Unfolding microbiota and volatile organic compounds of Portuguese Painho de Porco Preto fermented sausages

(Article begins on next page)

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Unfolding microbiota and volatile organic compounds of Portuguese *Painho de Porco Ibérico* fermented sausages

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Abstract

In the present study, bacterial and fungal diversity, as well as volatile profiles, of ready-to-eat Portuguese *Painho de Porco Ibérico* fermented sausages manufactured by two artisanal producers in the districts of Beja (producer A) and Evora (producer B) were studied. To this end, different selective growth media and a metataxonomic analysis were joined with Headspace-Solid Phase Micro Extraction-Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS). The results of the microbiological viable counts revealed active microbial populations of lactic acid bacteria (up to 8 Log cfu g^{-1}), coagulase negative cocci (up to 6 Log cfu g^{-1}), and eumycetes (up to 6 Log cfu g^{-1}). Bacterial populations were characterized by high relative frequencies of *Latilactobacillus sakei* (up to 72%) together with *Weissella* and *Staphylococcus equorum*. The mycobiota was mainly dominated by *Debaryomyces hansenii* (up to 55% of the relative frequency) and *Kurtzmaniella zeylanoides* (up to 24% of the relative frequency). Unexpected species as *Wickerhamomyces subpelliculosa* and *Zygosaccharomyces rouxii* were also detected. HS-SPME-GC/MS analysis allowed to identify a complex volatile profile showing more than 160 volatile organic compounds (VOCs). VOCs belonged to twelve classes, such as aldehydes, ketones and lactones, esters and acetates, alcohols, terpenoids, sulfur compounds, aliphatic hydrocarbons, aromatic hydrocarbons, nitrogen compounds, acids, furans and pyrans, and phenols. The analysis of VOCs composition provided evidence that samples from the two producers (A and B) were different, as confirmed by the Principal Component Analysis. Hence, it is likely that, although the production processes applied by the two producers were those commonly applied for the manufacturing of *painho*-type sausages, the environmental conditions, the raw materials used, as well as variations related with the empirical practice of the butchers, strongly influenced the final product. The results obtained in the present study represent a further advancement in the knowledge on the biodiversity and VOCs composition of Portuguese fermented sausages. To better understand the interactions occurring between the autochthonous microorganisms and the meat batter in *Painho de Porco Ibérico* fermented sausage, microbial and VOCs dynamics must be further deepened throughout the production process.

1. Introduction

Ethnic foods represent the core of the European gastronomy. Among these foods, traditional fermented sausages constitute part of the main diet in many European countries. The production of fermented sausages is still carried out on the basis of ancient recipes that dates back to the medieval times (Belleggia et al., 2020a). Indeed, during that period, the art of charcuterie was born based on the need to overcome spoilage of meat in the absence of refrigeration. Hence, on the basis of empirical procedures, meat (commonly from swine) was cut into small pieces, added with lard, salt and spices, stuffed into animal casings and left to ferment. The obtained sausage was stable at room temperature being characterized by low water content and acidic taste.

Although the ancients knew how to make delicious fermented meat products, what they did not know was that these delicacies were the result of complex interactions between the meat matrix, the added ingredients, the microorganisms naturally present in the raw materials, and the environmental conditions applied during fermentation (e.g., smoking, etc.) and ripening of the sausages.

Indeed, the modifications taking place during the fermentation of the stuffed meat batter are strongly influenced by the native meat enzymes coupled with microbial metabolic activities carried out mainly by lactic acid bacteria, coagulase negative cocci, end eumycetes (Kumar et al., 2017; Leroy, Geyzen, Janssens, De Vuyst, & Scholliers, 2013). The study of microbial populations occurring in traditional fermented sausages could represent a valid strategy for understanding their origin, thus preserving their typical features.

Today, charcuterie products represent some of the most known specialties in southern European countries as Italy, Spain, and Portugal. In this latter country more than 50 traditional meat products, including 26 major types of traditional/ethnic sausages, bagged hams, and other traditional meat products are manufactured (Marcos, Viegas, de Almeida, & Guerra, 2016). Among these, 36 are awarded with the Protected Geographical Indication (PGI) and 2 with the Protected Designation of Origin (PDO) status (Marcos et al., 2016). As reviewed by Marcos et al. (2016), Portuguese traditional sausages are mostly produced in the districts of Vila Real and Bragança (Northern region of Portugal) and in the districts of Evora, Beja, and Portalegre (Southern region of Portugal). The most known Portuguese sausages are represented by *alheira, cacholeira, chouriça, chouriço de sangue, chouriço preto, linguiça, morcela, paio preto*, and *painho* (Marcos et al., 2016).

Surprisingly, the microbiota and volatile compounds that characterize Portuguese fermented sausages are almost uninvestigated, thus justifying the need for in depth studies.

The *painho*-type fermented sausage is usually produced in the districts of Portalegre, Évora and Beja using swine meat and fat. In some municipalities, the addition of spices and white or red wine may confer to *painho* its peculiar sensory characteristics. Regarding the manufacturing process, differences in the resting time of the meat batter and in the smoking/drying phase may occur, thus leading to different end products. In more detail, the main meat cuts used in the manufacture of *painho* fermented sausages produced in the Evora district are usually swine loin, tenderloin, leg, neck, cheek, and tong. Moreover, salt, garlic, pepper paste, orange, white/red wine, and spicy paprika can be added to the meat batter. After mixing the raw materials, a resting time from 1 to 6 days follows. Then, the meat batter is stuffed into a fresh, thick pork casing and smoked/dried at low heat obtained with cork or holm oak wood for a period that ranges from 8 to 90 days. The *painho* fermented sausages manufactured in the Beja district are usually produced with swine leg, neck, lean, loin, tenderloin, and cheek. Other ingredients as salt, garlic, pepper paste, white wine, and sweet paprika can be added to the meat batter. The mixing of raw materials is followed by a resting time from 1 to 8 days. Then, the meat batter is stuffed into fresh pork casing and smoked/dried at low heat obtained with cork or holm oak wood for a period from 30 to 40 days.

The end product is a cylindrical and reddish-brown salami with a rosy-red slice. The length of the sausage varies between 15 and 20 cm, with a diameter of approximately 70 mm, and weight ranging from 550 and 600 g.

