Combined effect of enterocin AS-48 and high hydrostatic pressure to control food-borne pathogens inoculated in low acid fermented sausages

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ABSTRACT

The single and combined effects of enterocin AS-48 and high hydrostatic pressure (HHP) on Listeria monocytogenes, Salmonella enterica, and Staphylococcus aureus was investigated in fuet (a low acid fermented sausage) during ripening and storage at 7°C or at room temperature. AS-48 (148 AU g⁻¹) caused a drastic 5.5 log cfu g⁻¹ decrease in L. monocytogenes (P < 0.001) and a significant (P < 0.01) inhibition (-1.79 logs) for Salmonella at the end of ripening (10 d). After pressurization (400 MPa) and storage Listeria counts remained below 5 cfu g⁻¹ in all fquets containing AS-48 (pressurized or not). HHP alone had no anti-Listeria effect. HHP treatment significantly reduced Salmonella counts, with lowest levels in pressurized fquets with AS-48. S. aureus showed similar growth for all treatments and storage conditions. These results indicate that AS-48 can be applied alone to control L. monocytogenes and combined with HHP treatment to control Salmonella in fquets.

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1. Introduction

Fermentation is a traditional preservation technology that provides relatively stable meat products with typical sensorial characteristics. The type and manufacture of fermented sausages differ depending on the country. The consumption of low acid fermented sausages generally following a spontaneous fermentation, with a limited acid taste, is common among Mediterranean countries. Fuet is a low calibre fermented sausage made with pork meat, representative of this type of products. Meat and meat products may often act as vehicles for bacterial pathogens. Salmonella enterica, Staphylococcus aureus, and Listeria monocytogenes are three representative raw meat-borne pathogens, being responsible for an increasing number of food-borne outbreaks in Europe (151995 salmonellosis and 1554 listeriosis reported cases in 2007) (EFSA, 2007). S. aureus was responsible for 2053 reported cases of outbreaks caused by bacterial toxins in 2006 (EFSA, 2007). Although listeriosis has remained an uncommon infection with a few proportion of cases and outbreaks of illness, it is still of great concern for several reasons: the high mortality of L. monocytogenes infections (20–30%), its ubiquity in raw foods and its capacity to overcome different hurdles used in processed foods (Swaminathan, Cabanes, Zhang, & Cossart, 2007). It is also of great concern the increasing number of susceptible individuals (YOPIs: young, old, pregnant, and immunocompromised people). Therefore, it seems vital to find new methods to control L. monocytogenes, especially in ready-to-eat foods.

There is a growing trend to develop new alternative processing and preservation technologies, which could be applied in combination with chemical or natural/biological preservatives. The latter include lactic acid bacteria (LAB) and their antibacterial peptides, the bacteriocins. Several LAB bacteriocins (nisin, enterocin AS-48, enterocin A and B, sakacin, and pediocin AcH) have been tested, alone or in combination with other physical or chemical hurdles, in order to control bacterial pathogens in foods (reviewed by Gálvez, Abriouel, Lucas López, & Ben Omar, 2007). Enterocin AS-48 is a cationic circular bacteriocin produced by Enterococcus faecalis S-48, very stable to heat and pH. We have proved in vitro that its broad bactericidal activity affects most of the Gram-positive bacteria and some Gram-negative bacteria (Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1989). The efficacy of AS-48, alone or in combination with other hurdles (chemical preservatives and heat), to control some of the food-borne pathogenic (Salmonella spp., L. monocytogenes, S. aureus, Bacillus cereus) and food-
spoilage bacteria (*Bacillus* spp., *Paenibacillus* spp.) has been also verified in several foods such as milk and cheese, meat products, juices, and vegetables (Muñoz et al., 2004, 2007; Ananou, Garriga, et al., 2005; Ananou, Maqueda, Martínez-Bueno, Gálvez, & Valdivia, 2005; Ananou, Gálvez, Martínez-Bueno, Maqueda, & Valdivia, 2005; Grande et al., 2006a, 2006b, 2007a, 2007b).

