Listeria *Inactivation by the Combination of High Hydrostatic Pressure and Lactocin AL705 on Cured-Cooked Pork Loin Slices*

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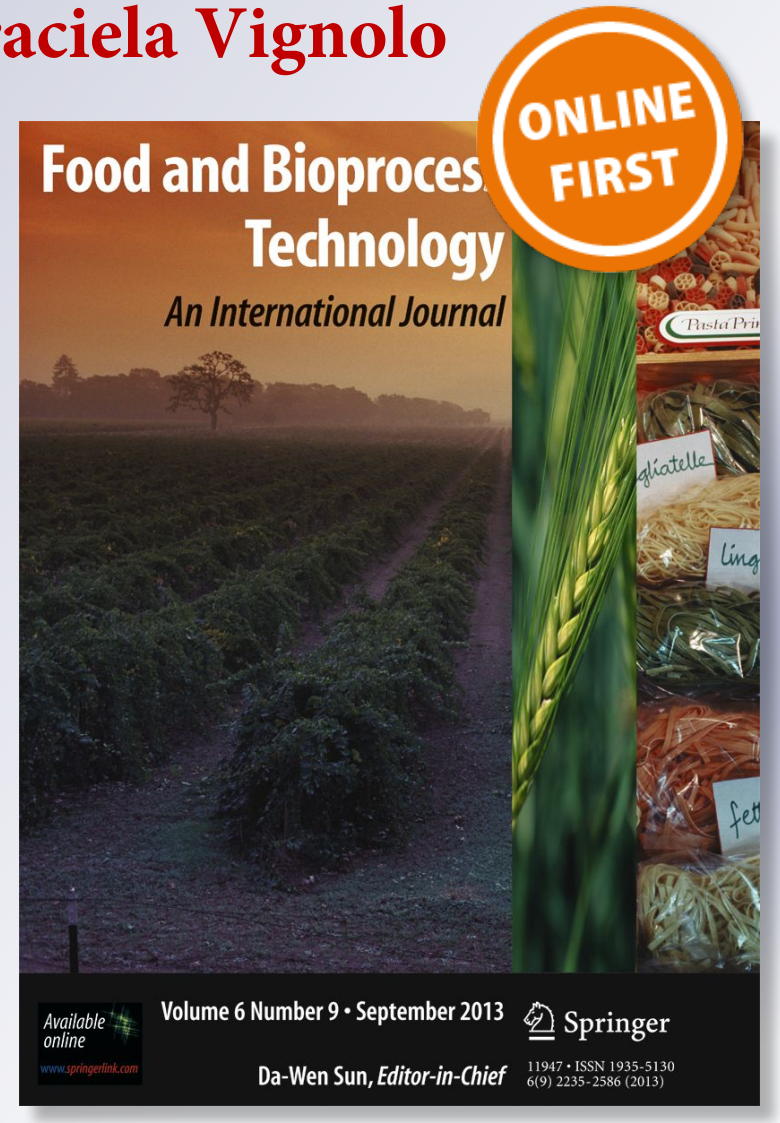

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ORIGINAL PAPER

Listeria Inactivation by the Combination of High Hydrostatic Pressure and Lactocin AL705 on Cured-Cooked Pork Loin Slices

Andrea Micaela Dallagnol[1] · Yanina Barrio[2] · Mariana Cap[2] · Natalia Szerman[2,3] · Patricia Castellano[4] · Sergio Ramón Vaudagna[2,3] · Graciela Vignolo[4]

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Abstract The effect of the bacteriocin lactocin AL705 in combination with high hydrostatic pressure (HHP) on the inactivation of *Listeria innocua* 7, a nonpathogenic indicator for *Listeria monocytogenes*, deliberately inoculated (ca. 6.4 log CFU/g) onto the surface of ready-to-eat (RTE) sliced cured-cooked pork loin, was evaluated. Nontreated pork slices (control) and treatments subjected to lactocin AL705 (105 AU/ml) and/or HHP (400 or 600 MPa) were prepared. *L. innocua* 7 was monitored at days 1, 20, and 40 of storage at 4 °C. The results showed a complete inhibition of *L. innocua* 7 after the combined treatment with lactocin AL705 and 600 MPa and no regrowing of cells up to 40-day storage. The treatment at 600 MPa alone was not enough to avoid regrowth of *L. innocua*. Ultrastructural cell damage was observed at the cytoplasm and cell membrane/wall levels with all treatments; however, complete cell lysis was observed only with the combined treatment. HHP in combination with lactocin AL705 provided a wider margin of safety as post-processing listericidal treatment of RTE cured-cooked meat products.

Keywords High hydrostatic pressure · Lactocin AL705 · *Listeria* · Cured-cooked pork loin · Cell ultrastructural changes

Vaudagna S.R. and Vignolo G. contributed equally to this work.

✉ Andrea Micaela Dallagnol
andymicd@gmail.com

[1] Posadas, Instituto de Materiales de Misiones (IMAM - CONICET) y Universidad Nacional de Misiones (UNaM), Ruta 12, Km 7.5, Posadas, Misiones, Argentina

[2] Instituto de Tecnología de Alimentos, Centro de Investigación de Agroindustria, Instituto Nacional de Tecnología Agropecuaria (INTA), Nicolas Repetto y de los Reseros s/n, Hurlingham, Buenos Aires, Argentina

[3] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

[4] Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, San Miguel de Tucumán, Tucumán, Argentina