To the authors' knowledge, no studies dealing with the microbiota and the volatile organic compounds (VOCs) of *Painho de Porco Ibérico* fermented sausage are available in the scientific literature. Hence, the aim of the present study was to obtain information on the bacterial and fungal diversity as well as volatile profiles of different batches of ready-to-eat *Painho de Porco Ibérico* fermented sausage manufactured by two artisanal producers located in the districts of Beja (Producer A) and Evora (Producer B). To this end, different selective growth media and a metataxonomic analysis were applied to study microbial populations, whereas VOCs were investigated by Headspace-Solid Phase Micro Extraction-Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS).

2. Materials and methods

2.1. Sampling

Twelve samples of ready-to-eat Portuguese *Painho de Porco Ibérico* (Figure 1) were purchased from two producers located in Portugal. For each producer, Portuguese fermented sausage samples were collected from three different production batches (two samples for each production batch). The samples of producer A were codified from P1 to P6 and were manufactured using the following ingredients: pork meat and fat (91.3%), salt, garlic, pepper, bell pepper, dextrose, dextrin, black pepper, pepper oleoresin, antioxidants (E300, E301) and preservatives (E252, E250). The samples of producer B were codified from P7 to P12 and were manufactured using the following ingredients: pork meat and fat (88%), chili paste, water, garlic, salt, emulsifiers (E450, E451), saccharose, antioxidants (E301, E331), preservatives (E250, E252), soy proteins and lactose. Each Portuguese *Painho de Porco Ibérico* fermented sausage sample consisted of 300 g of vacuum-packaged ready-to-eat product, and no starter cultures were added during their production processes. The samples were stored under refrigerated conditions and analyzed before their expiration date.

2.2. Physico-chemical analyses

Water activity (a_w) was measured in accordance with the standard ISO 21807:2004 method by means of Aqualab 4TE apparatus (Meter Group, Pullman, USA). The pH was measured by inserting at the core of each sample a pH meter equipped with a HI2031 solid electrode (Hanna Instruments, Padova, Italy). To measure total titratable acidity (TTA), 10 g of each sample were previously weighted and mixed with 90 mL of deionized water by means of a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy) at 260 rpm for 5 min. The TTA was expressed as the total volume (mL) of 0.1 N NaOH solution added to obtain a fixed pH of 8.3. Lactic acid and acetic acid contents were measured in accordance with the manufacturer's instructions of the D-/L-Lactic Acid (D-/L-Lactate) and Acetic Acid (ACS Manual Format) test kits (Megazyme, Bray, Ireland), respectively. The physico-chemical measurements were carried out in technical duplicate for each Portuguese fermented sausage sample and the results were reported as mean ± standard deviation.

2.3. Microbiological analyses

Ten g of each sample was weighted and homogenized with 90 mL of sterile peptone (1 g L^{-1}) (Oxoid, Milan, Italy) water by means of a Stomacher 400 Circulator apparatus (VWR International PBI) at 260 rpm for 5 min. The microbiological viable counts were performed following ten-fold serial dilutions on the same diluent solution. De Man Rogosa and Sharpe (MRS) agar (VWR Prolabo Chemicals, Leuven, Belgium) medium was supplemented with cycloheximide (250 mg L^{-1}), and was used to enumerate the presumptive lactic acid bacteria – the incubation period was 37 °C for 48-72 h. The Slanetz Bartley Agar (SBA) medium was used to enumerate the enterococci – the incubation period was 37 °C for 48 h. Mannitol Salt Agar (MSA) (VWR Prolabo Chemicals) medium was used

to enumerate the coagulase negative cocci – the incubation period was 37 °C for 24-48 h. Pseudomonas Agar Base (PAB) medium was added with cetrimide-fucidin-cephalosporin (CFC) selective supplement (VWR International, Milan, Italy), and was used to enumerate *Pseudomonadaceae* – the incubation period was 37 °C for 24-48 h. The Violet Red Bile Glucose Agar (VRBGA) (VWR Prolabo Chemicals) medium was used to enumerate the *Enterobacteriaceae* – the incubation period was 37 °C for 24 h. The Tryptone Sulfite Neomycin (TSN) agar medium was used to enumerate the sulfite-reducing clostridia as reported by Belleggia et al. (2020b) – the incubation period was 37 °C for 24 h under anaerobic conditions by means of the AnaeroGen 2.5 System (Oxoid). Rose Bengal Chloramphenicol Agar (VWR Prolabo Chemicals) medium was used to enumerate the eumycetes – the incubation period was 25 °C for 72 h. The microbiological analyses were carried out in technical duplicate for each Portuguese fermented sausage sample, and the results were reported as mean of Log cfu/g ± standard deviation.

Finally, the apparatus miniVIDAS was used to determine the presence or absence of *Listeria monocytogenes* and *Salmonella* spp. through the Enzyme-Linked Fluorescent Assay (ELFA), following, respectively, the standard AFNOR BIO 12/11–03/04 and AFNOR BIO 12/16–09/05 methods (Haouet et al., 2017).

2.4. DNA extraction and meta-taxonomic amplicon sequencing

DNA was extracted by using …………..

DNA was then quantified by using the QUBIT ds Kit and normalized at 5 ng uL^{-1}; 2.5 uL of the DNA was used for the amplification of the V3-V4 region of the 16S rRNA by using primers 16SF (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 16SR (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013) for the microbiota. While for the mycobiota the region D1-D2 of the 26S rRNA was amplified by using the primers NL4R (5'-GGTCCGTGTTTCAAGACGG-3') and LS2-MF (5'-GAGTCGAGTTGTTTGGGAAT-3') (Mota-Gutierrez, Ferrocino, Rantsiou, & Cocolin, 2019). Standard Illumina overhang adapter sequences were added to locus-specific sequences. PCR conditions consisted of 25 cycles (95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds) plus one additional cycle at 72 °C for 10 min as a final chain elongation. PCR products were then purified by using the Agencourt AMPure XP beads (Beckman Coulter Genomics) and tagged according to the Illumina Sequencing Library Preparation. Amplicons were quantified using Qubit dsDNA assay kit diluted to 4 nM, denatured with 0.2 N NaOH and spiked with 20% (v/v) of PhiX. The combination of pool library and PhiX were diluted to 8 pM, and paired end sequencing was performed on the MiSeq platform using the MiSeq Reagent Kit V2 (2×250 bp) (Illumina, San Diego, USA), following the standard Illumina sequencing protocol.

