, which results in limited application in the food industry. Therefore, the majority of the scientific work in HHP aims at the reduction of the applied pressure which could translate into lower wear of the equipment or shorter processing times resulting in higher product output and lower prices. This is normally achieved by following the principles of the "Hurdle Technology", as defined by Leistner (1992).

Freezing is an excellent preservation method to delay or prevent microbial growth and preserve the organoleptic characteristics of fresh products (Olivera & Salvadori, 2009; Leygonie et al., 2012). Rapid freezing methods are generally preferred to avoid the formation of large ice crystals, which can cause mechanical damage and downgrade the product's appearance (Zatirsky, 2011). HHP application has the potential to improve the kinetics of freezing and the characteristics of ice crystals formed in the product

(Sanz & Otero, 2014). Phase transitions of ice crystals during the pressurization of frozen food have been previously suggested to take place during the HHP treatment that results in cellular damage and increased microbial inactivation (Luscher et al., 2004; Shen et al., 2005; Su et al., 2014a). Several studies have proposed that the application of HHP on frozen products at low or subzero temperatures and atmospheric conditions can enhance the microbial inactivation and thus allow the usage of lower pressure treatments (Knorr et al., 1998; Fernández et al., 2007; Vaudagna et al., 2012; Bulut, 2014a; Bulut, 2014b; Albertos, 2016; Parlapani et al., 2019). The effectiveness of the combined HHP and freezing treatment depends on the characteristics of the product (pH, a_w, composition, etc.), on the type of the tested microorganism and on the process conditions (pressure level, holding time and temperature; Sanz & Otero, 2014; Choi, 2008; Park et al., 2008).

The application of liquid smoke condensates is lately used on meat, fish and poultry as an alternative modern technique for smoking of foods, which requires less time than traditional smoking, produces less wastes and polycyclic aromatic hydrocarbons (PAHs) to the environment or the smoked products (Hattula et al. 2001; EFSA, 2007; Lingbeck et., 2014). Smoked food products contain PAHs, but many researchers have demonstrated that using smoke flavorings instead of traditional smoking procedure led to no detectable carcinogenic PAHs or to high reduction of their concentration in the final product (Gomaa et al., 1993; Muratore et al., 2007; Dimitriadou et al., 2008; McDonald, 2015). Liquid smoke condensates are produced by the wood smoke generated from sawdust or untreated wood, by the controlled process of minimal oxygen pyrolysis (IOFI, 2012). The main compounds in liquid smoke are phenols, acids and carbonyls. In particular, phenolic compounds are responsible for the characteristic

aroma and flavor of a smoked product and the carbonyls can also give a sweet aroma and color by browning reaction (Theobald et al., 2012; Fasano et al., 2016).

It is known that phenols can damage the bacterial membrane while carbonyl compounds can penetrate the cell wall and inactivate enzymes of the cytoplasm and the cytoplasmatic membrane (Lingbeck et., 2014). There are numerous studies indicating that liquid smoke showed inhibitory effects against foodborne pathogens such as *L. monocytogenes*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Escherichia coli* etc. in RTE products (Montazeri et al., 2013; Suñen et al., 2001; Van Loo et al., 2012). Yousef and Courtney (2002), proposed that cell membrane damage, leads to their microbial death after HHP treatment. This can affect the cell membrane permeability, disrupt the protein structure and function, make the pathogen more vulnerable under the presence of antimicrobial agents, such as carbonyls or phenols, contained into liquid smoke condensates.

Smoked fish are normally RTE foods and HHP treatment could increase the product quality and further reduce the microbial load. Furthermore, it is possible to introduce a freezing stage prior to HHP treatment and reduce the microbial load even further, possibly reduce the pressurization times and still maintain a high-quality product (Vaudagna et al., 2012; Bulut, 2014a). To the best of the authors' knowledge, this is the first time that the application of HHP was investigated on a frozen product treated with liquid smoke against *L. monocytogenes*, a pathogen that has been previously associated with smoked fish.

2. Materials & methods

2.1. Bacterial cultures

Three different commonly used reference strains of *L. monocytogenes* (LO28, EGD-e and 10403S) were used in this study. LO28 is a serotype 1/2c, while EGD-e and 10403S both belong to serotype 1/2a. All bacterial strains were kept in cryovials supplemented with 7.0 % (vol/vol) dimethyl sulfoxide (DMSO; Sigma-Aldrich, Dorset, UK) at −80 ^{o}C. Before experiments, strains were streaked onto Brain Heart Infusion (BHI) agar (LABM, Lancashire UK) and incubated overnight at 37 ^{o}C. A single colony from each plate was transferred with a sterile loop in 3 mL of BHI (LAB M, Lancashire UK) and incubated overnight at 37 ^{o}C without shaking. To prepare the final working cultures, 20 mL sterile BHI were inoculated with the overnight culture and left for incubation at 37 ^{o}C for 24 h without shaking. Cultures from stationary phase were harvested by centrifugation (Heraeus™ Multifuge™ X 3 Centrifuge, Thermo Scientific, UK) at 9000 ×g for 5 min (room temperature), washed twice using sterile Phosphate-buffered saline (PBS pH 7.1; Oxoid, Basingstoke, UK) and re-suspended in the same medium. Further serial decimal dilutions were prepared for direct plating on BHI agar. The plates were incubated for 24 h at 37°C and viable cell number was expressed as log CFU/mL.

2.2. Liquid smoke extracts preparation

All liquid smoke fractions were provided by "Ruitenberg Ingredients B.V. (Twello, the Netherlands). The liquid smoke condensates coded as L9 and G6 were chosen as the most effective following preliminary experiments. L9 is a coloring product mainly used upon heating, containing about 350 g/l carbonyls and 1 g/l phenols, while G6 is a true smoke condensate fraction with high carbonyl content (160 g/l) and also lower phenol compounds (0.0 – 2.0 g/l). According to specifications, both extracts may contain a maximum content of 10 ppb (0.01 mg/kg) benzo[a]pyrene and 20 ppb (0.02 mg/kg)

benzo[a]anthracene (BaP), are derived from non-genetically modified organisms (non-GMO) and Generally Regarded as Safe (GRAS) by FDA and are also USDA approved. The working stock solutions of liquid smoke condensates were prepared with filtration through a 0.22 μm pore size membrane filter (Durapore, Millipore) directly to sterilize and finally to store at 2 °C.

2.3. Resistance of L. monocytogenes strains to liquid smoke fractions

Harvested cells were suspended into sterile Universal Polystyrene Containers (Sterilin, UK) with 10 mL liquid smoke solutions in BHI broth of final concentrations ranging from 0.25 % to 1.00 % (v/v) in 0.25 increments. The initial population was fixed at 10^9 – 10^{10} CFU/mL for each strain individually. Subsequently, the samples were left for incubation at 37 °C. Samples of 1 mL were taken after 0, 30, 60 and 180 min. Decimal dilution of samples were prepared in Maximum Recovery Diluent (MRD), (Oxoid, Basingstoke, UK), into sterile Eppendorf tubes, and 25 μL from each dilution and also directly from the BHI broth (zero dilution) were plated onto BHI agar using the spot plate method adapted from Laubacher and Ades (2008) and incubated for 24 h at 37 °C. The limit of detection was 1.60 log CFU/mL.

2.4. Sample preparation for HHP

2.4.1. Preparation of BHI broth samples

After 24 h incubation at 37 °C, 10 mL volumes of BHI containing about 10^9 – 10^{10} CFU/mL cell population, were placed into sterile Stomacher bags (Worthing, UK). Also, sterile BHI broth containing 3.80 % (w/v) NaCl was prepared. Subsequently, all samples were heat-sealed after removing all air as described by Bulut (2014b) and finally placed individually into a second plastic bag made of polyamide/polyethylene

and vacuum packed for 30 s using a Fresco 300 Packaging Machine (SousVidetools.com).

