FORMATION OF Zn-PROTOPORPHYRIN DURING THE ELABORATION PROCESS OF NON-NITRIFIED SERRANO DRY-CURED HAMS AND ITS RELATIONSHIP WITH LIPOLYSIS

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Abstract

This study assessed the ZnPP content, heme content, salt content, and instrumental color in the *biceps femoris* and *semimembranosus* muscles during the elaboration of Serrano dry-cured ham manufactured without the addition of nitrate and nitrite for 15 months. The effects of lipolysis and lipid oxidation on the content of Zn-protoporphyrin were also investigated in the *biceps femoris*. We found that the maximum formation of Zn-protoporphyrin occurred between resting and 6 months of processing, which coincides with temperature increase during processing and the end of salt equalization. Zn-protoporphyrin further increased in the *biceps femoris* until 9 months of processing but remained unchanged in the *semimembranosus*. Free fatty acid content increased till 6 months and then remained unchanged until the end of the process. These findings and those from an *in vitro* study reinforced the idea that the release of free fatty acids can promote the activity of the endogenous enzyme ferrochelatase and contribute to the formation of Zn-protoporphyrin from heme. However, the content of Zn-protoporphyrin decreased at the end of the processing, which may be due to the progression of lipid oxidation.

Keywords: porphyrin content, nitrite, heme, color, clean label, fatty acids

Highlights:

- The formation of Zinc protoporphyrin (ZnPP) in hams is coincident with salt equalization and lipolysis
- ZnPP content increases until 9 months of processing causing a decrease in heme content
- The presence of fatty acids can enhance Zn-chelatase activity
- Oxidation could be responsible for the decrease in heme and ZnPP at the end of the process

1. INTRODUCTION

The production of Serrano dry-cured ham traditionally involves the addition of nitrifying agents (nitrate and/or nitrite), whereas the omission of nitrifying agents is mandatory in the production of Italian PDO dry-cured Parma ham (EU Regulation, 2007). However, the growing demand for clean label products has led to the popularization of Serrano dry-cured ham in Spain without added nitrate and/or nitrite (Bou, Llauger, Arnau, Olmos, & Fulladosa, 2020). In addition to satisfying consumer demands, this omission may bring health benefits by preventing the potential formation of carcinogenic nitrosamines in nitrified meat products (Flores & Toldra, 2021).

Nitrosylmyoglobin, formed by the reaction of nitric oxide with muscle myoglobin, is responsible for the characteristic red color of hams produced with the addition of nitrifying agents (Pegg & Shahidi, 1997). Zn-protoporphyrin IX (ZnPP) is responsible for the characteristic red and stable chromophore of hams produced without nitrifying agents in which the porphyrin moiety complexes with Zn(II) instead of Fe(II) (Wakamatsu, Nishimura, & Hattori, 2004). Therefore, the latter and residual heme are the main pigments responsible for the color of non-nitrified dry-cured hams (Bou et al., 2020). Regardless of the addition or omission of nitrifying agents, the production of any type of dry-cured ham involves salting, resting, drying, and ripening steps. However, the elaboration procedure and raw materials for Italian Parma ham differ from those used during the production of Serrano ham. In general, Parma hams are made of legs from heavier pigs than those used in Serrano hams. In Serrano ham production, the legs are exposed to a faster salting period and, drying and maturing is carried out at higher temperatures than those used during Parma ham production. These differences can also influence the formation of ZnPP, as they can affect reactions and enzyme activities that contribute to the sensory characteristics of the final product (Toldrá, Flores, & Sanz, 1997). In this regard, several studies have suggested that the activity of the endogenous enzyme ferrochelatase plays a crucial role in the Fe-Zn transmetalation

of the porphyrin ring (Benedini, Raja, & Parolari, 2008; Khozroughi, Kroh, Schluter, & Rawel, 2018; Wakamatsu, Okui, Ikeda, Nishimura, & Hattori, 2004) and thus, may affect the final color of dry-cured hams.

The formation of ZnPP via ferrochelatase seems to occur throughout the ham production process despite the slight decrease in the activity of Zn-chelatase (catalyzes the incorporation of Zn into protoporphyrin IX) throughout the processing of Parma ham (Parolari, Aguzzoni, & Toscani, 2016; Parolari, Benedini, & Toscani, 2009). The activity of ferrochelatase is regulated by several factors (Becker, Westermann, Hansson, & Skibsted, 2012; Benedini et al., 2008; Chau, Ishigaki, Kataoka, & Taketani, 2010). For instance, Chau et al. (2010) reported that *in vitro*, the optimal pH for iron removal from porphyrin is 5.5–6.0, whereas that for zinc-ion insertion is 7.5–8.0. It has recently been reported that the optimal pH for the two-step reaction leading to ZnPP formation in porcine skeletal muscle is 5.5 and 4.75, depending on the muscle fiber type (Wakamatsu et al., 2019). These findings are in line with the finding of previous studies in non-nitrified Serrano dry-cured hams where raw hams with a *post mortem* pH ≥ 5.9 showed lesser ZnPP formation than those with *post mortem* pH of 5.4–5.6 (Bou et al., 2020). Therefore, intrinsic factors such as *post mortem* pH seem to play an important role in the enzymatic formation of ZnPP and should not be disregarded.

Certain extrinsic factors may also be crucial in ZnPP formation and the color of the final product. In *in vitro* studies, the addition of salt has been shown to promote the activity of ferrochelatase, leading to a higher content of ZnPP (Becker et al., 2012; Benedini et al., 2008). In commercial Parma hams, the content of ZnPP was positively correlated with salt content (Bou, Llauger, Arnau, & Fulladosa, 2018). However, Serrano hams exposed to a conventional salting period (1 d/kg of raw ham) and those exposed to a reduced salting period (0.5 d/kg of raw ham) showed no effect on the final content of ZnPP (Bou et al., 2020). In another *in vitro* study, the addition of certain free fatty acids (FFAs) and phospholipids has been shown to enhance Zn-chelatase activity and lead to increased ZnPP formation (Chau et al., 2010; Taketani, 1993). During the drying-curing process of hams, muscle lipases and

phospholipases increase FFA content (Andres, Cava, Martin, Ventanas, & Ruiz, 2005; Motilva, Toldra, Nieto, & Flores, 1993), and the activity of endogenous acid lipase is promoted by elevated salt content (Andres et al., 2005; Motilva et al., 1993). This explains the observed fatty acid decrease in both neutral and polar lipid fractions during the processing of dry-cured hams. This also corroborates the observed positive correlation between the content of n-3 FFA and ZnPP in the final product (Bou et al., 2020). However, the relationship between ZnPP content and lipolysis in dry-cured hams needs to be studied further.

It is important to note that the content of fatty acids at the end of production may be not representative of the extent of lipolysis because lipid oxidation also occurs during the elaboration process of dry-cured hams (Andres et al., 2005; Martin, Cordoba, Ventanas, & Antequera, 1999). The susceptibility to lipid oxidation is influenced by several factors, including the fatty acid degree of unsaturation, FFA content, and salt content (Decker and Xu, 1998). However, the ZnPP formation may also depend on alternative non-enzymatic mechanisms, which require further investigation (Akter, Shiraishi, Kumura, Hayakawa, & Wakamatsu, 2019; Grossi, do Nascimento, Cardoso, & Skibsted, 2014; Parolari et al., 2016). Indeed, certain processing conditions may affect the formation of ZnPP and may explain the higher content of ZnPP and instrumental redness observed in Serrano ham compared to Parma ham (Bou et al., 2020; Parolari et al., 2016). Therefore, the formation of ZnPP during the elaboration process of Serrano dry-cured hams must be investigated and its relationship with other parameters and processing factors must be established to further elucidate pigment formation pathways.

Therefore, in this study, we aimed to assess the formation of ZnPP during the different stages of non-nitrified Serrano dry-cured hams processing and determine its relationship with processing factors and other physicochemical parameters. For that reason, we assessed the ZnPP content, heme content, and instrumental color in the *biceps femoris* (BF) and *semimembranosus* (SM) muscles of ham following a typical elaboration process of 15 months. As FFA may play a crucial role on the formation of ZnPP, we

also determined the relationship between ZnPP formation and lipolysis in dry-cured ham elaborated without nitrifying agents by assessing the content of ZnPP, heme, instrumental color, total fatty acids, FFA, and hexanal content in the BF muscle.

2. MATERIAL AND METHODS

2.1 Reagents and standards

Chlorohemin (heme) from porcine was purchased from Panreac Quimica SLU (Barcelona, Spain). ZnPP, protoporphyrin IX, adenosine 5'-triphosphate dipotassium salt dihydrate, tritridecanoylglycerol, pentadecanoic acid, and tridecanoic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, United States). Hexanal was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The methanol used was of HPLC grade (Merck KGaA, Darmstadt, Germany) and the other reagents used in this study were of ACS-grade.

