

Application of protective cultures against *Listeria monocytogenes* and *Campylobacter jejuni* in chicken products packaged under modified atmosphere

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ABSTRACT To control the growth, or reduce the numbers, of food pathogens such as *Listeria monocytogenes* and *Campylobacter jejuni* in chicken products packaged under modified atmosphere (MAP), the effectiveness of protective cultures was evaluated in this study. *Leuconostoc pseudomesenteroides* PCK18 reduced the counts of *L. monocytogenes* by 1.22 log cfu/g in chicken burgers under MAP after 24 d. Furthermore, a reduction of 1.16 log cfu/g in *C. jejuni* together with a delay in the

growth of lactic acid bacteria was obtained in chicken legs inoculated with *Bifidobacterium longum* ssp. *longum* PCB133 and packaged under MAP after 9 d. The combination and concentration of gases in the MAP doubled the products' shelf-life in comparison with air-packaged samples in both experiments. In conclusion, this study has shown the effectiveness of 2 protective culture strains against 2 foodborne pathogens, resulting in safer products with a longer shelf-life.

Key words: *Leuconostoc pseudomesenteroides* PCK18, *Bifidobacterium longum* ssp. *longum* PCB133, chicken leg, chicken burger meat

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INTRODUCTION

Poultry and poultry products are well known to be reservoirs of foodborne pathogens such as *Listeria monocytogenes* and *Campylobacter jejuni* (Miettinen et al., 2001; Reiter et al., 2005; Mor-Mur and Yuste, 2009; Melero et al., 2011). In 2009, the number of listeriosis cases in the European Union (1,645 human cases) was much lower than the number of campylobacteriosis cases (198,252 human cases; EFSA and ECDC, 2011). However, although the incidence of listeriosis is low, it remains a major public health concern because of its severity and nonenteric nature (meningitis or meningococcal septicemia, and miscarriage) in target populations such as young children, the elderly, pregnant women, and other immunocompromised individuals (Meng and Doyle, 1998; Rovira et al., 2006; Mor-Mur and Yuste, 2009). In contrast, campylobacteriosis is commonly a self-limiting illness that affects an important number of patients per year. Although it tends to be self-limiting, it can also provoke long-term side-effects such as reactive arthritis and Guillain–Barré syndrome and may also contribute to the pathogenesis

of chronic gastrointestinal conditions such as inflammatory bowel disease and celiac disease (Riddle et al., 2012).

Chicken is a highly perishable product, even when stored in chilling conditions. In this respect, the positive effect of modified atmosphere packaging (MAP) to extend the shelf-life of food products, as well as to maintain the quality of fresh meat, vegetables, and fruits at chilling temperatures, is well known (Church and Parsons, 1995). Indeed, the shelf-life of poultry meat can be increased from 8 to 10 d to 15 to 17 d when packaged under 60 to 80% CO₂ (Jiménez et al., 1997; Sarantópoulos et al., 1998). However, the use of MAP to extend shelf-life by inhibiting “normal” spoilage microflora must take into account the opportunity it provides for the growth of food pathogens such as *L. monocytogenes*, which, because they are able to grow at refrigeration temperatures, require the use of chemical additives to ensure the safety of meat products (Wimpfheimer et al., 1990; Bell and Kyriakides, 2002). Moreover, raw chicken products are recognized as vehicles of cross-contamination when handled in the kitchen.

As consumers are nowadays demanding safe food products with minimal processing, the application of biopreservation techniques could be a natural alternative for food preservation (Stiles, 1996; Castellano et al., 2008). Biopreservation can be defined as a preservation method that improves the safety and stability of

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food products, without altering their sensory qualities, by using certain microorganisms, their metabolites, or both (Holzapfel et al., 1995; Lücke, 2000). Thus, lactic acid bacteria (LAB) have major potential for use as a protective culture because they are safe for consumption (generally recognized as safe, GRAS) and naturally dominate the microbiota of many foods (Castellano et al., 2008). Lactic acid bacteria can exert a bioprotective or inhibitory effect against other microorganisms as a result of their competition for nutrients; their production of bacteriocins or other antagonistic compounds such as organic acids, hydrogen peroxide, and enzymes (Castellano et al., 2008); or a combination of these. Indeed, a large number of studies have confirmed that both bacteriocins and bacteriocinogenic LAB are effective against *L. monocytogenes* (Hugas et al., 1998; Bredholt et al., 1999; Castellano and Vignolo, 2006; Dortu et al., 2008). Moreover, gram-negative bacteria have an inherent resistance to many microbicidal agents due to their outer membrane, which acts as an efficient barrier against macromolecules and hydrophobic substances. Despite this, some researchers have achieved promising results when applying protective culture or bacteriocins against *C. jejuni* (Lyon and Glatz, 1991; Chaveerach et al., 2004; Santini et al., 2010) and other gram-negative bacteria (Kalchayanand et al., 1998; Maragkoudakis et al., 2009).

