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Microbiota of Iberian dry-cured ham as influenced by chemical composition, high pressure processing and prolonged refrigerated storage

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ABSTRACT

The effect of high pressure processing (HPP) on the microbiota of ripened Iberian ham of different water activity, salt concentration and intramuscular fat content was investigated before and after a 5-month refrigeration period. At the beginning of the refrigeration period, the only significant effects of chemical composition were those of water activity on psychrotrophs and *Micrococcaceae* in untreated hams, and of the salt-in-lean ratio on lactic acid bacteria in HPP-treated hams. At the end of the refrigeration period, the only significant effect was that of intramuscular fat content on moulds and yeasts in HPP-treated samples. All microbial groups were significantly affected by HPP, with reductions ranging from 1.7 to 2.0 log cycles after treatment. A significant recovery of all microbial groups took place in HPP-treated hams during the refrigeration period, with increases ranging from 0.5 to 1.1 log cycles. In spite of this recovery, microbial levels in HPP-treated hams remained significantly lower than in untreated hams. *Staphylococcus* accounted for 93.4% of Iberian ham bacterial isolates, with *S. equorum* as the most abundant species. Representatives of the *Tetragenococcus*, *Carnobacterium* and *Streptomyces* genera, not previously reported in dry-cured ham, were also isolated. Most of the yeast isolates (75.0%) were identified as *Debaryomyces hansenii.*

1. Introduction

Iberian ham is a dry-cured meat product highly appreciated by consumers due to its high sensory quality, partly due to the characteristics of the raw material (acorn-fed autochthonous pig breed) and the length of the manufacturing process (Ruiz et al., 2002). The manufacturing process of Iberian ham basically consists in four stages: a conditioning step, a salting step, a post-salting step and a ripening period, which may last up to 24 months (Toldrá and Flores, 1998). During the salting step, a microbial community mostly consisting of microorganisms from the salt colonises the ham surface (Cornejo et al., 1992). During the post-salting step, salt diffusion to the inner zones and water loss result in a gradual predominance of the salt-tolerant microbiota (Blesa et al., 2008).

Micrococcaceae, moulds and yeasts have been reported as the dominant microorganisms in dry-cured ham (Huerta et al., 1988; Núñez et al., 1996a,b). *Micrococcaceae* are also the dominant microbial group in the salt used for the manufacture of dry-cured ham (Cordero and Zumalacárregui, 2000). In Iberian ham, most of the *Micrococcaceae* isolates belonged to the *Staphylococcus* genus, being *S. xylosus* followed by *S. equorum* the predominant species (Rodríguez et al., 1994). The presence of *S. equorum* in ham may have been underestimated because of its confusion with *S. xylosus* when isolates were identified by phenotypical and biochemical methods (Blaiotta et al., 2004; Landeta et al., 2011). Staphylococci play important roles in colour formation through their nitrate reductase activity, inhibition of rancidity through their antioxidant activity, and flavour development via the catabolism of branched-chain amino acids and pyruvate (Vermassen et al., 2016).

Moulds are considered beneficial in the ripening of dry-cured ham due to their positive effects on flavour and external appearance (Núñez et al., 1996a). However, production of mycotoxins should be considered a health hazard. Species of the *Penicillium*, *Aspergillus* and *Eurotium* genera were reported as the predominant moulds in Iberian ham, and members of the *Aureobasidium*, *Cladosporium*, *Curvularia* and *Syncephalastrum* genera were also detected (Núñez et al., 1996a). Among yeasts, *Debaryomyces hansenii* and *Candida zelanoides* were the predominant species in Iberian ham (Núñez et al., 1996b). *D. hansenii* showed an intense proteolytic activity against pork myofibrillar proteins (Rodríguez et al., 1998; Martín et al., 2001).

The meat industry tries to satisfy consumers demand for high quality, tasty, healthy, and safe meat products with an extended shelf life. In the specific case of dry-cured ham, consumers demand low salt and fat contents. Nevertheless, salt is an essential ingredient in the manufacturing process of dry-cured ham since it prevents microbial

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growth, reduces water activity (a_w), controls enzyme action, facilitates the solubilisation of some proteins and confers a typical salty taste to ham that enhances its flavour (Andrés et al., 2004). Salt reduction may increase microbiological risks and bring about technological problems (Armenteros et al., 2009). It has been shown that low salt Serrano ham had higher levels of volatile compounds coming from Strecker reactions and microbial metabolism than medium or high salt hams (Martínez-Onandi et al., 2016).

Although entire dry-cured ham is considered a shelf-stable product, the deboning, slicing and packaging operations characteristic of ready-to-eat products may increase the risk of microbial cross-contamination by spoilage and pathogenic microorganisms, compromising shelf life and safety. High pressure processing (HPP), a non-thermal preservation technology, is being widely used in the meat industry for controlling food spoilage and improving food safety, while retaining the characteristics of fresh, minimally processed foods. Microbial inactivation by HPP (Rendueles et al., 2011) depends on treatment conditions (pressure level, holding time and treatment temperature), factors related to the microorganism (species, strain, physiological status, etc.), and food composition (pH, a_w, etc.). HPP of Serrano ham resulted in significant reductions of total bacterial levels after treatment, from 1.5 to 2.5 log cycles (De Alba et al., 2015; Garriga et al., 2004; Martínez-Onandi et al., 2017a) while chemical composition significantly affected counts of aerobic mesophiles, psychrotrophs, moulds and yeasts in Serrano ham (Martínez-Onandi et al., 2017a). However, to our knowledge, there is no available information on the effect of Iberian ham chemical composition on its microbiota or on how chemical composition would modulate the effect of HPP on the microbiota.

The objective of the present study was to investigate the effect of chemical composition, HPP treatment and refrigerated storage for 5 months on the microbiota of ripened dry-cured Iberian ham, with particular interest on the microbial diversity of this meat product.

2. Materials and methods

2.1. Selection of Iberian hams

Two groups, each consisting of 30 Iberian hams, were selected from a batch of 120 hams produced at a processing plant in Extremadura (Spain) from 50% Iberian x Duroc pigs, reared at the same farm under the same conditions, and ripened for 24 months. Selection of hams was performed at the Institute of Food and Agricultural Research and Technology (IRTA, Monells, Spain) as previously described (Martínez-Onandi et al., 2017b). The first group of hams was analysed 3 days after HPP treatment and the second group after a 5-month refrigeration period, simulating commercial storage conditions.

