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 *Listeria 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 6°C experienced a quick growth of *L. monocytogenes*. Both antimicrobial packaging and pressurization delayed the growth of the pathogen. However, at 6°C 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 6°C of pressurized antimicrobial packed cooked ham resulted in *L. monocytogenes* levels below the detection limit (day 90). On the other hand, storage at 1°C controlled the growth of the pathogen until day 39 in non-pressurized ham, while antimicrobial packaging and storage at 1°C exerted a bacteriostatic effect for 60 days. All HPP lots stored at 1°C 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.

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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, 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.

The risk assessment for *L. monocytogenes* in deli meats carried out by the FSIS (Gallagher et al., 2003) indicated that the use of a combination of interventions in deli meats exposed to the environment after the lethal treatment had the greatest impact on lowering the risk of illness or death from *L. monocytogenes*. Therefore, the final rule for the control of *L. monocytogenes* (FSIS, 2003) includes three alternative approaches that establishments can take in the processing of RTE meat products during post-lethal exposure. The first alternative proposed the use of a post-lethal treatment and an antimicrobial agent; the second one, proposed the use of either a post-lethal treatment or an antimicrobial agent; and the third one, proposed the use of sanitation measures only.

Following these proposals, the objective of the present work was to compare the effectiveness of three strategies to control *L. monocytogenes* during refrigerated storage of sliced cooked ham: application of HPP, antimicrobial packaging and combined use of antimicrobial packaging and HPP, combined with two different storage temperatures.

### 2. Materials and methods

#### 2.1. Bacteriocin production

*Enterococcus faecium* CTC492, isolated from a meat product and producer of enterocins A and B (Aymerich et al., 1996; Casaus et al., 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 following additives (g/kg; SKW Biosystems, Rubi, Spain): 20 beef extract, 20 glucose, and 15 agar, was used to support soft TSBYE (TSBYE with 7.5 g/l agar) seeded with 20 μl of the overnight mixture of *L. monocytogenes*. Enterocin samples were serially diluted twofold with 50 mM phosphate buffer, pH 6. A 10 μl sample of each dilution was spotted onto soft TSBYE lawn. The plates were incubated overnight at 30 °C. An arbitrary unit (AU) was defined as the highest dilution showing growth inhibition of the indicator lawn, and bacteriocin activity was expressed as AU/ml.

#### 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 30 °C.

Bacteriocin activity was quantified by the agar spot test (Tagg et al., 1976). A solid medium composed of, in g/l, 20 beef extract, 20 glucose, and 15 agar, was used to support soft TSBYE (TSBYE with 7.5 g/l agar) seeded with 20 μl of the overnight mixture of *L. monocytogenes*. Enterocin samples were serially diluted twofold with 50 mM phosphate buffer, pH 6. A 10 μl sample of each dilution was spotted onto soft TSBYE lawn. The plates were incubated overnight at 30 °C. An arbitrary unit (AU) was defined as the highest dilution showing growth inhibition of the indicator lawn, and bacteriocin activity was expressed as AU/ml.

#### 2.3. Film manufacturing

Film forming solutions were obtained as suggested by Del Nobile et al. (2003) and Buonocore et al. (2005), with some modifications. Alginate solutions were obtained by stirring for 2 h at 80 °C, a 5% (w/v) alginic acid (Sigma-Aldrich) solution in distilled water. Glycerol (5%, v/v) was added as plasticizer, and the solution was stirred at ambient temperature for 30 min. After measuring the volume of the film blend, the active solution was obtained by adding the appropriate dilution from the stock solution of enterocins (409,600 AU/ml) to obtain a concentration of 2000 AU/cm². The solution was stirred at ambient temperature until completely dissolved. The films were manufactured by casting 3 ml of the prepared solutions onto sterile polystyrene dishes (28 cm²) and were dried under a biological safety cabinet. After drying, alginate films were reticulated by immersion in a 2% (w/v) calcium chloride solution. The thickness of the films obtained was measured by means of a Digimatic Micrometer (Mitutoyo, Japan). The value of the film thickness was obtained by averaging 10 measurements. The films obtained had an average thickness of 120 ± 10 μm.

