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Microbiota and volatilome of dry-cured pork loins manufactured with paprika and reduced concentration of nitrite and nitrate

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ABSTRACT

Dry-cured pork loin is a very popular meat product in Mediterranean countries. Pork-loin is manufactured rubbing curing salts, nitrite and nitrate, and spices on the surface of the loin which is then dry-cured or smoked for several months. Although nitrite-derived compounds are crucial for the microbiological safety and development of a distinct flavour, there have been recent concerns about the adverse health effects of nitrite-derived compounds driving to the reduction of curing agents in meat products. In this study, we have evaluated the differences in microbiota and aroma of dry-cured pork loins manufactured with or without paprika and reduced ingoing amounts of nitrate and nitrite. *Staphylococcus* dominated the microbiota of pork loins without paprika, regardless of the nitrite and nitrate reduction. On the contrary, the reduction of nitrite and nitrate in loins with paprika had an important effect on the microbiota. In these loins a codominance of *Staphylococcus* and *Bacillus* together by Enterobacteriaceae occurred. Moreover, paprika addition and reduction of nitrite and nitrate seemed to promote proliferation of lactic acid bacteria. Occurrence of these genera was correlated with the generation of free amino acids and their derived volatile compounds setting clear differences in the aroma profile of dry-cured loins.

1. Introduction

Dry-cured pork loin is one of the most valued dry-cured products (Lorido, Ventanas, Akcan, & Estévez, 2016). Loins are whole muscular pieces seasoned by rubbing a mixture of salt and curing agents, as well as spices such as paprika or garlic. Diffusion of compounds derived from spices, susceptibility to oxidation of the loin surface due to direct exposure to oxygen and surface microbial contamination have a significant impact on flavour and quality of cured loins (Muriel, Antequera, Petrón, Martín, & Ruiz, 2004). The impact of spices as contributors to specific aroma compounds in dry-cured meat products is well known (Ventanas, Ventanas, Estévez, & Ruiz, 2010). Paprika is used in meat products not only for its colour and flavour properties but also for its antioxidant characteristics (Cremer & Eichner, 2000; Aguirrezábal, Mateo, Domínguez, & Zumalacárregui, 2000). The microbial load of paprika may contribute to the development of a specific microbiota, promoting the ripening process of the meat product (Molnár et al. 2018). Moreover, free amino acids and residual nitrate in paprika may influence the generation of volatile compounds (Kehayoglou & Manoussopoulos, 1977).

Nitrite addition in cured meat products allows development of typical sensory characteristics, contributing to colour stabilization, aroma, taste, and reduction of lipid oxidation processes. In addition, nitrite controls growth of pathogens and spoilage microorganisms (Honikel, 2008, Sindelar & Milkowski, 2011). However, the demand for healthier meat products with natural ingredients and reduced ingoing amounts of curing agents has driven the search for alternatives to nitrite addition (Aquilani et al., 2018). The reduction of ingoing amounts of nitrate and nitrite in dry-cured meat products is determined by the presence or not of both agents (nitrite and nitrate) and the specific ripening conditions (Perea-Sanz, Montero, Belloch, & Flores, 2018). Recent studies have demonstrated that the reduction of nitrite content has a negative impact on the quality of dry-cured products (Hospital, Hierro, & Fernández, 2014, Hospital et al., 2015, Perea-Sanz, Montero, Belloch, & Flores, 2018, Perea-Sanz, Montero, Belloch, & Flores, 2019). The main consequences of nitrite reduction are the alteration of the aroma profile and growth of undesirable microorganisms (Hospital, Hierro, & Fernández, 2012, Hospiral, Hierro, Stringer, & Fernández, 2016, Christieans, Picgirard, Parafita, Lebert, & Gregori, 2018). The main alterations detected in the aroma profile of nitrate-reduced

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sausages were due to the generation of higher amounts of volatile compounds from amino acid degradation, such as dimethyl disulphide (toasted, garlic) and methional (cooked potato), as well as fruity odours (ethyl acetate, ethyl butanoate, ethyl-2-hydroxypropanoate, ethyl-2-methylbutanoate and ethyl-3-methylbutanoate) from esterase activity, and acetic acid (vinegar odour) and 2-butanone (fruity) from carbohydrate fermentation (Perea-Sanz et al., 2018).

The study of food microbiota has changed profoundly in the last years with the application of rDNA amplicon HTS (High-Throughput Sequencing) techniques (Ercolini, 2013). The occurrence and abundance of microorganisms present in different food products such as cheese, beef, fermented fish and meat products can be assessed by rDNA amplicon sequencing (Dolci, De Filippis, La Storia, Ercolini, & Cocolin, 2014; De Filippis, La Storia, Villani, & Ercolini, 2013, Osimani et al., 2019, Greppi et al., 2015, Juárez-Castelán et al., 2019). Several studies have demonstrated that the structure of the microbiota in the food products is largely affected by the animal origin of the raw ingredients, fermentation conditions, salt content, and addition of ingredients such as spices or herbs (Quigley et al., 2012, Nieminen et al., 2017). Detection of food-borne pathogens has also benefited from application of HTS techniques, revealing contamination by pathogenic microorganisms or monitoring their presence in food products (Chaillou et al., 2015, Masoud et al., 2011).

The composition of the microbial community present in dry-cured loins or similar meat products by HTS of 16S rDNA amplicons has not been investigated. However, the utilization of classical microbiological methods has revealed that Staphylococcaceae and Micrococcaceae are the predominant bacterial groups in cured pork loins (Yamanaka, Akimoto, Sameshima, Arihara, & Itoh, 2005), Spanish dry cured ham (Rodriguez et al., 1994) and dry-cured lacón (Lorenzo et al., 2012).

The aim of this study was to evaluate the differences in the microbiota and aroma profile of dry-cured loins manufactured with or without paprika and reduced ingoing amounts of nitrite and nitrate. Paprika, as source of microbiota, and nitrate concentration were taken into account in our study. Differences in microbiota and volatilome due to the origin of the different pork loins were minimized using four loins as biological replicates for each experiment.

2. Material and methods

2.1. Dry-cured loins manufacture

Dry-cured loins were manufactured with (LP) and without (L) paprika addition, containing three different ingoing amounts of nitrate and nitrite, and using four loins as replicates for each formulation (n = 24). Each loin consisting of the whole muscle (*Longissimus thoracis* et *lumborum*) was cut into two pieces of similar size. One piece was prepared with paprika and curing salts and the other piece was prepared only with curing salts. Processing started by rubbing the loin's surface with salt (NaCl 2.4% final), potassium nitrate and sodium nitrite, and optionally with paprika (*Capsicum annuum*) (9 g/kg) from POD (Protected Designation of Origin, Spain) "Pimenton de la Vera". The amount of salt, nitrite and nitrate was calculated per kg of loin weight. Three different type of loins depending on the amount of nitrate and nitrite were prepared: control loins (C) contained 150 mg/Kg of sodium nitrite and 150 mg/Kg of potassium nitrate, loins 25% reduced (N25) contained 113 mg/Kg of nitrite and nitrate, and loins 50% reduced (N50) contained 75 mg/Kg of nitrite and nitrate.

Seasoned loins were kept at refrigeration for 3 d, subsequently were stuffed in collagen casings (10 cm diameter) and kept at 4 °C during 6 d. Afterwards, the loins were dried and maturated at 11 °C and 70–80% relative humidity until a final mean weight loss of 38.5% was reached. At the end of the drying period, 43 d, the loins were placed on the clean bench and the central part of each loin aseptically sliced into pieces. A slice of 25 g was taken and immediately processed for microbiological analysis and DNA extraction. About two to three slices were taken for volatile analysis. These slices were wrapped in aluminium foil, vacuum packed, and stored at −80 °C. An additional slice was taken for colour measurement. Approximately 100 g of dry-cured loin were minced and used for moisture and pH analysis. The remaining loin was minced, vacuum packed and frozen at −20 °C for TBARS, protein, residual nitrate and nitrite, sodium chloride, and free amino acids analysis.