Introduction

To avoid contamination with pathogens, different alternatives have been used by food processors, including the application of post-packaging treatments and antimicrobial agent/process. Among the available food preservation methods, high hydrostatic pressure (HHP) stands as a nonthermal process able to inactivate and eliminate pathogenic and food spoilage microorganisms (Considine et al. 2008; Rendueles et al. 2011). This technology found enormous potential in the food industry, improving food safety, controlling food spoilage, and extending product shelf life without substantial modification of their nutritional, functional, and sensory properties (Hugas et al. 2002; Welti-Chanes et al. 2005). By means of high pressure processing, foods (liquids or solids) are subjected to pressures between 50 and 1000 MPa, depending on the desired objective (Welti-Chanes et al. 2005; Rendueles et al. 2011). The loss of viability of microbial cells through HHP is the result of a combination of injuries in the cell; when the accumulated damage exceeds the ability of the cell for reparation, death occurs. The cell membrane is the main target for HHP, its permeability and functionality being mainly affected; ribosomes, protein synthesis, and the structure of DNA-enzyme complexes were also reported as sensitive (Rendueles et al. 2011; Mota et al. 2013). Microbial resistance to HHP is highly variable, depending on the type of organism and the food matrix involved (Masana et al. 2015).

The effect of high pressure on a range of microorganisms, including *Escherichia coli* O157:H7, *Listeria innocua*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Campylobacter jejuni*, and *Yersinia enterocolítica*, has been

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reported (Ponce et al. 1998; Morgan et al. 2000; Garriga et al. 2004; Black et al. 2005; Jofré et al. 2009; Gunther et al. 2015). In addition, a number of studies have investigated the effectiveness of using HHP in combination with bacteriocins such as nisin, pediocin, lacticin 3147, and enterocin AS-48 for the inhibition of food-borne bacteria and spoilage by lactic acid bacteria (Morgan et al. 2000; Kalchayanand et al. 2004; Kaletunç et al. 2004; Black et al. 2005; Pérez Pulido et al. 2015). HHP treatment was reported to cause increased viability loss to bacterial cells; however, in the presence of bacteriocins, an increased cell death occurred, presumably because of the associated damages inflicted in the cell envelope (wall and membrane) of Gram-positive bacteria, while irreversible sublethal injury in the cell envelope allows the sensitization of Gram-negative bacteria to bacteriocins (Somolinos et al. 2008; Rendueles et al. 2011). Such technology has many advantages in food processing, including the reduction of energy costs and the production of safer, more palatable products (Marcos et al. 2008; Hereu et al. 2012).

Listeria is a genus with more thermoresistant members, some of them more pathogenic than many other nonsporing genera of food-borne pathogens. Among them, *L. monocytogenes* has been widely related with post-processing contamination (EFSA 2013; FSIS 2014). This pathogen has been isolated from a wide variety of ready-to-eat (RTE) foods and is responsible for several listeriosis outbreaks linked to the consumption of meat, poultry, dairy, fish, and vegetable products. The ability of *L. monocytogenes* to multiply in various foods at refrigeration temperatures, high pH and NaCl and low oxygen concentrations, and residual nitrite (McDonnell et al. 2013; Ribeiro and Destro 2014; King et al. 2016) makes its occurrence in RTE meat products of particular concern (Ross et al. 2009; Gómez et al. 2015). The ability of *L. monocytogenes* to contaminate finished products and colonize, persisting on industrial premises, is likely responsible for a large number of food recalls, redesigns, and/or rejections at great cost to producers (Ferreira et al. 2014). Since RTE products do not require further thermal treatment before consumption, the absence or low levels of pathogenic microorganisms are of paramount importance.

The production of antibacterial substances by the meat-borne *Lactobacillus curvatus* CRL705 has been previously reported (Castellano et al. 2003; Cuozzo et al. 2003). Antimicrobial effectiveness was demonstrated for lactocin 705 and the antilisteria lactocin AL705 applied to chilled meat slurry and vacuum-packed fresh meat as well as to activated polymers to control *L. innocua* on the surface of wieners (Castellano et al. 2004; Castellano and Vignolo 2006; Blanco Massani et al. 2014). Cured-cooked pork loin prepared by brine injection and cooking of *Longuissimus dorsi* muscle, which is marketed sliced and vacuum-packed as a RTE chilled product, was used in this study. The combination of HHP treatment and lactocin AL705 addition was assayed to inactivate *L. innocua* 7, a nonpathogenic indicator for *L. monocytogenes*, after processing and during chilled storage of vacuum-packed cured-cooked pork loin slices. Morphological and structural changes after treatments were also investigated by transmission electron microscopy (TEM).

Material and Methods

Bacterial Strains and Culture Conditions

L. innocua 7 obtained from the Unité de Recherches Laitieres et Genetique Appliqué, INRA (France), was grown for 24 h in trypticase soy broth (TSB) with 0.5% added yeast extract (YE) at 30 °C. The bacteriocin-producing *L. curvatus* CRL705 isolated from dry-cured sausages (Vignolo et al. 1993) and deposited at CERELA culture collection was grown overnight in De Man, Rogosa, and Sharpe (MRS) broth at 30 °C. Both microorganisms were stored in TSB-YE and MRS containing 20% (*v*/*v*) glycerol at −20 °C. Unless otherwise stated, all the media used were supplied by Biokar (Beauvais, France).