2.4.2. Preparation of trout samples

Raw and hot smoked trout (*Oncorhynchus mykiss*) fillets obtained from a local store (Reading, UK). Samples were cut uniformly in chunks of 5 g (approx. 35 mm × 45 mm × 10 mm), sprayed on with 70 % (v/v) alcohol and placed on a rack with no contact between the samples to evaporate the excess alcohol. A 3.80 % (w/v) NaCl sterile solution was prepared and 100 μL was added on raw and hot smoked trout chunks. The samples were then inoculated with 100 μL of the *L. monocytogenes* 10403S bacterial suspension which gave a final concentration of about 10^7 CFU/g. Subsequently, 100 μL of 0.50 % L9 or G6 liquid smoke extract was added on the chunks. As a control, 100 μL sterile water was added on the fish samples. All inoculations (salt, sterile water, liquid smoke and *L. monocytogenes* inoculum) were done to the upper surface of the chunks. A sterile spatula vas used to spread the inoculums on the surface of the chunks. The samples were then heat sealed and vacuum packed. Finally, the samples were separated into two groups and half were pressure-treated while the other half, were left overnight at −80 ^{o}C inside a high efficiency upright ultra-low temperature freezer (Eppendorf HEF U570, New Brunswick, Germany) before being pressure-treated.

All samples were microbiologically analyzed in triplicate while, non-treated samples, without pressure treatment or freezing, were also analyzed.

2.5. HHP treatment set up and operation mode

All samples were pressure-treated at 200 MPa for 15 min, as one of the lowest in the food industry. A laboratory-scale high-pressure system (Stansted Fluid Power Products

Ltd., Harlow, UK) with 40 mm internal diameter vessel and 250 ml working volume was used. The pressure transmitting medium (PTM) used was 30% (v/v) aqueous solution of mono propylene glycol (Sigma-Aldrich, Dorset, UK). A cooling circulator (Grant LTD6G, UK) was used to circulate the water containing mono propylene glycol as antifreeze, in the jacket surrounding the pressure vessel to control and maintain the processing temperature measured through the PTM inside the pressure chamber. Packed samples treated in room temperature were immediately placed into the pressure chamber. Frozen samples at −80 ºC were removed from the freezer and immediately placed in the working vessel which was kept at 0-1 ºC. The time needed to remove the samples from the freezer and place them in the HHP vessel was less than 20 s suggesting that the temperature was still close to −80 ºC. The pressure come-up time was about 100 MPa/10 s, while decompression time was less than 5 s. The chamber temperature was monitored and recorded by a K-type thermocouple mounted to the center of the top closure of the pressure chamber.

Untreated inoculated BHI broth (without liquid smoke), raw trout and smoked trout, vacuum packaged samples were used as control samples, and non-treated (without HHP treatment or without freezing) were used as blank samples.

2.6. Bacterial enumeration and evaluation of injury

To determine the microbiological count in broth samples, 100 µL were removed and serial decimal dilutions were prepared in 900 µL MRD (Oxoid, Basingstoke, UK), into sterile Eppendorf tubes. For fish samples, 5 g were removed aseptically using a sterile scalpel and placed into a sterile Stomacher bag (Worthing, UK) containing 45 mL MRD. Bags were stomached for 90 s and 25 µL were removed from the appropriate serial decimal dilution and were placed onto BHI agar using the spot plate method and

incubated for 24 h – 72 h at 37 ºC. The limit of detection for all fish samples was 2.60 log CFU/g. To verify the amount of sublethal injury, samples were also placed in parallel onto the selective medium Brilliance™ Listeria Agar (OXOID, Wesel, Germany) and plates were incubated at 37 ºC for 24 h – 72 h.

To calculate the percentage of sub lethally injured cells after freezing, HHP treatment and liquid smoke extracts (L9 or G6), the following equation (Dykes, 1999; Wang et al., 2017) was used based on the viable counts' differences on non-selective ($N_{non-selective}$) and selective media ($N_{selective}$):

$$\%\text{Sublethal Injury} = \frac{N_{\text{non-selective}} - N_{\text{selective}}}{N_{\text{non-selective}}} \times 100 \quad (1)$$

2.7. Experimental design

All samples were taken in triplicate (biological replicates) while each biological replicate was analyzed with two technical replicates. Data plotting and statistical analysis by calculating mean values was performed using Excel Microsoft® Office 365 (ver. 16.18). To assess the differences between the tested treatments one-way analysis (ANOVA) was performed and Tukey post hoc test was used to compare sample data with the use of the IBM® SPSS® statistics 19 software, for Windows (SPSS Inc.,U.S.). For data that showed a normal distribution, the Student's t-test was used to determine significance. Level of significance was defined as 5.00 %, and thus values with $P < 0.05$ were considered significant.

3. Results

3.1. Effect of liquid smoke extracts on L. monocytogenes strains

277 The behavior of the different strains in BHI broth, against the two liquid smoke extracts
278 (L9 and G6) was assessed (Fig. 1). The population of *L. monocytogenes* 10403S WT,
279 LO28 WT, and EGD-e WT strains after 180 min in BHI broth without added liquid
280 smoke (control), was 9.42, 9.22 and 8.89 log CFU/mL, respectively (Fig. 1). Following
281 exposure for 180 min, in BHI broth supplemented with 1.00 % (v/v) L9 or G6, *L.*
282 *monocytogenes* 10403S WT resulted in a significant drop in bacterial numbers that
283 reached below the detection limit of 1.60 log CFU/mL corresponding to a higher than
284 7.70 log reduction of CFU/mL (Fig. 1A). Furthermore, bacterial numbers of LO28 WT
285 and EGD-e WT dropped below the detection limit, after 180 min, in BHI broth
286 supplemented with 0.75 % and 1.00 % L9 corresponding to 7.50 and 7.12 log reduction
287 of CFU/mL, respectively. In contrast, to L9 the effect of G6 1.00 % was significantly
288 ($p < 0.05$) lower showing a reduction of 3.13 and 3.15 log CFU/mL, for LO28 and
289 EGD-e, respectively (Fig. 1B & C). After 1 h the results varied among the different *L.*
290 *monocytogenes* strains while again L9 0.75 % and 1.00 % was significantly more
291 effective than G6 for the same concentrations against LO28 WT and EGD-e WT.
292 Logarithmic reduction of LO28 WT and EGD-e WT after 60 min with 0.50 % and 0.75
293 % G6 was less than 0.50 log CFU/mL, while after exposure to 0.75 % L9, a reduction
294 of 1.57 and 1.53 log CFU/mL was reached, for LO28 WT and EGD-e WT, respectively
295 (Fig. 1B & C). The population of 10403S WT after 1 h of exposure to L9 0.50 %, 0.75
296 % and 1.00 % showed a reduction of 0.86, 1.69 and 2.49 log CFU/mL, respectively.
297 Against the highest concentration of 1.00 % G6 after 1 h, the counts of 10403S WT
298 reached a reduction of 0.83 log CFU/mL (Fig. 1A).
299 Our results showed that following a 30 min exposure, the antimicrobial activity was
300 similar for all the subjected strains with no significant population reduction that was
301 not higher than 0.50 log CFU/mL (Fig. 1A, B & C).

3.2. Effect of HHP and liquid smoke extracts on L. monocytogenes strains

As our aim was to use liquid smoke and freezing as additional hurdles to reduce the intensity of an HHP treatment, further experiments were carried out with shorter exposure times of 15 min (HHP treatment time) at 0.50 % (v/v) concertation (unless otherwise stated) of liquid smokes L9 and G6. The samples that were exposed to liquid smoke (L9 or G6) for 15 min without HHP treatment resulted in < 0.20 log reductions of CFU/mL, regardless of containing NaCl or not (Fig. 2; $p > 0.05$). Pressure treatment of BHI broth samples without liquid smoke (control) at 200 MPa for 15 min resulted in minor reductions of 0.15 and 0.65 log CFU/mL for the samples with NaCl and without NaCl, respectively (Fig. 2; $p > 0.05$).

