HIGH PRESSURE PROCESSING AND ANTIMICROBIAL BIODEGRADABLE PACKAGING TO CONTROL *LISTERIA MONOCYTOGENES* DURING STORAGE OF COOKED HAM

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Abstract

The efficiency of combining high pressure processing (HPP) and active packaging technologies to control *L. monocytogenes* growth during the shelf life of artificially inoculated cooked ham was assessed. Three lots of cooked ham were prepared: control, packaging with alginate films, and packaging with antimicrobial alginate films containing enterocins. After packaging, half of the samples were pressurized. Sliced cooked ham stored at 6ºC experienced a quick growth of *L. monocytogenes*. Both antimicrobial packaging and pressurization delayed the growth of the pathogen. However, at 6ºC the combination of antimicrobial packaging and HPP was necessary to achieve a reduction of inoculated levels without recovery during 60 days of storage. Further storage at 6ºC of pressurized antimicrobial packed cooked ham resulted in *L. monocytogenes* levels below the detection limit (day 90). On the other hand, storage at 1ºC controlled the growth of the pathogen until day 39 in non-pressurized ham, while antimicrobial packaging and storage at 1ºC exerted a bacteriostatic effect for 60 days. All HPP lots stored at 1ºC led to counts <100 CFU/g at day 60. Similar results were observed when combining both technologies. After a cold chain break no growth of *L. monocytogenes* was observed in pressurized ham packed with antimicrobial films, showing the efficiency of combining both technologies.

1. Introduction

The thermal treatment applied to processed ready-to-eat (RTE) meat products generally eliminates *Listeria monocytogenes*. However, products can be recontaminated by exposure to the environment during peeling, slicing, repackaging, and other procedures (FSIS, 2006). Moreover, prolonged shelf life at refrigeration temperatures may contribute to the survival and growth of *L. monocytogenes*, a pathogen capable of exponential growth at refrigeration temperatures (Duffy et al., 1994; Glass and Doyle, 1989; Rocourt and Cossart, 1997).

Interest in using high pressure processing (HPP) to improve safety and to extend the shelf-life of low-acid food products is increasing (Aymerich et al., 2005; Cheftel, 1996; Lucore et al., 2000). HPP is capable of inactivating microorganisms and endogenous enzymes, while maintaining nutrients and flavours (Ross et al., 2003). Several reports have dealt with the effect of HPP on *L. monocytogenes* in RTE meat products (Aymerich et al., 2005; Hayman et al., 2004). Overall, HPP is effective for inactivating vegetative cells of microorganisms. The hurdle concept is being studied in many applications because it often allows reduced intensity of any antimicrobial treatment while improving the overall antimicrobial protection (Sebranek and Houser, 2006). Preservation of RTE meat products with HPP requires the choice of the appropriate combined technologies to achieve the desired levels of microbial inactivation and shelf life extension (Raso and Barbosa-Canovas, 2003).

Recent studies have indicated that HPP inflicts sublethal injury on microorganisms, even at lower pressures than those required for their death (Patterson et al., 1995). Sublethally injured cells are more susceptible to antimicrobial compounds (Kalchayanand et al., 1994). The high levels of inactivation observed with the application of antimicrobial agents and HPP are believed to be due to the combined factors of destabilization of membrane structure or function, their specific modes of action being different (Kalchayanand et al., 1994; Masschalck et al., 2001). Following to sublethal stress, barrier functions of cell wall structures are impaired in the injured survivors, facilitating the contact between antimicrobials and cell membrane (Kalchayanand et al., 1994). Several studies have considered the combined effect of HPP and bacteriocins (Garriga et al., 2002; Kalchayanand et al., 1992; Kalchayanand et al., 1998). Antimicrobial packaging is a practical way of applying bacteriocins to meat products. Bacteriocins are added to the packaging materials and will be released to the meat surface during storage.