2.2 Dry-cured ham elaboration procedure and sampling

A total of 30 raw hams (12.25 ± 0.56 kg) from carcasses of 15 Large White x Landrace pigs were selected according to the pH of the *semimembranosus* (SM) muscle at 24 h *post mortem* from a commercial slaughterhouse. The pH was measured with a penetration electrode (Crison 52–32, Crison Instruments, SA, Alella, Spain) and averaged 5.48 ± 0.07. The average weight of the hams was 12.25 kg ± 0.56 kg. All hams were salted according to traditional procedures but without nitrifying agents. In brief, the hams were manually rubbed with 10 g salt/kg of raw ham and pile salted at 3 ± 2 °C and 85 ± 5% relative humidity (RH) for 12 days. After salting, hams were washed with cold water and post-salted (resting period) at 3 ± 2 °C and 85 ± 5% RH for 57 days (of a total of 69 days). Temperature was then increased to 8 °C and 80 ± 5% RH for 2 months and further at 16 ± 2 °C and 70 ± 2% RH until 9 months of processing. The hams were kept at 25 °C until the end of processing (15 months). Weight loss (%) was monitored throughout the elaboration period (6.5 ± 1.1, 17.8 ± 1.7, 23.8 ± 2.6, 26.7 ± 2.8, 30.0 ± 3.4,

32.4 ± 3.4, 33.7 ± 1.3, 36.3 ±1.4, 37.9 ± 1.4, after salting, resting, at 3 months, 4 months, 6 months, 8 months, 11 months, 13 months, and 15 months of processing, respectively).

The experimental design considered the distribution of the rear leg pairs during the elaboration process of non-nitrified Serrano hams to balance the effect of the animal (Supplementary Table). Six sampling periods were selected corresponding to the following processing stages: raw material stage (day 0), after salting (at day 12), after resting (at day 69 of processing), and after drying until reaching 6 months, 9 months, and 15 months of processing. At each sampling period, the BF and SM muscles were excised and sampled to evaluate the instrumental color and pH. Next, the samples were homogenized, and some aliquots were frozen at -80 °C for porphyrin content analysis, and the remaining sample was stored at -20 °C for other analyses.

2.3 In vitro ZnPP formation model system

Fresh meat homogenates (10 g/100 g of the BF muscle from Large White x Landrace pigs in MilliQ water) were prepared as previously described (Wakamatsu et al., 2019), with minor modifications. Pork meat homogenates were adjusted to pH 5.5 using diluted hydrochloric acid. To prevent microbial growth, 70 µg/mL penicillin G potassium, 250 µg/mL streptomycin sulfate, and 50 µg/mL gentamicin sulfate were added to the homogenate (Wakamatsu, Okui, et al., 2004). To this, sodium linoleate (75 and 300 mg / 100 g BF muscle) in the presence and absence of NaCl (4 g/100 g homogenate) was added. The experiment was performed in duplicates, and the solutions were incubated anaerobically at 25 °C for 4, 8, 12, and 18 days in darkness.

2.4 Physicochemical determinations

Water content (moisture) in the BF and SM samples was determined by drying at 103 ± 2 °C until a constant weight was reached. The chloride content was determined according to ISO 1841-2 using a potentiometric titrator 785 DMP Titrino (Metrohm AG, Herisau, Switzerland) and expressed as the NaCl content. The NaCl content was calculated on a fresh weight (FW) basis (Salt = g NaCl/100 g

sample) and dry matter (DM) basis (NaCl DM = g NaCl/(100 g sample - g H_2O)). The pH was determined using an S40 SevenMulti (Mettler-Toledo S.A.E., Barcelona, Spain) and an Inlab Solids Pro (Mettler-Toledo S.A.E.) probe. All analyses were performed in triplicates.

2.5 Instrumental color determinations

The color on the surface of 2 cm thick slices of the BF muscles was measured in quadruplicates. The Minolta Chroma Meter CR-400 colorimeter was used to measure color in regard to the CIELAB color space in terms of lightness (*L**), redness (*a**), and yellowness (*b**). The illuminant D65 and 10° standard observer was used.

2.6 Determination of Zn-protoporphyrin

ZnPP was quantitatively extracted (recovery > 96%) under subdued light conditions using ethyl acetate/acetic acid/dimethyl sulfoxide solvent mixture (10:2:1, v/v/v), in quadruplicates as described before (Bou et al., 2020). In brief, 1.5 grams of ground ham samples were weighed and homogenized using an UltraTurrax T25 model disperser (IKA Werke GmbH & Co. KG, Staufen, Germany) for 1 min at 9000 rpm with 10 mL of the solvent mixture. After extraction on ice for 20 min, the solution was centrifuged (1100 *g*, 15 min, 4 °C) and the supernatant obtained was filtered and collected in a volumetric flask. Aliquots were transferred to 96-microwell plates and fluorescence was measured using the Thermo Fisher Scientific Varioskan microplate reader (Waltham, Massachusetts, USA) with excitation at 416 nm and emission at 588 nm.

A stock ZnPP standard was prepared by dissolving 20 mg of ZnPP in 20 mL of dimethyl sulfoxide and then diluted with an ethyl acetate/acetic acid solution. Coefficients of determination (R^2) higher than 0.99 were obtained from linear regression analysis carried out using a calibration curve ranging from 0.01 to 8 mg/L prepared using ZnPP standard solutions prepared in the same solvent mixture. The excitation and emission spectra of the standards and samples were compared. The dry matter (DM) (ZnPP content DM = mg ZnPP /(kg sample - kg water)) and desalted DM (ZnPP content DM = mg

ZnPP/(kg sample - kg water - kg NaCl)) was calculated for each sample. Unless otherwise specified, the results are expressed based on the DM content. In the model system, the results were expressed as relative fluorescence units (RFU)/g of muscle.

2.7 Determination of heme content

Total heme pigments were determined in triplicates after extraction of heme under subdued light conditions using 90% (v/v) aqueous acetone containing HCl (0.24 M) as described before (Bou et al., 2020). Briefly, 1.5 g of ground ham samples were weighed under subdued light conditions into 50 mL capacity centrifuge tubes, and 200 µL of 0.5% (w/v) aqueous cysteine HCl solution plus 10 mL of the acidified acetone solution were added to each. The mixture was homogenized using an UltraTurrax T25 model for 1 min at 9000 rpm while the tube was immersed in ice. The sample was kept on ice for 1 h in the dark and then centrifuged for 15 min (1100 g at 4 °C). The supernatant was filtered through filter papers (grade 42) and collected in a volumetric flask, protecting it from light as much as possible. An aliquot was filtered through a 0.45 µm PTFE syringe filter and then injected (40 µL) into an Agilent 1100 series HPLC system (Waldbronn, Germany) equipped with a Luna C18 column (150 × 4.6 mm, 5 µm, 100 Å) from Phenomenex (Torrance, USA) and a UV/Vis detector set at 414 nm. Acetic acid (2%, v/v) and methanol (100%) were used as mobile phase A and B, respectively. Heme was eluted with a gradient in which phase B increased from 60% to 100% in 5 min and then maintained for 10 min at a constant flow rate of 1 mL/min. Coefficients of determination (R^2) higher than 0.99 were obtained from linear regression analysis carried out using a calibration curve ranging from 0.1 to 10 mg/L using porcine chlorohemin standard solutions prepared in acidified acetone. The total heme content was also expressed based on DM content (heme content DM = mg heme /(kg sample - kg water)).

2.8 Lipid analysis

The lipid fraction was determined in duplicates according to the Folch extraction procedure by homogenizing 1.5 g of minced muscle without subcutaneous fat in 30 mL of $CHCl_3$:methanol (2:1, v/v).

To determine the different lipid fractions, an aliquot of 30–40 mg of the total lipid extract was dissolved in 1 mL of $CHCl_3$:methanol (2:1, v/v) and loaded onto an aminopropyl column. The neutral lipids were eluted with 6 mL of $CHCl_3$:isopropanol (2:1, v/v), FFAs with 6 mL of diethyl ether:acetic acid (2%), and phospholipids with 6 mL of methanol:HCl (9:1, v/v). The fatty acid composition of the total lipid extract and the different eluted fractions were determined by gas chromatography after two-step methylation as described before (Bou, Codony, Tres, Baucells, & Guardiola, 2005). 1,2,3-Tritridecanoylglycerol was used as an internal standard for total fatty acid and neutral fatty acid quantification. Pentadecanoic and tridecanoic acids were used as internal standards for the quantification of phospholipids and FFAs, respectively. The results are expressed as mg/100 g dry matter.