The presence of *L. monocytogenes* in burger meat and *C. jejuni* in chicken cuts were detected in a previous study performed in a poultry processing plant (B. Mero, unpublished data). In light of these results, the aims of this study were to 1) use MAP to extend the shelf-life of commercial chicken burger meat and chicken legs and 2) apply *Leuconostoc pseudomesenteroides* PCK18 and *Bifidobacterium longum* ssp. *longum* PCB 133 as protective cultures against growth of *L. monocytogenes* and *C. jejuni* in chicken burger meat and chicken legs in MAP, respectively.

MATERIALS AND METHODS

Bacterial Strains

The *L. monocytogenes* strains CECT 5366 (Spanish Culture Collection), CECT 934, CECT 4032, and LTA0020, isolated from minced chicken meat, were used in this study. *Leuconostoc pseudomesenteroides* PCK18, provided by Probiotal (Novara, Italy), was used as a protective culture strain against *L. monocytogenes*.

The reference *C. jejuni* strain CECT7572 and strains LTA0034, LTA0036, and LTA0040 isolated from poultry products were used to test the effect of *Bifidobacterium longum* ssp. *longum* PCB133, which was also provided by Probiotal, as a protective culture.

Two different experiments (see below) were performed to evaluate the effect of MAP and protective cultures against *L. monocytogenes* and *C. jejuni* in chicken products. This protective and probiotic strain demonstrated very strong in vitro antimicrobial activ-

ity against *L. monocytogenes* and moderate against *C. jejuni* (Carlini et al., 2010).

Inocula Preparation

Listeria monocytogenes strains were grown at 37°C overnight in BHI broth (Oxoid, Basingstoke, Hampshire, UK) to achieve a viable cell population of 9 log cfu/mL. Previously frozen *C. jejuni* strains were streaked onto Columbia Blood Agar (Oxoid) supplemented with 5% horse blood (Oxoid) and the plates incubated at 41.5°C for 48 h under a microaerobic atmosphere (5% O₂, 10% CO₂, 85% N₂) generated using a CampyGen (Oxoid). Thus, one colony of each strain was transferred into BHI broth (Oxoid) and incubated as described above. For both pathogens, each culture was then centrifuged at 4,500 × *g* for 10 min at 4°C. The supernatant was decanted and the pellet suspended in sterile Ringer solution (Oxoid) by vortexing. This washing step was repeated 3 times. The suspension of washed cells was diluted twice in 9 mL of Ringer solution (Oxoid). For each pathogen, the 4 tubes containing 10 mL of the cell suspension for each strain were diluted with 960 mL of Ringer solution; then for *L. monocytogenes*, 20 mL of this cell suspension was mixed with 1.5 kg of meat per batch to obtain a final concentration of 3.10 log cfu/g. For *C. jejuni*, a 4-mL aliquot of the mixture was then sprayed onto the surface of the entire chicken leg using a hand-operated spraying bottle to a final concentration of 5.30 log cfu/g.

Protective culture inocula were prepared from a freeze-dried preparation of 9.28 log cfu/g and 8 log cfu/g for *Lc. pseudomesenteroides* PCK18 and *B. longum* ssp. *longum* PCB133, respectively. For *Lc. pseudomesenteroides* PCK18, a 20-mL aliquot of the resulting cell suspension was mixed with meat to achieve a final concentration of 5.20 log cfu/g. A final concentration, on the meat, of 4.36 log cfu/g for *B. longum* PCB133 was achieved by spraying 2 mL onto the entire chicken leg.

Experiment 1: Application of *Lc. pseudomesenteroides* PCK18 Against *L. monocytogenes* in MAP Chicken Meat Burger: Sample Inoculation

Minced chicken meat and fat were supplied by a poultry company in Burgos (Spain). Chicken meat burgers (80.23% meat, 11.46% fat, 5.39% water, and 2.92% additives) were prepared in the pilot plant. A closed commercial additive preparation (Taberner, Valencia, Spain) was diluted in water and mixed with the meat, fat, and inocula in a mixer at medium speed for 15 min. The additive preparation included salt, vegetable fiber, antioxidants (E325, E331, E300), starch, dextrose, spices, preservatives (E221, sulfite), and colorant (E120).

A total of 5 batches were prepared (Table 1), with batches C to LP5 being packaged under MAP. Four

meat burgers (50 g each) were placed in a polyethylene/ethylene vinyl alcohol/polystyrene tray (Sanviplast, Barcelona, Spain) with a gas permeability of O_2 TR = $0.99 \text{ cm}^3 \cdot \text{m}^{-2} \cdot \text{d}^{-1} \cdot \text{atm}^{-1}$; CO_2 TR = $0.55 \text{ cm}^3 \cdot \text{m}^{-2} \cdot \text{d}^{-1} \cdot \text{atm}^{-1}$ at 75% RH, 25°C; WV TR = $1.69 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1} \cdot \text{atm}^{-1}$. The trays were sealed with polyethylene terephthalate polyvinylidene chloride/polyethylene film (O_2 TR = $7 \text{ cm}^3 \cdot \text{m}^{-2} \cdot \text{d}^{-1} \cdot \text{atm}^{-1}$; CO_2 TR = $20 \text{ cm}^3 \cdot \text{m}^{-2} \cdot \text{d}^{-1} \cdot \text{atm}^{-1}$ at 65% RH, 23°C; water vapor transmission rate = $4 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1} \cdot \text{atm}^{-1}$ at 90% RH, 38°C). The gas mixture applied (50% CO_2 /10% O_2 /40% N_2) was prepared using a WITT-Gasetechnik device (WITT-Gasetechnik GmbH & Co KG, Witten, Germany). This atmosphere was selected because, according to previous results (not published), 50% CO_2 is the highest concentration of CO_2 that does not produce exudate and prevents also the growth of *L. monocytogenes* (Melero et al., 2012). Samples were stored at 4°C and analyzed at predetermined time intervals of 0, 1, 4, 7, 10, 14, 17, and 24 d poststorage. Gas atmosphere, pH, microbiological, and sensorial analyses were performed from 2 trays per batch on each sampling day (n = 6). Three meat burgers were analyzed before and after inoculation at d 0.