64 The risk assessment for *L. monocytogenes* in deli meats carried out by the FSIS (Gallagher et al.,
65 2003) indicated that the use of a combination of interventions in deli meats exposed to the
66 environment after the lethal treatment had the greatest impact on lowering the risk of illness or death
67 from *L. monocytogenes.* Therefore, the final rule for the control of *L. monocytogenes* (FSIS, 2003)
68 includes three alternative approaches that establishments can take in the processing of RTE meat
69 products during post-lethal exposure. The first alternative proposed the use of a post-lethal treatment
70 and an antimicrobial agent; the second one, proposed the use of either a post-lethal treatment or an
71 antimicrobial agent; and the third one, proposed the use of sanitation measures only.
72 Following these proposals, the objective of the present work was to compare the effectiveness of three
73 strategies to control *L. monocytogenes* during refrigerated storage of sliced cooked ham: application
74 of HPP, antimicrobial packaging and combined use of antimicrobial packaging and HPP, combined
75 with two different storage temperatures.

76 ## 2. Materials and methods

77 ### *2.1. Bacteriocin production*

78 *Enterococcus faecium* CTC492, isolated from a meat product and producer of enterocins A and B
79 (Aymerich et al., 1996; Casaus et al., 1997), was grown in modified MRS broth. The composition of
80 standard MRS was modified as follows: reduction of glucose to 0.5%, increase of Tween 80 (Sigma-
81 Aldrich, Saint Louis, MO, U.S.A) to 0.75%, without addition of beef extract. Enterocins A and B were
82 obtained from a 2 litre culture grown for 15 h at 30ºC. The cells were removed by centrifugation at
83 10000 g for 10 min at 4ºC, and 300 g/l of ammonium sulphate (Sigma-Aldrich) were added. The
84 protein precipitate was pelleted by centrifugation at 10000 g for 30 min and dissolved in 50 mM
85 phosphate buffer, pH 6. An additional heat treatment of 10 min at 100ºC was applied. The obtained
86 bacteriocins were stored at -80ºC.

87 ### *2.2. Bacteriocin assay*

88 The indicator strains, *L. monocytogenes* CTC1010, CTC1011, and CTC1034 were separately grown
89 overnight in Tryptic Soy Broth with 0.6% yeast extract (TSBYE; Merck, Darmstadt, Germany) at 30ºC.
90 Bacteriocin activity was quantified by the agar spot test (Tagg et al., 1976). A solid medium composed
91 of, in g/l, 20 beef extract, 20 glucose, and 15 agar, was used to support soft TSBYE (TSBYE with 7.5

92 g/l agar) seeded with 20 µl of the overnight mixture of *L. monocytogenes*. Enterocin samples were
93 serially diluted twofold with 50 mM phosphate buffer, pH 6. A 10 µl sample of each dilution was
94 spotted onto soft TSBYE lawn. The plates were incubated overnight at 30ºC. An arbitrary unit (AU)
95 was defined as the highest dilution showing growth inhibition of the indicator lawn, and bacteriocin
96 activity was expressed as AU/ml.

97 *2.3. Film manufacturing*

98 Film forming solutions were obtained as suggested by Del Nobile et al. (2003) and Buonocore et al.
99 (2005), with some modifications. Alginate solutions were obtained by stirring for 2 hours at 80ºC a 5%
100 (w/v) alginic acid (Sigma-Aldrich) solution in distilled water. Glycerol (5% v/v) was added as plasticizer,
101 and the solution was stirred at ambient temperature for 30 min. After measuring the volume of the film
102 blend, the active solution was obtained by adding the appropriate dilution from the stock solution of
103 enterocins (409,600 AU/ml) to obtain a concentration of 2,000 AU/cm^2. The solution was stirred at
104 ambient temperature until completely dissolved. The films were manufactured by casting 3 ml of the
105 prepared solutions onto sterile polystyrene dishes (28 cm^2) and were dried under a biological safety
106 cabinet. After drying, alginate films were reticulated by immersion in a 2% (w/v) calcium chloride
107 solution. The thickness of the films obtained was measured by means of a Digimatic Micrometer
108 (Mitutoyo, Japan). The value of the film thickness was obtained by averaging 10 measurements. The
109 films obtained had an average thickness of 120±10 µm.

