Research Note

Inhibition of *Listeria monocytogenes* and *Salmonella* by Natural Antimicrobials and High Hydrostatic Pressure in Sliced Cooked Ham

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MS 04-223: Received 28 May 2004/Accepted 19 September 2004

ABSTRACT

The effectiveness of nisin, lactate salts, and high hydrostatic pressure to inhibit the growth of *Listeria monocytogenes* and *Salmonella* in sliced cooked ham was studied through a combination of PCR-based detection methods, most probable number, and classical microbial enumeration techniques (International Organization for Standardization protocols). A synergistic effect to inhibit a cocktail of *Listeria monocytogenes* CTC1010, CTC1011, and CTC1034 was observed between potassium lactate, high hydrostatic pressure (400 MPa, 17°C, 10 min), and low storage temperature when sliced cooked ham was stored for 84 days at 1°C. The high hydrostatic pressure treatment also proved to be useful to inhibit a cocktail of *Salmonella enterica* serotypes London CTC1003, Schwarzengrund CTC1015, and Derby CTC1022.

Sliced cooked ham is a perishable meat product. The low salt content (2% on average), a pH around 6.0, and a water activity higher than 0.945 are only small hurdles to inhibit the usual types of microorganisms associated with postprocessing contamination. Vacuum and modified atmosphere packaging have usually been applied to improve shelf life. However psychrotrophic facultatively anaerobic foodborne pathogens like *Listeria monocytogenes* may still survive and/or grow (23). Repasteurization of cooked ham after slicing and packaging is not recommended because the consequent release of meat juices and fat decreases their acceptability to the consumer. Thus, alternatives to post-packaging treatments such as high hydrostatic pressure (HHP) and natural antimicrobials are being considered.

HHP is an alternative nonthermal food preservation method to avoid postprocessing contamination, especially for foods whose nutritional, sensory, and functional characteristics are thermosensitive. HHP kills or sublethally injures cells by disruption of the cell wall and membrane, dissociation of protein and ribosomal subunit structures, and loss of activity of some enzymes (22). Although it is well accepted by European consumers (8), few reports have dealt with the effect of HHP on microorganisms in the food matrices, i.e., pressurized milk and model meat products under refrigeration (10, 39).

Some bacterial preparations such as Flora Carn LC2 (Chr Hansen) and bacteriocinogenic lactic acid bacteria and/or their bacteriocins have been used as protective cultures, and their activity against *L. monocytogenes* has been proved in meat products (6, 17, 23). The enhanced antimicrobial activity of bacteriocins through HHP has been reported in cell suspensions, milk, and model meat products (2, 18, 24). Lactate salts are widely used in the meat industry for their capacity to improve flavor, safety, and shelf life, and several studies have reported antimicrobial activity against a number of spoilage and foodborne pathogenic microorganisms (20, 33). In Europe the use of lactate salts (sodium lactate [E325], potassium lactate [E326], calcium lactate [E327]) as food additives is allowed in all types of food, as stated in the EU directive 95/2/CE relative to food additives other than colors and sweeteners (14). In the United States, lactate is considered a validated antilisterial compound, and its use is regulated by the *Federal Register* (38).

*Salmonella* and *L. monocytogenes* are the most common causes of death due to foodborne diseases in the United States (31). The incidence of listeriosis is low (3.8% of the illness-related hospitalizations), and the infection dose is high, but the severity of the illness to at-risk populations, such as pregnant women and immunocompromised people, makes it responsible for 27.6% of the deaths associated with foodborne pathogens. In contrast, *Salmonella* is more frequent but less severe. It is responsible for 25.6% of the foodborne illness-related hospitalizations and 30% of the foodborne related deaths in the United States (31).

