Combined effect of natural antimicrobials and high pressure processing to prevent *Listeria monocytogenes* growth after a cold chain break during storage of cooked ham

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Abstract

The effect of high pressure processing (400 MPa for 10 min) and natural antimicrobials (enterocins and lactate-diacetate) on the behaviour of *L. monocytogenes* in sliced cooked ham during refrigerated storage (1ºC and 6ºC) was assessed. The efficiency of the treatments after a cold chain break was evaluated. Lactate-diacetate exerted a bacteriostatic effect against *L. monocytogenes* during the whole storage period (3 months) at 1ºC and 6ºC, even after temperature abuse. The combination of low storage temperature (1ºC), high pressure processing (HPP) and addition of lactate-diacetate reduced the levels of *L. monocytogenes* during storage by 2.7 log CFU/g. The most effective treatment was the combination of HPP, enterocins and refrigeration at 1ºC, which reduced the population of the pathogen to final counts of 4 MPN/g after 3 months of storage, even after the cold chain break.

Keywords: *L. monocytogenes*, enterocins, lactate, diacetate, high pressure processing, temperature

1. Introduction

A moderate increase of *L. monocytogenes* notifications in meat and meat products other than poultry were detected in 2005 (RASFF, 2005). Most of the notifications (65%) dealt with *L. monocytogenes* in meat products such as salami, ham and cooked ham, regarded as ready-to-eat (RTE) foods. The experience over the past 15 years points to recontamination as the primary source of *L. monocytogenes* in many commercial RTE foods (Tompkin, 2002). This fact, together with the growing demand for minimally processed RTE products poses a challenge to food safety and has led to the development of mild post-processing technologies to inhibit microbial growth while maintaining the freshness and quality of food. Within this context the combination of hurdles to inhibit pathogen growth have acquired high importance. Hurdle technology advocates the deliberate combination of existing and novel preservation techniques in order to establish a series of hurdles that no microorganisms present should be able to overcome (Leistner & Gorris, 1995).

High pressure processing (HPP) is a non-thermal food preservation technology for inactivating post-processing contaminants, especially for foods whose nutritional, sensory and functional characteristics are thermosensitive (Carlez, Rosec, Richard, & Cheftel, 1994). HPP kills and/or sub-lethally injures the cells by destroying the functionality of the cell wall and the cytoplasmic membrane, dissociating the proteins and the ribosomal subunit structures, and inactivating some enzymes (Hoover, Metrick, Papineau, Farkas, & Knorr, 1989). Several reports have dealt with the effect of HPP on *L. monocytogenes* in RTE meat products (Aymerich, Jofré, Garriga, & Hugas, 2005; Hayman, Baxter, O'Riordan, & Stewart, 2004). Pressurized sliced cooked ham is already commercially available in Spain.

Another strategy to prevent the growth of pathogens during storage is the addition of natural antimicrobials as food ingredients. Sodium and potassium lactate are natural compounds derived from lactic acid. Lactates act as a bacteriostat by increasing the lag phase of microorganisms. The addition of lactate to food products with neutral pH has proved to prolong its shelf-life (Houtsma, de Wit, & Rombouts, 1993). A synergistic inhibitory effect of lactate in inhibiting the growth of pathogenic organisms in meat products was observed when combined with diacetate (Mbandi & Shelef, 2002; Samelis, Bedie, Sofos, Belk, Scanga, & Smith, 2002). The EU and FDA permit the use of sodium lactate, potassium lactate and sodium diacetate in

meat products (EC, 1995; FSIS/USDA, 2000). Class IIa bacteriocins, described as being active against *Listeria* sp., are one of the most interesting groups of antimicrobial peptides produced by lactic acid bacteria used in food preservation (Drider, Fimland, Héchard, McMullen, & Prévost, 2006). Among class IIa bacteriocins, enterocins have proved to be effective for controlling *L. monocytogenes* growth in meat products. Enterocins can be applied to meat products in several ways: starter cultures, active packaging, in the meat batter and/or sprayed on the meat surface (Aymerich, Garriga, Ylla, Vallier, Monfort, & Hugas, 2000; Aymerich et al., 2005; Marcos, Aymerich, Garriga, & Monfort, 2006).

