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Metabolomic profiling of Iberian dry-cured ham: Preliminary approach to discriminate between hams from different commercial categories

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ABSTRACT

For more than 25 years, profuse research has aimed to discriminate Iberian-dry-cured hams produced from pigs differing in genetics (pure Iberian vs. crosses) and feeding background (natural resources vs. commercial feeds). Certain advanced MS-based analytical tools have been found useful to characterise and authenticate a variety of meat products. Here, for the first time, the metabolome (more than 3000 identified compounds) of Iberian dry-cured hams from the three most valuable categories, labeled as BLACK, RED and GREEN, is analysed using a Q-Exactive Orbitrap mass-spectrometry (MS) equipment. The chemical structure, plausible origin and role played by the 35 most abundant chemical species in Iberian dry-cured hams, are reported. Additionally, Iberian hams differing in genetic background namely, BLACK (100 % Iberian) and RED (50 % Iberian) were found to differ in 142 discriminating metabolites. Sixty-six distinctive metabolites were found in RED hams, produced from pigs fed on natural resources, while seventy discriminating metabolites were identified in GREEN hams, produced from pigs fed on concentrate. The method applied provided a preliminary metabolic fingerprinting of Iberian dry-cured hams, which may be helpful for authentication purposes.

1. Introduction

Iberian dry-cured ham is a highly appreciated meat product, which is commonly sold in delicatessen markets worldwide (Villanueva, Salazar-Ordoñez, Granado-Díaz, & Rodríguez-Entrena, 2021). The quality of meat and processed meats from rustic Iberian pigs highly depends on the genetic and feeding backgrounds of the animals (Estévez, Ventanas, Morcuende, & Ventanas, 2014). The genetic background of pigs is responsible for varying percentages of intramuscular fat (IMF), with this parameter having a significant impact on appreciated sensory properties of the final product such as marbling and juiciness (Fuentes, Ventanas, Ventanas, & Estévez, 2014). A high IMF content (around 12 %) is a typical feature in hams from Iberian pigs as compared to the more lean "*Serrano*" hams produced from industrial genotypes such as Large-White or Landrace (Lorido, Estévez, Ventanas, & Ventanas, 2015). Products from pure Iberian breed ("100 % IBÉRICO") are the most expensive owing to their recognition as the most appreciated in terms of quality. Introduction of Duroc genes as parental line (up to 50 %) is allowed by the Quality Regulation (BOE, 2014), but products from these cross-bred pigs are typically regarded as of lower quality ("50/75 % IBÉRICO"). The traditional feeding on acorns, on the other hand, has a crucial effect in terms of fat content, fatty acid composition and tocopherols content (González-Domínguez, Sayago, & Fernández-Recamales, 2020). The current regulation considers three feeding/rearing regimes. In the most traditional rearing system ("BELLOTA"), pigs are fed exclusively on acorns, grass and other natural resources while grazing the land in a free-range regime (Rodríguez-Estévez, García, & Gómez, 2009). A second option involves free-range reared pigs mainly fed on cereal-based mixed diets while they may have access to natural resources such as pasture ("CAMPO"). The third option implies an intensive rearing system in which pigs are exclusively fed on cereal- and legumes-based mixed diets ("CEBO").

Taken the above into account, the commercial categories established for Iberian dry-cured hams are as follows in order of decreasing quality and price: "BELLOTA 100% IBÉRICO (BLACK Label); "BELLOTA IBÉRICO" (RED Label); "CEBO DE CAMPO IBÉRICO" (GREEN Label);

Abbreviations: AGC, Automatic Gain Control; FC, Fold Change; FS, Full-Scan; GC-FID, Gas Chromatography – Flame Ionization Detector; GC-IMS, Gas Chromatography – Ion Mobility Spectrometry; GC–MS, Gas Chromatography – Mass Spectrometry; HPLC-MS, High Pressure Liquid Chromatography – Mass Spectrometry; IMF, Intramuscular Fat; MS, Mass Spectrometry; NIRs, Near Infrared Spectroscopy; NMR, Nuclear Magnetic Resonance; PCA, Principal Component Analysis; PLS-DA, Partial Least Square – Discriminant Analysis; QC, Quality Control; SNP, Single Nucleotide Polymorphisms.

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and "CEBO IBÉRICO" (WHITE Label). It is worth noting that the last three categories refer to products from 50 % or 75 % cross-bred Iberian pigs and that the main difference between BLACK and RED labels is the genetic background. On the other hand, the difference between the RED and GREEN categories are the feeding background since both belong to the same genetic background (whether 50 or 75 % Iberian).

Authentication of the commercial categories in Iberian dry-cured hams has been a challenge for more than thirty years. Substantial scientific efforts have been made in order to find reliable biomarkers of genetic purity and feeding backgrounds, which would, in turn, be able to discriminate between BLACK and RED hams (genetics) and between RED and GREEN (feeding), respectively. Some recent approaches have been made using GC–MS (Garrido-Fernández & León-Camacho, 2024; Sánchez, Antequera, Pajuelo, & Perez-Palacios, 2024), GC-IMS (Rodríguez-Hernández et al., 2023; Zhu, Zhu, & Sun, 2023), GC-FID (González-Domínguez et al., 2020), and by the application of some non-destructive technologies such as NMR (J. Zhang et al., 2018; Zhou et al., 2021) or portable NIRs (Hernández-Jiménez, Revilla, Hernández-Ramos, & Vivar-Quintana, 2024). Despite of the discriminating ability of the aforementioned methods, in most cases, the characterizing chemical species of each category remain unknown and/or the molecular basis of their discriminating nature remains indefinite.

Metabolomics involves the study of the complete set of small molecules (*metabolites*) in a given biological sample. In food science, metabolomics has a straightforward application in the thorough analysis of chemical species in complex food matrices. Among these metabolites, we may identify "characterizing" and "discriminating" chemical species, which may serve, for instance, to set up a metabolomic fingerprint for a specific food item (Garlito et al., 2023; Liu, Zhang, Chen, Harnly, & Sun, 2022; Nguyen et al., 2022). Owing to its high sensitivity and throughput of samples, a MS-based non-targeted metabolomic approach is particularly valuable in processed meats in order to identify chemical species from different origin including endogenous natural muscle components, xenobiotic species derived from feeding and/or inhalation and those formed during processing/storage. To the best of our knowledge, few MS-based metabolomics applications have been made on Chinese dry-cured hams, and they were aimed to analyze targeted metabolites in relation to quality traits (Li et al., 2025; Liao et al., 2022; Zhu et al., 2021).

This study was carried out to describe, for the first time, the metabolome of Iberian dry-cured hams, identify the most abundant metabolites and address their potential role in the final product. Furthermore, we made discriminating analyses between BLACK vs. RED (differing in genetic background) and RED vs. GREEN (differing in feeding system) ham categories, in order to establish a preliminary chemical fingerprinting of the three most valuable commercial categories of Iberian dry-cured hams.

2. Material and methods

2.1. Material

"Optima" MS-grade methanol, acetonitrile and formic acid were bought from Fisher Chemical (Massachusetts, USA). Chloroform and Isopropanol were purchased from Scharlab (Barcelona, Spain). Milli-Q water was obtained from a water purification system. Mixer Mill Retsch MM400 was purchased from Retsch (Düsseldorf, Germany). All other chemical compounds were purchased from Panreac (Panreac Química, S. A., Barcelona, Spain), Merck (Darmstadt, Germany), Fisher Scientific (San Jose, CA, USA) and Extrasynthese (Genay, France).

The three categories of Iberian dry-cured ham under study, namely, BLACK, RED and GREEN, were sampled from Spanish supermarkets as sliced and vacuum-packed dry-cured ham. Two commercial samples from each category were collected from six different manufacturers, totalling twelve samples per commercial category ($n = 12$). All samples belonged to the Denomination of Origin ("Jamón Dehesa de Extremadura") which double-check the accuracy of the commercial category of hams by technical on-farm inspections of animals and feeds. According to the information provided by the manufacturers, all commercial samples were treated with nitrified salt as the unique additives allowed to be used in the production of Iberian dry-cured hams (BOE, 2014).