Experiment 2: Application of *B. longum* ssp. *longum* PCB133 Against *C. jejuni* in MAP Chicken Legs: Sample Inoculation

A total of 80 chicken legs (right and left were selected at random) were removed from chicken carcasses a few hours after slaughter. These legs were divided into 5 batches and submitted to different treatments (see Table 1). To ensure the same volume of liquid was applied to all treated samples, batches J and B were also sprayed with 2 mL of distilled water. Batches A and C were sprayed with 4 mL of water. Two chicken legs from each batch were placed in the same type of tray

and film described in experiment 1 (see above). The gas mixture applied in this experiment was 30% CO_2 /70% N_2 . This atmosphere was selected because *Bifidobacteria* are anaerobic (Biavati and Mattarelli, 2006). Samples were stored at 4°C and analyzed at predetermined time intervals, namely 0, 1, 3, 6, and 9 d poststorage. Measures of pH and gas atmosphere and microbiological and sensorial analyses were performed from 2 trays per batch on each sampling day (n = 6). Three chicken legs were analyzed before and after inoculation at d 0.

Gas and pH Analysis

Gas analysis of the internal atmosphere was carried out on the same sampling days using a digital O_2/CO_2 analyzer (OXYBABY, WITT-Gasetechnik GmbH & Co KG) by withdrawing a 10-mL gas sample through a septum glued onto the surface of the pack using the analyzer's needle.

The pH analysis was performed after microbiological analysis. The pH was measured with a pin electrode of a pH meter (microPH2001, Crison, Barcelona, Spain) inserted directly into the sample. The result was expressed as the mean of 3 separate readings per tray.

Microbiological Analysis

Meat samples (25 g) were removed aseptically from each tray and homogenized in 225 mL of buffered peptone water (BPW, AES Laboratoire, Bruz, France) for 2 min in a sterile plastic bag using a laboratory blender (Stomacher 400, Seward, London, UK). Cell counts were determined by plating serial dilutions (1:10 in BPW) of the meat homogenate. To determine the total viable counts (TVC), and the LAB and *Lc. pseudomesenteroides* PCK18 counts, 1 mL of the appropriate dilution was inoculated using the pour-plated method on plate count agar (PCA, Pronadisa, Torrejón de Ardoz,

Table 1. Relationship between batches, inocula, and atmosphere used in both experiments

Batch	Experiment 1			Experiment 2		
	<i>Listeria monocytogenes</i> ¹	<i>Leuconostoc pseudomesenteroides</i> ¹	Atmosphere	<i>Campylobacter jejuni</i> ¹	<i>Bifidobacterium longum</i> ¹	Atmosphere
A	—	—	Air	—	—	Air
C	—	—	50% CO_2 /10% O_2 /40% N_2	—	—	30% CO_2 /70% N_2
P5	—	5.20	50% CO_2 /10% O_2 /40% N_2	—	—	—
L	3.10	—	50% CO_2 /10% O_2 /40% N_2	—	—	—
LP5	3.10	5.20	50% CO_2 /10% O_2 /40% N_2	—	—	—
B	—	—	—	—	4.36	30% CO_2 /70% N_2
J	—	—	—	5.30	—	30% CO_2 /70% N_2
JB	—	—	—	5.30	4.36	30% CO_2 /70% N_2

¹Log cfu/g.

Madrid, Spain; ISO, 2003), and 100 μL was streaked onto MRS (Oxoid), respectively. The plates were incubated at 30°C for 3 d (TVC), and 30°C for 48 to 72 h (LAB and *Lc. pseudomesenteroides* PCK18). The *B. longum* PCB133 counts were determined on de Man, Rogosa, and Sharpe agar (MRS) supplemented with 0.5% L-cysteine (MRSC; Sigma-Aldrich, Buchs, Switzerland) after incubation under anaerobic conditions, which were generated using an Anaerogen (AN0025A, Oxoid), for 48 h at 37°C.

Campylobacter jejuni counts were determined on modified charcoal-cefoperazone-deoxycholate agar after incubation at 41.5°C for 48 h under a microaerobic atmosphere. Suspected *Campylobacter* spp. colonies in naturally contaminated chicken legs were identified as *C. jejuni* using the tests recommended in ISO 10272-1:2006 (ISO, 2006).