2.9 Hexanal content

Hexanal determination was performed in duplicates. Samples were weighed (1 g) in 10-mL vials and placed in a CombiPAL injector autosampler (CTC Analytics, Zwingen, Switzerland) suitable for headspace solid-phase microextraction (HS–SPME) analysis. The sample vials were incubated at 40 °C for 15 min. A 75 µm carboxen/polydimethylsiloxane (CAR/PDMS) Stable Flex SPME fiber (Supelco, Bellefonte, PA) was inserted through the septum into the vial and exposed to the headspace. Vial penetration depth was set at 22 mm, and after 20 min of extraction, the SPME fiber was inserted into the injection port of the Agilent GC (Agilent, Santa Clara, CA) model 6850 coupled to a mass spectrometer model 5975C VL MSD. The injection penetration depth was set to 54 mm. The SPME fiber was desorbed at 240 °C for 10 min in the split-less mode. Chromatographic separation was performed on an Agilent J&W DB-5 MS column (30 m, i.d. 0.25 mm, film thickness 1 µm). The oven was held for 3 min at 40 °C, heated up to 200 °C at 5 °C/min, and then held for 10 min. Helium was used as the carrier gas at a flow of 0.8 mL/min. The electron impact source was 70 eV and the m/z range was 20–450. Hexanal identification was carried out by injecting a commercial standard and data were analyzed using the NIST 05 Mass Spectral Library and Mass Hunter software. An abundance of m/z 56 was used for the quantification. The results were expressed in area units (AU) × 10^{-3} g^{-1}.

2.10 Zn-chelatase activity

Zinc chelatase activity was determined in the meat samples based on the method described by (Parolari et al., 2009) with minor modifications. The homogenate (2.5 mL of 10% pork BF muscle) was mixed with 2.5 mL of ice-cold extraction buffer (pH = 8) containing tris-HCl 100 mM, glycerol 40% (w/v), KCl 1.6% (w/v), and Triton X-100 2% (w/v). This mixture was gently stirred for 30 min at 4 °C, then centrifuged at 27000 × *g* for 10 min, and the supernatant was filtered through a No. 597 Whatman filter paper and used for the enzyme assay (Zn-chelatase activity). The filtrate (300 µL) was incubated at 37 °C for 45 min in the dark with 250 µL of 400 µM $ZnSO_4$ in 360 mM Tris-HCl buffer (pH 8.0), 50 µL of 0.25 mM protoporphyrin IX in 360 mM Tris-HCl buffer (pH 8.0), and 200 µL of 25 mM adenosine 5'-triphosphate dipotassium salt dihydrate in NaCl 20% (w/v). Each filtrate was assayed against a blank obtained by adding 35 µL of 50 mM EDTA to the reaction mixture. After incubation, the enzymatic reaction was stopped by adding 35 µL of EDTA and placing the reaction tubes in an ice-cold bath for 5 sec. To this, 840 µL of ethanol 96% (v/v) was added and centrifuged at 26000 × *g* for 10 min. Aliquots of the clear supernatant obtained were transferred to 96-microwell plates and fluorescence was measured using a Varioskan microplate reader with excitation set at 420 nm and emission at 590 nm. A calibration curve for ZnPP was obtained in the range 0.2 to 12 µM by diluting a concentrated solution of ZnPP in tetrahydrofuran. The linear curve (R^2 = 0.996) was used to calculate the enzyme activity in the filtrate. Accordingly, one activity unit was expressed as the amount of enzyme per gram of fresh muscle catalyzing the formation of 1 nmol product (ZnPP) in 1 h.

2.11 Statistical analysis

A multivariate ANOVA was performed considering the different stages of the process and animals as factors. Differences between mean values were tested using Tukey's test. Pearson's correlation was calculated between ZnPP content, heme content, and physicochemical parameters. Principal component analysis (PCA) was used to examine the correlation between different variables.

Differences at $P < 0.05$ were considered statistically significant. All analyses were performed using the JMP13 statistical package (SAS Institute, Cary, North Carolina, USA).

3. RESULTS AND DISCUSSION

3.1 Physicochemical changes in the BF and SM muscles during the processing of hams

Moisture decreased and NaCl content (% FW) increased progressively throughout the elaboration of non-nitrified Serrano hams in both the BF and SM muscles (Table 1). NaCl (% DM) increased in the BF muscle throughout the process while that in the SM muscle occurred until 69 days due to the direct uptake of salt during the salting process, and probably due to migration from the more external g*racilis* muscle (Arnau, Guerrero, Casademont, & Gou, 1995), and then decreased due to migration to the inner muscles. With time, the NaCl:moisture ratio became similar in both muscles because of the tendency of NaCl to equilibrate (the NaCl:moisture ratio in the BF to that in the SM muscles was 0.34 after salting, 0.48 at the end of resting, 0.79 at 6 months, 0.94 at 9 months and 0.97 at 15 months). Changes in salt content were especially important from salting to 6 months and less important thereafter until the end of the process (Table 1). The loss of moisture was also faster in the external SM muscle, as expected. These findings in Serrano hams are in line with those of other studies (Andres et al., 2005; Arnau et al., 1995). In comparison to Parma hams, moisture content was similar to that seen in Serrano hams throughout the process, whereas salt content seemed to be slightly lower (Fantazzini, Gombia, Schembri, Simoncini, & Virgili, 2009; Parolari et al., 2016). The evolution of pH in both muscles was similar and, in general, increased with the elaboration process (Table 1), which was mainly attributed to proteolysis (Arnau et al., 1995).

The contents of ZnPP and heme were expressed on dry weight (DW) basis for better comparison during the elaboration process (Table 1). Durek et al. (2012) reported low ZnPP in fresh pork meat, mainly attributed to microbial growth. However, other authors have concluded that ZnPP formation in meat

is mainly due to the endogenous activity of ferrochelatase (Khozroughi et al., 2018). Our findings support this idea as ZnPP formation is also observed in the internal BF muscle, which has lower bacterial counts than the superficial muscles. ZnPP content tended to be higher in the BF and SM muscles during resting (69 days) than salting (12 days), however, ZnPP was significantly higher than in the initial stages only after 6 months. These findings are in line with the results reported in Parma hams (Parolari et al., 2009). However, these authors reported that in hams that had been matured for 6–12 months, the amount of ZnPP in the BF and SM muscles was approximately 4-to 6-fold and 4-to 5-fold, respectively, of the ZnPP content after the resting stage (90 days). The limited formation of ZnPP in the initial stages could be, in part, caused by the low activity of ferrochelatase at low temperatures (Benedini et al., 2008; Parolari et al., 2016).

The increase in temperature after resting may explain the marked increase and similar ZnPP content in both the SM and BF muscles after 6 months of processing (Table 1). ZnPP formation has previously been reported in Parma ham produced at 4 °C, but when produced at conventional temperatures (16 °C), ZnPP content has been reported to be higher (Parolari et al., 2016). Regardless of the processing temperature, Parolari et al. (2016) reported mean values of 32.8 and 23.8 mg ZnPP/kg dry weight in the BF and SM muscles, respectively after 6 months of processing and 19.5 and 16.5 mg ZnPP/kg dry weight in the BF and SM muscles, respectively after resting (90 days). These results suggest that the formation of ZnPP could be intense during the initial stages of the drying period in which the enzyme ferrochelatase has an elevated activity (Parolari et al., 2009). In general, it has been shown that the initial activity of the enzyme decreases after resting but is consistent thereafter until the end of the process (up to 18–20 months). In addition, Zn-chelatase activity seems to be lower in the SM muscle than in the BF muscle (Parolari et al., 2016; Parolari et al., 2009). This could be due to the presence of different types of fibers in the BF and SM and their different optimal pH for ZnPP formation (Wakamatsu et al., 2019). After 6 and 9 months of processing, ZnPP content in Serrano hams was higher than that reported in Parma ham at the same stage of processing (Parolari et al., 2016), probably

because of the different temperature profiles. In this regard, the positive effect of salt on enzyme activity should also be considered (Becker et al., 2012; Benedini et al., 2008; Parolari et al., 2016). In an *in vitro* study, Benedini et al. (2008) reported that increased salt addition to pork meat homogenates could be responsible for the higher activation of ferrochelatase. In this study, the highest level of salt addition was equivalent to a salt concentration of 8% in the hams. However, elevated concentrations of salt (≥ 50 g/L) can slow down or inhibit the formation of ZnPP (Becker et al., 2012). This explains not only the lower ZnPP content in the SM than in the BF muscle in this study but also in that reported in Parma hams (Parolari et al., 2016). ZnPP formation increased rapidly in the BF muscle until 9 months of processing, whereas in the SM muscle, this increase was less intense and not different from that after 6 months of processing. However, in Parma hams, the content of ZnPP in the BF muscle was similar at 6 and 9 months, whereas, in the SM muscle, there was an increase in the content of ZnPP (Parolari et al., 2016). Moreover, after 12 months of processing, the ZnPP content remained unchanged in both the SM and BF muscles. These results are also in line with previous studies in which non-significant increases were observed in the SM and BF muscles of purchased Parma hams aged ≥ 18 than in those aged ≤ 12 months (Parolari et al., 2009). However, we observed a marked decrease in ZnPP content in both the BF and SM muscles from 9 to 15 months of production (Table 1). The temperature should not exceed 20 °C during the elaboration of Parma hams however, in this study, the temperature was maintained at 16 °C for 9 months, and then raised to a temperature of 25 °C and maintained until the end of the process (15 months). High temperatures can negatively affect ZnPP content towards the end of processing. This feature and the effect of oxidation on the ZnPP content will be discussed in more detail in the following section.

