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Processing of dry-cured ham in a reduced-oxygen atmosphere: Effects on physicochemical and microbiological parameters and mite growth

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ABSTRACT

The effects of a reduced-oxygen atmosphere (ROA) ($[O_2] < 4.5\%$) during part or the whole of dry-cured ham processing on microbiological and physico-chemical parameters and mite growth were investigated in two independent experiments. In Experiment 1, six hams were processed in ROA and six in air for 275 days; in Experiment 2, where lower RH was used, six hams were processed in ROA for 289 days, six for 214 days in air + 75 days in ROA, and six in air for 289 days. Microbiological analyses during the process and physicochemical analyses in final products were carried out. The use of ROA during the whole process increased the L^* colour parameter in the subcutaneous fat and proteolysis index and decreased b^* in the external part of the subcutaneous fat and cholesterol oxide concentration. The use of ROA combined with low RH retarded microbial growth and prevented mite growth.

1. Introduction

The traditional dry-cured ham process is based on a curing stage followed by a resting period at below 5 °C and an ageing period where the temperature is gradually raised. During the processing, microbial growth (bacteria, yeasts and moulds), and lipid and protein oxidation occur on the surface of dry-cured hams, a phenomena facilitated by the fact that hams are processed in air. The typical growth of moulds on the surface of Spanish dry-cured hams occurs in the resting and later stages (Hugas, Arnau, & Roca, 1987) and may represent a health risk, since some of the moulds isolated are toxin producers (Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996; Núñez et al., 2000; Rojas, Jodral, Gosalvez, & Pozo, 1991; Sosa et al., 2002). Furthermore, moulds can be a health risk for workers (Bush, Portnoy, Saxon, Terr, & Wood, 2006). In addition, the growth of moulds in the inner parts of ham, especially in the area around the coxofemoral joint, may contribute to the development of off-odours in this area and adjacent muscles (Arnau, 1987). Mite infestation is also frequent in dry-cured ham and can lead to product rejection or to allergic reactions in sensitive consumers when ingested, handled or inhaled (Armentia et al., 1994). Despite the positive effect of lipid oxidation on flavour (Gandemer, 2009), there are concerns due to the formation of oxidation products, such as cholesterol oxides, which are detrimental to health (Bösinger, Luf, & Brandl, 1993; Finocchiaro & Richardson, 1983; Paniangvait, King, Jones, & German, 1995; Peng & Taylor, 1984). Lipid oxidation occurs mostly in outer and inner dry-cured ham muscles, such as the *semimembranosus* and *biceps femoris* (Andres, Cava, Ventanas, Muriel, & Ruiz, 2004). Additionally, protein breakdown can be affected by oxygen, since intermolecular protein–protein cross-links formed in highly oxidative conditions can make proteins less susceptible to enzymatic proteolysis (Davies, 2001). Both processes, lipid oxidation and protein breakdown, start in the cold phase (Díaz, 1993; Hortós, 1995) and the relationship between lipid and protein oxidation in dry-cured meat has been confirmed (Ventanas, Estevez, Tejeda, & Ruiz, 2006). Packaging under vacuum or in an atmosphere with a very low oxygen concentration has been used for decades to prevent mould growth and lipid oxidation in sliced dry-cured hams. Vacuum packaging during the resting period increases proteolysis and microbial counts and reduces oil drip, mould growth and weight loss during processing (Sánchez-Molinero & Arnau, 2008). However, the effect of a reduction of oxygen in the atmosphere during the resting and drying processes is not known.

The aim of this study was to evaluate, in two independent experiments, the effect of a reduced-oxygen atmosphere in two different relative humidities (in the range commonly used in dry-cured ham manufacture), on some physicochemical and microbiological parameters of dry-cured ham. The ROA was applied either throughout the whole process, or only in the last stages, where the risk of microbial growth in the area around the coxofemoral joint was considered high due to the high temperature and crack formation. The survival of mites added to the surface of the hams 25 days before the end of processing was also evaluated.

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2. Material and methods

2.1. Ham processing

2.1.1. Experiment 1: processing of hams in higher RH

A total of 12 hams obtained from six commercial carcasses, were selected at a local slaughterhouse at 24 h *post-mortem* (9.7 ± 0.5 kg, pH_{24} < 6.2 in *semimembranosus* muscle, mean pH_{24} 5.68 ± 0.15 and 2.2 ± 0.2 cm subcutaneous fat thickness) and partially skinned according to the typical Spanish V shape. After 48 h at 2–3 °C, they were thoroughly rubbed with a mixture of 35 g NaCl, 0.15 g $NaNO_2$ and 0.30 g KNO_3 per kg of green ham. The hams were placed individually in plastic bags (Cryovac® BB3050, Sealed Air) with the lean covered by the part of the salt mixture that had not adhered to the ham. Then, according to the experimental design (Table 1), some were assigned to a control treatment (C1) and packaged in air and the rest were assigned to an oxygen-reduced atmosphere (T1) where the gas composition was N_2 99.9 (±0.1)% and O_2 0.1 ± 0.1%, total gas volume being 10 l approximately. After 5 days of storage in a horizontal position at 2–3 °C, the bags were opened, the brine was discarded and each ham was rubbed once more with 30 g of NaCl per kg of green ham, repackaged and left as before for 16 days. Then, the hams were taken out of the bags, washed with water (temperature 14 °C) and, according to the experimental design (Table 1), were assigned to a control treatment (C1) and placed in a 2-m^3 drying chamber to be processed in air or to a treatment in reduced-oxygen (T1) in an identical chamber to be processed in an atmosphere with a reduced oxygen concentration. The hams were placed in such a way that the two hams from the same carcass were hung in the same location inside the assigned chamber. In order to obtain the reduced-oxygen atmosphere (ROA), immediately after the hams were placed inside the drying chamber, 300 oxygen scavengers (Tyvek LH3000, ATCO) were placed at the bottom of the drying chamber and nitrogen gas (Industrial Nitrogen, Carburos Metálicos) was introduced into the chamber with a flow rate of 25 l/min until the oxygen concentration dropped below 0.5%. The N_2 entering the chamber was previously hydrated by passing it through saturated NaCl solution. Simultaneously to the N_2 flushing, the drying chamber door was sealed with silicon. During flushing, the gases exited the drying chamber through a U-shaped tube that had the last 2 mm immersed in water. This whole procedure was repeated every time the drying chamber was opened for sampling or weight control purposes (every 25–35 days). In order to maintain reduced levels of oxygen during the entire drying stage, N_2 was injected daily into the chamber for a period of 6 h. Oxygen was analysed before and after nitrogen injection with a gas analyser (Checkmate II PBI Dansensor, Danemark). The oxygen concentrations before and after flushing were 3.5 ± 1.0% and 0.5 ± 0.2% respectively. HR was controlled by a hygrometer and monitored and registered continuously by a capacity probe (Testo, Hygrotest 600) connected to a data logger system (Desin Instruments, DAS-8000) located outside the chamber.

