Unravelling microbial populations and volatile organic compounds of artisan fermented liver sausages manufactured in Central Italy

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Abstract

The aim of the present study was to obtain information on the occurrence of bacteria and eumycetes in ready-to-eat fermented liver sausages manufactured by 20 artisan producers located in the Marche Region (Italy). To this end, culture-dependent analyses and metataxonomic sequencing were carried out. Furthermore, the characterization of the volatilome of the fermented liver sausages was evaluated via Gas Chromatography-Mass Spectrometry (GC-MS) analysis, together with physico-chemical determinations. Finally, the presence of hepatitis E virus (HEV) was also assessed via real-time-RT-(q)PCR assays. The results of microbial viable counts highlighted the presence of active microbial populations mainly composed by lactic acid bacteria, enterococci, coagulase-negative cocci, and eumycetes. *Enterobacteriaceae*, *Pseudomonadaceae*, or sulfite-reducing anaerobes, were not detected in the majority of the samples, thus attesting the high quality of raw materials and production processes. The metataxonomic analysis performed on the fermented liver sausages allowed major and minor microbial taxa to be identified, highlighting a main microbiota dominated by *Latilactobacillus sakei* in all the sample analyzed, reaching abundances up to 80%. *Staphylococcus xylosus* and *Staphylococcus equorum* were also detected. Among minority bacterial taxa, *Weissella* spp., *Leuconostoc* spp., *Macrococcus caseolyticus*, *Staphylococcus xylosus*, *Brochothrix thermosphacta, Staphylococcus succinus*, *Lactobacillus coryniformis*, *Lactiplantibacillus plantarum*, *Lactococcus garviae, Psychrobacter* spp., and *Carnobacterium viridans* were detected. The main mycobiota was composed by *Debaromyces hansenii* that was present in all samples with the highest frequency. Among minority fungal taxa, *Aspergillus* spp., *Penicillium* spp., *Kurtzmaniella zeylanoides*, *Candida* spp., *Yamadazyma* spp., *Scopulariopsis* spp., *Yarrowia* spp., and *Starmerella* spp. were detected. Interestingly, associations between some taxa and some physico-chemical parameters were also highlighted. The absence of HEV in all the samples attested a high level of safety. Finally, the most VOCs detected in the analyzed fermented liver sausages belonged to six classes, as: terpenoids, aldehydes, ketones, alcohols, esters and acids. Nitrogen compounds, sulphur compounds, phenols, hydrocarbons, lactones, furans and aromatic hydrocarbons were also identified. By plotting the correlation between microbiota, mycobiota and VOCs several significant relationships were observed. In view of obtaining a geographical indication status (e.g., PGI) for fermented liver sausages of the Marche Region, the data obtained could serve as reference in drawing up a production disciplinary. Such disciplinary should consider the microbiological and technological peculiarities of the products manufactured by each producer as well as provide common reference values in order to create uniform and recognizable products.

Keywords: *Latilactobacillus sakei*, *Debaromyces hansenii*, liver, metataxonomic analysis, traditional product.

1. Introduction

The production of salami through fermentation of meat represents one of the most ancient and effective methods to preserve such a perishable foodstuff. To make fermented sausages, meat is usually cut into pieces which size varies in accordance with the recipe. Shredded lard is usually added to the meat batter together with salt, spices, and other ingredients (e.g., curing salt, wine, blood, liver, sugar, herbs, starter cultures, antioxidants, etc.) that contribute to characterize the end product (Flores, & Piornos, 2021). The resulting batter is then stuffed into animal bowel or artificial casings and left to ferment at specific time, relative humidity and temperature, allowing fermentation to occur. The raw materials, together with the physico-chemical and microbiological modifications occurring during fermentation, strongly contribute to define sensory traits and safety of the product. It is noteworthy that the anaerobic conditions established in the stuffed meat allow the selection of key pro-technological microorganisms such as coagulase-negative cocci and lactic acid bacteria (Belleggia et al., 2020). The composition of the naturally occurring microbiota during manufacturing of fermented sausages can vary depending on the process parameters applied and on interaction with the raw materials, other ingredients, and environment.