2.4.1. Bioinformatic Analysis

After sequencing, *fastq* files were imported in QIIME version 2 (Bolyen et al., 2019). Sequence adapters and primers were trimmed by using *cutadapter*, while DADA2 algorithm (Callahan et al., 2016) was used to trim low quality reads, to remove chimeric sequences, and joined sequences shorter than 300 bp by using the DADA2 denoise paired plug-in of QIIME2. A total of 252.162 (12.608 reads/sample) for 16S and 752.468 (37.623 reads/sample) for 26S clean reads were generated and used for downstream analysis.

Amplicon sequence variants (ASVs) obtained by DADA2 were rarefied at the lowest number sequences/sample and used for taxonomic assignment using the QIIME feature-classifier plugin against the Greengenes 16S rRNA gene database for the microbiota and the manually build database for the mycobiota (Mota-Gutierrez et al., 2019). Taxonomy assignment for 16S and 26S was double checked on BLAST suite tools and ASVs tables display the highest taxonomic resolution reached.

Data generated by sequencing were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and are available under the BioProject Accession Number PRJNA788784.

2.5. SPME-GC/MS analysis of volatile components

Headspace volatiles from each sausage were analyzed by HS-SPME-GC/MS, using a 7890 Agilent GC system coupled to an Agilent 5975 (Agilent Technologies, Santa Clara, California, USA) inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler (Gerstel, Mülheim, Germany) as described by Belleggia et al. (2020a). Briefly, about 5 g collected from the core of the sausage, was shredded, and placed in a 20 mL headspace vial. The sample was stirred for 10 min at 45°C to accelerate equilibrium of headspace volatile compounds between the sample and the headspace. Then, volatile compounds extraction was carried out by injecting a 50/30 μm Divinylbenzene/Carboxen/PolyDiMethylSiloxane (DVB/Carboxen/PDMS) SPME fiber (Supelco, Bellefonte, PA) into the vial and exposing it to the headspace for 30 min at 45°C. Afterwards, the SPME fiber was desorbed directly into the injection port of the GC at 240 °C for 10 min in the splitless mode. Volatile compounds were separated using a capillary column HP Innowax (Agilent Technologies) (30 m x 0.25mm id. X 0.25 μm film thickness); the carrier gas was helium with a flow of 1mL min $^{-1}$. The temperature program of the GC oven was the following: 50 °C (hold 1 min), ramp to 110 °C at 6 °C min^{-1}, ramp to 180 °C at 20 °C min^{-1} (hold 3 min), and ramp to 220 °C at 5 °C min^{-1}. The injector, the quadrupole, the source and the transfer line temperature were maintained at 240 °C, 150 °C, 230 °C and 200 °C, respectively. Electron ionization mass spectra in full-scan mode were recorded at 70eV electron energy in the range 31-350 amu (Belleggia et al 2020a). Identification of volatile compounds was achieved by comparing mass spectra with the Wiley and Nist libraries (Wiley 7, NIST 05). The proportion of each compound was estimated dividing its mean area by the total area of the chromatogram and expressed as percentage. Blank experiments were carried out in two different modalities: blank of the fiber and blank of the empty vial. Controls were processed every 4 analyses of the experimental samples. All the analyses were performed in duplicate, and the results expressed as mean value of three technical replicates ± standard deviation.

2.6. Statistical analysis

Statistical differences among samples were determined through the Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) by means of one-way analysis of variance (ANOVA). The statistical analysis was performed with the software JMP Version 11.0.0 (SAS Institute Inc., Cary, NC).
For microbiota and mycobiota within-differences in batch or producers were evaluated by Wilcoxon matched pairs test, as appropriate.
Principal Component Analysis (PCA) was performed on VOCs profiles through the
Pairwise Spearman's non-parametric correlations performed by the *psyc* package of R were used to study the relationships between the relative frequency of ASVs (bacteria or fungi) and VOCs.

3. Results

3.1. Physical-chemical analyses

The physico-chemical measurements of the Portuguese fermented sausage samples are listed in Table 1. The a_w results ranged between 0.84 ± 0.01 (samples P5 and P6) and 0.91 ± 0.01 (samples P7, P10, P11). The statistical analysis showed significant differences between producers, attesting at 0.85 ± 0.01 (producer A) and 0.91 ± 0.01 (producer B). Regarding pH, values were comprised between 5.19

± 0.01 (sample P2) and 5.68 ± 0.01 (sample P12). The producers showed statistically significant variations, with 5.32 ± 0.10 (producer A) and 5.57 ± 0.12 (producer B). The TTA values varied from 7.1 ± 0.8 (sample P1) to 14.3 ± 0.4 mL of NaOH 0.1 N (sample P6). No statistical differences were determined between producer A and B, attesting at 9.63 ± 2.63 and 10.62 ± 1.91 mL of NaOH 0.1 N, respectively. Lactic acid contents ranged from 0.629 ± 0.182 (sample P2) and 1.973 ± 0.118 g 100 g^{-1} (sample P10). The producer A showed a statistically lower mean value compared to producer B, with 0.896 ± 0.239 and 1.871 ± 0.104 g 100 g^{-1}, respectively. Acetic acid contents were comprised between 0.013 ± 0.006 (sample P1) and 0.172 ± 0.017 g 100 g^{-1} (sample P9). The statistical analysis highlighted a significant difference between producers, with 0.022 ± 0.010 (producer A) and 0.140 ± 0.010 g 100 g^{-1} (producer B).

3.2. Microbiological analyses

The results of the microbiological viable counts of the Portuguese fermented sausage samples are listed in Table 2. The presumptive lactobacilli ranged from 5.12 ± 0.04 (sample P4) and 7.97 ± 0.05 Log cfu g^{-1} (sample P2). The statistical analysis showed significant differences between producers, attesting at 6.47 ± 1.19 (producer A) and 7.40 ± 0.36 Log cfu g^{-1} (producer B). The enterococci values were comprised between 1.39 ± 0.12 (sample P4) and 6.05 ± 0.02 (sample P8). The producer A mean value was significantly lower compared to the producer B one, with 2.73 ± 1.07 and 4.58 ± 1.05 Log cfu g^{-1}, respectively. As for coagulase negative cocci, the results varied from 2.99 ± 0.01 (sample P9) and 6.09 ± 0.04 Log cfu g^{-1} (sample P11). No statistical differences were highlighted between producer A and B, attesting at 5.59 ± 0.41 and 5.10 ± 1.04 Log cfu g^{-1}, respectively. A similar trend was observed for *Pseudomonadaceae*, *Enterobacteriaceae* and sulfite-reducing clostridia; such microbial groups were determined only in one production batch of the producer B. In detail, *Pseudomonadaceae* counts reached 3.95 ± 0.01 and 3.20 ± 0.05 Log cfu g^{-1} in samples P11 and P12, respectively; *Enterobacteriaceae* counts reached 3.25 ± 0.02 and 3.22 ± 0.01 Log cfu g^{-1} in samples P11 and P12, respectively; sulfite-reducing clostridia counts reached 1.76 ± 0.03 and 1.67 ± 0.10 Log cfu g^{-1} in samples P9 and P10, respectively. For such microbial groups, indeed, statistically significant differences were registered between producer A and producer B. Regarding eumycetes, the results ranged between 4.18 ± 0.31 (sample P9) and 6.01 ± 0.04 Log cfu g^{-1} (sample P11). No statistical variations were determined between producers, with 5.59 ± 0.33 (producer A) and 5.21 ± 0.80 Log cfu g^{-1} (producer B). Finally, the absence of *L. monocytogenes* and *Salmonella* in 25 g of product occurred in each sample.