2.2. Physicochemical parameters, amino acids and volatile profile analysis

2.2.1. Physicochemical analysis

Moisture was determined according to the official method for analysis of meat products (BOE, 1979) by dehydration at 100 °C until constant weight. Protein content was estimated measuring nitrogen by the Kjeldahl method and multiplying the nitrogen content by 6.25. pH was measured in minced loin and distilled water (1:1, p/v) (ISO 2917:1999) using a pH meter HI 99,163 (Hanna Instruments Inc.). Colour was determined in a colorimeter (CR-400/410, Konica Minolta Sensing Inc., Japan) with D65 illuminant (Olivares, Navarro, Salvador, & Flores, 2010).

Nitrate and nitrite residual content was determined using the method of Merino (2009) using zinc reduction. Results are expressed as mg/kg of sodium nitrite and sodium nitrate in dry loin (mg/Kg). Lipid oxidation was evaluated using the thiobarbituric acid reactive substances test (TBARS) as described in Corral, Salvador, & Flores (2013). Values were expressed as μg of malonaldehyde (MDA) per gram of loin (mg MDA/Kg). Cations (sodium) were analyzed by ion chromatography (Armenteros, Aristoy, Barat, & Toldrá, 2009). Chloride anion in sample solutions was determined using a Metrohm 761 Compact IC with a Metrohm 833 IC Liquid Handling Suppressor unit to improve the chromatographic signal. The guard column was A-Supp 4/5 (5.0 × 4.0 mm) and the analytical column Supp 5–250 (4.0 × 250 mm). The mobile phase consisted of 1 mM $NaHCO_3$ and 3.2 mM Na_2CO_3 with 30 mL/L acetone. The concentration of each ion was determined from respective calibration curves, using a set of standard solutions of Na^+ and Cl^- (Fluka, Switzerland; Sigma, St. Louis, MO, USA). The results (means of three determinations) were expressed as the percentage of NaCl in dry-cured loins.

2.2.2. Free amino acids examination

Free Amino Acid content in dry-cured loins was analysed by GC-FID using the Phenomenex kit with a Zebron ZBPAAC-MS 10 m × 0.25 mm column (Phenomenex, Torrance, CA, USA). Free amino acids were extracted from 5 g of minced dry-cured loin by homogenization with 20 mL of HCl 0.1 N. The extract was diluted ½ (v/v) and analysed using norvaline as internal standard following the instructions of the kit manufacturer. The derivatized free amino acids were separated by using a GC Agilent Technologies 7890B equipped with an automatic liquid sampler 7693A and a flame ionization detector (Agilent Technologies, Palo Alto, CA, USA). The helium carrier gas flow was constant (27 mL/min) during the run, and the column head pressure was 8.78 psi. The GC oven temperature was initially held at 110 °C and then raised to 320 °C at 32 °C/min. The inlet temperature was 250 °C and the detector was set at 320 °C. Samples of 2.5 μl were injected in split mode (15:1). Identification and quantification of compounds was based on retention times and peak area integration of the reference compounds (Phenomenex standard solutions and Sigma, St. Louis, MO). Calibration curves for each amino acid were obtained with the solutions of standard amino acids provided by Phenomenex at concentrations of 200 mM. In addition, several amino acids were prepared at higher concentrations to adjust the calibration curves to the levels present in meat products. The results were expressed in mg/100 g of dry matter (dm).

2.2.3. Analysis of volatile compounds

The volatile compounds were extracted from the headspace of minced dry-cured loin as described by Campus, Flores, Martinez, &

Toldrá (2008) and analysed using a gas chromatograph Agilent HP 7890 series II GC (Agilent Technologies, Palo Alto, CA, USA) with an HP 5975C mass selective detector (Agilent Technologies, USA) equipped with a Gerstel MPS2 multipurpose sampler (Gerstel, Germany). Extraction of headspace volatile compounds was performed using a solid-phase microextraction (SPME) with an 85 μm Carboxen/Polydimethylsiloxane (CAR/PDMS) fibre for automatic holder (Supelco, Bellefonte, PA, USA). Before each sample analysis, the fibre was preconditioned as indicated by the manufacturer. For each experiment, 4 g of minced dry-cured loin was weighted into a 20 mL headspace vial sealed with a PTFE faced silicone septum and 0.75 mg of BHT (Butyl hydroxy toluene) were added to avoid lipid oxidation. Before mincing, 3 mm of the external layer of the loin slice were removed to avoid the interference of paprika volatiles. Equilibration of the headspace was done maintaining the vial at 37 °C during 30 min. Then, the SPME fibre was exposed to the headspace while maintaining the sample at 37 °C during 3 h. The fibre was baked at 250 °C for 15 min before the extraction procedure. The compounds adsorbed by the fibre were desorbed in the injection port of the GC–MS for 5 min at 240 °C with the purge valve off (splitless mode). The analysis of volatile compounds in the GC–MS was done as described by Campus, Flores, Martinez, & Toldrá (2008). Linear retention index (LRI) of volatile compounds were calculated using the series of n-alkanes (Aldrich, Germany) (Van den Dool & Dec Kratz, 1963). Compounds were identified by comparison to mass spectra from NIH mass spectral database (NIST05), LRI and authentic standards. Authentic samples of the compounds were analysed under the same GC/MS conditions to provide LRI values. Identified volatile compounds were quantified in SCAN mode using either a total or extracted ion chromatogram (TIC or EIC) on an arbitrary scale. Results are expressed as the abundance area of volatile compounds (TIC $\times$ 10^5) per gram of dry-cured loin in dry matter. Each loin was analysed in duplicate.

2.3. Microbiological analysis

Loin samples (25 g) were finely sliced, blended with 225 mL of buffered peptone water (Pronadisa, Madrid, Spain) in a filtered bag (Scharlau, Barcelona, Spain) and homogenized for 30 s in a Pulsifier II (Microgen Biotech, Carlow, Ireland). The filtered homogenized samples were distributed into 5 mL aliquots, frozen using liquid nitrogen and stored at −80 °C until DNA extraction. Additional 1 mL aliquots of sample were used to prepare decimal dilutions which were subsequently spread in triplicates on appropriate media plates for microbial counts. Microbial counts were determined on the following media: total mesophilic bacteria on Plate Count Agar (Pronadisa, Madrid, Spain) at 30 °C for 3 days, lactic acid bacteria (LAB) on MRS Agar (Scharlau, Barcelona, Spain) at 30 °C for 3 days, Gram positive cocci on Mannitol Salt Agar (MSA) (Scharlau, Barcelona, Spain) at 30 °C for 3 to 5 days, Enterobacteriaceae on Violet Red Bile Agar with Glucose (VRBG) (Pronadisa, Madrid, Spain) at 37 °C for 24 h in anaerobiosis, and yeasts and moulds on Rose Bengal Agar with chloramphenicol (Scharlau, Barcelona, Spain) at 30 °C for 5 to 7 days. Microbial counts (CFU/g) found in the loin samples were represented in column charts using Excel (Microsoft, Redmond, USA).

