

# *Inactivation of Listeria monocytogenes in raw and hot smoked trout fillets by high hydrostatic pressure processing combined with liquid smoke and freezing*

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1 **Inactivation of *Listeria monocytogenes* in raw and hot smoked trout fillets by high**  
2 **hydrostatic pressure processing combined with liquid smoke and freezing.**

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32

33 **Abstract**

34 High hydrostatic pressure (HHP; 200 MPa for 15 min), in combination with liquid  
35 smoke and freezing was used to eliminate *Listeria monocytogenes* in BHI broth, raw  
36 and smoked trout. The bactericidal effect of liquid smoke (L9 and G6) solutions, HHP  
37 and their combinations were evaluated against *L. monocytogenes* LO28, EGD-e and  
38 10403S and further continued with the most resistant strain (10403S) to the combined  
39 treatment. For first time, a synergistic effect of liquid smoke and HHP was observed,  
40 further enhanced by freezing at  $-80\text{ }^{\circ}\text{C}$  prior to HHP. The effect of HHP and liquid  
41 smoke on *L. monocytogenes* 10403S, prior to freezing was highest in BHI compared to  
42 raw and smoked trout. However, major synergistic effect of HHP, liquid smoke and  
43 freezing was observed, reaching a 5.48 log CFU/g reduction when smoked trout was  
44 used. High levels of injury also occurred, among the treatments on *L. monocytogenes*  
45 10403S.

46

47 **Industrial relevance**

48 HHP is a novel, non-thermal technique that emerged in food protection within the last  
49 three decades and further knowledge needs to be generated. This paper illustrates for  
50 first time, the possibility of using a very low pressure in combination with liquid smoke  
51 and freezing to eliminate *L. monocytogenes*. It was demonstrated that treatment of trout  
52 samples with liquid smoke followed by freezing prior to pressurization at 200 MPa for  
53 15 min reduced the number of *L. monocytogenes* by more than 5 log CFU/g. Such a  
54 remarkable bacterial inactivation at a very low pressure (compared to common  
55 industrial practices) is a significant achievement that could allow production of safer  
56 and novel products by HHP at an affordable price, as the cost of equipment manufacture

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

59

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

62

### 63 **1. Introduction**

64 Fish and seafood are much appreciated because of their high nutritional value but are  
65 perishable and represent a high microbiological risk for the consumer (Novotny et al.,  
66 2004). According to the Food and Agriculture Organization (FAO, 2018), live, fresh or  
67 chilled seafood represent 45 % of the world's human consumption, while cured (dried,  
68 salted, in brine, fermented smoked) seafood represent 12 %.

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

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

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

85 Nowadays, consumers desire minimally processed food products with less additives,  
86 but with improved safety and shelf-life (Spence, 2006; Sun-Waterhouse et al., 2014;  
87 Moses et al., 2014). HHP can provide food products with high retention of nutritional  
88 quality and minimal effects on the organoleptic characteristics (Rastogi & Knorr, 2013).

89 During the last decades the use of this technique has been extended to various types of  
90 foodstuff, including fish and seafood (Elamin et al., 2015). Furthermore, several  
91 products processed with HHP are commercially available in regions of North America,  
92 Europe, Oceania and Asia. In seafood products, HHP is being used as a non-thermal  
93 technology for inactivation of bacterial pathogens and for reducing the spoilage  
94 microorganisms (Koutchma, 2014). One of the major disadvantages of HHP is the high  
95 cost of the initial investment which combined with the limited product output as a batch  
96 process, which results in limited application in the food industry. Therefore, the  
97 majority of the scientific work in HHP aims at the reduction of the applied pressure  
98 which could translate into lower wear of the equipment or shorter processing times  
99 resulting in higher product output and lower prices. This is normally achieved by  
100 following the principles of the “Hurdle Technology”, as defined by Leistner (1992).

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

107 (Sanz & Otero, 2014). Phase transitions of ice crystals during the pressurization of  
108 frozen food have been previously suggested to take place during the HHP treatment  
109 that results in cellular damage and increased microbial inactivation (Luscher et al.,  
110 2004; Shen et al., 2005; Su et al., 2014a). Several studies have proposed that the  
111 application of HHP on frozen products at low or subzero temperatures and atmospheric  
112 conditions can enhance the microbial inactivation and thus allow the usage of lower  
113 pressure treatments (Knorr et al., 1998; Fernández et al., 2007; Vaudagna et al., 2012;  
114 Bulut, 2014a; Bulut, 2014b; Albertos, 2016; Parlapani et al., 2019). The effectiveness  
115 of the combined HHP and freezing treatment depends on the characteristics of the  
116 product (pH,  $a_w$ , composition, etc.), on the type of the tested microorganism and on the  
117 process conditions (pressure level, holding time and temperature; Sanz & Otero, 2014;  
118 Choi, 2008; Park et al., 2008).