Among the alternative technologies used in food preservation, high hydrostatic pressure (HHP) treatment has emerged as a non-thermal food preservation method. It is energy efficient, and is employed to destroy contaminants using an isostatic pressure between 100 and 600 MPa. In general, HHP at low or moderate temperature destroys microbial vegetative cells and inactivates enzymes without changing the organoleptic characteristics and leaving vitamins intact. In addition, sublethally HHP-injured cells become more susceptible to some antimicrobial substances (Hugas, Garriga, & Monfort, 2002). This technology has been applied to a range of food products on a commercial scale, such as fruit juices, hummus, dips, jams, guacamole, oysters, or ready-to-eat meat products (Lau & Turek, 2007) and it is being assessed in many others, including different meat products (Garriga, Aymerich, Costa, Monfort, & Hugas, 2002; Garriga, Grébol, Aymerich, Monfort, & Hugas, 2004; Jofré, Garriga, & Aymerich, 2007). Since some foodborne pathogens may survive after mild HHP treatments, combinations of HHP with additional hurdles such as LAB bacteriocins (Garriga et al., 2002; Jofré, Aymerich, & Garriga, 2008; Jofré, Aymerich, Grébol, & Garriga, 2009; Kalchyanandan, Dunne, Stikes, & Ray, 2004; Marcos, Jofré, Aymerich, Monfort, & Garriga, 2008), lactate-diace tate (Marcos et al., 2008), lactoperoxidase system (Garcia-Graells, Valckx, & Michiels, 2000), or low pH (Garcia-Graells, Hauben, & Michiels, 1998) have been assayed with the aim of improving treatment efficacy.

In the present study we have investigated the antibacterial effects of enterocin AS-48 alone or in combination with HHP treatment against three meat-borne pathogens: *L. monocytogenes*, *S. enterica*, and *S. aureus*. A fermented traditional sausage, the fuet, was spiked with these pathogens and stored under different conditions, applying the bacteriocin as additive in the formulation and also in combination with HHP treatment.

2. Materials and methods

2.1. Bacterial strains

*E. faecalis* A-48-32 was used as an enterocin AS-48 producer. *E. faecalis* S-47 is routinely used as a bacteriocin-sensitive strain to determine the inhibitory activity of AS-48 preparations. The following strains, from the IRTA co-authors collection, were inoculated in the meat mixture as AS-48 target bacteria: *L. monocytogenes* (CTC1010, CTC1011, CTC1034), *S. enterica* ser. Derby CTC1022, *S. enterica* sp. enterica ser. London CTC1003, *S. enterica* sp. enterica ser. Schwarzengrund CTC1015, and *S. aureus* (CTC1008, CTC1019, CTC1021). Previously, the susceptibility of strains to AS-48 was determined by the agar well diffusion method as described below.

2.2. Enterocin AS-48 preparation

*E. faecalis* A-48-32 was cultivated for 8 h at 30°C in BHI broth plus 0.5% glucose. Bacteriocin was recovered by cation exchange chromatography on carboxymethyl Sephadex CM-25 (as described by Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003). Eluted fractions were dialyzed against distilled water through a 2000-Da cut-off membrane and sterilized by filtration through a 0.22-μm pore size Millex GV filter (Millipore, Belford, MA, USA). Fractions were tested for bacteriocin activity against the indicator strain *E. faecalis* S-47 and the multi-strain mixture of *L. monocytogenes* used to inoculate the sausage mixture by the agar well diffusion method using stainless steel cylinders of 8 mm (outer) diameter (Gálvez, Maqueda, Valdivia, Quesada, & Montoya, 1986). The titre of bacteriocin, obtained from a two-fold dilution series, was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and it was expressed in arbitrary units (AU) per millilitre. Usually, from one liter culture we get about 20 ml of concentrated bacteriocin.