The present study was undertaken to assess the effect of HPP, natural antimicrobials and storage temperature against *L. monocytogenes* in sliced cooked ham during refrigerated storage. Moreover, the efficiency of the treatments was evaluated by studying if *L. monocytogenes* was able to overcome the applied hurdles after a cold chain break.

2. Materials and methods

2.1. Enterocin production

Enterococcus faecium CTC492, isolated from a meat product and producer of enterocins A and B (Aymerich, Holo, Havarstein, Hugas, Garriga, & Nes, 1996; Casaus, Nilsen, Cintas, Nes, Hernandez, & Holo, 1997), was grown in modified MRS broth. The composition of standard MRS was modified as follows: reduction of glucose to 0.5%, increase of Tween 80 (Sigma-Aldrich, Saint Louis, MO, U.S.A) to 0.75%, and no addition of beef extract. Enterocins A and B were obtained from a 2 litre culture grown for 15 h at 30ºC. The cells were removed by centrifugation at 10,000 g for 10 min at 4ºC, and 300 g/l of ammonium sulphate (Sigma-Aldrich) were added. The protein precipitate was pelleted by centrifugation at 10,000 g for 30 min and dissolved in 50 mM phosphate buffer, pH 6. A heat treatment of 10 min at 100ºC was applied. The obtained bacteriocin was stored at -80ºC.

2.2 Bacteriocin assay

The indicator strains, *L. monocytogenes* CTC1010, CTC1011, and CTC1034 were separately grown overnight in Tryptic Soy Broth with 0.6% yeast extract (TSBYE; Merck, Darmstadt, Germany) at 30ºC. Bacteriocin activity was quantified by the agar spot test (Tagg, Dajani, &

78 Wannamaker, 1976). A solid medium composed by beef extract, 20 g/l; glucose, 20 g/l and
79 agar, 15 g/l, was used to support soft TSBYE (TSBYE with 7.5 g/l agar) seeded with 20 µl of the
80 overnight mixture of *L. monocytogenes*. Enterocin samples were serially diluted twofold with 50
81 mM phosphate buffer, pH 6. Ten µl of each dilution were spotted onto soft TSBYE lawn. The
82 plates were incubated overnight at 30ºC. An arbitrary unit (AU) was defined as the highest
83 dilution showing growth inhibition of the indicator lawn, and bacteriocin activity was expressed
84 as AU/ml.
85
86 *2.3. Cooked ham manufacturing*
87 Cooked ham was prepared with pork shoulder and the following additives (g/Kg; SKW
88 biosystems, Rubi, Spain): water, 115; sodium chloride, 20.7; dextrose, 5.8; sodium tri-
89 polyphosphate, 5.8; carragenate, 2.3; sodium nitrite, 0.1 and L-ascorbate, 0.6. Pork shoulder
90 meat was minced in a cutter (Teqmaq, Spain) to a particle size of 4 mm. Three independent lots
91 were prepared: a control (lot C), a lot containing 2,400 AU/g of enterocins (lot E), and a lot
92 containing 1.4% potassium lactate and 0.1% sodium diacetate (lot L-D), added as 2.5 %
93 PURASAL Opti.Form 4 (Purac Biochem, Gorinchem, The Netherlands). Ingredients were
94 homogenized in a mixer (model 35P, Tecnotrip S.A., Terrassa, Spain) for 30 min. The meat
95 batter was stuffed into impermeable plastic casings (Prolan SV 150) using a stuffing machine
96 (model H15, Tecnotrip). The product was cooked in an oven at 75ºC until internal temperature
97 reached 72ºC (total cooking time was 2.6 h). Whole pieces of cooked ham were stored at 1ºC
98 before slicing.
99
100 *2.4. Sample preparation and high pressure processing*
101 Cooked ham was sliced at 1.5 mm thickness after aseptic removal of plastic casings. Slices
102 were inoculated with the appropriate dilution of an overnight culture of a 3-strain cocktail of *L.*
103 *monocytogenes* (CTC1010, CTC1011, CTC1034) to reach an inoculation level of 10^4 CFU/g.
104 Slices were vacuum packed in polyamide-polyethylene bags (Sacoliva, Castellar del Vallès,
105 Spain).
106 Half of the samples were non-pressurized and half were subjected to high pressure processing
107 (HPP). Pressurisation was performed at 400 MPa for 10 min at 17ºC. HPP was carried out in an