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

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

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

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

152

## 153 **2. Materials & methods**

### 154 *2.1. Bacterial cultures*



155 Three different commonly used reference strains of *L. monocytogenes* (LO28, EGD-e  
156 and 10403S) were used in this study. LO28 is a serotype 1/2c, while EGD-e and 10403S  
157 both belong to serotype 1/2a. All bacterial strains were kept in cryovials supplemented  
158 with 7.0 % (vol/vol) dimethyl sulfoxide (DMSO; Sigma-Aldrich, Dorset, UK) at –80  
159 °C. Before experiments, strains were streaked onto Brain Heart Infusion (BHI) agar  
160 (LABM, Lancashire UK) and incubated overnight at 37 °C. A single colony from each  
161 plate was transferred with a sterile loop in 3 mL of BHI (LAB M, Lancashire UK) and  
162 incubated overnight at 37 °C without shaking. To prepare the final working cultures, 20  
163 mL sterile BHI were inoculated with the overnight culture and left for incubation at 37  
164 °C for 24 h without shaking. Cultures from stationary phase were harvested by  
165 centrifugation (Heraeus™ Multifuge™ X 3 Centrifuge, Thermo Scientific, UK) at 9000  
166 ×g for 5 min (room temperature), washed twice using sterile Phosphate-buffered saline  
167 (PBS pH 7.1; Oxoid, Basingstoke, UK) and re-suspended in the same medium. Further  
168 serial decimal dilutions were prepared for direct plating on BHI agar. The plates were  
169 incubated for 24 h at 37°C and viable cell number was expressed as log CFU/mL.

170

## 171 *2.2. Liquid smoke extracts preparation*

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

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

184

### 185 *2.3. Resistance of L. monocytogenes strains to liquid smoke fractions*

186 Harvested cells were suspended into sterile Universal Polystyrene Containers (Sterilin,  
187 UK) with 10 mL liquid smoke solutions in BHI broth of final concentrations ranging  
188 from 0.25 % to 1.00 % (v/v) in 0.25 increments. The initial population was fixed at  $10^9$   
189 –  $10^{10}$  CFU/mL for each strain individually. Subsequently, the samples were left for  
190 incubation at 37 °C. Samples of 1 mL were taken after 0, 30, 60 and 180 min. Decimal  
191 dilution of samples were prepared in Maximum Recovery Diluent (MRD), (Oxoid,  
192 Basingstoke, UK), into sterile Eppendorf tubes, and 25 µL from each dilution and also  
193 directly from the BHI broth (zero dilution) were plated onto BHI agar using the spot  
194 plate method adapted from Laubacher and Ades (2008) and incubated for 24 h at 37 °C.  
195 The limit of detection was 1.60 log CFU/mL.

196

### 197 *2.4. Sample preparation for HHP*

#### 198 *2.4.1. Preparation of BHI broth samples*

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

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

206

#### 207 2.4.2. Preparation of trout samples

208 Raw and hot smoked trout (*Oncorhynchus mykiss*) fillets obtained from a local store  
209 (Reading, UK). Samples were cut uniformly in chunks of 5 g (approx. 35 mm × 45 mm  
210 × 10 mm), sprayed on with 70 % (v/v) alcohol and placed on a rack with no contact  
211 between the samples to evaporate the excess alcohol. A 3.80 % (w/v) NaCl sterile  
212 solution was prepared and 100 µL was added on raw and hot smoked trout chunks. The  
213 samples were then inoculated with 100 µL of the *L. monocytogenes* 10403S bacterial  
214 suspension which gave a final concentration of about 10<sup>7</sup> CFU/g. Subsequently, 100 µL  
215 of 0.50 % L9 or G6 liquid smoke extract was added on the chunks. As a control, 100  
216 µL sterile water was added on the fish samples. All inoculations (salt, sterile water,  
217 liquid smoke and *L. monocytogenes* inoculum) were done to the upper surface of the  
218 chunks. A sterile spatula was used to spread the inoculums on the surface of the chunks.  
219 The samples were then heat sealed and vacuum packed. Finally, the samples were  
220 separated into two groups and half were pressure-treated while the other half, were left  
221 overnight at -80 °C inside a high efficiency upright ultra-low temperature freezer  
222 (Eppendorf HEF U570, New Brunswick, Germany) before being pressure-treated.  
223 All samples were microbiologically analyzed in triplicate while, non-treated samples,  
224 without pressure treatment or freezing, were also analyzed.

225

#### 226 2.5. HHP treatment set up and operation mode

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

229 Ltd., Harlow, UK) with 40 mm internal diameter vessel and 250 ml working volume  
230 was used. The pressure transmitting medium (PTM) used was 30% (v/v) aqueous  
231 solution of mono propylene glycol (Sigma-Aldrich, Dorset, UK). A cooling circulator  
232 (Grant LTD6G, UK) was used to circulate the water containing mono propylene glycol  
233 as antifreeze, in the jacket surrounding the pressure vessel to control and maintain the  
234 processing temperature measured through the PTM inside the pressure chamber.  
235 Packed samples treated in room temperature were immediately placed into the pressure  
236 chamber. Frozen samples at  $-80\text{ }^{\circ}\text{C}$  were removed from the freezer and immediately  
237 placed in the working vessel which was kept at  $0\text{-}1\text{ }^{\circ}\text{C}$ . The time needed to remove the  
238 samples from the freezer and place them in the HHP vessel was less than 20 s suggesting  
239 that the temperature was still close to  $-80\text{ }^{\circ}\text{C}$ . The pressure come-up time was about  
240 100 MPa/10 s, while decompression time was less than 5 s. The chamber temperature  
241 was monitored and recorded by a K-type thermocouple mounted to the center of the top  
242 closure of the pressure chamber.