The HHP treatment in combination with L9 or G6 caused a significant population reduction of all *L. monocytogenes* strains tested. As expected, the results among the different strains varied with the highest log reduction of 4.75 log CFU/mL and 3.85 log CFU/mL achieved for *L. monocytogenes* LO28 supplemented with 0.50 % (v/v) G6 without and with NaCl, respectively (Fig. 2; $p < 0.05$). HHP treatment combined with the most effective extract G6 resulted in 2.64 and 2.97 log CFU/mL reduction in number of *L. monocytogenes* EGD-e for the samples without salt and with salt, respectively (Fig. 2; $p > 0.05$). Pressure treatment for *L. monocytogenes* 10403S in BHI supplemented with G6 resulted in log reductions of 2.56 and 1.80 CFU/mL for the samples with and without salt, respectively (Fig. 2; $p < 0.05$).

HHP treatment of BHI broth samples with L9 (no NaCl) reduced the number of *L. monocytogenes* LO28, EGD-e and 10403S by 2.10, 1.40 and 1.12 log CFU/mL, respectively (Fig. 2; $p < 0.05$). When BHI broth samples supplemented with L9 and NaCl were pressure treated under the same conditions, 2.55, 1.42 and 1.44 log CFU/mL

reductions were obtained in number of *L. monocytogenes* LO28, EGD-e and 10403S, respectively (Fig. 2).

Under the conditions tested, *L. monocytogenes* 10403S was the most resistant to the combined treatment compared to the other two strains. Therefore, further experiments were carried out by using *L. monocytogenes* strain 10403S as test microorganism.

3.3. Application of HHP and freezing at −80 °C in broth, raw trout and hot smoked trout samples.

Following the investigation of the combined effect of HHP and liquid smoke, we investigated the possibility of applying these technologies in frozen broth, raw and hot smoked trout at −80 °C as an additional hurdle to further increase the inactivation of *L. monocytogenes* 10403S. As seen in Fig. 3, the samples containing liquid smoke L9 or G6 with or without salt showed no significant differences in the absence of pressure treatment and the log reduction remained below 0.50, regardless of the samples being frozen or not. The effect of HHP without addition of liquid smoke, on *L. monocytogenes* 10403S was statistically significant ($p < 0.05$) but minimal (0.73 log CFU/mL). On the other hand, HHP combined with freezing at −80 °C, without liquid smoke, resulted in 2.35 log CFU/mL reduction (Fig. 3). Similar results were obtained for the samples, supplemented with NaCl where 3.00 log reduction increased from 0.51 log CFU/mL to 3.17 CFU/mL as a result of freezing the samples before the pressure treatment (Fig. 3; $p > 0.05$).

For the samples that were at room temperature before the pressure treatment, addition of salt to liquid smoke increased the log reduction from 1.85 to 3.07 CFU/mL for L9 and from 2.53 to 3.07 CFU/mL for G6 (Fig. 3; $p < 0.05$). However, when the pressure-treated samples with or without NaCl, before freezing supplemented with 0.50 % (v/v)

G6, a reduction of 3.07 and 2.53 log CFU/mL, was obtained (Fig. 3; $p > 0.05$). Addition of salt to the samples containing liquid smoke which were then frozen at −80 °C before pressure treatment caused a slight decrease in log reductions for L9 (from 3.23 to 2.94 log CFU/mL) and made no significant difference for G6 (from 2.94 to 2.90 log CFU/mL).

The application of HHP treatment in combination with smoke on raw rainbow trout chunks resulted in a limited reduction in number of *L. monocytogenes* 10403S in both the presence and absence of salt. As seen in Fig. 4, maximum log reduction remained below 0.42 log CFU/g at all conditions tested for the samples that were at room temperature before the pressure treatment. HHP treatment of raw rainbow trout chunks frozen at −80 ºC before the pressure treatment resulted in significant reduction in number of *L. monocytogenes* 10403S for all the treatments (Fig. 4). HHP treatment of frozen raw rainbow trout chunks without liquid smoke, containing or not salt, reduced the population of *L. monocytogenes* 10403S by 1.60 and 1.37 log CFU/g, respectively (Fig. 4; $p > 0.05$). In the absence of salt, treatment of samples with L9 and G6 and then freezing before the pressure treatment increased the bacterial death to 1.93 and 1.80 log (CFU/g), respectively (Fig. 4). In the presence of salt, pressure treatment of frozen samples with L9 and G6 resulted in 1.54 and 1.68 log (CFU/g) reductions, respectively (Fig. 4; $p > 0.05$).

Subsequently, we assessed the effect of HHP treatment in combination with liquid smoke on *L. monocytogenes* 10403S inoculated on hot smoked trout. In the absence of freezing, pressure treatment of inoculated hot smoked trout samples resulted in only 0.25 log reduction of CFU/g (Fig. 5). Furthermore, the addition of liquid smoke on the surface of the fish without freezing, after HHP, also resulted in a minimal effect of 0.29 and 0.26 log CFU/g for L9 and G6, respectively (Fig. 5; $p > 0.05$). In addition, also

freezing at −80 ºC by itself resulted in no reduction of CFU/g (data not shown). HHP treatment of inoculated hot smoked trout chunks (with no liquid smoke) after they were frozen at −80 ºC did not cause a significant reduction (0.46 log CFU/g) in the numbers of *L. monocytogenes* 10403S. However, the same samples, upon supplementation with 0.50 % (v/v) L9 or G6, resulted in log reduction of CFU/g that was beyond 5.48 logs (n=6, Fig. 5; $p < 0.05$).

3.4. Sublethal injury of L. monocytogenes 10403S WT *strain*

The percentage of sub lethally injured cells in BHI broth and on raw and smoked trout chunks, without freezing, was quite high for the samples containing L9 or G6 without added NaCl. More specifically, when G6 combined with HHP, prior freezing, caused the highest level of injury on bacterial cells, while freezing did not cause any significant changes. The percentage of injury after HHP and without freezing at −80 ºC for BHI broth and raw trout chunks was 51.49 % ± 19.82 % and 32.79 % ± 10.11 %, respectively showing no significant differences among the treatments, prior freezing ($p > 0.05$; Table 1).

It is noteworthy to highlight that freezing at −80 ºC in combination with HHP, caused high injury levels. The maximum level of bacterial injury effect observed for pressure-treated cells in BHI broth. The injury for BHI broth samples without and with salt, after freezing, without liquid smoke additives was significantly different in comparison with the samples without freezing (20 ºC) and equal with 55.98 % ± 8.64 % and 55.23 % ± 3.93 % (Table 1; $p < 0.05$). Pressure-treated BHI broth samples with freezing and L9 or G6 without NaCl content, compared with the same samples prior freezing, showed no significant differences (Table 1; $p > 0.05$). Though the percentage of injury for BHI broth, with NaCl and L9, before freezing was 15.12 % ± 4.81 %, while after freezing

was increased significantly and was equal with 55.86 % ± 1.24 % (Table 1; $p < 0.05$). Table 1 also illustrates that G6 addition in BHI broth caused significant changes to the percentage of sub lethal injury of *L. monocytogenes* for the samples with or without salt.

The *L. monocytogenes* cells inoculated on the surface of raw trout chunks, also revealed high injury for all treated samples as shown in Table 1. For raw trout chunks prior freezing, without NaCl, significant differences observed only among the samples with G6 (32.79 % ± 10.11 %) and without liquid smoke (10.87 % ± 3.56 %). Concurrent application of freezing with HHP treatment on raw trout, caused sublethal injury of *L. monocytogenes* cells, equal with 36.11 % ± 5.58 % and 26.92 % ± 5.61 % for the samples supplemented with L9, containing or not NaCl, respectively (Table 1; $p < 0.05$). However, pressure-treated raw trout samples after freezing, containing or not salt, showed no significant differences (Table 1).