industrial hydrostatic pressurization unit (Alstom, Nantes, France) with a chamber volume of 320 l and diameter of 280 mm. The pressurization fluid was water, the come up time was 13.5 min, the pressure release time was 1.33 min and the adiabatic heat generated was 5ºC.

2.5. Refrigerated storage, temperature abuse and sampling

After high pressure processing samples were stored at 1ºC or 6ºC for three months. During refrigerated storage samples were submitted to a temperature abuse simulating a cold chain break during the shelf-life of cooked ham. Temperature abuse, which consisted of maintaining the samples for 24 h at room temperature, was performed at days 40 and 60 of storage for samples stored at 6ºC and 1ºC, respectively. Three different packages from each lot (C, E, L-D), temperature (1 ºC, 6ºC) and treatment (non-pressurized, pressurized) combinations were sampled at days 0 (after packaging), 1 (after HPP), 21, 42, 63, and 84 of storage.

2.6. Enumeration of L. monocytogenes

At each selected time, 25 g of cooked ham were 10-fold diluted in sterile buffered peptone water (BPW) (AES Laboratoires, Combourg, France). The solution was homogenized for 1 min in a Masticator (IUL Instruments, Barcelona, Spain). After appropriate dilutions, enumeration of *Listeria* was performed by spread plating on Palcam agar (Merck, Darmstadt, Germany) with supplement SR0150 (Merck) incubated at 30 °C for 72 h. Five colonies of each plate were confirmed by PCR. Two μl of a colony suspended in 25 μl of distilled water were used for the assay. For expected counts under 10 CFU/g, the most probable number (MPN) technique was also used. Serial dilutions of 3 BPW tubes of three successive dilutions were incubated at 37ºC for 40 hours. BPW was used as a non-selective media in order to recover the sublethally injured bacteria. The growth of *L. monocytogenes* in MPN tubes was determined by PCR. To determine presence/absence of the pathogens, homogenates were incubated at 37ºC for 40 h.

2. 7. Pre-PCR treatment of samples and *PCR reactions*

As a pre-PCR treatment, a Chelex® 100-based DNA purification was performed using 1.4 ml of enriched homogenates or MPN tubes showing growth. Briefly, the pellet from the 1.4-ml enriched culture was dissolved in 300 μl of 6% Chelex® 100 (BioRad, Munich, Germany),

incubated at 56ºC for 20 min, boiled for 8 min, and cooled on ice. Thorough mixing between each step was needed. Cell debris were centrifuged at 13,000 g for 5 min and the supernatant used for PCR reaction.

A validated species-specific PCR assay was used (D'Agostino et al., 2004). Two μl of DNA, 2 μl of 10× PCR buffer (Invitrogen, Merelbeke, Belgium), 2.5 mM $MgCl_2$, 150 mM each dNTP, 0.3 mM each primer (Lip1 (5'-gatacagaaacatcggttggc) and Lip2 (5'-gtgtaacttgatgccatcagg), 1 mg/ml BSA, and 1 U of Taq DNA polymerase (Invitrogen) and 1pg of the corresponding internal amplification control. The PCR program was: 2 min at 94ºC and 40 cycles of 30 s at 94ºC, 30 s at 64ºC, 1 min at 74ºC and a final extension of 5 min at 74ºC. The sensitivity of both PCR reactions is 10 CFU/reaction (D'Agostino et al., 2004; Malorny, Tassios, Radstrom, Cook, Wagner, & Hoorfar, 2003).