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

246

#### 247 *2.6. Bacterial enumeration and evaluation of injury*

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

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

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

262

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

263

## 264 2.7. Experimental design

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

274

## 275 3. Results

### 276 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).

302

303 *3.2. Effect of HHP and liquid smoke extracts on L. monocytogenes strains*

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

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

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

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

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

332

333 *3.3. Application of HHP and freezing at -80 °C in broth, raw trout and hot smoked*  
334 *trout samples.*

335 Following the investigation of the combined effect of HHP and liquid smoke, we  
336 investigated the possibility of applying these technologies in frozen broth, raw and hot  
337 smoked trout at -80 °C as an additional hurdle to further increase the inactivation of *L.*  
338 *monocytogenes* 10403S. As seen in Fig. 3, the samples containing liquid smoke L9 or  
339 G6 with or without salt showed no significant differences in the absence of pressure  
340 treatment and the log reduction remained below 0.50, regardless of the samples being  
341 frozen or not. The effect of HHP without addition of liquid smoke, on *L. monocytogenes*  
342 10403S was statistically significant ( $p < 0.05$ ) but minimal (0.73 log CFU/mL). On the  
343 other hand, HHP combined with freezing at -80 °C, without liquid smoke, resulted in  
344 2.35 log CFU/mL reduction (Fig. 3). Similar results were obtained for the samples,  
345 supplemented with NaCl where 3.00 log reduction increased from 0.51 log CFU/mL to  
346 3.17 CFU/mL as a result of freezing the samples before the pressure treatment (Fig. 3;  
347  $p > 0.05$ ).

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



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

357 The application of HHP treatment in combination with smoke on raw rainbow trout  
358 chunks resulted in a limited reduction in number of *L. monocytogenes* 10403S in both  
359 the presence and absence of salt. As seen in Fig. 4, maximum log reduction remained  
360 below 0.42 log CFU/g at all conditions tested for the samples that were at room  
361 temperature before the pressure treatment. HHP treatment of raw rainbow trout chunks  
362 frozen at  $-80\text{ }^{\circ}\text{C}$  before the pressure treatment resulted in significant reduction in  
363 number of *L. monocytogenes* 10403S for all the treatments (Fig. 4). HHP treatment of  
364 frozen raw rainbow trout chunks without liquid smoke, containing or not salt, reduced  
365 the population of *L. monocytogenes* 10403S by 1.60 and 1.37 log CFU/g, respectively  
366 (Fig. 4;  $p > 0.05$ ). In the absence of salt, treatment of samples with L9 and G6 and then  
367 freezing before the pressure treatment increased the bacterial death to 1.93 and 1.80 log  
368 (CFU/g), respectively (Fig. 4). In the presence of salt, pressure treatment of frozen  
369 samples with L9 and G6 resulted in 1.54 and 1.68 log (CFU/g) reductions, respectively  
370 (Fig. 4;  $p > 0.05$ ).

371 Subsequently, we assessed the effect of HHP treatment in combination with liquid  
372 smoke on *L. monocytogenes* 10403S inoculated on hot smoked trout. In the absence of  
373 freezing, pressure treatment of inoculated hot smoked trout samples resulted in only  
374 0.25 log reduction of CFU/g (Fig. 5). Furthermore, the addition of liquid smoke on the  
375 surface of the fish without freezing, after HHP, also resulted in a minimal effect of 0.29  
376 and 0.26 log CFU/g for L9 and G6, respectively (Fig. 5;  $p > 0.05$ ). In addition, also

377 freezing at  $-80\text{ }^{\circ}\text{C}$  by itself resulted in no reduction of CFU/g (data not shown). HHP  
378 treatment of inoculated hot smoked trout chunks (with no liquid smoke) after they were  
379 frozen at  $-80\text{ }^{\circ}\text{C}$  did not cause a significant reduction ( $0.46\text{ log CFU/g}$ ) in the numbers  
380 of *L. monocytogenes* 10403S. However, the same samples, upon supplementation with  
381  $0.50\text{ }\%$  (v/v) L9 or G6, resulted in log reduction of CFU/g that was beyond  $5.48\text{ logs}$   
382 ( $n=6$ , Fig. 5;  $p < 0.05$ ).

383

#### 384 *3.4. Sublethal injury of L. monocytogenes 10403S WT strain*

385 The percentage of sub lethally injured cells in BHI broth and on raw and smoked trout  
386 chunks, without freezing, was quite high for the samples containing L9 or G6 without  
387 added NaCl. More specifically, when G6 combined with HHP, prior freezing, caused  
388 the highest level of injury on bacterial cells, while freezing did not cause any significant  
389 changes. The percentage of injury after HHP and without freezing at  $-80\text{ }^{\circ}\text{C}$  for BHI  
390 broth and raw trout chunks was  $51.49\text{ }\% \pm 19.82\text{ }\%$  and  $32.79\text{ }\% \pm 10.11\text{ }\%$ , respectively  
391 showing no significant differences among the treatments, prior freezing ( $p > 0.05$ ; Table  
392 1).