In fact, numerous variations in terms of processing methods and ingredients for the production of fermented sausages are applied around the world and especially in Southern Europe, thus allowing the production of an ample variety of fermented meat sausages like, for example, the well-known *chorizo*, *salsichon*, and *androlla* in Spain, a*lheira*, *painho*, and *cacholeira* in Portugal, *Salame Milano*, *Salame di Cremona* PGI, *Salame Piacentino* PDO, *Soppressa* TP, *Ciauscolo* PGI, and *Salame Napoli* in Italy (Aquilanti, Garofalo, Osimani, & Clementi, 2016) *salami aeros* and *loukanika* in Greece (Vignolo, Fontana, & Fadda, 2010), *Petrovac* sausage in Serbia (Milićević, Tomović, Danilović, & Savic, 2021).

Given the great variety and uniqueness of European salami, the European Union food quality policy has been aimed at protecting the names of specific products to valorize their distinctive characteristics. In such a context, food product names can be awarded by a "geographical indication" if they have a specific link to the place where they are made, thus allowing consumers to trust and distinguish quality products. Geographical indications of food products include Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), Geographical Indication (GI), and Traditional Speciality Guaranteed (TGI). In Italy, the Ministry of Agricultural, Food and Forestry Policies (MiPAAF, 2021) has also focused on niche products of limited production. With the aim of valorizing Italian food products, in which agricultural or livestock products are still processed according to ancient recipes, a list of traditional Italian foods, including meat products, is published once per year. Among food categories included in the list, fermented meat products constitute one of the major categories, being part the of Italian gastronomic culture since the 12th century. Fermented sausages containing liver are included in the list of traditional Italian food products with different names, as *salamelle di fegato* (Abruzzo Region), *mazza fegato* (Emilia Romagna Region), *ammazzafegato* (Toscana Region), and *mazzafegato – salsiccia matta* (Marche Region), thus attesting the vocation of Central Italy to charcuterie.

In the Marche Region, the method for the production of fermented liver sausages follows both empirical and traditional local techniques. Indeed, as there are no official guidelines yet for defining procedures, ingredients and product parameters, the production process can frequently be subjected to modifications based on the manufacturer.

Fermented liver sausages are generally prepared with blends of pork meat cuts, liver (from 15% to 30%), and offal (heart, tongue, etc.). The main ingredients are chopped and subsequently grinded to obtain a homogeneous meat batter. Moreover, salt, spices, flavourings, and additives, can be added at different ratios. Once uniformly mixed, the meat batter is stuffed into pork gentle casings that are previously desalted and washed with water or wine. The liver sausages are then left to dry under controlled conditions for 5 days from 20 to 18 °C and from 90 to 75% relative humidity. Ripening is carried out at 15 °C and 75% of relative humidity for at least 50 days. Fermented liver sausages are renowned for their peculiar reddish dark brown colour and their tender consistency. Such jewel of the Italian gastronomy is appreciated for its metallic (ferrous) taste conferred by liver, and its spreadability.

Although physico-chemical and technological features of liver sausages to be eaten cooked have already been investigated (Hugo, & Hugo, 2015), to the authors' knowledge, a lack of information on the microbiota and volatile compounds (VOCs) occurring in fermented sausages containing liver is evidenced in the scientific literature. Hence, the present study was aimed to get a first insight into the occurrence of bacteria and

eumycetes in ready-to-eat fermented liver sausages manufactured by 20 artisan producers located in the Marche Region. To this end, a combined approach based on the use of selective growth media and metataxonomic sequencing was adopted. Physico-chemical parameters and the characterization of the volatilome of the fermented liver sausages, by Gas Chromatography-Mass Spectrometry (GC-MS) analysis, were also evaluated.
It is noteworthy that, fermented sausages containing pork liver could constitute a risk for the safety of consumers since liver can be the vehicle of the hepatitis E virus (HEV), being such organ the main site of HEV replication (Colson et al., 2010; Di Bartolo, Angeloni, Ponterio, Ostanello, & Ruggeri, 2015; Martin-Latil, Hennechart-Collette, Guillier, & Perelle, 2014; Said et al., 2014). Hence, in order to evaluate the presence of HEV in the analyzed samples, its detection via real-time-RT-(q)PCR assay was also carried out.

2. Materials and methods

2.1. Sampling

Sixty samples of spontaneously fermented liver sausages from 20 different producers located in the Marche region were collected at production plants. In more detail, 3 samples of the same production batch were obtained from each producer. Fermented liver sausages were labelled as follows: from A1, A2, A3 (producer 1) to V1, V2, V3 (producer 20). Although all sausages were prepared based on the use of swine meat and liver, the list of other ingredients slightly differed depending on the producer. Therefore, the different formulations of the fermented liver sausages under study are reported in Table 1. Each fermented liver sausage sample consisted of at least 150 g of whole end product, collected aseptically and stored under refrigeration (+4 °C) until use. All the analyses described in the present study were conducted before sausages expiration date.