2.2. Sampling and high pressure processing

Two slices (approximately 150 g) from the cushion (mainly composed of the *Biceps femoris*, *Semimembranosus* and *Semitendinosus* muscles) were obtained from each ham and individually vacuum-packaged. HPP treatment 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 was applied to one of the two slices whereas the other slice served as untreated control. Ham slices were maintained at 4 °C until microbiological analysis, which was carried out after 3 days or 5 months of storage at 4 °C. After microbiological analysis, samples for chemical determinations were maintained at −35 °C until they were analysed.

2.3. Physicochemical determinations

Representative ham homogenates were obtained with a mechanical grinder (IKA Labortechnik, Staufen, Germany). Chloride content was determined by the Volhard method (AOAC, 2000) and intramuscular fat (IMF) content by the Folch method (Folch et al., 1957). Water activity (a_w) was measured using an AquaLab Series 3 equipment (Decagon, Devices, Inc., Pullman, WA, USA). Analyses 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 mesophilic bacteria, psychrotrophs, *Enterobacteriaceae*, lactic acid bacteria, enterococci, *Micrococcaceae*, coagulase-positive staphylococci, and moulds and yeasts were respectively enumerated on Plate Count Agar (PCA, Biolife), PCA, Violet Red Bile Glucose Agar (VRBG, Biolife), MRS Agar (Biolife), Kanamycin Aesculin Azide Agar (KAA, Oxoid, Basingstoke, Hampshire, UK), Mannitol Salt Agar (MSA, Oxoid), Baird-Parker agar with rabbit plasma fibrinogen (RPF) Supplement II (BP + RPF, Biolife), and Sabouraud Dextrose Agar (SDA, Oxoid) as previously described (Martínez-Onandi et al., 2017a). Microbial counts were expressed in log cfu per gram of ham. The presence of *L. monocytogenes* and *Salmonella* spp. in 25 g of ham was investigated by pre-enrichment, enrichment and selective isolation on specific media as previously described (Martínez-Onandi et al., 2017a).

2.5. Isolation of microorganisms and DNA extraction

Representative bacterial isolates from different solid culture media (40 colonies from PCA incubated at 30 °C, 30 from MSA, 20 from BP + RPF, 15 from PCA incubated at 8 °C and 15 from MRS at the beginning of the storage period and a similar number at the end of the storage period) and 24 representative yeast isolates from SDA plates were randomly selected. Selection of previously numbered colonies was performed by using an aleatory number generator application (http://numbergenerator.org/). They were purified by streaking them three times on the same medium. Isolates thus obtained were stored at - 40 °C in liquid cultures supplemented with glycerol (20% w/v).

Total genomic DNA from pure cultures in the late exponential growth phase was isolated either with the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, Tres Cantos, Spain) or with the Yeast DNA Extraction kit (Thermo Fisher Scientific, Madrid, Spain), following the manufacturer's recommendation.

2.6. Molecular identification of isolates

An approximately 800 basepair (bp) region of the *16S rDNA* gene was amplified using the universal primers W01 and 800R as previously described (Campos et al., 2011). Amplified PCR products from 96 bacterial isolates were purified using the GenElute PCR clean-up kit (Sigma-Aldrich) and sequenced at the Genomic Unit (Complutense University CAI, Madrid, Spain). The obtained sequences (forward and reverse) were compared to those deposited in databases BLAST (http://blast.ncbi.nlm.nih.gov/) and RDP (http://rdp.cme.mus.edu). With the obtained sequences, a partial amplified rDNA restriction analysis (partial ARDRA) was developed to identify the 131 remaining bacterial isolates. A first digestion with restriction endonuclease *Kpn*I (Thermo Fisher Scientific, Madrid, Spain) classified them in two groups (digestible or non-digestible with *Kpn*I). *Kpn*I-digestible amplicons were subjected to the action of endonucleases *Mbo*I and *Ava*II, and were classified in three different groups. Amplicons with no *Kpn*I site and those digested with *Ava*II were subjected to the action of endonuclease *Taq*I. Restriction fragment patterns were analysed in 2% (wt/vol) agarose in 0.5 × Tris-borate-EDTA buffer using a 100-bp DNA ladder (Thermo Fisher Scientific) as molecular size marker.

For the 24 yeast isolates, the region spanning the internal transcribed spacers (ITS1 and 2) and the 5.8S rDNA gene was amplified using primers ITS1 and ITS4 as described (Guillamón et al., 1998). Purification, sequencing and comparison of the obtained sequences were performed as above described.

2.7. Statistical analysis

Microbial log counts were analysed using the SPSS 19.0 statistical package (S.P.S.S. Inc., Chicago, IL, USA). Hams were divided in three groups (low, medium and high values) for each compositional parameter. The mean ± 0.5 standard deviation (SD) criterion was used to establish these three groups (Martínez-Onandi et al., 2016). One-way analysis of variance (ANOVA) was carried out with each of the compositional parameters (a_w, salt concentration, salt-in-lean ratio, intramuscular fat content) as main effect and ham as random effect or with HPP treatment as main effect and ham as fixed effect. Means were compared by Tukey's test, with the significance assigned at $P < 0.05$.

3. Results

3.1. Effect of ham chemical composition

In the first group of 30 Iberian hams, analysed at the beginning of the 5-month refrigeration period, a_w ranged from 0.845 to 0.912, with a mean value of 0.876 (SD, 0.015). In untreated hams, a_w value significantly influenced counts of psychrotrophs and *Micrococcaceae*, with the highest levels of these microbial groups in high a_w hams, while in HPP-treated hams it had no significant effect on any of the microbial groups (Table 1).

NaCl concentration of hams ranged from 3.02% to 5.62%, with a mean value of 4.27% (SD, 0.67%). Salt concentration did not significantly influence the counts of any microbial group in either untreated or HPP-treated hams (Table 1). Salt-in-lean (S/L) ratio of hams ranged from 0.036 to 0.063, with a mean value of 0.049 (SD, 0.005). The S/L ratio did not affect the counts of any microbial group in untreated hams but it influenced the counts of lactic acid bacteria in HPP-treated hams, with the highest levels of this microbial group in hams of low S/L ratio (Table 1).