Preparation of Lactocin AL705 Bacteriocin

L. curvatus CRL705 was inoculated (2%) in 1 l of MRS broth and incubated at 30 °C for 24 h. Cells were removed by centrifugation (11,200×*g* for 10 min at 4 °C) (Sorvall RC-3C, Sorvall Instruments, USA); the supernatant was collected, and proteins were precipitated with ammonium sulfate (Anedra, Argentina) up to 60% saturation. This process was carried out at 4 °C for 30 min under mild stirring. Proteins were then pelleted by centrifugation (21,952×*g* for 30 min at 4 °C) (Sorvall RC-3C), dissolved in 20 ml of potassium phosphate buffer (20 mM, pH 6.5), and heat treated (65 °C for 30 min). The semi-purified bacteriocin (lactocin AL705) was stored at 2 °C; the activity of the bacteriocin solution was determined by the critical dilution method according to Vignolo et al. (1993). Briefly, plates with a bottom layer of 1.5% TSB-YE agar and a top layer of 0.7% TSB-YE soft agar freshly inoculated with 10 μl of an overnight indicator culture of *L. innocua* 7 were prepared. After solidification, serial two-fold dilutions of the bacteriocin solution (30 μl) were filled in wells (5 mm) cut into the plates and incubated at 30 °C for 24 h. The antimicrobial activity, defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn, was expressed in activity units per milliliter (AU/ml). Sterile MRS broth was used as control. The presence of lactocin AL705 activity on slices of cured-cooked pork loin was qualitatively evaluated by removing 100 μl of exudate fluid from packaged slices, centrifuged at 13,000 rpm for 5 min at room temperature (Eppendorf 5417C, Germany), and the supernatant (5 μl) spotted on semi-solid TSB plates freshly inoculated

(0.1%) with *L. innocua* 7 (Castellano et al. 2010). Plates were incubated at 30 °C for 24 h, and the presence of inhibition zones was observed.

Cured-Cooked Pork Loin Preparation

Cured-cooked pork loins were prepared as reported by Szerman et al. (2013). Briefly, pre-selected pork loins (*Longissimus dorsii*, 48 h post-slaughter, weight 1386.36 ± 116.25 g, pH 5.58 ± 0.07) were injected to 120% over the original weight using an automatic multi-needle injector (36 needles, FRICOR, Buenos Aires, Argentina) with a brine solution containing sodium chloride (NaCl, Dos Anclas, Argentina), sodium tripolyphosphate (STPP, N15-16 Chemische Fabrik Budenheim R.A Oetker, Budenheim), potassium chloride (KCl, Dos Anclas, Argentina), sodium nitrite ($NaNO_2$, General Chemical, Tecnoalimenti, Argentina), and liquid smoke flavoring. The final concentrations of the ingredients and additives in the injected muscles were 0.60% NaCl, 0.50% STPP, 1.20% KCl, and 150 ppm $NaNO_2$. Four cured-cooked pork loins were used for the complete experiment. The loins were then tumbled intermittently for 60 min (9 rpm, 5 min on/10 min off) at 1.5 ± 0.5 °C in a Lance Industries tumbler (model LT-15, Allenton, USA), vacuum-packed in Cryovac BB2800 bags (Sealed Air, Buenos Aires, Argentina), and stored at 1.5 ± 0.5 °C during 18 h. Finally, the pork loins were cooked in an electric oven (Kerres-1600 EL, Backnang, Germany) at 180 °C until reaching a core end temperature of 72 °C. After cooking, the loins were vacuum packaged and stored at 1.5 ± 0.5 °C for 18 h to facilitate subsequent slicing. Then, the cooked loins were sliced (1.5–2.0 mm × 40 mm × 70 mm; 6.54 ± 0.45 g/slice) using a slicer machine (Berkel Rotterdam Type 834, Netherlands) and kept for further experiments. To avoid meat contamination, ethanol (70%) was used for slicer and dissection area cleaning and disinfection.

Inoculation of Listeria, Bacteriocin Addition, and High Hydrostatic Pressure Treatment

A completely randomized design with a 2 × 3 factorial arrangement was applied. The studied factors were the concentration of lactocin AL705 added to the cured-cooked pork slices (0 and 105 AU/cm^2) and the pressure level (0.1, 400, and 600 MPa) as shown in Table 1. The obtained pork slices were randomly assigned to the following treatments: untreated (control) (C0), lactocin AL705 (105 AU/cm^2) (L0), 400 MPa (C400), lactocin AL705 + 400 MPa (L400), 600 MPa (C600), and lactocin AL705 + 600 MPa (L600). Three samples (slices) from each treatment were analyzed at each testing time (1, 20, and 40 days); 216 slices were prepared for the complete experiment, and two independent trials were carried out.

Table 1 Factorial arrangement (2 × 3) used to evaluate the effect of lactocin AL705 and HHP treatments on cured-cooked pork loin slices

Lactocin AL705 (AU/cm)	HHP (MPa)		
	0.1	400	600
0	C0	C400	C600
105	L0	L400	L600

C0 untreated slices (control), *L0* lactocin AL705, *C400* 400 MPa, *L400* lactocin AL705 + 400 MPa, *C600* 600 MPa, *L600* lactocin AL705 + 600 MPa

L. innocua 7 was used to inoculate 54 pork slices. The inoculum was prepared from an overnight culture (*ca.* 2 × 10^9 CFU/ml), washed twice with saline solution (NaCl 0.85%), and diluted (1:10) with the same solution to attain 2 × 10^8 CFU/ml. The washed cells were used to separately inoculate the surfaces of the pork loin slices by spreading (140 μl each) *ca.* 4 × 10^6 CFU/g (1 × 10^6 CFU/cm^2) which were allowed to dry for 2–3 min. Then, the semi-purified lactocin AL705 solution (100 μl) was spread over the surface of inoculated pork slices using a sterile spatula allowing its absorption. Slices were then individually vacuum packaged (Cryovac BB2800, Sealed Air, Buenos Aires, Argentina) and subjected to pressurization. HHP treatment was performed in a Stansted Fluid Power Ltd. system (Iso-Lab FPG9400:922, Stansted, UK) at 400 and 600 MPa for 5 min at 5 °C. After treatments, the pressurized and nonpressurized samples were stored at 4 ± 0.5 °C during 1, 20, and 40 days.