2.4. Analysis of microbiota by 16S rRNA gene amplicon sequencing

2.4.1. DNA extraction

DNA was extracted from 2.5 mL of homogenate using the methodology proposed by Mendoza, Neef, Vignolo, & Belloch (2017) with some modifications. In summary, samples were thawed and centrifuged at 12,000 rpm for 10 min (MiniSpin, Eppendorf, Spain), the pellet was washed with saline solution (0.9% salt) and again centrifuged. Lysis buffer (400 μl) (2% Triton 100X, 1% SDS,100 mM NaCl, 10 mM Tris HCl pH 8, 1 mM EDTA pH 8) was added and vortexed until complete suspension of the pellet, then 0.3 g of glass beads were added and mixed by vortexing. Samples were kept in ice and phenol:chloroform:isoamyl alcohol (25:24:1) (400 μl) at 4 °C was added, the mixture was homogenized three times for 30 s in a Mini BeadBeater 8 (Biospec Products, USA). After homogenization, 400 μl TE (10 mM Tris, 1 mM EDTA pH 7.5) was added and thoroughly mixed by vortexing. Samples were kept in ice for 15 min and centrifuged at 4 °C, 14,000 rpm for 10 min (Centrifuge 5810R, Eppendorf, Spain). After centrifugation approximately 700 μl of the upper layer were collected in a clean tube. When <700 μl were collected, fresh TE (400 μl) (10 mM Tris, 1 mM EDTA pH 7.5) was added to the phenol:chloroform:isoamyl alcohol containing tubes, thoroughly mixed by vortexing and again centrifuged at 4 °C, 14,000 rpm for 10 min. The upper layer was collected in a clean tube. DNA was precipitated adding 1 mL of iced (-20 °C) absolute ethanol and centrifuged at 12,000 rpm for 10 min (MiniSpin, Eppendorf, Spain). Precipitated DNA was washed twice with 70% ethanol and centrifuged at 12,000 rpm for 5 min (MiniSpin, Eppendorf, Spain). All centrifugations were done at room temperature except indicated. DNA was extracted repetitively until three DNAs showing similar quality and quantity measurements in a NanoDrop ND-1000 (Thermo-Fisher, Waltham, USA) were obtained from each loin sample. Subsequently, the three selected DNAs from each sample were pooled for PCR amplification. Paprika samples of 50 mg were taken and DNA extracted and pooled as above.

2.4.2. PCR of the V4-V5 region of the 16S rRNA gene and amplicon sequencing

PCR amplification was carried out using fusion primers consisting of a 33 nucleotide overhang required for MiSeq Illumina sequencing and primers 533F and 907R used for amplification of the target sequence V4-V5 of the 16S rRNA gene (Yang, Wang, & Qiah, 2016). Primer sequence for 533F was 5′-GTGCCAGCMGCCGCGGTAA-3′ and for 907R 5′-CCGTCAATTCCTTTRAGTTT-3′. PCR reactions were carried out in 25 μl final volume containing 2.5 μl of 10x buffer, 100 mM deoxynucleotides, 0.3 mM of each primer, 1.5 mM of MgCl2, 0.15 units of Taq polymerase (Takara Bio Inc., Kusatsu, Japan) and 60 ng of pooled DNA. PCR reactions were setup on ice and then directly heated to denaturation temperature in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). PCR was done under the following conditions: initial denaturation at 94 °C for 2 min, 22 cycles of denaturation at 94 °C for 25 s, annealing at 56.5 °C for 25 s, extension at 72 °C for 20 s, with a final extension at 72 °C for 7 min and a holding step at 4 °C. PCR amplification of each pooled DNA was done in triplicate. A negative PCR control was prepared without DNA template. Presence of the expected DNA band was visualized using 5 μl of each PCR reaction in 1% agarose gel containing RedSafe (iNtRON Biotechnology Inc., Gyeonggi-do, Korea) at 5% dilution in 1xTAE buffer. Fragment length was estimated by comparison with a 100 bp DNA ladder (Invitrogen, Carlsbad, USA). After electrophoresis, PCR products containing the desired band were pooled and concentrated (IR Micro-Cenvac, N-BIOTEC, Gyeonggi-do, Korea) to a 20 μl final volume. PCR products were then visualized by electrophoresis using the same conditions as above, and DNA bands of the expected size range were excised from the gel and purified using the High Pure PCR Purification kit (Roche, Basel, Switzerland). PCR products were analysed by quality, integrity and concentration using a Bioanalyzer 2100 with high-sensitivity chip (Agilent, Santa Clara, USA). Library preparation of paired ends and sequencing was done at the Genomic Service of the SCSIE (University of Valencia, Spain) using a MiSeq (Illumina, San Diego, CA) and sequencing strategy PE250 (paired-ends 2x250). The datasets of raw reads are freely accessible from the EBI database under the project accession number PRJEB36593.

2.4.3. Data processing

Analysis of reads was based on the standard operating procedure for MiSeq data developed for 16S rDNA microbial analysis with mothur (Schloss et al., 2009). In summary, paired-end reads were used to build a list of dataset pairs. Samples representing biological replicates were merged under the same sample name. Pairs of reads, forward and

reverse, were used to make contigs (contiguous sequences). Sequences were filtered by size range (400–420 bp) and absence of base ambiguities. Unique sequences were aligned against an *E. coli* reference of the V4-V5 16S rRNA region and further filtered. Unique sequences that differed by <1 in 100 bases were clustered together and chimeric sequences were removed. Sequences were classified using the Silva reference database (release 138, Dec 2019) (https://www.arb-silva.de/documentation/release-138/) (Quast et al., 2013) and non-bacterial sequences were removed. Sequences were clustered at 3% cut-off and classified into OTUs. The dataset was normalized by sub-sampling all samples down to the sample with the fewest reads. Rarefaction curves and diversity metrics (sobs, ACE, Chao, Simpson, Shannon and Goods' coverage estimators) were calculated. Differences in bacterial community composition between samples was represented in a PCA (Principal Components Analysis) using R. Bacterial composition of the samples was expressed as relative abundance and represented in column charts using Excel (Microsoft, Redmond, USA). OTUs present in ≥ 0.5% of total reads in any sample were selected and the representative sequence of each OTU compared (BLASTN) against the NCBI Reference for 16S rRNA sequences (refseq_rna) for further classification.

2.5. Statistical analysis

Physicochemical data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2018, Addinsoft, Barcelona, Spain). The experimental unit was the loin. The data were analysed using the linear mixed model and included nitrate and nitrate content and paprika addition as fixed effects. When a significant effect in the treatment group was detected ($P < 0.05$), least squares means (LSM) were compared using the Tukey test. In addition, principal component analysis (PCA) was performed to evaluate the relationships among variables (amino acid and volatile compounds contents) and formulations. Heatmaps were done using XLSTAT 2018.

Effect of paprika addition and nitrite/nitrate reduction on microbiota composition, as well as correlations between microbiota and amino acids or volatile compounds were analysed using R (R Core Team, 2013). Pearson's index correlations for co-occurrence were established between the bacterial taxa selected by ≥ 0.5% representation in at least one loin and free amino acids and volatiles. Correlograms were plotted in R.

3. Results and discussion

3.1. Physicochemical parameters, free amino acids and volatile profile analysis

3.1.1. Loins quality parameters

Chemical composition values are shown in Table 1. No significant differences were found due to nitrate and nitrate reduction and/or paprika addition in dry-cured loins. On the other hand, paprika addition affected colour parameters, reducing the luminosity (L*) and increasing the redness (a*) and yellowness (b*) as observed in other meat products (Sánchez-Escalante, Torrescano, Djenane, Beltrán, & Roncales, 2003, Bazan-Lugo, García-Martínez, Alfaro-Rodriguez, & Totosaus, 2012). TBARS values were lower in paprika loins (LP) than in loins without paprika (L) supporting the antioxidant effect of paprika observed in dry sausages (Aguirrezábal, Mateo, Domínguez, & Zumalacárregui, 2000). Whereas residual nitrite levels were very low, residual nitrate levels were high. Residual nitrate concentration seemed to be related to the ingoing amount of nitrate and nitrite mixtures (Gratacós-Cubarsí et al., 2013), as it can be generated from the oxidation of nitrite to nitrate by microbiological means (Honikel et al., 2008; Mainar et al., 2017). Moreover, in LP loins the concentration of nitrate was higher than in L loins indicating the paprika contribution to the overall nitrate content. Analysis of nitrate content in paprika revealed the presence of 1888 mg/Kg of sodium nitrate. Therefore, the quantity of paprika added to each loin (9 g paprika/Kg) contributed with 17 mg/Kg of sodium nitrate to the LP loins.

3.1.2. Free amino acids content

Regarding the free amino acids content in dry-cured loins (Fig. 1; Table S1), no significant differences were found due to nitrite/nitrate reduction except for threonine, which was significantly higher in the LP-N50 loins. On the other hand, a significant increase in the majority of free amino acids was found in LP loins. This increase of the total amino acid content in LP loins with respect to the L loins was of 26, 25 and 49 % in LP-C, LP-N25 and LP-N50, respectively. The higher concentration of free amino acids in LP loins could be explained by the presence of free amino acids in paprika (Cremer & Eichner, 2000). The analysis of free amino acids in the paprika used for manufacturing of the LP loins yielded 1216 mg/100 g paprika, meaning that the contribution of paprika (9 g paprika/kg loin) to free amino acids content in LP loins was about 10.9 mg amino acids/100 g loin. However, the mean difference in free amino acids between L and LP loins was 523 mg/100 g loin (dm).