Early and sensitive detection is a critical issue in public health policy. The identification of *Salmonella* and *L. monocytogenes* by the International Organization for Standardization standards is laborious and time consuming and can last up to 7 to 10 days (3, 4). In recent years, PCR-based methods have been reported as rapid, specific, and sensitive...
alternatives and have been increasingly used to identify several bacterial species from food and clinical samples (30, 32). Many primer sets have been used for the detection of *Salmonella* or *L. monocytogenes*, but they differ in sensitivity and accuracy. Recently, as part of the European Commission-funded project (FOOD-PCR QRLT 1999-00226), PCR-based methods for the detection of both pathogens have been evaluated and validated by full-scale interlaboratory collaborative assays (15, 29). Proper validation based on consensus criteria is an absolute prerequisite for successful adoption of a robust PCR-based diagnostic methodology (21).

The aim of this study was to examine the effectiveness of nisin, lactate salts, and HHP to inhibit the growth of *L. monocytogenes* and *Salmonella* in sliced cooked ham. The behavior of *L. monocytogenes* and *Salmonella* during storage was analyzed through a combination of classical microbiological enumeration techniques and validated PCR assays.

**MATERIALS AND METHODS**

**Products.** Cooked ham was prepared with minced pork shoulder (4% fat, 73 to 74% moisture) and the following additives (g/kg; SKW Biosystems, Rubi, Spain): water, 115; salt, 20.7; dextrose, 5.8; sodium tripolyphosphate, 5.8; carragenate, 2.3; NaNO2, 0.1; and 1-ascorbate, 0.6. Pork shoulder meat was minced in a cutter (Teqmaq, Spain) to a particle size of 4 mm. Ingredients were homogenized in a mixer (model 35P Technotip S.A., Terrassa, Spain) for 30 min and stuffed using a stuffing machine (model H15, Technotrip) into impermeable plastic film (Prolan SV 150) to facilitate the homogenization of ingredients and natural antimicrobials and to standardize the slices (sliced at 1.5 mm thick). The product was cooked in an oven at 75°C until internal temperature reached 72°C (total cooking time 2.6 h). Three independent batches were prepared: batch 1 (control), batch 2 (800 arbitrary units of nisin A [Nisaplin; Aplin and Barrett Ltd., UK]), and batch 3 (3% Purusal P/H Pure 60 [1.8% potassium lactate]; Purac Biochem, Holland).

**Inoculation of foodborne pathogens and pressurization treatment.** A high level (3 × 10^4 CFU/g) and a low level (3 × 10^2 CFU/g) of a cocktail of three *L. monocytogenes* strains (CTC1010, CTC1011, and CTC1034) and three *Salmonella enterica* subsp. *enterica* (serotypes London CTC1003, Schwarzengrund CTC1015, and Derby CTC1022) were spread between two slices of the different cooked ham batches. Inoculum cocktails were prepared by diluting −80°C stored stocks in 0.1% peptone, and 0.85% NaCl and 140 μl were used for inoculating each pair of slices. Slices were vacuum packaged in triple plastic bags of PET/PE with oxygen permeability <50 (cm^3/m^2/24 h) and water vapor permeability <15 (mg/m^2/24 h) (Sacoliva S.L., Barcelona, Spain). HHP treatment was performed in an industrial hydrostatic pressurization unit (Alstom, Nantes, France [chamber volume of 320 liters and diameter of 280 mm]) at 400 MPa for 10 min at 17°C. The pressurization fluid was water, the come-up time was 17.5 min, the pressure release time was 1.5 min, and the adiabatic heat generated was 5°C. After treatment, the pressurized and nonpressurized samples were stored at 1°C or 6°C to study the effect of temperature during a 3-month refrigerated storage.

**Microbial analysis and PCR pretreatment of the samples.** Triplicates of each batch were analyzed before pressurization and 24 h, 3, 6, and 12 weeks after the HHP treatment by combining classical microbiological techniques and validated PCR protocols (FOOD-PCR project: http://www.pcr.dk). For expected counts higher than 10 CFU/g, brilliant green agar plates (Difco, Sparks, Md.) and Palcam plates (Merck, Darmstadt, Germany) were used for enumeration of *Salmonella* and *L. monocytogenes*, respectively. Five colonies of each plate were confirmed by validated species-specific PCR (FOOD-PCR European project). Two microliters of a 10-fold dilution of the colony suspension in 25 μl of distilled water was used for the assay. For expected counts under 10 CFU/g, the most-probable-number (MPN) technique was used. Serial dilutions of three or five buffered peptone water tubes of three successive dilutions were incubated at 37°C for 40 h. Buffered peptone water was used as a nonselective media in order to recover the sublethally injured bacteria and not to underestimate counts. The growth of *L. monocytogenes* and *Salmonella* in MPN tubes was determined by the validated species-specific PCR assays. In case of doubt, both methods were applied.