Listeria monocytogenes was enumerated according to ISO 11290-2:1998 (ISO, 1998) for both naturally and artificially contaminated samples on Chromogenic *Listeria* agar (Oxoid) supplemented with OCLA (ISO) Selective Supplement (SR 0226E, Oxoid) and Brilliance *Listeria* Differential Supplement (SR 0228E, Oxoid), using the spread plate method. Plates were incubated at 37°C for 24 to 48 h, and ISO 11290-1:1998 (ISO, 1996) was followed for the detection of *L. monocytogenes* in naturally contaminated samples.

Sensorial Analyses

Sensorial analyses were performed by an 8-member trained sensory panel. Before opening the package, the panel evaluated meat color and presence of molds for chicken burger meat, whereas meat color, shiny (a surface that reflects the light), and slime (thick, wet substance that covers the surface of the samples) were evaluated for chicken legs. Immediately after each pack was opened, the sample was also assessed for off-odor intensity and for the general rejection in both products. The samples were scored on a 5-point hedonic scale where 1 corresponds to no modification and 5 to high modification with respect to d 0. When the general rejection score was higher than 3, the meat was considered unacceptable (Kotzekidou and Bloukas, 1996). On d 0, chicken burger meat and a chicken leg were evaluated by the panelists before inoculation to know the initial characteristics of the products.

Statistical Analyses

A one-way ANOVA was performed to determine significant differences among samples. Least significance difference test was used with a 95% significance level, and normal distribution test of data was realized previously. All the analyses were performed using the Statgraphics Plus 5.1 for Windows statistical package (Statgraphics.Net, Madrid, Spain).

RESULTS

Experiment 1: Application of *Lc. pseudomesenteroides* PCK18 Against *L. monocytogenes* in MAP Chicken Burger Meat

The CO₂ level increased slightly but significantly ($P < 0.05$), whereas the O₂ and N₂ levels decreased ($P < 0.05$; data not shown), throughout the study period. A slight decrease in pH (from 6.04 to 6.24 to 5.76 to 6.04; $P < 0.05$) was observed in all batches at the end of the storage period.

Listeria monocytogenes counts remained constant throughout the storage period in the burgers without protective culture added (batch L, $P > 0.05$), thus suggesting a bacteriostatic effect of the MAP used against *L. monocytogenes* (Figure 1). Moreover, a decrease of 1.22 log cfu/g was found from d 0 to 24 in the burgers with *L. monocytogenes* and the protective culture (batch LP5, $P < 0.05$). In this batch, the major count reduction (0.92 log cfu/g) was obtained between d 0 and 4. Furthermore, a reduction of 0.89 log cfu/g ($P < 0.05$) was achieved at the end of the study in batch LP5 compared with batch L.

A TVC value of 7 log cfu/g was established as the upper acceptability limit for fresh chicken meat (Senter et al., 2000). The TVC reached 7.26 log cfu/g at d 10 in samples packaged in air (A), whereas it reached 7.18 to 8.17 log cfu/g between d 17 and 24 for the remaining batches (Table 2). As far as LAB is concerned, batch A achieved a value of 7.22 log cfu/g at d 14, whereas the remaining batches reached a similar value between d 17 and 24, except in the burgers with *L. monocytogenes* and the protective culture (batch LP5), which did not reach such level during the entire study period.

Sensorial analyses results showed slight modifications (scored less than 3) in color, odor, and general rejection in all batches using MAP along the storage period.

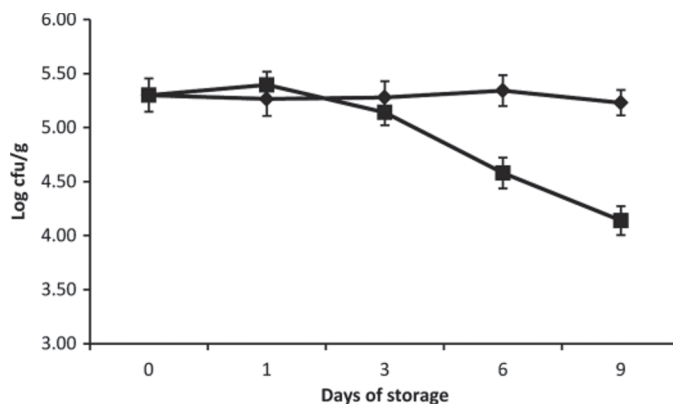


Figure 1. *Listeria monocytogenes* counts on modified-atmosphere-packaged chicken burger meat at 4°C for 24 d. Batch L was inoculated only with *L. monocytogenes*; batch LP5 was inoculated with *L. monocytogenes* and *Lc. pseudomesenteroides* PCK18.