3.3. Metataxonomic analysis

The microbiota and mycobiota of the fermented sausages under study were analyzed considering differences between producers or individual batches. Alpha diversity index did not show significant differences by considering producers or batch as a discriminant factor (data not shown). Microbiota of sausages was mainly dominated by *Latilactobacillus sakei* at 72% of the relative frequency on average in producer A, and 47% in producer B. *Weissella* was detected with a relative frequency of 7% and 1% in producer A and B, respectively. The presence of *Clostridium* was detected at a relative frequency of 5% and 10% in producer A and B, respectively, and *Staphylococcus equorum* at a relative frequency of 1% and 5% in producer A and B, respectively (Figure 2). *Lactobacillus* was detected with higher frequencies in samples from Producer B (10%) than producer A (1% of the relative frequency). Several ASVs were found to be characteristic of each producer. In more detail, *Bacillus* and *Weissella* were associated with samples from producer A, whereas *Lactobacillus, Corynebacterium, Escherichia,* and *Levilactobacillus brevis* were associated with samples from producer B ($P < 0.05$). A change in microbiota as a function of the batches was also observed. *Bacillus, Kurthia, Lachnospiraceae, Methylobacteriaceae,* and *Weissella* were mostly associated with batch 3 of producer A, whereas *Enterococcus, Lactobacillus, Leuconostoc mesenteroides,* and

Staphylococcus were associated with batch 1 of producer B. *Staphylococcus equorum* was associated with batch 2 of producer B, whereas *Latilactobacillus sakei* with batches 1 and 2 of producer A ($P < 0.05$).

Regarding the mycobiota, alpha diversity index did not show significant differences by considering producers or batch as a discriminant factor (data not shown). Mycobiota of sausages was mainly dominated by *Debaryomyces hansenii* (29% and 55% of the relative frequency on average in producer A and B, respectively), *Kurtzmaniella zeylanoides* (24% and 7 % of the relative frequency on average in producer A and B, respectively), *Wickerhamomyces subpelliculosa* (20% and 0.7% of the relative frequency on average in producer A and B, respectively) and *Zygosaccharomyces rouxii* (15% and 0.4% of the relative frequency on average in producer A and B, respectively) (Figure 3). Producer B displayed the predominance of *Starmella apicola* (20%), *Geotricum* and *Galactomyces* at 5% of the relative frequency, respectively (Figure 3). Few ASVs were found to be associated with a specific producer, in particular, *Geotricum*, *Galactomyces* and *Starmella apicola* were associated with producer B ($P < 0.05$). *Saccharomyces cerevisiae* and *Alternaria* were associated with batch 3 from producer A ($P < 0.05$).

3.4. Volatile components

The SPME-GC/MS analysis allowed to identify more than 160 volatile components in Portuguese *Painho de Porco Ibérico* fermented sausages, showing a complex volatile profile of the samples analyzed (Supplementary Table 1).

The volatile components belonged to twelve classes, such as aldehydes, ketones and lactones, esters and acetates, alcohols, terpenoids, sulfur compounds, aliphatic hydrocarbons, aromatic hydrocarbons, nitrogen compounds, acids, furans and pyrans and phenols.

Samples from Producer A were characterized mainly by alcohols (40.6%), esters and acetates (19.7%), acids (9.9%), aldehydes (8.1%) and terpenoides (7.6%). Samples from producer B were characterized mainly by alcohols (18.6%), phenols (18.2%), acids (15.1%), ketones and lactones (13.4%) (Figure 4).

Among aldehydes, acethaldehyde, 2-methylbutanal, 3-methylbutanal, hexanal and benzenacetaldehyde were found in all the samples, also if the samples P5 and P6 had the highest percentage.

Among ketones, 2-butanone and acetoin were detected in all the samples, while samples from Producer B were characterized also for the presence of 3-methyl-2-cyclopenten-1-one, 2-methylcyclopentanone, 2-hydroxy-3-methyl-2-cyclopente-1-one and butyrolactone.

Esters and acetates were detected mainly in the samples from Producer A. Those detected in the high percentage were ethylacetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl sorbate. Alcohols were found in the highest amount in the samples from Producer A. The main alcohols detected were ethanol, isobutanol, isoamyl alcohol and phenethyl alcohol. Terpenoids were detected only in the samples from Producer A. The main terpenoids were a-pinene, beta-pinene, sabinene, limonene, caryophillene, derived probably from the spices used in the production of the sausages.

Sulfur compounds were found in all the samples. In particular, 1-propene-3-methylthio, allyl disulfide and 1-Propene, 3,3'-thiobis-. Low percentages of aliphatic and aromatic hydrocarbons were found in all the samples. Instead, only samples from Producer B were characterized for the presence of some nitrogen compounds, such as pyridine, methylpyrazine and 3-methoxypiridine.

Acids were detected in all the samples. Acetic acid was the acid found in the highest percentages in all samples followed by hexanoic, pentanoic and butanoic acids.

Furans and pyrans were found mainly in the samples from Producer B. The most representative ones were 2-furanmethanol and 2-acetyl furan. Phenols characterized mainly samples from Producer B. The main representative ones were 2-methoxyphenol, phenol, 4-methyl phenol and 3-methyl phenol.

The analysis of VOCs composition provided evidence that samples from different production areas (A and B) were different, as confirmed by the Principal Component Analysis (PCA) (Figure 5).

