Modelling the piezo-protection effect exerted by lactate on the high pressure resistance of *Listeria monocytogenes* in cooked ham

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Abstract

Food safety is often based on the application of several preservative (hurdle) factors whose combination must be smartly selected. The aim of the present study was to evaluate the effect of lactate and diacetate on the high pressure processing (HPP) inactivation of three *L. monocytogenes* strains (CTC1011, CTC1034 and Scott A) in sliced cooked ham. Inoculated vacuum-packed slices of cooked ham formulated without organic acids and with lactate, diacetate or the combination of both were pressurized at 400 MPa for different holding times and the inactivation kinetics were characterised by fitting primary and secondary models. The shape of the inactivation curves for *L. monocytogenes* depended on both product formulation and strain. Interestingly, lactate caused a dose-dependent piezo-protection in all three strains, as the HPP inactivation rate decreased in cooked ham formulated with increasing amounts of lactate and in comparison with the control product. The design, validation and implementation of HPP requires a tailor-made approach, considering product formulation and selection of strain/s.

Keywords: High hydrostatic pressure; Pressurization; Food Safety; Meat Products; Pathogens; Organic Acids.

1. Introduction

Listeria monocytogenes is a foodborne pathogen that can cause listeriosis, a severe human illness often associated with the consumption of ready-to-eat (RTE) products, particularly those favoring the growth of the pathogen during the refrigerated storage. Among RTE food, cooked meat products commercialized in a convenient format (i.e. sliced, diced, and packaged) are particularly considered of high risk according to the risk assessments developed by several organizations worldwide (EFSA BIOHAZ Panel, 2018; FDA/USDA, 2013).

Food safety criteria regarding *L. monocytogenes* in RTE products differ between countries, *e.g.* EU and USA. Regulation (EC) 2073/2005 (European Commission, 2005) establishes a maximum of 100 cfu/g of *L. monocytogenes* during the shelf-life of the product, whereas in USA a zero-tolerance policy is imposed (FSIS, 2003), which means the presence of the pathogen is not allowed either in product or on food contact surfaces. The zero-tolerance poses a challenge for the meat industry to comply with such regulation due to the technical difficulties for the control and complete eradication of *L. monocytogenes.*

To accomplish with the microbiological criteria for *L. monocytogenes* in RTE food control measures can be implemented. The purpose of these measures is (i) to minimize the occurrence of the pathogen in raw materials, (ii) to reduce its levels by applying lethality or post-lethality treatments (PLT) and/or (iii) to prevent its increase (either by recontamination or growth) through the use of antimicrobial agents (AMA) or processes, among others. In this framework, there are regulations such as those of the USA and Canada (FSIS, 2003; Health Canada, 2011) that follow a risk-based approach to identify alternative operating methods for an effective control of *L. monocytogenes* in post-lethality exposed RTE products, classifying the manufacturers according to the risk associated with their products. In the USA, the Listeria Rule establishes that the safest operating procedures are those validated as alternative 1, which rely on the combination of alternatives 2a and 2b. Alternative 2 consists in the application of a PLT to reduce or eliminate the contamination (Alternative 2a), or an AMA to reduce or inhibit the growth of *L. monocytogenes* (Alternative 2b, considered of higher risk than alternative 2a). The higher risk

occurs when operation procedures rely exclusively on sanitation and good manufacturing practices (i.e. Alternative 3) (FSIS, 2003).

High pressure processing (HPP) is a non-thermal technology usually used as in-package PLT particularly interesting for products exposed to microbial contamination after the lethality treatment (i.e. during slicing and packaging operations). HPP is a widespread application in the meat industry. The microbial inactivation during HPP depends on technological factors (pressure, time and temperature) as well as food intrinsic factors (e.g. pH, a_w and food preservatives), either by favoring *L. monocytogenes* inactivation in case of low pH, or by exerting a protective effect in case of low a_w (Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2012; Rendueles, Omer, Alvseike, Alonso-Calleja, Capita, & Prieto, 2011). Due to this product-specific lethal effect of HPP, the application of HPP as a PLT of RTE food products must be validated to reduce at least 1 log of *L. monocytogenes* in the product and an increased level of control is considered when a 2 log reduction of *L. monocytogenes* is documented (FSIS, 2014, 2015).