Table 2. Microbiological counts during the storage period for chicken burger meat packaged in air and modified atmosphere, and inoculated or not with *Listeria monocytogenes* and *Leuconostoc pseudomesenteroides* PCK18

Batch	Days of storage ¹							
	0	1	4	7	10	14	17	24
TVC²								
A	6.66 (0.15) ^{e,A}	6.69 (0.20) ^{e,A}	6.67 (0.23) ^{e,A}	6.62 (0.28) ^{e,A}	7.26 (0.19) ^{d,A}	7.56 (0.13) ^{c,A}	8.15 (0.08) ^{b,A}	8.33 (0.10) ^{a,A}
C	6.66 (0.15) ^{bc,A}	6.69 (0.48) ^{b,A}	6.54 (0.23) ^{bc,AB}	6.43 (0.36) ^{bc,AB}	6.35 (0.28) ^{c,B}	6.35 (0.16) ^{c,C}	6.37 (0.22) ^{c,C}	7.18 (0.25) ^{c,D}
P5	6.68 (0.23) ^{b,A}	6.76 (0.12) ^{b,A}	6.38 (0.26) ^{c,B}	6.22 (0.19) ^{d,C}	6.06 (0.15) ^{d,C}	6.78 (0.06) ^{b,B}	6.75 (0.13) ^{b,B}	7.53 (0.27) ^{a,C}
L	6.66 (0.15) ^{cd,A}	6.73 (0.23) ^{c,A}	6.60 (0.28) ^{cd,AB}	6.49 (0.20) ^{d,AB}	6.45 (0.07) ^{b,B}	6.20 (0.18) ^{e,D}	6.48 (0.18) ^{d,C}	8.17 (0.20) ^{a,A}
LP5	6.68 (0.23) ^{b,A}	6.68 (0.26) ^{b,A}	6.70 (0.20) ^{b,A}	6.44 (0.16) ^{cd,AB}	6.49 (0.22) ^{c,B}	6.32 (0.19) ^{d,C}	6.42 (0.16) ^{cd,C}	7.79 (0.26) ^{a,B}
LAB² and <i>Lc. pseudomesenteroides</i> PCK18								
A	4.55 (0.28) ^{f,B}	4.50 (0.21) ^{f,D}	4.58 (0.30) ^{f,B}	4.96 (0.34) ^{e,C}	6.78 (0.29) ^{d,A}	7.22 (0.34) ^{c,A}	7.84 (0.21) ^{b,A}	8.13 (0.12) ^{a,A}
C	4.55 (0.28) ^{cd,B}	4.34 (0.27) ^{d,D}	4.57 (0.48) ^{cd,B}	4.93 (0.26) ^{c,C}	4.91 (0.14) ^{c,C}	5.42 (0.30) ^{b,D}	5.63 (0.25) ^{b,C}	7.14 (0.23) ^{a,C}
P5	5.20 (0.24) ^{e,A}	5.50 (0.21) ^{d,A}	5.43 (0.16) ^{d,A}	5.54 (0.25) ^{d,A}	5.52 (0.09) ^{d,A}	5.91 (0.38) ^{c,B}	6.43 (0.26) ^{b,B}	7.14 (0.29) ^{a,C}
L	4.55 (0.28) ^{f,B}	4.88 (0.08) ^{e,C}	4.68 (0.48) ^{ef,B}	5.24 (0.21) ^{d,B}	5.38 (0.17) ^{d,B}	5.60 (0.17) ^{c,C}	6.20 (0.13) ^{b,B}	7.48 (0.11) ^{a,B}
LP5	5.20 (0.24) ^{e,A}	5.10 (0.20) ^{c,B}	5.16 (0.18) ^{c,A}	5.24 (0.14) ^{c,B}	5.19 (0.13) ^{c,B}	5.78 (0.15) ^{b,B}	5.80 (0.34) ^{b,C}	6.70 (0.15) ^{a,D}

^{a-f}Means with a different lowercase letter in the same row are significantly different ($P < 0.05$).

^{A-D}Means with a different capital letter in the same column are significantly different ($P < 0.05$).

¹Data are expressed as mean log cfu/g (SD). Each mean represents n = 6 measurements.

²TVC = total viable count; LAB = lactic acid bacteria.

However, in batch A the panel scored over 3 at d 10 due to the presence of molds in burger meat.

Experiment 2: Application of *B. longum* ssp. *longum* PCB133 Against *C. jejuni* in MAP Chicken Legs

No changes ($P > 0.05$) in the concentration of gases were observed during the storage period for all the batches under MAP. The pH of all samples on d 1 post-inoculation ranged between 6.13 and 6.36. There were no differences ($P > 0.05$) in any batch along the storage period. However, at d 9, differences in pH were observed between batches. In that sense the pH of batches B, J, and JB (6.31, 6.23, and 6.24, respectively) was lower ($P < 0.05$) than that for the noninoculated batches A and C (6.48 and 6.46, respectively).

The effect of *B. longum* PCB133 against *C. jejuni* is shown in Figure 2. A reduction ($P < 0.05$) in inoculated *C. jejuni* counts was observed between d 6 and 9 in chicken legs with the pathogen and protective culture (batch JB) in comparison with batch J (reduction of 0.76 and 1.09 log cfu/g, respectively). At the end of the study, a reduction ($P < 0.05$) of *C. jejuni* counts of 1.16 log cfu/g was obtained in comparison with the level of inoculum (5.30 log cfu/g). Furthermore, no differences ($P > 0.05$) in *C. jejuni* counts were observed for batch J during the 9-d study period. Batches A, C, and B were all naturally contaminated with a level of *C. jejuni* between 1.50 and 2.82 log cfu/g (data not shown).