2.2. Extraction of metabolites from Iberian dry-cured ham

Dry-cured ham slices were cut into smaller portions and subsequently chopped. One hundred mg of each sample were individually weighed in 2 mL vial. Immediately, samples were mixed with 400 μL of methanol and 400 μL of chloroform. Homogenization of the sample was made by vortexing 30 s each sample and then using a Mixer Mill Retsch MM400 with five steel balls per tube during 1 min. Subsequently, eight hundred μL of milli-Q water was added as a polar solvent. Agitation through vortex for about 30 s led to a biphasic aqueous/organic solution, with both phases being carefully separated into two different vials. Both (aqueous and organic) vials were evaporated using a speedvac (MiniVac Gyrozen, Korea) (2000 rpm, 1.5 h, 30 °C). The pellets were resuspended with 150 μL of acetonitrile:isopropanol:milli-Q (65 %:30 %:5 %) in case of the organic extract, and with 150 μL of acetonitrile:milli-Q (80 %:20 %) in case of the aqueous extract. After centrifugation (750 xg, 10 min, 4 °C), fifty microliters of the upper phase were transferred into high-pressure liquid chromatography (HPLC) vials. According to this procedure, two vials per sample, one with polar metabolites (aqueous extract) and another with non-polar metabolites (organic extract), were prepared. In addition to these samples, a blank sample was prepared by subjecting 100 μL of milli-Q water to the aforementioned procedure. A flow-chart of the extraction procedure is available as supplementary material (Fig. S1).

2.3. Metabolites separation and identification using Q-Exactive MS-Orbitrap

The mixture of metabolites extracted from both phases were separated in a Dionex UltiMate 3000 RSLCnano system and identified using a Q-Exactive High Resolution Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). For the organic extract, the column used was C18 Accucore ™ Aq (150 mm × 2.1 mm, 2.6 μm, Thermo Fisher Scientific, San Jose, CA, USA). The total run time was 20 min. The flow rate was set at 400 μL/min, and mobile phases were Optima HPLC-MS grade water (A) and Optima HPLC-MS grade acetonitrile (B), both with 0.1 % Optima HPLC-MS grade formic acid. For non-polar metabolites, the HPLC program was as follows: 0–1 min (2 % B, isocratic), 1–14 min (95 % B, increasing linearly), 14–16 min (95 % B, isocratic), 16–16.1 min (2 % B, decreasing linearly) and 16.1–20 min (2 % B, isocratic). For the aqueous extract, the column used was HPLC Accucore ™ HILIC (150 mm × 3 mm, 2.6 μm, ThermoFisher, San Jose, CA, USA). Total run time was 15 min. The flow rate was set at 500 μL/min, and mobile phases were Optima HPLC-MS grade water (A), and B, Optima HPLC-MS grade acetonitrile, both with 0.1 % Optima HPLC-MS grade formic acid. For polar metabolites, the HPLC program was as follows: 0–1 min (99 % B, isocratic), 1–3 min (85 % B, decreasing linearly), 3–6 min (50 % B, decreasing linearly), 6–9 min (5 % B, decreasing linearly), 9–10 min (5 % B, isocratic), 10–10.5 (99 % B, increasing linearly) and 10.5–15 (99 % B, isocratic). The HPLC program for the separation of both, polar, and non-polar metabolites, is displayed in Fig. S2 (Supplementary material).

For the HPLC-MS/MS analysis of metabolites from both, the aqueous and organic extracts, a pool of all samples (quality control sample, QC) was injected for the aligning of small shifts in retention times, mass accuracy, for avoiding signal drift and carry over effects, as well as for normalizing peak areas. In that fashion, QC runs covered as many as possible MS matches between metabolites from samples and databases from Compound Discoverer (Thermo Fisher Scientific, San Jose, CA, USA). Once this procedure was done, samples were run, and every 6-

sample injections, the QC was again injected to further validate the stability of the HPLC-MS system. Eight μL of each sample were injected for HPLC-MS Orbitrap analysis in a random, non-grouped order. Blank samples made from milli-Q water subjected to the aforementioned extraction procedures were also prepared and injected to prepare an exclusion list of compounds.

The ionization source was operated in the positive and negative switching ionization modes for the HILIC run and only positive for the C18 run. The flow rates of the sheath gas, auxiliary gas and sweep gas (N_2) were set to 50, 25 and 3 arbitrary units, respectively. The capillary spray source was set at 4 kV while the capillary temperature was fixed at 350 °C. A data-dependent analysis (DDA) was carried out in the first four QC injections using a Top5 approach, where the 5 most abundant ions in each iteration were selected for fragmentation and therefore, identified. For this methodology, resolution was set at 17500 FWHM, automatic gain control (AGC) at 1×10^5 and mass/charge relation was established between 53.4 and 800 (m/z). For the remaining samples, full-scan (FS) analysis was made using a resolution of 70,000 FWHM, an AGC of 1×10^6 and the same m/z range. A positive identification was confirmed for discriminating metabolites comparing MS data with those from available standard compounds. The equipment was weekly calibrated using both Pierce LTQ Velos ESI Positive Ion Calibration Solution and Pierce LTQ Velos ESI Negative Ion Calibration Solution (Thermo Fisher Scientific, San Jose, CA, USA).

The mass data acquired were processed with Compound Discoverer software (ThermoFisher, San Jose, CA, USA). This software was used as a pure compound identifier, sorting them by their abundance on the samples. ChemSpider was used as a main database searcher with more than 10 databases (including Human Metabolome Database, FooDB, KEGG or BioCyc). Among the main settings used for aligning, identifying and comparing, the metabolites found in every group were a maximum shift of 1 min and mass tolerance lower than 5 ppm.

2.4. Data analysis

Analyses were performed in 12 samples from each Iberian ham category. The distribution of raw data was determined using the Shapiro-Wilk normality test. Metabolites were statistically assessed using the MetaboAnalyst software (https://www.metaboanalyst.ca/), establishing standard deviation as statistical filter for the 40 % of the non-informative variables and the pareto scaling for normalizing the raw data. The 35 most abundant chemical species (Table 1) of all dry-cured hams were listed as characterizing metabolites and discussed below as such. Additionally, Principal Component Analyses (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were used as multivariate analyses. We carried out Dendrogram and Heatmap as hierarchical cluster analyses to facilitate the visualization differences between groups. PCA was conducted to assess differences between these groups. Furthermore, discriminating metabolites among the three commercial categories of Iberian dry-cured hams were identified using PLS-DA and Volcano plots methods, enabling a more detailed examination of the metabolome. Volcano plot combined results from fold change (FC) analysis and *t*-tests as one-factor statistical method used into one single study using a P value threshold <0.05 and a fold change threshold >2. The 25 most abundant discriminating metabolites between groups using this methodology were listed for discussion in Tables 2 and 3.