110 *2.4. Cooked ham manufacturing*

111 Cooked ham was prepared with pork shoulder and the following additives (g/Kg; SKW Biosystems,
112 Rubí, Spain): water, 115; sodium chloride, 20.7; dextrose, 5.8; sodium tripolyphosphate, 5.8;
113 carragenate, 2.3; $NaNO_2$, 0.1; and L-ascorbate, 0.6. Pork shoulder meat was minced at -1ºC in a
114 cutter (Teqmaq, Spain) to a particle size of 4 mm. Ingredients were homogenized in a mixer (model
115 35P, Tecnotrip S.A., Terrassa, Spain) for 30 min. The meat batter was stuffed into 90 mm diameter
116 plastic casings (Prolan, Proveedora Hipano Holandesa, S.A., Sant Boi de Llobregat, Spain) using a
117 stuffing machine (model H15, Tecnotrip). The product was cooked in a steam oven (Doleschal,
118 Dordal,S.A., Santa Perpètua de la Mogoda, Spain) at 85ºC until internal temperature reached 75±2ºC,

(temperature probe TM 65, Crison Instruments S.A., Barcelona, Spain). Whole pieces of cooked ham were stored at 1ºC for 24 h before slicing.

2.5. Sample preparation and high pressure processing

Cooked ham was sliced at 7 mm thickness after removal of plastic casings and cut with a mould to fit film size. Slices were inoculated with 10^4 CFU/g of a 3-strain cocktail of *L. monocytogenes* (CTC1010, CTC1011, CTC1034). An overnight culture of each strain was prepared by inoculation into TSBYE broth followed by incubation at 30 °C .Slices were placed between two films and packed under vacuum in polyamide-polyethylene bags (Sacoliva, Castellar del Vallès, Spain). Three independent lots were prepared: a control without film, a lot packed with alginate control films (AC), and a lot packed with alginate films containing 2,000 AU/cm^2 of enterocins (AE).

Half of the samples were non-pressurized and half were subjected to high pressure processing (HPP). Pressurization 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.

2.6. Refrigerated storage and temperature abuse

After high pressure processing samples were stored at 1ºC or 6ºC for two months. After 60 days of refrigerated storage, samples stored at 1ºC were submitted to temperature abuse, consisting of maintaining the samples for 24 h at room temperature (c. 20ºC), simulating a cold chain break during the shelf-life of cooked ham. The behaviour of *L. monocytogenes* at 6ºC was further studied until day 90.

2.7. Enumeration of L. monocytogenes

During refrigerated storage of vacuum-packed cooked ham sampling was performed at days 0 (after packaging), 1 (after HPP), 4, 8, 15, 22, 30, 39, 50 and 60. After the cold chain break for the product at 1ºC and during further refrigeration at 6ºC, cooked ham was sampled at days 77 and 90.

At each selected time, 20 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

146 Masticator (IUL Instruments, Barcelona, Spain). After appropriate dilutions, enumeration of *Listeria*
147 was performed by spread plating on Palcam agar (Merck, Darmstadt, Germany) with supplement
148 SR0150 (Merck) incubated at 30ºC for 72 h. To determine presence/absence of the pathogen,
149 homogenates were incubated at 37ºC for 40 h and spread plated on Palcam agar.
150 Three different packages from each lot (C, AC, AE), temperature (1ºC, 6ºC) and treatment (non-
151 pressurized, pressurized) combinations were sampled at each sampling time.

152 *2.8. Statistical analysis*

153 Data were subjected to analysis of variance using the General Linear Model procedure from the SAS
154 statistical package (SAS © System for Windows, Release 8.2, SAS Institute, Cary, NC, USA).
155 The model included lot, storage temperature, storage time, and their interaction as fixed effects.
156 Differences between effects were assessed by the Tukey test ($p<0.05$).

157 **3. Results and discussion**

158 No significant differences in *L. monocytogenes* numbers ($p>0.05$) were observed between control (C)
159 and the alginate control (AC) lots throughout storage (data not shown). These results evidence that
160 packaging with alginate films in itself had no effect on the behaviour of *L. monocytogenes.* Cooked
161 ham packed with control films (AC) supported a quick growth of *L. monocytogenes* at 6ºC, reaching
162 the maximum growth (8.6 log CFU/g) in 22 days (Fig. 1). The ability of *L. monocytogenes* to grow
163 exponentially in cooked ham refrigerated at 6ºC reflects the necessity of applying additional hurdles to
164 refrigeration in order to prevent the growth of *L. monocytogenes* in contaminated food.
165 Antimicrobial packaging is a promising form of active packaging especially for meat products (Kerry et
166 al., 2006). Moreover, the effectiveness of alginate films containing bacteriocins against food-borne
167 pathogens has recently been demonstrated (Millette et al., 2007). In the present study, vacuum
168 packaging with alginate films containing 2,000 AU/cm^2 of enterocins (AE), significantly delayed the
169 growth of *L. monocytogenes* at 6ºC (Fig. 1). Antimicrobial packaging was able to inhibit the growth of
170 the pathogen during the first days of storage, extending the lag phase of *L. monocytogenes* until day
171 8. Although there was further growth, the pathogen did not exceed inoculated levels for 15 days in the
172 AE lot. This finding is consistent with previous observations in cooked ham contaminated with *L.*
173 *monocytogenes* packed with alginate films containing 2,000 AU/cm^2 of enterocins (Marcos et al.,