Intramuscular fat content (IMF) of hams ranged from 4.63% to 18.59%, with a mean value of 11.96% (SD 3.56%). The IMF content did not significantly influence the counts of any microbial group in either untreated or HPP-treated hams (Table 1).

In the second group of 30 Iberian hams, analysed after 5 months at 4 °C, a_w ranged from 0.859 to 0.890, with a mean value of 0.875 (SD, 0.008). Values of a_w did not significantly influence the counts of any microbial group in either untreated or HPP-treated hams (Table 2).

Salt concentration ranged from 3.51% to 5.35%, with a mean value of 4.48% (SD, 0.50%). Salt concentration did not significantly influence the levels of any microbial group in either untreated or HPP-treated hams (Table 2). The S/L ratio of hams ranged from 0.042 to 0.061, with a mean value of 0.051 (SD, 0.005). The S/L ratio did not significantly influence the levels of any microbial group in either untreated or HPP-treated hams (Table 2).

IMF content ranged from 8.87% to 16.62%, with a mean value of 12.38% (SD, 2.12%). The IMF content did not affect the counts of any microbial group in untreated hams, although it significantly influenced the counts of moulds and yeasts in HPP-treated hams, with the highest levels of this microbial group in low and medium fat content hams (Table 2).

3.2. Effect of high pressure processing

Counts of the different microbial groups in untreated and HPP-treated hams, at the beginning and the end of the 5-month refrigerated storage period, are shown in Table 3. No *Enterobacteriaceae*, enterococci or coagulase-positive staphylococci (grey-black shiny colonies surrounded by an opaque halo of precipitation) were detected in untreated or HPP-treated hams, although some microbial growth was recorded in BP + RPF plates (grey colonies without an opaque zone). At both times, all the analysed microbial groups were significantly ($P < 0.05$) affected by the HPP treatment. Reductions of 1.78, 2.00, 2.04, 1.74 and 1.87 log cfu g^{-1} were recorded after HPP treatment for aerobic mesophiles, psychrotrophs, lactic acid bacteria, *Micrococcaceae*, and moulds and yeasts, respectively. Neither *L. monocytogenes* nor *Salmonella* spp. were detected after enrichment of untreated or HPP-treated Iberian ham 25 g samples.

At the end of the 5-month refrigeration period, counts of aerobic mesophiles, psychrotrophs, lactic acid bacteria, *Micrococcaceae* and moulds and yeasts were respectively 0.53, 1.02, 1.34, 0.93 and 0.81 log cfu g^{-1} lower in HPP-treated ham samples than in untreated samples. *L. monocytogenes* and *Salmonella* spp. were not detected after refrigerated storage in any of the untreated or HPP-treated Iberian ham samples.

3.3. Effect of prolonged refrigerated storage

During the 5-month refrigeration period, mean counts of aerobic mesophiles and moulds and yeasts significantly decreased (- 0.36 and - 0.31 log cfu g^{-1}, respectively), those of lactic acid bacteria significantly increased (+0.39 log cfu g^{-1}), and those of psychrotrophs and *Micrococcaceae* (- 0.04 and + 0.28 log cfu g^{-1}, respectively) did not vary significantly in untreated Iberian ham. In contrast, significant increases were recorded for mean counts of aerobic mesophiles (+0.89 log cfu g^{-1}), psychrotrophs (+0.94 log cfu g^{-1}), lactic acid bacteria (+1.09 log cfu g^{-1}), *Micrococcaceae* (+0.53 log cfu g^{-1}) and moulds and yeasts (+0.75 log cfu g^{-1}) in HPP-treated Iberian ham. In spite of those increases, the levels of all microbial groups in HPP-treated ham remained significantly lower than in untreated Iberian ham at the end of the refrigeration period. Since no significant differences in chemical composition between hams at the beginning and the end of the refrigerated storage were found, the observed differences were attributed to the effect of the prolonged refrigerated storage on microbial viability.

3.4. Microbial diversity

A fragment of approximately 800 bp of the *16S rDNA* gene was amplified by PCR using as template the DNA from 113 bacterial isolates from untreated and HPP-treated ham samples at the beginning of the refrigeration period and from 114 bacterial isolates from ham samples at the end of the refrigeration period. Sequencing of 96 amplicons identified isolates as follows: 90 belonged to the genus *Staphylococcus*, 3 to *Tetragenococcus*, 2 to *Enterococcus* and 1 to *Carnobacterium*. Among *Staphylococcus* isolates, the following species were found: *S. equorum* (45 isolates), *S. epidermidis* (21 isolates), *S. pasteuri/warneri* (22 isolates), *S. hominis* (1 isolate) and *S. saprophyticus* (1 isolate). Species belonging to other genera were *T. solitarius* (2 isolates), *T. koreensis* (1 isolate), *E. faecalis* (1 isolate), *E. faecium/hirae* (1 isolate), and *C. divergens* (1 isolate). Isolates from all sequenced *Staphylococcus* species, with the only exception of *S. saprophyticus*, possessed a *Kpn*I site in the amplicon, whereas isolates from all other genera did not have this site. A first digestion with endonuclease *Kpn*I allowed us to classify isolates in two groups: those with a *Kpn*I site or those without it. It was found that 218 of the 227 isolates (96.0%) had a *Kpn*I site. It was considered that those isolates supposedly belonged to the genus *Staphylococcus*. Digestions with endonucleases *Mbo*I and *Ava*II resulted in three to four and two to three restriction fragments, respectively (Fig. 1). The obtained patterns could be ascribed to the species *S. equorum/hominis*, *S. epidermidis* or *S. pasteuri/warneri* (Fig. 1). To differentiate between *S. equorum* and *S. hominis*, amplicons digestible with AvaII were subjected to the action of endonuclease *Taq*I. Those with a *Taq*I site belonged to the species *S. hominis* (data not shown). The most abundant species was *S. equorum* (144 isolates), followed by *S. epidermidis* (39 isolates) and *S.*

Table 1

Levels[1] of the main microbial groups in untreated and HPP-treated ripened Iberian hams as influenced by chemical composition, at the beginning of the 5-month refrigeration period.