393 It is noteworthy to highlight that freezing at  $-80\text{ }^{\circ}\text{C}$  in combination with HHP, caused  
394 high injury levels. The maximum level of bacterial injury effect observed for pressure-  
395 treated cells in BHI broth. The injury for BHI broth samples without and with salt, after  
396 freezing, without liquid smoke additives was significantly different in comparison with  
397 the samples without freezing ( $20\text{ }^{\circ}\text{C}$ ) and equal with  $55.98\text{ }\% \pm 8.64\text{ }\%$  and  $55.23\text{ }\% \pm$   
398  $3.93\text{ }\%$  (Table 1;  $p < 0.05$ ). Pressure-treated BHI broth samples with freezing and L9  
399 or G6 without NaCl content, compared with the same samples prior freezing, showed  
400 no significant differences (Table 1;  $p > 0.05$ ). Though the percentage of injury for BHI  
401 broth, with NaCl and L9, before freezing was  $15.12\text{ }\% \pm 4.81\text{ }\%$ , while after freezing

402 was increased significantly and was equal with  $55.86 \% \pm 1.24 \%$  (Table 1;  $p < 0.05$ ).

403 Table 1 also illustrates that G6 addition in BHI broth caused significant changes to the

404 percentage of sub lethal injury of *L. monocytogenes* for the samples with or without

405 salt.

406 The *L. monocytogenes* cells inoculated on the surface of raw trout chunks, also revealed

407 high injury for all treated samples as shown in Table 1. For raw trout chunks prior

408 freezing, without NaCl, significant differences observed only among the samples with

409 G6 ( $32.79 \% \pm 10.11 \%$ ) and without liquid smoke ( $10.87 \% \pm 3.56 \%$ ). Concurrent

410 application of freezing with HHP treatment on raw trout, caused sublethal injury of *L.*

411 *monocytogenes* cells, equal with  $36.11 \% \pm 5.58 \%$  and  $26.92 \% \pm 5.61 \%$  for the

412 samples supplemented with L9, containing or not NaCl, respectively (Table 1;  $p <$

413  $0.05$ ). However, pressure-treated raw trout samples after freezing, containing or not

414 salt, showed no significant differences (Table 1).

415 As seen in Table 1, the differences of sub lethal injury of *L. monocytogenes* cells found

416 in smoked trout chunks without liquid smoke, submitted in freezing at  $-80\text{ }^{\circ}\text{C}$ , prior to

417 HP treatment ( $38.59 \% \pm 3.15 \%$ ) were significant comparing with the same samples

418 stayed at  $20\text{ }^{\circ}\text{C}$  ( $14.31 \% \pm 1.23 \%$ ) (Table 1;  $p < 0.05$ ). When the smoked trout

419 supplemented with L9 or G6 without NaCl, prior freezing and followed by HHP

420 treatment, no significant differences observed, while after freezing no injury on

421 bacterial cells detected (Table 1). During the investigation of the treatments on hot

422 smoked trout fillets, no extra NaCl added to an already salted product to better evaluate

423 the real conditions.

424

425 **4. Discussion**

426 The current study investigates the behavior of *L. monocytogenes* strains in a liquid  
427 medium and hot smoked or raw trout upon the application of different treatments, such  
428 as HHP, freezing and smoking. In the current work, only one type of HHP treatment  
429 (200 MPa for 15 min), was used, which is one of the lowest, if not the lowest in the  
430 food industry (Koutchma, 2014; Khan et al., 2017). The first hurdle introduced in  
431 combination with HHP was liquid smoke. Two water-soluble liquid smoke condensates  
432 (L9 and G6) were used, as common preservatives for protein-based foods, such as  
433 seafood. The smoke extracts against all three strains was effective in a dose and  
434 exposure time-related pattern. Nowadays traditional smoking is primarily used to  
435 endow the typical characteristics of aroma, flavor and color to the smoked products  
436 (Theobald et al., 2012; Fasano et al., 2016) but the controlled use of liquid smoke  
437 extracts can inhibit the growth of foodborne pathogens (Lingbeck et al., 2014). There  
438 are several studies, presenting the *in vitro* antimicrobial effect of liquid smokes against  
439 foodborne pathogens, such as *Aeromonas hydrophila* (Sofos et al., 1988; Suñen et al.,  
440 2003), *Yersinia enterocolitica* (Suñen et al., 2001), pathogenic *Escherichia coli*  
441 (Schmid-Hempel & Frank, 2007; Van Loo et al., 2012) and *L. monocytogenes* (Martin  
442 et al., 2010; Pittman et al., 2012; Morey, et al., 2012). In previous work Vitt et al.  
443 (2001), tested 5 commercial liquid smoke extracts at varying concentrations to estimate  
444 the inhibitory effect on *L. innocua* and *L. monocytogenes* and found that 0.50 % liquid  
445 smoke inhibited *L. innocua* in BHI broth. This is in agreement with our work where  
446 0.50 % liquid smoke resulted in minor but measurable reductions in the *L.*  
447 *monocytogenes* numbers suggesting that such a concentration would be inhibitory in  
448 our experimental setup. It is well established that great differences may occur in stress  
449 resistance between individual strains and therefore the most tolerant strains should be  
450 considered to better assess, the food safety risks posed by tolerant strains and develop