The BF muscle had higher heme content than SM muscle, which may be because of higher myoglobin content (Lindahl, Lundström, & Tornberg, 2001) and the lower water exudation during salting in the BF muscle. In the BF and SM muscles, the heme content showed a tendency to decrease, whereas ZnPP showed a slight tendency to increase until the resting stage (Table 1). After 6 months of processing,

the heme content was lower than that initially found in both muscles. The heme content tended to decrease until the end of processing (6-15 months); however, owing to the high variability between the hams, a significant difference in heme content over time could not be established. In general, the heme content trended to be opposite to that of ZnPP and decreased progressively during the elaboration of hams (Table 1). The sum of heme and ZnPP content in the BF muscle up to 6 months of the process was in agreement with the initial heme content (121 mg/kg and 127 mg/kg, respectively). The Zn-chelatase activity of ferrochelatase was higher at the beginning of the process and appears to have been maintained from 6 to 20 months of drying (Parolari et al., 2009). Thus, it can be assumed that the formation of ZnPP mainly originated from heme, and ferrochelatase may have played a crucial role in this process.

However, the sum of porphyrin content (heme + ZnPP) after 9 months of processing in the BF muscle was slightly higher than the initial heme content (227 µmol/kg and 195 µmol/kg, respectively, which represents 116% and 179% of the initial porphyrin content, calculated on a dry matter basis and desalted dry matter basis, respectively). Likewise, the initial content of porphyrins in the SM muscle (123 µmol/kg) was constant until resting and then increased after 6 months (165 µmol/kg, representing 134% of the initial content on dry matter basis) and 9 months (180 µmol/kg, representing 156% of the initial content on dry matter basis) of processing. These findings could be, in part, attributed to the high variability of porphyrin content in the analyzed samples. However, several authors have suggested that ZnPP can be formed from sources other than heme (De Maere et al., 2016; Wakamatsu et al., 2019). Wakamatsu et al. (2019) reported that the formation of ZnPP in porcine muscles at pH 4.75 was not caused by direct Fe-Zn substitution in the porphyrin ring and thus, was independent of heme content. Moreover, it was found that the increase in ZnPP during the processing of a non-nitrified dry fermented sausage was greater than the decrease in heme (De Maere et al., 2016). Therefore, the existence of alternative mechanisms, as suggested by other authors, should be considered and investigated (De Maere et al., 2016; Grossi et al., 2014; Parolari et al., 2016). For

instance, Parolari et al. (2016) reported the non-enzymatic formation of ZnPP in dry-cured ham produced at 4°C. In addition, Akter et al. (2019) reported that different proteins may be involved in pigment formation. These alternative mechanisms could explain the higher porphyrin content up to 9 months. At the end of the process, the porphyrin content in the BF and SM muscles accounted for approximately 83% and 75% of the initial porphyrin content; both heme and ZnPP content decreased (Table 1). The decrease in ZnPP in Serrano hams could be related to the temperature increase to 25 °C from 9 months until the end of the process and oxidation. This is discussed further in the following section.

In terms of color changes, lightness decreased with processing time (Table 1). This finding is in line with other studies and is explained by the decrease in light scattering, which is in turn affected by pH, water holding capacity, salt content, and protein structure alteration (del Olmo, Calzada, & Nuñez, 2013; Hughes, Clarke, Purslow, & Warner, 2020; Pérez-Alvarez et al., 1997). In the SM muscle, there was considerable dehydration during the salting and drying stages, which may explain the decrease in lightness after the resting stage. A similar trend was observed in the BF muscle. Given that the BF is a more internal muscle (related to salt uptake and water loss), these changes may have occurred later. Even though we did not have data at 9 months of processing, it seems that lightness values remained relatively unchanged upon drying. Parolari et al. (2016), in Parma hams, reported similar lightness values after 3, 6, 9, and 12 months of processing in the BF muscle, whereas decreased lightness after an uptrend at 6–9 months, in the SM muscle. These results were attributed to the uneven moisture changes.

The BF muscle showed higher redness and yellowness values with longer processing periods (Table 1). This trend in redness and yellowness values is in close agreement with previous findings (Parolari et al., 2016). Despite this, redness values remained unchanged until 6 months in the BF muscle and only increased at 15 months of processing. In the SM muscle, redness increase occurred faster, which could be attributed to the muscle location and the mass transfer processes discussed previously. In general,

non-nitrified Serrano hams show lower lightness and higher redness than Parma hams (Bou et al., 2020; Parolari et al., 2016; Parolari et al., 2009). The increased redness of Serrano hams seems to correlate with the increased ZnPP content (Bou et al., 2020; Parolari et al., 2016; Parolari et al., 2009). Therefore, although the presence of heme compounds is not negligible, the characteristic red color of Serrano dry-cured hams attained without the addition of nitrifying agents is mainly attributed to the presence of ZnPP (Bou et al., 2018; Bou et al., 2020). Yellowness is known to be influenced by fat oxidation, internal reflectance, and the distribution of myoglobin compounds in cured products (del Olmo et al., 2013; Gou et al., 2012). Considering that porphyrin content at the end of processing was lower than the initial content, it is possible that the loss of water compensated for the loss of porphyrins and caused increased redness at the end of the process.

3.2 Hexanal content, fatty acid composition, and correlations between parameters in the BF muscle

FFAs may influence ZnPP formation (Chau et al., 2010; Taketani, 1993). However, unsaturated FFAs are prone to oxidation (Decker and Xu, 1998). This makes it difficult to interpret the relationship between lipolysis and ZnPP in certain meat products, such as in dry-cured hams (Bou et al., 2020). Therefore, lipid oxidation should also be considered when studying FFA release. Table 2 shows the hexanal content in the BF muscle. Despite being similar in the initial stages, oxidation seemed to occur rapidly and reached the maximum values after 6 months of processing. Thereafter, the hexanal content decreased progressively and reached values similar to those recorded in the initial stages. Hexanal is widely used as a lipid oxidation marker for several reasons, including its correlation with rancidity and its abundance and greater stability than unsaturated alkenes (Barriuso, Astiasarán, & Ansorena, 2013). However, hexanal and other products of lipid oxidation can interact with muscle proteins (Pignoli, Bou, Rodriguez-Estrada, & Decker, 2009). This explains the initial increase followed by decrease of lipid oxidation compounds, such as malondialdehyde, in Serrano hams (del Olmo, Calzada, & Nunez, 2016).

Besides, several aldehydes can react with myoglobin and hydroxynonenal has been shown to enhance metmyoglobin formation by binding covalently to specific histidine residues in the protein (Faustman, Sun, Mancini, & Suman, 2010). Reinforcing these findings, the maximum levels of oxidation (6–9 months) coincided with the maximum levels of ZnPP and heme, followed by a marked decrease in porphyrins and hexanal at the end of the process (Tables 1 and 2). The interaction between lipid oxidation compounds, porphyrins, and other matrix compounds may have led to the formation of tertiary oxidation products and may explain the observed hexanal decrease during processing.