2006). The bacterial counts in the AE lot were 1.6 to 3 logarithms lower than in the AC lot until day 30; no significant differences between both lots ($p>0.05$) were observed thereafter. In the AE lot, *L. monocytogenes* reached the stationary phase (8.3 log CFU/g) at day 39 (Fig. 1). Therefore, antimicrobial packaging (alginate films containing 2,000 AU/cm^2) of sliced cooked ham stored at 6ºC significantly delayed the growth of *L. monocytogenes*. The growth, although delayed, was evident, demonstrating the necessity of introducing additional hurdles such as the application of a non-thermal treatment. Non-thermal technologies such as gamma irradiation, have been applied together with antimicrobial packaging as a method for controlling *L. monocytogenes* growth (Ouattara and Sabato, 2001). To our knowledge this is the first report about the combined use of antimicrobial packaging and high pressure processing (HPP) technologies.

High pressure processing (400 MPa, 10 min, 17ºC) of control samples produced an immediate reduction of *L. monocytogenes* population of 3.4 log units. The levels obtained after HPP (0.6 log CFU/g) were maintained for 8 days in the AC lot stored at 6ºC (Fig. 1). Until day 8 differences of 3.7-4.4 logarithms were observed between pressurized and non-pressurized ham packed with control films. Further storage of the pressurized AC lot allowed the growth of *L. monocytogenes*, though, no increase ($p>0.05$) from inoculated levels (10^4 log CFU/g) was achieved after 22 days. Throughout this period, the pressurized AC lot showed lower counts of *L. monocytogenes* ($p<0.05$) compared with the non-pressurized AE lot. The comparison of both technologies evidences that pressurization was more effective in controlling *L. monocytogenes* at 6ºC than antimicrobial packaging. Although it improved pathogen control, HPP alone (400 MPa for 10 min) was not able to prevent pathogen growth (Fig. 1).

Preservation by combined technologies generally enhances antimicrobial effects with the resultant extended shelf life (Leistner, 2000; Raso and Barbosa-Canovas, 2003). Figure 1 shows the good performance of the combined application of antimicrobial packaging and HPP technologies (pressurized AE lot). After pressurization the levels obtained in the AE lot (0.6 log CFU/g) were maintained, allowing an extension of the lag phase of *L. monocytogenes* until day 22. A slight increase of the pathogen was observed afterwards, reaching counts of 1.4-1.8 log CFU/g, which were maintained until the end of storage (day 60). After two months of storage, the counts of the pathogen were 6.8-7.3 logarithms lower in the pressurized AE lot compared with other lots.

By day 60 of the storage period both pressurized and non-pressurized AC lots, and the non-pressurized AE lot had already reached the stationary phase (8.2-8.8 log CFU/g). The performance of