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

454 In the present work, the application of HHP at 200 MPa for 15 min on BHI broth  
455 samples (without liquid smoke) resulted in a minor log reduction of CFU/mL with no  
456 significant differences among *L. monocytogenes* strains. The presence or absence of  
457 salt in BHI broth did not have any effect, while both liquid smoke extracts enhanced  
458 the lethal effect of HHP. The liquid smoke and HHP showed a synergistic effect while  
459 the addition of salt had no major effect for most of the samples, with only exception the  
460 samples of *L. monocytogenes* LO28 and EGD-e with or without salt and G6, where the  
461 differences observed, were significantly different ( $p < 0.05$ ; Fig. 2). This demonstrates  
462 that it is possible to enhance the antimicrobial effects of HHP with the use of smoke or  
463 vice-versa. Exposure of *L. monocytogenes* to liquid smoke (L9 or G6) in BHI broth for  
464 30 min resulted in a minor reduction, less than 0.50 log CFU/mL (Fig. 1A, B & C).  
465 From all three strains, LO28 represents a normal strain of *L. monocytogenes*, while  
466 EGD-e is a lab strain with extreme resistance to oxidative stress and 10403S an  
467 extremely acid resistant strain which might be linked to its increased resistance to the  
468 combined HHP and liquid smoke treatment. However, the combined treatment of  
469 extremely low HHP and low levels of liquid smoke is able to bring about more than  
470 4.00 log reduction of CFU/ml for a normal *L. monocytogenes* strain, such as LO28 and  
471 this is promising.

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

476 under HHP without freezing is major. This could be possibly due to the state of liquid  
477 smoke under freezing conditions, which went through phase changes while it has  
478 freezing point lower than water (Ledford, 1981) but further work is required to  
479 investigate this. When freezing is performed properly, without multiple freezing steps,  
480 food quality is minimally affected (Oosterhuis, 1981; Evans, 2009). Redmond et al.  
481 (2003), used a liquid nitrogen cryogenic environmental chamber to freeze mashed  
482 potatoes, where the pots of potato placed at  $-90\text{ }^{\circ}\text{C}$  reached an internal temperature of  $-$   
483  $25\text{ }^{\circ}\text{C}$  within 24 min compared to 78 min for samples frozen at  $-30\text{ }^{\circ}\text{C}$  with similar  
484 results for fresh mashed potato.

485 Regarding the microbial safety, it is well established that freezing can induce high rates  
486 of injury to the bacterial population. Jiang et al. (2011), revealed that, frozen storage at  
487  $-18\text{ }^{\circ}\text{C}$  as a pretreatment, followed by chill storage at  $4\text{ }^{\circ}\text{C}$  in combination with surface  
488 application of antimicrobials on roasted turkey, reduced significantly the initial  
489 population of *L. monocytogenes*. Golden et al. (1988), mentioned that, after 2 weeks at  
490  $-18\text{ }^{\circ}\text{C}$ , the injury level of *L. monocytogenes* strains, reached up to 82 %. Our results  
491 for *L. monocytogenes* after freezing, followed by HHP treatment, revealed high injury  
492 levels, for BHI broth samples (Fig. 3).

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

501 experiments, also on these raw trout experiments we observed that liquid smoke elicited  
502 hardly any antimicrobial effect under freezing temperature for the samples without  
503 HHP (Fig. 4). However, when we added this low concentration of liquid smokes (0.50  
504 %) on already smoked trout chunks we observed an impressive 5.48 log reduction of  
505 CFU/g following freezing at  $-80^{\circ}\text{C}$  and HHP (Fig. 5). In this case the increased  
506 concentration of smoke contributed the above major synergistic effect while in its  
507 absence, the observed reduction was less than 0.50 log (CFU/g) (Fig. 5). We also  
508 demonstrate that this major synergistic effect could be used to significantly decrease  
509 the HHP intensity which could translate to major reduction in economic costs.

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

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

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

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

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

578 As a general observation, freezing process as a pretreatment before high pressure did  
579 not affect the color of our final product (raw and smoked trout). Other studies looked  
580 at the differences on the color of aquacultured King salmon (*Oncorhynchus*  
581 *tshawytscha*) fillets treated with combined liquid smoke and HHP treatment at 200 –  
582 600 MPa (Kong et al., 2015), frozen shrimps processed at 250 MPa up to 10 min  
583 (Parlapani et al., 2019) and the effect of HHP on rainbow trout (Erkan et al., 2010b) or  
584 even smoked fish (Erkan et al., 2011). They reported that HHP did not change  
585 significantly the color of the product. This might be important since sensory appearance  
586 plays an important role for the purchase decisions by the consumers (Garber et al.,  
587 2003).

588

## 589 **5. Conclusion**

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

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

613

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619

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906 **Legends to figures**

907

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

915

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

922

923 **Figure 3.** Effect of HHP on *L. monocytogenes* 10403S WT in BHI broth supplemented  
924 with 0.50 % (v/v) G6 or L9 with or without NaCl, being at room temperature (20 °C)  
925 and after freezing at -80 °C. Columns represent mean values three replicates analyzed  
926 twice (two technical replicates); error bars represent the standard error (n=6). Values  
927 followed by the same lowercase letter within treatment are not significantly different ( $p$   
928  $> 0.05$ ). Different uppercase letters represent significant differences among treatments  
929 with or without freezing ( $p < 0.05$ ). HHP processing temperature measured through  
930 PTM was set at 0 °C at the start of pressurization.