#### 2.4. Cooked ham manufacturing

Cooked ham was prepared with pork shoulder and the following additives (g/kg; SKW Biosystems, Rubi, Spain): water, 115; sodium chloride, 20.7; dextrose, 5.8; sodium tripolyphosphate, 5.8; carrageenate, 2.3; NaNO₂, 0.1; and L-ascorbate, 0.6. Pork shoulder meat was minced at -1 °C in a cutter (Tecmaq, Spain) to a particle size of 4 mm. Ingredients were homogenized in a mixer (model 35P, Tecnotrip S.A., Terrassa, Spain) for 30 min. The meat batter was stuffed into 90 mm diameter plastic casings (Prolan, Provedeora Hipano Holandes, S.A., Sant Boi de Llobregat, Spain) using a stuffing machine (model H15, Tecnotrip). The product was cooked in a steam oven (Doleschal, Dordal, S.A., Santa Perpètua de la Mogoda, Spain) at 75 °C until internal temperature reached 72 ± 2 °C (temperature probe TM 65, Crison Instruments S.A., Spain).
Barcelona, Spain). Whole pieces of cooked ham were stored at 1 °C 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, and 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 (AC) films, and a lot packed with alginate films containing 2000 AU/cm² of enterocins (AE).

Half of the samples were non-pressurized and half were subjected to HPP. Pressurization was performed at 400 MPa for 10 min at 17 °C. HPP was carried out in an industrial hydrostatic pressurization unit (Alstom, Nantes, France) with a chamber volume of 3200 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 5 °C.

2.6. Refrigerated storage and temperature abuse

After HPP samples were stored at 1 or 6 °C for 2 months. After 60 days of refrigerated storage, samples stored at 1 °C were submitted to temperature abuse, consisting of maintaining the samples for 24 h at room temperature (ca. 20 °C), simulating a cold chain break during the shelf life of cooked ham. The behaviour of L. monocytogenes at 6 °C 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 1 °C and during further refrigeration at 6 °C, 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 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. To determine presence/absence of the pathogen, homogenates were incubated at 37 °C for 40 h and spread plated on Palcam agar.

Three different packages from each lot (C, AC, AE), temperature (1 and 6 °C) and treatment (non-pressurized and pressurized) combinations were sampled at each sampling time.

2.8. Statistical analysis

Data were subjected to analysis of variance using the general linear model procedure from the SAS statistical package (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

No significant differences in L. monocytogenes numbers ($P>0.05$) were observed between control (C) and the AC lots throughout storage (data not shown). These results evidence that packaging with alginate films in itself had no effect on the behaviour of L. monocytogenes. Cooked ham packed with control films (AC) supported a quick growth of L. monocytogenes at 6 °C, reaching the maximum growth (8.6 log CFU/g) in 22 days (Fig. 1). The ability of L. monocytogenes to grow exponentially in cooked ham refrigerated at 6 °C reflects the necessity of applying additional hurdles to refrigeration in order to prevent the growth of L. monocytogenes in contaminated food.

Antimicrobial packaging is a promising form of active packaging especially for meat products (Kerry et al., 2006). Moreover, the effectiveness of alginate films containing bacteriocins against food-borne pathogens has recently been demonstrated (Millette et al., 2007). In the present study, vacuum packaging with alginate films containing 2000 AU/cm² of enterocins (AE), significantly delayed the growth of L. monocytogenes at 6 °C (Fig. 1). Antimicrobial packaging was able to inhibit the growth of the pathogen during the first days of storage, extending the lag phase of L. monocytogenes until day 8. Although there was further growth, the pathogen did not exceed inoculated levels for

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**Fig. 1.** Growth of L. monocytogenes in sliced cooked ham packed with control alginate films ( ), and alginate films containing 2000 AU/cm² of enterocins ( ), submitted (black line) or not (grey line) to HPP and stored at 6 °C.
Preservation by combined technologies generally enhances antimicrobial effects with the resultant extended shelf life (Leistner, 2000; Raso and Barbosa-Canovas, 2003). Fig. 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 2 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 the combination of HPP and antimicrobial packaging was assessed during further storage. Table 1 shows the counts obtained until day 90 of storage at 6°C of sliced cooked ham. The pressurized AE lot not only maintained but also reduced the counts during further storage. It is important to highlight that after 3 months of storage at 6°C, pressurization of sliced cooked ham packed with alginate films containing 2000 AU/cm² was not only able to prevent the growth of L. monocytogenes but also to reduce the counts to below the detection limit (5 CFU/g).