Samples from Producer A (P1-P6) were clearly separated from samples obtained from Producer B (P7-P12) on the basis of the main class of compounds detected. The first two principal components (PC) explained 83.58% of the total variance. Acids, phenols, ketones and lactones and sulfur compounds positively loaded on PC1, whereas alcohols, terpenoids, esters and acetates and positively load on PC2. Only the sample P5 and P6 differed from the other samples from producer A. In fact, as determined by the two PCs (factor) P5 and P6 were entirely located in the higher left section of the graph, whereas P1, P2, P3 and P4 were located in the lower left section of the graph.

3.5. Correlation analysis

A different pattern of associations was observed between the two producers. In sausages from producer A (Figure 6, *P* < 0.01) a positive relationship was found between *Staphylococcus equorum* and acetic acid, acetoin, pentanoic acid, 2-butanone and 1-propene-3,3-thiobios, whereas a negative relationship with ethanol was observed. *L. sakei* had a negative relationship with 3-methylbutanal, beta pinene, delta 3-carene, gamma terpinene, and propanic acid. *L. sakei* was associated with ethyl sorbate. In addition, *Weissella* had a positive relationship with propanoic acid. Regarding mycobiota, *Pencillium* and *Yarrowia deformans* had a positive relationship with pentanoic acid, propanoic acid, 2-methyl- (CAS), and a negative relationship with ethanol and ethyl,2-methylbutanone. *Debaryomyces hansenii* was associated with phenol, whereas negatively associated with ethyl,2-methylbutanone (Figure 6, *P* < 0.01).

Plotting the correlation between ASVs of producer B and VOCs (Figure 7, *P* < 0.01), a positive relationship of *L. sakei* with isoamyl acetate was observed, whereas it had a negative relationship with phenol 2,4-dimethyl. *S. equorum* displayed positive correlations with phenol compounds, whereas negative correlations with 1-propene-3-3 methylthio, benzene acetaldehyde, ethyl butanoate, ethyl acetate and hexanoic acid were observed. *Clostridium* showed a positive correlation with benzonitrile. *Weissella* showed negative correlations with 2,3-dimethyl-2-cyclopenten-1-one and 2-acetyl-5-methylfuran, whereas a positive correlation with 2-butanol was observed. Regarding the correlation among mycobiota and volatilome profiles, *Debaryomyces hansenii* showed a negative relationship with hexanal, whereas *Yarrowia deformans* had a positive correlation with 2,3-dimethyl-2-cyclopenten-1-one (Figure 7, *P* < 0.01).

4. Discussion

To the authors' knowledge, only a few papers are actually dealing with the physico-chemical characterization of dry fermented sausages *Painho de Portalegre* and *Painho da Beira Baixa* (Dias et al. 2020; Roseiro et al. 2008); moreover, one research paper deals with physico-chemical and microbiological traits (viable counts) of a fermented sausage, named *Paio do Alentejo* (Elias, & Carrascosa, 2010), that can be considered similar to *Painho de Porco Ibérico*. Of note, no metataxonomic analysis related to *Painho de Porco Ibérico* fermented sausage is published in the scientific literature yet. Hence, the results of the present study will be discussed in comparison with data obtained by Dias et al. (2020), Roseiro et al. (2008), and Elias & Carrascosa (2010), moreover, data will be compared with those reported by other studies dealing with Portuguese fermented sausages.

Regarding the a_w values, detected in the analyzed samples, data were in accordance with those reported by Roseiro et al. (2008) for *Painho de Portalegre*. The a_w values observed in the present study were slightly lower than those reported by Teixeira, Fernandes & Pereira (2020) for naturally fermented *alheira* sausages produced in North of Portugal, that attested between 0.94 and 0.96. The a_w values were similar with those reported by Belleggia et al. (2020a) for Portuguese *cacholeira* blood sausages that showed values between 0.81 and 0.92, depending on the production batch. Moreover, a_w values detected in the analyzed *Painho de Porco Ibérico* samples from producer B were very similar to those reported by Cadavez et al. (2016) for ripened *salpicão*, a naturally fermented

Portuguese sausage, that attested at about 0.91-0.92. It is noteworthy that low a_w value represents one of the key parameters for the assurance of the safety of fermented sausages. Interestingly, as reported by Werlang, Vieira, Cardoso, & de Freitas Costa (2021), no growth of *Salmonella* is observed in fermented sausages with a_w values of 0.896. Moreover, in accordance with Regulation (EC) 2073/2005, products with pH $\leq$ 5.0 and $a_w \leq$ 0.94 are unable to support the growth of *L. monocytogenes*.

As for pH values detected in the analyzed *Painho de Porco Ibérico* samples from the two producers, data were in accordance with those reported by Roseiro et al. (2008) for *Painho de Portalegre*, and similar with those reported by Teixeira et al. (2020) for *alheira* sausages, that varied between 5.40 and 6.00. The values of pH detected in the present study were also in accordance with those reported by Elias & Carrascosa (2010) for *Paio do Alentejo* fermented sausages. The pH and the TTA are strongly affected by the metabolic activity of lactic acid bacteria. Low pH values are usually reached in the stuffed meat batter after fermentation, with subsequent inhibition of pathogenic and spoilage microorganisms. Of note, proteolytic microbiota and endogenous proteases, that produce peptides, free amino acids, amines, and ammonia, are responsible for a slight rise in pH at the end of ripening (Elias & Carrascosa, 2010), thus explaining the average pH values (between 5.32 and 5.57) detected in the present study.

Lactic and acetic acids were also detected in all the samples, associated with the occurrence of both hetero- and homofermentative lactic acid bacteria. Data collected in the present study for lactic and acetic acid in samples from producer A were in accordance with those reported by Belleggia et al. (2020a) for *cacholeira* blood sausages which were around 0.97 and 0.021, respectively. Interestingly, lactic and acetic acid values were notably higher in samples from producer B, thus explaining the highest average TTA values detected in the same samples.

The counts of lactic acid bacteria reflected the presence of an active microbiota in all the analyzed fermented sausages. The detected values were in accordance with those reported by Dias et al. (2020) for naturally fermented *Painho da Beira Baixa* sausages that attested at about 7 Log cfu g^{-1}. Moreover, values between 5.0 and 7.9 Log cfu g^{-1} were detected by Elias & Carrascosa (2010) in *Paio do Alentejo* sausages. The pro-technological activity of lactic acid bacteria, through the production of organic acids (mainly lactic and acetic acid), represents the key factor that allow the transformation of the raw meat into salami. As reviewed by Barcenilla, Ducic, López, Prieto, & Álvarez-Ordóñez (2022), lactic acid bacteria are able to transform carbohydrates into lactic acid and to produce other compounds with biologically active functions as diacetyl, acetoin, polyols, hydrogen peroxide, antifungal and antibacterial peptides (e.g., bacteriocins), and flavor precursors. Moreover, as reviewed by Fadda, López, & Vignolo (2010), during sausage fermentation, lactic acid bacteria contribute to the degradation of meat proteins through the synergistic action of endogenous meat proteases, and acid-induced changes. The pH reduction produced by lactic acid bacteria metabolism also leads to the coagulation of fibrillar meat proteins, resulting in enhanced firmness and cohesiveness of the end product, thus facilitating the slicing of salami (Cruxen et al., 2019).