Only chicken legs with protective culture (batches B and JB) were analyzed for the presence of *B. longum* PCB133 counts because these samples were inoculated with this strain. The results showed no significant differences ($P > 0.05$) between B and JB samples during the study (Table 3).

The TVC results are presented in Table 3. As in experiment 1, a TVC value of 7 log cfu/g was used as the upper acceptability limit for fresh chicken meat. Chicken legs packaged in air (batch A) reached a level

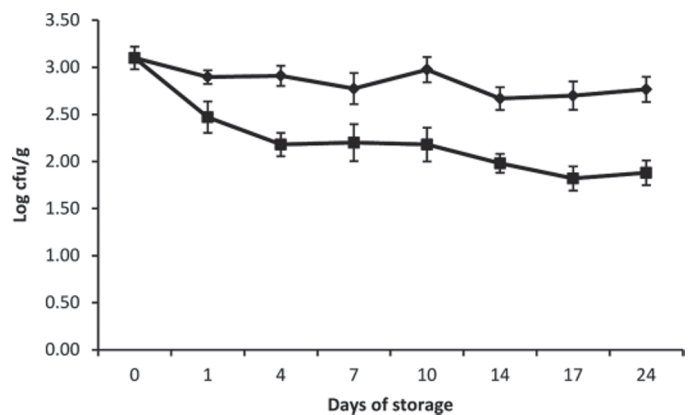


Figure 2. *Campylobacter jejuni* counts on modified-atmosphere-packaged chicken legs at 4°C for 9 d. Batch J was inoculated only with *C. jejuni*; batch JB was inoculated with *C. jejuni* and *Bifidobacterium longum* PCB133.

Table 3. Microbiological counts during the storage period for chicken legs packaged in air and modified atmosphere, and inoculated or not with *Campylobacter jejuni* and *Bifidobacterium longum* PCB133

Batch	Days of storage ¹				
	0	1	3	6	9
TVC ²					
A	4.50 (0.11) ^{e,C}	5.01 (0.15) ^{d,AB}	6.72 (0.31) ^{c,A}	8.17 (0.03) ^{b,A}	9.00 (0.12) ^{a,A}
C	4.50 (0.11) ^{e,C}	4.87 (0.26) ^{d,B}	5.35 (0.12) ^{c,C}	7.02 (0.02) ^{b,B}	8.03 (0.23) ^{a,B}
B	4.91 (0.15) ^{d,A}	4.88 (0.35) ^{d,B}	5.36 (0.57) ^{c,C}	6.30 (0.17) ^{b,D}	7.11 (0.16) ^{a,C}
J	4.74 (0.09) ^{e,B}	5.32 (0.29) ^{d,A}	6.02 (0.54) ^{c,AB}	7.00 (0.26) ^{b,B}	7.88 (0.27) ^{a,B}
JB	5.04 (0.23) ^{cd,A}	4.87 (0.20) ^{d,B}	5.47 (0.32) ^{c,BC}	6.54 (0.28) ^{b,C}	7.33 (0.43) ^{a,C}
LAB ²					
A	4.15 (0.23) ^{d,A}	3.95 (0.15) ^{e,BC}	5.37 (0.31) ^{c,AB}	7.00 (0.14) ^{b,A}	7.50 (0.30) ^{a,A}
C	4.15 (0.23) ^{cd,A}	3.80 (0.93) ^{d,C}	4.82 (0.42) ^{c,B}	6.09 (0.09) ^{b,BC}	7.45 (0.15) ^{a,A}
B	4.15 (0.23) ^{c,A}	4.25 (0.34) ^{c,AB}	5.27 (0.61) ^{b,AB}	5.89 (0.67) ^{b,C}	6.57 (0.32) ^{a,B}
J	4.15 (0.23) ^{e,A}	4.59 (0.22) ^{d,A}	5.51 (0.68) ^{c,A}	6.33 (0.02) ^{b,B}	7.61 (0.12) ^{a,A}
JB	4.15 (0.23) ^{e,A}	4.49 (0.27) ^{d,AB}	5.45 (0.39) ^{c,AB}	6.06 (0.30) ^{b,C}	6.82 (0.41) ^{a,B}
<i>B. longum</i> PCB133					
B	4.36 (0.10) ^{a,A}	4.08 (0.13) ^{bc,B}	4.26 (0.21) ^{a,A}	4.00 (0.17) ^{bc,A}	4.15 (0.09) ^{b,A}
JB	4.36 (0.10) ^{b,A}	4.30 (0.08) ^{b,A}	4.45 (0.25) ^{a,A}	3.96 (0.36) ^{c,A}	4.06 (0.15) ^{c,A}

^{a-e}Means with a different lowercase letter in the same row are significantly different ($P < 0.05$).