Chemical parameter	Untreated ham						P[2]	HPP-treated ham						P[2]
Microbial group	Low		Medium		High			Low		Medium		High		
a_w[3]	(n = 6)		(n = 17)		(n = 7)			(n = 6)		(n = 17)		(n = 7)		
Aerobic mesophiles	2.82	± 0.54	3.33	± 0.92	3.46	± 0.74	ns	1.55	± 0.70	1.36	± 0.59	1.73	± 1.07	ns
Psychrotrophs	2.64	± 0.74^{b}	3.19	± 0.73^{ab}	3.39	± 0.91^{a}	*	1.33	± 0.49	1.09	± 0.29	1.07	± 0.27	ns
Lactic acid bacteria	2.59	± 0.51	3.02	± 0.78	2.84	± 0.71	ns	0.76	± 0.92	0.93	± 0.78	0.73	± 1.22	ns
Micrococcaceae	2.59	± 0.75^{b}	3.21	± 0.70^{a}	3.20	± 0.57^{a}	*	1.08	± 0.29	1.41	± 0.54	1.43	± 0.93	ns
Moulds and yeasts	2.92	± 0.48	3.08	± 0.45	3.14	± 0.49	ns	1.33	± 0.65	1.19	± 0.50	1.07	± 0.27	ns
Salt concentration[4]	(n = 11)		(n = 10)		(n = 9)			(n = 11)		(n = 10)		(n = 9)		
Aerobic mesophiles	3.53	± 1.03	2.98	± 0.83	3.23	± 0.40	ns	1.52	± 0.93	1.42	± 0.61	1.51	± 0.68	ns
Psychrotrophs	3.27	± 1.04	2.94	± 0.78	3.16	± 0.43	ns	1.05	± 0.21	1.10	± 0.31	1.28	± 0.46	ns
Lactic acid bacteria	3.03	± 0.95	2.89	± 0.65	2.73	± 0.47	ns	1.15	± 1.00	0.77	± 0.69	0.57	± 0.96	ns
Micrococcaceae	3.25	± 0.60	2.90	± 0.86	3.09	± 0.67	ns	1.43	± 0.82	1.40	± 0.50	1.20	± 0.46	ns
Moulds and yeasts	3.08	± 0.66	2.97	± 0.33	3.15	± 0.28	ns	1.39	± 0.70	1.10	± 0.31	1.06	± 0.24	ns
S/L ratio[5]	(n = 12)		(n = 9)		(n = 9)			(n = 12)		(n = 9)		(n = 9)		
Aerobic mesophiles	3.44	± 1.03	3.04	± 0.86	3.23	± 0.40	ns	1.53	± 0.91	1.40	± 0.60	1.51	± 0.68	ns
Psychrotrophs	3.17	± 1.08	3.04	± 0.69	3.16	± 0.43	ns	1.04	± 0.20	1.11	± 0.32	1.28	± 0.46	ns
Lactic acid bacteria	2.99	± 0.93	2.93	± 0.66	2.73	± 0.47	ns	1.20	± 0.93^{a}	0.66	± 0.72^{b}	0.57	± 0.96^{b}	*
Micrococcaceae	3.18	± 0.65	2.96	± 0.86	3.09	± 0.67	ns	1.44	± 0.79	1.39	± 0.50	1.20	± 0.46	ns
Moulds and yeasts	3.12	± 0.56	2.90	± 0.46	3.15	± 0.28	ns	1.23	± 0.56	1.28	± 0.57	1.06	± 0.24	ns
IMF content[6]	(n = 9)		(n = 10)		(n = 11)			(n = 9)		(n = 10)		(n = 11)		
Aerobic mesophiles	3.18	± 0.69	3.18	± 0.92	3.40	± 0.88	ns	1.42	± 0.63	1.40	± 0.94	1.62	± 0.66	ns
Psychrotrophs	2.93	± 0.98	3.29	± 0.81	3.14	± 0.64	ns	1.06	± 0.24	1.15	± 0.37	1.18	± 0.39	ns
Lactic acid bacteria	2.81	± 0.67	2.96	± 0.66	2.90	± 0.86	ns	0.77	± 0.76	0.78	± 1.08	0.97	± 0.90	ns
Micrococcaceae	3.17	± 0.75	3.05	± 0.72	3.04	± 0.72	ns	1.28	± 0.46	1.40	± 0.82	1.37	± 0.56	ns
Moulds and yeasts	2.99	± 0.30	3.14	± 0.47	3.05	± 0.57	ns	1.06	± 0.24	1.10	± 0.31	1.39	± 0.70	ns

[1] Levels are expressed in log cfu g^{-1}, mean ± SD of duplicate determinations.

[2] Significance in the analysis of variance: ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

[3] Low a_w was < 0.868, medium a_w was within the range 0.868–0.883, and high a_w was > 0.883.

[4] Low salt concentration was < 3.93%, medium salt concentration was within the range 3.93–4.60%, and high salt concentration was > 4.60%.

[5] Low salt-in-lean (S/L) ratio was < 0.045, medium S/L ratio was within the range 0.045–0.052, and high S/L ratio was > 0.052.

[6] Low intramuscular fat (IMF) content was < 10.18%, medium IMF content was within the range 10.18–13.75%, and high IMF content was > 13.75%.

pasteuri/warneri (25 isolates). The last two species were more frequently found in both untreated and HPP-treated Iberian ham samples at the beginning of the refrigeration period than at the end of the refrigeration period (Table 4).

However, six supposedly *Staphylococcus* isolates (digested with *Kpn*I) which could not be digested with *Mbo*I or *Ava*II and three isolates which could not be digested with *Kpn*I were sequenced. Isolates with the *Kpn*I site were identified as *Kocuria palustris* (2 isolates), *Brachybacterium conglomeratum* (2 isolates) and *Streptomyces flavofungini/lomondensis* (2 isolates). Those without a *Kpn*I site turned out to be *Tetragenococcus halophilus* (1 isolate), *Enterococcus faecium* (1 isolate) and *Kocuria rhizophila* (1 isolate).