Among AMA, organic acids and their salts (e.g. lactate, diacetate) are frequently used by the meat industry within a natural biopreservation strategy (Pérez-Rodríguez, Carrasco, Bover-Cid, Jofré, & Valero, 2017), with levels varying from 1.5 to 3.0% of lactate, added alone or together with diacetate at levels from 0.10 to 0.25% (Mbandi & Shelef, 2001; Mellefont & Ross, 2007a; Porto-Fett et al., 2010). The efficacy of an AMA mainly depends on the type and amount of antimicrobial added and its mode of application (Aymerich, Garriga, Jofré, Martín, & Monfort, 2006). AMA should limit the growth of *L. monocytogenes* over the shelf-life of the product and they must be validated to allow no more than 2 log growth of *L. monocytogenes* (FSIS, 2014, 2015). In the framework of the implementation of the EU microbiological criteria (European Commission, 2005), a product (e.g. formulated with an AMA) belongs to the RTE food category that does not support the growth of *L. monocytogenes* when no more than 0.5 log units of pathogen growth is observed throughout the product shelf-life (EURL Lm, 2019). Several studies report the survival and growth capacity of *L. monocytogenes* in the presence of AMA, especially organic acids and their salts (Bover-Cid, Serra-Castelló, Dalgaard, Garriga, & Jofré, 2019; Mellefont &

86 Ross, 2007b). However, the interaction between HPP and organic acids or their salts has been
87 scarcely studied. According to the hurdle technology (Leistner, 2000), an increased effectiveness
88 in controlling *L. monocytogenes* survival/growth (synergistic or additive effect) should be
89 expected with the intelligent combination of hurdles. Despite this, data available in literature
90 suggested that the addition of lactate in meat products, such as cooked ham and dry-cured ham,
91 increases the HPP resistance of *L. monocytogenes* causing a piezo-protection that reduces the
92 efficacy of the HPP (Table 1).

93 In this framework, the present study aimed to evaluate the potential piezo-protective effect of
94 organic acid salts used as AMA to formulate cooked meat products treated by HPP. A modeling
95 approach was applied in order to quantitatively characterize the HPP-inactivation kinetics of three
96 different *L. monocytogenes* strains inoculated on cooked ham formulated without or with natural
97 antimicrobials often used by the meat industry, i.e. potassium lactate (food additive EU code: E-
98 326) and sodium diacetate (E-262) and thus, to quantify the potential piezo-protective effect of
99 organic acid salts towards *L. monocytogenes* HPP-inactivation in cooked ham.

100 **2. Material and methods**

101 2.1 Preparation of cooked ham

102 Cooked ham was prepared as in previous works (Bover-Cid et al, 2019; Hereu et al., 2012) with
103 pork shoulder minced in a cutter to a particular size of 4 mm, and the following additives (g/Kg):
104 water, 115; salt, 20.7; dextrose, 5.8; sodium tri-polyphosphate, 5.8; carrageenan, 2.3; $NaNO_2$, 0.1
105 and L-ascorbate, 0.6. Five batches of cooked ham were prepared by adding different types and
106 amounts of organic acid salts, and consisted of:

107 (i) 1.4 % of potassium lactate corresponding to 2.4% of HiPure product (Corbion®,
108 Montmeló, Spain) added in the product formulation;
109 (ii) 2.8% of potassium lactate corresponding to 4.7% of HiPure product (Corbion®,
110 Montmeló, Spain) added in the product formulation;
111 (iii) 0.1% of sodium diacetate (Grama Aliment SL, Les Preses, Spain);

(iv) 1.4% potassium lactate and 0.1% sodium diacetate corresponding to 2.5% of Optiform (Corbion®, Montmeló, Spain) added in the product formulation;

(v) a control batch was prepared without the addition of organic acids.

Though the addition of lactate and/or diacetate may influence the sensory characteristics of the product, the type and the amount of organic acid salts studied in the present work are within the ranges applied by the meat industry, thus resulting in products with sensory characteristics accepted by the consumers (Mellefont & Ross, 2007a; Porto-Fett et al., 2010).

Ingredients were homogenized in a mixer (model 35P, Tecnotrip S.A., Terrassa, Spain) for 30 min and stuffed using a stuffing machine (model H15, Tecnotrip S.A., Terrassa, Spain) into impermeable plastic film (Prolan SV 150, PHH, San Boi de Llobregat, Spain). The product was cooked in an oven at 75 ºC until internal temperature reached 72 ºC (total cooking time *ca*. 2.6 h).

In agreement with previous reports (Mellefont & Ross, 2007a), the addition of lactate and/or diacetate did not significantly change the physicochemical parameters of cooked ham compared to the control batch and the manufactured product had a pH of 6.04 ± 0.04 and a_w of 0.974 ± 0.003.

2.2 Inoculation of *L. monocytogenes* in sliced cooked ham and HPP

L. monocytogenes strains used in this study were the strains CTC1011 (serotype 1/2c) and CTC1034 (serotype 4b) both isolated from meat products and belonging to the Institute of Agriculture and Food Research and Technology (IRTA)-Food Safety Program's collection, as well as the strain Scott A (serotype 4b), a clinical isolate frequently included in HPP inactivation studies (van Boeijen, Moezelaar, Abee, & Zwietering, 2008). Cultures were prepared by growing each strain in Brain Heart Infusion (BHI) broth (Beckton Dickinson, Sparks, Md., USA) at 37 °C for 7 h and subsequently at 37 ºC for 18 h (i.e. till the stationary phase of growth was reached) in two consecutive subcultures. Final cultures were preserved frozen at −80 °C in the growth medium supplemented with 20% glycerol until their use. Freezing conditions expose cells to concentrated solutes, which cause an osmotic stress similar to that caused by dry environments