Thermal and photochemical degradation of porphyrin compounds may cause color fading (Adamsen, Moller, Hismani, & Skibsted, 2004; Pegg & Shahidi, 1997). However, considering the redness observed, this does not seem to have occurred in our study (Table 1). The development of lipid oxidation has been reported to not only correlate strongly with heme oxidation but also reduce the extractability of heme proteins wherein lipid oxidation products, mainly aldehydes, can induce an association between heme compounds and myofibrillar proteins (Chaijan & Undeland, 2015). The spectral observation of the extracts used to measure ZnPP content showed the characteristic spectra of ZnPP with an increase in the peaks corresponding to the Soret band (420 nm) and Q-band regions until 9 months of processing, followed by a decrease after 15 months of processing (Supplementary Figure). The observed decrease in the spectra supports the lower extractability of porphyrin compounds and/or porphyrin ring degradation. In a previous study on Serrano hams, the sum of ZnPP and heme at the end of the process (12 months) was higher in hams with higher *post-mortem* pH than in hams with low *post-mortem* pH (Bou et al., 2020) due to increased myoglobin oxidation at low pH. Furthermore, Wakamatsu et al. (2019), in an *in vitro* study, found that myoglobin concentration was decreased at pH 4.75 but not at pH 5.5. Although the latter pH is closer to that found in hams, it is worth noting that the authors studied the degradation of myoglobin within a shorter time. Further studies are needed in this regard. Nevertheless, it is likely that oxidation processes may contribute to the loss of and/or reduce the recovery of porphyrins in hams.

Table 2 also shows the fatty acid content from the total lipid fraction of the BF muscle. The fatty acid profile obtained is characteristic of Spanish dry-cured hams with oleic acid being the most abundant, followed by palmitic acid, linoleic acid, and stearic acid (Andres et al., 2005; Jimenez-Colmenero, Ventanas, & Toldra, 2010). A decrease in saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids have reported during processing (Andres et al., 2005). However, the fatty acid content remained, in general, unchanged during processing. Only 18:3 n-3 and 20:2 n-6 were increased after 6 months and then decreased owing to storage. As the relative percentages remained unchanged (data not shown), these controversial results could be attributed to the different fat content of the individual animals used in this study.

Table 3 shows the FFA content and fatty acid compositions corresponding to the polar fraction. FFAs increased until 6 months and then remained unchanged until the end of processing. This increase could be attributed to the activity of acid lipase, which is the major lipolytic enzyme during the processing of hams (Motilva et al., 1993; Toldrá et al., 1997). Muscle acid lipase activity is strongly activated by salt (6–8%), whereas neutral and basic lipases that are more active at the initial stages are inhibited (Motilva & Toldrá, 1993; Motilva et al., 1993). Our results corroborate those of Motilva and Toldrá (1993), who reported the maximum release of fatty acids after 5 months of processing and a similar steady evolution until the end of processing (15 months). It has been suggested that even though the amount of FFAs remains fairly constant from 5 to 15 months, the content at the end of the process depends on the ripening temperature (del Olmo et al., 2016). The steady evolution of FFA content after 6 months seems to be at the expense of fatty acids from the polar fraction. We found that from 6 to 15 months, there was a tendency towards a decrease in polar fatty acids, however, this decrease was only significant for 16:1 n-7, 20:1 n-9, 20:2 n-6, and 22:6 n-3. These findings suggest that phospholipids are major contributors to FFA release and that a compensatory mechanism between lipolysis and oxidation phenomena exists, as suggested by other authors (Andres et al., 2005; Martin et al., 1999).

PCA was carried out to assess the relationships between the studied parameters (Figure 1). The correlations between the most relevant relationships from the PCA are shown in Table 4. ZnPP was plotted against heme and was mainly explained by the first component (52.1% of the variance). In addition, fatty acid content from the polar fraction was plotted against FFA and salt content, in similar coordinates. The ZnPP content was also localized close to the salt content and free 18:2 n-6, 22:6 n-3, and 20:4 n-6. However, fatty acid content corresponding to the total lipid fraction, hexanal content, and redness were determined by the second component (18.4% of the variance). These relationships help explain changes that typically occur during the processing of dry-cured hams. In this regard, temperature and diffusion of salt may have contributed to the activation of lipases, which explains the relationship between FFAs and salt content (e.g., 20:4 n-6; r = 0.909, $P < 0.001$). The addition of salt has also been reported to activate ferrochelatase (Benedini et al., 2008). This could explain the strong correlation between ZnPP and salt in the BF muscle (r = 0.855, $P < 0.0001$). Salt uptake was faster in the SM muscle, and until 6 months, we did not observe major differences in ZnPP content when compared to the BF muscle. This explains the weak correlation between ZnPP and salt content in the SM muscle (r = 0.515, $P = 0.0036$) and reinforces the idea that the formation of ZnPP may be influenced more by temperature. Moreover, when dry-cured hams were exposed to different salting times to obtain different final salt contents (BF muscle 5.7% vs. 4.0% fresh weight basis), salt and ZnPP content at the end of the process were negatively correlated (Bou et al., 2020). The correlation between salt and hexanal (r = 0.386, $P < 0.035$) and that between hexanal and lipolysis (sum of FFA) (r = 0.598, $P = 0.001$) seem to be of lesser importance. In previous studies, positive correlations between ZnPP and various fatty acids at the end of the process, in particular, stearic acid, linoleic acid, and arachidonic acid, have been reported (Bou et al., 2020). These findings are in line with our findings that suggest a high correlation between ZnPP and 18:2 n-6 (r = 0.885, $P < 0.001$), 20:4 n-6 (r = 0.893, $P < 0.001$), and the sum of FFA (r = 0.886, $P < 0.001$), suggesting the role of lipolysis in the formation of ZnPP. This is also supported by the reported formation of ZnPP in the presence of fatty acids and phospholipids *in*

vitro (Chau et al., 2010; Taketani, 1993). In this study, the FFA content remained almost unchanged until the end, but a compensatory mechanism between lipolysis and oxidation may explain the slight decrease in certain fatty acids from the polar fraction.

3.3 Effect of linoleic acid and salt on ZnPP formation in the BF muscle model system

Overall, the high correlation between FFAs and ZnPP content reinforces the idea that in the production of dry-cured hams without nitrites, lipolysis may play a crucial role in the formation of pigment. To understand the potential mechanisms leading to this, the effect of linoleic acid and salt addition on Zn-chelatase activity and formation of ZnPP, was assessed in a model system. Enzymatic activity was measured in 10% BF muscle homogenates after preparation. The initial Zn-chelatase activity in the BF muscle was 79 ± 1.1 nmol g^{-1} muscle h^{-1} while with the addition of linoleic acid (75 mg/100 g muscle), the activity was 107 ± 8.9 nmol g^{-1} muscle h^{-1} and 94 ± 5.0 nmol g^{-1} muscle h^{-1} in the absence and presence of added salt, respectively. When linoleic acid was added at a higher amount (300 mg/100 g muscle), Zn-chelatase activity was below the detection limit. These results agree with the formation of ZnPP in the homogenates (Figure 2). The addition of linoleic acid at a lower level enhanced the formation of ZnPP, regardless of the amount of salt, however, the promoting effect of linoleic acid was not observed when added at a higher concentration. This experiment also showed that the addition of salt at a fresh weight concentration similar to that found in hams may reduce the formation of ZnPP with the equivalent addition of linoleic acid. In a similar experiment, Becker et al. (2012) reported a possible “salting in” effect wherein salt addition at 3% facilitated the reaction between heme proteins and ferrochelatase. In contrast, a “salting out” effect was reported when salt was added at 5%, causing the inhibition of ferrochelatase. Therefore, it is likely that the boundary level for the inhibition of Zn-chelatase activity is close to the studied salt level of addition (4%). Accordingly, further studies should also consider muscle salt uptake and final salt content and their effects on ZnPP formation.

In this study, Zn-chelatase activity was not determined during Serrano ham processing, but it has been previously shown to decrease gradually during the elaboration of dry-cured hams (Parolari et al., 2016; Parolari et al., 2009). At the initial stages, salt may play an important role in the activation of lipases (Motilva et al., 1993) and the onset of lipolysis may, in turn, enhance the formation of ZnPP. However, at the end of the process, high salt and FFA contents could be detrimental to ZnPP formation and promote oxidation reactions.

4. CONCLUSIONS

During the production of non-nitrified Serrano hams, the formation of ZnPP after resting increased significantly and simultaneously in the BF and SM muscles. This increase could be attributed to the increased temperatures and salt uptake, which promoted the conversion of heme to ZnPP. The presence of FFAs, which increased up to 6 months of processing further promoted ZnPP formation. These findings and those from *in vitro* studies suggest that lipolytic processes may play an important role in the enzymatic formation of ZnPP. However, the degree of lipolysis and other factors, such as salt content, can also play a role in determining ZnPP content. Oxidation is favored in the presence of FFAs as well as salt and explains the decreased content of ZnPP and heme after 15 months of processing. Thus, controlling the temperature and salt uptake during the elaboration of dry-cured hams may be crucial for optimal color development. Further studies are needed to confirm these findings and evaluate the formation of ZnPP under determined endogenous (e.g., pH) and exogenous (e.g., salt, temperature) conditions that typically occur during the industrial process, along with the examination of the stability of the different porphyrins during the elaboration and storage of hams.