Eighteen of the 24 yeast isolates (75.0%) were identified as *Debaryomyces hansenii* (Table 4). The remaining isolates were members of the genera *Moniliella* (2 isolates), *Rhodotorula* (2 isolates), *Cryptococcus* (1 isolate) and *Ustilago* (1 isolate).

4. Discussion

In the present study, microbial counts in individual samples of untreated Iberian ham ranged from 2.0 to 5.7 log cfu g^{-1} while average counts were close to 3 log cfu g^{-1} for all the investigated microbial groups. Those levels were similar to the counts reported for *Micrococcaceae* in Iberian ham (Rodríguez et al., 1994) and for aerobic mesophiles in Parma ham (Hinrichsen and Pedersen, 1995) and Serrano ham (De Alba et al., 2015; Martínez-Onandi et al., 2017a). They were lower than the microbial counts reported for entire Serrano ham, with levels of aerobic mesophiles ranging from 3 to 8 log cfu g^{-1} and of lactic acid bacteria from 2 to 6 log cfu g^{-1}, depending on sample location and time of post-salting (Blesa et al., 2008), and than the average counts (4.6 or 4.8 log cfu g^{-1}) found in vacuum-packaged Serrano ham slices (Clariana et al., 2011; Garriga et al., 2004), although in the latter works differences could be partly explained by microbial contamination of ham during slicing and packaging.

Microorganisms, particularly staphylococci, play an important role in the sensory characteristics of dry-cured ham through their enzymatic activities (Rodríguez et al., 1994; Vermassen et al., 2016). Volatile compounds of microbial origin accounted for more than 6% of the volatile fraction of Iberian ham (Martínez-Onandi et al., 2017b; 2018), in which differences in chemical composition were considered to affect microbial growth and metabolism (Martínez-Onandi et al., 2017a).

High a_w values are generally favourable for microorganisms. However, the only microbial groups significantly affected in the present study were psychrotrophs and *Micrococcaceae*. Otherwise, low a_w values increase the resistance of microorganisms to HPP, as shown for *Staphylococcus aureus* and lactic acid bacteria after HPP at 600 MPa for 6 min in three meat products (marinated beef, cooked ham and dry-cured ham) of different a_w (Hugas et al., 2002). In the present study, counts of microbial groups in HPP-treated hams were not significantly influenced by a_w values.

Salt is an essential element in the manufacturing process of dry-cured ham (Toldrá and Flores, 1998). It is the main source of

Table 2
Levels[1] of the main microbial groups in untreated and HPP-treated ripened Iberian hams as influenced by chemical composition, at the end of the 5-month refrigeration period.

Chemical parameter	Untreated ham			P^2	HPP-treated ham			P^2
Microbial group	Low	Medium	High		Low	Medium	High	
a_w[3]	(n = 11)	(n = 9)	(n = 10)		(n = 11)	(n = 9)	(n = 10)	
Aerobic mesophiles	2.99 ± 1.25	2.94 ± 0.68	2.75 ± 0.51	ns	2.41 ± 1.16	2.13 ± 0.92	2.53 ± 1.01	ns
Psychrotrophs	2.97 ± 1.08	2.97 ± 0.84	3.32 ± 0.74	ns	2.39 ± 1.26	1.89 ± 0.84	1.89 ± 1.03	ns
Lactic acid bacteria	3.32 ± 1.14	3.29 ± 0.63	3.23 ± 0.89	ns	1.98 ± 1.44	1.50 ± 1.20	2.28 ± 1.00	ns
Micrococcaceae	2.82 ± 1.30	2.88 ± 0.54	2.73 ± 1.25	ns	1.92 ± 1.19	1.62 ± 0.93	2.07 ± 1.14	ns
Moulds and yeasts	2.64 ± 0.89	2.69 ± 0.58	2.94 ± 0.73	ns	2.22 ± 1.22	1.66 ± 0.90	1.87 ± 0.70	ns
Salt concentration[4]	(n = 12)	(n = 7)	(n = 11)		(n = 12)	(n = 7)	(n = 11)	
Aerobic mesophiles	2.64 ± 0.60	3.19 ± 0.40	2.99 ± 1.25	ns	2.35 ± 1.05	2.32 ± 0.86	2.41 ± 1.16	ns
Psychrotrophs	3.17 ± 0.86	3.14 ± 0.71	2.97 ± 1.08	ns	1.83 ± 0.97	2.00 ± 0.89	2.39 ± 1.26	ns
Lactic acid bacteria	3.18 ± 0.83	3.38 ± 0.65	3.32 ± 1.14	ns	2.00 ± 1.14	1.76 ± 1.20	1.98 ± 1.44	ns
Micrococcaceae	2.69 ± 1.15	3.00 ± 0.53	2.82 ± 1.30	ns	1.89 ± 1.11	1.80 ± 0.99	1.92 ± 1.19	ns
Moulds and yeasts	2.81 ± 0.73	2.84 ± 0.56	2.64 ± 0.89	ns	1.72 ± 0.72	1.85 ± 0.94	2.22 ± 1.22	ns
S/L ratio[5]	(n = 10)	(n = 9)	(n = 11)		(n = 10)	(n = 9)	(n = 11)	
Aerobic mesophiles	2.75 ± 0.51	2.94 ± 0.68	2.99 ± 1.25	ns	2.53 ± 1.01	2.13 ± 0.92	2.41 ± 1.16	ns
Psychrotrophs	3.32 ± 0.74	2.97 ± 0.84	2.97 ± 1.08	ns	1.89 ± 1.03	1.89 ± 0.84	2.39 ± 1.26	ns
Lactic acid bacteria	3.23 ± 0.89	3.29 ± 0.63	3.32 ± 1.14	ns	2.28 ± 1.00	1.50 ± 1.20	1.98 ± 1.44	ns
Micrococcaceae	2.73 ± 1.25	2.88 ± 0.54	2.82 ± 1.30	ns	2.07 ± 1.14	1.62 ± 0.93	1.92 ± 1.19	ns
Moulds and yeasts	2.94 ± 0.73	2.69 ± 0.58	2.64 ± 0.89	ns	1.87 ± 0.70	1.66 ± 0.90	2.22 ± 1.22	ns
IMF content[6]	(n = 10)	(n = 11)	(n = 9)		(n = 10)	(n = 11)	(n = 9)	
Aerobic mesophiles	2.58 ± 0.94	3.19 ± 0.91	2.89 ± 0.68	ns	2.27 ± 1.03	2.52 ± 1.04	2.28 ± 1.08	ns
Psychrotrophs	3.13 ± 0.70	2.99 ± 1.08	3.16 ± 0.91	ns	1.88 ± 1.09	2.35 ± 1.11	1.95 ± 1.03	ns
Lactic acid bacteria	3.36 ± 1.08	3.40 ± 1.05	3.05 ± 0.37	ns	1.90 ± 1.32	2.11 ± 1.31	1.76 ± 1.14	ns
Micrococcaceae	2.80 ± 1.39	2.86 ± 1.11	2.75 ± 0.68	ns	1.80 ± 1.03	2.03 ± 1.19	1.80 ± 1.10	ns
Moulds and yeasts	2.59 ± 0.70	2.82 ± 0.76	2.85 ± 0.80	ns	2.13 ± 1.08 ^{a}	2.15 ± 1.02 ^{a}	1.46 ± 0.70 ^{b}	*