The temperature and relative humidity conditions during treatment are shown in Table 2.

2.1.2. Experiment 2: processing of hams in lower RH

Eighteen hams from nine carcasses with the following characteristics: 9.4 ± 0.6 kg, pH_{24} < 6.2 in the *semimembranosus* muscle, mean pH_{24} 5.63 ± 0.23 and 1.6 ± 0.1 cm subcutaneous fat thickness were processed by the procedure in Experiment 1. They were assigned to a control treatment (C2), a treatment in ROA for 289 days (T21) and a treatment in air for 214 days + 75 days in ROA (T22) as shown in Table 1. C2 hams and T22 hams remained in bags with an air atmosphere throughout the salting stage and moved to the drying chamber with air after washing, while T21 hams remained in bags with N_2 99.9 (±0.1)% and O_2 0.1 ± 0.1% throughout salting and moved to the drying chamber with ROA. T22 hams were moved to the drying chamber with ROA at 214 days. The oxygen concentrations in the drying chamber with ROA before and after flushing were as in Experiment 1. Temperature and relative humidity conditions are shown in Table 2. After 264 days of processing, all hams were subjected to infestation with mobile forms of *Tyrophagus putrescentiae*, mites commonly found in dry-cured ham companies (Cantoni, D'Aubert, & Calcinardi, 1970).

2.2. Physicochemical analyses

2.2.1. Sampling procedure and sequence of analyses

At the end of the process, the instrumental colour of the external surface of subcutaneous fat was measured and averaged over three zones (only in Experiment 2). Then, the hams were deboned and transversal cuts were made at A (level of coxofemoral joint), B and C (Fig. 1) to obtain slice AB (2.0 mm thick) and slice BC (2.5 cm thick). The parts of *semimembranosus* and *biceps femoris* muscles from each slice were removed. The muscles from slice AB were used for colour stability analyses in air (only in Experiment 2) and the muscles from slice BC for pH distribution analysis (Experiment 1) and colour changes during storage (Experiment 2). Then, all samples were vacuum packaged in plastic bags (50 μm polyamide/100 polyethylene multilayer, Sacoliva, S.L., Spain) and stored for 15 days at 2–4 °C. After this period, all the packages were opened, the colour of samples from slice BC was measured, and then all the samples were minced, vacuum packaged and stored at 2–4 °C for further physicochemical analyses.

In Experiment 2, 10-g samples of *gracilis* muscle up to 3 mm depth were taken and minced for analyses of superficial a_w and 20-g samples from the whole surface of the *gluteus medius* muscle (up to 3 mm depth) for cholesterol oxide analysis.

2.2.2. Instrumental colour analyses

All colour measurements were done with a Minolta Chromameter CR-200 with C illuminant and 10° standard observer (specular component included) in the CIELAB space [lightness (L^*), redness (a^*), yellowness (b^*)] (Commission Internationale de l'Eclairage, 1976). For colour stability (Experiment 2), the colour measurements were performed on *biceps femoris* and *semimembranosus* muscles from slice AB, and averaged over three zones at time 0 h (fresh cut) and after 10, 20, 30, 60 and 120 min. During this period, the samples were kept in a chamber at 20 ± 2 °C with an average illuminance of 460 lx (light source: Philips TL-D 36 W/54 Daylight) and covered, to avoid surface drying, with a food grade PVC film (oxygen permeability: 20.000 cm^3/m^2/24 h; water-vapour transmission rate 2000 g/m^2/24 h; Macopal, S.L., Lliçà de Vall, Spain). Colour was also evaluated in *biceps femoris* and *semimembranosus* muscles from slice BC (averaged over three zones) just after slicing

Table 1
Experimental design for Experiments 1 and 2.

	Treatment	Days in air	Days in ROA[b]	Hams assigned to each treatment[a]
Experiment 1	C1	275	0	1l 2r 3l 4r 5l 6r
	T1	0	275	1r 2l 3r 4l 5r 6l
Experiment 2	C2	289	0	7l 8r 9l 10r 11l 12r
	T21	0	289	7r 8l 9r 13l 14r 15l
	T22[c]	214	75	10l 11r 12l 13r 14l 15r

[a] Hams with the same number come from the same carcass. l = left ham, r = right ham.
[b] ROA: reduced-oxygen atmosphere.
[c] After 214 days of processing, the hams were taken from the drying chamber with air and moved to the drying chamber with ROA.