931

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

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943 **Figure 5.** Effect of HHP on *L. monocytogenes* 10403S WT, in smoked trout fillets,  
944 supplemented with 0.50 % (v/v) G6 or L9, being at room temperature (20 °C) and after  
945 freezing at -80 °C. Columns represent the mean values of three replicates analyzed  
946 twice (two technical replicates); error bars represent the standard error (n=6). Values  
947 followed by the same lowercase letter within treatment are not significantly different ( $p$   
948  $> 0.05$ ). Different uppercase letters represent significant differences among treatments  
949 with or without freezing ( $p < 0.05$ ). HHP processing temperature measured through  
950 PTM was set at 0 °C at the start of pressurization.

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952

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 °C.

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

956 Lm: *Listeria monocytogenes*10403S WT

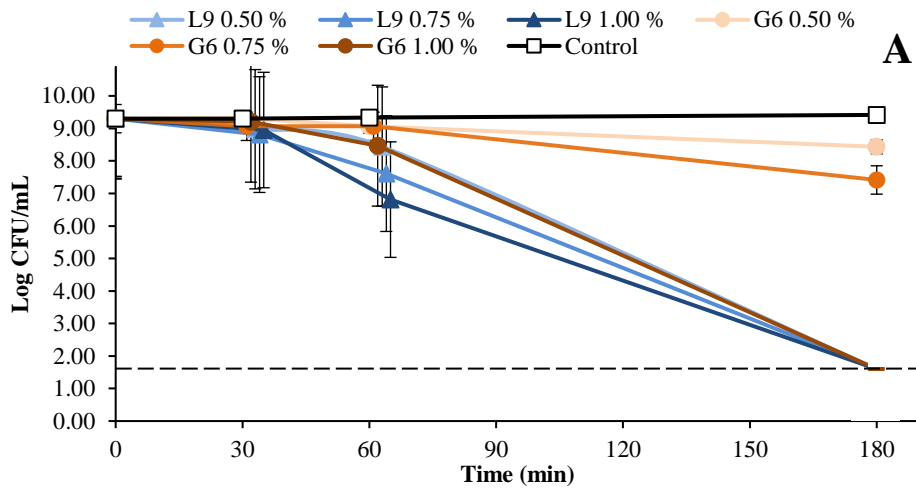
957 Values (mean ± standard deviation. n=6) followed by different uppercase letter in the same column are  
 958 significantly different (P < 0.05).

959 Values (mean ± standard deviation. n=6) followed by different lowercase letter in each row are  
 960 significantly different (P < 0.05).

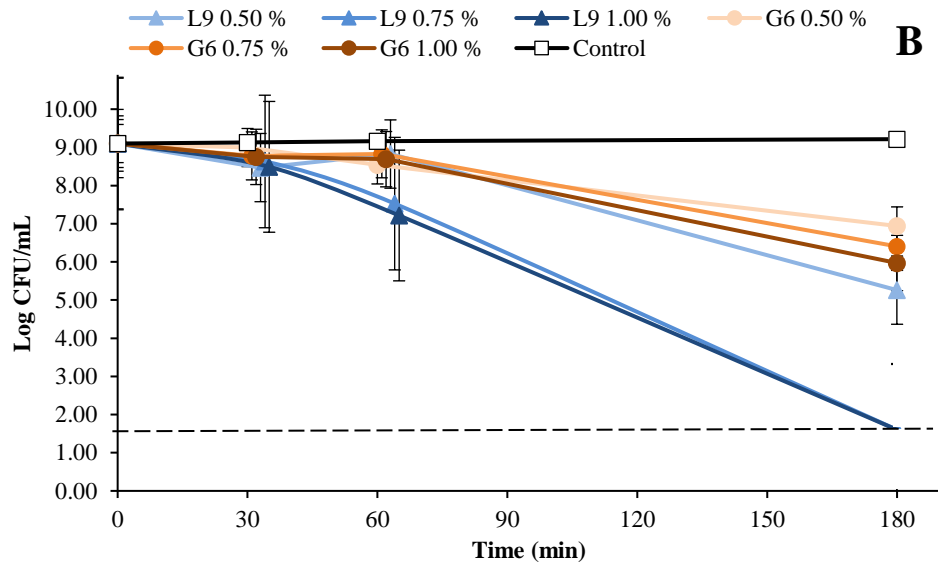
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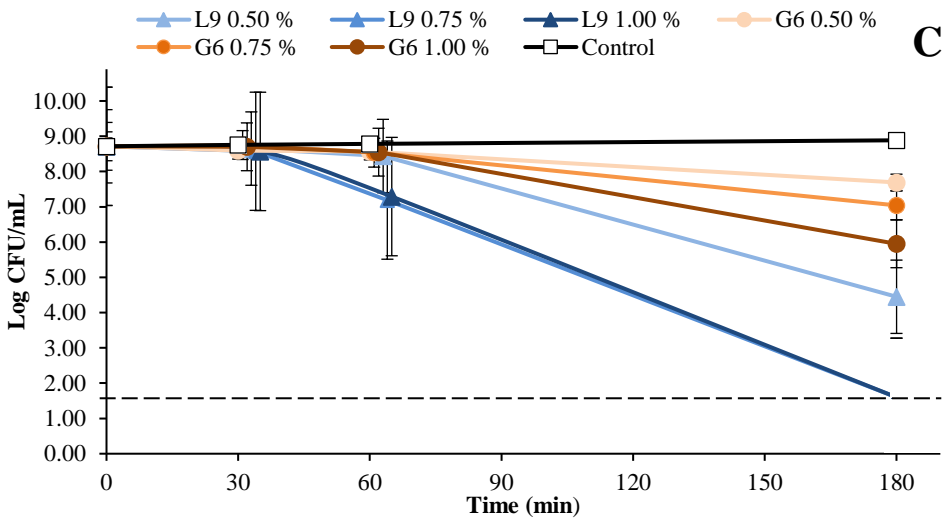
963 **FIG 1**