As for enterococci, the counts were similar with those reported by Belleggia et al. (2020a) in *cacholeira* blood sausages produced in Portugal which were around 3-4 Log cfu g^{-1}. In fermented sausages, enterococci are responsible for low acidification, proteolytic and lipolytic activities and carbohydrate fermentation, although the exact role of these microorganisms is still poorly understood (Correia Santos, Fraqueza, Elias, Barreto, & Semedo-Lemsaddek, 2017). Interestingly, Correia Santos et al. (2017), that studied enterococci belonging mainly to the species *Enterococcus faecalis* and *Enterococcus faecium*, isolated from Portuguese meat-sausages (*Catalão*, *Chouriço-preto*, *Linguiça*, *Paio*, and *Salsichao*), showed their ability to produce a wide spectrum of enzymes (e.g., esterase, esterase lipase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, phosphatase acid, naphthol AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase, etc.), thus highlighting their role in the definition of sensory traits in artisanal fermented sausages.

Coagulase-negative cocci represent the second main group of microorganisms responsible for the physico-chemical modifications occurring during meat fermentation. The counts detected in the

present study were similar with those detected by Elias & Carrascosa (2010) in *Paio do Alentejo* and slightly higher than those reported by Dias et al. (2020) for naturally fermented *Painho da Beira Baixa* sausages that attested at about 4 Log cfu g^{-1}. Coagulase-negative cocci detected in the analyzed *Painho de Porco Ibérico* samples were also higher than those detected by Belleggia et al. (2020a) in *cacholeira* blood sausages that attested at about 3-4 Log cfu g^{-1}. Coagulase-negative cocci naturally occur in the raw materials used to produce fermented sausages as well as in the production environment (Ojha, Kerry, Duffy, Beresford, & Tiwari, 2015). In fermented sausages, such microbial group contribute to stabilization of color, degradation of peroxide and reduction of nitrates to nitrites. Moreover, proteases and lipases of coagulase-negative cocci release aromatic compounds and organic acids that strongly affect the sensory traits of the end product (Iacumin, Comi, Cantoni, & Cocolin, 2006). Of note, potassium nitrate (E252) or sodium nitrate (E251), acting as precursors of nitrite, are usually added in fermented sausages. Nitrate is converted to nitrite during fermentation by the nitrate reductase enzyme synthesized by many coagulase-negative cocci. The resulting color-generating nitric oxide molecule, interacting with muscle-based myoglobin, produces the nitrosomyoglobin with subsequent color stabilization of cured meat (Sánchez Mainar, Stavropoulou, & Leroy, 2017).

Enterobacteriaceae, Pseudomonadaceae as well as sulfite-reducing anaerobes were not detected in samples from producer A, whereas low counts of *Enterobacteriaceae, Pseudomonadaceae* and sulfite-reducing anaerobes were generally detected in samples from producer B. Of note, the values detected in some samples collected from producer B suggested a lack of hygiene practices during production.

Finally, active eumycetes populations were detected in all the samples. The values were in accordance with those reported by Elias & Carrascosa (2010) for *Paio do Alentejo* that were between 3.7 and 5.6 Log cfu g^{-1} but were notably higher than those reported by Dias et al. (2020) for naturally fermented *Painho da Beira Baixa* sausages that attested at about 1 Log cfu g^{-1}. As reported by Flores, Corral, Cano-García, Salvador, & Belloch (2015), meat origin can exert an influence on yeast species at the beginning of the processing. Moreover, during fermentation, yeasts protect sausages from the detrimental effect of oxygen, facilitate the drying process, produce proteases and lipases whose activities affect flavor development. In addition, in fermented sausages, yeasts produce an increase in pH and lactate utilization, thus contributing to a more pleasant (low acidic) taste of the end product (Flores et al., 2015). As reported by Magistà, Susca, Ferrara, Logrieco, & Perrone (2017), pro-technological mold species, with white or greyish fungal felt (e.g., *Penicillium*), exert beneficial effects on flavor and taste of fermented sausages through lactate oxidation, proteolysis, degradation of amino acids, lipolysis, β-oxidation, and catalase activity. Moreover, the felt of mold protects sausages against light and facilitates skin peeling (Magistà et al., 2017).

Metataxonomic analysis allowed the identification of major and minor microbial species. To the authors' knowledge, no previous studies regarding the biodiversity of *Painho de Porco Ibérico* fermented sausages are available in the scientific literature, hence, data will be discussed taking into consideration similar fermented meat products.

L. sakei was the major bacterial species detected in all the samples, irrespective of the producer. The occurrence of such lactic acid bacterium has already been detected in almost all fermented sausages produced in southern European countries (Aquilanti, Garofalo, Osimani, & Clementi, 2016), thus confirming its peculiar adaptation and competitiveness in the meat environment. In fermented sausages, this key microorganism has positively been correlated with carbohydrate metabolism and with the occurrence of acetoin (Ferrocino et al., 2018). Indeed, *L. sakei* is a facultative heterofermentative lactic acid bacterium that produces lactic acid from homolactic fermentation of hexoses, and acetic acid from heterolactic fermentation of pentoses. Moreover, endo and exo-peptidases (e.g., dipeptidase, aminopeptidase, tripeptidase, X-prolyl-dipeptidylpeptidase, arginine aminopeptidase, etc.) produced by *L. sakei* actively contribute to the hydrolysis of the sarcoplasmic proteins (Landeta, Curiel, Carrascosa, Muñoz, & de las Rivas, 2013), thus contributing to increase the amount of free amino acids in the meat batter with improved final sensory traits of fermented sausages (Flores, & Toldrá, 2011). In addition, the enzyme catalase produced by *L. sakei,* exerting

antioxidant activity, is able to counteract fermented meats rancidity (Hertel, Schmidt, Fischer, Oellers, & Hammes, 1998). Moreover, as reported by Leroy & De Vuyst (2016), *L. sakei* is able to use alternative non-glucidic compounds, such as arginine and nucleosides, naturally occurring in meat, as energy sources, hence, also this feature can be relevant for bacterial dominance in fermented sausages. Regarding the safety of fermented sausages, the positive features of *L. sakei* are not only related to the production of organic acids with bactericidal or bacteriostatic effects but also to its ability to produce bacteriocins, as, for example, sakacin A, P and K that are active against *L. monocytogenes* (Työppönen, Petäjä, & Mattila-Sandholm, 2003).