Besides, although L. monocytogenes is able to grow during refrigerated storage, temperature control has proved to be essential in reducing the risk of the pathogen. Refrigeration at 1°C of sliced cooked ham packed with control films showed no growth of L. monocytogenes for 39 days (Fig. 2). Those results agree with previous studies where refrigeration at 1°C effectively controlled the growth of the pathogen on sliced cooked ham for 40 days (Marcos et al., in press). Fig. 2 shows an increase of 1.4 log CFU/g from inoculated levels during further storage of the

Table 1: Evolution of L. monocytogenes population during extended storage of sliced cooked ham at 6 and 1°C

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>AC</th>
<th>HPP</th>
<th>AE</th>
<th>HPP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
<td>HPP</td>
<td>NP</td>
<td>HPP</td>
</tr>
<tr>
<td><strong>6°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>8.83 ± 0.09 a</td>
<td>8.25 ± 0.10 a</td>
<td>8.34 ± 0.20 a</td>
<td>1.49 ± 1.22 b</td>
</tr>
<tr>
<td>77</td>
<td>8.56 ± 0.07 a</td>
<td>8.32 ± 0.05 a</td>
<td>8.46 ± 0.17 a</td>
<td>1.02 ± 0.55 b</td>
</tr>
<tr>
<td>90</td>
<td>8.39 ± 0.07 a</td>
<td>8.24 ± 0.17 a</td>
<td>8.47 ± 0.09 a</td>
<td>0.60 ± 0.00 b</td>
</tr>
<tr>
<td><strong>1°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60³</td>
<td>5.42 ± 0.13³ a</td>
<td>0.60 ± 0.00³ c</td>
<td>4.39 ± 0.30³ b</td>
<td>0.60 ± 0.00 c</td>
</tr>
<tr>
<td>77</td>
<td>8.41 ± 0.08³ a</td>
<td>6.62 ± 0.10³ b</td>
<td>6.50 ± 0.07³ b</td>
<td>0.60 ± 0.00 c</td>
</tr>
<tr>
<td>90</td>
<td>8.47 ± 0.28³ a</td>
<td>8.53 ± 0.40³ a</td>
<td>7.38 ± 0.03³ a</td>
<td>0.60 ± 0.00 b</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD (n = 3) in log CFU/g.

AC: Samples packed with control alginate films; AE: samples packed with alginate films containing 2000 AU/cm² of enterocins; NP: non-pressurized samples; HPP: pressurized samples.

x–z: Within each storage temperature means with different letters in the same column are significantly different (P < 0.05).
a–c: Within time of storage means with different letters in the same row are significantly different (P < 0.05).

³Product stored at 1°C submitted to temperature abuse (24 h at ca. 20°C) at day 60.
product. At the end of storage, the counts in the AC lot stored at 1 °C were 3.4 logarithms lower than at 6 °C.

At 1 °C, packaging of cooked ham with films containing enterocins (AE lot) gave lower counts \((P < 0.05)\) than in the control lot throughout storage. Fig. 2 shows \(L.\) monocytogenes numbers as being 1 logarithm lower than inoculated levels from day 30 until day 39, and its recovery to initial counts by the end of storage. Thus, antimicrobial packaging applied as an additional hurdle to low refrigeration temperature (1 °C) was effective for controlling \(L.\) monocytogenes during storage of 60 days. The importance of refrigeration temperature should be noted: storage at 1 °C became a key factor in preventing \(L.\) monocytogenes growth. However, commercial and home refrigerator temperatures may run at higher temperatures (Bakalis et al., 2003; Sergelidis et al., 1997), and application of additional technologies, such as HPP and antimicrobial packaging, would assure the safety of contaminated foods during its shelf life. Pressurization of the AC lot followed by refrigeration at 1 °C effectively reduced inoculated numbers of \(L.\) monocytogenes around the detection limit, with this level being maintained throughout storage. Similarly, during the whole storage the pressurized AE lot stored at 1 °C presented levels around 0.6 log CFU/g for 60 days (Fig. 2). Absence of \(L.\) monocytogenes was not achieved in any of the studied lots. At 1 °C no differences \((P < 0.05)\) were observed between the pressurized AC and AE lots from day 8 onward, suggesting that at the lower temperature of storage, antimicrobial packaging did not give additional protection against \(L.\) monocytogenes to pressurized samples.

In order to assess the efficiency of these two technologies, a temperature abuse was performed after 2 months of storage. After 24 h at room temperature, the temperature was reset at 1 °C 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 1 °C 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 in the US and Spanish market. Among the studied strategies the combination of HPP and storage at 1 °C 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 (6 °C), antimicrobial packaging with alginate films containing enterocins was necessary to fulfil the microbiological criteria.

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