Table 2
Processing conditions for Experiments 1 and 2.

Stage	Experiment 1				Experiment 2			
	Time (days)	Accumulated time (days)	Temperature (°C)	RH (%)	Time (days)	Accumulated time (days)	Temperature (°C)	RH (%)
Salting	21	21	2–3	–[a]	21	21	2–3	–[a]
Resting (R1)	20	41	2–4	70–85	35	56	3–5	70–80
Resting (R2)	7	48	6–8	70–85	8	64	6–8	70–80
Drying 1 (D1)	37	85	12–14	65–80	57	121	12–15	60–70
Drying 2 (D2)	65	150	12–14	65–80	50	171	15–17	60–70
Drying 3 (D3)	60	210	19–21	65–75	43	214	19–20	60–70
Drying 4 (D4)	56	266	23–25	50–60	50	264	22–24	50–60
Drying 5 (D5)	9	275	25–27	50–60	25	289	24–26	50–60

[a] The hams were packaged in plastic bags.

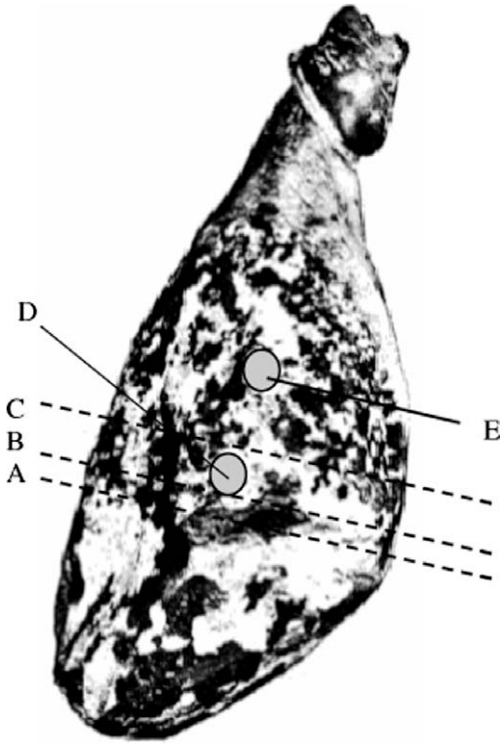

Fig. 1. Sampling zones. A, B, C: zones where transversal cuts were made. D: sampling area of *adductor* surface for microbiological analysis (swab method, Experiment 1). E: sampling area of *gracilis* surface for microbiological analysis (swab method, Experiment 1).

and after 15 days of vacuum packaged storage at 2–4 °C in darkness.

2.2.3. pH distribution analysis

The pH distribution was carried out on slice BC using a pH-metre (Sharlau Science 8424) with a penetration probe (Crison Ingold LoT406-M3-S7/25). The probe was inserted at three points in the *semimembranosus* muscle: external (1–2 mm from surface), internal (3–4 mm from the junction with *biceps femoris* muscle) and central (midway between the other two points) and at 3 points in the *biceps femoris* muscle: external (1–2 mm from subcutaneous fat), internal (3–4 mm from the junction with *semimembranosus* muscle) and central (midway between these two points).

2.2.4. Other physicochemical analyses

The following analyses were performed on the minced samples: water activity (a_w), was measured at 25 °C with a Novasina AW-Sprint-TH 500 instrument (Axair Ltd., Pfäffikon, Switzerland); pH (Experiment 2); water content by drying at 103 ± 2 °C until constant weight (AOAC, 1990); NaCl with a chloride analyser Technicon™ (Berman, 1976); total nitrogen (TN) by the Kjeldahl method (ISO 937: 1978) and expressed as protein content percentage (TN × 6.25); non-protein nitrogen content (NPN) by precipitation of proteins with trichloroacetic acid (Gáspár, 1984), followed by determination of the nitrogen in the extract by the Kjeldahl method. With these values, a proteolysis index (PI) was calculated by the formula $PI = 100 \times NPN \times TN^{-1}$. For cholesterol oxide determination, the method described by García-Regueiro and Maraschiello (1997) was followed substituting the purification by thin layer chromatography by a solid phase extraction method based on aminopropylsilica (Petrón, García Regueiro, Martín, Muriel, & Antequera, 2003). Only the cholesterol oxides identified (by gas chromatography coupled to mass spectrometry) were reported. Weight losses were analysed throughout the process.

2.3. Microbiological analyses

2.3.1. Sampling procedure

The swab method was used in Experiment 1 at 21 days and the dissection method was followed in the rest of the sampling. In the swab method, an area of *adductor* and *gracilis* muscles (Fig. 1) delimited by a steel circle of 7.0 cm diameter was aseptically sampled with two sterile cotton swabs previously moistened with a dilution medium consisting of 0.1% Bacto Peptone (Difco Laboratories, Detroit, MI), and 4.0% NaCl (Merck, Darmstadt, Germany) at pH 7.0. Then, the swabs were put into a tube containing 10 ml of dilution medium and shaken for 30 s. In the dissection method, 10-g samples were taken under aseptic conditions and blended for 1 min in a laboratory homogenizer (Stomacher Labblender) in 90 ml of dilution medium. The dissection method used samples taken from a maximum depth of 2 mm from the lean surface during processing (at 85 and 200 days in Experiment 1 and at 21, 100 and 210 days in Experiment 2) and with samples taken from *adductor* muscle around the coxofemoral joint and from *rectus femoris* and *semitendinosus* muscles of the finished product.