204 the combination of HPP and antimicrobial packaging was assessed during further storage. Table 1
205 shows the counts obtained until day 90 of storage at 6ºC of sliced cooked ham. The pressurized AE lot
206 not only maintained but also reduced the counts during further storage. It is important to highlight that
207 after three months of storage at 6ºC, pressurization of sliced cooked ham packed with alginate films
208 containing 2,000 AU/cm^2 was not only able to prevent the growth of *L. monocytogenes* but also to
209 reduce the counts to below the detection limit (5 CFU/g).
210 Besides, although *L. monocytogenes* is able to grow during refrigerated storage, temperature control
211 has proved to be essential in reducing the risk of the pathogen. Refrigeration at 1ºC of sliced cooked
212 ham packed with control films showed no growth of *L. monocytogenes* for 39 days (Fig. 2). Those
213 results agree with previous studies where refrigeration at 1ºC effectively controlled the growth of the
214 pathogen on sliced cooked ham for 40 days (Marcos et al., in press). Figure 2 shows an increase of
215 1.4 log CFU/g from inoculated levels during further storage of the product. At the end of storage, the
216 counts in the AC lot stored at 1ºC were 3.4 logarithms lower than at 6ºC.
217 At 1ºC, packaging of cooked ham with films containing enterocins (AE lot) gave lower counts ($p<0.05$)
218 than in the control lot throughout storage. Figure 2 shows *L. monocytogenes* numbers as being 1
219 logarithm lower than inoculated levels from day 30 until day 39, and its recovery to initial counts by the
220 end of storage. Thus, antimicrobial packaging applied as an additional hurdle to low refrigeration
221 temperature (1ºC) was effective for controlling *L. monocytogenes* during storage of 60 days. The
222 importance of refrigeration temperature should be noted: storage at 1ºC became a key factor in
223 preventing *L. monocytogenes* growth. However, commercial and home refrigerator temperatures may
224 run at higher temperatures (Bakalis et al., 2003; Sergelidis et al., 1997), and application of additional
225 technologies, such as HPP and antimicrobial packaging, would assure the safety of contaminated
226 foods during its shelf life. Pressurization of the AC lot followed by refrigeration at 1ºC effectively
227 reduced inoculated numbers of *L. monocytogenes* around the detection limit, with this level being
228 maintained throughout storage. Similarly, during the whole storage the pressurized AE lot stored at
229 1ºC presented levels around 0.6 log CFU/g for 60 days (Fig. 2). Absence of *L. monocytogenes* was
230 not achieved in any of the studied lots. At 1ºC no differences ($p<0.05$) were observed between the
231 pressurized AC and AE lots from day 8 onward, suggesting that at the lower temperature of storage,
232 antimicrobial packaging did not give additional protection against *L. monocytogenes* to pressurized
233 samples.

In order to assess the efficiency of these two technologies, a temperature abuse was performed after two months of storage. After 24 h at room temperature, the temperature was reset at 1ºC until day 90. After the cold chain break, day 77, *L. monocytogenes* increased 3 log CFU/g in the control lot, reaching the stationary phase (Table 1). Antimicrobial packaging, though, allowed a lower increase (2.1 log CFU/g) after temperature abuse. Packaging with films containing enterocins slowed down the growth rate of the pathogen, and showed final counts as being 1 logarithm lower than packaging with control films. Table 1 shows the fatal effect of temperature abuse on the pressurized AC samples. After the cold chain break the population grew from 0.6 to 6.6 log CFU/g at day 77, reaching maximum growth (8.5 log CFU/g) at day 90. These results evidence the capability of pressure injured cells to recover under favourable growth conditions, while on the contrary, no growth of *L. monocytogenes* was observed in the pressurized AE lot after the cold chain break (day 77). Further storage at 1ºC of the product submitted to temperature abuse resulted in a slight increase until 1.7 log CFU/g. Thus the combination of antimicrobial packaging with HPP proved to be able, not only to control and reduce the numbers of *L. monocytogenes*, but also to overcome temperature abuse.

HPP is a technology already applied to sliced cooked ham which is commercially available on the U.S and Spanish market. Among the studied strategies the combination of HPP and storage at 1ºC complied with the Regulation (EC) 2073/2005 (EC, 2005) for RTE foods being able to support the growth of *L. monocytogenes*, limited to 100 CFU/g at the end of its shelf life. However, at a higher refrigeration temperature (6ºC), antimicrobial packaging with alginate films containing enterocins was necessary to fulfil the microbiological criteria.

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351 **Figure captions**

352

353 **Figure 1**. Growth of *L. monocytogenes* in sliced cooked ham packed with control alginate films (○),
354 and alginate films containing 2,000 AU/cm^2 of enterocins (△), submitted (black line) or not (grey line)
355 to HPP and stored at 6ºC.