As for *Weissella*, mainly detected in samples from producer A, the occurrence of this lactic acid bacterium in fermented sausages is almost uncommon, although it has been found with low frequency in Chinese dry-cured or smoked-cured sausages (Wang, Zhang, Ren, & Zhan, 2018; Zhang et al., 2021). *Weissella* has also been found by Belleggia et al. (2020a) as minority species in Portuguese *cacholeira* blood sausages and by Pini, Aquilani, Giovannetti, Viti, & Pugliese (2020) in Italian Cinta Senese dry-fermented sausages. As reported by Zhang (2021), *Weissella* species can be considered as functional lactic acid bacteria due to their ability to produce useful metabolites, as organic acids and bacteriocins, with positive effects on the sensory traits and safety of fermented sausages.

Regarding the presence of *S. equorum*, this coagulase-negative bacterium has already been detected in fermented meat sausages produced in southern European countries as France, Italy, Greece, Portugal, and Spain (Aquilanti et al., 2016). Indeed, its occurrence has been reported in many Italian fermented sausages, including *Salame Piacentino*, traditional *Piedmontese* sausages, *Ciauscolo*, *Pitina*, *Soppressata molisana*, Soppre*ssata del Vallo di Diano*, *Salame di Senise*, *Salame Fabriano*, French and Greek dry fermented sausages, *Chorizo*, and *Androlla* (Aquilanti et al., 2016; Cardinali et al., 2018; Iacumin et al., 2020). The nitrate reductase activity exerted by *S. equorum* in fermented sausages allows the reduction of the nitrate added to the meat batter to nitrite, thus limiting lipid oxidation (Cruxen et al., 2019). Of note, catalase enzymes produced by *S. equorum* are pivotal in preventing unwanted color modifications caused by peroxide-forming lactic acid bacteria and in reducing the oxidation of fat in fermented sausages (Iacumin, Manzano, & Comi, 2012).

The presence of *Clostridium* in some samples partly confirms the results of viable counts. In fermented sausages, clostridia represent unwanted microorganisms that can be responsible for serious food poisoning (e.g., *Clostridium botulinum*) and spoilage (e.g., cold-tolerant clostridia as *Clostridium estertheticum* and *Clostridium gasigenes*) (Luong et al., 2020). Clostridia can reach the raw material via cross-contamination and their presence in the end product may indicate the use of low-quality meat or insufficient use of nitrate.

As for the mycobiota, *D. hansenii* was one of the major yeast species detected in the analyzed samples. This halotolerant species can be found in different niches with low water activity (Medina-Córdova, Rosales-Mendoza, Hernández-Montiel, & Angulo, 2018). *D. hansenii* has already been detected by Belleggia et al. (2020a; 2020c) as dominant yeast in Portuguese *cacholeira* blood sausages and in *Ciauscolo* salami. Moreover, Murgia, Marongiu, Aponte, Blaiotta, Deiana, & Mangia (2019) reported the dominance of *D. hansenii* among the yeasts detected in *Salsiccia Sarda*. As reported by Andrade, Córdoba, Casado, Córdoba, & Rodríguez (2010), peptidases and proteases produced by *D. hansenii* are responsible for a high proteolytic activity in fermented sausages, thus affecting the final flavor of the product. Indeed, Cano-Garcia, Rivera-Jiménez, Belloch, & Flores (2014) demonstrated that *D. hansenii* is able to produce aldehydes, ketones, alcohols, sulfur compounds, and methyl and ethyl esters, with these latter compounds able to increase consumer acceptance of fermented sausages. Interestingly, *D. hansenii* strains isolated from dry-cured meats showed antagonistic effect against toxigenic *Penicillium* and *Aspergillus* species, thus suggesting a potential biological activity as control agent of fungal contamination (Medina-Córdova et al., 2018).

Regarding *K. zeylanoides*, this psychrotrophic yeast has already been detected as minority species by Belleggia et al. (2020a; 2020c) in *cacholeira* sausages, in *Ciauscolo* and other Italian sausages, as well as in dry-cured Iberian ham (Wen, Sun, Li, Chen, & Kong, 2021). In fermented sausages, *K.*

zeylanoides, which occurrence usually increases at the end of ripening stage, is responsible for lipolysis due to the production of lipases (Mendoza, Padilla, Belloch, & Vignolo, 2014).

As for *W. subpelliculosa*, to the authors' knowledge, the occurrence of this yeast species has never been reported in fermented sausages before, although other *Wickerhamomyces* species have already been detected in Chinese traditional sausages (Tang et al., 2019). Of note, Pimentel et al. (2021) reported that this yeast, commonly detected in fruits, vegetables, flowers, and traditional fermented beverages, is able to modulate the intestinal human microbiota promoting the increase of beneficial microorganisms, hence, it is likely that a similar effect could be hypothesized also for the strains detected in the analyzed *Painho de Porco Ibérico* samples.

Z. rouxii has rarely been detected in fermented sausages. Interestingly, *Zygosaccharomyces* with potential features as aroma enhancer has been isolated from *Nanx Wudl*, a Chinese Dong fermented pork by Mi et al. (2021).

The SPME-GC/MS analysis identified the major and minor volatile components in the analyzed *Painho de Porco Ibérico* samples. To authors' knowledge, this is the first study on the characterization of volatile compounds responsible for the aroma in naturally fermented *Painho de Porco Ibérico* sausages.

The aromatic profile of the samples differed mainly according to their area of origin as a consequence of supposed differences due to the raw meat and ingredients used (especially spices), microbial metabolism, endogenous enzymatic activities and process parameters.

The volatile fraction of the fermented sausages from Beja district (producer A) were mainly dominated by alcohols, esters and acetates, aldehydes, acids and terpenoids, whereas the aroma profile of the samples from Evora district (producer B) appears mainly composed of alcohols, acids, ketones and acetates, phenols, furans and pyrans.