3. Results and discussion

3.1. Metabolome of Iberian-dry-cured ham: Most abundant chemical species

A total metabolomic profiling from all Iberian dry-cured hams was carried out, resulting in 3215 identified compounds using the Q-Exactive Orbitrap MS technology. Among all these compounds, Table 1 shows the 35 most abundant metabolites in all samples under study. Qualitatively, the most abundant compounds were free proteinaceous amino acids (12) with most of them (8) being recognized as essential amino acids. Some of them, such as lysine, methionine, threonine and tryptophan are regarded as the most likely limiting amino acids in humans (Paoletti, Courtney-Martin, & Elango, 2024) and hence, its occurrence in dry-cured ham as abundant components may be regarded as a finding of relevant nutritional value. While free amino acids are common components in swine skeletal muscle, the abundance of these species in dry-cured hams is enhanced throughout processing owing to the proteolytic activity of endogenous enzymes. In particular, cathepsins (acid lysosomal proteases) break down proteins into peptides, which are hydrolyzed, in turn, by aminopeptidases and carboxypeptidases into free amino acids (Toldrá & Flores, 1998). Not only these free amino acids are involved in biosynthesis of proteins since some of them are also precursors of bioactive molecules such as neurotransmitters (tryptophan) or endogenous antioxidant defenses (methionine) (Estévez et al., 2020). Renowned active dipeptides, such as carnitine and carnosine, were also listed as some of the most abundant metabolites in Iberian dry-cured ham. Carnosine displays key roles in counteracting oxidative stress (Estévez et al., 2020) while carnitine enables the transportation and β-oxidation of fatty acids into the mitochondria facilitating lipid catabolism (Pekala et al., 2011). The supplementation of the latter has been proposed for loss weight purposes in human beings (Talenezhad, Mohammadi, Ramezani-Jolfaie, Mozaffari-Khosravi, & Salehi-Abargouei, 2020). Muscle-related nitrogen-containing metabolites such as creatinine and creatine were also found. Creatine is regarded as a conditionally essential nutrient and is directly implicated in energy metabolism and ATP synthesis in cells (Wu, 2020). Creatinine, the waste metabolite of creatine, has been proposed as biomarker of the length of the dry-curing process as its concentration increases throughout the ripening process (Mora, Hernández-Cázares, Sentandreu, & Toldrá, 2010). Interestingly, we found, among the most abundant metabolites, a compound that was tentatively identified as a creatinine dimer (m/z 227.12). At MS^2, this metabolite was fragmented into two identical ions of m/z 114.06, which had, in turn, an MS spectra and fragmentation pattern identical to genuine creatinine. To our knowledge, such metabolite has not been described before in biological samples, which may be due to the short life of such waste metabolite, which is readily excreted through urine. In dry-cured hams, the accretion of creatinine throughout the ripening process plus the molecular crowding effect caused by dehydration would facilitate the formation of this chemical species. Choline, another common constituent of skeletal muscle, was found to be the most abundant metabolite in all hams under study. Choline, a conditionally essential nutrient that occurs in many biomolecules such as phospholipids or neurotransmitters, is implicated in early stages of brain development and its supplementation protects against the onset of non-alcoholic fatty liver (Zeisel, 2000). Methylated and acetylated forms of histidine, identified as relevant molecules in brain and skeletal muscle (Holeček, 2020), are described as remarkable metabolites for the first time in dry-cured ham. Two lysine degradation products, namely piperidine and piconilamide, are also found as characterizing metabolites of Iberian dry-cured ham. The former is a well-known specific Strecker aldehyde of lysine while piconilamide along with amino adipic acid and piperideine-6-carboxylate/pipecolic acid (also found in the metabolome of dry-cured ham) are recognized degradation products of this essential amino acid. Interestingly, all the aforementioned lysine degradation products are different products from the oxidative deamination of protein-bound lysine, which leads to the formation of a unique product, the aminoadipic semialdehyde, also known as allysine (Xue et al., 2019). Allysine, identified as a major carbonyl protein in biological systems, including meat and muscle tissue (Estévez, 2011), when free, may undergo spontaneous cyclization to yield piperideine-6-carboxylate/pipecolic acid that would, in turn, lead to piperidine through decarboxylation. The present results indicate that oxidative degradation products from lysine, with some of them being

Table 1
Most abundant metabolites in Iberian dry-cured ham by using non-targeted metabolomic analysis sorted by decreasing order in abundance.

Order	Metabolites	Formula	m/z	Abundance	Chemical structure	Plausible origin	Biological relevance
1	Choline	C5 H13 N O	104.10683	9.22E+09	Quaternary salt of ammonium	Skeletal muscle component	Essential nutrient
2	Creatinine	C4 H7 N3 O	114.06608	8.73E+09	Cycled degradation product from creatine	Skeletal muscle component	Waste compound from energy metabolism (Wu, 2020)
3	Leucine	C6 H13 N O2	132.10178	5.97E+09	Non-essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins. Energy metabolism (Duan et al., 2016)
4	Phenylalanine	C9 H11 N O2	166.08617	5.60E+09	Non-essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins
5	Proline	C5 H9 N O2	116.07045	5.59E+09	Non-essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins. Redox homeostasis (Vettore, Westbrook, & Tennant, 2021)
6	3,5-Dihydro-4H-pyrazolo [3,4-*d*]pyrimidin-4-one	C5 H4 N4 O	137.04569	2.45E+09	Pyrimidine derivative	Indefinite	Unknown
7	Creatine	C4 H9 N3 O2	132.07662	2.14E+09	Glycine derivative having methyl and amidino groups	Skeletal muscle component	Conditionally essential nutrient. Energetic metabolism (Wu, 2020)
8	2-Methylamino-butyric acid	C5 H11 N O2	118.08609	2.10E+09	γ-amino acid	Microbial metabolite (gut)	Linked to butyric acid and a gamma-aminobutyric acid. Improves intestinal barrier function (Sun et al., 2020)
9	2-[(1E)-1,3-Butadien-1-yl]-1H-pyrrole	C8 H9 N	120.0807	1.83E+09	Pyrrole derivative	Indefinite	Unknown
10	Valine	C5 H11 N O2	118.08608	1.76E+09	Essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins
11	Histidine	C6 H9 N3 O2	156.07671	1.50E+09	Essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins
12	Arginine	C6 H14 N4 O2	175.11885	1.43E+09	Non-essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins
13	Piperidine	C5 H11 N	86.09633	1.13E+09	Heterocyclic amine	Skeletal muscle component	Lysine degradation intermediate
14	Indoline	C8 H9 N	120.08068	1.12E+09	Heterocyclic organic compound. Reduced form of indol	Tryptophan-derived microbial metabolite(gut)	Improves intestinal barrier function (Lee et al., 2015)
15	Carnitine	C7 H15 N O3	162.11233	1.09E+09	Dipeptide (Lysine and methionine)	Skeletal muscle component	Transport fatty acids into mitochondria. Energy metabolism (Pkala et al., 2011)
16	Methionine	C5 H11 N O2 S	150.05826	8.58E+08	Essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins, lipid metabolism and glutathione precursor (Martínez et al., 2017)
17	Tryptophan	C11 H12 N2 O2	205.09704	7.53E+08	Essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins and precursor of neurotransmitters and other biologically active metabolites (Sève, 1999)
18	Creatinine dimer	C8 H14 N6 O2	227.12488	7.42E+08	Creatinine adduct	Indefinite	Unknown
19	Threonine	C4 H9 N O3	120.06543	7.34E+08	Essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins
20	Stearic acid	C18 H34 O2	283.26287	3.50E+08	ω-9 Saturated free fatty acid	Skeletal muscle component. Lipolysis from acylglycides	Structural (acylglycerides) and fuel molecule
21	Indoleacrylic acid	C11 H9 N O2	188.07049	5.79E+08	Indol-derived alpha,beta-unsaturated monocarboxylic acid. Acrylic acid as functional group	Produced by *Peptostreptococcus* species on gut (Vasquez et al., 2022)	Suppresses gut inflammation and displays anti-inflammatory and antidiabetic proprieties
22	Carnosine	C9 H14 N4 O3	227.11365	5.01E+08	Dipeptide (Alanine and histidine)	Skeletal muscle component	Antioxidant (Aldini et al., 2021)
23	Lysine	C6 H14 N2 O2	147.11268	4.84E+08	Essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins
24	3,4-Diaminopyridine	C5 H7 N3	110.07121	4.38E+08	Pyridine derivative	Skeletal muscle component	Muscle contraction
25	Piperidine-2-carboxylic acid/Pipecolic acid	C6 H11 N O2	130.0862	3.87E+08	Carboxylic acid derivative of piperidine	Oxidative degradation of lysine	Lysine oxidation product
26	Tyrosine	C9 H11 N O3	182.08115	3.79E+08	Non-essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins
27	Linoleic acid	C18 H30 O2	279.23164	5.99E+08	ω-6 essential free fatty acid	Skeletal muscle component. Lipolysis from acylglycides	Structural (acylglycerides) and fuel molecule
28	2-Oleoylglycerol	C21 H40 O4	356.2925	3.13E+08	Monoacylglycerol with oleic acid	Lipolysis of acylglycides	Structural (acylglycerides) and fuel molecule
29	Serine	C3 H7 N O3	106.04982	3.04E+08	Non-essential amino acid	Skeletal muscle component. Proteolysis	Biosynthesis of proteins
30	Oleic acid	C18 H32 O2	281.24725	3.00E+08	ω-9 free fatty acid	Skeletal muscle component. Lipolysis from acylglycides	Structural (acylglycerides) and fuel molecule

(*continued on next page*)

Table 1 (*continued*)

Order	Metabolites	Formula	m/z	Abundance	Chemical structure	Plausible origin	Biological relevance
31	N-Acetyl-L-histidine	C8 H11 N3 O3	198.08722	2.65E+08	Acetylated amino acid	Derived from essential amino acid	Protection and stability of proteins
32	Phenylacetylene	C8 H6	103.05415	2.59E+08	Decarboxylated form of phenylpropiolic acid	Indefinite	Unknown
33	N(tau)-Methyl-histidine	C7 H11 N3 O2	170.09232	2.05E+08	Methylated amino acid	Derived from essential amino acid	Biodegradation/turnover of proteins
34	Tetrahydrothiophene carboxylic acid	C5 H8 O2 S	133.03166	2.31E+08	S-Heterocycle	Indefinite	Significantly negatively associated with lean pigs (Song et al., 2022)
35	Picolinamide	C6 H6 N2 O	123.0552	2.17E+08	Amide of pipecolic acid	Indefinite	Unknown

Formula and *m/z* are values acquired by Compound Discoverer 3.3 software (Thermo Fisher Scientific, San Jose, CA, USA). Chemical species having a specific role are identified and references provided, otherwise, knowledge has been obtained by Fennema's Food Chemistry (Damodaran, 2007).