Sample Analysis

Microbiological Analysis

L. innocua 7 was enumerated by homogenization of meat slices with 0.1% (*w*/*v*) peptone solution (1:10) for 2 min in a Stomacher 400 Lab Blender (Seward Medical, UK). Tenfold serial dilutions prepared with saline solution were used to inoculate (0.1 ml) Palcam (Biokar) agar plates. The plates were incubated at 37 °C for 48 h, and the results were expressed as log CFU per square centimeter (CFU/cm^2). Total aerobic mesophilic bacteria (TAMB) and lactic acid bacteria (LAB) initially present (control) and after treatments were enumerated in cured-cooked pork loin slices by deep seeding plate count agar (PCA) and MRS (Britania, Argentina) agar plates with 1 ml of each dilution. The plates were incubated at 30 °C for 48 h, and the results were expressed as log CFU per gram (CFU/g). To avoid interferences on the PCA medium with *L. innocua* 7, noninoculated slices were used as control.

pH and a_w Determinations

The pH was measured in homogenates of cured-cooked pork loin slices (5 g of meat slices in 25 ml distilled water pH 7.0) with a pH meter (Thermo Orion 710A+, Beverly, MA, USA) equipped with a combination pH electrode (Thermo Orion Model 9172BN) and an ATC probe (Thermo Orion, Beverly MA, USA). The water activity (a_w) was determined by using an AquaLab Series 3 instrument (Decagon Devices, USA). Samples were cut into pieces of <0.5 cm and placed into the AquaLab sample cup. Before use, the water activity meter was calibrated and measurements were taken at room temperature (25 °C).

Ultrastructural Changes in Listeria Cells as Determined by Transmission Electron Microscopy

TEM analyses were performed to evaluate the ultrastructural changes produced by lactocin AL705 and/or HHP on *Listeria* cells. *L. innocua* 7 cells were grown for 12 h in TSB-YE broth at 30 °C to *ca.* 1×10^9 CFU/ml. Cells were then harvested by centrifugation; the supernatant was discarded, and cells were suspended in one volume of PBS 0.1 M (pH 7.4). The suspension was distributed (40 ml) in sterile 50-ml conical centrifuge tubes (UltraCruz, Santa Cruz Biotechnology, Texas, USA) and subjected to the different treatments (C0, L0, C400, and L400). For L0 and L400, lactocin AL705 semi-purified solution (105 AU/ml) was added prior to vacuum packaging, whereas C400 and L400 samples were pressurized (400 MPa) as previously described. All samples were incubated at 4 ± 1 °C for 2 h, and *Listeria* viability was assessed using Palcam agar medium. For TEM, cells were harvested by centrifugation and fixed with Karnovsky's solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer) at 4 ± 1 °C for 2 h. The fixing solution was eliminated from cells with three consecutive washes with PBS. The cells were post-fixed with 1% (*w*/*v*) osmium tetroxide in phosphate buffer at 4 ± 1 °C for 2 h. Dehydration was performed in ethanol in a series of 50% (2×) and 70, 80, 90, and 100% (3×) dilutions for 10 min each and finally dried with 100% acetone. After the washing and dehydration steps, the pellets were infiltrated in 100% epoxy resin (Spurr 1969). After hardening, thin sections were stained with uranyl acetate and Reynolds' lead citrate (Reynolds 1963). These sections were examined in a transmission electron microscope (Joel 1200 EX II, USA).

Statistical Analysis

Data were compared by analysis of variance (ANOVA) and Tukey test using Minitab software (Minitab Statistic Program, release 8.21; Minitab Inc., Philadelphia, PA, USA). Mean values plus standard deviation (SD) were presented.

