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Prevention of spoilage by enterocin AS-48 combined with chemical preservatives, under vacuum, or modified atmosphere in a cooked ham model

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ABSTRACT

The effect of enterocin AS-48 in controlling *Lactobacillus sakei, Brochothrix thermosphacta*, and *Staphylococcus carnosus* in a cooked ham system has been studied. AS-48 alone showed activity against *Lactobacillus*, with 60 μg g^{-1} reducing lactobacilli below detection levels from the beginning to end of storage at 5 °C. Combinations of 40 μg g^{-1} AS-48 with nitrate/nitrite, pentasodium tripolyphosphate, sodium pyrophosphate, sodium acetate, and sodium lactate reduced *L. sakei* below detection levels from the beginning to end of storage. Even 20 μg g^{-1} of enterocin combined with tripolyphosphate permanently eliminated *L. sakei*. Enterocin AS-48 (40 μg g^{-1}) was also active against *B. thermosphacta* and *S. carnosus*, reducing both bacteria by more than 3 log in the cooked ham. Modified atmosphere (40% CO_2/60% N_2) packaging affected neither bacterial growth nor AS-48 activity against any of the three bacteria. In contrast, storage under vacuum remarkably increased the growth and the inhibitory activity of the enterocin against *B. thermosphacta* and especially against *L. sakei*.

1. Introduction

Meats and meat products are good media for the growth of microorganisms. Spoilage in meats by mesophilic or psychrotrophic microorganisms can occur at room temperature or during cold storage of the products. In this respect, cooked ham, due to its characteristics (low salt content of approx. 2%, pH near 6, and $a_w > 0.945$), is more prone to spoilage than many other meat products. In addition, in cooked ham marketing, ham is sliced and packaged, and then it can become contaminated with spoilage and pathogenic microorganisms and consequently have its shelf life greatly shortened.

Lactic acid bacteria (LAB) have been found to be major contributors to meat-foods spoilage, with *Lactobacillus* spp. being of special concern (Borch, Kant-Muemansb, & Blixt, 1996; Nychas, Marshall, & Sofos, 2007). Although many strains of *Lactobacillus sakei* are often used as starters in cured meats, some strains are described as responsible for meat spoilage due to ropy slime production (Aymerich, Garriga, Costa, Monfort, & Hugas, 2002; Najjari, Ouzari, Boudabous, & Zagorec, 2008). *Brochothrix thermosphacta* is also an important meat and fish spoilage bacterium able to grow both under aerobic and anaerobic conditions (Borch et al., 1996; Holley, 2000). Meats spoiled by *B. thermosphacta* develop an offensive, sour-sweet odour associated mainly with acetoin (Pin, García de Fernando, & Ordóñez, 2002). Staphylococci are microorganisms commonly found on fresh, processed, and even vacuum-packaged meats through contamination from their more common habitat, the skin of animals (Nychas et al., 2007); *Staphylococcus carnosus* is amongst the most frequent species of coagulase negative staphylococci associated with meat products (Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2002).

One strategy in food preservation, designed as biopreservation, is based on the use of microorganisms and/or their natural products. Amongst the antimicrobial substances produced by LAB are the antimicrobial peptides known as bacteriocins. The application of LAB bacteriocins in food technology is currently intended through the combination of these antimicrobials with physical and chemical treatments (Gálvez, Abriouel, Lucas-López, & Ben Omar, 2007). This approach can overcome major challenges for the present-day food industry by mitigating economic losses due to food spoilage and avoiding the transmission of microbial pathogens through the food chain. At the same time, it has the potential of satisfying growing consumer demand for foods that are ready-to-eat, fresh-tasting, nutrient and vitamin rich, and minimally-processed.

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Enterocin AS-48 is a cationic circular bacteriocin produced by *Enterococcus faecalis* S-48 with broad bactericidal activity against most Gram-positive bacteria, including several pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Mycobacterium* spp., *Bacillus cereus*, and some Gram-negative bacteria (Abriouel, Maqueda, Gálvez, Martínez-Bueno, & Valdivia, 2002; Abriouel, Valdivia, Gálvez, & Maqueda, 1998; Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1989). The features of AS-48 (a broad spectrum of antimicrobial activity, stability across a wide range of temperatures and pH, and sensitivity to digestive proteases) (Gálvez, Maqueda, Valdivia, Quesada, & Montoya, 1986; Samyn et al., 1994) make it a promising alternative to chemical preservatives for use as a biopreservative in foods. In fact, AS-48 has been shown to be effective in the control of various food-borne pathogens in dairy, meat, and vegetable products (Ananou et al., 2010; Cobo Molinos et al., 2008; Muñoz et al., 2007) and also against several spoilage bacteria in vegetable and vegetable-derived foods (Grande et al., 2006, 2007). The purpose of the present work was to test the efficacy of AS-48, applied alone or in combination with licensed chemical preservatives or vacuum or modified atmosphere (MA) packaging, in the control of *L. sakei, B. thermosphacta*, and *S. carnosus,* in a cooked ham meat system.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. faecalis A-48-32 (Martínez-Bueno, Gálvez, Valdivia, & Maqueda, 1990) was used as an AS-48 producer. *E. faecalis* S-47 from our collection was used as the standard indicator strain for bacteriocin activity assays. *L. sakei* CTC 245 was obtained from the IRTA (Institute for Agro-Food Research and Technology, Monells, Girona, Spain) collection. *S. carnosus* CECT 4491 and *B. thermosphacta* CECT 847 were supplied by the Spanish Type Culture Collection (CECT). For meat inoculation, *S. carnosus* and *B. thermosphacta* were grown overnight on brain heart infusion (BHI) and *L. sakei* on Man Rogosa Sharpe broth (MRS, Scharlau, Barcelona, Spain) at 28 °C, washed in a sterile saline solution, and then inoculated in the meat mixture at the selected concentration. All strains were cultivated routinely on BHI at 28 °C, and stored at 4 °C on BHI-agar slants.