Table 2
Twenty-five most abundant discriminating compounds between BLACK and RED dry cured hams sorted by decreasing abundance in the ham of higher quality (BLACK).

Metabolite	Formula	m/z	Abundance in BLACK	Abundance in RED	log2(FC)	raw.pval
(3E)-3-Penten-2-amine	C5 H11 N	86.09633	1.70E+09	0.00E+00	3.7102	4.57E-11
Threonine	C4 H9 N O3	120.06543	1.14E+09	5.56E+08	1.0351	2.76E-08
β-Alanine	C3 H7 N O2	90.0549	4.85E+08	0.00E+00	3.0918	5.62E-12
Pyrrolidine	C4 H9 N	72.08076	1.34E+08	2.66E+07	2.3286	6.86E-17
1-acetyl-3-piperidinol	C7 H13 N O2	144.10188	6.01E+07	0.00E+00	2.9863	7.96E-11
Citrulline	C6 H13 N3 O3	176.10286	4.26E+07	9.27E+06	2.1994	9.92E-11
bis(Hydroxymethyl)nitramine	C2 H6 N2 O4	123.04024	3.40E+07	0.00E+00	3.8188	1.03E-09
O-Nitro-serine	C3 H6 N2 O5	151.03522	3.01E+07	2.58E+05	6.8661	1.75E-09
3-Amino-1-methyl-2-pyrrolidinone	C5 H10 N2 O	115.08653	2.75E+07	0.00E+00	3.4105	2.84E-09
2,3-Diformylthiophene	C6 H4 O2 S	141.00039	2.56E+07	0.00E+00	3.0822	4.50E-17
2,4-Nonadienal	C9 H14 O	139.11169	2.51E+07	0.00E+00	3.4156	1.29E-03
Dihydrothymine	C5 H8 N2 O2	129.06571	2.09E+07	0.00E+00	7.9066	9.32E-06
Homocysteine-thiolactone	C5 H9 N O2 S	148.04261	1.93E+07	8.23E+06	1.2297	2.40E-03
Methyl-2-octynoate	C9 H14 O2	155.10658	1.56E+07	0.00E+00	4.9637	2.36E-02
Hypotaurine	C2 H7 N O2 S	110.027	1.16E+07	1.02E+06	3.503	9.87E-10
N-[2-(Methylsulfonyl)ethyl]acetamide	C5 H11 N O3 S	164.03877	1.09E+07	0.00E+00	2.9025	2.95E-11
S-Vinyl-cysteine	C5 H9 N O2 S	148.04262	7.80E+06	0.00E+00	3.8146	3.57E-04
4-(Methylthio)-2-oxobutyric acid	C5 H8 O3 S	149.0267	7.67E+06	0.00E+00	2.7412	4.15E-13
Alanine	C3 H7 N O2	90.05497	6.54E+06	1.64E+05	5.3149	7.46E-09
Cyclohexanone	C6 H10 O	99.08038	5.22E+06	0.00E+00	3.2848	8.44E-05
N-Nitrosopyrrolidine	C4 H8 N2 O	101.07091	5.14E+06	0.00E+00	2.538	7.97E-21
Dimetiletanolamina	C4 H11 N O	90.09129	2.02E+06	0.00E+00	2.6473	2.35E-16
N-acetylthreonine	C6 H11 N O4	160.06156	1.91E+06	0.00E+00	2.539	4.55E-18
3-Hydroxy-proline	C5 H9 N O3	130.05101	1.66E+06	0.00E+00	2.888	1.02E-09
2-aminosuberic acid	C8 H15 N O4	188.09287	1.64E+06	0.00E+00	3.1008	5.93E-12

This table also shows their significance (p-value), their ratio (log2(FC)), their formula and their estimated m/z.

rarely identified in processed foods, are remarkable components of the metabolome of Iberian dry-cured ham.

Metabolites from the lipid fraction of muscle were also listed as some of the most abundant metabolites in Iberian dry-cured ham. Stearic, oleic, and linoleic acids, the most abundant fatty acids in pork and dry-cured hams (Jiménez-Colmenero, Ventanas, & Toldrá, 2010), appeared in the list of most abundant metabolites. 2-Oleoylglycerol, a monoacylglycerol with an esterified oleic acid, was also found as a characterizing metabolite in Iberian dry-cured ham. The occurrence of all the aforementioned lipid-derived molecules is expected since the concentration of free fatty acids and partially hydrolyzed acylgycerols has been proposed to increase as the ripening of hams progresses (Andrés, Cava, Ventanas, Muriel, & Ruiz, 2004; Pajuelo et al., 2023). Interestingly, we also identified as characterizing chemical species, microbial metabolites likely produced by gut microbiota, absorbed and subsequently accumulated in porcine adipose tissue. Indoleacrylic acid (Vasquez, Oh, Song, & Kang, 2022), 2-methylamino-butyric acid (Sun et al., 2020) or indoline (Lee, Wood, & Lee, 2015) are some examples of microbial metabolites with potential benefits in terms of bioactivity. Indoleacrylic acid, for instance, is known to display in vivo anti-inflammatory and anti-diabetic properties and it is able to mitigate food allergies (Pei et al., 2024).

3.2. Discrimination between ham categories: BLACK vs. RED

Metabolomic profiles of hams from different categories were compared for the identification of discriminating compounds for authentication purposes. In particular, when comparing BLACK vs. RED dry-cured hams, a total of 142 discriminating chemical species were identified by *m/z* (50–200) with 57 being significantly more abundant in the BLACK group and 85 being more abundant in the RED group. Table 2 shows the 25 most abundant discriminating metabolites between BLACK and RED Iberian dry-cured hams sorted by decreasing concentration in the ham of highest quality (BLACK). Most of these metabolites are whether free amino acids (threonine, alanine), intermediates in the degradation of free amino acids ([3E]-3-penten-2-amine, pyrrolidine, 1-acetyl-3-piperidinol or 2,3-thiophenedicarboxaldehyde) or formed as a result of the involvement of those amino acids in further reactions (i.e. *N*-acetylthreonine, 3-hydroxy-L-proline). Plausibly, most of these species are formed as a result of the reactivity of free amino acids with other degradation products from muscle components during the long ripening process of dry-cured hams. In fact, the formation of odor- and flavor-active compounds such as pyrrolidine (decarboxylation of proline), thiophenes (degradation of sulfur amino acids) or piperidines/piperidinols (oxidative deamination of lysine), is known to be favored during

Table 3
Twenty-five most abundant discriminating compounds between RED and GREEN dry cured hams sorted by decreasing abundance in the ham of higher quality (RED).