2.2. Physico-chemical analysis

The pH value was determined through the insertion of a pH meter equipped with a HI2031 solid electrode (Hanna Instruments, Padova, Italy) at the core of fermented liver sausages.
The water activity (a_w) was determined through an Aqualab 4TE apparatus (Meter Group, Pullman, USA) in accordance with ISO 21807:2004 standard method.
The salt (sodium chloride) content was determined through gravimetric analysis in conformity with the Italian method ISTISAN 96/34 described by Istituto Superiore di Sanità.
Lipid oxidation was monitored by determining the peroxide value (PV, mEq peroxide kg sample^{-1}) according to AOAC method 965.33 (AOAC, 1990).
The total titratable acidity (TTA) was determined weighting and homogenizing 10 g of each fermented liver sausage sample with 90 mL of deionized water in a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy) at 260 rpm for 3 min. The TTA results were expressed as the mL of a 0.1 N sodium hydroxide (NaOH) solution necessary to obtain a stationary endpoint of 8.3.
The lactic acid and acetic acid contents were determined through the D-/L-Lactic Acid (D-/L-Lactate) and Acetic Acid (ACS Manual Format) test kits (Megazyme, Bray, Ireland) following the manufacturer's instructions.
For each fermented liver sausage sample, the analyses were conducted in three technical replicates and the results were reported as mean ± standard deviation.

2.3. Microbiological analysis

The microbiological viable counts were performed by adding 90 mL of peptone (Oxoid, Milan, Italy) water (1 g/L) to 10 g of each fermented liver sausage sample. Subsequently, the serial ten-fold dilutions were set up to determine the presence and concentration of the following microorganism groups: (i) presumptive lactic acid bacteria on De Man, Rogosa and Sharpe (MRS) agar (VWR Prolabo Chemicals, Leuven, Belgium), supplemented with 250 mg/L of cycloheximide and incubated at 37 °C for 48 h; (ii) enterococci on Enterococcus selective agar (Thermo Fisher Scientific, Buchs, Switzerland), incubated at 37 °C for 48 h; (iii) coagulase negative staphylococci on Mannitol Salt Agar (MSA) (VWR Prolabo Chemicals), incubated at 37 °C for 24-48 h; (iv) *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA) (VWR Prolabo Chemicals), incubated at 37 °C for 24 h; (v) *Pseudomonadaceae* on Pseudomonas Agar Base (PAB) (VWR Prolabo Chemicals), added with cetrimide-fucidin-cephalosporin (CFC) selective supplement (VWR

International, Milan, Italy) and incubated at 30 °C for 24-48 h; (vi) sulfite-reducing clostridia: for such microbial group, homogenates were treated in a water bath at 80 °C for 10 min and cooled in ice; aliquots of the serial ten-fold dilutions of the treated samples were inoculated in Tryptone Sulfite Neomycin (TSN) agar (Liofilchem, Teramo, Italy) and incubated at 37 °C for 24 h under anaerobic conditions by means of the AnaeroGen 2.5 System; (vii) eumycetes on Rose Bengal Chloramphenicol Agar (VWR Prolabo Chemicals), incubated at 25 °C for 72 h. For each fermented liver sausage sample, the analyses were conducted in three technical replicates and the results were reported as mean of Log of colony forming units (cfu) per g ± standard deviation.
Finally, a miniVIDAS apparatus (bioMérieux, Marcy l'Etoile, France) was used to assess the presence/absence of *Listeria monocytogenes* and *Salmonella* spp. through the enzyme-linked fluorescent assay (ELFA) method, in accordance with the AFNOR BIO 12/11–03/04 and AFNOR BIO 12/16–09/05 standard methods, respectively (Haouet et al., 2017).

