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Microbiota of high-pressure-processed Serrano ham investigated by culture-dependent and culture-independent methods

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ABSTRACT

The microbiota of Serrano dry-cured ham of different chemical composition, subjected or not to high-pressure processing (HPP), was investigated using culture-dependent and culture-independent methods. Microbial counts were submitted to analysis of variance with physicochemical parameters (a_w, NaCl concentration, salt-in-lean ratio and intramuscular fat content) or HPP as main effects. In untreated hams, physicochemical parameters significantly affected counts of aerobic mesophiles, psychrotrophs, and moulds and yeasts. NaCl concentration and fat content influenced the levels of four and three of the five studied microbial groups, respectively, whereas no influence of a_w was stated. The HPP treatment had a significant effect on counts of all investigated microbial groups. Culture-independent methods showed the presence of bacteria such as *Staphylococcus equorum*, *Staphylococcus succinus*, *Bacillus subtilis* and *Cellulosimicrobium* sp., moulds like *Penicillium commune*, *Aspergillus fumigatus*, *Sclerotinia sclerotiorum*, *Eurotium athecium* and *Moniliella mellis*, and yeasts like *Debaryomyces hansenii* and *Candida glucosophila*. Absence of *B. subtilis* bands and weaker bands of *E. athecium* were recorded for HPP-treated hams.

The higher microbial levels found in lean ham might result in a quicker deterioration. HPP treatment confirmed its suitability as a procedure to control spoilage microorganisms. DGGE did not seem to be sensitive enough to highlight changes caused by HPP treatment in the microbiota of ham, but contributed to the detection of microbial species not previously found in ham.

1. Introduction

Serrano ham is a Spanish dry-cured meat product highly appreciated worldwide, with an annual production exceeding 125 million kg. The particular sensory characteristics of Serrano ham are mostly due to the activity of muscle proteinases and lipases (Toldrá and Flores, 1998) throughout a ripening period which may last up to 18 months.

The manufacturing process of Serrano ham starts with a salting step, during which a microbial community primarily consisting in microorganisms present in the salt colonizes the ham surface (Cornejo et al., 1992). During Serrano ham ripening, the growth of most microorganisms in raw meat is limited by the environmental conditions they face (low a_w and pH in the product, and low relative humidity and low to medium temperatures in the cold room). Only microbial groups adapted to those adverse conditions, in particular to the low a_w values, will survive throughout ripening of Serrano ham. Some of those microorganisms are proteolytic and/or lipolytic and certain species can reduce nitrates to nitrites, thus contributing to the sensory properties of ham (Cordero and Zumalacárregui, 2000). According to these authors, *Micrococcaceae* was the most abundant microbial group in salt used in the manufacture of dry-cured ham. Gram-positive, catalase-positive cocci were identified as the predominant microorganisms in different types of dry-cured ham (Giolitti et al., 1971; Langlois and Kemp, 1974; Rodríguez et al., 1994). Most *Micrococcaceae* isolates from Iberian dry-cured ham belonged to the genus *Staphylococcus*, with *S. xylosus* as the predominant species, although a remarkable diversity of *Staphylococcus* and *Micrococcus* species throughout ripening was detected (Rodríguez et al., 1994). *Staphylococcus* and *Micrococcus* isolates from ham possess enzymatic activities such as nitrate reductase, catalase, lipases and proteinases (Giolitti et al., 1971) which could influence ham sensory characteristics.