Metabolite	Formula	m/z	Abundance in RED	Abundance in GREEN	log2(FC)	raw.pval
Lysine	C6 H14 N2 O2	147.11268	6.63E+08	1.65E+07	5.3258	2.68E-07
3-Amino-2-hydroxypropanal	C3 H7 N O2	90.05491	3.86E+08	0.00E+00	3.0962	3.14E-08
1-(2-Piperidinyl)-1,2-ethanediol	C7 H15 N O2	146.1175	4.78E+07	0.00E+00	2.8746	4.98E-14
4-Aminophenol	C6 H7 N O	110.05999	3.36E+07	6.94E+06	2.2741	6.38E-03
Pyrrolidine	C4 H9 N	72.08076	2.66E+07	0.00E+00	2.6767	1.96E-16
Agmatine/(4-aminobutil)guanidine	C5 H14 N4	131.12906	2.40E+07	9.15E+06	1.3933	1.22E-03
Isethionic acid	C2 H6 O4 S	124.99142	1.56E+07	0.00E+00	6.0972	5.48E-03
2-(Diethylamino)ethanol	C6 H15 N O	118.1226	1.51E+07	6.22E+06	1.2803	1.62E-02
n-Hexanamide	C6 H13 N O	116.10695	1.25E+07	3.96E+06	1.6639	9.18E-03
(1*R*,2*R*,3*R*,4*S*,5*R*)-4-Amino-5-sulfanyl-1,2,3-cyclopentanetriol	C5 H11 N O3 S	166.05322	1.06E+07	0.00E+00	2.9154	1.17E-12
3-Amino-3-methyl-2-pyrrolidinone	C5 H10 N2 O	115.08652	1.06E+07	0.00E+00	4.249	1.71E-06
Lactic Acid	C3 H6 O3	89.02446	9.98E+06	3.25E+06	1.6171	2.35E-09
6-Azidocaproic acid	C6 H11 N3 O2	158.09234	8.97E+06	1.50E+06	2.583	2.61E-06
N-(2-aminoethyl)ethyleneurea	C5 H11 N3 O	130.09749	8.71E+06	0.00E+00	3.2223	2.31E-06
7-Deazaguanine	C6 H6 N4 O	151.06139	8.38E+06	0.00E+00	2.9195	4.50E-10
Homocysteine-thiolactone	C5 H9 N O2 S	148.04261	8.23E+06	0.00E+00	3.6546	2.30E-08
Hex-2-ulose	C6 H12 O6	179.05614	8.02E+06	1.39E+06	2.5257	4.45E-07
HMBOA hexose	C8 H17 N O2	160.13314	7.62E+06	0.00E+00	2.8619	1.48E-13
2S,3R,4R,5R-3,4,5-Trihydroxypipecolic acid	C6 H11 N O5	176.05642	7.53E+06	0.00E+00	3.1449	5.6203E-07
3-(Methylamino)-1-butanol	C5 H13 N O	104.10691	6.72E+06	0.00E+00	3.2605	1.01E-08
1,4,5,6-Tetrahydrocyclopenta[*c*]pyrazole-3-carbohydrazide	C7 H10 N4 O	167.09269	6.68E+06	0.00E+00	3.1247	6.8701E-12
Cyclododecanone	C12 H22 O	183.17428	6.64E+06	3.24E+06	1.0348	0.034986
Gluconic acid	C6 H12 O7	195.051	6.63E+06	0.00E+00	4.4805	9.15E-03
1-Carbamoylproline	C6 H10 N2 O3	159.07635	6.43E+06	3.02E+06	1.0891	2.20E-05
1-Pentylamine	C5 H13 N	88.11201	5.41E+06	0.00E+00	7.163	0.0042245

This table also shows their significance (p-value), their ratio (log2(FC)), their formula and their estimated m/z.

long drying processes. The concurrence of severe proteolysis and enduring lipid oxidation in an environment with decreasing water activity and hence, molecular crowding, explains this high reactivity (Gonzalez & Ockerman, 2000; Toldrá, Aristoy, & Flores, 2009; Toldrá & Flores, 1998). Thiophene related compounds, such as 2,3-diformylthiophene, are derived mainly from Maillard reaction between cysteine and ribose (Van Boekel, 2006) providing dry-cured meats with a meaty flavor, especially in those subjected to mild temperatures and long ripening periods (Li, Belloch, & Flores, 2021). Other discriminating compounds such as 2,4-nonadienal (lipid-derived alkadienal), methyl-2-octynoate (fatty acid methyl ester) and cyclohexanone, are also known as potent odorants (Gao et al., 2023) and hence, their occurrence in BLACK hams only may contribute to sensory outcomes. BLACK label hams from the present study had a higher concentration of IMF than RED ones (20.60 ± 2.51 vs. 16.52 ± 1.98; $P < 0.05$). Hams with higher lipid content are typically ripened for longer time, owing to a more slowly dehydration process, which would explain a higher abundance of certain metabolites of intricate formation. Current trends in Spanish meat processing plants involve ripening Iberian hams of the highest quality (BLACK label) for 5–6 years vs. the 2–3 years for the more standard drying process (Segura-Borrego et al., 2022). Yet, this long ripening could also be plausibly behind the increased formation in these hams of nitroso compounds with potential toxicological implications such as N-nitrosopyrrolidine, bis(hydroxymethyl)nitramine and O-Nitro-L-serine. Yet, the toxicological concern of these compounds is yet to be defined because even if they can be regularly found in cured (nitrite-cured) meats, the doses are normally within safe ranges (Hospital et al., 2024). The lack of an accurate quantification of individual chemical species in the current non-targeted approach calls for the necessity to quantitatively assess the occurrence of nitrosative reactions in cured and long-ripened dry-cured hams.

Further to the potential influence of processing conditions on the metabolome of BLACK and RED hams, the genetic background may have also played a role in the occurrence of other discriminating metabolites such as dihydrothymine, citrulline, homocysteine-thiolactone, hypotaurine and 2-aminosuberic acid. The latter dicarboxylic acid has a role as human metabolite as it is accumulated in blood and tissues as a result of a congenital impaired metabolism of oleic acid (Ranea-Robles & Houten, 2023). Purebred Iberian pigs are known to display a unique transcriptome profile, which is responsible for their distinct lipid metabolism (Villaplana-Velasco et al., 2021). Since this metabolite was only found in hams from pure-bred Iberian pigs (BLACK), the possibility that this compound could be related to the unique genetic background and metabolism of pure-bred pigs deserves deeper examination. Dihydrothymine, citrulline and hypotaurine are naturally occurring intermediate metabolites in the synthesis of thymine, arginine and taurine, respectively. The supplementation of citrulline in pigs leads to benefits in terms of growth performance, microbiota composition and meat quality (Du et al., 2023). Homocysteine-thiolactone, hypotaurine, as well as its final product, taurine, are found in skeletal muscle and display, among others, functions as antioxidant defenses against oxidative stress (Estévez et al., 2020). These three metabolites were between 5 and 10 times more abundant in BLACK hams than in the RED counterparts, providing strength to the hypothesis of the role played by the genetic background in the synthesis and metabolism of relevant sulfur-containing biomolecules in the skeletal muscle of Iberian pigs.

3.3. Discrimination between ham categories: RED vs. GREEN

Metabolomics was also carried out between RED and GREEN groups to identify discriminating metabolites in hams from pigs submitted to different feeding regimes. A total of 66 chemical species were found to be more abundant in RED hams and 70 were, conversely, more abundant in hams with GREEN label ($P < 0.05$). Table 3 shows the 25 most abundant discriminating metabolites between RED and GREEN Iberian dry-cured hams sorted by decreasing concentration in the ham of highest quality (RED).