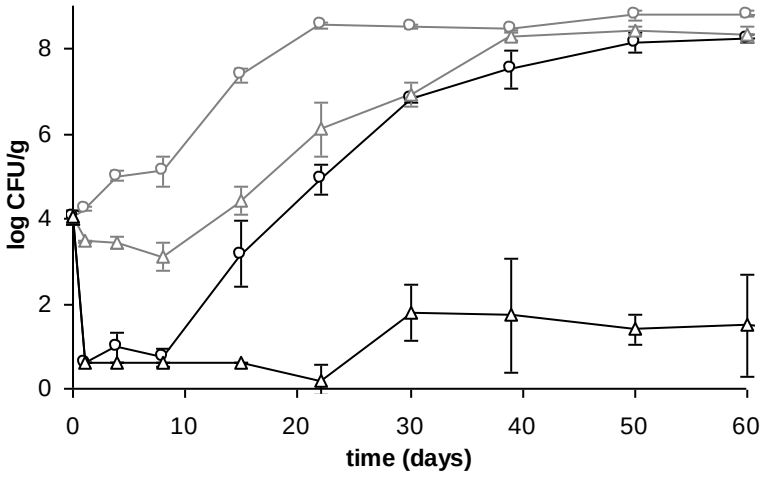
356

357 **Figure 2**. Growth of *L. monocytogenes* in sliced cooked ham packed with control alginate films (○),
358 and alginate films containing 2,000 AU/cm^2 of enterocins (△), submitted (black line) or not (grey line)
359 to HPP and stored at 1ºC.

360

361 **Figure 1**.

362



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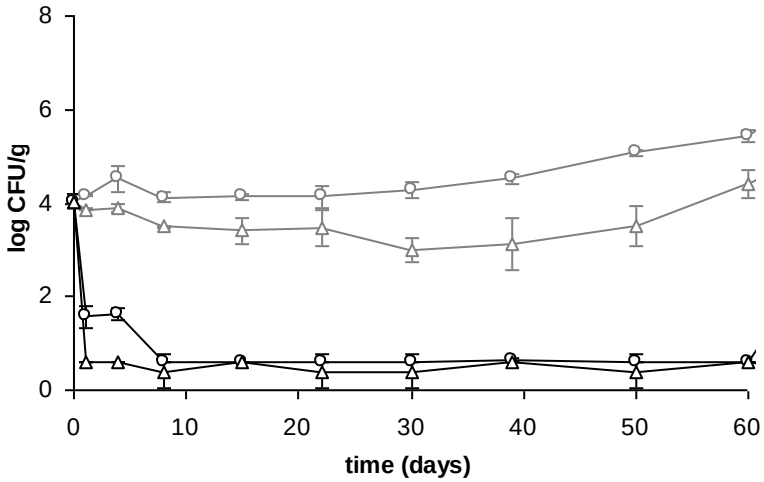
367 **Figure 2**.



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370 **Table 1.** Evolution of *L. monocytogenes* population during extended storage of sliced cooked ham at
371 6ºC and 1ºC.
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		AC		AE	
	time (days)	NP	HPP	NP	HPP
6ºC:	60	8.83±0.09 a	8.25±0.10 a	8.34±0.20 a	1.49±1.22 b
	77	8.56±0.07 a	8.32±0.05 a	8.46±0.17 a	1.02±0.55 b
	90	8.39±0.07 a	8.24±0.17 a	8.47±0.09 a	0.60±0.00 b
1ºC*:	60*	5.42±0.13^{y} a	0.60±0.00^{z} c	4.39±0.30^{z} b	0.60±0.00 c
	77	8.41±0.08^{x} a	6.62±0.10^{y} b	6.50±0.07^{y} b	0.60±0.00 c
	90	8.47±0.28^{x} a	8.53±0.40^{x} a	7.38±0.03^{x} a	0.60±0.00 b

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374 Values are given as mean ± SD (n=3) in log CFU/g.
375 * Product stored at 1ºC submitted to temperature abuse (24h at c. 20ºC) at day 60.
376 AC: samples packed with control alginate films; AE: samples packed with alginate films containing 2,000 AU/cm^2
377 of enterocins; NP: non-pressurized samples; HPP: pressurized samples.
378 x-z: within each storage temperature means with different letters in the same column are significantly different
379 ($p<0.05$).
380 a-c: within time of storage means with different letters in the same row are significantly different ($p<0.05$).
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