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Table 1. Changes in the physicochemical parameters, ZnPP and heme in BF and SM muscles during the different stages of the elaboration of nitrite-free Serrano hams[1]

Muscle	Stage (time)	Moisture (%)	NaCl (% FW)	NaCl (% DM)	pH	ZnPP (mg/kg)	Heme (mg/kg)	L	a	b
BF	Raw ham	74.9 ± 1.0a	0.2 ± 0.0d	0.8 ± 0.1c	5.6 ± 0.21b	3 ± 1c	124 ± 22a	48.1 ± 3.1a	4.6 ± 1.4b	5.7 ± 2.0b
	Salting (12 days)	72.7 ± 0.6b	0.9 ± 0.4cd	3.3 ± 1.5bc	5.7 ± 0.05ab	5 ± 3c	111 ± 34a	48.3 ± 3.4a	6.1 ± 2.3b	5.9 ± 0.5b
	Resting (69 days)	71.6 ± 0.5b	1.8 ± 0.4c	6.2 ± 1.5b	5.9 ± 0.13a	10 ± 8c	93 ±18ab	46.1 ± 2.6ab	6.4 ± 1.3b	5.6 ± 1.4b
	Drying (6 months)	67.6 ± 1.2c	3.4 ± 0.9b	10.5 ± 2.3a	5.7 ± 0.04ab	60 ± 15b	61 ± 13bc	44.2 ± 3.5ab	6.2 ± 2.0b	4.1 ± 2.2a
	Drying (9 months)	63.9 ± 1.1d	4.0 ± 0.5ab	11.1 ± 1.1a	5.9 ± 0.02a	96 ± 23c	48 ± 14c	-	-	-
	Drying (15 months)	59.0 ± 1.4e	4.7 ± 0.4a	11.5 ± 0.8a	5.9 ± 0.07a	67 ± 16b	39 ± 10c	42.5 ± 0.8b	12.0 ± 0.9a	6.2 ± 0.5a
SM	Raw ham	74.2 ± 0.9a	0.2 ± 0.0c	0.9 ± 0.1c	5.5 ± 0.12d	2 ± 1c	78 ± 16a	51.9 ± 1.6a	2.8 ± 0.9c	6.2 ± 1.5ab
	Salting (12 days)	71.1 ± 4.5a	2.6 ± 1.2b	8.7 ± 2.9ab	5.6 ± 0.11c	4 ± 1c	71 ± 23ab	51.5 ± 3.3a	4.3 ± 2.7c	6.8 ± 1.0a
	Resting (69 days)	65.4 ± 1.6b	3.4 ± 0.2ab	10.0 ± 0.5a	5.8 ± 0.11b	11 ± 4bc	65 ± 15ab	44.5 ± 4.3b	5.4 ± 1.5bc	4.6 ± 1.7abc
	Drying (6 months)	56.2 ± 3.8c	3.6 ± 0.9a	8.1 ± 1.6ab	5.7 ± 0.10b	63 ± 14a	42 ± 12bc	40.3 ± 3.9b	7.5 ± 0.7b	1.6 ± 2.5c

Drying (9 months)	54.2 ± 1.4c	3.6 ± 0.3a	7.9 ± 0.6ab	5.8 ± 0.05ab	79 ± 22a	35 ± 10c	-	-	
Drying (15 months)	47.5 ± 2.6d	3.9 ± 0.3a	7.5 ± 0.2b	5.9 ± 0.03a	31 ± 8b	29 ± 14c	38.4 ± 2.2b	10.7 ± 0.8a	3.6 ± 0.7bc

[1] Results expressed as means ± standard deviation (n = 5). Different letters in the same column (a–e) indicate significant differences within a muscle ($p \leq 0.05$). The content in NaCl (% FW) and NaCl (% DW) are expressed on a fresh and dry weight basis, respectively. Zn-protoporphyrin (ZnPP) and heme are expressed on a dry weight basis.

Table 2. Hexanal (10^3 AU/g) and fatty acid content (mg /100 g dry matter) corresponding to total lipid fraction in BF muscle during the elaboration process of nitrite-free Serrano hams[1]

	Raw ham	Salting (12 days)	Resting (69 days)	6 months	9 months	15 months
Hexanal	7 ± 3a	235 ± 185a	550 ± 283a	3112 ± 855c	1631 ± 854b	117 ± 15a
14:0	103 ± 54a	119 ± 50a	105 ± 42a	161 ± 62a	124 ± 52a	127 ± 26a
16:0	2443 ± 842a	2723 ± 709a	2527 ± 614a	3398 ± 1063a	2689 ± 754a	2721 ± 327a
16:1 n-7	265 ± 137a	327 ± 129a	290 ± 131a	379 ± 161a	295 ± 135a	341 ± 56a
18:0	1380 ± 383a	1444 ± 270a	1389 ± 247a	1879 ± 516a	1546 ± 304a	1434 ± 171a
18:1 n-9	3974 ± 1546a	4771 ± 1556a	4452 ± 1257a	5634 ± 2068a	4431 ± 1460a	4811 ± 684a
18:1 n-7	424 ± 171a	524 ± 282a	485 ± 165a	565 ± 214a	456 ± 151a	505 ± 97a
18:2 n-6	1562 ± 425a	1682 ± 15a	1595 ± 108a	2104 ± 312a	1676 ± 130a	1603 ± 276a
18:3 n-3	24 ± 14a	37 ± 15ab	34 ± 8ab	59 ± 25b	42 ± 8ab	38 ± 8ab
20:1 n-9	48 ± 24a	73 ± 25a	68 ± 28a	96 ± 49a	71 ± 29a	73 ± 23a
20:2 n-6	23 ± 12a	40 ± 9ab	37 ± 8ab	58 ± 22b	42 ± 9ab	41 ± 9ab
20:3 n-6	25 ± 12a	36 ± 8a	36 ± 4a	38 ± 8a	38 ± 3a	38 ± 7a
20:4 n-6	374 ± 116a	416 ± 44a	409 ± 41a	406 ± 52a	396 ± 42a	399 ± 37a
22:6 n-3	21 ± 12a	33 ± 5a	31 ± 8a	31 ± 5a	32 ± 5a	31 ± 5a
Total	10668 ± 3541a	12226 ± 3011a	11460 ± 2483a	14808 ± 4414a	11836 ± 2920a	12162 ± 1409a

[1] Results expressed as means ± standard deviation (n = 5). Different letters (a–b) indicate significant differences between different dry-cured ham stages of the elaboration process ($p \leq 0.05$).

Table 3. Fatty acid content (mg /100 g dry matter) corresponding to the free fatty acid and polar fractions of BF muscle during the elaboration process of nitrite-free Serrano hams[1]

	Free fatty acids fraction						Polar fraction					
	Raw ham	**Salting (12 days)**	**Resting (69 days)**	**6 months**	**9 months**	**15 months**	**Raw ham**	**Salting (12 days)**	**Resting (69 days)**	**6 months**	**9 months**	**15 months**
14:0	3 ± 0a	3 ± 0a	3 ± 0a	5 ± 1b	4 ± 1ab	4 ± 0ab	17 ± 1a	15 ± 1a	14 ± 0a	18 ± 13a	11 ± 0a	10 ± 0a
16:0	66 ± 10a	60 ± 8a	77 ± 7a	122 ± 13b	117 ± 15b	117 ± 5b	161 ± 43b	161 ± 45b	117 ± 34ab	63 ± 48ab	45 ± 17a	30 ± 11a
16:1 n-7	8 ± 1a	6 ± 1a	6 ± 2a	9 ± 3a	8 ± 3a	9 ± 1a	19 ± 1d	18 ± 2cd	17 ± 1c	12 ± 0b	11 ± 0ab	10 ± 0a
18:0	46 ± 8ab	33 ± 3a	47 ± 6ab	92 ± 9c	87 ± 10c	85 ± 3c	147 ± 37b	160 ± 46b	119 ± 34ab	58 ± 32ab	37 ± 14a	26 ± 9a
18:1 n-9	71 ± 14ab	55 ± 17a	61 ± 9a	113 ± 28c	106 ± 31bc	110 ± 7c	107 ± 32c	112 ± 32c	85 ± 24bc	43 ± 23ab	27 ± 7a	17 ± 4a
18:1 n-7	10 ± 1a	9 ± 2a	11 ± 2a	22 ± 5b	21 ± 4b	22 ± 3b	42 ± 8c	43 ± 10c	36 ± 8bc	23 ± 9ab	17 ± 3a	13 ± 2a
18:2 n-6	68 ± 8a	64 ± 15a	88 ± 19a	163 ± 21b	157 ± 17b	162 ± 16b	245 ± 53c	251 ± 83c	184 ± 54bc	88 ± 48ab	54 ± 17a	30 ± 8a
18:3 n-3	4 ± 1ab	3 ± 1a	4 ± 0ab	4 ± 1ab	4 ± 0av	4 ± 0ab	17 ± 1b	18 ± 5b	14 ± 0ab	12 ± 0a	11 ± 0a	10 ± 0a