As seen in Table 1, the differences of sub lethal injury of *L. monocytogenes* cells found in smoked trout chunks without liquid smoke, submitted in freezing at −80 ^{o}C, prior to HP treatment (38.59 % ± 3.15 %) were significant comparing with the same samples stayed at 20 ^{o}C (14.31 % ± 1.23 %) (Table 1; $p < 0.05$). When the smoked trout supplemented with L9 or G6 without NaCl, prior freezing and followed by HHP treatment, no significant differences observed, while after freezing no injury on bacterial cells detected (Table 1). During the investigation of the treatments on hot smoked trout fillets, no extra NaCl added to an already salted product to better evaluate the real conditions.

4. Discussion

The current study investigates the behavior of *L. monocytogenes* strains in a liquid medium and hot smoked or raw trout upon the application of different treatments, such as HHP, freezing and smoking. In the current work, only one type of HHP treatment (200 MPa for 15 min), was used, which is one of the lowest, if not the lowest in the food industry (Koutchma, 2014; Khan et al., 2017). The first hurdle introduced in combination with HHP was liquid smoke. Two water-soluble liquid smoke condensates (L9 and G6) were used, as common preservatives for protein-based foods, such as seafood. The smoke extracts against all three strains was effective in a dose and exposure time-related pattern. Nowadays traditional smoking is primarily used to endow the typical characteristics of aroma, flavor and color to the smoked products (Theobald et al., 2012; Fasano et al., 2016) but the controlled use of liquid smoke extracts can inhibit the growth of foodborne pathogens (Lingbeck et al., 2014). There are several studies, presenting the *in vitro* antimicrobial effect of liquid smokes against foodborne pathogens, such as *Aeromonas hydrophila* (Sofos et al., 1988; Suñen et al., 2003), *Yersinia enterocolitica* (Suñen et al., 2001), pathogenic *Escherichia coli* (Schmid-Hempel & Frank, 2007; Van Loo et al., 2012) and *L. monocytogenes* (Martin et al., 2010; Pittman et al., 2012; Morey, et al., 2012). In previous work Vitt et al. (2001), tested 5 commercial liquid smoke extracts at varying concentrations to estimate the inhibitory effect on *L. innocua* and *L. monocytogenes* and found that 0.50 % liquid smoke inhibited *L. innocua* in BHI broth. This is in agreement with our work where 0.50 % liquid smoke resulted in minor but measurable reductions in the *L. monocytogenes* numbers suggesting that such a concentration would be inhibitory in our experimental setup. It is well established that great differences may occur in stress resistance between individual strains and therefore the most tolerant strains should be considered to better assess, the food safety risks posed by tolerant strains and develop

methods to control the growth and survival of the pathogen in the final product (Lunden et al., 2008; Carpentier & Cerf, 2011; Ferreira et al., 2014; Heir et al., 2018). The liquid smokes used contain high carbonyls and low phenolic compounds.

In the present work, the application of HHP at 200 MPa for 15 min on BHI broth samples (without liquid smoke) resulted in a minor log reduction of CFU/mL with no significant differences among *L. monocytogenes* strains. The presence or absence of salt in BHI broth did not have any effect, while both liquid smoke extracts enhanced the lethal effect of HHP. The liquid smoke and HHP showed a synergistic effect while the addition of salt had no major effect for most of the samples, with only exception the samples of *L. monocytogenes* LO28 and EGD-e with or without salt and G6, where the differences observed, were significantly different ($p < 0.05$; Fig. 2). This demonstrates that it is possible to enhance the antimicrobial effects of HHP with the use of smoke or vice-versa. Exposure of *L. monocytogenes* to liquid smoke (L9 or G6) in BHI broth for 30 min resulted in a minor reduction, less than 0.50 log CFU/mL (Fig. 1A, B & C). From all three strains, LO28 represents a normal strain of *L. monocytogenes*, while EGD-e is a lab strain with extreme resistance to oxidative stress and 10403S an extremely acid resistant strain which might be linked to its increased resistance to the combined HHP and liquid smoke treatment. However, the combined treatment of extremely low HHP and low levels of liquid smoke is able to bring about more than 4.00 log reduction of CFU/ml for a normal *L. monocytogenes* strain, such as LO28 and this is promising.

Subsequently, we introduced freezing prior to HHP treatment. Freezing combined with HHP showed to exert antimicrobial effects as shown previously (Bulut, 2014a; 2014b; Su et al., 2014b). The liquid smoke enhanced the antimicrobial effects of HHP and freezing, but this additional effect was not major. In contrast the impact of liquid smoke

under HHP without freezing is major. This could be possibly due to the state of liquid smoke under freezing conditions, which went through phase changes while it has freezing point lower than water (Ledford, 1981) but further work is required to investigate this. When freezing is performed properly, without multiple freezing steps, food quality is minimally affected (Oosterhuis, 1981; Evans, 2009). Redmond et al. (2003), used a liquid nitrogen cryogenic environmental chamber to freeze mashed potatoes, were the pots of potato placed at −90 ºC reached an internal temperature of -25 ºC within 24 min compared to 78 min for samples frozen at −30 ºC with similar results for fresh mashed potato.

Regarding the microbial safety, it is well established that freezing can induce high rates of injury to the bacterial population. Jiang et al. (2011), revealed that, frozen storage at −18 ºC as a pretreatment, followed by chill storage at 4 ºC in combination with surface application of antimicrobials on roasted turkey, reduced significantly the initial population of *L. monocytogenes*. Golden et al. (1988), mentioned that, after 2 weeks at −18 ºC, the injury level of *L. monocytogenes* strains, reached up to 82 %. Our results for *L. monocytogenes* after freezing, followed by HHP treatment, revealed high injury levels, for BHI broth samples (Fig. 3).

Moreover, when *L. monocytogenes* 10403S was inoculated on the surface of sterile raw trout chunks and subjected to HHP a limited lethal effect (< 0.40 log reduction of CFU/g) was observed without freezing which was significantly lower than that observed in BHI cultures. This is possibly due to constituents on the trout that might protect the cells from the combined effect of HHP. The type, composition and the matrix of the tested food seems to have an impact on the resistance of bacteria at HHP (Bucur et al., 2018). Freezing also resulted to higher injury levels for *L. monocytogenes* cells on raw trout chunks compared to those without freezing. As seen in BHI

experiments, also on these raw trout experiments we observed that liquid smoke elicited hardly any antimicrobial effect under freezing temperature for the samples without HHP (Fig. 4). However, when we added this low concentration of liquid smokes (0.50 %) on already smoked trout chunks we observed an impressive 5.48 log reduction of CFU/g following freezing at −80^{o}C and HHP (Fig. 5). In this case the increased concentration of smoke contributed the above major synergistic effect while in its absence, the observed reduction was less than 0.50 log (CFU/g) (Fig. 5). We also demonstrate that this major synergistic effect could be used to significantly decrease the HHP intensity which could translate to major reduction in economic costs.

Working with cold-smoked salmon, Vitt et al. (2001), observed that a much higher concentration than 0.50 % of liquid smoke was required to inhibit *L. innocua* or *L. monocytogenes*, possibly due to the salmon proteins affecting the activity of the inhibitory phenolic compounds. Also, Painter et al., (1998), proposed three mechanisms for the inhibitory action of the carbonyls, were Type A involving the isolation of amino acids or ammonia by condensation with the carbonyl compounds, Type B is effective against putrefactive bacteria or molds and Type C, were through chemical changes the substrate becomes less approachable and open to the microbial enzymes. Nevertheless, the exact mechanism of inactivation remains unknown and both phenols and carbonyls seem to inhibit bacteria.