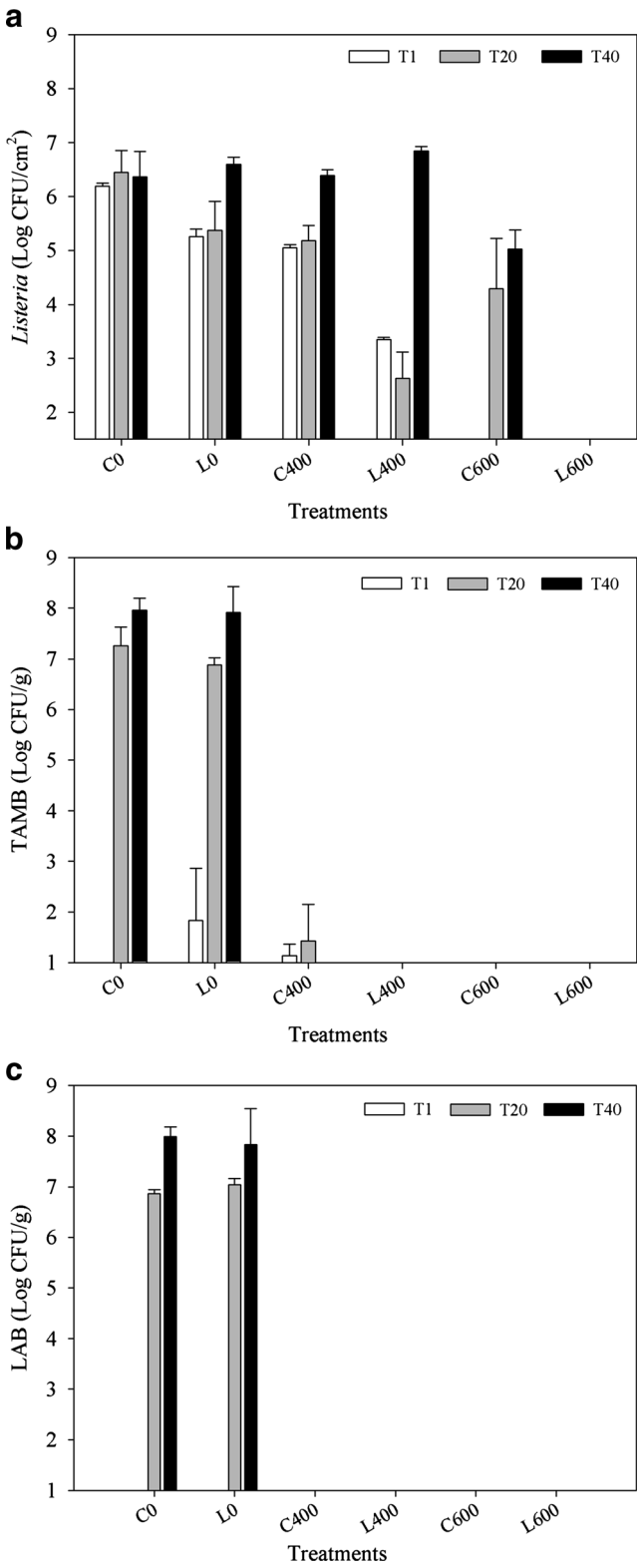
Results and Discussion

Microbiological and Physicochemical Changes in Cured-Cooked Pork Loin Slices

Sliced cured-cooked pork loin is a ready-to-eat product of which safe shelf life can be compromised due to cross-contamination during slicing and packaging operations. In spite of improvements made by good manufacturing practices, such as appropriate cleaning, sanitation and hygiene programs, and effective temperature control throughout the food production, distribution, and storage chains, the ongoing presence of *L. monocytogenes* is still high (EFSA 2013). In this study, the effect of semi-purified lactocin AL705 produced by *L. curvatus* CRL705 combined with HHP treatments on *L. innocua* 7 inoculated on vacuum-packaged cured-cooked pork loin slices during chilled storage at 4 ± 0.5 °C was evaluated. High *Listeria* inoculum was used (*ca.* 6.4 log CFU/g) in order to validate treatments as a pasteurization method. *L. innocua* 7 was used as a pathogen model instead of *L. monocytogenes* because of their similar growth rate, final population at 4 °C, and sensitivity to lactocin AL705 as was previously reported (Castellano et al. 2004). No significant differences were observed between *Listeria* species suggesting that both microorganisms associated with meat might be reduced by a similar amount of bacteriocin (Castellano et al. 2004; Blanco Massani et al. 2014).

When cured-cooked pork loin slices were inoculated with *L. innocua* 7 (C0), counts ranged between 6.3 and 6.5 log CFU/cm^2 throughout the 40-day storage at 4 °C (Fig. 1a). In the presence of lactocin AL705 alone (L0), although a minor count (5.3 to 5.4 log CFU/cm^2) in *Listeria* cells up to 20 days was observed, a regrowth to 6.5 log CFU/cm^2 was produced at 40 days of storage. These results indicated a bactericidal effect of lactocin AL705 on *Listeria* up to 20 days at 4 °C, while its growth has been previously prevented on vacuum-packaged raw beef discs during 36 days at 2 °C (Castellano and Vignolo 2006). It is known that the effectiveness of *Listeri*a inhibition by bacteriocins is dependent on several factors such as storage temperature (Mataragas et al. 2006), meat product composition (presence of additives), and distribution of bacteriocin molecules in the meat matrix as well as bacteriocin diffusion rate (Ananou et al. 2005). In addition, the high initial *L. innocua* 7 inoculum (> 6 log CFU/cm^2) used in this study and the spontaneous resistance development to class IIa bacteriocins (such as lactocin AL705) previously reported (Castellano et al. 2001; Gravesen et al. 2002) may account for the *Listeria* outgrowth observed at 40 days.

Similarly, HHP treatments at 400 and 600 MPa for 5 min were not enough to eliminate all *Listeria* during storage at 4 °C (Fig. 1a). When the 400 MPa treatment was applied (C400), a decrease of about 1 log CFU/cm^2 on *Listeria* counts



◀ **Fig. 1** Counts of *Listeria innocua* 7 (**a**), total aerobic mesophilic bacteria (TAMB) (**b**), and lactic acid bacteria (LAB) (**c**) on vacuum-packaged cured-cooked pork loin slices stored at 4 ± 0.5 °C under different treatments. Control (*C0*): untreated slices and treated with lactocin AL705 (*L0*), 400 MPa (*C400*), lactocin AL705 + 400 MPa (*L400*), 600 MPa (*C600*), and lactocin AL705 + 600 MPa (*L600*). Values are mean ± standard deviation from triplicate sampling and two independent experiments. The minimum level of detection was 2 $\log_{10}$ CFU/cm^2 (Listeria) and 1 $\log_{10}$ CFU/g (TAMB and LAB)

was observed at 20 days while a regrowth up to 6.4 log CFU/cm^2 was also produced at the 40-day storage; this result was identical to that obtained with lactocin AL705 alone. On the other hand, although the number of survivors was initially reduced below the detection limit when the 600 MPa treatment was applied (C600), *L. innocua* 7 reached 4.4 and 5.2 log CFU/cm^2 at 20- and 40-day storage, respectively. This result agrees with the generally accepted fact that the degree of inactivation is directly related to the level of pressure applied (Garriga et al. 2004). The ability of *L. monocytogenes* to recover after a 600 MPa treatment was reported for cooked chicken stored during 21 days at 8 °C (Patterson et al. 2011) and cheese stored during 14 days at 4 °C (Tomasula et al. 2014). However, the efficacy of HHP at 600 MPa against *L. monocytogenes* and Gram-negative food pathogens on convenience meat products during 120 days of storage at 4 °C was reported (Jofré et al. 2009); the more severe HHP treatment conditions (6 min/31 °C) and lower initial pathogen load (3.5 log CFU/g) may be accounted for the obtained results. The initial contamination level highly affects *L. monocytogenes* reduction by HHP; the time to inactivation increased with increasing inoculum level and decreasing pressure magnitude from 1.5 min at 700 MPa/2 log CFU/ml to 15 min at 450 MPa/6 log CFU/ml as was reported by Youart et al. (2010). Indeed, according to this developed model, more than 7 min would have been necessary to inactivate the *Listeria* inoculum used in this study. On the other hand, it is known that different microorganisms react with different degrees of resistance to HHP treatment; vast HHP process sensitivity differences among bacterial species and even strains may occur (Considine et al. 2008).