Three pretreatments were considered: (i) 10-fold dilution of the enrichment broth (i.e., positive MPN tube), (ii) centrifugation of the enriched culture at 9,500 × g for 2 min and resuspension in 1:50 initial volume, and (iii) Chelex 100–based DNA extraction. Briefly, the pellet from the 2-ml enriched culture was dissolved in 300 μl of 6% Chelex 100 (Bio-Rad, Richmond, Calif.), incubated at 56°C for 20 min, boiled for 8 min, and cooled on ice. Thorough mixing between each step was needed. Cell debris were centrifuged at 18,000 × g for 5 min, and the supernatant was used for PCR reaction.

**PCR reactions.** Species-specific PCR assays recently validated as part of the European Commission-funded project (FOOD-PCR; http://www.pcr.dk) were performed. For *Salmonella* spp. the reaction conditions were 2 μl of DNA, 2 μl of 10× PCR buffer (Invitrogen, Merelbeke, Belgium), 1.5 mM MgCl2, 200 mM each deoxyxynucleoside triphosphate (dNTP), 0.4 mM of each primer (139 [5′-gttaatatacgcagctggcgca] and 141 [5′-tcgaaacctgaagaaggagac]), 1 mg/ml bovine serum albumin, and 0.75 U of platinum *Taq* DNA polymerase (Invitrogen), and 300 copies of the corresponding internal amplification control. The PCR program was as follows: 1 min at 95°C and 38 cycles of 30 s at 95°C, 30 s at 64°C, 30 s at 72°C, and a final extension of 4 min at 72°C. For *L. monocytogenes* the program was 2 μl of DNA, 2 μl of 10× PCR buffer (Invitrogen), 2.5 mM MgCl2, 150 mM each dNTP, 0.3 mM each primer (Lip1 [5′-gtcagagagaatactgggtgcc] and Lip2 [5′-gtaacttggatgcatcgg]), 1 mg/ml bovine serum albumin, 1 U of platinum *Taq* DNA polymerase (Invitrogen), and 1 pg of the corresponding internal amplification control. The PCR program was 2 min at 94°C and 40 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 74°C, and a final extension of 5 min at 74°C. The sensitivity of both PCR reactions is 10 CFU per reaction (15, 29).

**RESULTS**

The effect of storage temperature, inocula, the presence of nisin and potassium lactate with or without HHP at 400 MPa for 10 min at 17°C on the inhibition of the growth of *L. monocytogenes* and *Salmonella* were assayed. The combination of International Organization for Standardization protocols and PCR-validated protocols (FOOD-PCR) enabled the enumeration of *L. monocytogenes* and *Salmonella* in two working days. This reduced the sampling time and workload of the microbiological analysis, as opposed to the traditional confirmation methods, which require a minimum of 7 days for unequivocal identification.
Effect of initial contamination level and storage temperature. The initial contamination level (3 × 10^2 and 3 × 10^4 CFU/g) did not affect the survival of *Salmonella* under refrigerated storage in any of the batches (data not shown) or the growth of *L. monocytogenes* in the cooked ham containing lactate and stored at 6°C (Fig. 1B). In contrast, in the nisin and in the control batches stored at 6°C, the growth of *L. monocytogenes* was higher in the batch with low initial contamination level and counts of 10^6 CFU/g were achieved in both treatments after 41 days of storage at 6°C (Fig. 1).