140 occurring in the food industry (e.g. clean and dry food contact surfaces). Additionally, some
141 industrial processes to prepare sliced RTE products include a pre-freezing step to facilitate the
142 slicing process (Hereu et al., 2012; Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2014).
143 Cooked ham was sliced aseptically in slices of 12-14 g (1.5 mm thick). The frozen cultures thawed
144 at room temperature were used to independently inoculate each strain at 1% v/w to achieve *ca.*
145 10^7 CFU/g. The inoculum was spread on the surface of the slices with a sterile spreader until
146 absorbed (<1 min in a biosafety cabinet).
147 Inoculated slices were individually vacuum-packaged (EV-15-2-CD; Tecnotrip, Terrassa, Spain)
148 in plastic bags of PET/PE (oxygen permeability <50 cm^3/m^2/24 h and water vapor permeability
149 <15 mg/m^2/24 h; Sacoliva S.L., Barcelona, Spain).
150 HPP was performed in a Wave6000/120 industrial equipment (Hiperbaric, Burgos, Spain) at 400
151 MPa and holding times of 0, 2.5, 3.75, 5, 6.25, 7.5, 8.5, 9.5 and 10 min. According to the data
152 recorded through the SCADA system of the HPP equipment, the come-up time was 2.0 min and
153 the pressure release time was almost immediate (<2s). The pressurization fluid was water, and the
154 initial temperature was set at 13 ºC. After pressurization, the samples were kept for 2 h at room
155 temperature before *L. monocytogenes* analysis. The HPP treatments applied to cooked ham are
156 known to have no or minimal impact on the physico-chemical and sensory characteristics of
157 cooked meat products (e.g. Hereu et al., 2012; Olmo, Calzada, & Nuñez, 2014; Vercammen et
158 al., 2011), which was confirmed by the visual observation of samples before the microbiological
159 analysis (data not shown).
160 2.3 Microbiological analysis
161 Each sample (12-14 g) was aseptically minced, 1/10 diluted in Tryptic Soy Broth (Becton,
162 Dickinson) supplemented with 0.6% yeast extract (TSBYE) and homogenized for 1 min in a bag
163 blender (Smasher, Biomerieux, France). Samples were kept at room temperature for 1 hour
164 following the ISO recommendations before preparing the appropriate serial dilutions in 0.1%
165 Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85% NaCl. Samples were then
166 spread plated on Chromogenic Listeria Agar (Oxoid Ltd., Basingstoke, UK) and incubated at 37
167 ºC for 48 h. Duplicate or triplicate analysis for each batch and strain was performed.

168 The presence/absence of the pathogen was investigated in samples with expected concentration
169 of *L. monocytogenes* below the quantification limit. Ten-g samples were 1/10 diluted in TSBYE,
170 homogenized and incubated at 37 ºC for 48 h. After enrichment, the presence of *L. monocytogenes*
171 was investigated by plating on Chromogenic Listeria Agar (Hereu et al., 2012; 2014). For
172 modelling purposes, presence below the quantification limit was assumed as 1 cfu/g and absence
173 as 0.1 cfu/g.
174 The absence of detectable levels of spoilage specific organisms (i.e. lactic acid bacteria) in cooked
175 ham slices was checked by plating 1 ml of the homogenized 1/10 dilution into MRS (de Man,
176 Rogosa and Sharpe) agar plates (Merck KGaA, Darmstadt, Germany), which were incubated at
177 30 °C for 72 h under anaerobiosis.
178
179 2.4 Data analysis
180 *L. monocytogenes* counts were log transformed and the pathogen inactivation calculated as
181 log N/N_0. To estimate the kinetic inactivation parameters, the primary inactivation Weibull model
182 (Eq. 1) was fitted to the inactivation data (log N/N_0) along HPP holding time, using the nls2 and
183 nls functions from the respective nls2 and nls packages of R (R Core Team, 2013).

184 $$\log N/N_0 = (\log N/N_0)_i - \left(\frac{t}{\delta}\right)^p \qquad \text{Eq. 1}$$

185 where $(\log N/N_0)_i$ is a fixed value representing the average value of the initial bacterial
186 inactivation of 3 replicates at t = 0 (i.e. a cycle of pressure come-up and release without holding
187 time), δ is the holding time (min) required for the first log reduction, p is a dimensionless
188 parameter describing the shape of the inactivation curve and t is the holding time (min) during
189 HPP. The characteristics of the Weibull model in terms of flexibility (being able to fit most typical
190 survivor curves depending on the p parameter, i.e. p<1 concave; p=1 linear and p>1 convex), its
191 parsimony and meaningfulness of the δ parameter (i.e. close to widely known decimal reduction
192 time, D) to be used in secondary modeling are the reasons reported to recommend this model for
193 fitting microbial inactivation curves associated with food processing and preservation treatments
194 (van Boekel, 2002).