20:1 n-9	3 ± 1ab	2 ± 1a	2 ± 1a	4 ± 1c	4 ± 1bc	3 ± 0bc	16 ± 2d	14 ± 0c	14 ± 0c	12 ± 0b	11 ± 0ab	10 ± 0a
20:2 n-6	2 ± 1a	2 ± 1a	2 ± 1a	5 ± 0b	4 ± 1b	4 ± 0b	20 ± 2dc	20 ± 2dc	18 ± 1c	12 ± 0b	11 ± 0ab	10 ± 0a
20:3 n-6	4 ± 0a	4 ± 0a	5 ± 1a	8 ± 1a	18 ± 22a	8 ± 1a	26 ± 3bc	26 ± 3bc	22 ± 2b	13 ± 2a	11 ± 0a	10 ± 0a
20:4 n-6	22 ± 3a	19 ± 3a	28 ± 5a	60 ± 9b	59 ± 7b	64 ± 7b	107 ± 21b	128 ± 43b	91 ± 24b	39 ± 12a	28 ± 5a	21 ±5a
22:6 n-3	5 ± 1a	5 ± 1a	5 ± 0a	7 ± 1b	7 ± 1b	7 ± 1b	24 ± 2c	25 ± 4c	21 ± 3c	14 ± 2b	11 ± 1ab	10 ± 0a
Total	308 ± 36a	263 ± 45a	336 ± 35a	608 ± 69b	592 ± 75b	595 ± 28b	948 ± 199b	990 ± 258b	752 ± 175b	407 ± 163a	285 ± 61a	204 ± 40a

[1] Results expressed as means ± standard deviation (n = 5). Different letters (a–d) indicate significant differences between different dry-cured ham stages of the elaboration process ($p \leq 0.05$).

Table 4. Pearson's coefficients of physicochemical variables and free fatty acids content in the BF muscle during the elaboration of nitrite-free Serrano hams.

	ZnPP	Heme	pH	NaCl	Moisture	a*	Hexanal	16:0	18:0	18:1 n-9	18:2 n-6	20:4 n-6	22:6 n-3	Sum FFA
ZnPP	1.000	-0.734	0.441	0.855	-0.800	-0.339	0.519	0.878	0.893	0.722	0.885	0.893	0.848	**0.886**
	<.0001	<.0001	0.015	<.0001	<.0001	0.067	0.003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Heme		1.000	-0.462	-0.844	0.829	0.197	-0.342	-0.778	-0.765	-0.558	-0.765	-0.812	-0.776	-0.766
		<.0001	0.010	<.0001	<.0001	0.297	0.065	<.0001	<.0001	0.001	<.0001	<.0001	<.0001	<.0001
pH			1.000	0.536	-0.560	0.011	0.037	0.351	0.305	0.260	0.372	0.422	0.331	0.357
			<.0001	0.002	0.001	0.954	0.845	0.057	0.102	0.166	0.043	0.020	0.074	0.053
NaCl%				1.000	-0.945	-0.027	0.386	0.855	0.862	0.673	0.900	0.909	**0.820**	**0.876**
				<.0001	<.0001	0.887	0.035	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Moisture					1.000	-0.046	-0.190	-0.781	-0.776	-0.636	-0.822	-0.859	-0.769	-0.803
					<.0001	0.810	0.315	<.0001	<.0001	0.000	<.0001	<.0001	<.0001	<.0001
a*						1.000	-0.251	-0.111	-0.146	-0.054	-0.084	-0.070	-0.189	-0.117
						<.0001	0.180	0.559	0.440	0.777	0.659	0.714	0.319	0.539
Hexanal							1.000	0.621	0.619	0.544	0.579	0.541	0.505	0.598
							<.0001	0.000	0.000	0.002	0.001	0.002	0.005	0.001
16:0								1.000	0.974	0.854	0.976	0.958	0.884	0.986
								<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
18:0									1.000	0.832	0.967	0.958	0.901	0.980
									<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
18:1 n-9										1.000	0.793	0.797	0.767	0.894
										<.0001	<.0001	<.0001	<.0001	<.0001
18:2 n-6											1.000	0.970	0.880	0.975
											<.0001	<.0001	<.0001	<.0001
20:4 n-6												1.000	0.938	0.967
												<.0001	<.0001	<.0001
22:6 n-3													1.000	0.901
													<.0001	<.0001
Sum FFA														1.000
														<.0001

3 Values correspond to correlations and p-values below. See Table 1 and Figure 1 for abbreviations and units. ZnPP
4 and heme contents are expressed on dry matter basis whereas NaCl content on fresh weight basis.
5

Supplementary Table. Sampling design: distribution of ham pairs[1] (p1-p15) during the elaboration process of nitrite-free Serrano hams

Sampling stage	Ham pairs
Raw material	p1, p2, p3, p4, p5
After salting (12 days)	p1, p6, p7, p8, p9
After resting (69 days)	p2, p6, p10, p11, p12
6 months of processing	p3, p7, p10, p13, p14
9 months of processing	p4, p8, p11, p13, p15
15 months of processing	p5, p9, p12, p14, p15

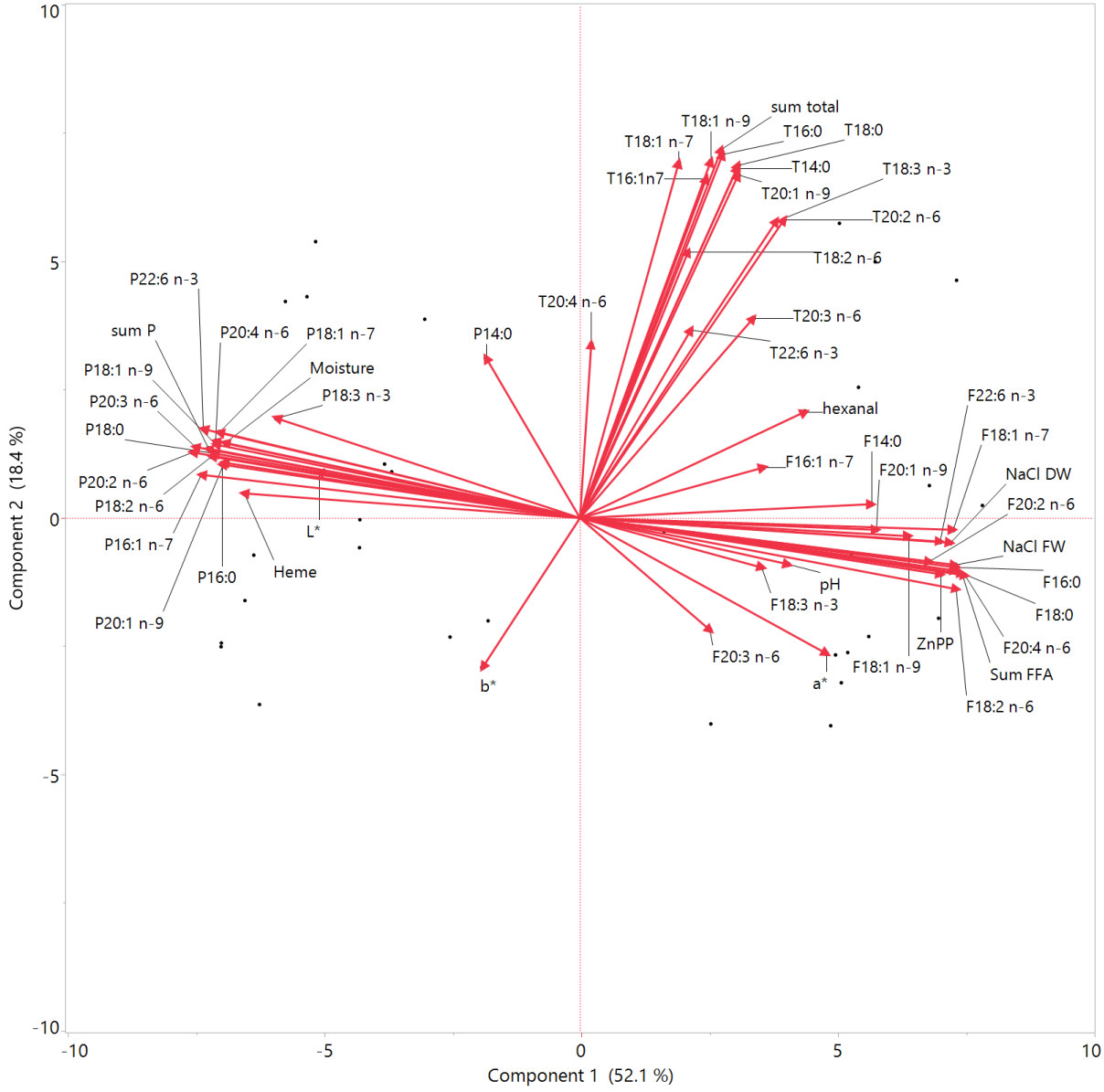
[1]Thirty legs from 15 animals were elaborated in pairs (p) and strategically sampled throughout the different processing stages to enable paired statistical comparisons.