The growth of *L. monocytogenes* during refrigerated storage was dependent on the temperature used. After 21 days at 6°C and without HHP treatment, *L. monocytogenes* showed counts of 10^8 CFU/g in the nisin and control batches, while at 1°C the counts were significantly lower (*P* < 0.0001), with values of 10^3 CFU/g (Fig. 1B). However, at the end of the refrigerated storage period (3 months) at 1°C, control batches and the samples containing nisin with high initial inocula level achieved the same level as the samples stored at 6°C (Fig. 1B). The batch containing potassium lactate allowed growth of *L. monocytogenes* when stored at 6°C, while at 1°C *L. monocytogenes* did not grow and remained at initial levels independent of the inocula level (Fig. 1A).

In samples not treated with HHP, *Salmonella* did not grow during the refrigerated storage period either at 1°C or 6°C in any of the treatments assayed (data not shown).

Effect of natural antimicrobials and HHP. In nonpressurized batches, the addition of nisin at a concentration of 800 AU/g did not significantly reduce the growth of *L. monocytogenes* during the storage period when compared with the control batch. Moreover, when considering the batches with low initial inocula level, after 3 months of storage at 1 or 6°C the counts were significantly higher (*P* < 0.0001) than those in the control batch (Fig. 1). The inactivation of *L. monocytogenes* was significantly higher in the batch containing potassium lactate (*P* < 0.0001). Potassium lactate at a concentration of 1.8% (3% Purasal HI Pure 60) was able to inhibit the growth of *L. monocytogenes* until the end of vacuum storage when stored at 1°C and the initial levels remained until the end of the storage period; however, after 3 months of storage at 6°C, *L. monocytogenes* was able to grow to 10^6 CFU/g and 10^8 CFU/g from low or high initial contamination levels, respectively (Fig. 1). The behavior of *Salmonella* was not significantly different (*P* > 0.15) in the treatments containing antimicrobials (nisin or lactate) when compared with the control batch with high and low initial inocula. The counts remained at the initial levels during the storage period either at 1°C or 6°C (data not shown).

A high hydrostatic posttreatment of the vacuum-packaged sliced cooked ham at 400 MPa for 10 min at 17°C was immediately able to reduce the number of viable cells of *L. monocytogenes* (Fig. 2) and *Salmonella* (data not shown). The counts of *Salmonella* significantly diminished after treatment (*P* < 0.0001) and remained at this level until the end of the storage period either at 1°C or 6°C, with levels under 4 MPN/g at the end of the storage period. Besides, more than 50% of the samples containing nisin recorded an absence of *Salmonella* in 25 g during the storage period (data not shown). The growth of *L. monocytogenes* significantly diminished (*P* < 0.0001) after the HHP treatment, and the growth was inhibited in all the treatments until the 42nd day of storage at 1°C or 6°C. After this period and when stored at 6°C, *L. monocytogenes* was able to grow and achieved counts of 10^6 CFU/g in the control batch while in the nisin batch significantly lower levels (*P* < 0.0001) were obtained (3.5 × 10^4 CFU/g) (Fig. 2). In the potassium lactate samples, the amount of the reduction of *L. monocytogenes* counts after HHP was significantly lower (*P* < 0.0001) compared with the nisin and control batches; however, *Listeria* was not able to recover during the storage period and the counts maintained until the end of the storage at 6°C at ≤21 MPN/g and even significantly diminished at 1°C (*P* < 0.0001). Very low counts (1 MPN/
g) of *L. monocytogenes* were obtained in sliced cooked ham when combining lactate, HHP, and 1°C refrigeration (Fig. 2), and a synergistic effect between the three factors was observed (significant interaction, *P* < 0.0001).

**DISCUSSION**

HHP associated with antimicrobials and low storage temperature may represent an efficient nonthermal treatment for avoiding postprocessing contamination in cooked ham. Sliced cooked ham has been described as a good candidate for HHP since color, texture, and flavor did not change with pressurization treatments (13). Moreover, the thermodurability of the selected antimicrobials allowed their presence at the most critical step, slicing, when the total microflora is very low and may facilitate the growth of a particular strain.