2.3. Fuet manufacture, inoculation with food-borne pathogens and bacteriocin treatment and ripening

Fresh meat was bought directly from a local cutting plant and was tempered overnight in a cold chamber at −1/0°C before sausage manufacture. The sausage mixture contained lean pork (50%) and backfat pork (50%) ground through a 6 mm plate, mixed in a kneading machine together with the following ingredients (in g kg⁻¹): sodium chloride, 20 g kg⁻¹; black pepper, 2.5 g kg⁻¹; sodium nitrite 0.1 g kg⁻¹; potassium nitrate 0.1 g kg⁻¹; dextrose, 2 g kg⁻¹; and sodium ascorbate, 0.5 g kg⁻¹; water-diluted pathogen cocktails 2.3 ml kg⁻¹.

The meat batter was inoculated by adding a *L. monocytogenes*, *S. enterica* and *S. aureus* multi-strain cocktail prepared by diluting the −80°C stocks in water (35 ml) to achieve a final concentration of ca. 10⁶ cfu g⁻¹. Afterwards, enterocin AS-48 (80 ml of AS-48 preparation with an activity of approx. 2.2 × 10⁹ AU ml⁻¹) or water were added to obtain the AS-48 batch (148 AU g⁻¹) against *E. faecalis* S-47 and control batch, respectively. Each batch weighed 12 kg. After this, meat batters were stuffed in 28 mm-diameter collagen casings to prepare the raw *fuets* (approx. 200 g each).

Raw *fuets* were dipped in a *Penicillium candidum* suspension (Danisco, France) prepared according to manufacturer instructions, hung in a Sanyo MLR-350 H climate chamber (Sanyo, Osaka, Japan), and ripened for 10 d at 15°C and 72% relative humidity (RH).

2.4. Hydrostatic pressure (HHP) treatment

After the 10-d ripening period, *fuets* (112–131 g) were vacuum packed in polyamide–polyethylene plastic bags (Sacoliva, Castellar del Vallès, Spain) and subjected to pressure treatment (day 11). Pressure (400 MPa for 10 min at 17°C) was applied in an industrial hydrostatic pressurization unit (Alstom, Nantes, France) with a 3201 volume and 280 mm diameter chamber. Pressurization fluid was water. The come-up time was about 9 min and the pressure release time was 1.5 min. After pressure treatment, plastic bags were removed and both non-pressurized and pressurized *fuets* were stored in macroperforated bioriented polypropylene co-extruded bags at room temperature (RT: 22°C, 51% RH) or under refrigeration (7°C, 83% RH) in a climate chamber (MLR-350H, Sanyo). These storage conditions represent the main European consumer habits (Tradisausage EU project QLK1-CT-2002-02240). Three different *fuets* from each treatment (pressurized and non-pressurized) were analysed every sampling time.

2.5. Microbiological analyses

*Fuet* samplings were performed at days 0, 4, 8, and 10 (before HHP treatment), 11 (immediately after HHP treatment), 18, 24, and 30 (storage). At each selected time, the casing was removed and 25 g of *fuets* were ten-fold diluted in tryptic soy broth with 0.6% yeast extract, TSBYE (BD Becton, Dickinson and Company, NJ, USA). The mixture was homogenized for 1 min in a Masticator Classic (JUL S.A. Barcelona, Spain) and serially diluted. The remaining TSBYE homogenate was enriched for 48 h at 37°C.
Bacterial enumeration was done by spread-plating in the following media: Chromogenic Listeria agar (CLA, Oxoid, Madrid, Spain) (37°C for 24–48 h); Brilliant Green agar (BGA, Difco, Sparks, MD, USA) for Salmonella, (37°C for 24 h); Mannitol Salt agar (MSA, Oxoid) for Staphylococci (37°C for 72 h), and Baird Parker agar (BPA, Oxoid) for S. aureus (37°C for 48 h). Lactic acid bacteria were enumerated on de Man Rogosa Sharpe agar (MRS, Merck, Madrid) in double-layered poured plates incubated anaerobically at 30°C for 72 h.