To quantitatively characterize the effect of lactate on the kinetic inactivation parameters (δ and p), polynomial models were developed for each strain. The fit using different transformations of kinetic parameter estimates (δ and p), including square root, inverse, ln and log were assessed. Stepwise regression was carried out to obtain equations with only the significant parameters using R software (R Core Team, 2013).

Besides the classical two-step modelling approach described above, the one-step or global regression procedure was applied by integrating the primary Weibull model into the polynomial secondary models (Eq. 2) for the kinetic inactivation parameters and fitting it to the entire data set of inactivation values (n=225) for HPP cooked ham formulated without and with different concentrations of potassium lactate.

$$\log N/N_0 = (\log N/N_0)_i - \left(\frac{t}{(a+b*LAC^2)}\right)^p \qquad \text{Eq. 2}$$

where $(\log N/N_0)_i$ is a fixed value representing the average value of the initial bacterial inactivation of 3 replicates at t = 0, t is the holding time (min), p is a dimensionless parameter describing the shape of the inactivation curve (i.e. p<1 concave; p=1 linear and p>1 convex) and *lactate* is the concentration of potassium lactate added (%). The parameters a and b are the coefficients estimates of the regression describing the effect of lactate (LAC) on the time for the first log reduction (δ).

The statistical goodness of fit of the primary models was assessed by means of residual sum of squares (RSS) and root mean of square error (RMSE). The RSS was derived by summing the squared differences between the experimental (observed) data and the value provided by the model (fitted data). The RMSE was calculated as the square root of the Mean Sum of Squared Errors (MSSE), which were derived by dividing the RSS by the number of degrees of freedom (i.e. the number of data points minus the number of parameters and initial values used). For the secondary models the adjusted determination coefficient (R^2_{adj}) as in Eq. 3 was also considered (Spiess & Neumeyer, 2010). The F-test was applied to assess the need of different models for the three *L. monocytogenes* strains studied (Zwietering, Jongenburger, Rombouts, & van't Riet, 1990).

222

223 $$R^2_{adj} = \frac{(n-1)\cdot R^2 - k + 1}{n-k}$$

224 Where R^2 is the coefficient of determination, i.e. 1−RSS/SSTO, with SSTO being the sum of the
225 squared differences between the experimental (observed) values and the mean of these
226 experimental values.

227 **3. Results and discussion**

228 3.1 *L. monocytogenes* behavior in pressurized cooked ham without organic acids

229 The high pressure inactivation kinetics of the 3 tested strains of *L. monocytogenes* in cooked ham
230 formulated without organic acids are shown in Figure 1 (a,b,c) with fitted kinetic parameters of
231 the Weibull model shown in Table 2. As expected, inactivation of *L. monocytogenes* was higher
232 as the holding time increased from 0 to 10 min. However, inactivation curves of different shape
233 were found for the different strains. *L. monocytogenes* CTC1011 showed a convex shape with a
234 considerable shoulder described by a δ value, i.e. the holding time needed for the first log
235 reduction, of almost 6 min. The pronounced shape of the inactivation curve (p >3) observed for
236 holding times higher than 6 min was due to the virtually total inactivation of the inoculated
237 pathogen (not detected) in some samples. At higher holding times (> 6 min), the inactivation of
238 CTC1011 was 3 log higher than CTC1034 and Scott A. Inactivation of CTC1034 followed a
239 linear curve trend, with a p parameter close to 1, resulting in almost constant effect of HPP in the
240 inactivation kinetics across 10 min of holding time. The δ found for CTC1034 had a value close
241 to 4 min, indicating that CTC1034 was more sensitive to HPP at lower holding times than
242 CTC1011.

243 The concave shape for the inactivation curve (p<0.5) of the clinical isolate *L. monocytogenes*
244 Scott A resulted in much lower holding time to achieve the first log reduction (δ = 0.7 min)
245 compared to CTC1011 and CTC1034. However, the shape was compatible with the occurrence
246 of a resistant tail for holding times higher than 6 min. Thus, Scott A was the most sensitive strain
247 to HPP at lower holding times but also the most resistant to HPP at higher times.