2.4. Microbial DNA extraction, sequencing and bioinformatics

Aliquots of 1 mL were collected from the first dilution (10-1) of each fermented fish sausage sample and were centrifuged at 14,000 rpm for 10 min. The supernatants were discarded, and the pellets were treated for the total microbial DNA extraction by means of the E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's instructions.
A total of 60 DNA samples (3 for each producer) were quantified using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and standardized at 5 ng μL^{-1}. Two µl of each DNA was amplified for microbiota analysis by using the primers and condition for the amplification of the V3-V4 region of the 16S rRNA gene as described by Klindworth et al. (2013). The mycobiota was studied by the amplification of the D1-D2 domain of the 26S according to Mota-Gutierrez, Ferrocino, Rantsiou, & Cocolin (2019). Pair-end sequencing (2X250bp) was performed with a MiSeq Illumina instrument (Illumina) with V2 chemistry according to the manufacturer's instructions.
Raw reads were analyzed by using the Quantitative Insights Into Microbial Ecology QIIME2 (Bolyen et al. 2019). Primers and adapters were first trimmed by using Cutadapter and then quality filtered using the DADA2 algorithm (Callahan et al., 2016). Low-quality bases, chimeric sequences, and sequences shorter than 300 bp were filtered out by using the dada2 denoise-paired plug in of QIIME2. Amplicon Sequence Variants (ASVs) generated by DADA2 were rarefied at the lowest sequences per samples and used for taxonomic assignment using the QIIME feature-classifier plugin against the Greengenes 16S rRNA gene database for the microbiota and the manually built database for the mycobiota (Mota-Gutierrez et al., 2019).
Taxonomy assignment at the highest taxonomic resolution reached for 16S and 26S was confirmed by double checking on BLAST suite tools. QIIME2 diversity script was used to perform alpha diversity analysis. The data generated by sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under the Bioprojects Accession Number PRJNA776119.

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. (2020). 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. The temperature program of the GC oven was the following: 50 °C (hold 1 min), ramp to 110 °C at 6 °C/min, ramp to 180 °C at 20 °C/min (hold 3 min), and ramp to 220 °C at 5 °C/min. 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 2020). 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. HEV analysis

Virus extraction and detection was performed as describe by Di Pasquale et al. (2019). Briefly, approximately 10 g of each fermented liver sausage sample was homogenised using a mechanical disruptor osterizer. After adding 10 µL of process control virus and 7 mL of TRIZOL Reagent (Life Technologies, Carlsbad, Canada), 5 g of sample was homogenized. Following mechanical disruption and centrifugation of the sample, the recovered supernatant was added of 1.4 mL of chloroform, vortexed for 15 s, and then incubated at room temperature for 15 min. Thereafter, the sample was again centrifuged, and the aqueous phase retained.
A total of 1 mL of the virus preparations of each sample was used for the nucleic acid extraction. The nucleic acids were extracted from the samples using the NucliSENS® easyMAG system (BioMérieux, Marcy I'Etoile, France) according to the manufacturer's instructions.
HEV detection was carried out using real-time-RT-(q)PCR assay, using the RNA UltraSense™ One-Step qRT-PCR System (Life Technologies) and the QuantStudio7 flex real-time PCR System (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).
Viral stock of recombinant Mengovirus (strain vMC_0) and HEV-EC RNA were kindly provided by Istituto Superiore di Sanità, National Reference Laboratory for Foodborne Viruses, Rome, Italy.

2.7. Statistical analyses

The statistical analysis of microbiological and physico-chemical data was performed to determine differences among fermented liver sausage samples through the JMP v11.0.0 software (SAS Institute Inc., Cary, NC). To this end, the Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used by one-way analysis of variance (ANOVA).
Water activity, pH, acetic acid, NaCl, lactic acid and total titratable acidity data obtained from the chemical analysis of the samples were clustered in quartiles (Q1-Q4) in order to create group of samples (Supplementary Table 1). Variables were compared by the Kruskal-Wallis and Bonferroni's correction for multiple comparisons was applied; a *P* value of 0.05 or lower was considered as statistically significant. Shannon index were visualized by Box plots representing the interquartile range between the first and the third quartile, with the error bars showing the lowest and the highest value. ASVs table and metabolomic data were then imported in R in order to performed spearman correlation visualized by the *corr.plot* function of R.