In general, Gram-positive bacteria are more resistant to pressure than Gram-negative bacteria, and cocci are more resistant than rod-shaped bacteria. In addition, the type of substrate and composition of the food can have a dramatic effect on the sensitivity of microorganisms to HHP. Carbohydrates, proteins, lipids, and other food constituents can confer a protective effect; some foods appear to give more protection than others, and this may be due to the ability of rich media to provide essential vitamins and amino acids to stressed cells (Considine et al. 2008). Furthermore, a controversial influence of NaCl and nitrite in cured meat products may be suggested, not only because these chemicals could act as an extra hurdle with an additive effect on *Listeria* growth inhibition but also because salt content seemed to exert a considerable baroprotective effect against HHP inactivation of microorganisms mainly due to the water activity reduction (Myers et al. 2013).

Based on bacterial counts, antilisteria bacteriocin (lactocin AL705) and HHP single treatments were not able to inhibit the proliferation of *Listeria* strain under the assayed conditions; therefore, combined treatments involving bacteriocin solution + HHP treatments were assayed. The results showed a pronounced initial effect (L400 and L600) compared to lactocin AL705 and HHP alone, suggesting a synergistic effect of both hurdles on cured-cooked pork loin slices. While reductions of *L. innocua* 7 population by 2.7 and 3.5 log CFU/cm^2 were found in samples treated with lactocin AL705 + 400 MPa (L400) at 1 and 20 days of storage, respectively, the regrowth of *Listeria* cells up to control values was produced at the 40-day storage at 4 °C. However, the complete elimination of *L. innocua* 7 immediately after the combined treatment of lactocin AL705 + 600 MPa (L600) was produced on cured-cooked pork loin slices, *Listeria* counts resulting below the detection limit (1×10^2 CFU/cm^2). Similar results were reported by the combination of nisin and 600 MPa on RTE sliced dry-cured ham, which resulted in the reduction of *L. monocytogenes* immediately after HHP treatment and throughout 60 days at 8 °C (Hereu et al. 2012). Moreover, HHP treatment at 600 MPa for 8 min in combination with enterocin AS-48 was found as the most effective strategy for improving the microbiological safety of fuet-type sausages, rice pudding, and cherimoya pulp (Ananou et al. 2010; Pérez Pulido et al. 2012). In the present study, the combined activities of lactocin AL705 and HHP resulted in substantial cell death, which exceeded that observed with either of these individual treatments. The combination of both hurdles (biopreservation and HHP) was able to provide a wider margin of safety for the control of both Gram-positive and Gram-negative bacteria during the storage of high pH and a_w RTE products.

When lactocin AL705 activity in exudates from pork slices previously treated with the combined strategy was qualitatively evaluated, residual activity was detectable in all samples, this being higher at 1-day storage at 4 °C than at 20 and 40 days (Table 2). HHP seemed to have no effect on the activity of bacteriocins, since similar results were observed in exudates after treatment at 400 or 600 MPa (5 min, 4 °C). As reported by Rendueles et al. (2011), low-molecular-weight compounds such as bacteriocins are hardly affected by HHP; thus, residual bacteriocins in the food matrix would continue to exert their inhibitory effect after treatment with nonthermal processing hurdles, thereby inactivating and preventing the recovery of any sublethally injured cells (Morgan et al. 2000). Indeed, *L. innocua* 7 injured cells resulting from the C600 treatment (HHP alone) were observed to recover during storage time, whereas those from the L600 treatment (HHP + lactocin AL705) did not.

TAMB and LAB naturally present in cured-cooked pork loin slices and those after treatment with lactocin AL705 and HHP were assessed. Absence or counts near the detection limit (10 CFU/g) for both bacterial groups were found for unpressurized control samples (C0 and L0) after 1 day of incubation at 4 °C; counts around 7.0 and 8.0 log CFU/g after 20 and 40 days, respectively, were then reached by both populations (Fig. 1b, c). Although very low or absence of counts for TAMB and LAB, respectively, was observed in samples treated with 400 MPa during refrigerated storage, a complete bacterial elimination was observed in samples treated at 600 MPa alone or both HHP treatments combined with lactocin AL705. Similarly, HHP at 600–650 MPa for 5–6 min at 20 and 31 °C was effective for avoiding the growth of psychrotrophs, *Enterobacteriaceae*, and LAB with a potential as spoilage microorganisms in different commercial meat products (Garriga et al. 2004; Vaudagna et al. 2012). Additionally, similar results to the present study were reported when nisin and pressurization (300–400 MPa, 10 min, 17 °C) were evaluated in a meat model system during storage at 4 °C for 61 days in which slime-producing LAB were maintained below the detection limit (Garriga et al. 2002; Black et al. 2005).

In this study, although no significant differences ($p > 0.05$) among samples were recorded regarding treatments, the pork slices exhibited a slight variation in pH values; the combined treatment (L400 and L600) exhibited a significant reduction in pH units after the 40-day storage (Table 2). Concerning a_w values, they ranged between 0.982 and 0.989, higher a_w being obtained for L600 sample after 40 days. In general, low water activity protects cells against pressure, decreasing HHP effectiveness as reported for different meat products by Aymerich et al. (2005). Because of the high water activity of the pork loin slices, TAMB and LAB from cross-contamination during processing grew quickly at 8.0 log CFU/g in control and bacteriocin-treated samples in 40 days, whereas all pressurized samples showed very low or lack of associated spoilage microorganisms, contributing to freshness maintenance of the product.