248 The need of different holding times to achieve the first log reduction (δ = 0.5-6 min) and the
249 differences in the shape (concave, linear, convex) proved that inactivation curves, and thus, their
250 piezo-resistance, were highly dependent on the *L. monocytogenes* strain. The strain-specific
251 resistance to HPP could be related with the strain membrane composition and properties to
252 withstand pressure (Jung, Lee, Lee, Kim, & Ahn, 2013).
253 To the best of the author's knowledge, few studies are available describing the impact of HPP on
254 the *L. monocytogenes* membrane. Although that, for gram-negative bacteria it has been shown
255 that the bacterial membrane integrity is often compromised with the application of HPP, leading
256 to morphological and physiological changes (Ma et al., 2019). Within this framework, Klotz et
257 al. (2010) hypothesized that the differences in pressure resistance observed between two strains
258 of *E. coli* in the range of 100 to 700 MPa were related to the dissimilar ability of their membranes
259 to withstand pressure. More specifically, for some *Salmonella* strains, Tamber (2018) found that
260 the higher the proportion of cyclopropane fatty acids in the bacterial membrane the higher the
261 resistance to HPP,. These results were in agreement with those reported by Charoenwong et al.
262 (2011) in which the cyclopropane fatty acid synthase had a decisive role on the HPP resistance of
263 *E. coli*. On the other hand, HPP was shown to induce an elongation of *L. monocytogenes* cells,
264 leading to an increased permeability of the membrane and to a rupture of the internal cellular
265 structure (Jung et al., 2013). In this line, the degree of pressure resistance has been related with
266 the ability of the cells to repair ion leaks of the membrane (Na^+, K^+, Mg^{2+} and Ca^{2+}) after
267 decompression (Farkas & Hoover, 2000; Ma et al., 2019).
268 Besides this, some authors showed that the genetic features of each strain play a role in *L.*
269 *monocytogenes* HPP resistance. Karatzas et al. (2003) reported that some piezo-tolerant isolates
270 of *L. monocytogenes* had a mutation in the CtsR gene, leading to a loss of its function and to an
271 increased expression of Clp proteases (which prevent harmful accumulation of damaged proteins)
272 that confer resistance of *L. monocytogenes* cells to HPP. Moreover, the resistance to HPP
273 observed for some *L. monocytogenes* strains was not related with mutation in the CtsR gene,
274 which suggest that other mechanisms may confer resistance to HPP such as proteins involved in
275 stress responses which are mainly regulated by the RpoS gene (Karatzas, Valdramidis, & Wells-

Bennik, 2005; Landini, Egli, Wolf, & Lacour, 2014; Chen, Neetoo, Ye, & Joerger, 2009; Gayán, Cambré, Michiels, & Aertsen, 2017; Gayán, Rutten, Van Impe, Michiels, & Aertsen, 2019)

Some authors also described the importance of strain ability to accumulate compatible solutes to withstand pressure, such as a proline, whose synthesis is strain-dependent and conditioned by the food matrix components (Bartlett, 2002; Considine, Sleator, Kelly, Fitzgerald, & Hill, 2011).

Overall, the different mechanisms described above involved in *L. monocytogenes* resistance to HPP could lead to a different degree of pressure resistance depending on the temporal frame along the HPP; such strain specific resistance can explain the different shapes shown by the inactivation curves.

3.2 *L. monocytogenes* behavior in pressurized cooked ham with organic acids

The presence of salts of organic acids in the cooked meat products did not modify the shape (convex, linear or concave) of the inactivation curves of the *L. monocytogenes* strains compared to those found in cooked ham without organic acids (Figure 1). However, the extent of the inactivation and the corresponding inactivation kinetic parameter values differed depending on the type and concentration of added organic acid salt (Table 2).

The addition of lactate increased the HPP resistance of all the strains. In all cases, the inactivation was lower than in control products and the inactivation kinetic curve moved upwards (Table 2, Figure 1 d, e, f, g, h, i). This fact empirically confirms that lactate exerts a piezo-protective effect on *L. monocytogenes* inactivation in cooked ham in a strain and dose-dependent magnitude. In addition, this finding is in accordance with previous studies in which HPP was systematically reported to be less effective when lactate was used as antimicrobial in the formulation of meat products or the packaging (Table 1).

At the maximum holding time assessed (10 min), inactivation of *L. monocytogenes* strains in control batches (without organic acids) was higher than in products with 1.4% of added lactate. At this holding time, the difference in inactivation in observed values when comparing control and 1.4% lactate ham was of 0.5, 1.46 and 1.29 log for CTC1011, CTC1034 and Scott A, respectively. In products with 2.8% of added lactate, the difference in *L. monocytogenes*