Table 1
Physicochemical parameters of dry cured loins L (without paprika) and LP (with paprika) manufactured with different amounts of curing agents.

	L loins						LP loins									
	L-C[1]		L-N25		L-N50		LP-C		LP-N25		LP-N50		RMSE[2]	P_N[3]	P_P	P_{NxP}
Moisture (%)	51.29		55.07		50.70		57.17		57.31		56.76		3.39	ns	ns	ns
Protein (%)	35.35		33.59		34.77		33.85		34.08		35.66		2.16	ns	ns	ns
pH	5.58		5.53		5.49		5.61		5.58		5.63		0.10	ns	ns	ns
L*[4]	57.29		56.27		56.31		53.55		52.50		52.43		2.26	ns	***	ns
a*	19.62		19.12		18.93		20.82		20.42		19.51		0.98	ns	*	ns
b*	9.21	ab	8.00	c	8.29	bc	10.07	a	9.21	ab	9.86	a	0.51	**	***	ns
TBARS (mg MDA/kg)	0.47	a	0.42	ab	0.36	bcd	0.28	d	0.32	cd	0.39	abc	0.04	ns	***	***
NaCl (%)	3.42		3.14		3.73		3.39		3.18		3.15		0.38	ns	ns	ns
NaNO2 (mg/Kg)	0.06		0.24		0.18		0.30		0.00		0.41		0.29	ns	ns	ns
NaNO3 (mg/Kg)	157.05	a	111.20	bc	72.00	c	180.62	a	140.04	ab	94.03	c	29.60	***	*	ns

[1] C: Nitrate loins manufactured without reduction in curing agents (150 mg/Kg KNO3 and 150 mg/Kg NaNO2), N25: loins manufactured with 25% reduction in curing agents (113 mg/Kg and KNO3 and 113 mg/Kg NaNO2) and N50: loins manufactured with 50% reduction in curing agents (75 mg/Kg and KNO3 and 75 mg/Kg NaNO2).

[2] RMSE: Root Mean Square Error.

[3] P_N: *P* value of nitrite and nitrate reduction, P_P: *P* value of paprika addition, P_{NxP}: *P* value of interaction between nitrite and nitrate reduction and paprika addition.
*** : $P < 0.001$;
** : $P < 0.01$;
* : $P < 0.05$; ns: P greater than 0.05. Different letters in the same row indicate significant differences among dry cured loins.

[4] Color parameters: L*, a* and b*.

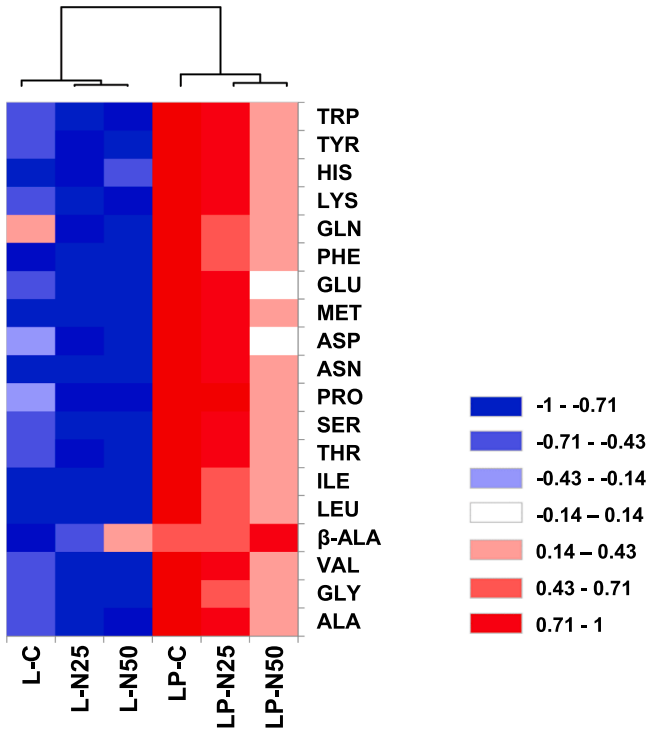

Fig. 1. Heatmap representing differences in free amino acid concentration (mg/100 g of dry matter) between L and LP loins. L-C and LP-C loins were manufactured without reduction in curing agents (150 mg/Kg KNO_3 and 150 mg/Kg $NaNO_2$), L-N25 and LP-N25 loins were manufactured with a 25% reduction in curing agents (113 mg/Kg KNO_3 and 113 mg/Kg $NaNO_2$), and L-N50 and LP-N50 loins were manufactured with a 50% reduction in curing agents (75 mg/Kg KNO_3 and 75 mg/Kg $NaNO_2$).

Consequently, only a small percentage of free amino acids in LP loins could be explained by paprika addition. The higher concentration of amino acids in LP loins with respect to L loins could be alternatively explained by a distinctly active proteolysis during the manufacturing process, namely the proteolytic activity of the microbiota in paprika (Molnár et al., 2018, Armenteros, Aristoy, Barat, & Toldrá, 2009, Zhou et al., 2017)

3.1.3. Volatile compounds profile

The production of volatile organic compounds (Total VOCs) in the dry-cured loins was strongly affected by paprika addition (Fig. 2; Table S2). The 57 identified volatile compounds (VOCs) could be classified by their origin from lipid autoxidation, amino acid catabolism, carbohydrate fermentation, microbial esterification and lipid beta-oxidation (Corral, Salvador, & Flores, 2013). Most VOCs of microbial origin were present in higher amount in LP than in L loins with the exception of those produced during microbial esterification. The antioxidant effect of paprika is appreciable in the lower TBARS (Table 1), which is in agreement with the lower abundance of VOCs originating from lipid autoxidation in LP loins. Exceptions were found in case of 1-butanol and propanoic acid, which concentrations were higher in all LP loins. The concentration of VOCs generated by catabolism of amino acids, carbohydrates fermentation and lipid beta-oxidation was higher in LP loins, suggesting a probable role of the paprika microbiota in VOCs generation (Molnár et al., 2018). The most significant exception was 1-penten-3-ol which was found in higher amounts in L loins.

3.2. Viable counts of microorganisms

Bacteria colony counts in all media were significantly higher in LP respect to L loins, but no differences were found in case of fungi counts (Fig. 3). Counts of aerobic mesophilic bacteria, salt tolerant bacteria and lactic acid bacteria in L loins were higher than those observed by Aliño et al. (2010). Colony counts of yeasts in LP loins are comparable with those found by other authors (Campus, Flores, Martínez, & Toldrá, 2008, Higuero, Moreno, Lavado, Vidal-Aragón, & Cava, 2020). Differences in colony counts between LP and LP-N25 or LP-N50 loins were not significant, as previously found by Higuero, Moreno, Lavado, Vidal-Aragón, & Cava (2020). Differences in bacteria colony counts between L and LP loins could be mainly attributed to the addition of paprika (Table S3), although proliferation of mesophilic bacteria was favoured by reduction of nitrite and nitrate concentration. Bacteria counts were higher in the paprika sample except for lactic acid bacteria and fungi.

Lactic acid bacteria counts were significantly higher in LP than L loins, although these bacteria were absent in paprika. The origin of these bacteria must be found in the environment of the pilot plant (Talon, Leroy, & Lebert, 2007), and the higher counts in LP loins could be due to the reducing microenvironment in the surface of the loins coated with paprika. The only related physicochemical parameter different between L and LP loins supporting our findings is TBARS, indicative of lipid oxidation, which was lower in LP loins (Table 1). Moreover, several species in the genus *Lactobacillus* display a significant reduction capability (Brasca, Morandi, Lodi, & Tamburini, 2007). These factors, together with the paprika acting as a physical barrier for oxidation, could explain the higher counts of lactic acid bacteria in LP loins.