In nonpressurized samples, potassium lactate was able to delay the growth of *L. monocytogenes* significantly even at a higher refrigeration temperature (6°C), although it was necessary to decrease the refrigeration temperature to 1°C to inhibit the growth of the pathogen. Without HHP treatment, the combination of lactate, 1°C, and vacuum packaging reduced *L. monocytogenes* 5 log units when compared with the control batch. These results are in contrast to the bacteriostatic antilisterial effects claimed by Bacus and Bontenbal (7) in frankfurters with 2% lactate and stored at 4.4°C, but they are in agreement with the reported effectiveness of 2 to 2.5% of lactate to delay but not to inhibit the growth of *Listeria* in unsmoked bratwurst, smoked wieners, cooked roast beef stored at 4.5°C (20, 35, 36), and the enhanced antilisterial activity at lower storage temperatures (16, 20, 37). As previously described, a positive antilisterial interaction between nisin and HHP was recorded at the end of the storage time at 6°C (12, 25). *L. monocytogenes* is a psychrotrophic microorganism that can even grow at 0.4°C (9). In accordance with the results of Garriga et al. (19) in meat models stored at 4°C, the best treatment to diminish the counts of *Salmonella* efficiently in spiced sliced cooked ham was HHP. Although nisin has been applied at the maximum intake dosage allowed for a child of 30 kg consuming 100 g every day (1,780 IU/g) (11), the absence of *Salmonella* was only achieved in some of the samples of the nisin batch treated with HHP. Nisin is an amphiphilic peptide that adsorbs to the food matrix and is sensitive to proteases and pasteurization (1, 5). The previously reported synergistic effects of nisin and HHP in gram-negative bacteria (18, 27) were not conclusive, probably due to a critical level of active bacteriocin.

The triple hurdle combination technology including HHP, 1°C, and potassium lactate at 1.8% gave the best results for the control of the growth of *L. monocytogenes* in sliced cooked ham, and a synergistic effect was noted. Immediately after HHP, the drop of *Listeria* counts was significantly greater in the control and nisin batches than in the lactate batch, although in this study this could not be attributed to the suggested capability of lactate to reduce the water activity and the subsequent lower antimicrobial effectiveness of HHP (26, 34). However, the lactate batches reduced the counts to 1 MPN/g at the end of the storage period thus minimizing the tailing problems that are associated with HHP treatments. The synergistic effect observed in this study was in accordance with the hurdle technology and the concept of multitarget preservation introduced by Leistner (28). Different hurdles directed at different target sites should be more effective in the preservation of a particular food system because the homeostasis of the microorganisms is disturbed in several ways (28). In this study, the combination of (i) HHP that destabilizes the structural and functional integrity of the cytoplasmic membrane, induces protein denaturation, and inhibits genetic mechanisms (22) with (ii) potassium lactate, which is reported to lower the water activity, acidify the cytoplasm after entering the cell, and chelate the iron from meat (34), and (iii) the storage at 1°C could explain why this triple treatment was the best. Several authors have reported a combination of different synergistic factors such as low pH and antimicrobials (nisin, lysozyme, lactoperoxidase system, enterocin, sakacin, and pediocin) with HHP in vitro, in milk, and in meat model systems (2, 18, 19, 26, 27), but this is the first report showing a synergistic effect between HHP, lactate, and a low storage temperature.

From the safety point of view, the use of PCR-based detection methods and their combination with International Organization for Standardization standards proved to be very useful and fast. Thus the assayed validated PCR protocols may be considered as convenient alternatives to traditional methods in challenge tests and a way to speed up and increase the microbial controls in the hazard analysis critical control point plans.

**ACKNOWLEDGMENTS**

This study has been supported by The Ministry of Science and Technology of Spain (CICYT) within the project number AGL2002-03496 “Food safety in meat products: application of emerging preservation techniques and evaluation of pathogens through development of new molecular techniques.” The authors thank Elena Avelli for her secretarial support.

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ERRATUM

In the article “Inhibition of Listeria monocytogenes and Salmonella by Natural Antimicrobials and High Hydrostatic Pressure in Sliced Cooked Ham,” by Aymerich et al., that appears in the Journal of Food Protection 68(1):173–177, Figures 1 and 2 are reversed. The legends for the figures are accurate and correspond to the text.