3. Results

3.1. Physico-chemical analyses

The data collected from the physico-chemical measurements of fermented liver sausages are reported in Table 1. The pH mean values were comprised between 4.85 ± 0.06 from samples of producer L and 5.70 ± 0.11 from samples of producer A. The a_w data showed the lowest mean value of 0.772 ± 0.005 from samples of producer O, and the highest mean value of 0.912 ± 0.003 from samples of producer A, whereas the salt (NaCl) concentration data showed the lowest mean value of 1.67 ± 0.02 g/100 g from samples of producer M, and the highest mean value of 3.88 ± 0.05 g/100 g from samples of producer S. Regarding the peroxide values, the lowest mean value was registered from samples of producer A with 5.0 ± 0.0 meq O_2/kg of fat, and the highest mean value was registered from samples of producer O with 41.5 ± 0.07 meq O_2/kg of fat. The TTA mean values ranged between 18.07 ± 1.42 from samples of producer U and 39.83 ± 1.65 mL of 0.1 N NaOH from samples of producer L. The lactic acid content data showed the lowest mean value of 0.163 ± 0.079 g/100 g from samples of producer C, and the highest mean value of 2.279 ± 0.143 g/100 g from samples of producer L, whereas the acetic acid content data showed the lowest mean value of 0.002 ± 0.003 g/100 g from samples of producer U, and the highest mean value of 0.047 ± 0.003 g/100 g from samples of producer H.

3.2. Microbiological analyses

The results of the microbiological viable counts of fermented liver sausages are listed in Table 2. The counts of presumptive lactobacilli were comprised between 6.80 ± 0.30 of samples from producer Q and 8.76 ± 0.12 Log cfu/g of samples from producer I. As for enterocci, the lowest mean values belonged to samples from producers L and R with counts lower than 1.00 Log cfu/g, whereas the highest mean value was reached from samples of producer Q with 5.71 ± 0.84 Log cfu/g. The coagulase-negative cocci counts ranged between 3.00 ± 0.07 of samples from producer P and 7.18 ± 0.30 Log cfu/g of samples from producer N. Low counts of Enterobacteriaceae and Pseudomonadaceae were detected among fermented liver sausages samples; however, the highest mean values of Enterobacteriaceae and Pseudomonadaceae counts were 4.18 ± 0.47 of samples from producer U and 5.10 ± 0.22 Log cfu/g of samples from producer M, respectively. The sulfate-reducing anaerobes were not detected in any producer except for producer M with 2.27 ± 0.18 Log cfu/g. Finally, the counts of total eumycetes were comprised between 4.18 ± 0.54 of samples from producer H and 6.47 ± 0.19 Log cfu/g of samples from producer N. No samples revealed the presence of *L. monocytogenes* or *Salmonella* spp. in 25 g of product.

3.3. Microbiota composition

A total of 1,121,517 high quality reads were used for the downstream analysis with an average of 19,009 sequences/sample and an estimate sample coverage of 99%. Shannon diversity as a function of the producer showed that samples from producers D, H, and S had the highest richness, whereas those from producers L, P, U, and V the lowest (supplementary Figure 1). Data of a_w, pH, NaCl, lactic acid, acetic acid, and total titratable acidity were clustered in quartiles (Q1-Q4) to perform the statistical comparison (supplementary Table 1). Samples with the highest value of NaCl (belonging to Q4) and pH (Q3 and Q4 quartiles) showed the highest microbial richness, whereas samples with the highest lactic acid content and total titratable acidity showed the lowest microbial richness (supplementary Figure 1, $P<0.05$). Microbiota composition (obtained from the average of triplicates for each producer) showed a core microbiota dominated by *Latilactobacillus sakei* in all the analyzed samples, reaching an abundance above 80% in samples from 16 producers, and 77% and 50 % in samples from four producers, respectively (Figure 1). *Staphylococcus xylosus* was observed at 15% only in samples from two producers (S and I), at about 10% in samples from three producers (Q, V, and C), and from 1 to 7% in samples from nine producers. *Staphylococcus equorum* was found at 40% in samples from producer B, at about 10% in samples from two producers (C and N), and from 1 to 5% in samples from nine producers. *Weissella* was observed at 18% in samples from producer D and 5% in samples from producer H. *Leuconostoc* was observed at 5% only in samples from three producers (A, H and S). The amplicon sequence variants (ASVs) of the different chemical characteristics listed above were then analyzed. The ASVs based on a_w clusters showed the highest frequency of *Clostridium* in Q3 and Q4 groups of samples, while *Macrococcus caseolyticus* and *Staphylococcus xylosus* were associated with samples belonging to cluster Q1 (supplementary Table 1, $P < 0.05$). Highest pH values seemed to favor the presence of *Leuconostoc*, *S. equorum* and *Brochothrix thermosphacta*. *Clostridium* was then associated with samples belonging to Q2, whereas the presence of *Staphylococcus succinus* and *Macrococcus caseolyticus* was associated with samples clustered in Q2 range. Acetic acid showed the association with *Lactobacillus coryniformis* and *Weissella confusa*. *Lactiplantibacillus plantarum*, *Lactococcus garviae* and *Weissella* where associated with samples that display the highest level of NaCl (Q4 groups). Samples belonging to the Q1 range of lactic acid were associated with *Staphylococcus equorum,* whereas *Lactiplanibacillus plantarum, Lactobacillus* and *Leuconostoc* were associated with samples belonging to Q2. Regarding the total titratable acidity, *Psychrobacter* was associated with samples that display the lower range for this parameter (Q1), *Carnobacterium viridans* was associated with Q2 group and *Enterococcus* with Q4 group (supplementary Table 1, $P < 0.05$).