2.3.2. Microbial counts

Serial dilutions were made and plated onto appropriate culture media to determine aerobic halotolerant microorganisms in PCA agar (Merck, Darmstadt, Germany) containing 4% NaCl at 30 °C for 72 h; Gram-positive catalase-positive cocci in Mannitol Salt Agar (MSA, Difco Laboratories) at 30 °C for 72 h; Lactic-acid bacteria in Lactobacilli MRS Agar (Difco Laboratories) double-layered plates at 30 °C for 72 h in an anaerobic atmosphere; yeasts and moulds in 2% dextrose Saboureaud Agar with oxytetracycline supplement (Merck, Darmstadt, Germany) at 25 °C for 5 days; *Brochrotrix Thermosphacta* in agar STAA (Oxoid) with supplement (streptomicine sulphate, talium acetate and ciclohexymide) at 22–25 °C for 2 d; *Enterobacteriaceae* in agar VRBD (Merck) at

30 °C for 24 h; Sulphite reducing clostridia in agar SPSS (Merck) at 37 °C for 24 h in an anaerobic atmosphere.

2.3.3. Evaluation of mould growth

Mouldiness (extension of the area of the lean covered by moulds) (Sánchez-Molinero & Arnau, 2008) was evaluated visually by a panel consisting of six selected and trained assessors with a 0–10 non-structured scale, i.e. 0 for absence of the descriptor and 10 for maximum intensity (Amerine, Pangborn, & Roessler, 1965). When moulds were not observed, on the surface, the growth of moulds in the Saboureaud agar media was evaluated.

2.4. Presence of mites

The presence of mites on the ham surface was evaluated visually throughout the process.

2.5. Statistical analyses

A least square analyses of variance (ANOVA) was carried out for each parameter, using the GLM (General Linear Models) procedure from the SAS statistical package (SAS Institute, 1999). The model included the treatment and the carcass (block) as fixed effects. The means for each treatment were obtained by the *least square means* procedure of the same software. For frequency data a χ^2 test (Meilgaard, Civille, & Car, 1990) was performed to find out whether there was an effect of the treatment on frequency.

3. Results

3.1. Experiment 1: processing of hams in high RH

3.1.1. Physico-chemical parameters

Weight losses, length, width and thickness during processing were not affected by the treatment ($P > 0.1$) (results not shown). Mean weight losses for C1 and T1 respectively were 4.3% and 4.4% at 21 days, 18.4 and 18.2% at 72 days, 27.2% and 27.6% at 186 days, and 33.6% and 33.0% at the end of the processing. The results for physico-chemical parameters in *semimembranosus* and *biceps femoris* muscles are shown in Table 3. In *semimembranosus* muscle, the hams processed in reduced-oxygen atmosphere (T1) had lower protein ($P < 0.05$), higher moisture content, a_w ($P < 0.1$), proteolysis index and pH values in the external and medium part ($P < 0.05$) than control hams. In *biceps femoris* T1 hams had higher values of proteolysis index ($P < 0.05$). The carcass factor had a significant effect ($P < 0.05$) on NaCl content (expressed in all forms), protein content, proteolysis index, a_w, and pH in the medium part of *semimembranosus* muscles and in moisture, NaCl, protein, proteolysis index and a_w of *biceps femoris* muscles (results not shown).

3.1.2. Microbiological parameters

Superficial aerobic halotolerant microorganisms and Gram-Positive catalase-positive cocci counts were lower than 3.0 $\log_{10}$ cfu/cm^2 after the salting step and did not differ between ROA and control hams. At 85 days, ROA hams had significantly higher counts than control hams of aerobic halotolerant microorganisms ($P < 0.001$), Gram-positive catalase-positive cocci ($P < 0.01$) and yeasts ($P < 0.10$). Then, the counts in control hams increased and at 200 days only yeasts were slightly higher in ROA than in controls (Table 4).

The surface covered with moulds was larger in T1 hams than in control hams at 85 and 200 days. All microbial counts on *semitendinosus* and *rectus femoris* were lower than 2.0 $\log_{10}$ cfu/g (results not shown). In *adductor* muscles, control hams had higher counts than ROA hams of aerobic halotolerant microorganisms and Gram-positive catalase-positive cocci ($P < 0.01$) but did not differ in lactic-acid bacteria and yeasts counts ($P > 0.10$). *Brochothrix thermosphacta*, *Enterobacteriaceae* and sulphite reducing clostridia were not detected in any sample.

No carcass effect was observed for any microbiological parameter at any sampling time. Mites were observed during processing on the surface of control hams, but not on the surface of ROA hams.

3.2. Experiment 2: processing of hams in low RH

3.2.1. Physico-chemical parameters

Weight losses, length, width and thickness of dry-cured hams during processing were not affected by the treatments ($P > 0.1$) (results not shown). Mean weight losses for C2, T21 and T22 respectively were 3.8%, 3.9% and 4.1% at 21 days; 22.0%, 21.5% and 22.1% at 90 days; 31.8%, 32.1% and 32.0 at 210 days and 36.4%, 37.1% and 36.6% at the end of processing.

The results for physico-chemical parameters in *semimembranosus* and *biceps femoris* muscles are shown in Table 5. The proteolysis index in both muscles was higher in T21 than in T22 and C2, although the differences were only significant in *biceps femoris* ($P < 0.05$). The carcass factor had a significant effect ($P < 0.05$) on

Table 3
Experiment 1: physico-chemical parameters (*least square means*) in finished product.