Our results showed that HHP treatment (200 MPa, 15 min) or treatment with 0.50 % liquid smoke on their own could not inactivate *L. monocytogenes* in already smoked trout fillets (< 0.30 log CFU/g reduction). However, when HHP treatment was carried out after freezing, the samples that were treated with liquid smoke showed a synergistic effect indicated by an impressive 5.48 log CFU/g reduction (Fig. 5). This was more than 5.00 log reduction of CFU/g of the most tolerant *L. monocytogenes* strain, in

compliance with USFDA (2000) requirements for novel technologies. It should be stated that levels of smoke in this experiment could be close to the highest limits as we added this low concentration of 0.50 % (manufacturer's maximal dosage is 2.91 % for processed fish) to an already smoked product. We decided to add a small amount of liquid smoke to an already smoked product, to further enhance antimicrobial effects without affecting the organoleptic characteristics. Hot smoked products are frequently contaminated with *L. monocytogenes* and due to the long period of storage and the lack of antagonistic microflora (Lianou & Sofos, 2007), the pathogen can grow and multiply during storage at refrigeration temperatures, creating an increased risk of infection for the consumers (Jemmi & Keusch, 1992). In a recent study, Hokkanen et al. (2018), analyzed 80 smoked fish products using an accredited gas chromatography-tandem MS to determine benzo[*a*]pyrene and PAH4 levels and found that the mean concentrations were below the maximum levels. This confirms that the levels of smoke in our experiment could be even lower than the maximum, or if higher not significantly higher than the maximum. Visciano et al. (2008), showed that after smoking treatment of trout fillets, with traditional flue gas smoking and use of liquid smokes, the PAH concentrations did not increase.

Our experiments indicate a major synergistic effect of all three hurdles used in this work and this is highly important as it could be exploited in further work which could set the actual parameters, resulting in a process that could be used by the food industry. It should be stated here that *L. monocytogenes* is of particular concern to ready-to-eat smoked fish products (Acciari et al., 2017; Rodrigues et al., 2017). HHP lethality depends on both food composition and technological parameters and these factors can act synergistically (Bucur et al., 2018). Interestingly, by the combination of freezing and liquid smoke we managed to reduce the levels of pressure to one of the lowest used

551 in the industry which can translate into reduced costs for HHP which, however, still
552 remains expensive in comparison with other conventional methods (Elamin et al.,
553 2015).
554 It is well known that bacterial cells can be injured by stresses such as freezing (El-Kest
555 and Marth, 1991). In our experiments, after freezing followed by HHP treatment, the
556 sub lethally injured *L. monocytogenes* cells (without liquid smoke) in smoked trout
557 fillets, were doubled comparing with the samples without freezing, something that
558 could presumably explain the synergistic effect of freezing, in combination with HHP
559 (Table 1).
560 Regarding the overall safety of the product after the addition of the liquid smoke
561 extracts it is important to mention that some of the compounds maybe contained into
562 the liquid smoke flavorings such as benzo[*a*]pyrene (B*a*P), benzo[a]anthracene or other
563 PAHs are considered as potentially genotoxic and carcinogenic (Alomirah et al., 2011;
564 McDonald, 2015). Therefore, the maximum allowable concentrations need to be
565 considered for the final product. The maximum acceptable level for benzo[*a*]pyrene
566 (B*a*P) is 2 μg/kg wet weight for smoked meats, poultry and seafood established by
567 European Commission, (E.C., 2011), while for benzoates in general is 200 mg/kg for
568 smoked fish as established by the Codex Alimentarius Commission and adopted in
569 2018 (Alimentarius, C., 2015) and for PAHs is 5 μg/kg (Varlet et al., 2007). The WHO
570 in 1994, stated that phenol intake must not exceed the 100 μg/kg body weight per day,
571 while there is no available data for maximum levels of carbonyls. According to
572 specifications, both liquid smoke extracts used in this study comply with the
573 Regulations. There are numerous studies showing that during smoking with liquid
574 smoke extracts instead of traditional smoking, the PAHs are no detectable or at much
575 lower concentrations than the maximum acceptable concentration in the final product

(Gomaa et al., 1993; Muratore et al., 2007; Varlet et al., 2007; Dimitriadou et al., 2008; McDonald, 2015).

As a general observation, freezing process as a pretreatment before high pressure did not affect the color of our final product (raw and smoked trout). Other studies looked at the differences on the color of aquacultured King salmon (*Oncorhynchus tshawytscha*) fillets treated with combined liquid smoke and HHP treatment at 200 – 600 MPa (Kong et al., 2015), frozen shrimps processed at 250 MPa up to 10 min (Parlapani et al., 2019) and the effect of HHP on rainbow trout (Erkan et al., 2010b) or even smoked fish (Erkan et al., 2011). They reported that HHP did not change significantly the color of the product. This might be important since sensory appearance plays an important role for the purchase decisions by the consumers (Garber et al., 2003).

5. Conclusion

Combination of HHP treatment with natural preservatives such as liquid smoke extracts and freezing was able to reduce considerably *L. monocytogenes* population on different matrices, thus reinforcing food safety. High pressure treatment at 200 MPa for 15 min on its own caused a maximum reduction of 0.73 log CFU/mL in number of *L. monocytogenes* in BHI broth and 0.06 and 0.25 log CFU/g on raw and hot smoked trout, respectively. Freezing prior to HHP treatment enhanced the lethal effect in the presence of liquid smoke extracts. When hot smoked trout fillets were treated with liquid smoke condensates (L9 and G6) and freezing, followed by HHP, a remarkable 5.48 log CFU/g reduction was obtained, which shows a major synergistic effect with levels of liquid smoke that comply with USFDA requirements for novel technologies.

There are no references in the literature reporting such a high level of *L. monocytogenes* inactivation after a treatment at as low as 200 MPa. This high level of inactivation occurred by the addition of 0.50 % liquid smoke to trout fillets and freezing before the HHP treatment. Even though freezing at very low temperatures, requires an additional investment, products need to be kept there for just a few hours. In addition, lower costs could be achieved by the low intensity of high pressures used. Also, deep freezing processes are appropriate for small scale production and development of products because of the capital cost for the nitrogen freezer is very low compared to mechanical refrigeration. Furthermore, we clearly demonstrate that with this approach we can produce new and safe RTE products at significantly reduced pressures, which could reduce remarkably the operational costs. Future studies should focus on better understanding of HHP/subzero temperature on food components and microbial kinetics, before its validation and acceptance from the food industry.

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Legends to figures

Figure 1. Population reduction of *L. monocytogenes* strains: (A) 10403S WT, (B) LO28 WT, (C) EGD-e WT, grown in BHI broth and exposed to liquid smoke fractions L9 or G6, ranging from 0.50 % to 1.00 % (v/v) in increments of 0.25 for 0 min, 30 min, 60 min and 180 min. Control samples were grown in BHI broth without liquid smoke. Values represent the mean values of three replicates analyzed twice (two technical replicates); error bars represent the standard error (n=6). The dashed horizontal line represents the detection limit of 1.60 logs CFU/mL.

Figure 2. Effect of HHP on *L. monocytogenes* 10403S WT, LO28 WT and EGD-e WT in BHI broth supplemented with 0.50 % (v/v) G6 or L9 with or without NaCl. Columns represent the mean values of three replicates analyzed twice (two technical replicates); error bars represent the standard error (n=6). Values followed by the same lowercase letter within treatment are not significantly different ($p > 0.05$). Different uppercase letters represent significant differences among strains ($p < 0.05$).

Figure 3. Effect of HHP on *L. monocytogenes* 10403S WT in BHI broth supplemented with 0.50 % (v/v) G6 or L9 with or without NaCl, being at room temperature (20 ^{o}C) and after freezing at −80 ^{o}C. Columns represent mean values three replicates analyzed twice (two technical replicates); error bars represent the standard error (n=6). Values followed by the same lowercase letter within treatment are not significantly different ($p > 0.05$). Different uppercase letters represent significant differences among treatments with or without freezing ($p < 0.05$). HHP processing temperature measured through PTM was set at 0 ^{o}C at the start of pressurization.