2.6. PCR reactions

Taqman-based real-time PCR was used to investigate the presence/absence of L. monocytogenes and Salmonella in enriched TSBYE homogenates when plate counts were below 5 cfu g⁻¹. Before PCR, the pellet from 1 ml of enriched broth was resuspended in 0.3 ml of Chelex® 100 (BioRad, Madrid, Spain), boiled for 8 min, cooled on ice, and centrifuged at 13,000g for 5 min. Then, extracted DNA was precipitated with 0.1 volume of sodium acetate 3 M, pH 5.2, and 2.5 volumes of ethanol. The pellet was resuspended in 1 mM Tris–HCl, pH 8, and 0.01 mM EDTA, and finally 2 μl of extracted DNA were used for PCR. Real-time PCR assays previously described by Malorny et al. (2004) and by Rodríguez-Lázaro, Jofré, Aymerich, Hugas, and Pla (2004) were used for the detection of Salmonella and L. monocytogenes, respectively, using a 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

2.7. pH and water activity (a_w)

pH was measured with a Crison Basic 20 pH-meter with a Crison penetration 52–32 electrode (Crimon Instruments, Alella, Spain). a_w was measured at 25°C using a Novasina Thermoconstanter TH-500 (Novasina, Pfäffikon, Switzerland).

2.8. Statistical analyses

Statistical analyses were performed using the SPSS-PC 15.0 software (SPSS, Chicago, Ill., USA). Data relating to microbiological counts, a_w, and pH throughout the ripening, HHP treatment, and storage of fuets were subjected to ANOVA. The application of HHP or the presence or absence of enterocin AS-48 were used as factors with the following categories: non-pressurized fuets without AS-48 (control), fuets with AS-48, pressurized fuets without AS-48 and pressurized fuets with AS-48.

3. Results

3.1. Sensitivity of the bacterial strains to enterocin AS-48

None of the strains of S. aureus or Salmonella were sensitive to the AS-48 preparation in vitro (results not shown). In contrast, all L. monocytogenes strains were even more sensitive to AS-48 than the previously tested L. monocytogenes CECT 4032 strain (22,200 AU ml⁻¹ for any of the CTC strains versus 12,800 AU ml⁻¹ for CECT strain) (results not shown).

3.2. Effect of enterocin AS-48 alone or combined with HHP treatment on the bacterial pathogens inoculated in fuets

L. monocytogenes was able to grow in control fuets during ripening (Fig. 1), increasing from an initial value of 2.72 log units to 6.59 log units at the end of ripening (10 d). However, after 4 days the addition of AS-48 (148 AU g⁻¹) caused a significant (P < 0.001) reduction of 3.96 log units in the number of viable L. monocytogenes compared to control fuets. Application of HHP treatment alone only caused a slight reduction of 0.58 log units on the L. monocytogenes population. During storage at RT of fuets without AS-48, pressurized or not, L. monocytogenes counts experienced a slight but continuous decline, probably due to the progressive dehydration, measured by the decrease of a_w (Fig. 5C), of fuets under storage conditions. However, the reductions in L. monocytogenes counts achieved in control fuets were not sufficient to meet safety criteria (European Commission, 2007) except for pressurized fuets in the last period (24–30 d) of storage at 7°C, in which counts decreased to approx. 1 log unit (Fig. 1B). In contrast, with AS-48 containing fuets, pressurized or not, L. monocytogenes counts remained at levels achieved after ripening (below 1 log cfu g⁻¹) during the entire storage period at 7°C as well as at RT (Fig. 1A, B). S. enterica counts also increased in control fuets during ripening, but to a lesser extent (from 2.65 to 3.90 log units at day 10) compared to L. monocytogenes (Fig. 2). The presence of AS-48 caused a significant reduction (P < 0.01) of 1.87 log units at the end of the ripening period. The application of HHP treatment at day 11 reduced Salmonella counts in control fuets by 2.08 log units. In the batch supplemented with AS-48, the application of HHP treatment caused an additional reduction of 0.64 log units in the Salmonella population with respect to counts determined in controls at the same time (P < 0.01). During further storage, Salmonella counts steadily decreased in all types of fuets and at both temperatures. Although the differences were not significant (P > 0.05), the lowest counts attained during storage always corresponded to pressurized
and especially to pressurized AS-48 treated fuet, being below 0.5 log units after 11–18 d storage.