Parameter[c]	Muscle *semimembranosus*			Muscle *biceps femoris*		
	Treatments [d]		SEM[e]	Treatments		SEM
	C1	T1		C1	T1	
Moisture (%)	*44.44*[b]	*45.96*[a]	0.59	58.63	58.95	0.29
NaCl (%)	5.88	5.98	0.17	7.20	6.81	0.35
NaCl (% DM)	10.47	11.19	0.38	17.37	16.58	0.75
NaCl/H_2O (%)	13.14	13.16	0.46	12.30	11.58	0.68
Protein (%)	41.63[a]	39.78[b]	0.46	26.40	27.04	0.63
Protein (% DM)	74.98	73.58	0.81	63.85	65.81	0.60
Proteolysis index	20.02[a]	22.01[b]	0.58	26.35[b]	28.08[a]	0.57
pH						
Internal	5.69	5.72	0.11	5.80	5.76	0.05
Medium	5.67[b]	5.73[a]	0.03	5.75	5.73	0.05
External	5.49[b]	5.61[a]	0.04	5.64	5.65	0.04
a_w	*0.857*[b]	*0.864*[a]	0.005	0.887	0.889	0.001

[a,b] For each muscle, values in a row with different superscripts are significantly different ($P < 0.05$, except those in italics with $P < 0.1$).
[c] All the composition parameters expressed as percentages are on wet basis except for those that are expressed on a dry-matter basis (DM).
[d] C1: hams processed in air; T1: hams processed in a reduced-oxygen atmosphere.
[e] SEM: standard error of mean, $n = 6$.

Table 4
Experiment 1: microbial counts (*least square means*) and mouldiness during processing[c].

	21 days			85 days			200 days			275 days		
	Treatments[d]		SEM[e]	Treatments		SEM	Treatments		SEM	Treatments		SEM
	C1	T1		C1	T1		C1	T1		C1	T1	
AHM	2.6	2.7	0.4	6.5[b]	8.7[a]	0.6	7.7	8.4	0.3	6.8[a]	4.3[b]	0.8
GPCPC	2.7	2.8	0.4	6.7[b]	9.0[a]	0.6	7.9	8.2	0.5	6.2[a]	4.1[b]	0.7
LAB	<1.0	<1.0	–	<1.0	<1.0	–	<1.0	<1.0	–	<1.0	<1.0	–
Yeasts	<0.6	<0.6	–	*5.0*[a]	*5.5*[b]	0.4	*6.4*[b]	*6.9*[a]	0.5	3.5	3.6	0.7
Mouldiness[f]	0.0	0.0	–	3.1[b]	6.6[a]	0.5	5.3[b]	8.7[a]	0.4	1.5	2.6	0.6

[a,b] For each sampling time, values in a row with different superscripts are significantly different ($P < 0.05$ except those in italics with $P < 0.1$). AHM: aerobic halotolerant microorganisms; GPCPC: Gram-positive catalase-positive cocci; LAB: lactic-acid bacteria.
[c] Samples were taken from ham surface at 21 days (swab method, results expressed in $\log_{10}$ cfu/cm^2), 85 days and 200 days (dissection method, results expressed in $\log_{10}$ cfu/g) and from *adductor* muscle around coxofemoral joint at 275 days (dissection method, results expressed in $\log_{10}$ cfu/g).
[d] C1: hams processed in air; T1: hams processed in a reduced-oxygen atmosphere.
[e] SEM: standard error of mean, $n = 6$.
[f] Surface of lean covered by moulds evaluated visually.

Table 5
Experiment 2: physico-chemical parameters (*least square means*) in finished product.

Parameter[c]	Muscle *semimembranosus*				Muscle *biceps femoris*			
	Treatments[d]			SEM[e]	Treatments			SEM
	C2	T21	T22		C2	T21	T22	
Moisture (%)	42.03	42.46	42.80	0.49	57.59	56.85	56.76	0.46
NaCl (%)	5.87	6.12	6.16	0.18	8.95	9.60	9.21	0.22
NaCl (% DM)	10.13	10.64	10.77	0.19	22.06	22.75	21.84	0.62
NaCl/H_2O (%)	14.01	14.50	14.47	0.16	15.86	17.28	16.65	0.42
Protein (%)	45.24	44.59	44.14	0.32	29.59	29.28	29.08	0.47
Protein (% DM)	78.04	77.49	77.17	0.38	69.77	67.86	67.25	0.53
Proteolysis index	18.92	19.59	19.17	0.30	27.18[a]	28.17[b]	27.01[a]	0.34
pH	6.00	5.92	5.90	0.07	6.00	5.95	5.95	0.04
a_w whole muscle	0.849	0.846	0.859	0.005	0.883	0.880	0.884	0.004
a_w superficial[f]	0.816	0.817	0.820	0.003	–	–	–	–

[a,b] For each muscle, values in a row with different superscripts are significantly different ($P < 0.05$).
[c] DM = dry matter.
[d] C2: hams processed in air for 289 days; T21: hams processed in a reduced-oxygen atmosphere for 289 days; T22: hams processed in air for 214 days and in a reduced-oxygen atmosphere for 75 days.
[e] SEM: standard error of mean, $n = 6$.
[f] Samples of ham surface (down to 3-mm depth in *gracilis* muscle).