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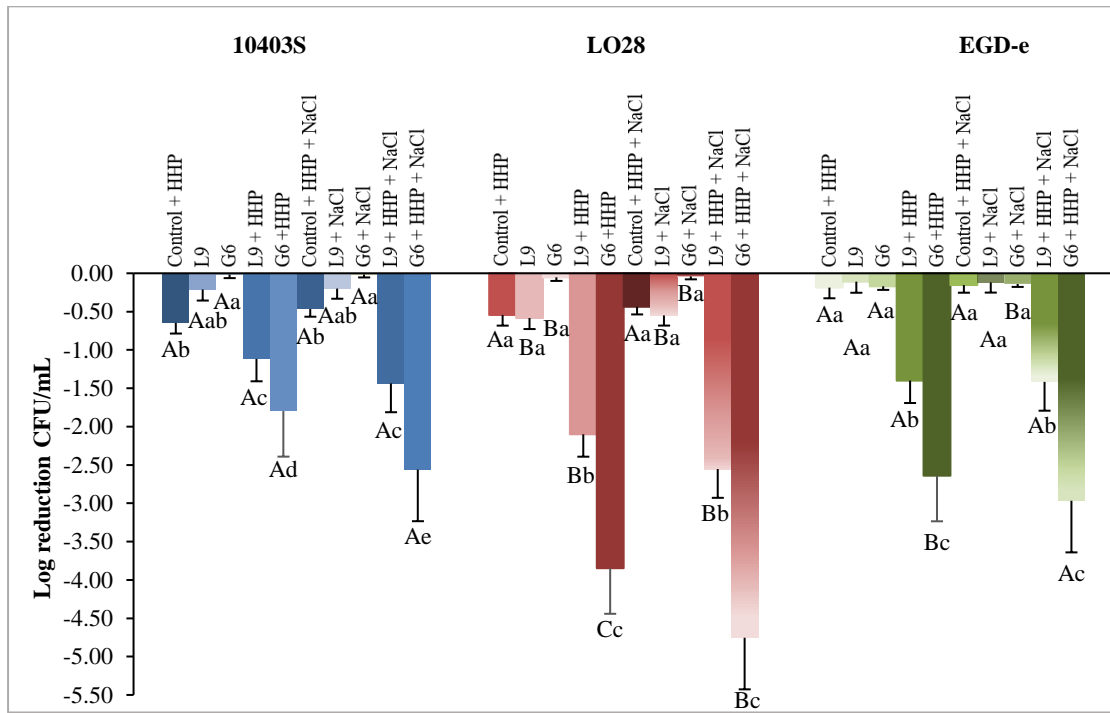


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968 **FIG 2**

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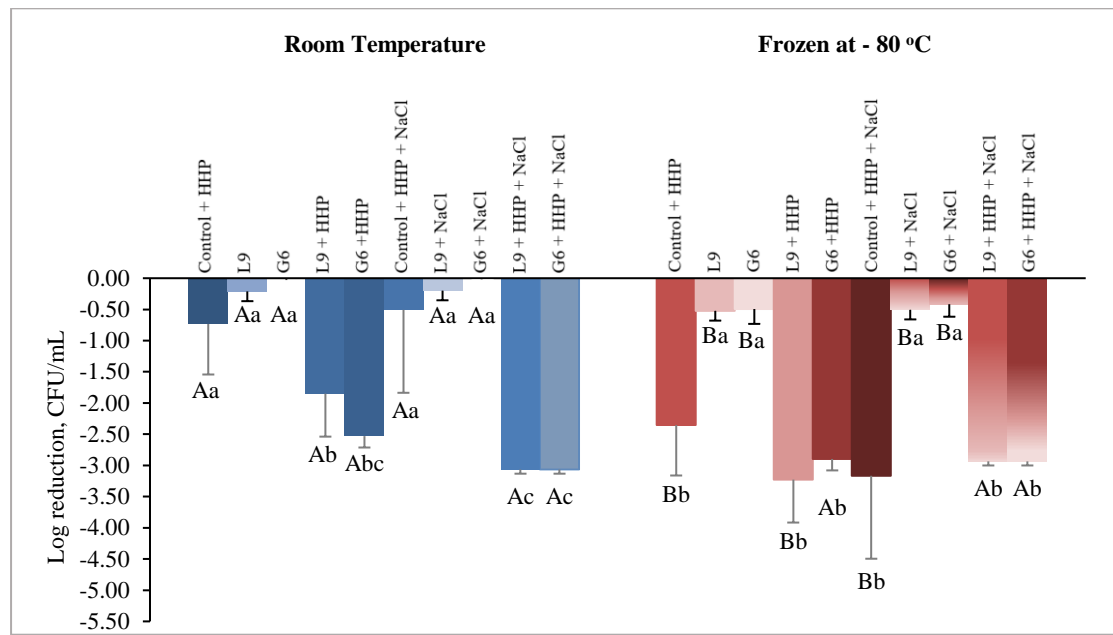
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984 **FIG 3**