Enterobacteriaceae counts were significantly higher in LP loins, which is in agreement with the elevated count of Enterobacteriaceae in paprika (Fig. 3). Moreover, in both L and LP loins Enterobacteriaceae counts were higher in loins reduced in nitrite and nitrate, although these differences were not significant. Nevertheless, these findings would confirm previous studies regarding the influence of nitrite and nitrate reduction in Enterobacteriaceae counts (Hospital et al., 2015).

3.3. Analysis of microbiota in loins and paprika

A total of 1,099,322 raw sequences from the amplified V4-V5 region of the 16S rRNA gene were obtained by MiSeq sequencing of total DNAs extracted from dry-cured loins (Table S4). From these, 776,350 reads (70.6%) passed the cleaning filters applied using the MiSeq SOP of mothur (https://www.mothur.org/wiki/MiSeq_SOP). Removal of lineages other than bacteria identified a large number or reads (69,206, 8.9% of all reads) pertaining to the chloroplast lineage in LP loins (92% of chloroplast reads) originating from the paprika used in the manufacturing of these loins. The large number of chloroplast reads removed from the analysis of microbiota in LP loins explains the lower numbers of bacterial high quality reads (HQR-B) in these samples. Similar percentage of reads classified as chloroplast have also been reported in the analysis of microbiota from spiced poultry sausages (Chaillou et al., 2015).

3.3.1. Bacterial richness and diversity

Differences in the number of HQR-B reads between samples has a significant impact on alpha diversity indices (Kim et al., 2017), therefore these were estimated on a subsample of 3988 reads corresponding to the lowest number of reads in the paprika sample (Table S4). Alpha diversity and evenness estimators indicated that the microbial community in LP loins and paprika was much more diverse and richer than in L loins. The low Shannon index estimated for L loins indicate that most reads belong to a few sequence types. Differences in bacterial community composition (beta diversity) between loins supports these findings (Fig. 4). L loins clustered together demonstrating that differences in microbiota composition were not significant. On the contrary, large differences in beta diversity were found among LP loins. Those containing the normal amount of nitrite and nitrate (LP-C) clustered close to L loins indicating a similar bacterial community composition. Moreover, the intermediate Shannon index (alpha diversity) (Table S4) in LP-C loins is consistent with a lower diversity than in LP-N25 and LP-N50 loins. This would

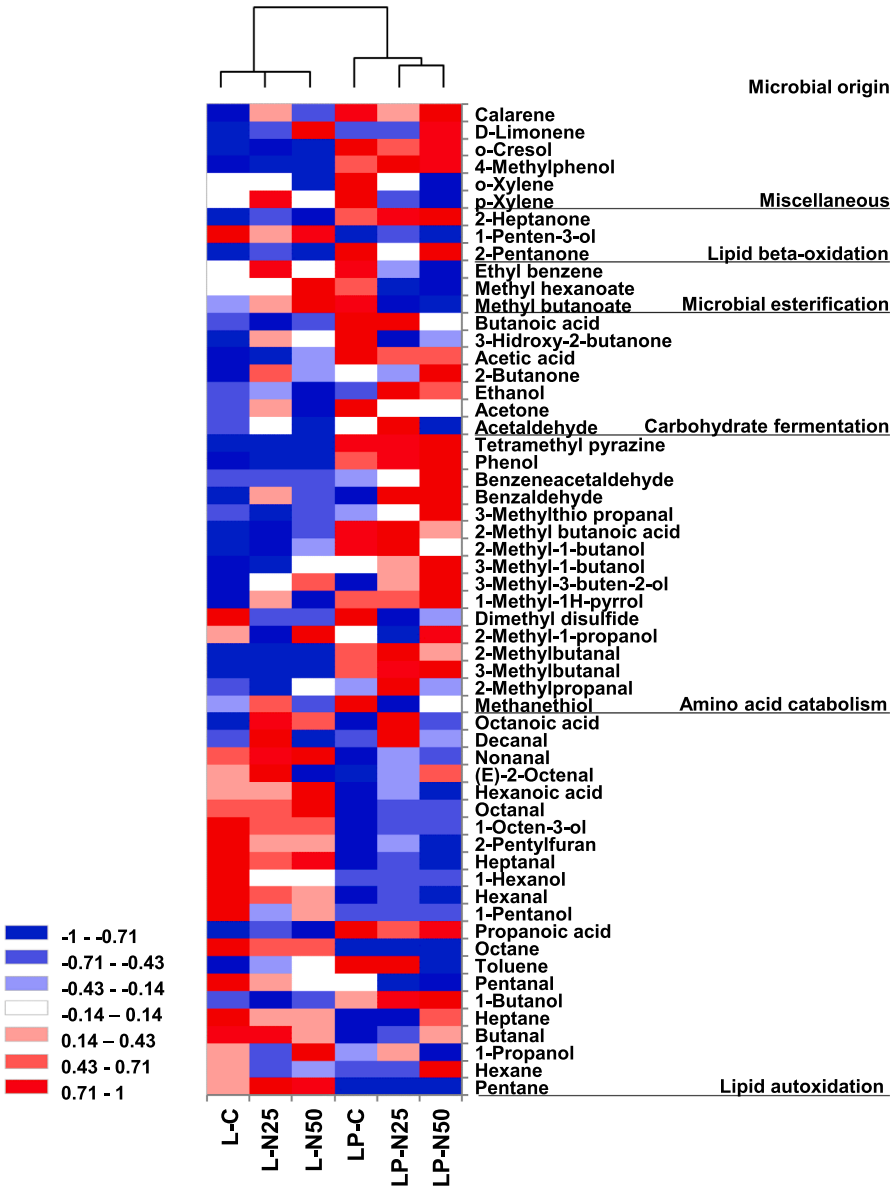

Fig. 2. Heatmap representing differences in VOCs generation (abundance area TIC × 10^5 per gram of dry matter) between L and LP loins. L-C and LP-C loins were manufactured without reduction in curing agents (150 mg/Kg KNO_3 and 150 mg/Kg $NaNO_2$), L-N25 and LP-N25 loins were manufactured with a 25% reduction in curing agents (113 mg/Kg KNO_3 and 113 mg/Kg $NaNO_2$) and L-N50 and LP-N50 loins were manufactured with a 50% reduction in curing agents (75 mg/Kg KNO_3 and 75 mg/Kg $NaNO_2$). Volatile compounds are grouped by their origin.

indicate that in LP-C loins the standard amount of nitrite and nitrate controls microbial growth. On the contrary, reduction of nitrite and nitrate as in LP-N25 and LP-N50 loins favours proliferation of the autochthonous microbiota. The highest Shannon values in these loins (Table S4) and their separated position in the PCA graph (Fig. 4) indicate that, in addition to the paprika microbiota introduced, the low nitrate and nitrite conditions favour growth of different microbiota compared to L-C loins or paprika. Finally, sequencing depth was assessed calculating the coverage and rarefaction curves. The high coverage values for all samples indicated that the majority of microbiota in the loins is represented in the results (Table S4). The rarefaction curves (Figure S1) showed that in L and LP-C loins saturation was approached faster than in paprika and LP loins with reduced nitrite and nitrate concentration.

3.3.2. *Composition of the bacterial community*

Applying a 97% sequence similarity criterion for OUT definition against the SILVA 138 release as reference data set, 776,350 high quality bacterial reads of the V4-V5 16S rRNA region were classified into 4,586 distinct OTUs (Table S5). A summary of OTU number and percentage of bacterial high quality reads by sample type classified and unclassified into taxa, number of phyla and genera per loin type is shown in Table 2. The number of phyla and genera was consistently much higher in LP than in L loins (Table S6). The number of OTUs classified up to genera level accounted for more than 98% of reads in L loin samples and more than 90% in LP loin samples (Table 2; Table S7). Unclassified OTUs (ucOTUs) represented a very low percentage of reads in all loins, in total just 1.96% of all reads in 4047 ucOTUs (Table 2; Table S8), and it was higher in LP (average 6.55%) than in L loins (average 0.96%).