NaCl content, NaCl/moisture ratio and a_w in both muscles, in moisture of *biceps femoris* and in superficial a_w (results not shown). Cholesterol oxide concentrations in the *gluteus medius* are shown in Table 6. 25-Hydroxycholesterol and 7-ketocholesterol were not observed in any ham, and α-epoxycholesterol was detected, but it was not possible to quantify due to the low signal/noise ratio. The concentration of the rest of cholesterol oxides was lower than 1 mg/kg. When ROA was used during the whole process (T21), the concentration of 7β-hydroxycholesterol, β-epoxycholesterol and cholestanetriol was significantly reduced ($P < 0.05$) by 13.41%, 33.97% and 35.16% respectively. However, there was no significant effect on 7α-hydroxycholesterol ($P > 0.1$). Carcass factor was significant ($P < 0.05$) for all the oxides detected except cholestanotriol ($P > 0.1$) (results not shown). The external part of subcutaneous fat had higher L ($P < 0.1$) and lower b^* values ($P < 0.05$) in T21 than in C and T22 hams (Table 7). The colour immediately after slicing was not affected by the treatment and suffered small changes during 120 min of exposure (results not shown). During this period, for *semimembranosus* and *biceps femoris* respectively, L^* mean values changed from 32.58 to 33.60 and from 40.25 to 39.82, a^* mean values from 14.45 to 12.97 and from 17.53 to 16.64 and b^* mean values from 6.14 to 6.21 and from 8.03 to 8.02. Regarding the changes in colour due to cold storage, at the end of this period redness in the *semimembranosus* was higher ($P < 0.05$) in C2 hams than in T21 and T22 hams (Table 7). The change of L value during cold storage was higher in T21 hams in both muscles, although the differences were not significant. Carcass factor had a significant effect on all parameters in *semimembranosus* muscles at $t = 0$ and on L and a^* values at 15 days after slicing (results not shown).

Table 6
Experiment 2: cholesterol oxides identified (*least square means*) in finished product: (ppm).

Compound	Treatments[c]		SEM[d]
	C2	T21	
7α-Hydroxycholesterol	0.326	0.323	0.015
7ß-Hydroxycholesterol	0.179[a]	0.156[b]	0.008
ß-Epoxycholesterol	0.209[a]	0.138[b]	0.019
Cholestanetriol	0.129[a]	0.083[b]	0.011

[a,b] Values in a row with different superscripts are significantly different ($P < 0.05$).
[c] C2: hams processed in air; T21: hams processed in a reduced-oxygen atmosphere.
[d] SEM: standard error of mean, $n = 6$.

3.2.2. Microbiological parameters

Microbial counts were lower in Experiment 2 than in Experiment 1, except at the end of salting (Table 8). Superficial microbial counts after salting were not affected by the atmosphere ($P > 0.1$).

Table 7
Experiment 2: colour parameters in *semimembranosus*, *biceps femoris* muscles (after cutting and after 15-day storage vacuum packaged) and on the external surface of subcutaneous fat at the end of process.

	0 days				15 days			
	Treatments[c]			SEM[d]	Treatments			SEM
	C2	T21	T22		C2	T21	T22	
Subcutaneous fat								
L^*	63.28[b]	66.23[a]	63.76[b]	0.81				
a^*	4.20	4.64	4.54	1.20				
b^*	37.45[b]	34.19[a]	38.48[b]	0.96				
Semimembranosus								
L^*	32.13	32.63	32.86	1.48	40.81	43.81	42.11	1.66
a^*	14.94	13.69	14.62	0.79	9.53[b]	8.18[a]	8.17[a]	0.35
b^*	6.02	6.34	6.05	0.62	1.56	0.36	−0.02	1.63
Biceps femoris								
L^*	41.11	39.38	40.51	2.46	52.13	55.11	49.96	6.68
a^*	17.53	17.70	18.11	1.39	11.02	9.28	11.90	3.20
b^*	7.85	8.05	8.21	0.96	2.15	1.56	2.13	3.87

[a,b] For each muscle, values in a row with different superscripts are significantly different ($P < 0.05$).
[c] C2: hams processed in air for 289 days; T21: hams processed in a reduced-oxygen atmosphere for 289 days; T22: hams processed in air for 214 days and in a reduced-oxygen atmosphere for 75 days.
[d] SEM: standard error of mean, $n = 6$.

At 100 days, control hams had significantly ($P < 0.05$) higher superficial counts of aerobic halotolerant microorganism, Gram-positive catalase-positive cocci and yeasts than hams in ROA. In the control hams, the counts of yeasts were higher than of Gram-positive catalase-positive cocci, whereas the hams in ROA registered similar numbers for both microbial groups. At 210 days, an increase was observed only in the aerobic halotolerant microorganisms and Gram-positive catalase-positive cocci counts in hams in ROA. However, they were still significantly lower than in hams processed in air ($P < 0.05$). In control hams, yeast counts at this point were slightly lower than at 100 days, and similar to the aerobic halotolerant counts. Moulds were not observed on the surface of any ham during the process, although the presence of moulds in the culture medium (Saboureaud) was detected on all samples from C2 hams. At the end of the process, T21 hams showed the lowest ($P < 0.05$) counts of aerobic halotolerant microorganisms, Gram-positive catalase-positive cocci and yeasts in *adductor* muscle. In this zone, moulds were detected in C2 and T22 hams but not in T21. All microbial counts on *semitendinosus* and *rectus femoris* were lower than 2.0 $\log_{10}$ cfu/g (results not shown). *Brochrothrix thermosphacta*, *Enterobacteriaceae* and sulphite reducing clostridia were not observed at any point. No significant effect of carcass factor was observed for any parameter at any time. At the end of the process, mites were only observed on control hams.

4. Discussion

4.1. Effect of a reduced-oxygen atmosphere on the physico-chemical parameters of dry-cured ham

As shown by the difference in proteolysis index of *biceps femoris* muscle in both experiments, the drying in ROA facilitated proteolysis, which agrees with the findings of Motilva, Rico, and Toldrá (1993) who described a higher activity of cathepsin B, H and B + L in more reductive condition and with Lund et al. (2007), who observed a decrease in tenderness when fresh meat was packed in oxygen rich atmospheres. One possible explanation could be that intermolecular protein–protein cross-links formed in highly oxidative conditions make proteins less susceptible to enzymatic proteolysis (Davies, 2001).