304 inactivation compared with the product without lactate was enhanced, indicating a piezo-
305 protection effect due to the organic acid. For a holding time of 10 min, the difference in
306 inactivation reached values of 2.51, 1.75 and 2.35 log for CTC1011, CTC1034 and Scott A,
307 respectively, being relevant from a microbiological point of view (> 0.5 log) for holding times
308 higher than 5 min. Diacetate had the opposite effect on *L. monocytogenes* inactivation compared
309 to lactate as sensitized *L. monocytogenes* cells in front of the deleterious effects of HPP. For all
310 studied strains, an enhanced HPP inactivation was observed as shown by the down left shift of
311 the inactivation curves (Figure 1 j, k, l; Table 2) in comparison to the one obtained for control
312 cooked ham. Time for the first log reduction (δ) was reduced by 13, 31 and 20 % in strains
313 CTC1011, CTC1034 and Scott A, respectively, compared with the δ found in cooked ham without
314 organic acids.
315 Interestingly, when organic acids salts (lactate and diacetate) were combined, the effects
316 described above for each organic acid added alone were almost neutralized (Figure 1 m, n, o;,
317 Table 2) and the *L. monocytogenes* inactivation curve was not statistically different (p-
318 value>0.05) from that obtained in control cooked ham, indicating that for each *L. monocytogenes*
319 strain, a common inactivation model could be used for control and 1.4 % lactate plus 0.1 %
320 diacetate batches.
321 The mechanism by which the bacterial inactivation due to HPP is affected when salts of organic
322 acids are added in the culture medium or food product has been scarcely studied. In the present
323 study, while lactate was found to protect *L. monocytogenes* from HPP-inactivation, diacetate
324 enhanced the lethal effect of pressure, indicating that probably *L. monocytogenes* used different
325 mechanisms to respond to lactate and diacetate stresses. In the particular case of lactate, it has
326 been hypothesized that the piezo-protection is related with the a_w decrease as a consequence of
327 the addition of lactate in the product formulation (Shelef, 1994), but in the present study, the
328 addition of organic acid salts did not change the physicochemical parameters of cooked ham
329 compared to the control batch (Section 2.1), and thus, this seems unlikely to be the reason for the
330 observed piezo-protection exerted by lactate on *L. monocytogenes*. Stasiewicz et al. (2011)
331 reported that genes encoding membrane systems involved in ion transport and permeability were

altered during adaptation of *L. monocytogenes* to growth on potassium lactate and diacetate. However, in the present study, *L. monocytogenes* was not grown in the presence of organic acids before the HPP, but was short-term exposed to the organic acid salts of the ham formulation from the moment of inoculation until pressurization of the samples (*ca*. 30 min). Therefore, molecular mechanisms behind a long-term adaptation and a short-term exposure to organic acids may not be the same. On the other hand, transcriptional activation of the general stress and oxidative stress responses have also been reported to be mechanisms used for bacteria for adaptation to organic acids' stress (Suo, Gao, Baranzoni, Xie, & Liu, 2018) and to HPP (Jofre, Garriga, & Aymerich, 2007; Bowman, Bittencourt, & Ross, 2008). Maybe, these genes and proteins could also play a role in *L. monocytogenes* inactivation.

Although the mechanisms involved in the piezo-protective effect of lactate have not been studied, cross-resistance effects between different stresses applied simultaneously have been described by some authors. Higher resistance of *L. monocytogenes* Scott A strain to HPP in semi-skimmed milk than in buffer was reported by Karatzas & Bennik (2002), showing a cross-resistance effect of HPP with the food matrix components, though no specific piezo-protective compound was identified. The HPP-induction of genes encoding cold-shock proteins suggested a cross-resistance with other stresses such as heat stress (Bowman et al., 2008). In another study carried out on brain heart infusion (BHI) broth, pre-exposure of *L. monocytogenes* H7858 strain to organic acid salts (i.e. lactate) induced a cross-protection (i.e. reducing the sensitivity) against other food antimicrobials (nisin and ε–polylysine), being associated with the VirR-mediated genes (Kang, Wiedmann, Boor, & Bergholz, 2015). Additional genomic and transcriptomic studies would be necessary to understand the molecular basis of the piezo-protective effect of lactate on *L. monocytogenes* HPP inactivation.

3.3 Quantification of the dose-dependent piezo-protection of lactate

Despite the available data (Table 1) indicate that lactate protects *L. monocytogenes* from HPP inactivation, to the authors' knowledge, the quantification of this piezo-protective effect has not been performed before. This issue was addressed in the present study through a secondary and

360 global modelling approach. Results are reported in Figure 2, which shows the effect of lactate on
361 Weibull inactivation kinetic parameters, δ and p.
362 The value of δ increased with increasing lactate concentration in a strain-dependent manner,
363 especially for CTC1034 and Scott A. Despite of the transformations (square root, inverse, ln and
364 log) assessed, none of the δ transformations contributed to develop a polynomial model with a
365 better goodness of fit (data not shown). Therefore, non-transformed δ values obtained in the
366 primary modelling were used to develop the models, being the independent term and the quadratic
367 term of the polynomials statistically significant. F-test confirmed the need of three models to
368 quantify the impact of lactate on δ for the three *L. monocytogenes* strains.
369 The p parameter values, which determine the shape of the inactivation curve, were not
370 significantly dependent on lactate concentration as shown in Figure 2. A lack of fit was obtained
371 by fitting polynomials to p values and thus, a fixed value of p for each *L. monocytogenes* strain
372 could be used to describe the shape of the inactivation curve in all the concentrations of lactate.
373 Global fitting of the global model (Eq. 2) to 75 inactivation data points (log N/N_0) for each *L.*
374 *monocytogenes* strain resulted in readjusted values of the terms describing the inactivation
375 parameters δ and p (Table 3) describing satisfactorily the lactate dose-dependent relationship
376 magnitude of the inactivation but also the piezo-resistance characteristics of each strain.
377 The developed models are particularly useful to assess HPP efficacy and find the processing
378 parameters needed to achieve a specific *L. monocytogenes* inactivation and ultimately to comply
379 with the safety standards requested by, for instance, international organizations regarding *L.*
380 *monocytogenes* in ready-to-eat foods. Under the zero tolerance of the USA administration or the
381 British Retail Council (BRC) Certification, a HPP-based post-lethality treatment has to be
382 validated to achieve at least 1 log reduction of the pathogen level. When validated for a 2 log
383 reduction an "increased level of control" is recognized by these institutions (FSIS, 2015; BRC
384 Global Standards, 2018).
385 Using the developed model, the minimum treatment time at 400 MPa necessary to achieve a 2 log
386 reduction and thus, to increase the expected level of control towards *L. monocytogenes*, can be
387 estimated depending on the product formulation (Table 4). Interestingly, for CTC1011 strain, a