3.4. Mycobiota composition

A total of 2,933,567 high quality reads were used for the downstream analysis with an average of 49,721 sequences/sample and an estimate sample coverage of 99%. Regarding alpha diversity samples belonging to producers F, H and Q showed the highest Shannon richness, whereas samples from producers A, N, R, and U showed the lower mycobiota richness (supplementary Figure 2). Regarding the chemical determinations, the samples that had the highest level of NaCl and total titratable acidity (belonging to cluster Q4) showed the highest microbial richness, whereas samples with highest a_w value displayed the lower richness (supplementary Figure 2).

Twenty-six ASVs were detected in all the samples analyzed, however a few taxa dominated the mycobiota (Figure 2). The core mycobiota was composed by *Debaromyces hansenii* that was present in all samples with the highest abundance. Only samples from producers B, C, and H displayed a lower presence of this yeast (38.76%, 31.73%, 36.98% of the relative frequency respectively). *Aspergillus* was detected at 43.99% in samples from producer B and at 53.98% in samples from producer O. *Penicillium* was found at various percentages from 0.11% in sample from producer N to 42.99% in samples from producer V. *Kurtzmaniella zeylanoides* was detected in samples from producer I (0.04 %) and, with the highest frequency, in samples from producer P (25.71 %). Samples from producer F showed the presence of *Candida* spp. (8.33%), *Candida metapsilosis* (23.79%), *Yamadazyma atlantica* (6.25%), and *Yamadazyma triangularis* (3.55%). *Scopulariopsis* spp. was abundant in samples from producer G (3.45%), whereas samples from producer H showed the presence of *Yarrowia deformans* and *Yarrowia lipolytica* at a relative frequency of 11.10% and 6.22%, respectively. *Starmerella* spp. was found in samples from producers O and Q, reaching 3.17% and 1.01% of the relative frequency, respectively (Figure 2).
Mycobiota signature was observed taking into the account the cluster in quartiles (Q1-Q4) based on a_w, pH, acetic acid, NaCl, lactic acid and total titratable acidity (supplementary Table 1). *Debaryomyces hansenii* exhibited the highest frequency in samples belonging to Q4 cluster based on a_w, unlike *Aspergillus* and *Yamadazyma triangularis* which showed the highest frequency in the Q1 cluster. *Kurtzmaniella zeylanoides* was associated with samples belonging to Q2 cluster (supplementary Table 1, $P < 0.05$). *Penicillium roqueforti* was associated with samples belonging to Q4 based on pH range, whereas *Galactomyces candidum* was associated with cluster Q3 and *Yarrowia deformans* with cluster Q1. Regarding acetic acid, *Debaromyces* was associated with Q1 cluster, *Starmerella* spp. with Q3 cluster, and *Geotrichum* with Q4 cluster. *Galactomyces candidum* was associated with the highest NaCl concentrations (Q4 cluster), whereas *Candida alimentaria* was observed mainly in Q1 cluster. Regarding the total titratable acidity, *Candida galli* showed the association with cluster Q4 (supplementary Table 1, $P < 0.05$).