In Experiment 2, the oxygen concentration did not affect the proteolysis index in *semimembranosus*, in contrast to what was observed in *biceps femoris*, probably because the activity of enzymes in the former was lower than the latter due to its lower water content. The higher water content of *semimembranosus* in T1 hams ($P < 0.1$) compared to C1 hams probably also contributed to the difference in proteolytic index between treatments, since a higher water content favours proteolytic enzyme activity (Schivazappa, Virgili, & Parolari, 1992). The higher mould growth (Martín, Córdoba, Aranda, Guía Córdoba, & Asensio, 2006) and microbial counts (Bermell, Molina, Miralles, & Flores, 1992; Rodríguez, Núñez, Córdoba, Bermúdez, & Asensio, 1998) found in C1 hams could also have contributed to the differences in proteolytic index, although probably to a lesser extent, since in dry-cured ham, the proteolytic activity of enzymes is considered to be more important than that of microorganisms' (Verplaetse, 1994). The higher water content and a_w of *semimembranosus* in T1 hams compared to C1, was probably due to a slightly higher mean RH in the drying chamber with ROA, since the humidity range (70–85%) was sufficient to cause these differences in the water content of hams (Arnau, Gou, & Comaposada, 2003). The higher mould growth in T1 hams could also have contributed to the difference in moisture, since moulds may reduce water loss due to the mycelic cover (Hammes & Knauf, 1993; Jessen, 1995). The higher water content of T1 hams was the cause of their lower protein content. The higher pH values on the surface of ROA hams could be due to greater proteolysis and higher microbial growth. These results agree with Arnau et al. (2003), who ob-

Table 8
Experiment 2: microbial counts (*least square means* of $\log_{10}$ cfu/g) during processing[c].

	21 days			100 days			210 days			289 days			
	Atmosphere[d]		SEM[e]	Atmosphere		SEM	Atmosphere		SEM	Treatments[f]			SEM
	Air	ROA		Air	ROA		Air	ROA		C2	T21	T22	
AHM	5.1	4.7	0.4	5.7[a]	1.7[b]	0.6	5.3[a]	4.0[b]	0.3	5.3[a]	2.5[b]	5.5[a]	1.6
GPCPC	4.9	4.8	0.5	4.2[a]	2.0[a]	0.6	4.8[a]	4.0[a]	0.2	5.2[a]	2.6[a]	5.2[a]	1.6
LAB	<1.0	<1.0	–	<1.0	<1.0	–	<1.0	<1.0	–	<1.0	<1.0	<1.0	–
Yeasts	1.9	2.0	0.3	5.2[a]	2.1[a]	0.6	4.5[a]	1.8[a]	0.3	4.0[a]	1.3[a]	3.5[a]	0.8
Moulds[g]	0	0	–	6	0	–	6	0	-	5	0	3	–

[a,b] For each sampling time, values in a row with different superscripts are significantly different $P < 0.05$). AHM: Aerobic halotolerant microorganisms; GPCPC: Gram-positive, catalase-positive cocci; LAB: lactic-acid bacteria.
[c] All samples taken with the dissection method from ham surface, except at 289 days (finished product), taken from *adductor* muscle around coxofemoral joint and expressed as $\log_{10}$ cfu/g. AHM: aerobic halotolerant microorganisms; GPCPC: Gram-positive catalase-positive cocci; LAB: lactic-acid bacteria.
[d] ROA: processed in reduced-oxygen atmosphere; air: processed in air.
[e] SEM: standard error of mean, $n = 6$.
[f] C2: hams processed in air for 289 days; T21: hams processed in a reduced-oxygen atmosphere for 289 days; T22: hams processed in air for 214 days and in a reduced-oxygen atmosphere for 75 days.
[g] Number of samples which caused mould growth when plated into Saboreaud agar, $n = 6$.

served higher pH values in hams when the relative humidity during resting changed from 78 ± 3% to 85 ± 3%.

Even though the samples were taken on the surface, the levels of cholesterol oxides were lower than 1 mg/kg which is considered the risk value for human consumption (Paniangvait et al., 1995). The concentration of 7β-hydroxycholesterol and cholestanetriol were similar to those obtained by Vestergaard and Parolari (1999) in Parma ham. These authors also detected 25-hydroxycholesterol and 7-ketocholesterol, probably due to the different analytical methodology used. The processing of hams in ROA for the whole process reduced 7β-hydroxycholesterol, β-epoxycholesterol and cholestanetriol, probably due to the lower presence of hydroperoxides produced by fatty acid oxidation in ROA hams, which could be one of the factors influencing its formation (Maraschiello, Esteve, & García-Regueiro, 1998). The presence of 7-hydroxycholesterol is attributed, to some extent, to the effect of sample treatment because the formation of this compound by cholesterol oxidation should be lower than the formation of 7ß-hydroxycholesterol (García-Regueiro & Maraschiello, 1997).

The reduction of L^* and increase of b^* are typical of non enzymatic browning (Rampilli & Andreini, 1992), which in the case of subcutaneous fat can be produced by reaction of carbonyl compounds from lipid oxidation with amino acids (Lillard, 1987). Maillard reactions have maximum activity at a_w between 0.5 and 0.7 (O'Brien & Morrisey, 1989) which according to the drying conditions is the range expected on the fat surface during much of the processing. In T21, lipid oxidation was expected to be less intense, so the amount of carbonyl compounds formed was probably smaller, causing less browning. The fat colour in T22 hams and in C2 hams was similar, so at the time when the hams were changed from an air atmosphere to an ROA, the amount of carbonyl compounds and their precursors would be sufficient for the browning reactions to continue.

The low penetration of oxygen into the lean (Lawrie, 1991) could account for the absence of differences in colour among treatments. The usual development of white film on cut surface, due to tyrosine crystallization (Arnau, Guerrero, Hortós, & García-Regueiro, 1996), was shown by the increase in L^* observed in *semimembranosus* and *biceps femoris* muscles during the 15 days of storage under vacuum, which could also cause the decrease in a^* and b^* values. The higher values of L^* registered by hams of T21 conformed with their higher proteolysis indexes, although the differences in L^* were not significant ($P > 0.1$) due to the high variability in the data.