388 1.2 min increase in the pressurization time increases the inactivation of the pathogen from 1 log
389 to 2 log in all lactate concentrations. Conversely, lower pressurization times are required to reduce
390 1 log of CTC1034 and Scott A strains in cooked ham formulated without lactate (2.72 and 1.77
391 min, respectively) but more than 1 extra minute is needed to achieve the 2 log reduction (2.7 and
392 1.8 additional min, respectively), showing higher resistance to higher holding times for these
393 strains (Table 4). Moreover, differences in time for the first and second log reduction increment
394 with increasing lactate concentrations, being necessary more than 11 min of pressurization
395 (holding time) to achieve a 2 log reduction of the levels of the strains CTC1034 and Scott A in
396 products with 2.80% of lactate.
397
398 **4. Conclusions**
399 The quantitative modelling approach allowed the characterisation of the lethal effect of HPP on
400 *L. monocytogenes*, showing strain-dependent inactivation curves including convex (i.e. with a
401 shoulder of survival cells during the first minutes of the treatment), linear (i.e. constant
402 inactivation along treatment time) and concave (i.e. indicating the occurrence of a tail of resistant
403 cells), which can be probably related with different molecular mechanisms of response to HPP
404 depending on the strain. Interestingly, the presence of lactate exerted a notable and dose-
405 dependent piezo-protective effect on *L. monocytogenes* in cooked ham but did not modify the
406 strain-specific shape of the inactivation curve. The results showed that for the selection of the
407 pathogen strain to be used for validating HPP, both the HHP duration and lactate concentration
408 (as piezo-protective factor) are of paramount importance. As a result of this work, a versatile *L.*
409 *monocytogenes* pool consisting of strains with different inactivation characteristics was obtained,
410 which can be used in HPP validation studies for cooked meat products formulated either without
411 or with organic acids.
412 This study emphasizes that the design, validation and implementation of high-pressure processing
413 requires a tailor-made approach, considering the specific product formulation and the selection of
414 the most appropriate strain/s.
415

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596

Figure captions

Figure 1. Inactivation of *L. monocytogenes* strains (CTC1011, CTC1034 and Scott A) in cooked ham formulated without (control) and with organic acids (OA) pressurized at 400 MPa for different holding times. Symbols represent the experimental observed inactivation ($\log N/N_0$) data and lines the fit of the Weibull model.

Figure 2. Effect of lactate on the holding time for the first log reduction (δ, plot a) and the shape parameter of the inactivation curve (p, plot b) of each *L. monocytogenes* strain. Diamonds, square and circle symbols represent the kinetic parameters of strains CTC1011, CTC1034 and Scott A, respectively. Secondary model fits for δ are shown in dashed lines.

607 **Table 1.** High pressure inactivation of *L. monocytogenes* in meat products formulated without and with lactate reported in literature.

RTE product	*L. monocytogenes* strain	% Lactate (mode of application)[a]	HPP Treatment	HPP INACTIVATION (log reduction)			Reference
				Control (no lactate)	With Lactate	Difference (control-lactate)	
Cooked ham	Cocktail (CTC1010, CTC1011 and CTC1034)	1.40 (MB)	400MPa, 10 min	3.10	2.51	*0.59*	Marcos et al. (2008)
		1.80 (MB)	400MPa, 10 min	0.66	0.59	*0.07*	Aymerich et al. (2005)
		1.80 (IL)	400MPa, 10 min	1.76	1.50	*0.26*	Jofré et al. (2007)
		1.80 (MB)	600MPa, 5 min	3.79	3.71	*0.08*	Jofré et al. (2008)
Dry-cured ham	Cocktail (CECT4031, CTC1011 and CTC1034)	2.60 (B)	600MPa, 5 min	1.10	0.80	*0.30*	Stollewerk et al. (2012)
		2.60 (B)	600MPa, 5 min	1.60	0.22	*1.38*	Stollewerk et al. (2014)
Cooked turkey	Non-specified	1.80 (MB)	350MPa, 2 min	0.85	0.54	*0.31*	Lerasle et al. (2014)
		1.80 (MB)	350MPa, 8 min	1.42	0.81	*0.61*	Lerasle et al. (2014)
		1.80 (MB)	350MPa, 14 min	1.96	1.20	*0.76*	Lerasle et al. (2014)

608 [a]: Mode of application: B: during salting step; IL: active packaging (surface); MB: meat batter (additive in the product formulation)

609

Table 2. Estimated kinetic parameters resulting from fitting the Weibull model to *L. monocytogenes* inactivation data (log N/N_0) on different formulations of cooked ham pressurized at 400 MPa.