Moulds and yeasts are one of the major microbial groups during dry-cured ham processing and may contribute to the development of flavour characteristics (Lücke, 1986). Moulds belonging to the genera *Eurotium*, *Penicillium* and *Trichoderma* and yeasts identified as *Debaryomyces marama* were isolated from surface samples of Iberian dry-cured ham (Monte et al., 1986). In another study, mould isolates from surface samples of Iberian dry-cured ham mostly belonged to the genera *Penicillium*, *Aspergillus* and *Eurotium*, although members of the genera *Aureobasidium*, *Cladosporium*, *Curvularia* and *Syncephalastrum* were also detected (Núñez et al., 1996a). Yeasts isolated from the

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same surface samples of Iberian dry-cured ham were mainly identified as *Candida zeylanoides* and *Debaryomyces hansenii*, with *C. blankii*, *C. intermedia*, *Pichia carsonii* and *Rhodotorula rubra* also found sporadically (Núñez et al., 1996b). Some of the *D. hansenii* isolates showed high proteolytic activity against raw pork myofibrillar proteins (Martín et al., 2001; Rodríguez et al., 1998).

Consumers demand high quality, tasty, healthy, natural, convenient and safe meat products with an extended shelf life. On top of that, meat products should contain less salt, fat, acid and chemical preservatives (Aymerich et al., 2008). Producers in the meat industry try to adapt to consumers' demand for healthier food products elaborating products with low salt and fat contents. In dry-cured ham, salt contributes to microbial stability by lowering a_w, enhances protein solubilization, affects proteolysis, lipolysis and lipid oxidation, improves product texture and directly contributes to flavour (Toldrá and Flores, 1998). A reduction in salt content may increase microbial risk and cause technological problems, resulting in more pronounced rancid, fatty and buttery aroma notes than those of hams of high salt content (Coutron-Gambotti et al., 1999).

Although whole dry-cured ham is considered a shelf-stable product due to its salt content (up to 8–10% on total weight) and water activity (a_w, usually below 0.90), the trend for convenient products has resulted in an increasing percentage of hams being deboned, sliced and vacuum packaged at the processing plants (Morales et al., 2006). All these post-process operations increase the risk of microbial cross-contamination by pathogens and spoilage microorganisms, which could affect the safety and compromise the shelf life of the product. The presence and survival of *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* in ham packages from different manufacturers has been reported (Ng et al., 1997). In a ready-to-eat product as Serrano ham, these pathogens, which are difficult to eradicate from processing plants, may pose a safety risk to consumers.

High-pressure processing (HPP), a non-thermal technology that causes destruction of microbial vegetative cells and inactivation of certain enzymes, with minor changes in the sensory characteristics of meat products (Garriga et al., 2004), is being increasingly used for the decontamination of ready-to-eat meat products (Aymerich et al., 2008). Food composition influences the effect of HPP on microorganisms. Low a_w values and/or high solute concentration exert a baroprotective effect and reduce the extent of bacterial inactivation induced by HPP (Patterson, 2005). A significant reduction of at least 2 log units for spoilage microorganisms in vacuum-packed sliced dry-cured ham was recorded after HPP at 600 MPa for 6 min (Garriga et al., 2004). Also, the effect of HPP at 450 MPa for 10 min on *Listeria monocytogenes* in Serrano and Iberian dry-cured hams was investigated (Morales et al., 2006), with differences in pathogen lethality between types of ham ascribed by the authors to NaCl concentration. HPP of foods may result in the appearance of sublethally injured survivors unable to grow on culture media, in particular when plated on selective agars. Non-culturable bacteria have been detected in HPP-treated blood sausage, goat milk curd and cooked ham using culture-independent molecular techniques (Campos et al., 2011; Diez et al., 2008; Han et al., 2011).

HPP of Serrano ham and its chemical composition are known to affect the formation of volatile compounds during ripening (Martínez-Onandi et al., 2016). However, to our knowledge, there is no available information on the effect of chemical composition on the microbiota of ham or how this chemical composition would modulate the effect of HPP on microorganisms. The objective of the present study was to investigate Serrano ham microbiota before and after HPP as well as the effect of ham chemical composition on the microbiota by means of culture-dependent and culture-independent methods.