2.8. Statistical analysis

Data were analysed using the GLM procedure from the SAS statistical package (SAS © System for Windows, Release 8.2, SAS Institute, Cary, NC, USA).

The model included lot, storage temperature, storage time, and their interaction as fixed effects. Differences between effects were assessed by the Tukey test ($p<0.05$).

3. Results and discussion

3.1. Refrigerated storage

Refrigeration of sliced cooked ham at 6ºC allowed the growth of *L. monocytogenes* in control lot from inoculated levels (4.5-5 log CFU/g) to a value of 8.6 log CFU/g at day 21 of storage (Fig. 1). By contrast, no growth of *L. monocytogenes* ($p<0.05$) was observed during refrigeration in any lot of cooked ham stored at 1ºC (Fig. 2). Thus, refrigeration at the lowest temperature in itself proved to have a bacteriostatic effect against *L. monocytogenes*. The lower growth limit of *L. monocytogenes* in sterile foods having a neutral pH and high nutrient content is reported to be about 0ºC (ICMSF, 1996). Therefore, if the storage temperature was sufficiently low, the lag phase of *L. monocytogenes* would be longer than the shelf-life of the product and, even if the organism was theoretically able to grow, no growth would occur before consumption. However, in a product with a long shelf-life such as vacuum packed cooked ham, growth even at low

168 temperature could occur before spoilage of the product. Besides, it is unrealistic to think of
169 maintaining those low temperature values throughout the distribution chain. Temperature
170 measurements from domestic and retail refrigerators have proved that food products are
171 commonly stored at abusive temperatures. Sergelidis, Abrahim, Sarimvei, Panoulis,
172 Karaioannoglou, & Genigeorgis (1997) reported that 55% and 32% of investigated domestic and
173 retail refrigerators, respectively presented temperatures ≥9ºC. Temperatures from 2 to 12ºC
174 were found by Bakalis, Giannakourou, & Taoukis (2004) in domestic refrigerators. This
175 evidence suggests the need to apply further hurdles to chilled storage, such as addition of
176 antimicrobials to ham formulation, to prevent *L. monocytogenes* growth.
177 The addition of enterocins (Fig. 1) to the meat batter did not produce any difference with control
178 lot ($p>0.05$) in cooked ham stored at 6ºC. These results evidence that enterocins added to ham
179 formulation in a concentration of 2,400 AU/g were not effective in preventing the pathogen
180 growth. In a previous work, though, 1,600 AU/g of enterocins spread on sliced cooked ham
181 delayed growth of *L. innocua* (Aymerich et al., 2000). The higher efficiency of a lower enterocin
182 concentration could be explained either by the variation in susceptibility of *Listeria* strains to
183 bacteriocins (Ennahar, Deschamps & Richard, 2000) or by the fact that spreading would permit
184 a more direct contact between enterocins and *Listeria* cells. Moreover, the activity of
185 bacteriocins added to meat formulations could be affected by many factors such as adsorption
186 by proteins and interactions with fat (Drider et al., 2006; Hugas, Garriga, Aymerich, & Monfort,
187 2002)
188 The addition of 1.4 % potassium lactate and 0.1% sodium diacetate to the formulation of ham
189 exerted a bacteriostatic effect against *L. monocytogenes* during storage both at 1ºC and 6ºC
190 (Fig. 1 & 2). At day 21 counts in L-D lot were 4 logarithms lower than in the control lot at 6ºC.
191 The bacteriostatic effect of L-D was also reported by Blom et al. (1997) in cooked ham stored at
192 4ºC with 2% and 0.25% of sodium lactate and sodium diacetate, respectively. Mbandi & Shelef
193 (2002) also observed a listeriostatic effect of 2.5% lactate and 0.2% diacetate in beef bologna
194 stored at 5ºC.
195 High pressure processing (400 MPa, 10 min at 17ºC) produced an immediate reduction of *L.*
196 *monocytogenes* in a range of 2.5-3.4 logarithms from inoculated levels (Figs. 3 & 4). After HPP,
197 *L. monocytogenes* started to grow in the control lot stored at 1ºC, reaching counts of 3 log