The carcass effect observed on physico-chemical parameters could be explained by differences in initial pH and subcutaneous and intramuscular fat (which affect salt uptake and drying kinetics) and in proteolytic potential, which affects the proteolysis index. The carcass effect on cholesterol oxides was probably due to differences in fat composition among carcasses (Gandemer, 1998).

4.2. Effect of a reduced-oxygen atmosphere on the microbiological parameters and mite growth in dry-cured ham

At the end of salting Gram-positive catalase-positive cocci were the dominant group, especially in Experiment 2. Although their growth is facilitated in aerobic conditions, the atmosphere had no significant effect on their numbers, probably because the counts of Gram-positive, catalase-positive cocci and halotolerant flora does not usually change or slightly decreases during salting (Carrascosa, Marín, & Cornejo, 1989; Hugas et al., 1987; Marín Riaño, Carrascosa, & Cornejo, 1993).

The evolution of the microbial flora differed in the first and second experiments. In Experiment 1, Gram-positive catalase-positive cocci were the predominant flora and the counts during the drying period were higher than in Experiment 2 due to the lower a_w of hams in the latter. At 85 days the counts of Gram-positive catalase-positive cocci were higher in T1 hams than in C1 hams probably because the slightly higher water content on the surface of hams in ROA was more important than the reduction in oxygen content. The increase in temperature at 150 days from 12–14 to 19–21 °C caused the increase of the Gram-positive, catalase-positive cocci counts in C1 hams, but they were always lower than in ROA hams.

In Experiment 2, the lower RH caused yeasts to be the main microbial group (Blickstad, 1984) instead of Gram-positive, catalase-positive cocci, whose counts were lower than found by Hernández and Huerta (1993) and Rodríguez et al. (1994). The reduction in the number of yeasts and Gram-positive catalase-positive cocci observed between 21 and 100 days in ROA hams, compared to hams dried in air, was due to the combination of low oxygen content, RH and temperature and high NaCl content (Blickstad, 1984).

The reduction in the oxygen concentration did not prevent surface mould growth, as some *Penicillium* and *Aspergillus* are able to grow at oxygen concentrations lower than 1% (Ooraikul, 1991). However, the simultaneous reduction of RH and oxygen content could have had a synergistic effect which would explain the absence of mould growth in *adductor* muscle in T21, in agreement with Magan and Lacey (1984). The mould growth observed in T22 could be due to the lack of effectiveness of the oxygen reduction applied in the last steps of drying, when moulds had probably already grown in this muscle.

Lactic-acid bacteria counts were very low due to the low superficial a_w, low temperature and because no sugars were added. Generally, the counts of Lactic-acid bacteria are very variable, usually decreasing during resting, then increasing at the beginning of the drying period, when the temperature increases, and finally decreasing as the a_w decreases (Carrascosa et al., 1989; Francisco et al., 1981; Hugas et al., 1987; Marín Riaño et al., 1993; Silla, Molina, Flores, & Silvestre, 1989).

Brochotrix thermosphacta, which is a bacteria usually present in vacuum packed meat, was not detected at any point, in agreement with Hugas et al. (1987). However, higher counts were obtained by Cantoni, Comi, and Fagnani (1983) in defective hams.

The counts in *semitendinosus* and *rectus femoris* muscles were lower than found by others (Carrascosa et al., 1989; Hugas et al., 1987; Marín Riaño et al., 1993; Silla et al., 1989) inside the ham. This could be due to the lower counts obtained from the beginning of the process, the lower a_w and the lower drying temperature. However, in *adductor* muscle bacterial growth was higher due to the cracks that formed which facilitated oxygen diffusion and the sinovial liquid in the joint that increases the moisture content (Hugas et al., 1987). In this area, the aerobic salt-tolerant bacteria were the main flora, and the lactic-acid bacteria showed counts below 10 cfu/g, in agreement with Serra et al. (2007). In Experiment 1, C1 hams had higher Gram-positive catalase-positive cocci counts than T1 hams in this area at 275 days because, apart from the atmosphere effect, the lower water content on the surface facilitated the formation of cracks in the *adductor* muscle, which were visible when the aitch was removed. In Experiment 2, T21 hams had low bacterial counts in this area due to the combination of low a_w and low oxygen concentration. The oxygen concentration in *adductor* muscle in T21 hams was probably lower than on the ham surface, which rendered microbial growth more difficult. The use of a ROA during the last step of the drying (T22) was not enough to reduce the number of microorganisms probably because when the ROA was applied they had reached their stationary phase. The reduction of moulds on the surface and in the *adductor* muscle was probably due to the combination of the low humidity and low oxygen content.

The absence of mites in C2 hams during most of the process can be explained by the low RH applied and the lack of moulds, which stimulate mite growth (Arnau, Hugas, & Monfort, 1987). The use of ROA for 25 days eliminated and prevented the growth of mobile forms of *T. putrescentiae* in previously contaminated hams, which agrees with the results of others using low oxygen atmospheres (Guerrero & Arnau, 1995; Lorenzo & Flores, 1988; Riudavets, Castañé, Alomar, Pons, & Gabarra, 2009).

5. Conclusions

The use of an atmosphere with a reduced oxygen content during the whole process increased the proteolysis index, reduced cholesterol oxides concentration and, when combined with low relative humidity, retarded the growth of bacteria and fungi and prevented mite growth. The effects of this atmosphere on sensory traits should be further investigated.

Disclaimer

The views expressed in this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

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