2. Materials and methods

2.1. Selection and manufacture of Serrano hams

Serrano ham manufacture was carried out at the Institute of Food and Agricultural Research and Technology (IRTA, Monells, Spain). Thirty green hams were selected at commercial slaughterhouses from animals of different genotype in order to obtain a wide range of fat contents. Twenty one hams were from Large White x Landrace animals and nine hams from animals with a minimum of 50% Duroc breed. Fat content of entire hams was determined using a magnetic resonance sensor technology (JMP Ingenieros, Sotés, Spain). Homogeneous hams in terms of weight and pH were used in experiments. Average weight of hams was 11.77 ± 0.66 kg whereas the pH in the semimembranosus muscle at 24 h post-mortem ranged from 5.4 to 5.9. Hams were manually rubbed with the following mixture (per kg of raw ham): 10 g NaCl, 1.0 g dextrose, 0.5 g ascorbic acid, 0.15 g KNO_3 and 0.15 g $NaNO_2$. Afterwards, hams were individually salted with excess of salt at 3 ± 2 °C and 85 ± 5% RH for 0.6 to 1.5 days/kg of raw ham in order to obtain a wide range of salt contents. After salting, hams were washed with cold water, weighed and hung in a cold room at 3 °C and 75–80% RH to rest. Temperature was progressively increased up to 20 °C during ripening. The process was finished when a total weight loss of 36% was achieved.

2.2. Sampling and high pressure processing

The superficial part of the cushion was removed with a sterile knife. Afterwards, the whole cushion (mainly composed of the *Biceps femoris*, *Semimembranosus* and *Semitendinosus* muscles) from each ham was extracted with another sterile knife and two slices (approximately 150 g) were obtained and individually vacuum-packaged. One of the slices was HPP-treated at 600 MPa for 6 min at 21 °C (pressure build up time, 2.5 min; pressure release time <2 s) in a 120 L capacity Wave 6000 equipment (Hiperbaric, Burgos, Spain) at IRTA, whereas the untreated slice served as control. Ham slices were held at 4 °C prior to analysis, which was carried out within 3 days of HPP.

2.3. Physicochemical determinations

Water activity (a_w) was determined using an AquaLab Series 3-equipment (Decagon Devices, Inc., Pullman, WA, USA). Chloride content was analysed by the Volhard method (AOAC, 2000) and intramuscular fat content by a previously described method (Folch et al., 1957). All determinations were performed in triplicate.

2.4. Microbiological analysis

Representative ham samples (10 g) were aseptically taken and homogenized with 90 mL of a sterile saline peptone solution (Maximum recovery diluent, Biolife, Milano, Italy) in a Colworth Stomacher 400 (A. J. Seward Ltd., London, UK) for 3 min. Serial dilutions were prepared and plated in duplicate onto appropriate culture media. Aerobic mesophiles were enumerated on Plate Count Agar (PCA, Biolife) after incubation for 48 h at 30 °C, psychrotrophs on PCA after incubation for 7 days at 8 °C, *Enterobacteriaceae* in double-layered plates of Violet Red Bile Glucose Agar (VRBG, Biolife) after incubation for 24 h at 37 °C, lactic acid bacteria (LAB) on MRS Agar (Biolife) after anaerobic incubation for 72 h at 30 °C, enterococci on Kanamycin Aesculin Azide Agar (KAA, Oxoid, Basingstoke, Hampshire, UK), after incubation for 24 h at 37 °C, *Micrococcaceae* on Mannitol Salt Agar (MSA, Oxoid) after incubation for 36 h at 37 °C, coagulase-positive staphylococci on Baird-Parker agar with rabbit plasma fibrinogen (RPF) Supplement II (BP + RPF, Biolife) after incubation for 24 h at 37 °C, and moulds and yeasts on Sabouraud Dextrose Agar (SDA, Oxoid) after incubation for 5 days at 25 °C. Microbial counts were expressed as log cfu per gram of ham. Presence of *L. monocytogenes* was investigated in 25 g of each