[1] Levels are expressed in log cfu g^{-1}, mean ± SD of duplicate determinations.
[2] Significance in the analysis of variance: ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, P 0.001.
[3] Low a_w was < 0.871, medium a_w was within the range 0.871–0.879, and high a_w was > 0.879.
[4] Low salt concentration was < 4.23%, medium salt concentration was within the range 4.23–4.72%, and high salt concentration was > 4.72%.
[5] Low salt-in-lean (S/L) ratio was < 0.048, medium S/L ratio was within the range 0.048–0.054, and high S/L ratio was > 0.054.
[6] Low intramuscular fat (IMF) content was < 11.32%, medium IMF content was within the range 11.32–13.44%, and high IMF content was > 13.44%.

Table 3
Levels[1] of the main microbial groups in untreated and HPP-treated ripened Iberian hams as influenced by HPP treatment and refrigerated storage at 4 °C for 5 months.

Microbial group[2]	Storage period	Untreated hams (n = 30)	HPP-treated hams (n = 30)
Aerobic mesophiles	0 months	3.26 ± 0.84 ^{aA}	1.48 ± 0.75 ^{bB}
	5 months	2.90 ± 0.88 ^{aB}	2.37 ± 1.04 ^{bA}
Psychrotrophs	0 months	3.13 ± 0.81 ^{aA}	1.13 ± 0.34 ^{bB}
	5 months	3.09 ± 0.90 ^{aA}	2.07 ± 1.08 ^{bA}
Lactic acid bacteria	0 months	2.89 ± 0.73 ^{aB}	0.85 ± 0.92 ^{bB}
	5 months	3.28 ± 0.91 ^{aA}	1.94 ± 1.25 ^{bA}
Micrococcaceae	0 months	3.09 ± 0.72 ^{aA}	1.35 ± 0.62 ^{bB}
	5 months	2.81 ± 1.09 ^{aA}	1.88 ± 1.10 ^{bA}
Moulds and yeasts	0 months	3.06 ± 0.47 ^{aA}	1.19 ± 0.49 ^{bB}
	5 months	2.75 ± 0.75 ^{aB}	1.94 ± 0.99 ^{bA}

[1] Levels are expressed in log cfu g^{-1}, mean ± SD of duplicate determinations.
[2] Means within the same row with different lower-case superscripts differ significantly at $P < 0.01$. Means for the same microbial group within the same column with different upper-case superscripts differ significantly at $P < 0.01$.

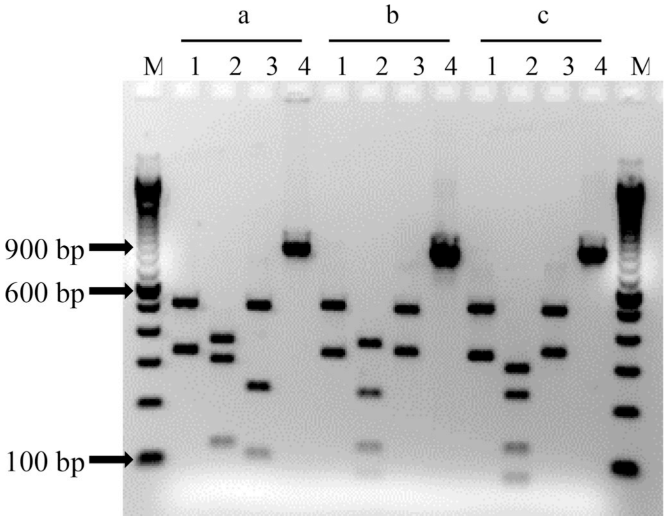

Fig. 1. Patterns obtained by partial amplified rDNA restriction analysis (ARDRA) with endonucleases *Kpn*I (1), *Mbo*I (2), *Ava*II (3). Undigested sample (4). *Staphylococcus equorum/hominis* (a), *S. epidermidis* (b), and *S. pasteuri/warneri* (c).

Micrococcaceae in dry-cured ham and exerts a selective pressure on the types and levels of microorganisms (Cordero and Zumalacárregui, 2000). *Micrococcaceae* was the predominant microbial group in Parma ham (Hinrichsen and Pedersen, 1995) and Serrano ham (Carrascosa et al., 1988; Blesa et al., 2008) and, according to our results, *Micrococcaceae* were also the predominant microbial group in Iberian ham. In fact, most bacterial isolates from PCA or MRS were identified as staphylococci. Salt concentration did not influence any of the microbial groups in the present study, what might be explained by the narrow

Table 4
Identification at the genus and species level of 227 bacterial isolates and 24 yeast isolates from untreated and HPP-treated Iberian hams at the beginning and the end of the 5-month refrigeration period.