198 CFU/g at day 42 (Fig. 3). In the L-D lot, though, the pathogen was maintained at the levels
199 achieved after HPP throughout 60 days of storage at 1ºC. Higher counts of *Listeria* ($p<0.05$)
200 were observed in L-D than in C lot until day 21, while at day 42, the counts in C lot reached the
201 same levels as in L-D. Pressurized samples containing enterocins maintained levels of 1.90 log
202 CFU/g during storage at 1ºC. At day 42, E lot presented lower levels of *L. monocytogenes*
203 ($p<0.05$) than the other lots. The higher levels of *L. monocytogenes* observed in pressurized L-
204 D with respect to C and E lots could be related with the protective effect of L-D against pressure
205 inactivation of *L. monocytogenes* as suggested by Aymerich et al. (2005). The product stored at
206 6ºC, presented counts of *Listeria* in a range of 2.1-2.6 log CFU/g at day 21, with no significant
207 differences between lots.
208
209 *3.2. Cold chain break and further refrigerated storage*
210 To assess whether the antimicrobial treatments applied could effectively control the growth of
211 the pathogen after a cold chain break, temperature abuse was applied at days 40 and 60 of
212 storage to samples stored at 6 ºC and 1ºC, respectively.
213 Non-pressurized C and E lots stored at 1ºC allowed a rapid growth of *L. monocytogenes* after
214 temperature abuse, reaching about 7 log CFU/g at day 63 (Fig. 2). Thus, 2,400 AU/g of
215 enterocins added to the formulation of ham were not effective to limit *L. monocytogenes* growth
216 at abusive temperatures. C and E lots stored at 6ºC already showed counts of 8 log CFU/g
217 before temperature abuse (Fig. 1). Aymerich et al. (2000) observed growth of *L. innocua* to
218 numbers exceeding 10^7 CFU/g after 37 days at 7ºC in sliced cooked ham stored with 1,600
219 AU/g of enterocins spread over slices, while a higher concentration (4,800 AU/g) limited the
220 growth without inhibiting it completely.
221 By contrast, *Listeria* counts remained stable, even after the cold chain break, in L-D lot stored at
222 both 1ºC and 6ºC. Thus, the addition of L-D in cooked ham was the only treatment that
223 effectively controlled *L. monocytogenes* in non-pressurized samples. With the same product
224 formulation Aymerich et al. (2005) also found that a concentration of 1.8% of potassium lactate
225 was able to inhibit the growth of *L. monocytogenes* for 3 months at 1ºC. However, they
226 observed a growth to 10^8 CFU/g after 3 months of storage at 6ºC. Therefore, in the present
227 work we confirmed the improved bacteriostatic effect against *L. monocytogenes* of lactate