^{A-D}Means with a different capital letter in the same column are significantly different ($P < 0.05$).

¹Data are expressed as mean log cfu/g (SD). Each mean represents $n = 6$ measurements.

²TVC = total viable count; LAB = lactic acid bacteria.

of more than 7 log cfu/g between d 3 and 6, whereas legs with no protective culture added (batches C and J) reached this value at d 6 and those with protective culture added (batches B and JB) at d 9. The behavior of all batches regarding LAB was similar to that observed for TVC, although they reached a level of 7 log cfu/g around 3 d later. Furthermore, batches B and JB did not reach 7 log cfu/g at the end of the study.

Sensory analyses showed that batch A got a score of 3.5 in general rejection, whereas MAP batches did not reach this score at the end of the study. Regarding the MAP batches, those inoculated with *B. longum* PCB133 get 2.25 for this parameter. Meat color, odor, and shiny received higher scores in batch A (3.00, 3.50, and 2.50, respectively) than in MAP batches that scored less than 2.25 for those parameters. The presence of slime was only observed in batch A at d 9.

DISCUSSION

Poultry meat is a highly perishable product that is susceptible to contamination with foodborne pathogens such as *L. monocytogenes* and *C. jejuni* (Miettinen et al., 2001; Reiter et al., 2005; Mor-Mur and Yuste, 2009; Melero et al., 2011). To control both quality and safety during storage, hurdle technology (MAP and protective culture) has been applied in this study to extend the shelf-life of both fresh chicken meat burgers and chicken legs. This technology involves the addition of *Lc. pseudomesenteroides* PCK18 and *B. longum* PCB133 as protective cultures to reduce the level of *L. monocytogenes* and *C. jejuni*, respectively.

Experiment 1 has shown that *L. monocytogenes* did not grow in the atmosphere selected; however, we cannot conclude that this fact could only be due to the effect of the MAP because these products had sulfite

as preservative. However, some authors have reported that this substance does not inhibit gram-positive bacteria in a similar manner to gram-negative bacteria (Roberts and Mcweeny, 1972; Banks et al., 1985). The bacteriostatic effect of a CO₂-enriched atmosphere to protect against *L. monocytogenes* in chicken products is similarly controversial. It seems clear that a 100% CO₂ atmosphere inhibits the growth of *L. monocytogenes* completely (Hart et al., 1991; Sheridan et al., 1995). However, other authors have found that CO₂ concentrations in the range of 60 to 90% do not prevent its growth (Zeitoun and Debevere, 1991; Marshall et al., 1992). Irkin and Esmer (2010) have reported a bacteriostatic effect when an atmosphere of 20% CO₂/80% N₂ is used in ground chicken breast. In contrast, *L. monocytogenes* growth was observed when 30% CO₂/70% N₂ was applied in raw chicken breast (Shin et al., 2010). However, the fact that most of these studies used a single strain of *L. monocytogenes* instead of a cocktail of strains suggests that the observed growth variability could be due to different strain behavior.

An oxygen-free atmosphere was chosen for experiment 2 because *Bifidobacteria* are anaerobic (Biavati and Mattarelli, 2006). *Campylobacter* have also been found to be sensitive to oxygen at concentrations above 10%, whereas CO₂ has been found to stimulate their growth (Bolton and Coates, 1983). Boysen et al. (2007) have reported that *C. jejuni* inoculated onto chicken fillets survived significantly longer in the presence of 100% N₂ and 70/30% N₂/CO₂ than in the presence of 70/30% O₂/CO₂. The survival of *C. jejuni* inoculated onto chicken legs was scarcely affected by the use of an 80% CO₂ atmosphere when compared with an 80% O₂ atmosphere, where a reduction of 1.2 log cfu/g was achieved (Rajkovic et al., 2010). Thus, the MAP used in this experiment appears not to be responsible for the

reduction in *C. jejuni* count; on the contrary, it could be responsible for its relatively high survival.

To the best of our knowledge, no previous studies concerning the application of protective cultures against *L. monocytogenes* and *C. jejuni* in fresh chicken products under MAP have been reported in the literature.

Leuconostoc pseudomesenteroides PCK18 (5.20 log cfu/g inoculated) reduced the concentration of *L. monocytogenes* by 1.22 log cfu/g in MAP chicken burger meat at the end of the study. In a similar study, Hugas et al. (1998) found a reduction in the concentration of *L. innocua* of around 1 log cfu/cm² in raw chicken breast inoculated with 6 log cfu/cm² of *Lactobacillus sakei* CTC494 and packaged under 20% CO₂/80% O₂ of around 1 log after 7 d. Likewise, Dortu et al. (2008) reported only a bacteriostatic effect when a combination of 2 protective cultures is applied to chicken breast in comparison with the reduction of 2 log found when the same combination is applied to beef. This lesser effect in chicken meat can be explained by the fact that diffusion of the protective culture and its antimicrobial substances may be limited in minced meat and the fact that some of these substances can bind to meat and fat particles (Schillinger et al., 1991; Dortu et al., 2008). Larger reductions of *L. monocytogenes* due to the antimicrobial activity of a protective culture have been obtained in studies carried out in MRS broth or on the surface of meat pieces (Castellano et al., 2008; Maragkoudakis et al., 2009), where both the protective culture and the target bacteria find themselves in a water-rich environment as a result of the inoculation procedure. Such an environment facilitates the diffusion of these antimicrobial substances, whereas once the protective culture is entrapped in the meat matrix its efficacy decreases (Schillinger et al., 1991; Dortu et al., 2008). It is remarkable that the reduction of *L. monocytogenes* was obtained in this study upon mixing the protective culture and pathogen in the meat batter.