3.5. Volatile components

The volatile compounds of the sausages manufactured by the 20 producers were identified through SPME-GC/MS technique. The volatile class abundances of fermented liver sausages are depicted in Figure 3, whereas the data of each volatile compound for each producer are listed in Supplementary Table 2. The most numerous compounds belonged to six classes, being terpenoides (52), aldehydes (22), ketones (17), alcohols (16), esters (13) and acids (12). Nitrogen compounds (7), sulphur compounds (6), phenols (5), hydrocarbons (5), lactones (4), furans (2) and aromatic hydrocarbons (1) were also identified (Tab oppure Suppl. Material VOCs). In all the samples, the proportion of terpenoides prevailed over other compounds, varying between 93.4% (samples from producer B) and 39.6% (samples from producer I), with exception of the sample from producer H having the lowest percentage of terpenoides (6.9%). Samples from producers B, T, M, D, and L were characterized by the highest percentage of total terpenoids area while samples from producers H, I, O, Q, and S were characterized by the lowest. In general, after terpenoids, the most prevalent compounds were in order aldehydes with an area percentage that varied between 1.8% - 32.2%, acids (1.1 - 21.3%), ketones (1.1 - 29.5%), hydrocarbons (0.9% - 7.2%) and alcohols (0.1-7.4%) (Figure 3). Among terpenoids, α-pinene, α-thujene, β-pinene, sabinene, delta-3- carene, α-phellandrene, limonene, β-phellandrene, cymene, α-terpinolene, α-copaene, linalool, caryophyllene and carvone were found in all the samples. Limonene was the terpenoid with the highest area percentage in almost all the analyzed samples. Also, beta-pinene, sabinene, delta 3-carene and caryophyllene were found in moderate percentage in all the samples. Some terpenoides appeared only in one or two samples. After terpenoids, aldehydes were the class of compounds found in high percentage in almost all the samples. In detail, samples from producers H, O, U, S, and R were characterized by the highest percentage of total aldehydes area, whereas samples from producers M, D, B, and V by the lowest. Hexanal, 2-methylbutanal, 3-methylbutanal, nonanal, 2-octenal, benzaldehyde were the most important aldehydes found in all the samples. Hexanal was the aldehyde with the highest area percentage in almost all the samples, mainly in samples from producers O, P, R, S, and U. Samples from producer H were characterized for high area percentage of benzeneacetaldehyde and benzaldehyde. Ketones occurred in low percentage in all the samples, with the exception of samples from producers I and H that were characterized by a higher area percentage of 29.5% and 16.8%, respectively. Among ketones, only 2-propanone, 2-butanone, 2,3-octanedione and 2-nonanone were found in all the samples. Also, acetoin was found in 16 samples, but the samples characterized by the highest percentage area were those from producer I.
Regarding acids, samples from producers H, I, A, O, and S were characterized by the highest area percentage,

whereas samples from producers B, T, and M by the lowest. Acetic, pentanoic, hexanoic, octanoic, nonanoic and decanoic acids were found in all the samples, although with different percentage areas. Other acids were also present in more than half of the samples. In most samples, acetic acid accounted for the largest percentage followed by valeric and hexanoic acid.

Low area percentage of esters was found in all the samples. The most common esters were ethyl acetate, ethyl isovalerate, ethyl hexanoate, ethyl decanoate, found in 11, 7, 12 and 14 samples, respectively. Samples from producers I and H were the sample with the higher total area percentage of esters.

Almost all the samples were characterized for a low percentage of alcohol compounds. The alcohols most commonly detected were 1-hexanol, 1-octanol, phenetylalcohol, 1-pentanol, ethanol occurring in 19, 18, 14, 14 and 11 different samples of sausages. Only three samples had a higher area percentage of alcohol compounds i.e. samples from producers H (7.4%), Q (5.4%), and I (4.1%). In particular, samples from producer H were characterized mainly from isoamyl alcohol, 2-butanol and phenetyl alcohol; samples from producer Q from 1-pentanol and 1 –hexanol and samples from producer I from ethanol.

Low area percentage of hydrocarbons was found in all the sample with the exception of samples from producer H and Q that were characterized from higher amount of 7.2% and 5.9%, respectively. The most representative hydrocarbons were hexane, heptane and octane, detected in all the samples.

Very low area percentages were accounted for furans, lactones, nitrogenous compounds, phenols and sulphur compounds.

Among furans, 2-pentylfuran was detected in almost all the samples with the exception of samples from producers D, M, and V, whereas 2-ethyl-5-methylfuran was found only in samples from producers B, C, and E.

Among lactones, gamma butyrolactone was found in almost all the samples while gamma nonalactone, 5-pentyl-2 (5H)- furanone and gamma caprolactine were detected in less than half of the samples.