The most abundant phylum found in all samples, loins and paprika, was *Firmicutes* which represented in average more than 90% of reads in L and LP loins and paprika (Fig. 5; Table S6). In pure loins without paprika it comprised more than 99.9% of the microbiota. Other phyla, namely Proteobacteria, Actinobacteria and Bacteroidota, were present in most loins but in lower percentages and represented the major differences between L and LP loins (Fig. 5). The remaining phyla were represented by a small number of OTUs and a very small percentage of reads (Table S6). These results are in agreement with previous studies showing Firmicutes and Proteobacteria among the most abundant phyla in bacterial communities of meat products (Juárez-Castelán et al., 2019, Chen, Fegana, Kocharunchittb, Bowman, & Diffy, 2020).

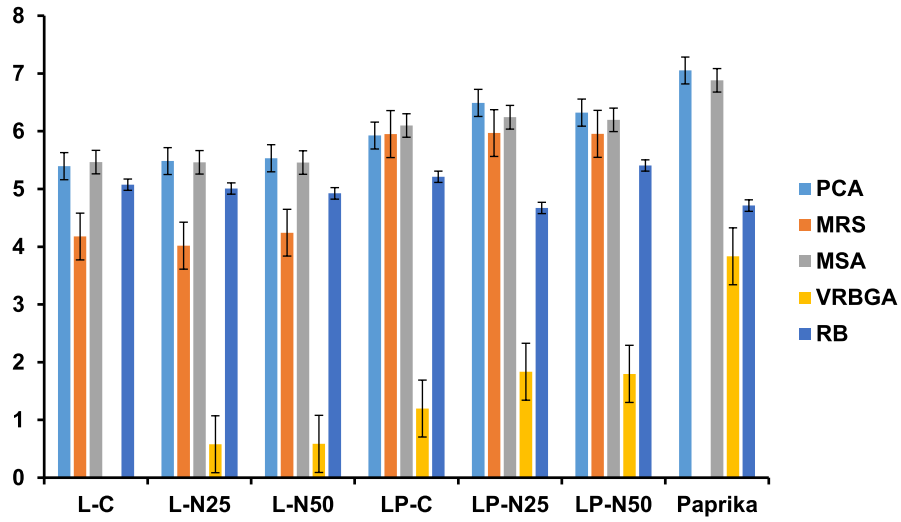

Fig. 3. Microbial counts (CFU/g) in the loin and paprika samples. Colonies were counted in plates of PCA, MRS, AMS, VRBGA and RB at the end of the loins manufacturing process.

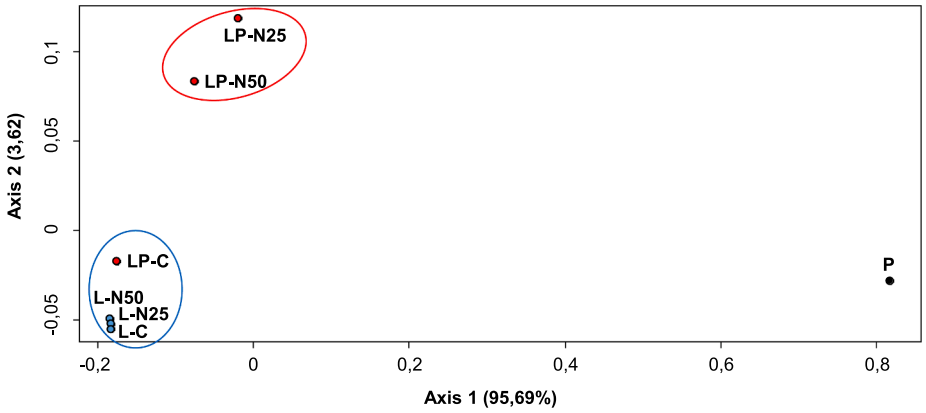

Fig. 4. PCA analysis showing the similarity in OTU composition between samples L and LP at different concentrations of nitrite and nitrate paprika (P). L-C and LP-C loins were manufactured without reduction in curing agents (150 mg/Kg KNO_3 and 150 mg/Kg $NaNO_2$), L-N25 and LP-N25 loins were manufactured with a 25% reduction in curing agents (113 mg/Kg KNO_3 and 113 mg/Kg $NaNO_2$) and L-N50 and LP-N50 loins were manufactured with a 50% reduction in curing agents (75 mg/Kg KNO_3 and 75 mg/Kg $NaNO_2$).

Table 2
Number of OTUs per sample classified or not classified into genera, percentage or reads in each category, and number of phyla and genera found in each sample.

	L.C	L. N25	L. N50	LP.C	LP. N25	LP. N50	Paprika
OTUs	1594	752	865	1098	817	520	148
OTUs cG [1]	22	51	36	252	302	179	59
%HQR-B cG [2]	99.13	99.10	98.88	97.60	90.30	92.45	93.15
OTUs ucG [3]	1572	701	829	846	515	341	89
%HQR-B ucG [4]	0.87	0.90	1.12	2.40	9.70	7.55	6.85
Phyla	4	6	5	12	18	12	5
Genera	8	32	19	129	151	99	32

[1]OTUs cG, number of OTUs classified at genus level, [2]%HQR-B cG, percentage of bacterial high quality reads representing OTUs cG, [3]OTUs ucG, number of OTUs not classified at genus level, [4]%HQR-B ucG, percentage of bacterial high quality reads representing uCG OTUs.

Bacterial diversity in loins was further investigated at genus level (Fig. 6a). Initially, 13 OTUs complied with the ≥ 0.5% abundance selection criteria (Table S9). Four of these OTUs were classified only at higher taxon level than genus using the SILVA 138 database. Blasting (BLASTN) of the sequence representing each OTU against the Reference rRNA sequences database at NCBI reclassified three OTUs at genus level. Only one OTU representing the Enterobacteriaceae remained classified at family level. Most genera were represented by only one OTU except for *Staphylococcus* and *Bacillus* represented by 2 OTUs (Table S9). Finally, bacterial diversity in loins was reduced to 11 taxa comprising in total between 88% and 98% of the total reads (Table S9). The by far most abundant genus in all loins was *Staphylococcus* followed by *Bacillus*. The genus *Staphylococcus* is normally found in meat products manufactured without starter addition, but its presence has been associated with environmental contamination (Stellato et al., 2016). *Bacillus* was the most abundant genus found in paprika, confirming previous results by Molnár et al. (2018). Moreover, *Bacillus* is usually not found in meat products (Greppi et al., 2015), except in paprika spiced sausages (Juárez-Castelán et al., 2019). Fig. 6a shows that *Staphylococcus* was dominant in L and LP-C loins, whereas in the LP-N25 and LP-N50 loins a co-dominance of *Staphylococcus* and *Bacillus* occurred. In LP-C loins, the low percentage of *Bacillus* and other genera present in paprika suggested that the high amount of nitrite and nitrate in these loins was a limiting factor for their growth. In accordance, the decrease in curing agents in LP-N25 and LP-N50 loins improved growth of *Bacillus* and other genera.

A correlation analysis between factors "paprika addition" and "nitrite/nitrate reduction" and the abundance of each bacteria is represented in Fig. 6b. Paprika addition appeared highly correlated with a decrease of *Staphylococcus* and the rise of other bacterial genera resulting in an increase of the microbiota diversity (Table S4). The highest concentration of nitrite and nitrate was negatively correlated with the presence of all bacterial genera except *Staphylococcus*. A clear

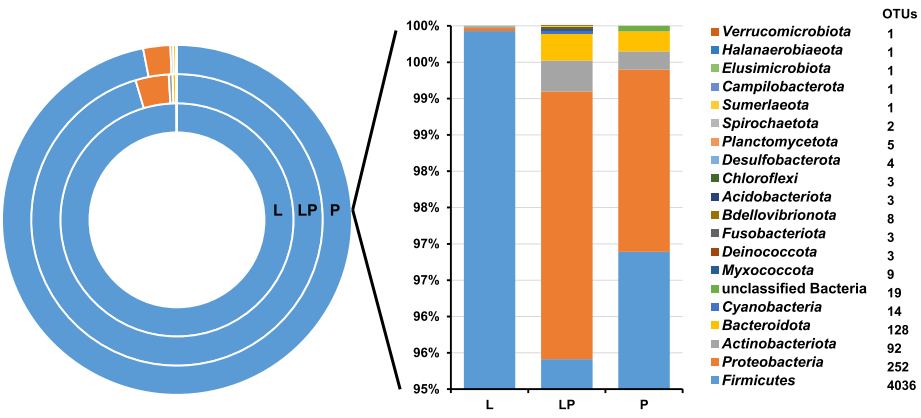

Fig. 5. Relative abundance of OTUs at phyla level in L and LP loins and paprika (P). Donut chart represents 100% of the reads, and column chart represents the upper 5% (95–100%) of the reads.