228 combined with diacetate for long storage periods at higher refrigeration temperatures. These
229 results are consistent with those of Schlyter, Glass, Loeffelholz, Degnan, & Luchansky (1993)
230 and Vogel, Ng, Hyldig, Mohr, & Gram (2006) who observed greater antilisterial activity when L
231 and D were incorporated than when only one compound was used.
232 At the end of storage (84 days), despite the cold chain break, lower counts ($p<0.05$) of *L.*
233 *monocytogenes* were observed in pressurized sliced cooked ham than in non-pressurized only
234 when the product was stored at 1ºC. Among pressurized samples, no increase above initial
235 numbers was observed in any of the lots after three months of storage at 1ºC. After the great
236 initial reduction of *L. monocytogenes* produced by HPP, C lot supported a gradual growth
237 throughout storage at 1ºC, reaching the inoculated levels at day 84 (Fig. 3). In L-D and E lots a
238 reduction of initial population of 2.5 and 4.6 logarithms, respectively, was observed during
239 storage at 1ºC. The counts obtained after HPP in L-D lot (2.7 log CFU/g) were maintained
240 during subsequent storage of the product, even after temperature abuse. In E lot, though, an
241 additional reduction was observed afterwards until final counts of 4 MPN/g. Therefore it seems
242 that the stress produced by HPP and storage at 1ºC to *L. monocytogenes* enhanced the
243 listericidal effect of enterocins. Kalchayanand, Sikes, Dunne, & Ray (1994, 1998) reported the
244 synergistic effect of HPP and bacteriocins, and its efficiency in meat products and meat
245 homogenates has also been demonstrated (Aymerich et al., 2005; Garriga, Aymerich, Costa,
246 Monfort, & Hugas, 2002).
247 It is important to highlight that despite the break of the cold chain, no further growth of *L.*
248 *monocytogenes* was observed in pressurized cooked ham when seasoned with natural
249 antimicrobials, and the storage temperature was 1ºC. However, a different behaviour was
250 observed when the product was stored at 6ºC (Fig. 4). In pressurized C lot, a 5-log increase
251 was observed from day 21 to day 42 after temperature abuse. In pressurized E lot, although the
252 levels of the pathogen were maintained around 2 log CFU/g immediately after the cold chain
253 break (day 42), during subsequent storage *L. monocytogenes* achieved values of 7 log CFU/g
254 at day 63. The presence of enterocins in the product delayed the recovery of the pathogen,
255 nonetheless its antimicrobial effect was not powerful enough to prevent its growth when stored
256 at 6ºC. Finally, the bacteriostatic effect of L-D could overcome temperature abuse for 63 days in
257 pressurized samples, the difference being of about 5 logarithms compared with C and E lots at

6ºC. During further refrigeration, *L. monocytogenes* reached initial counts (day 84). Therefore in the pressurized product stored at 6ºC, only L-D was able to control *L. monocytogenes* growth.

From the above results it can be concluded that the addition of L-D, regardless of storage temperature, exerted a bacteriostatic effect against *L. monocytogenes* during the shelf-life of cooked ham. Taking into account that L-D is a GRAS additive commercially available, the use of L-D could be advisable in RTE cooked ham, when we consider the risk of a cold chain break and the fact that during transportation and at retail level refrigeration is out of the manufacturer's direct control and often deviates from specifications. Nevertheless the combination of different hurdles (pressurization, storage at 1ºC and natural antimicrobials) was necessary to reduce the population of *L. monocytogenes*. The actual legal framework (EC, 2005) lays down the limit of *L. monocytogenes* in RTE foods able to support its growth to 100 CFU/g. From our results, the treatment consisting of pressurization, storage at 1ºC and addition of enterocins accomplished the safety criterion when high initial contamination levels are considered.

Acknowledgements

This work was funded by Ministry of Science and Technology (project MCYT AGL2002-03496), and the National Institute for Food and Agricultural Research, INIA (project CPE 03-012-C3B). B. Marcos is recipient of a pre-doctoral grant awarded by INIA.

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370 smoked salmon and has no adverse sensory effects. *Journal of Food Protection, 69*, 2134-
371 2142.
372
373

374 **Figure Captions**

375

376 **Figure 1**. *L. monocytogenes* counts of non-pressurized sliced cooked ham during storage at
377 6ºC of control (C), enterocin (E), and lactate-diacetate (L-D) lots. Bars indicate standard
378 deviation of triplicate samples.

379

380 **Figure 2.** *L. monocytogenes* counts of non-pressurized sliced cooked ham during storage at
381 1ºC of control (C), enterocin (E), and lactate-diacetate (L-D) lots. Bars indicate standard
382 deviation of triplicate samples.

383

384 **Figure 3**. *L. monocytogenes* counts of pressurized (400 MPa for 10 min) sliced cooked ham
385 during storage at 1ºC of control (C), enterocin (E), and lactate-diacetate (L-D) lots. Bars indicate
386 standard deviation of triplicate samples.