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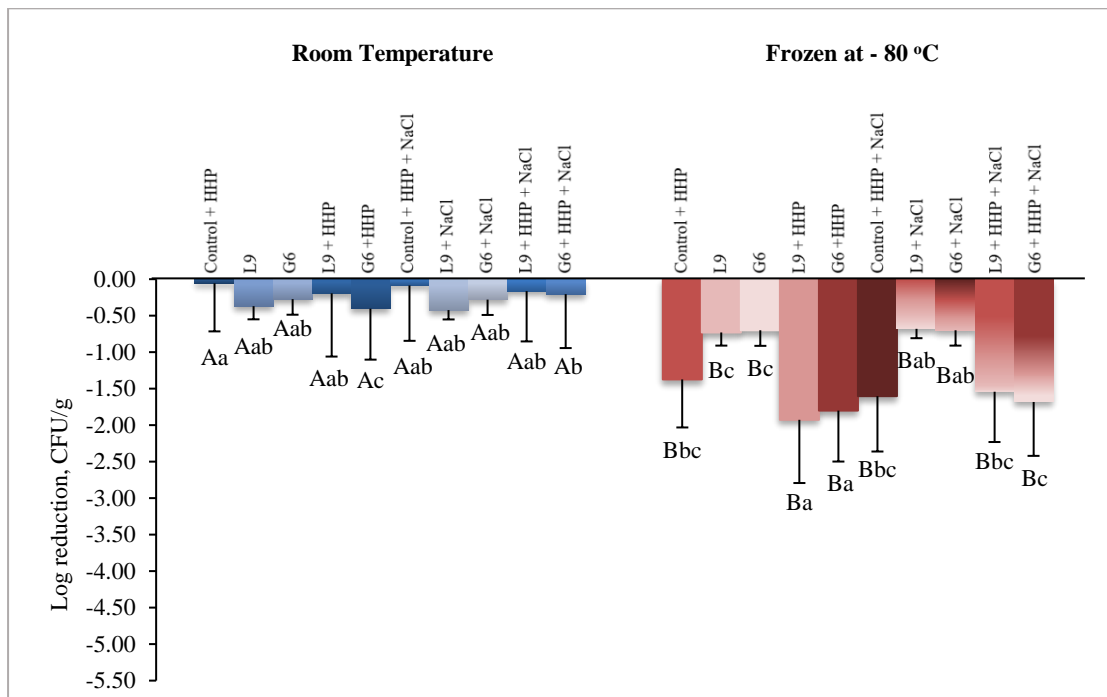
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1001 **FIG 4**

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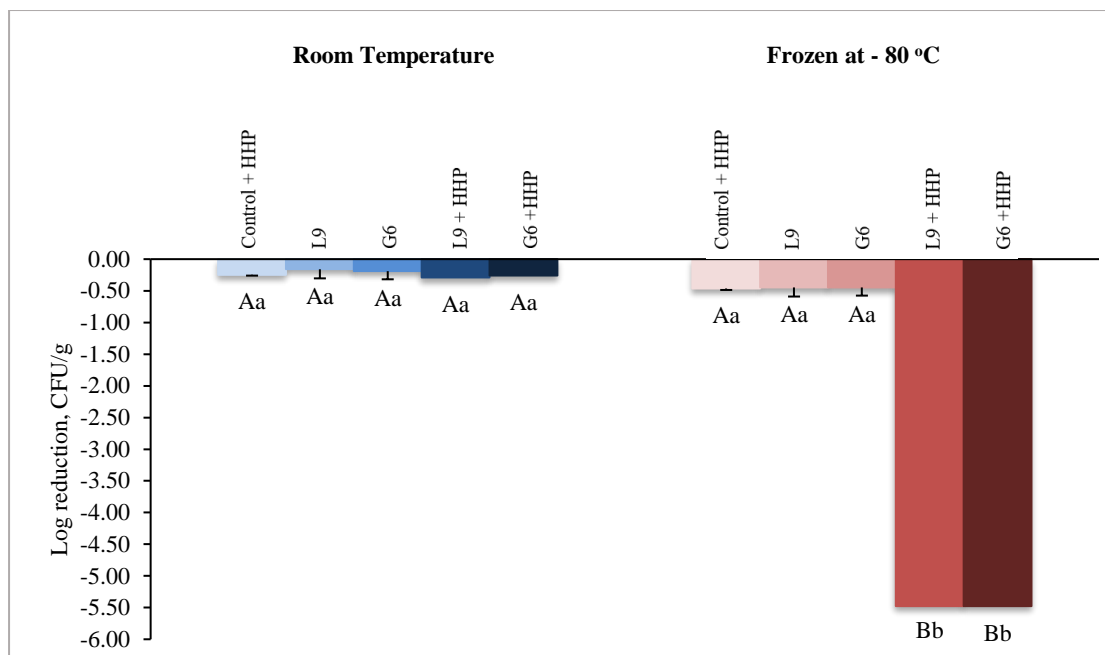
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1014 **FIG 5**



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1024 **Supplementary materials**



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1026 **Raw trout samples before HHP at 200 MPa for 15 min.**

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1030 **Raw trout samples after HHP at 200 MPa for 15 min.**

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1034 **Smoked trout samples before HHP at 200 MPa for 15 min.**



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1036 **Smoked trout samples after HHP at 200 MPa for 15 min.**

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