2.2. Preparation of bacteriocin AS-48

AS-48 was produced by culturing the strain *E. faecalis* A-48-32 in a food-grade whey-derived substrate, Esprion 300 (E-300) (DMV Int., Veghel, Netherland), supplemented with 1% glucose as described by Ananou et al., 2008.

2.3. Manufacture of cooked ham

The cooked ham was prepared with lean pork meat. The meat was coarsely ground through a 12 mm plate and brined. The brine solution contained (in g kg^{-1}): NaCl, 12; sodium ascorbate, 0.30; sodium phosphate, 2; sodium glutamate, 1 (all from Scharlau); and water, 30. The ingredients were homogenized by hand during 5 min and the mix was divided into two batches, to one of which was added AS-48. Then both batches were cooked in a bath until the internal temperature reached 65 °C and then inoculated with approximately 10^4 CFU g^{-1} of the selected bacteria (*L. sakei* CTC 245, *S. carnosus* CECT 4491, and/or *B. thermosphacta* CECT 847) and subdivided into four batches: A) a control batch; B) a batch to which was added AS-48 (20, 40, or 60 μg g^{-1}); C) a batch with added different preservatives (only in the case of *L. sakei* experiments): pentasodium tripolyphosphate 0.5% (STPP, E-451i), sodium nitrate/nitrite 0.015 or 0.007% (E-251/E-250), sodium pyrophosphate 0.15% (E-450i), sodium acetate 0.2% (E-262), sodium lactate 2% (E-325), potassium benzoate 0.1% (E-211), potassium sorbate 1% (E-202); D) a batch to which both AS-48 and preservative were added. Although cooked ham is usually manufactured with nitrate/nitrite as a preservative in the brine solution, in our experiments we eliminated this preservative with the aim of studying the precise effect of the combination of AS-48 with each specific compound, used individually. The pH of the final product in all batches was 6.0–6.2. All the batches were subdivided into two portions and stored in sterile screw cap bottles at 5 °C or, exceptionally, at 15 °C. Two complete, independent experiments were carried out. Preservatives were from Sigma (Sigma–Aldrich Chemie Gmbh, Steinheim, Germany).

For *L. sakei* sublethal heat treatments, two batches were separated, one from the meat mixture with added AS-48 (20 μg g^{-1}) and the other without AS-48. Afterwards, each batch was divided into a further two batches. One batch of each type of meat mixture was heat-treated (60 °C for 2 min each) to produce a sublethal cell injury to *L. sakei* (killing approx. 99%). Finally, four independent batches were established: an unheated control batch without AS-48, a second unheated batch containing enterocin AS-48, a third batch with sublethal heat without AS-48, and a final batch heated and containing AS-48 (20 μg g^{-1}). Two independent experiments were carried out.

2.4. Packaging conditions

To investigate the influence of packaging conditions on the antimicrobial effect of AS-48 (40 μg g^{-1}) against the spoilage bacteria in cooked ham, samples with and without AS-48 were stored in polyamide-polyethylene plastic bags under different conditions: atmospheric, vacuum, or MA (40% CO_2/60% N_2) at 5 °C for 60 d. Vacuum and MA conditions were achieved with a Tecnotrip EVT-10-2-CD-SC (Barcelona, Spain) machine. All samples were stored at 5 °C for 60 d.

2.5. Microbiological sampling and analysis

Samples from each treatment were withdrawn in duplicate at selected times (0, 1, 7, 15, 30, and 60 days) to determine viable counts of *L. sakei*, *S. carnosus*, and *B. thermosphacta.* For the microbiological determinations, 10 g were aseptically removed and mixed (1:10) with dilution medium (0.1% peptone, 0.85% NaCl). Homogenization was done in a Masticator blender (IUL, Barcelona, Spain) for 1 min followed by serial 10-fold dilution and plating on the respective selective media: Man Rogosa Sharpe agar plus 0.04% sodium azide for *L. sakei*, incubated under anaerobic conditions, mannitol salt agar (MSA, Scharlau) for *S. carnosus* and STAA agar (Oxoid LTD, Basingstoke, Hampshire, England) with added STAA supplement (Oxoid) for *B. thermosphacta,* both incubated under aerobic conditions. All bacteria were incubated at 28 °C for 48–72 h before counting the colonies.

2.6. Bacteriocin extraction

Bacteriocin was extracted at selected times from cooked ham (as described by Garriga, Aymerich, Costa, Monfort, & Hugas, 2002). Briefly, cooked ham samples were homogenized (1:10) in sodium acetate 50 mM, EDTA 100 mM, and Triton X 100 0.2% at pH 5 in a blender for 1 min, boiled for 10 min, cooled, and filtered through a filter paper. The bacteriocin in the liquid phase was precipitated with 300 g L^{-1} ammonium sulphate and the pellet was dissolved in phosphate buffer (50 mM, pH 7.2). The sample was heated at 80 °C