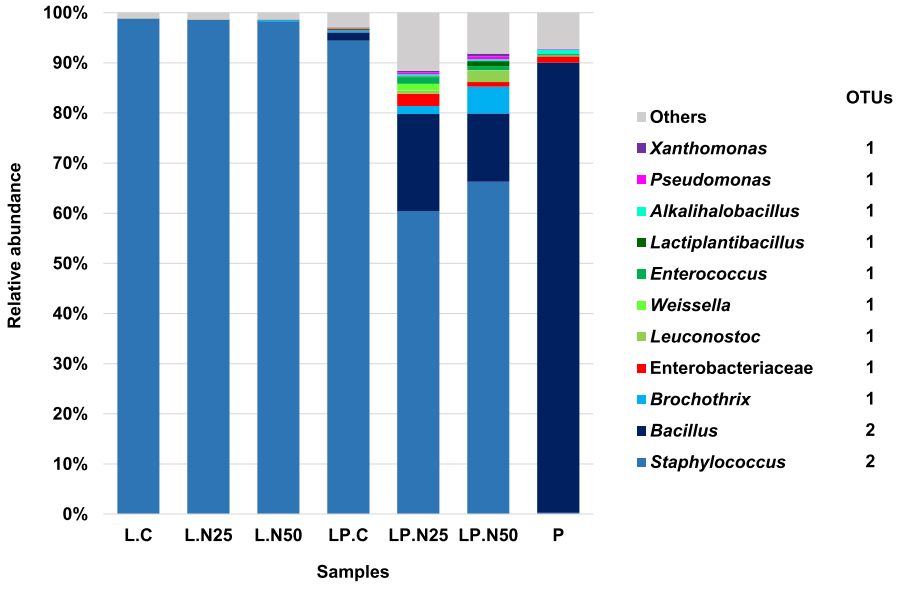

Fig. 6a. Relative abundance of OTUs at genus level (except in the case of the family Enterobacteriaceae) in L and LP loins and paprika (P). OTUs showing relative abundance ≥ 0.5% of reads in at least one sample were taken into account. The remaining OTUs are grouped in "Others".

correlation appeared to exist between nitrite and nitrate reduction and the occurrence of beneficial lactic acid bacteria *Leuconostoc* and *Lactiplantibacillus* (Fig. 6b; Table S9). Lactic acid bacteria, frequent starters in sausages (Polka, Rebecchi, Pisacane, Morelli, & Puglisi, 2015, Greppi et al., 2015), were above 0.1% only in LP loins and paprika (Fig. 6a). Moreover, the correlation between paprika addition and lactic acid bacteria was also positive (Fig. 6b; Table S9). This indicates that both factors, paprika addition and nitrite/nitrate reduction, favour growth of lactic acid bacteria.

Correlation analysis also indicated that an elevated percentage of *Staphylococcus* in loins appeared to be strongly and negatively correlated with *Bacillus, Enterococcus* and other genera. On the contrary, the increase of *Bacillus* in LP loins was associated with the increase of Enterobacteriaceae, *Enterococcus*, *Alkalihalobacillus*, *Pseudomonas* and *Xanthomonas*. Genus *Brochothrix,* frequently associated with spoilage in meat products (Stellato et al., 2016), was predominantly present in LP loins and more abundant in nitrite and nitrate reduced LP loins (Fig. 6a). Moreover, this correlation analysis confirms that both factors, paprika addition and nitrite/nitrate reduction, might play a role in the proliferation of these bacterial groups (Fig. 6b).

3.3.3. *Correlation between microbiota, free amino acids and VOCs*

Analysis of free amino acids in loins had revealed higher amounts of most amino acids in LP loins (Fig. 1). Additionally, most VOCs originated by microbial metabolism seemed to be more abundant in LP loins (Fig. 2). Taking these results into account, we studied the correlation between free amino acids and VOCs concentration and the abundance of different bacterial genera (Figs. 7a and 7b).

The correlogram in Fig. 7a indicated that paprika addition was strongly associated with an increase of free amino acids. Previous analysis of free amino acids in paprika showed that this might not be a result of paprika addition by itself, but the consequence of the proteolytic activity of the paprika-introduced microbiota in the LP loins. Correlation analysis between microbiota and free amino acids seems to confirm this hypothesis, as most bacteria associated with paprika addition in LP loins (Fig. 6b) present a high correlation with free amino acids. Previous studies seem to corroborate this idea as *Bacillus*, the most abundant genus in paprika (Molnár et al., 2018), is one of the most important bacterial sources of proteases (Contesini, Rodrigues de Melo, & Harumi Sato, 2018). On the contrary, *Staphylococcus* was inversely correlated with free amino acids.

Several *Staphylococcus* species are added as starters due to their ability to transform branched-chain amino acids into flavour compounds

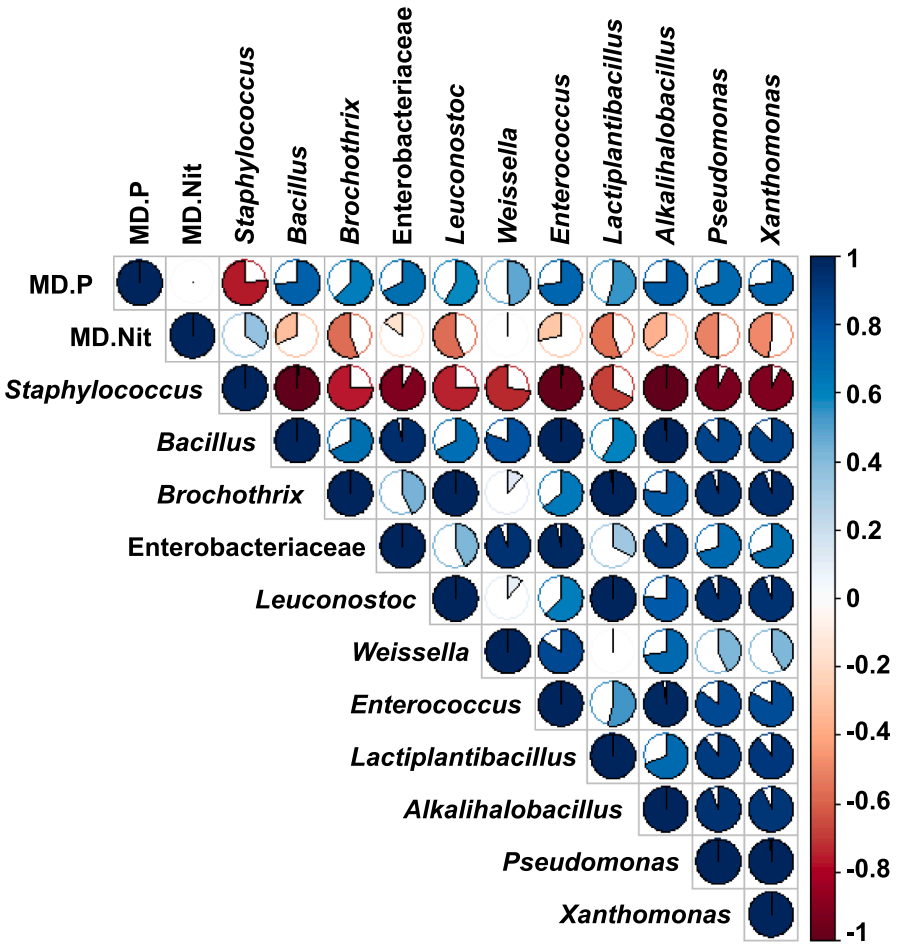

Fig. 6b. Correlogram showing the Pearson's index correlation among the genera showing relative abundance $\geq$ 0.5% of reads in at least one sample. MD.P and MD.Nit are the factors "paprika addition" and "nitrate reduction" affecting the microbiota composition in the loins. The Pearson's index correlation ranges from −1 to 1, with strong positive and strong negative correlation indicated in blue and red, respectively. Degree of circle filling is proportional to the index value, e.g., half-filling corresponds to values of −0.5 and 0.5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