As per the metabolomic profiling of hams differing in genetic backgrounds (BLACK vs. RED), the feeding regimes also affected the metabolome of hams, with amino acids and modified amino acids being the most abundant discriminating chemical species. Lysine was found at significantly higher concentrations in RED hams than in the GREEN counterparts ($P < 0.05$) and lysine oxidation products such 1-(2-piperidinyl)-1,2-ethanediol and 2S,3R,4R,5R-3,4,5-trihydroxypipecolic acid were characterizing metabolites of RED hams. Unfortunately, the information available on the formation and potential role of these chemical species in processed foods is scarce. It is worth noting that both are hydroxyl derivatives of lysine oxidation end-product pipecolic acid,

originally identified as major component of the metabolome of dry-cured hams in the present study. Conversely, pyrrolidine, the decarboxylation product from proline is a well-known flavor-active component of dry-cured hams (Luna, Aparicio, & García-González, 2006; Toldrá & Flores, 1998). This species, which appeared to be more abundant in BLACK than in RED hams, is now absent in hams from the GREEN group, suggesting that high-quality products may correlate with increased pyrrolidine levels in dry-cured hams. Furthermore, the occurrence of pyrrolidine only in hams from pigs fed on acorns (BLACK and RED) suggests that outdoor feeding practices could be influencing its accretion in ripened hams. The feeding background of Iberian pigs is known to significantly impact the availability of amino acids and the metabolic pathways involved in their degradation (Martin, Antequera, Ventanas, Benitez-Donoso, & Córdoba, 2001). Other interesting example of a key indicator of the feeding background is the deamination product of taurine, isethionic acid, found only in RED hams. Performing a non-targeted metabolomic analysis, similar to the one employed in the present study, Muroya, Oe, Nakajima, Ojima, and Chikuni (2014) and Muroya (2023) identified isethionic acid in postmortem muscles from pigs and cattle, respectively. While this metabolite is identified as a bioactive compound with protective effects against liver disorders, its occurrence in foods and its role as micronutrient is poorly understood (Wang et al., 2023). Another sulfur-containing metabolite that may be an indicator of extensive feeding is the methionine degradation product, homocysteine-thiolactone. Since it was already identified as a discriminating compound between BLACK vs. RED hams, its abundance seems to be influenced by the genetic background. Yet, its occurrence is directly dependent on outdoor feeding since no trace of homocysteine-thiolactone was found in hams from the GREEN group. Agmatine, also known as (4-aminobutil)guanidine is a biogenic amine formed as a result of arginine decarboxylation (Juraj et al., 2017) and its accumulation in cured and processed meat has been correlated with the length of the ripening period (Lorenzo, Martínez, Franco, & Carballo, 2007). According to our results, agmatine may also be affected by the feeding background, as its abundance in RED dry-cured hams was almost three-fold higher than in the GREEN counterparts.

Other relevant group of discriminating metabolites are distinctly dependent on microbial metabolism. The microbial population growing on the surface of dry-cured hams is well known to play a major role on quality and safety of these ripened meat products (Estévez et al., 2014). Yet, according to our results, the impact of surface microorganisms on the occurrence of metabolites in the core of the product seems to be negligible. Conversely, the nature and origin of microbial metabolites strongly suggests the likely impact of the feeding background on the microbiota of Iberian pigs. Acorn and grass, rich in polyphenols, fiber, and unsaturated fats, have been proposed to foster a more diverse gut microbiota, with increased abundances of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* (Fernández et al., 2020). This is the favorable environment for the production of HMBOA hexose, an organic compound known to be formed in the gut from the amino acid valine (Zhang et al., 2024). Since this metabolite was 7-fold more abundant in RED hams than in the GREEN counterparts, it is plausible to ascribe these differences to a distinct valine metabolism by the microbiota from pigs with different feeding backgrounds. In RED hams, valine was mainly converted after microbial digestion to HMBOA hexose, whereas on the GREEN group, other valine byproducts were identified as discriminating such as L-(+)-valinol, found at 14-fold higher concentrations in this latter group of hams. HMBOA hexose is also naturally found in cereals such as wheat, rye and maize (Sutour et al., 2024), so the higher abundance of this metabolite in hams from pigs exclusively fed outdoors (RED) may also be a consequence of this particular feeding regime. The occurrence of another microbial metabolite, 7-deazaguanine, only in RED dry-cured hams, may also be attributed to the interplay between the feeding regime and the gut microbiota. 7-Deazaguanine and its derivatives are a relatively unknown group of chemical species, which are known to be synthesized by a number of bacteria (de Crécy-Lagard et al., 2024). In the present study, we originally report the role of certain microbial metabolites as characterizing chemical species in dry-cured ham. Further studies would provide further insight into the role of feeding backgrounds on microbiota of Iberian pigs and the fate of certain metabolites that may be relevant for authentication purposes.

Finally, it is worth recalling that the official method for discrimination between hams from pigs differing in feeding backgrounds (natural resources "RED" vs. mixed diets "GREEN") involved the analysis of the fatty acid profile of adipose tissues since acorns provide extensively reared pigs with genuinely high levels of oleic acid (Ventanas, Ventanas, Tovar, García, & Estévez, 2007). Yet, feeding Iberian pigs with oleic acid enriched mixed diets led to Iberian hams of similar compositional characteristics and hence, such method was revoked in 2007. Interestingly, we found certain straight- and short-chain fatty acids naturally found in natural resources as precursors of discriminating compounds between RED and GREEN hams. Caproic/hexanoic acid, found in several green leaf plants and particularly concentrated in nuts, is stable against oxidative reactions and readily accumulated in adipose tissues from pigs (Hetényi, Bersényi, & Hullár, 2024). Two of the most abundant discriminating metabolites in RED hams, n-hexanamide and 6-azidocaproic acid, have a caproic core. This remarkable finding supports the report made by Pugliese et al. (2009) who stated that pigs fed under free-range conditions on acorn or chestnut had more hexanoic acid than those reared outdoors and fed a commercial mixed diet.

3.4. *Metabolomic profiling as a tool for Iberian dry-ham authentication*

PCA was carried out with data from the three categories to assess the extent to which the full metabolic profile would be a useful tool to discriminate between groups of Iberian hams. The results (Fig. 1A) show that the first two principal components, explaining the 34.5 % and 24.1 % of the variance, respectively, enabled clear discrimination between hams of the highest quality BLACK and the RED/GREEN counterparts, which strengthens the importance of genetics in the metabolomic profiling of Iberian hams. Although PCA was not able to differentiate among all three categories of dry-cured hams, in a subsequent PLS-DA in which discriminating compounds were loaded (Fig. 1B), the distinction between the three groups was fully accomplished. The principal component explained 33.6 % of the variances whereas the second most explanatory component reached 12.3 %. It is worth noting the dispersion between the projected GREEN hams in the similarity map as compared to the nearby position of BLACK and RED hams. This is consistent with the more flexible criteria for GREEN hams category, which are mainly based on extensive feeding on cereal-based mixed diets. For this same reason, GREEN hams are also known to be highly variable in terms of quality since some pigs may have certain access to natural resources and finished with mixed diets while others are exclusively fed using commercial feed mixtures of variable composition.

To confirm the efficacy of the metabolic profiling of Iberian hams in discriminating between categories, a dendrogram with its corresponding hierarchical cluster was designed using all available data (Fig. 1C). A dendrogram consists of U-shaped lines that connect samples in a hierarchical tree. The height of each U-shaped line connecting samples in a hierarchical tree represents the similarities between the samples being connected. In this case, the dendrogram shows a clear separation between BLACK and RED/GREEN dry-cured hams categories in a first hierarchical level. In order to add robustness to the results already shown, a heatmap is presented in Fig. 1D where clear differences are displayed between the 3 categories of dry-cured hams. Each block refers to the abundance of one metabolite from one sample. The samples are genuinely sorted by their category after statistical analysis.

When performing PCAs between comparisons of interest (BLACK vs. RED and RED vs. GREEN), a clear differentiation between groups was obtained. For the BLACK/RED contrast, PCA revealed that the first two principal components accounted for 54.8 % of the total variance for PC1