A considerable production of different alcohols was detected in all the samples, mainly in the samples from producer A. The most abundant alcohol compounds were ethanol, a contributor to a weak grilled and acetaldehyde-like aroma characteristics, followed by isoamyl alcohol and phenethyl alcohol. Most alcohols are derived from the metabolism of carbohydrates by microorganisms but are also considered as markers of secondary oxidation of fatty acids. Other authors found ethanol as the most abundant alcohol in Macedonian fermented sausages (Sulejmani, & Demiri, 2019), Thai fermented sausages (Rotsatchakul, Visesanguan, Smitinont, & Chaiseri, 2009), and fermented hams (Kim et al., 2016).

The presence of ester compounds, as also highlighted by the Rzepkowska, Zielińska, Ołdak, & Kołożyn Krajewska (2017), can contribute to the fruity aroma notes associated with a high acceptance of traditional dry sausages. The samples from producer A were characterized by a higher percentage of esters and acetates in respect to the samples from producer B, including five main compounds, as ethylacetate, ethyl hexanoate, ethyl butanoate, ethyl octanoate, and ethyl decanoate. As also evidenced by Karwowska, Kononiuk, Borrajo, & Lorenzo (2021), the origin of ester compounds in fermented sausages can be related with the activity of different microbial groups including lactic acid bacteria, coagulase-negative cocci, yeasts and molds. Indeed, as reported by metataxonomic analysis, samples from producer A were dominated by the highest percentage of different bacterial species, as *L. sakei* and *Weissella,* and eumycetes species as *K. zeylanoides*, *W. subpelliculosa*, *Z. rouxii*, *S. cerevisiae* and *Alternaria*.

Moreover, as reported by Cano-Garcia et al. (2014) also *D. hansenii* species can contribute to esters formation in sausages. Indeed, as highlighted by the metataxonomic analysis, all sausage samples were dominated by *Debaryomyces hansenii*.

Low percentage of aldehydes were detected in all the samples, with the exception of P5 and P6 that differed from the other samples by high amount of hexanal, nonanal, acethaldehyde, and 3-methylbutanal. Also, the PCA clearly separated these samples from the others. In particular, acetaldehyde and 3-methylbutanal may arise from the amino acids valine and threonine metabolism, whereas hexanal and nonanal are typical breakdown products of hydroperoxides produced during

oxidative degradation of lipids, as also highlighted in some *Paínho de Portalegre* dry fermented sausages (Partidário, Padilha, Roseiro, Silva & Santos, 2006).

All the samples, regardless from their origin, were characterized by moderate percentages of acids, as acetic, butanoic, pentanoic, and hexanoic acid. Among those, acetic acid was found at the highest percentage in all the sausage samples. This result was in accordance with results reported by Montanari et al. (2018) for other dry fermented sausages. As also highlighted by Bis-Souza et a., (2019) acetic and butanoic acids have a high influence in the aroma of fermented sausages.

Interestingly, terpenoids were detected only in the samples from Beja district (producer B). Terpenes are important volatile compounds which origin is related to the spices used in the preparation of the meat batter. Furthermore, terpenes in sausages can derive from animal feed and, therefore, they may be important for determining the geographical origin of the sausage. The main terpenes detected were delta 3-carene, α-pinene, β-pinene, limonene, caryophyllene, and sabinene. Different molecules among these compounds were detected in black pepper and paprika (Dosoky, Satyal, Barata, da Silva, & Setzer, 2019; Cirlini et al., 2019). Obviously, the variability and the percentage of the terpenes detected among the samples from producer A could depend on the different kind and quantity of spices added during the production of the sausages.

Sulfur compounds, occurred in all samples, were likely related with the use of garlic in the formulations.

Nitrogen compounds, phenols and furans, and pyrans were detected mainly in the samples from Producer B. Usually, furans and phenols are the main volatile compounds derived from smoking. In detail, 2-methylphenol, phenol and 2-furanmethanol were the main compounds detected in the *Painho de Porco Ibérico* fermented sausages from producer B, as also reported for other smoked products, including Slovenian sausages, Spanish *chorizo* sausages and other sausages of the Mediterranean area (Barbieri et al., 2021; Pereira et al., 2019).

5. Conclusions

In the present study, the microbial diversity and VOCs composition in *Painho de Porco Ibérico* fermented sausages were successfully studied. In more detail, although a core microbiota mainly represented by *L. sakei* and *D. hansenii* was observed in all the analyzed samples, minor microbial taxa characterized the products with different extent based on the producer. Unexpected species as *W. subpelliculosa* and *Z. rouxii* were also detected. Of note, as revealed by PCA, the occurrence of VOCs differed in accordance with producer. Hence, it is likely that, although the production processes applied by the two producers were those commonly applied for the manufacturing of *painho*-type sausages, the environmental conditions, the raw materials used, as well as variations related with the empirical practice of the butchers, strongly influenced the final product. The results obtained in the present study represent a further advancement in the knowledge on the biodiversity and VOCs composition of Portuguese fermented sausages. To better understand the interactions occurring between the autochthonous microorganisms and the meat batter in *Painho de Porco Ibérico* fermented sausage, microbial and VOCs dynamics should be further deepened throughout the production process.

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FIGURE CAPTIONS

Figure 1. Slices of Portuguese *Painho de Porco Ibérico* fermented sausages from producer A (on the left) and producer B (on the right).

Figure 2. Incidence of the bacterial taxonomic groups detected by sequencing.

Only ASVs with an incidence above 0.5% in at least two samples are shown.

Figure 3. Incidence of the fungal taxonomic groups detected by sequencing.

Only ASVs with an incidence above 0.5% in at least two samples are shown.

Figure 4. Average percentage of the classes of compounds found in *Painho de Porco Ibérico* samples from producer A (samples P1-P6) and producer B (samples P7-P12).

Figure 5. Principal Component Analysis (PCA) of VOCs composition of *Painho de Porco Ibérico* fermented sausage from producer A (samples P1-P6) and B (samples P7-P12).

Figure 6. Spearman's correlation between microbiota and VOCs. Only significant associations are shown ($P < 0.01$).

The intensity of the colors represents the degree of correlation between the bacteria and VOCs, as measured by Spearman's correlation, where the blue color represents a positive degree of correlation and red a negative correlation.

Figure 7. Spearman's correlation between mycobiota and VOCs. Only significant associations are shown ($P < 0.01$).

The intensity of the colors represents the degree of correlation between the fungi and VOCs, as measured by Spearman's correlation, where the blue color represents a positive degree of correlation and red a negative correlation.