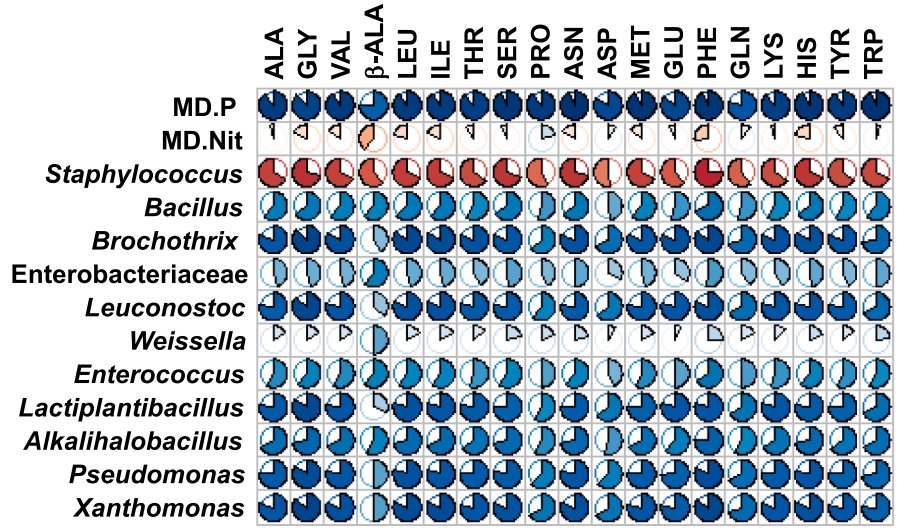

Fig. 7a. Correlogram showing the Pearson's index rank among the genera showing relative abundance $\geq$ 0.5% of reads in at least one sample and free amino acids amounts detected in each sample. Degree of circle filling is proportional to the index value, e.g., half-filling corresponds to values of −0.5 and 0.5 MD.P and MD.Nit are the factors paprika addition and nitrate reduction.

(Talon, Leroy, & Lebert, 2007, Cocolin, Dolci, & Rantsiou, 2011, Mainar et al., 2017). Our results show that *Staphylococcus* was related to the generation of compounds derived from lipid oxidation and microbial esterification (Fig. 7b), but a positive correlation in case of VOCs generated from amino acid metabolism was not found. A positive correlation was found between aldehydes and alcohols derived from amino acid metabolism and genera *Leuconostoc* and *Lactiplantibacillus* among others. This would be in agreement with previous findings by Papamanoli, Tzanetakis, Litopoulou-Tzanetaki, & Kotzekidou (2003) about the important role of lactic acid bacteria in aroma generation from

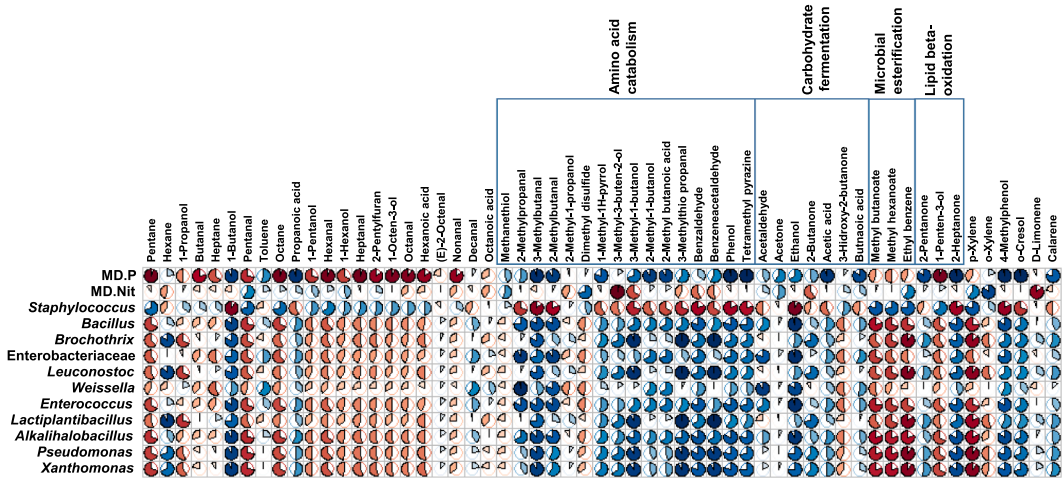

Fig. 7b. Correlogram showing the Pearson's index rank among the genera showing relative abundance ≥ 0.5% of reads in at least one sample and VOCs detected in each sample. VOCs are classified according to their origin. Degree of circle filling is proportional to the index value, e.g., half-filling corresponds to values of −0.5 and 0.5. MD.P and MD.Nit are the factors paprika addition and nitrate reduction.

amino acids. Moreover, *Leuconostoc* has been associated with carbohydrates utilization (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015), which is in agreement with the generation of ethanol and other fermentative VOCs by this genus (Fig. 7b).

3.4. Conclusions

Paprika addition did not have a direct effect on the general physicochemical parameters of dry-cured loins, however, it strongly impacted bacterial community composition and VOCs generation. Free amino acid concentration and VOCs generation was different in loins without and with paprika (L and LP). The higher concentration of free amino acids in LP with respect to L loins indicated a higher proteolytic activity in LP loins probably due to the proliferation of paprika microbiota. The higher concentration of VOCs, derived from amino acid catabolism, carbohydrate fermentation and lipid beta oxidation, in LP loins clearly indicated a decisive role of the paprika-derived microbiota in VOCs generation. Bacterial community composition analysis by amplicon sequencing of region V4-V5 16S rDNA indicated higher species richness and diversity in LP relative to L loins. Microbiota of loins was strongly affected by paprika addition and additionally by nitrite and nitrate reduction. Interestingly, this nitrite/nitrate reduction effect was differently pronounced in L versus LP loins. In the L-N25 and L-N50 samples compared to the L-C samples, staphylococci shrank from approximately 99 to 98.5 and 98.2%, accompanied by an increase of Proteobacteria and Actinobacteria by a factor of 5 to about 0.05%, whereas Bacteroidota appeared at abundances of up to 0.001% (Tab S5 and Tab S6). In LP loins the effect of nitrite/nitrate reduction was much more profound. *Staphylococcus* abundance decreased from 94% in LP-C loins to values between 60% and 65% in LP-N loins. Microbiota in both LP-N loins was characterised by a strong rise of *Bacillus*, the dominant genus in paprika. Thus, the reduction in nitrite and nitrate concentration affected the composition of bacterial microbiota only minimally in L loins, but did it profoundly in LP loins. The combined effect of paprika addition, as a kind of "seeder effect", and nitrite and nitrate reduction acted in a modulating way. Differences in microbial community composition between L and LP loins shaped clearly generation of free amino acids and VOCs. *Staphylococcus* was correlated with VOCs generated by microbial esterification. Lactic acid bacteria, present in larger percentages in LP loins, were related to generation of VOCs derived from amino acid metabolism.

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CRediT authorship contribution statement

Carmela Belloch: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Alexander Neef:** Formal analysis, Writing – review & editing. **Clarissa Salafia:** Investigation. **José Javier López-Diez:** Investigation. **Mónica Flores:** Conceptualization, Formal analysis, Writing – review & editing, Supervision.

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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110691.

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