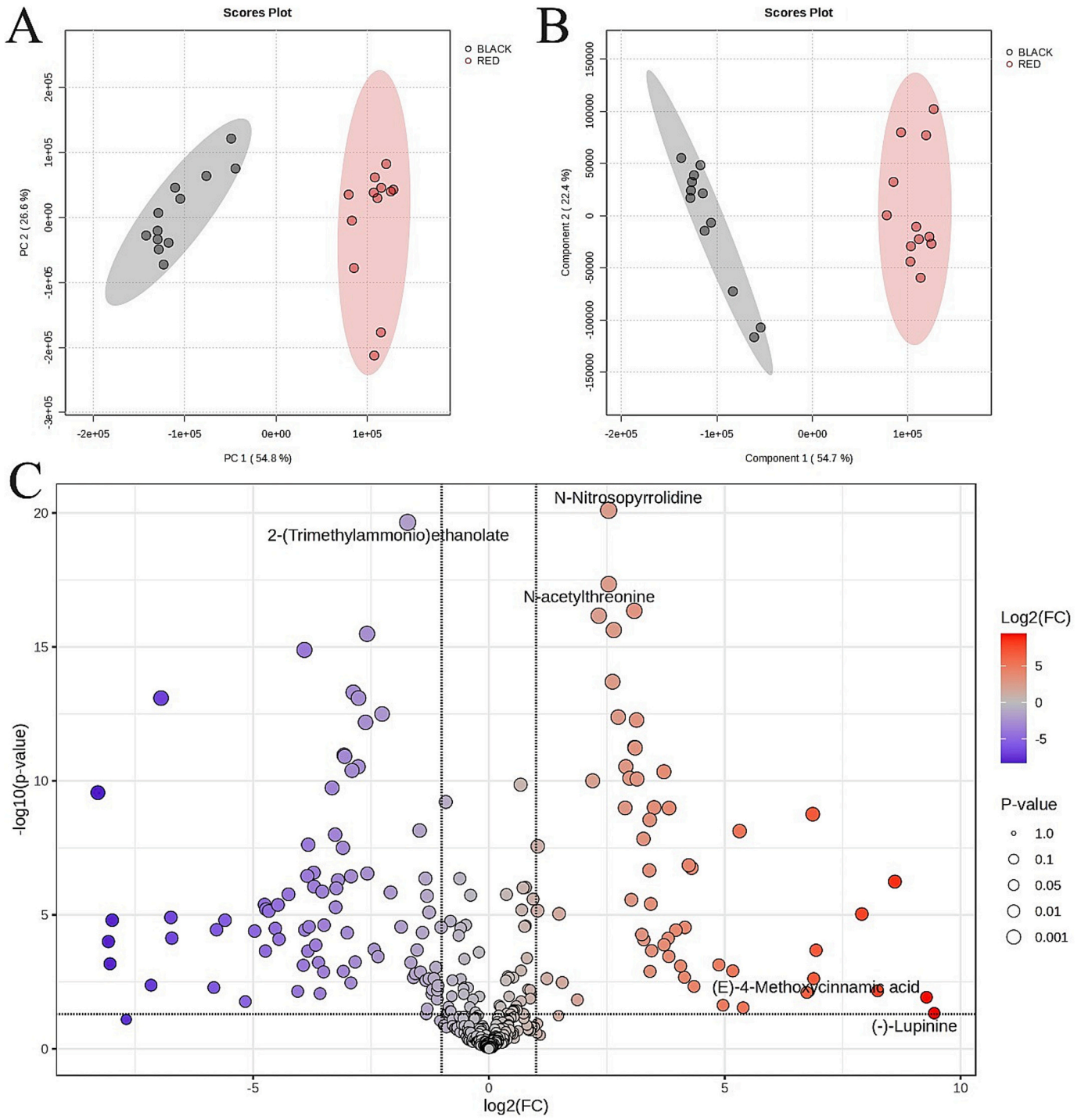

Fig. 1. Visual discrimination of Iberian dry-cured hams from different commercial categories (BLACK, RED and GREEN) based on various statistical analyses. A) Principal Component Analysis (PCA); B) Partial least squares-discriminant analysis (PLS-DA). C) Dendrogram. This representation allows to appreciate the differences in similarity (x axis) between groups. D) Heatmap shows the differences in concentrations for each compound. Samples are automatically hierarchized by the software in x axis and clustered together. Each block refers to the abundance of one metabolite from one sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and 26.6 % for PC2 (Fig. 2A), indicating a strong contribution of these components to the overall data structure. To further explore metabolic differences between BLACK- and RED-labeled dry-cured hams, we applied a supervised multivariate statistical approach using PLS-DA. This model showed that the first two latent variables explained 54.7 % of the variance for component 1 and 22.4 % for component 2 (Fig. 2B), showing a clear discrimination between the two groups. The robustness of this separation was further supported by the corresponding Volcano plot (Fig. 2C), highlighting some metabolites that contributed to the observed differences.

To investigate the metabolic differences between RED- and GREEN-labeled dry-cured hams we followed the same methodology as for the previous comparison. The PCA was conducted to explore variance within the dataset and assess the actual grouping of samples. The first two principal components explained 55.8 % of the total variance for PC1 and 14.0 % for PC2 (Fig. 3A), indicating a moderate separation between

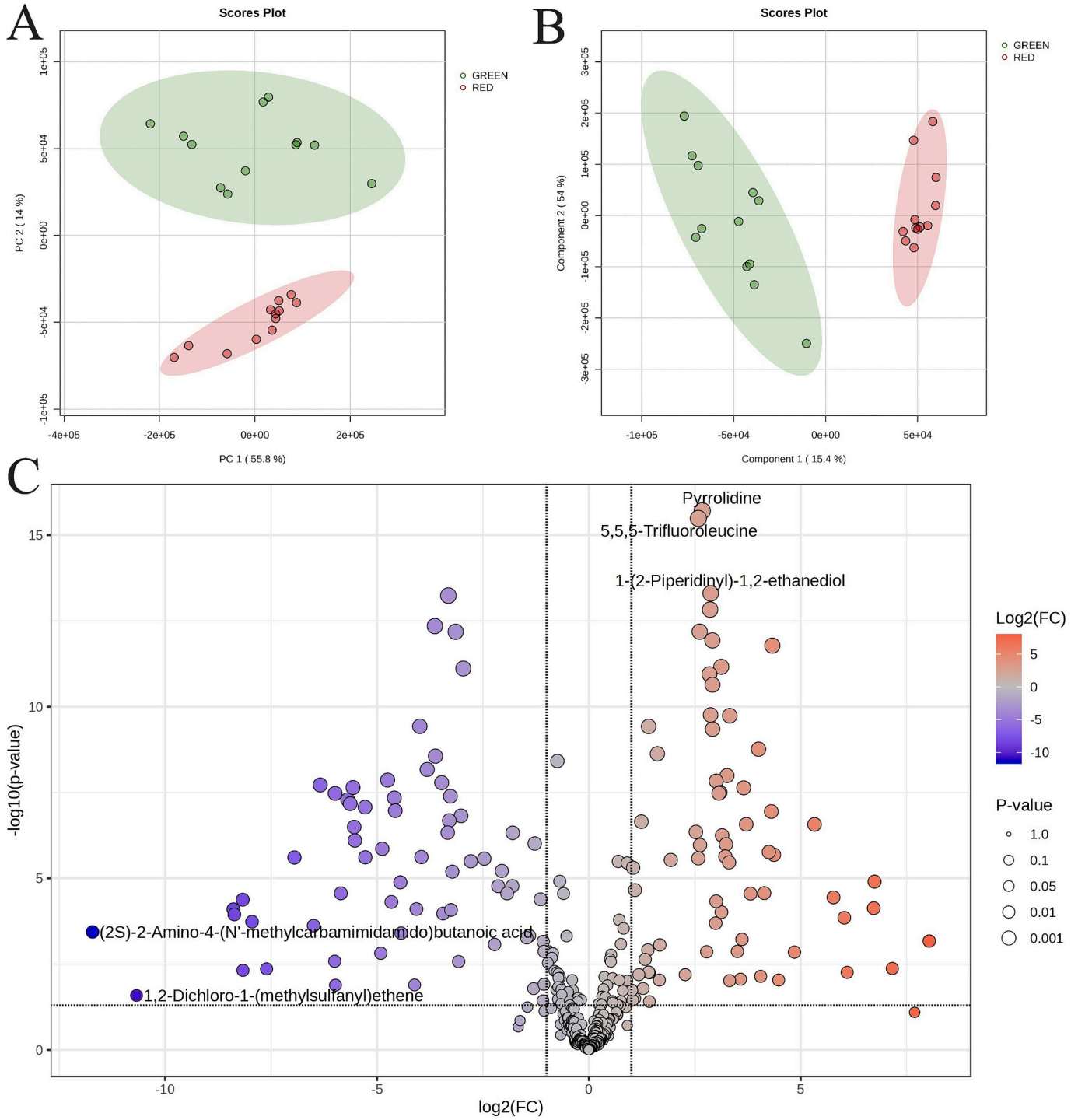

Fig. 2. Statistical analysis comparing BLACK vs RED groups. A) PCA; B) PLS-DA. C) Volcano plot shows the upregulated and downregulated compounds in red (FC > 2) and blue (FC < 2), respectively, following the conditions of a *p*-value of *t*-test <0.05. Name of the most significant compounds of either group are written in the graphic. The metabolites that are not significantly discriminating are represented as grey dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the two groups, likely influenced by differences in feeding practices. To enhance discrimination, PLS-DA was applied, which maximizes class separation by identifying key metabolites contributing to the observed variation. The PLS-DA model revealed that the first two latent variables accounted for 15.4 % of the variance in component 1 and 54.0 % in component 2 (Fig. 3B), demonstrating a clear distinction between RED- and GREEN-labeled hams. This differentiation suggests that diet, specifically acorn-based pasture feeding versus cereal-based feed, plays a crucial role in shaping the metabolic profile of the final product. Further statistical validation is provided by the Volcano plot (Fig. 3C), which highlights some metabolites contributing to differentiating the two groups. These results underscore the impact of dietary composition on the metabolomic fingerprint of dry-cured hams.