Genus	Species	Media[1]	0 months		5 months	
			Untreated	HPP-treated	Untreated	HPP-treated
Staphylococcus	*equorum*	a	38	12	46	48
	epidermidis	b	13	23	3	–
	hominis	c	–	1	2	–
	pasteuri/warneri	b	8	13	4	–
	saprophyticus	d	1	–	–	–
Enterococcus	*faecalis*	e	–	1	–	–
	faecium/hirae	f	–	2	–	–
Carnobacterium	*divergens*	g	–	1	–	–
Kocuria	*palustris*	d	–	–	2	–
	rhizophila	e	–	–	–	1
Tetragenococcus	*halophilus*	d	–	–	1	–
	koreensis	h	–	–	1	–
	solitarius	h	–	–	2	–
Brachybacterium	*conglomeratum*	i	–	–	–	2
Streptomyces	*flavofungini/lomodensis*	e	–	–	–	2
Debaryomyces	*hansenii*	j	2	3	5	8
Moniliella	*nigrescens*	j	2	–	–	–
Cryptococcus	*magnus*	j	–	1	–	–
Rhodotorula	*mucilaginosa*	j	–	–	–	2
Ustilago	*cynodontis/sparsa*	j	–	–	–	1

[1] Isolated from the following culture media: a, PCA incubated at 30 °C, PCA incubated at 8 °C, MSA and BP + RPF; b, PCA incubated at 30 °C, MRS, MSA and BP + RPF; c, PCA incubated at 30 °C and MSA; d, MSA; e, PCA incubated at 30 °C; f, PCA incubated at 30 °C and MRS; g, PCA incubated at 8 °C; h, MRS; i, BP + RPF; j, SDA.

range of salt concentration in Iberian hams (3.02–5.62% for the first group of hams and 3.51–5.35% for the second group) in comparison with the wider range of salt concentration (2.87–7.91%) found in a previous study on Serrano ham (Martínez-Onandi et al., 2017a).

Intramuscular fat contributes to the flavour, odour and texture of dry-cured ham. It is especially important in the perception of juiciness in Iberian dry-cured ham since this product is strongly dehydrated (Lorido et al., 2015). Higher levels of aerobic mesophiles, *Micrococcaceae*, halophiles and yeasts in the fat than in the lean of Italian hams were found during the first 4 months at 8 °C, but those differences did not persist at the end of ripening (Giolitti et al., 1971). In the present study, higher levels of moulds and yeasts were recorded in HPP-treated hams of low and medium fat content at the end of the refrigeration period. In Serrano ham, higher levels of aerobic mesophiles, psychrotrophs, and moulds and yeasts were recorded in untreated hams of low fat content than in those of medium or high fat content (Martínez-Onandi et al., 2017a).

Reductions in the counts of the different microbial groups shortly after the application of the HPP treatment ranged from 1.7 to 2.0 log cycles (Table 3). They were generally higher than the respective reductions reported for Serrano ham of similar microbial load, which ranged from 0.4 to 2.0 log cycles (Martínez-Onandi et al., 2017a). HPP treatment of sliced Serrano ham of higher microbial load at 600 MPa for 6 min resulted in a slightly higher reduction, of 2.6 log cfu g^{-1} (Clariana et al., 2011; Garriga et al., 2004). HPP causes microbial death due to a multiplicity of damage accumulated in different cell locations (Rendueles et al., 2011). In the present study, differences in microbial counts between untreated and HPP-treated Iberian ham samples diminished at the end of the 5-month refrigeration period (Table 3). In a previous study, counts of aerobic mesophiles in sliced Serrano ham treated at 600 MPa were around 3 log cfu g^{-1} after storage at 4 °C for 4 months while the levels of lactic acid bacteria and yeasts remained under the detection limit (Garriga et al., 2004). The higher fat content and a_w of Iberian ham might be responsible for the differences observed with respect to Serrano ham. In vacuum-packed “cecina”, a dry-cured meat product, a treatment at 500 MPa for 5 min lowered counts of aerobic mesophiles, lactic acid bacteria, *Micrococcaceae*, moulds and yeasts by 2 log cfu g^{-1}. After 7 months at 6 °C, differences between untreated and HPP-treated “cecina” samples ranged from 0.33 log cfu g^{-1} for lactic acid bacteria to 1.9 log cfu g^{-1} for aerobic mesophiles (Rubio et al., 2007).

Gram-positive catalase-positive cocci were identified as the predominant microorganisms along ripening of different types of dry-cured ham (Carrascosa et al., 1988; Giolitti et al., 1971; Hinrichsen and Pedersen, 1995; Huerta et al., 1988). In the present study, 93.4% of the identified isolates belonged to the genus *Staphylococcus*, in agreement with previous reports for Iberian ham (Rodríguez et al., 1994) and “lacón”, a dry-cured pork product (Lorenzo et al., 2012). In the cited works, about 70% of the isolates were identified as *S. xylosus* and about 8% as *S. equorum* using biochemical methods. However, the identification of isolates from these two species by biochemical methods is less reliable than by using molecular methods (Blaiotta et al., 2004; Landeta et al., 2011). In Serrano ham, DGGE allowed the identification of *S. equorum* and *S. succinus* (Martínez-Onandi et al., 2017a). In the present study, 63.4% of all isolates were *S. equorum*, followed by *S. epidermidis* (17.2%) and *S. pasteuri/warneri* (11.0%). The species *S. aureus*, able to produce enterotoxin D in Serrano ham (Dora et al., 2011), was not detected in the present study. A few other members of the family *Micrococcaceae* were found among the bacterial isolates (two *Kocuria palustris* isolates and one *K. rhizophila* isolate). Our results are in agreement with those of previous works on *Micrococcaceae* in Iberian ham (Rodríguez et al., 1994), salt used for salting of hams (Cordero and Zumalacárregui, 2000) and “lacón” (Lorenzo et al., 2012).

In the present study, isolation of members of the genera *Tetragenococcus*, *Carnobacterium* and *Streptomyces* from dry-cured ham is reported for the first time. Although their contribution to the biochemical and sensory characteristics of dry-cured ham remains unknown, it is now proven that they are able to adapt to the stressful conditions of the ham dry-curing process and to survive throughout long ripening and storage periods.