Ultrastructural Changes on *Listeria* Cells

In a separate trial, TEM was used to study the ultrastructural changes brought about by lactocin AL705 and HHP on *L. innocua* 7 cells in a buffer solution. *L. innocua* 7 viability in TSB-YE broth prior to treatments showed similar counts (ca. 9.3 log CFU/ml) for untreated samples (C0) and those added with lactocin AL705 (105 AU/ml) after 2 h at 4 °C, whereas treated samples showed a 1 log *Listeria* reduction in C400 (400 MPa) and 5 log in L400 (400 MPa + lactocin AL705) samples (data not shown). TEM results (Fig. 2) showed nontreated cells (C0) exhibiting a typical structure of Gram-positive bacteria with a thick and uniform wall where the cytoplasmic membrane was tightly adhered and the cytoplasm showing lighter patches that would reflect as vacuoles (Fig. 2a). After 2 h of exposure to the bacteriocin solution

Table 2 pH values, water activity (a_w), and antimicrobial activity of lactocin AL705 during treatments of cured-cooked pork loin slices stored at 4 ± 0.5 °C

Treatments	pH			a_w			Lactocin AL705 activity		
	1 day	20 days	40 days	1 day	20 days	40 days	1 day	20 days	40 days
C0	6.38 ± 0.03	6.39 ± 0.04	6.36 ± 0.05	0.986 ± 0.001	0.984 ± 0.001	0.986 ± 0.002	−	−	−
L0	6.25 ± 0.05	6.41 ± 0.10	6.26 ± 0.07	0.986 ± 0.003	0.983 ± 0.001	0.986 ± 0.003	++	+	+
C400	6.35 ± 0.06	6.34 ± 0.04	6.36 ± 0.02	0.984 ± 0.002	0.987 ± 0.002	0.984 ± 0.002	−	−	−
L400	6.32 ± 0.06	6.32 ± 0.10	6.28 ± 0.01	0.988 ± 0.002	0.987 ± 0.005	0.985 ± 0.002	++	+	+
C600	6.39 ± 0.06	6.36 ± 0.03	6.45 ± 0.10	0.987 ± 0.004	0.982 ± 0.001	0.984 ± 0.000	−	−	−
L600	6.52 ± 0.02	6.47 ± 0.06	6.21 ± 0.05	0.985 ± 0.003	0.987 ± 0.003	0.989 ± 0.001	++	+	+

No significant difference ($p > 0.05$) was observed among treatments. Values are mean ± standard deviation from triplicate sampling and two independent experiments

C0 untreated slices (control), *L0* lactocin AL705, *C400* 400 MPa, *L400* lactocin AL705 + 400 MPa, *C600* 600 MPa, *L600* lactocin AL705 + 600 MPa, ++/+ qualitative inhibition halo size, − absence of inhibition halo

(105 AU/ml) in PBS, a dramatic change in the ultrastructure of *Listeria* cells was induced (Figs. 2b and 3a). The enlargement of an electron-transparent layer between the plasmatic membrane and the outer cell wall layer was observed; chromatin condensation and mesosome-like membranous formations protruding into the cytoplasm indicated the severe injury of the cytoplasmic membrane with pore formation and subsequent lysis. These findings may be related to the viability loss (1 log cycle) of *L. innocua* 7 on pork slices at 20 days and the regrowth of noninjured and/or sublethally injured cells produced thereafter (Fig. 1a, L0). When HHP alone was applied at 400 MPa, the separation between the cell wall and cytoplasm membrane (Fig. 2c) was higher than that produced by lactocin AL705 alone (Fig. 2b). However, the cellular morphology was not really affected and a part of the HHP-treated population retained its membrane integrity, this being in agreement with that previously reported for *L. monocytogenes* (Tholozan et al. 2000; Huang et al. 2015). This heterogeneity of the treated cells suggests that under favorable conditions, cellular repair may occur as shown