Figure Captions:

Figure 1. Principal components analysis biplot of non-nitrified Serrano dry-cured hams. ZnPP, Zn-protoporphyrin; NaCl FW, percentage of NaCl on fresh weight basis; NaCl DW, percentage of NaCl on dry weight basis. Fatty acids preceded with F, P, and T correspond to the free fatty acid fraction, polar fraction, and total lipid extract, respectively.

Figure 2. Effect of the addition of linoleic acid (LA) at two levels (75 mg/100 g *biceps femoris* (BF) muscle and 300 mg/100 g BF muscle) with and without the presence of NaCl (4 g/100 g homogenate) on *in vitro* Zn-protoporphyrin (ZnPP) formation in 10% pork BF muscle homogenates. Homogenates were adjusted to pH 5.5 and incubated anaerobically in the dark for 18 days at 25 °C.

Supplementary Figure. Comparison of the spectra of the extracts (ethyl acetate/acetic acid/dimethyl sulfoxide solvent mixture; 10:2:1, v/v/v) used to measure Zn-protoporphyrin from the *biceps femoris* muscles during dry-cured hams processing.



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28 Figure 1

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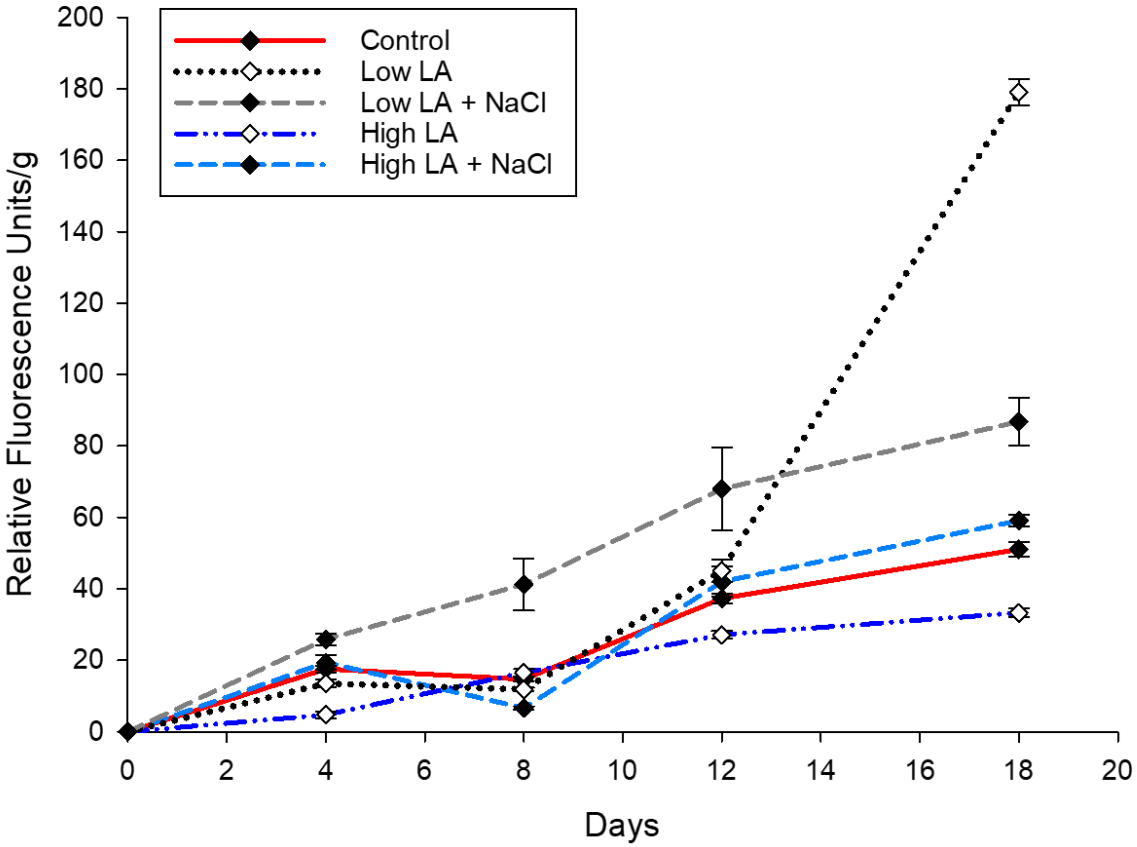
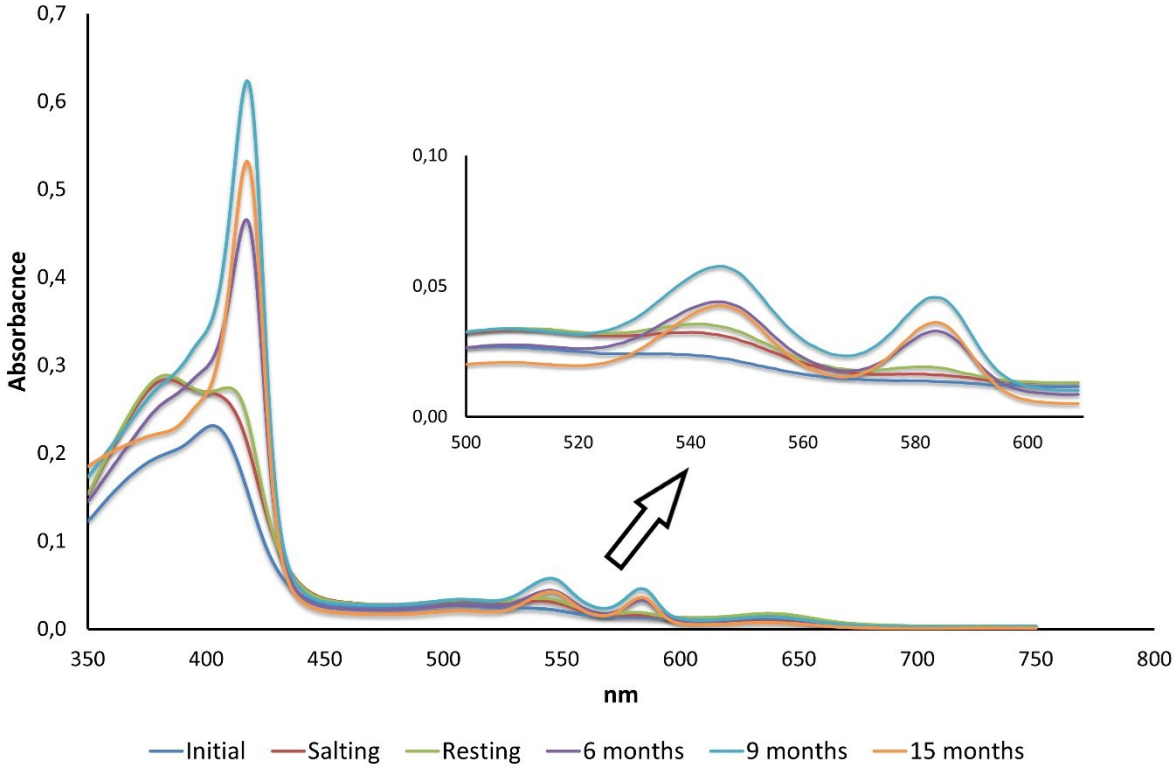

Control
Low LA
Low LA + NaCl
High LA
High LA + NaCl
Relative Fluorescence Units/g
Days
0
20
40
60
80
100
120
140
160
180
200
0
2
4
6
8
10
12
14
16
18
20

Figure 2



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33 Suppl. Figure