The genus *Tetragenococcus* is the only genus of lactic acid bacteria that can thrive at salt concentrations as high as 18% NaCl and at high pH values (Röling and van Verseveld, 1997). Two of the three species found in this work (*T. koreensis* and *T. halophilus*) are typically isolated from salt-rich foods and condiments such as kimchi, fish sauce, anchovy pickles and soy sauce. *T. koreensis* was recently isolated from a traditional Italian raw fermented sausage (Amadoro et al., 2015). *T. koreensis* and *T. solitarius* are the only two species from this genus able to grow in MRS (Justé et al., 2012), as it was the case for our isolates. In the present study, *T. halophilus* was isolated from plates of MSA, which contains 7.5% NaCl.

Carnobacterium species are able to grow in raw meat and meat products at temperatures close to 0 °C, and are among the dominant lactic acid bacteria in cooked meats (Samelis et al., 2000). This genus had not been associated with dry-cured ham and, to our knowledge, its resistance to HPP treatment had not been reported. In the present study, the *C. divergens* isolate came from a HPP-treated Iberian ham sample and was able to grow on PCA at 8 °C.

Enterococcus species are present in many foods due to their ability to grow at low a_w, in the presence of 6.5% NaCl, and at temperatures ranging from 10 to 45 °C. Although they can play a beneficial role in the ripening process and aroma development of meat and dairy products, enterococci have been also associated with the spoilage of meat and meat products, the production of biogenic amines and the carriage of virulence and antibiotic resistance factors (Foulquié Moreno et al., 2006). They have been detected in spoiled dry-cured ham (Marín et al., 1992). The resistance of a virulent *E. faecalis* strain in dry-cured ham to HPP was quite remarkable, with treatments of 750 MPa for at least 9.5 min being needed to achieve a 4 log-cycle reduction (Belletti et al., 2013a). In the present study, the three *Enterococcus* isolates came from HPP-treated Iberian ham samples.

Although selected strains of *Streptomyces griseus* are used as starter cultures in the manufacture of fermented sausages (Candogan et al., 2009), the presence of the genus *Streptomyces* in dry-cured ham had not been reported, to our knowledge. In the present study, two *Streptomyces flavofungini/lomodensis* isolates were obtained from HPP-treated samples of Iberian ham, a fact which points to the baroresistance of this species.

It must be noted that Gram-negative species were not detected among Iberian ham bacterial isolates, a fact which can be associated with their low salt tolerance, although some genera of *Enterobacteriaceae* have been isolated from spoiled dry-cured hams (Belletti et al., 2013b). The absence of spore-forming bacterial genera such as *Bacillus*, which includes salt tolerant species able to survive under stressful conditions, could be explained by their low or null prevalence in the processing environment of Iberian ham. However, the presence of *B. subtilis* in Serrano ham has been reported (Martínez-Onandi et al., 2017a).

D. hansenii, which accounted for 75.0% of yeast isolates, can be found in habitats and foods of low a_w and products of high sugar content. It is the most commonly isolated yeast in meat fermentations (Breuer and Harms, 2006). It has been isolated from surface samples of Iberian ham together with *Candida zeylanoides* (Núñez et al., 1996b; Gallardo et al., 2014) and from the internal samples of Serrano ham (Martínez-Onandi et al., 2017a). Our 75.0% percentage is similar to the 80.9% previously obtained by molecular identification techniques (Gallardo et al., 2014) and lower than the 99.0% obtained using classical identification techniques (Núñez et al., 1996b). *D. hansenii* contributes to the generation of peptides and free amino acids from raw pork myofibrillar proteins (Martín et al., 2001; Rodríguez et al., 1998) and to the formation of volatile compounds and the inhibition of lipid oxidation products during ripening of dry-fermented sausages (Flores et al., 2004). Its isolation in the present study from both untreated and HPP-treated samples indicates a certain baroresistance.

Members of the genera *Rhodotorula* (Núñez et al., 1996b; Gallardo et al., 2014) and *Cryptococcus* (Gallardo et al., 2014) have been previously isolated from Iberian ham. Its isolation in the present study from HPP-treated samples indicates a certain baroresistance. The low number of yeast isolates (24 in total) might have precluded the identification of members of the genus *Candida*, that have been previously isolated from Iberian ham (Núñez et al., 1996b; Gallardo et al., 2014).

The microbial diversity detected in the present study by means of culture-dependent techniques does not show a complete image of the microorganisms present in Iberian dry-cured ham. Microbial cells belonging to species with particular nutritional requirements or in a non-cultivable physiological status may be overlooked by plate counting methods. Culture-independent next generation sequencing stands out as a powerful tool to characterize complex microbial communities under *in situ* conditions. Its use in further research on the subject would widen the knowledge on taxonomical diversity and relative abundance of the microbial genera present in Iberian dry-cured ham. However, it should be borne in mind that the success and result quality of PCR-based sequencing can be affected by numerous factors such as DNA extraction method, amplicon targeted, primers selected, thermal cycling conditions, sequencing method, bioinformatics pipeline, etc. (Lee et al., 2017). The microbial diversity of the same sample could considerably differ depending on the choice of techniques.

5. Conclusions

Water activity, salt concentration and intramuscular fat content had a moderate effect on Iberian ham microbiota, with a_w as the most influential parameter. High a_w hams had the highest counts of psychrotrophs and *Micrococcaceae*. HPP treatment significantly reduced the levels of all the studied microbial groups and, although a recovery was observed during refrigerated storage, counts in HPP-treated samples remained 0.5 to 1.3 log cycles lower than in untreated samples after 5 months at 4 °C. Concerning the biodiversity of Iberian ham microbiota, isolates belonging to the genus *Staphylococcus* represented 93.4% of all bacterial isolates, with *S. equorum* as the most frequently isolated species. The presence of microorganisms not previously found in dry-cured ham, such as members of genera *Tetragenococcus*, *Carnobacterium* and *Streptomyces*, here reported is noteworthy. *Debaryomyces hansenii*, representing 75.0% of all yeast isolates, was the most frequently isolated yeast species. The high proportion of *Staphylococcus* and *Debaryomyces* among isolates from HPP-treated Iberian ham samples indicates a certain degree of baroresistance of these two genera.

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Appendix A. Supplementary data

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