The S. aureus population showed a 4 d lag period and then increased from 2.83 to 5.79 log units at the end of ripening, both in controls and in the bacteriocin-treated samples (Fig. 3). Application of HHP treatment did not reduce viable cell counts significantly in any of the samples. Furthermore, no significant differences were observed between controls and AS-48 treated fuet after pressurization in any of the manufacturing or storage conditions. Therefore, neither AS-48, pressurization, nor the combined treatment seemed to affect the viability of S. aureus, at least under the conditions tested ($P > 0.05$).

3.3. Effect of enterocin AS-48, alone or combined with HHP treatment on endogenous LAB and staphylococci

In control fuet, the initial indigenous staphylococci counts of 2.00 log cfu g$^{-1}$ decreased to 1.10 log cfu g$^{-1}$ at the end of ripening (10 d) (Fig. 4A, B). During storage at RT, they experienced a continuous decrease. In contrast, the endogenous LAB population in control fuet was 3.69 log units at the beginning and increased to 7.19 log units at the end of ripening. A delay in the growth of indigenous populations that was especially significant ($P < 0.01$) in the LAB population was observed during ripening in the AS-48 batch from the beginning of the process (Fig. 4C, D).

In ripened control fuet, the application of HHP treatment caused an immediate significant reduction of 1.06 log units ($P < 0.01$) in the LAB population. However, viable counts recovered to similar values to those of the controls at 18 d storage (at 7 °C or RT). Until the end of storage, no significant differences ($P > 0.05$) in LAB or staphylococci counts were detected between the different types of fuet. HHP had a low effect (if any) on the viability of Staphylococci at any of the storage temperatures.

3.4. pH and water activity evolution

The pH profile was very similar in all fuet, with values around 5.92 at the end of ripening. During storage, pH values increased slightly to approx. 6.5 at the end of storage. Changes in pH values were very similar in all types of fuet and for all storage temperatures, except for minor insignificant ($P > 0.05$) differences between fuet held at 7 °C or at RT at 18 d of storage (Fig. 5A, B).

The profiles of $a_w$ were very similar in fuet batches during ripening, and during storage at the same temperature (Fig. 5C, D). However, after ripening, $a_w$ decreased much more in the fuet held at RT. Significant differences ($P < 0.01$) only were detected at the end of storage at room temperature (24 d) between pressurized fuet (AS-48 treated or untreated) and unpressurized control fuet.

Regarding fuet weight-loss no differences were observed among batches at the end of ripening period (37–41%).