Figure 4. Effect of HHP on *L. monocytogenes* 10403S WT, in raw trout fillets, supplemented with 0.50 % (v/v) G6 or L9 with or without NaCl, with or without NaCl, at room temperature (20 ºC) and after freezing at −80 ºC. Columns represent the mean values of three replicates analyzed twice (two technical replicates); error bars represent the standard error (n=6). Values followed by the same lowercase letter within treatment are not significantly different ($p > 0.05$). Different uppercase letters represent significant differences among treatments with or without freezing ($p < 0.05$). HHP processing temperature measured through PTM was set at 0 ºC at the start of pressurization.

Figure 5. Effect of HHP on *L. monocytogenes* 10403S WT, in smoked trout fillets, supplemented with 0.50 % (v/v) G6 or L9, being at room temperature (20 ºC) and after freezing at −80 ºC. Columns represent the mean values of three replicates analyzed twice (two technical replicates); error bars represent the standard error (n=6). Values followed by the same lowercase letter within treatment are not significantly different ($p > 0.05$). Different uppercase letters represent significant differences among treatments with or without freezing ($p < 0.05$). HHP processing temperature measured through PTM was set at 0 ºC at the start of pressurization.

953 **Table 1.** Percentage (%) of injury of *L. monocytogenes* 10403S WT, in BHI broth, raw and
954 smoked trout chunks, pressurized at 200 MPa for 15 min with liquid smoke extracts G6 or L9
955 0.50 % (v/v) in combination with or without freezing at −80 ºC.

	Without freezing (20 ºC)			With freezing at -80 ºC		
Inoculum	BHI Broth	Raw trout	Smoked trout	BHI broth	Raw trout	Smoked trout
- NaCl						
Lm	$^{B}3.26 \pm 4.23^{a}$	$^{B}10.87 \pm 3.56^{b}$	$^{A}14.31 \pm 1.23^{b}$	$^{A}55.98 \pm 8.64^{d}$	$^{A}38.58 \pm 8.37^{cd}$	$^{B}38.59 \pm 3.15^{c}$
Lm + L9	$^{AB}26.58 \pm 12.81^{abc}$	$^{AB}14.41 \pm 5.25^{a}$	$^{A}19.47 \pm 9.47^{ab}$	$^{A}43.79 \pm 17.56^{cd}$	$^{A}26.92 \pm 5.61^{bcd}$	$^{A}0.00 \pm 0.00^{d}$
Lm + G6	$^{A}51.49 \pm 19.82^{ab}$	$^{A}32.79 \pm 10.11^{a}$	$^{A}24.16 \pm 7.31^{a}$	$^{A}37.08 \pm 18.88^{ab}$	$^{A}36.64 \pm 15.24^{ab}$	$^{A}0.00 \pm 0.00^{d}$
+ NaCl						
Lm	$^{B}3.00 \pm 3.27^{a}$	$^{AB}29.13 \pm 13.93^{b}$	-	$^{A}55.23 \pm 3.93^{c}$	$^{A}38.95 \pm 10.75^{bc}$	-
Lm + L9	$^{AB}15.12 \pm 4.81^{a}$	$^{B}10.94 \pm 0.89^{a}$	-	$^{A}55.86 \pm 1.24^{c}$	$^{A}36.11 \pm 5.58^{b}$	-
Lm + G6	$^{A}25.24 \pm 22.14^{ab}$	$^{B}8.98 \pm 2.06^{a}$	-	$^{A}49.42 \pm 10.92^{b}$	$^{A}35.13 \pm 10.14^{b}$	-

956 Lm: *Listeria monocytogenes*10403S WT
957 Values (mean ± standard deviation. n=6) followed by different uppercase letter in the same column are
958 significantly different ($P < 0.05$).
959 Values (mean ± standard deviation. n=6) followed by different lowercase letter in each row are
960 significantly different ($P < 0.05$).
961
962

FIG 1

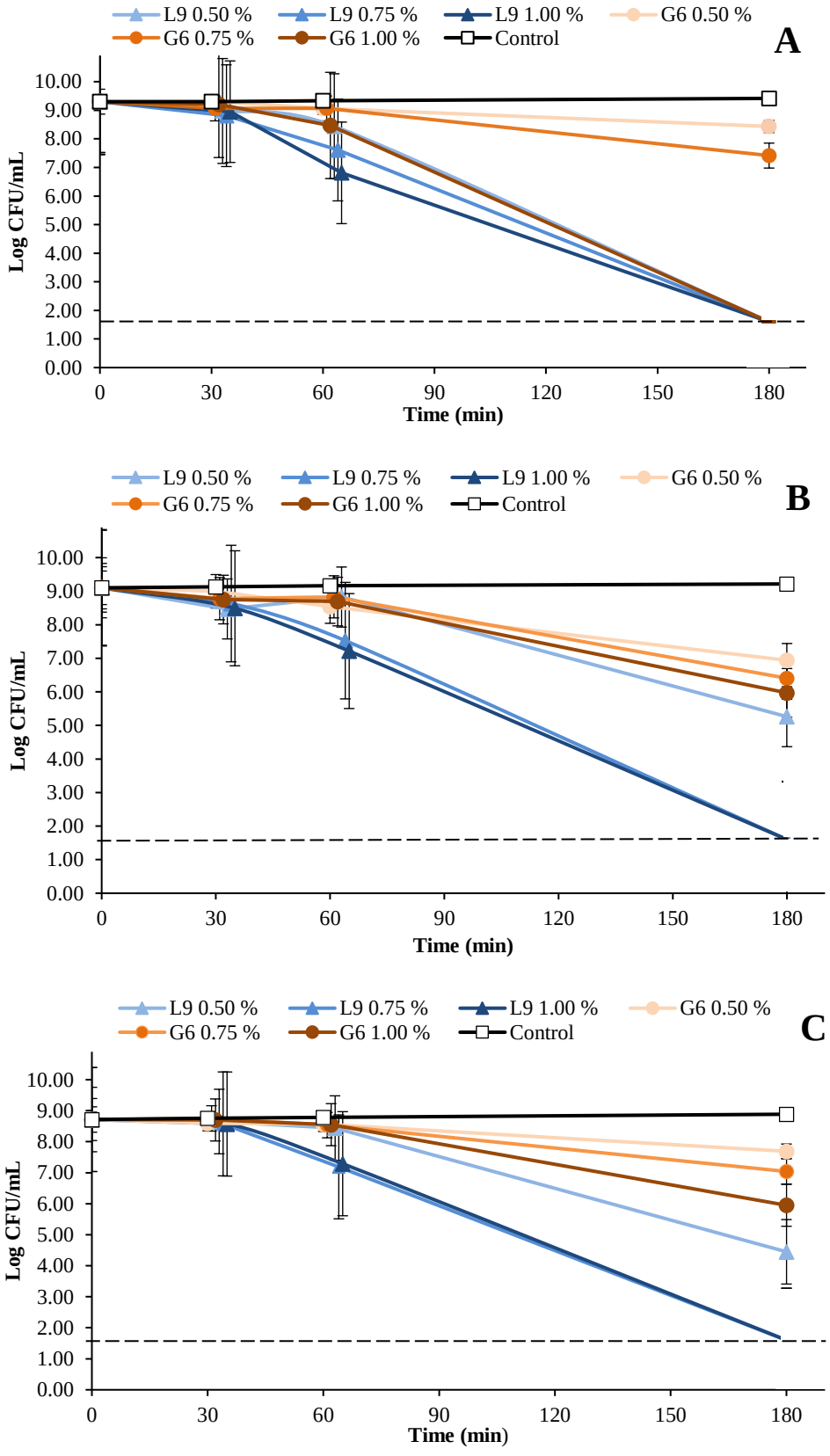

L9 0.50 %
L9 0.75 %
L9 1.00 %
G6 0.50 %
G6 0.75 %
G6 1.00 %
Control
A
B
C
Log CFU/mL
Time (min)
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
0
30
60
90
120
150
180