Gram-negative bacteria are insensitive to bacteriocins produced by gram-positive bacteria due to their outer membrane, which prevents these substances from reaching the cytoplasmic membrane (Castellano et al., 2008). A study published in 1994 pointed out that *Bifidobacteria* inhibit a range of both gram-positive (*L. monocytogenes*, *Clostridium perfringens*) and gram-negative bacteria (*Campylobacter*, *Escherichia coli*) by excreting a particular metabolite, with the most potent effect generally being exerted by *Bifidobacterium infantis* and *B. longum* (Gibson and Wang, 1994). Many other anti-*Campylobacter* strains have been tested in vitro (Chaveerach et al., 2004; Jones et al., 2008; Messaoudi et al., 2012), in fermented products (Pavlović et al., 2006), or in vivo as probiotic strains added to poultry feed (Neal-McKinney et al., 2012; Willis and Reid, 2008). In this sense, Santini et al. (2010) reported the marked in vitro and in vivo anti-*Campylobacter* activity of *B. longum* PCB133. Thus, after daily administration for 2 weeks, *C. jejuni* numbers were found to be significantly lower (by 1 log) in the chicken group fed

with the probiotic strain. However, no reports concerning the effect of a protective culture against *C. jejuni* in MAP chicken meat have been found in the literature. In this study, a 1.16 log cfu/g reduction of *C. jejuni* was obtained when *B. longum* PCB133 was inoculated onto fresh chicken legs at d 9.

The addition of *Lc. pseudomesenteroides* PCK18 and *B. longum* PCB133 as protective cultures had no effect on the pH of the samples inoculated as other authors have reported with the addition of *Lactobacillus curvatus* and *Lactobacillus plantarum* on raw meat and poultry meat (Castellano and Vignolo, 2006; Maragkoudakis et al., 2009). In this study, the shelf-life of chicken burger meat was extended from 10 d to 17 to 24 d upon packaging under the selected atmosphere. Likewise, the shelf-life of fresh chicken legs was extended from 3 d (air-packaged) to 6 to 9 d (MAP-packaged). This extension of the shelf-life in terms of TVC is in agreement with other authors who found that atmospheres with a CO₂ concentration above 30% allow the extension of the shelf-life (Jiménez et al., 1997; Patsias et al., 2008).

Although no inhibition of LAB was observed in any of the chicken meat burger samples, a concentration of 6 to 7 log cfu/g was achieved at d 10 for the air-packaged samples (A) and at d 17 for almost all the MAP samples. The LAB are the predominant bacteria in meat preserved under MAP, and in this respect the results presented in this study are in agreement with previous studies (Kakouri and Nychas, 1994; Jiménez et al., 1997; Patsias et al., 2006). This LAB behavior can be explained by a lack of competition with other microorganisms inhibited by MAP. In the case of fresh chicken legs, the MAP batches became spoiled at least 3 d later than the air-packed batch. A greater effect of the protective culture against indigenous LAB was also observed in experiment 2 in comparison with experiment 1. Likewise, the batches inoculated with the protective culture (B and JB) showed a delay in the growth of LAB, thus resulting in a longer shelf-life (by 3 d) than for the air-packaged batch. Cheikhyoussef et al. (2010) described an antimicrobial activity of *B. infantis* against several LAB in vitro. A similar beneficial effect of a protective culture against spoilage bacteria has also been reported in other studies. For example, the shelf-life of sliced, vacuum-packed cooked ham inoculated with *L. sakei* B-2 was 35 d, compared with 15 d for the control product, due to the inhibition of the undesirable indigenous LAB (Hu et al., 2008). In another study, sliced pariza, vacuum-packed and inoculated with *Lactobacillus alimentarius*, was found to be acceptable up to 51 and 42 d when inoculated with 1.6×10^3 and 1.6×10^5 cfu-cm⁻² of slice, respectively, whereas control pariza had a shelf-life of only 23 d (Kotzekidou and Bloukas, 1998).

In conclusion, no previous studies concerning the effect of a protective culture against *L. monocytogenes* and *C. jejuni* in MAP chicken meat products have been found. Thus, *Lc. pseudomesenteroides* PCK18 showed anti-*Listeria* activity when both microorganisms were

inoculated in chicken burger meat. Moreover, an anti-*Campylobacter* activity of *B. longum* PCB133 has also been observed together with a delay in the growth of LAB in chicken legs. Further studies are, however, required to determine the mechanism underlying these antimicrobial activities. In both cases, the MAP used doubles the shelf-life of products in comparison with their air-packaged counterparts.

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