Dry-cured ham is a highly prized delicacy renowned for its distinct flavors and textures. Its authenticity is closely tied to the geographic origin, genetic background of the livestock, and the feeding practices

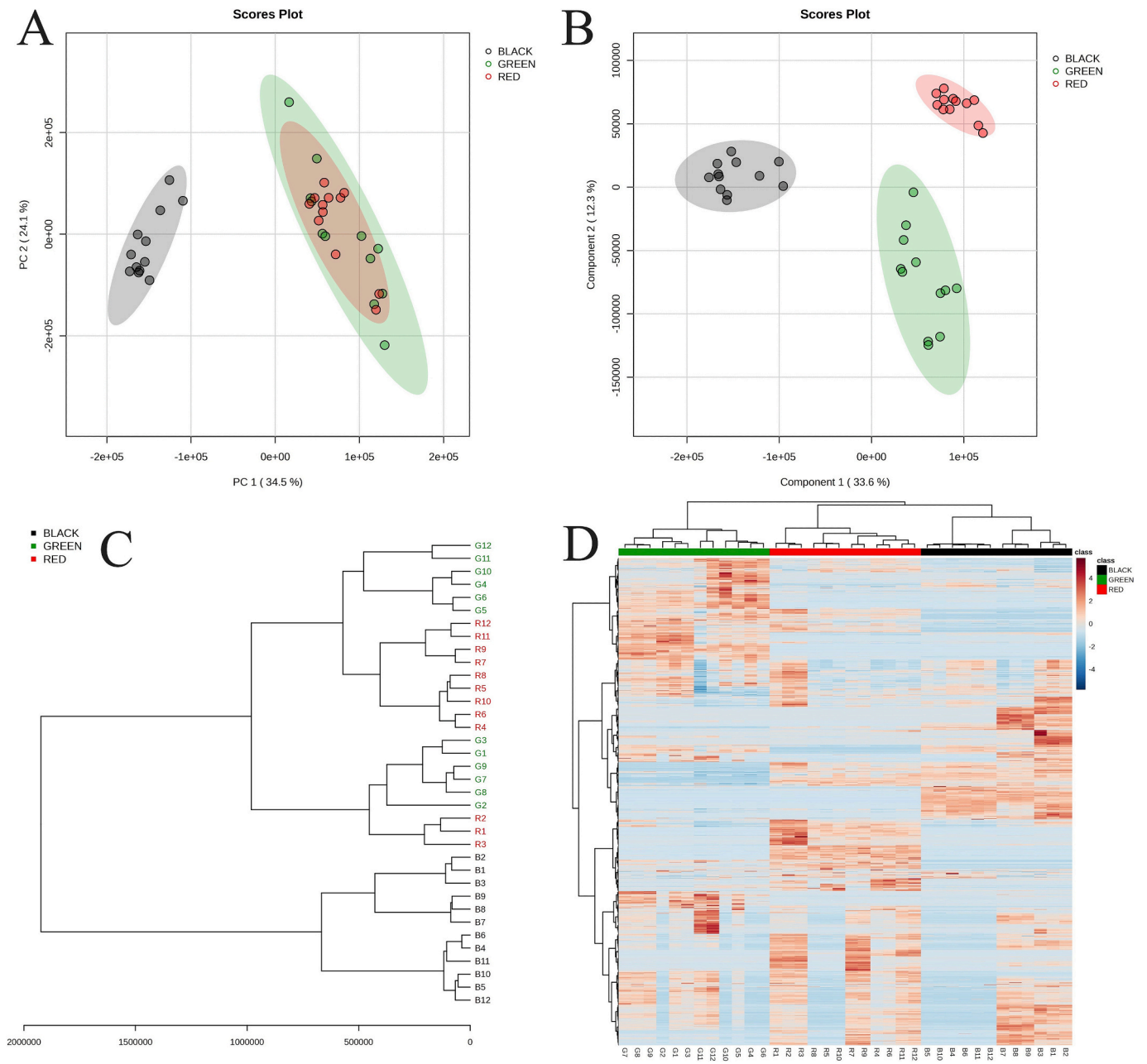

Fig. 3. Statistical analysis comparing RED vs GREEN groups. A) PCA; B) PLS-DA. C) Volcano plot shows the upregulated and downregulated compounds in red (FC > 2) and blue (FC < 2), respectively, following the conditions of a p-value of t-test <0.05. Name of the most significant compounds of either group are written in the graphic. The metabolites that are not significantly discriminating are represented as grey dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

employed in its production. To safeguard the reputation of this gourmet product and protect consumers from food fraud, several scientific approaches have been developed to authenticate dry-cured ham. Genetic markers, such as single nucleotide polymorphisms (SNPs), have been instrumental in establishing the authenticity of dry-cured ham based on the genetic background of the animals used. Studies have shown that the genetic profile of pigs can be linked to specific geographical regions and breeds (Muñoz et al., 2020). These markers help to prevent fraud and ensure that dry-cured ham is produced from the specified breeds, such as Iberian or Duroc pigs, which are known for their high-quality meat. The diet and feeding practices of the animals involved in dry-cured ham production have a significant impact on the quality and flavor of the final product. Various techniques have been used to ensure the authenticity of these feeding practices. Most of them are tested on the final product by gas chromatography – ion mobility spectrometry (GC-IMS) (Arce et al., 2009; Arroyo-Manzanares et al., 2018; Hernández-Jiménez et al., 2021; Martín-Gómez, Arroyo-Manzanares, Rodríguez-Estévez, & Arce, 2019). The only technique that enables a quality control at the farm level involves a biopsy of back fat and a subsequent analysis of the volatilome by gas chromatography (Rodríguez-Hernández et al., 2022). While genetic analysis could be highly accurate in identifying breed and geographic origin, it may not provide information about the animal's diet.

In this research, we approach the identification of discriminant compounds of different varieties of dry-cured ham with the use of a HR-MS Q Exactive Orbitrap. The precision and sensitivity of this equipment along with the ability to analyze a wide range of compounds offers an authentication methodology that could be used to avoid fraud and

guarantee authentication.

4. Conclusions

This study provides a comprehensive metabolomic characterization of Iberian dry-cured hams, revealing key and original insights into characterizing chemical species, including their likely origin, and plausible role in the final product. The occurrence of essential amino acids, bioactive peptides and other essential micronutrients highlights the nutritional relevance of Iberian dry-cured ham. Overall, this study demonstrates the utility of metabolomic profiling as a powerful tool for Iberian ham authentication, offering a scientific basis for quality control and potential regulatory applications in the meat industry. Nevertheless, the identification of the precise origin of metabolites linked to different genetic and/or feeding backgrounds should be identified as a limitation of the present study. The lack of control of multiple variables affecting the metabolome of commercial samples hinders the possibility of a more accurate discussion of results. Future research should focus on refining quantification methods and exploring the biological implications of identified metabolites, particularly those linked to genetic and dietary influences. As the dry-cured ham industry faces challenges related to geographic origin, genetic background, and feeding practices, the insights gained from this study contribute to the ongoing efforts to combat food fraud and ensure the quality and authenticity of this esteemed meat product.

Consent form

The authors declare that no consent form is applicable as the paper does not involve experimentation with human beings.

Author statement

The authors declare that NO AI was used during the preparation of this paper.

CRediT authorship contribution statement

Víctor Caballero: Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Guadalupe Sánchez-Terrón:** Writing – review & editing, Software, Methodology, Data curation. **Mario Estévez:** Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2025.109854.

Data availability

Data will be made available on request.

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