FIG 2

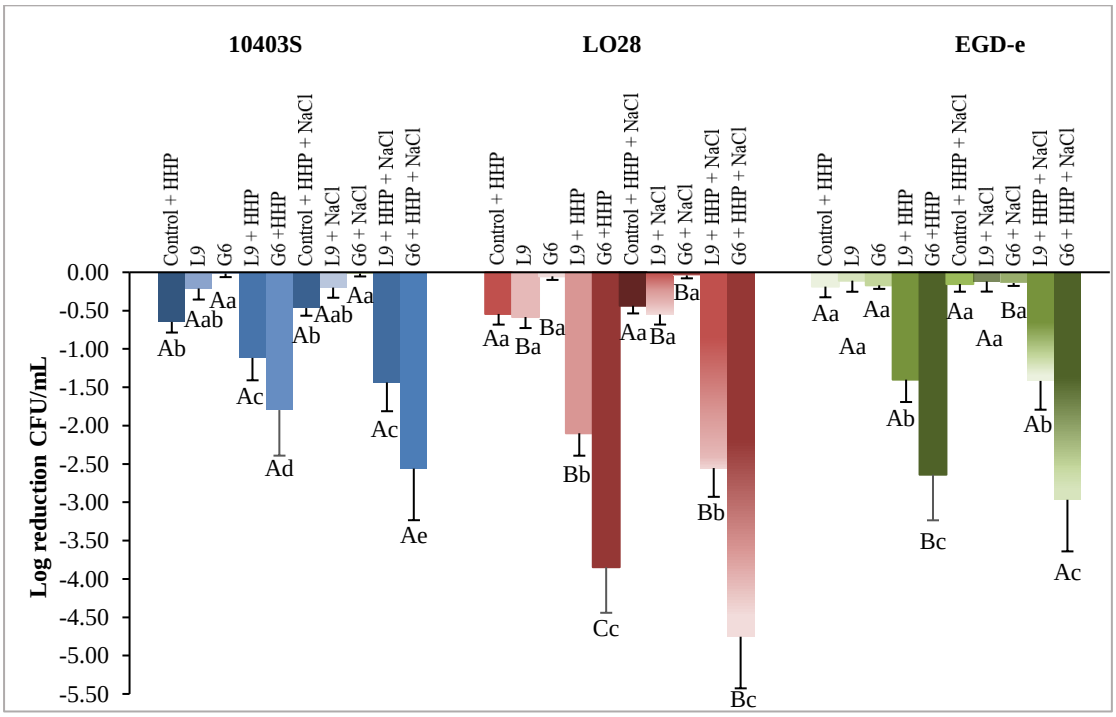

10403S
LO28
EGD-e
Log reduction CFU/mL
0.00
-0.50
-1.00
-1.50
-2.00
-2.50
-3.00
-3.50
-4.00
-4.50
-5.00
-5.50
Control + HHP
L9
G6
L9 + HHP
G6 +HHP
Control + HHP + NaCl
L9 + NaCl
G6 + NaCl
L9 + HHP + NaCl
G6 + HHP + NaCl
Ab
Aab
Aa
Ac
Ad
Ae
Aa
Ba
Bb
Cc
Bc

FIG 3

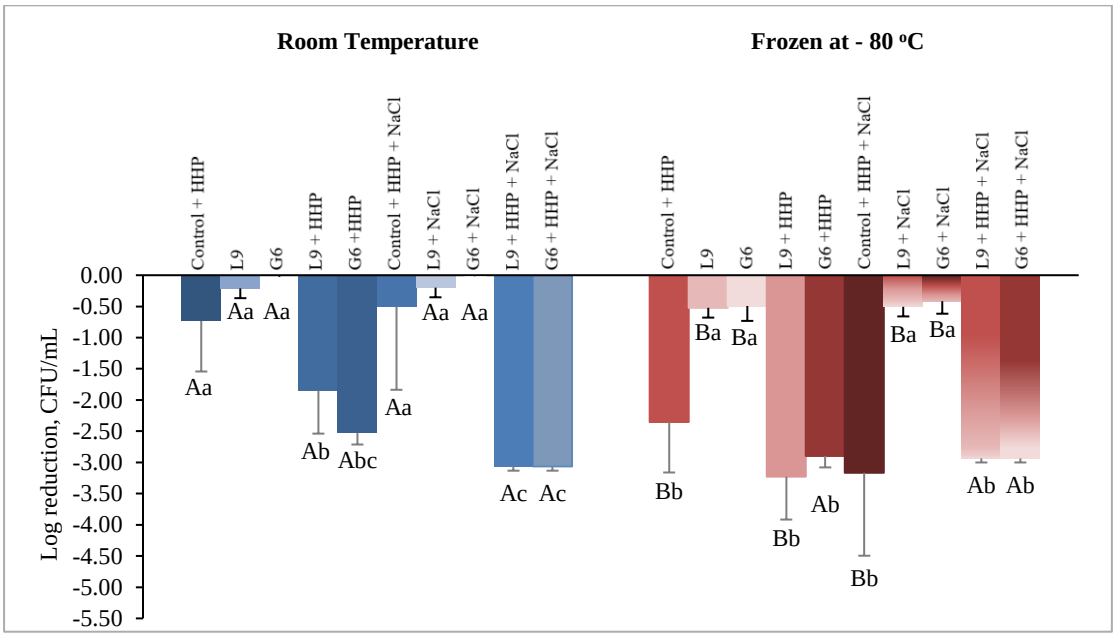

Room Temperature
Frozen at - 80 ºC
Log reduction, CFU/mL
0.00
-0.50
-1.00
-1.50
-2.00
-2.50
-3.00
-3.50
-4.00
-4.50
-5.00
-5.50
Control + HHP
L9
G6
L9 + HHP
G6 +HHP
Control + HHP + NaCl
L9 + NaCl
G6 + NaCl
L9 + HHP + NaCl
G6 + HHP + NaCl
Aa
Aa
Aa
Ab
Abc
Aa
Aa
Aa
Ac
Ac
Bb
Ba
Ba
Bb
Ab
Bb
Ba
Ba
Ab
Ab