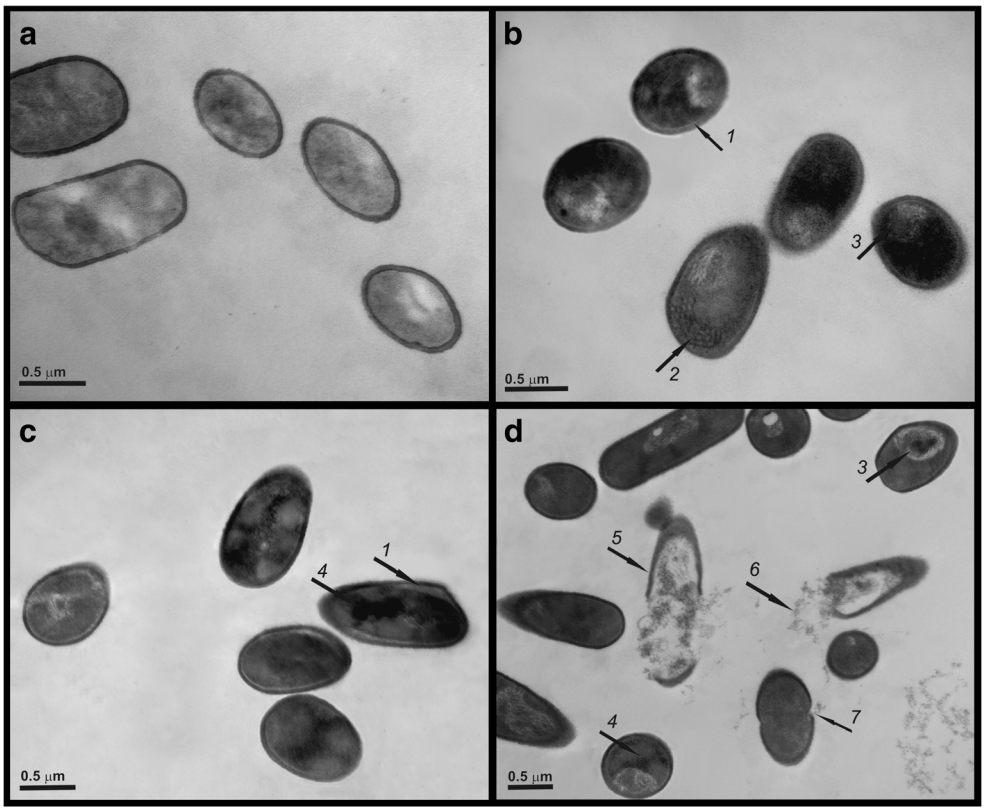


Fig. 2 TEM micrographs of *Listeria innocua* 7 cells. **a** Control (*C0*): untreated cells. **b** Cells treated with lactocin AL705 (*L0*). **c** Cells treated at 400 MPa and 5 °C for 5 min (C400). **d** Cells treated with 105 AU/ml lactocin AL705 + 400 MPa (L400). *Arrows* indicate electron-transparent area between the cytoplasm membrane and cell wall (*1*), mesosome-like membranous formation protruding into the cytoplasm (*2*), nuclear content condensation (*3*), protein condensation (*4*), lysed cells (*5*), plasmatic material outside the cell (*6*), and membrane rupture (*7*). *Magnification bar* in micrometers

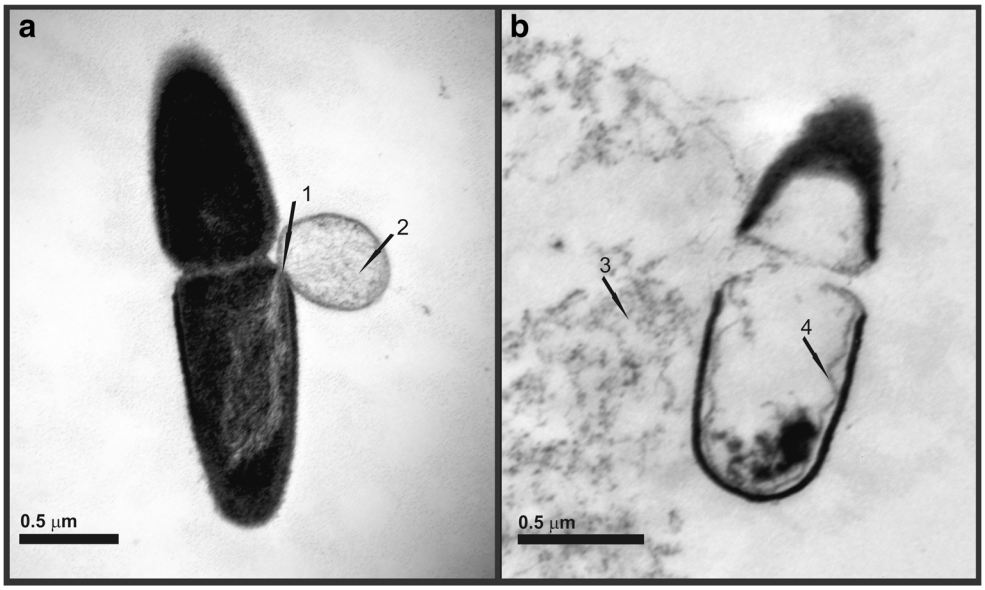


Fig. 3 TEM micrographs of *Listeria innocua* 7 cells with higher magnification. **a** Cells treated with lactocin AL705 (*L0*) and **b** cells treated with lactocin AL705 + 400 MPa (*L400*). *Arrows* indicate pore at septum level (*2*), cytoplasm material protruding from the cell through the pore (*2*), plasmatic material outside the cell (*3*), and waviness of the plasmatic membrane (*4*). *Magnification bar* in micrometers

by the increase in *L. innocua* 7 numbers at 40 days of storage (Fig. 1a, C400). Indeed, piezotolerance for *L. monocytogenes* strains was previously reported (Ritz et al. 2001; Kalchayanand et al. 2004).

On the other hand, HHP treatment at 400 MPa for 5 min in the presence of lactocin AL705 and the subsequent incubation during 2 h showed a more noticeable ultrastructural damage and numerous lysed cells (Figs. 2d and 3b). Thickened and disrupted cell walls with increased roughness and the lack of cytoplasm were observed, this pattern of abnormalities being evident in almost all the cells scanned under TEM. These observations may be correlated with *Listeria* reductions which occurred after exposure of the pork slices to 600 MPa + lactocin AL705 (Fig. 1a). *Listeria* cell disintegration after this combined treatment, as shown in Fig. 3b, may be the consequence of autolytic enzyme activity causing hydrolysis of the cell wall, as was reported for *Leuconostoc mesenteroides* (Kalchayanand et al. 2002).

Conclusion

This is the first study involving lactocin AL705 and high HHP (400–600 MPa, 5 min at 5 °C) levels as a nonthermal method for the eradication of *Listeria*. The treatment at 600 MPa alone, generally accepted as an effective treatment in cured-cooked meat products, was not enough to avoid *L. innocua* 7 regrowth in this study. However, the synergistic effect of both hurdles (lactocin AL705 and HHP at 600 MPa) was effective in reducing 6 log CFU/g (6D) of *L. innocua* 7 in vacuum-packed cured-cooked pork loin slices, assuring no regrowth of injured cell during refrigerated storage at 4 °C. Severe morphological damages to the cell wall/membrane and cytoplasmic components were produced by the combined treatment and evidenced by transmission electron microscopy. HHP associated with lactocin AL705 and low storage temperatures may represent an effective nonthermal pasteurization method for decreasing bacterial load and controlling post-processing regrowing of injured cells in refrigerated RTE cured-cooked meat products.

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