4. Discussion

In the present work we have investigated the efficacy of enterocin AS-48 in fuet to control L. monocytogenes, S. enterica, and S. aur-
The results presented here show that AS-48 was very effective in inhibiting the development of *L. monocytogenes* throughout the ripening and storage periods, regardless of storage temperature and HHP treatment. In contrast, in a previous study carried out in model sausages, the application of 225 AU g⁻¹ failed to keep *L. monocytogenes* CECT 4032 below detection levels after 9 days of incubation, requiring up to 450 AU g⁻¹ (Ananou, Garriga, et al., 2005). However, the meat...
fermentation was performed in closed petri dishes (i.e. no dehydration) and microbial populations were determined for only 9 days. Also, due to the higher sugar concentrations added in the meat mixture, *Listeria* reached much higher levels (up to $10^6$ cfu g$^{-1}$ in control batch). The increased efficacy of AS-48 in *fuets* can be attributed to greater susceptibility of the *L. monocytogenes* strains tested (which were almost two-fold more susceptible when assayed in *vitro* than strain CECT 4032) and also to environmental parameters such as processing conditions, formulation, pH, or $a_w$, which were different in the previously used meat model system. Application of HHP treatment (400 MPa) had no immediate inhibitory effect on *L. monocytogenes* in *fuets* and neither did the combined application of AS-48 and HHP improve the results achieved with the inclusion of enterocin in the meat mixture. It has been reported that the ability of bacteria to survive HHP treatments can be greatly increased in nutritionally rich media containing substances like carbohydrates, proteins, and fat, such as meat, for instance (Simpson & Gilmour, 1997). Previous studies have reported very different inactivation rates for *L. monocytogenes*, depending on the matrix used. In phosphate buffer, *Styles, Hoover, and Farkas* (1991) reported a reduction of more than 7 log cfu g$^{-1}$ at 340 MPa compared to 1.5 log in milk. Other authors (Carlez, Rosse, Richard, & Chefelt, 1993) obtained a negligible decrease at 325 MPa in minced beef muscle, but when they applied 400 MPa, the drop was greater than 5 log units. Garriga et al. (2002) reported a reduction from 9 log cfu g$^{-1}$ to below 2 log cfu g$^{-1}$ after 400 MPa treatment in a meat homogenate and Jofré et al. (2007) reported a reduction from 4 log cfu g$^{-1}$ to below 1 log cfu g$^{-1}$ in cooked ham pressurized at 600 MPa. In *fuets*, the combined application of enterocins A and B and HHP (400 MPa), reduced the counts of *L. monocytogenes* from 2.7 log cfu g$^{-1}$ to below 0.5 log cfu g$^{-1}$ after 24 days of ripening and storage at room temperature (Jofré et al., 2008). The $a_w$ is also a factor reported as influencing the inhibitory effect of HHP and it has been observed that low $a_w$ decreases the susceptibility of bacteria to HHP although inhibiting the subsequent recovery of sublethally damaged cells (US/FA/CFSAN, 2000). Fluid viscosity has also been described to be a major environmental parameter affecting bacterial inactivation by HHP, as opposed to water activity (Diels, Callewaert, Wuytack, Masschalck, & Michiels, 2005). Because the conditions of the present experiments are of high dryness (high viscosity) and low $a_w$, the detected resistance of *L. monocytogenes* to HHP treatment could be attributed to any of both factors. In any case, the heterogeneous results reported in the literature emphasize the importance of experimenting with true food matrices because results obtained in buffers or synthetic media cannot always be extrapolated and applied to real situations.