FIG 4

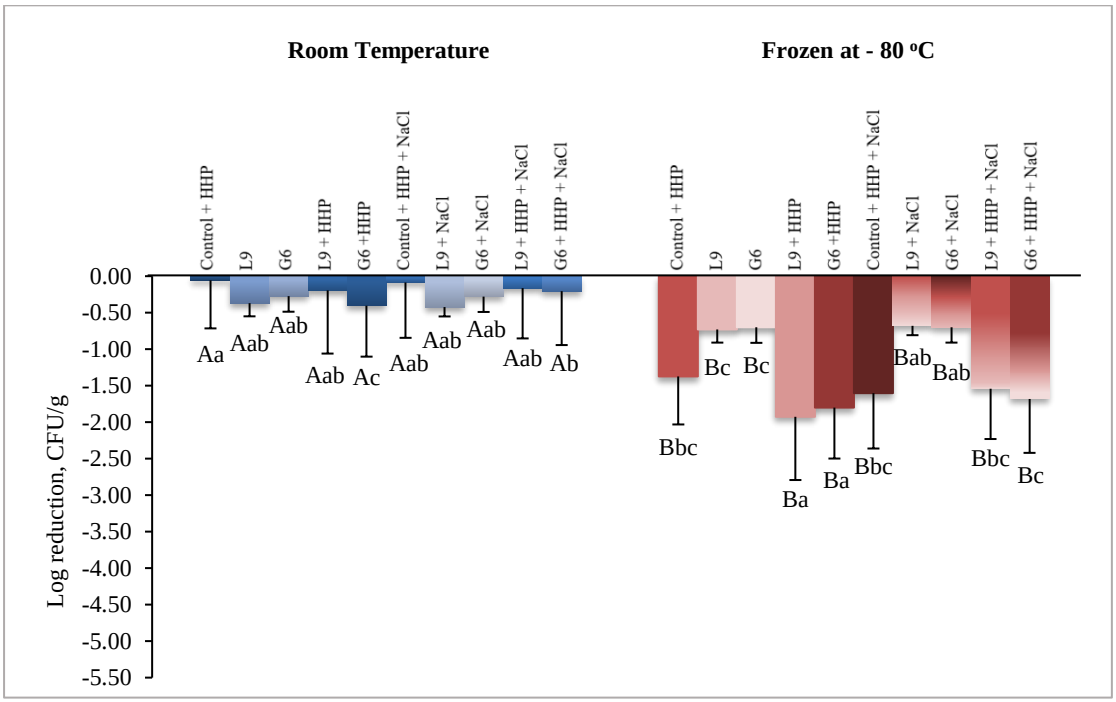
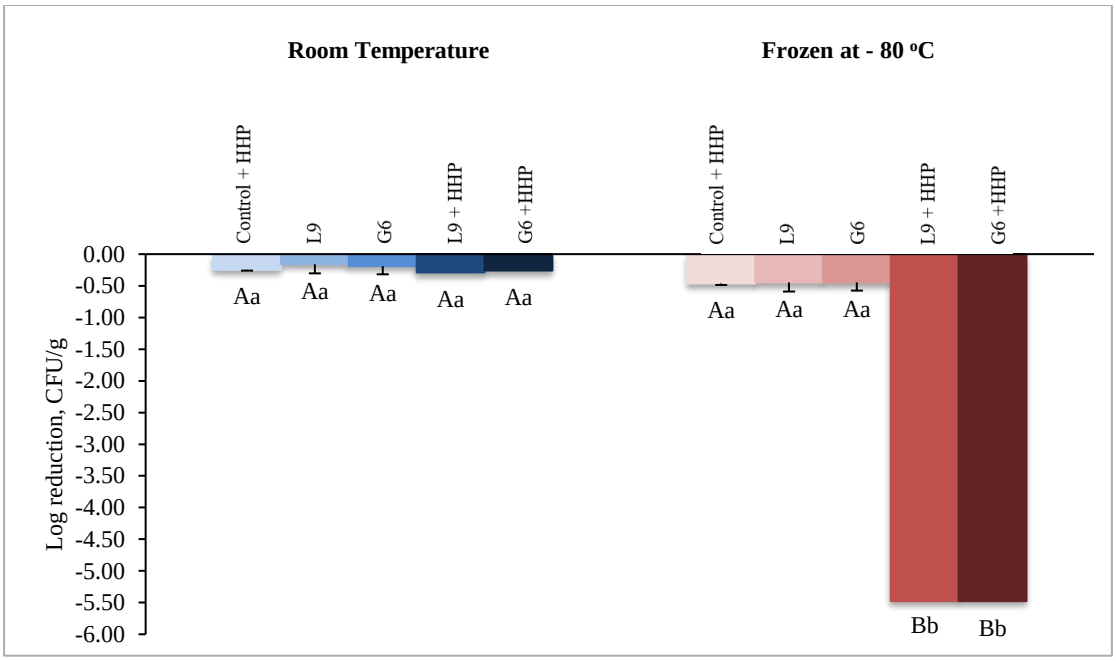
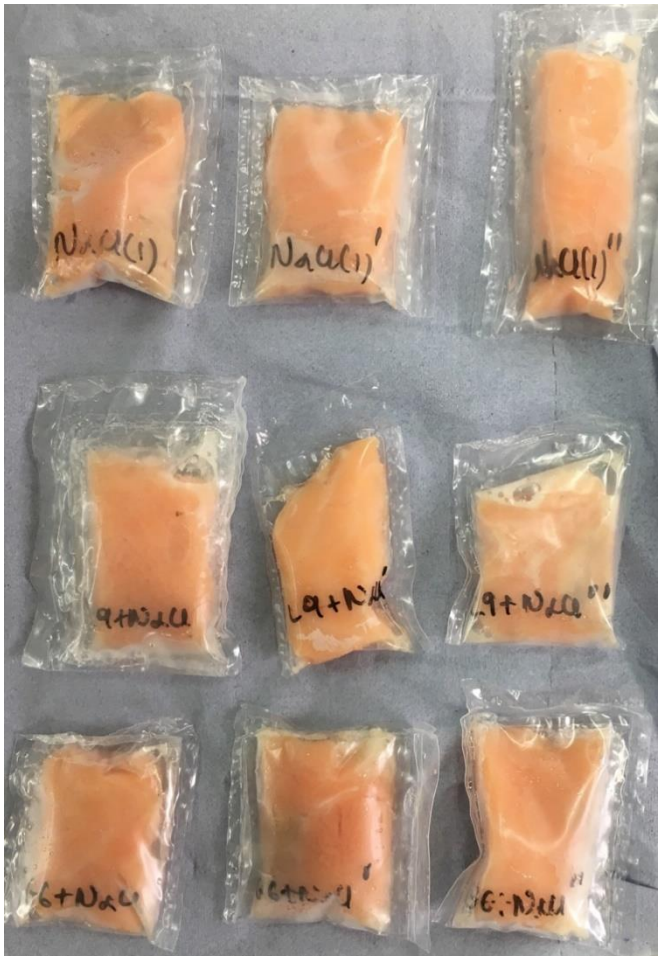

Room Temperature
Frozen at - 80 °C
Log reduction, CFU/g
0.00
-0.50
-1.00
-1.50
-2.00
-2.50
-3.00
-3.50
-4.00
-4.50
-5.00
-5.50
Control + HHP
L9
G6
L9 + HHP
G6 +HHP
Control + HHP + NaCl
L9 + NaCl
G6 + NaCl
L9 + HHP + NaCl
G6 + HHP + NaCl
Aa
Aab
Aab
Aab
Ac
Aab
Aab
Aab
Aab
Ab
Bbc
Bc
Bc
Ba
Ba
Bbc
Bab
Bab
Bbc
Bc

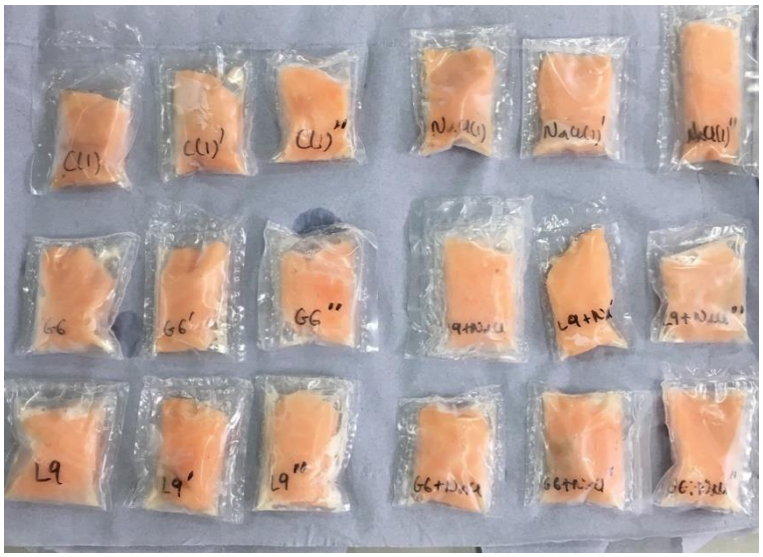
FIG 5


Room Temperature
Frozen at - 80 ºC
Control + HHP
L9
G6
L9 + HHP
G6 +HHP
Log reduction, CFU/g
0.00
-0.50
-1.00
-1.50
-2.00
-2.50
-3.00
-3.50
-4.00
-4.50
-5.00
-5.50
-6.00
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Bb
Bb

Supplementary materials

Raw trout samples before HHP at 200 MPa for 15 min.





Raw trout samples after HHP at 200 MPa for 15 min.

Smoked trout samples before HHP at 200 MPa for 15 min.

Smoked trout samples after HHP at 200 MPa for 15 min.