Experimental conditions			Kinetic parameters[a]			Goodness of fit[b]	
Added lactate (%)	**Added diacetate (%)**	***L. monocytogenes* strain**	**$(\log N/N_0)_i$**	**δ (min)**	***p***	**RSS**	**RMSE**
-	-	CTC1011	0.03	5.98	3.62	5.691	0.497
-	-	CTC1034	-0.14	3.89	1.29	6.113	0.516
-	-	Scott A	-0.32	0.70	0.47	19.669	0.946
1.40	-	CTC1011	-0.09	7.00	5.04	11.131	0.696
1.40	-	CTC1034	-0.10	4.62	1.43	28.949	1.122
1.40	-	Scott A	-0.09	0.85	0.40	11.099	0.695
2.80	-	CTC1011	-0.10	7.39	4.48	6.561	0.534
2.80	-	CTC1034	-0.18	7.48	1.17	3.991	0.417
2.80	-	Scott A	-0.13	2.48	0.41	7.961	0.588
-	0.10	CTC1011	-0.18	5.21	2.95	12.884	0.748
-	0.10	CTC1034	-0.12	2.67	1.01	18.786	0.904
-	0.10	Scott A	-0.35	0.56	0.45	30.172	1.145
1.40	0.10	CTC1011	-0.10	6.34	4.02	11.839	0.717
1.40	0.10	CTC1034	-0.10	3.97	1.08	6.619	0.536
1.40	0.10	Scott A	-0.32	1.28	0.51	12.914	0.749

[a] $(\log N/N_0)i$ is the average value of the initial bacterial inactivation of 3 replicates at $t = 0$, δ: holding time for the first log reduction; p: shape of the inactivation curve

[b] n=25 data points (log N/N_0) of each combination of conditions were included for fitting. RSS: residual sum of squares; RMSE: root mean squared error; R^2_{adj}: adjusted coefficient of determination.

616 **Table 3.** Parameter estimates for the global regression model for the inactivation of *L.*
617 *monocytogenes* in cooked ham pressurized at 400 MPa formulated with lactate, obtained for
618 three *L. monocytogenes* strains (CTC1011, CTC1034 and Scott A).

Strain	Kinetic parameters				Goodness of fit		
	$(\log N/N_0)_i$	δ(min)		p	RSS	RMSE	R^2_{adj}
		a	b				
CTC1011	-0.05	6.42	0.11	4.25	24.667	0.577	0.920
CTC1034	-0.14	3.94	0.39	1.35	39.737	0.733	0.659
Scott A	-0.18	0.53	0.29	0.43	38.715	0.728	0.622

619 [a] *(*log $N/N_0)i$ is a fixed value representing the average value of the initial bacterial inactivation
620 of 3 replicates at holding time $t = 0$ (HPP treatment consisting in pressure come-up followed by
621 an immediate pressure release), δ: pressure holding time to cause the first log reduction; p:
622 shape of the inactivation curve (dimensionless).

623 [b] RSS: residual sum of squares; RMSE: root mean squared error; R^2_{adj}: adjusted coefficient of
624 determination.

625

626 **Table 4**. High pressure holding times necessary to cause the 1st and 2nd log reduction[a] of *L.*

627 *monocytogenes* strains (CTC1011, CTC1034 and Scott A) in cooked ham HP treated at 400

628 MPa and at different lactate concentrations predicted from global models of Table 3.

	Time for 1st log reduction (min)			Time for 2nd log reduction (min)		
Lactate (%)	**CTC1011**	**CTC1034**	**Scott A**	**CTC1011**	**CTC1034**	**Scott A**
0.0	6.34	3.52	0.33	**7.51**[a]	6.24	2.10
0.5	6.37	3.61	0.38	**7.54**	6.40	2.39
1.0	6.44	3.88	0.52	**7.63**	6.87	3.26
1.5	6.58	4.32	0.75	**7.79**	7.65	4.72
2.0	6.76	4.93	1.07	8.01	**8.74**	6.75
2.5	7.00	5.73	1.48	8.29	**10.15**	9.37
2.8	7.17	6.29	1.77	8.49	11.14	**11.22**

629 [a]: numbers in bold highlight the longest holding time of HPP to achieve 2 log reduction for each
630 lactate concentration. It facilitates the identification of the most resistant strain depending on the
631 lactate added in the cooked ham.

632