In contrast to *L. monocytogenes*, *fuets* was revealed not to be a beneficial environment for supporting growth of *Salmonella*, as shown by the counts remaining unchanged for the first 8 d of ripening. Also, in this case AS-48 was active against *Salmonella* and although differences were not significant (except for day 10) *Salmonella* populations in AS-48-added *fuets* always remained below those of the control. It is worth noting that the three different serotype strains used in this challenge were not susceptible to the bacteriocin in *vitro*. This resistance was also observed in previous experiments carried out in culture media and buffer where *S. choleraesuis* LT2 was completely resistant to AS-48, even at concentrations ten-fold higher; only the combination of the enterocin with treatments de-stabilizing the outer membrane rendered *Salmonella* susceptible to AS-48 (Abriouel, Valdivia, Gálvez, & Maqueda, 1998). It is possible that the improved inhibitory activity of AS-48 against *Salmonella* in *fuets* could be caused by the synergistic interaction with other inhibitory factors present in this food product such as nitrate/nitrite, pepper, or the low $a_w$. In contrast to *L. monocytogenes*, HHP treatment caused further inactivation of *Salmonella*, reducing the viable counts of the pathogen by 2.08 and 0.64 log units in control and AS-48 *fuets*, respectively, after pressurization (11 d). The sensitivity of *Salmonella* and higher resistance of *L. monocytogenes* to HHP treatment are coherent with reports ascertaining that the effectiveness of HHP is dependent on the microbial type. In this regard, *Gram-negative bacteria* have generally been found to be more sensitive to HHP than *Gram-positive bacteria* (Hugas et al., 2002). In a previous study, also dealing with low acid fermented sausages treated over the same times and pressures as in this work, Garriga et al. (2005) reported the absence of *Salmonella* in pressurized batches. In the present work, *Salmonella* counts also decreased (although more slightly) in non-pressurized AS-48 added *fuets* during storage, probably due to a decrease in $a_w$. When lower pressure (300 MPa) was applied before ripening of sausages, the population of *Salmonella* (same strains as in the present work) underwent a 2-log reduction during the ripening process (27 days), recording low counts of the pathogen, 3 MPN g$^{-1}$ (Marcos, Aymérich, & Garriga, 2005). Interestingly, given the low AS-48 concentration used in this challenge test, the results obtained suggest that *L. monocytogenes* and *Salmonella* could be efficiently eliminated in naturally contaminated products (probably less densely contaminated), by either the same or higher pressures and AS-48 concentrations, as pointed out in previous studies (Ananou, Garriga, et al., 2005; Jofré et al., 2007; Mendoza, Maqueda, Gálvez, Martínez-Bueno, & Valdivia, 1999).

*S. aureus* was the most resistant pathogen to AS-48 and also to HHP treatment under the conditions assayed in the present work. In fact, after a 5-day lag period, *S. aureus* grew in both control and enterocin batches, reaching at the end of ripening populations that exceeded $10^6$ cfu g$^{-1}$, which are considered necessary to accumulate an intoxicating toxin dose (US/FA/CFSAN, 2003). The low anti-staphylococcal activity displayed by AS-48 in this experiment could be attributed to the ability of the pathogen to grow in low acid fermented sausages, but, mainly, to the non-susceptibility of the *S. aureus* inoculated strains to AS-48. In fact, the lower efficacy displayed by AS-48 against *S. aureus* agrees with our former results obtained in buffer, which revealed *S. aureus* was much more susceptible to AS-48 at pH 5 than at neutral pH (Ananou, Valdivia, Martínez Bueno, Gálvez, & Maqueda, 2004). The poor effect of pressurization could be due to its Gram-positive cell wall and cocoid morphology. Previous studies have already shown that *S. aureus* is among the most resistant vegetative cells to HHP treatment (Hugas et al., 2002; Jofré et al., 2007) and that their inactivation by HHP is highly dependent on the food matrix (Jofré et al., 2009).

More worrying was the delaying effect of AS-48 on LAB growth. However, the addition of the bacteriocin did not influence the evolution of pH in the products since similar values were recorded for control and bacteriocin-treated batches. The increase in pH observed at the end of ripening and during storage is usual for mould ripened sausages (Leistner, 1995) and the decrease in $a_w$ was also expected for this type of product.

## 5. Conclusions

Our results indicate that enterocin AS-48 (148 AU g$^{-1}$) applied alone can be slightly effective against *Salmonella* at the end of ripening but very effective for the inhibition of *L. monocytogenes* in low acid fermented sausages, from the beginning of ripening until the end of the process, as well as during storage at both temperatures studied. This is an important finding, since the pH of this type of food is near neutral and storage at room temperature may facilitate the proliferation of many pathogens and spoiling bacteria. HHP treatment is less effective in this food system than in other meat products such as cooked ham, possibly due to the lower $a_w$. As suggested by the results presented here, further experiments
with higher concentrations of AS-48 combined or not with higher HHP treatments will be necessary to increase the safety of these food products.

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References