387

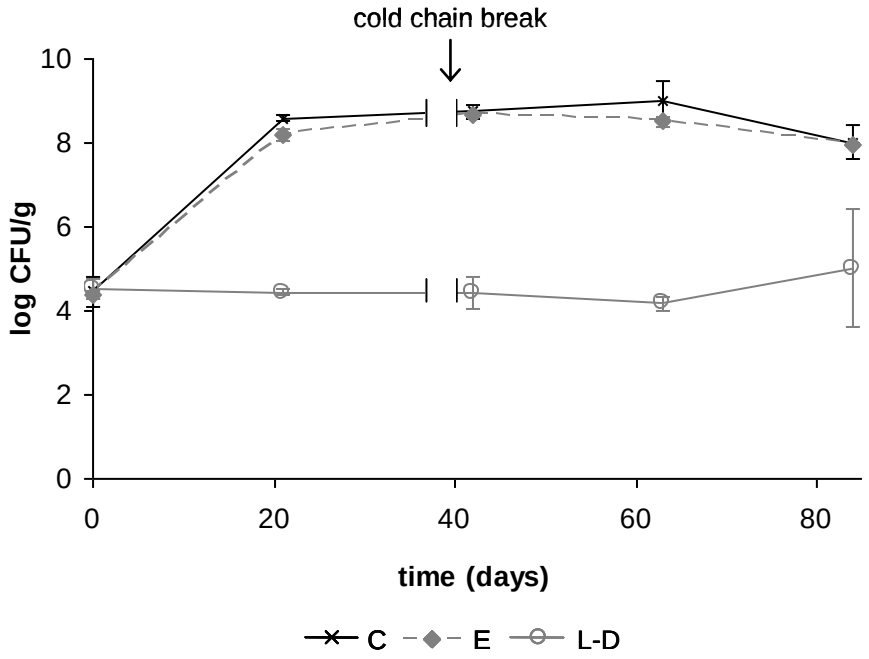
388 **Figure 4**. *L. monocytogenes* counts of pressurized (400 MPa for 10 min) sliced cooked ham
389 during storage at 6ºC of control (C), enterocin (E), and lactate-diacetate (L-D) lots. Bars indicate
390 standard deviation of triplicate samples.

391

392

393 **Figure 1**. *L. monocytogenes* counts of non-pressurized sliced cooked ham during storage at
394 6ºC of control (C), enterocin (E), and lactate-diacetate (L-D) lots. Bars indicate standard
395 deviation of triplicate samples.

396



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400 1ºC of control (C), enterocin (E), and lactate-diacetate (L-D) lots. Bars indicate standard
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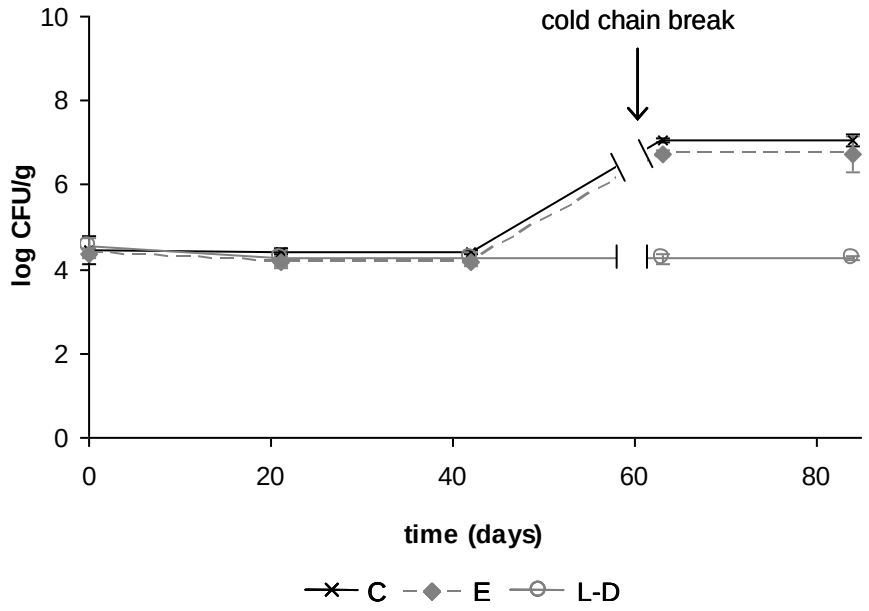

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406 during storage at 1ºC of control (C), enterocin (E), and lactate-diacetate (L-D) lots. Bars indicate
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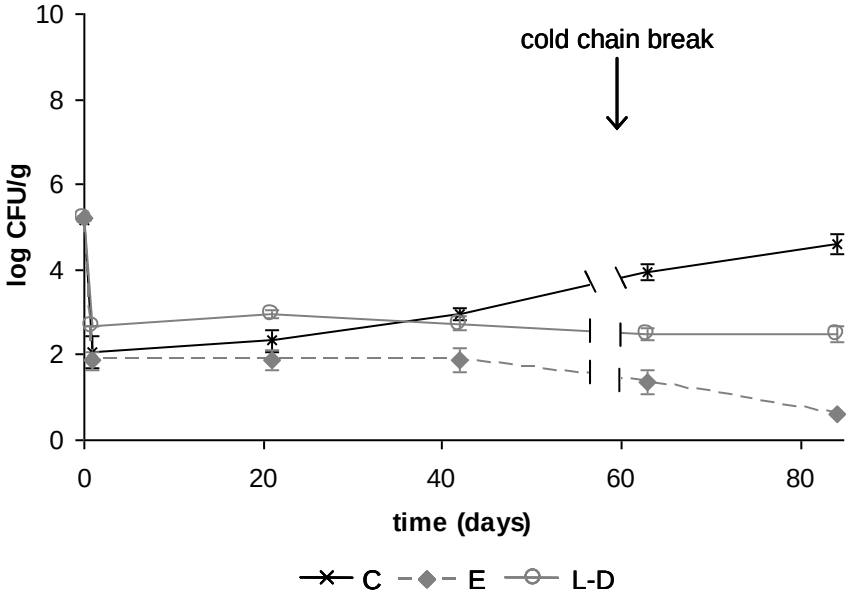

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412 during storage at 6ºC of control (C), enterocin (E), and lactate-diacetate (L-D) lots. Bars indicate
413 standard deviation of triplicate samples.

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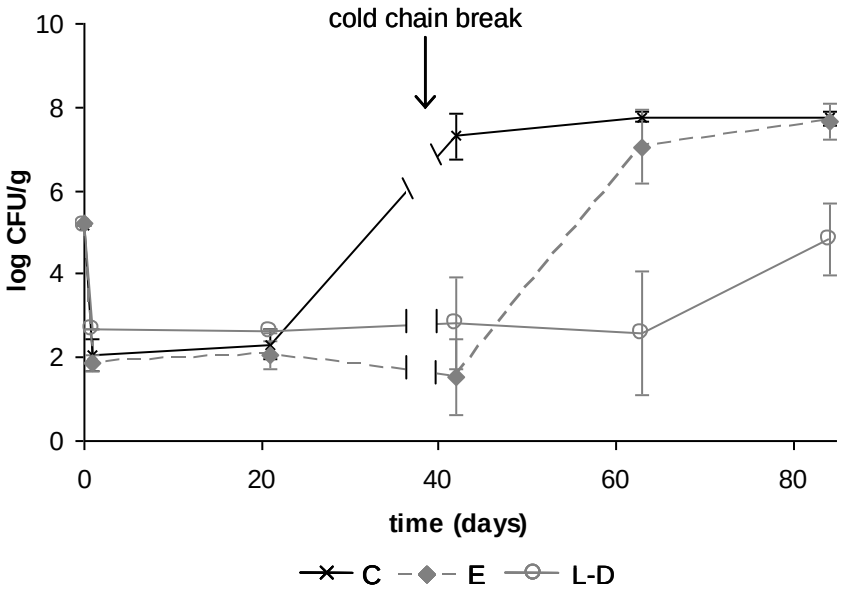

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