Nitrogenous compounds were detected in low percentage and in very low samples. The most representative nitrogenous compounds were 2,5-dimethyl-pyrazine, 2,6-dimethyl-pyrazine and trimethylpyrazine, detected in samples from producers A, B, C, D, H, I, L, and V.

Phenol and 4-methylphenol were detected in all the samples, whereas guaiacol was accounted just in samples from producers F, G, and T. Eugenol and methyleugenol characterized 11 and 9 samples.

The most common sulfur compounds were allyl methyl sulphide, detected in more than half samples, and methionol, diallyldisulfide and dimethylsulfone detected in low samples.

Benzene, 1,3-(1,1-dimethylethyl) was the only aromatic hydrocarbon found in trace amounts in the samples analyzed.

3.6. Correlation analysis

By plotting the correlation between microbiota, mycobiota and VOCs several significant relationships were observed (Figure 4, $P < 0.05$).

In particular, isolvaleric and propanoic acids were highly correlated with the presence of lactic acid bacteria such as *Lact. plantarum*, *L. coryniformis*, *Levl. brevis*, *Weissella confusa and Leuconostoc spp.* Among ketones, acetoin was highly correlated with *Lactobacillus* spp., *Leuconostoc* spp., *Levl. brevis*. and *Weissella*. Regarding the core microbiota communities *L. sakei* was found positively correlated with pentanal, 2-heptenal 1−octen−3−one, sabinene, alpha-pinene, and alpha thujene. The presence of *S. xylosus* was correlated with isolvaleric acid, linalool, and alpha terpinolene. *S. equorum* displayed the highest number of negative correlations with most of all detected VOCs. *Weissella* and *Leuconostoc* were found positively correlated with acetoin, isolvaleric acid, and propanoic acid. Regarding the mycobiota, the core *D. hansenii* ASV was correlated with cymene and beta phellandrene, whereas *C. galli* displayed several positive associations as those with acetic acid, ethyl acetate, octane, hexane, 3−methylbutanal, 2−methylbutanal, and 2−propanone. *Y. lipolytica* was correlated with acetic acid, 1-pentanol, benzeneacetaldehyde, and acetoin. Finally, *Starmerella* was associated with ethanol, 2-propanone, and with several terpenoids compounds.

3.7. Real-time-RT-(q)PCR assay results

In this study, the presence of HEV RNA by real-time RT-PCR in liver fermented sausages samples was investigated. Samples from the 20 producers were tested. Samples with threshold cycle (Cq) above 40 and no evidence of amplification were considered negative. All reactions were run in duplicate, and no positive samples were detected.

To evaluate the correctness of the HEV RNA extraction procedure, every sample was spiked with 10 µL of Mengovirus ($3x10^4$ TCID50/ml), used as process control virus. Extraction efficiency was assessed through the recovery of Mengovirus by comparing the Cq value of Mengovirus RNA obtained in spiked samples (Sample + Mengo) with the Cq value of the first point of a Mengovirus RNA standard curve (Mengo T.Q) by applying this formula:

$$X = 10^{(\Delta Cq / s)} * 100$$

Where:
X= extraction efficiency; ΔCq = Cq (Sample + Mengo) – Cq (Mengo T.Q.); "s" is the slope of the Mengovirus standard curve.
The extraction efficiency was greater than 1% for each sample and so, all the results were considered acceptable.
Furthermore, a control for RT-PCR inhibition was also performed. Briefly, HEV external control RNA (EC-RNA) was added to each sample. The Cq value obtained in samples with external control RNA (Sample + EC-RNA) was then compared with the Cq value obtained from the analysis of the EC-RNA alone, to calculate the ΔCq as follows: ΔCq = Cq (Samples + EC-RNA) – Cq (EC-RNA).
Values with ΔCq< 2 were considered valid (negative for inhibitors presence).
In the present study, the different ΔCq values obtained ranged from ΔCq=0.18 to ΔCq=1.98 and so, all the results were considered acceptable.
Considering all the above-mentioned aspects, no HEV contamination was present in all the samples.

4. Discussion

Traditional foods represent a gastronomic heritage of undisputed value that have to be preserved. Hence, the study of production processes and factors involved in the manufacturing of such foods is the first step to understand their link with the territory and the society, in order to accurately pass them on to future generations. To the authors' knowledge, there is a lack of information in the scientific literature on the physico-chemical parameters, volatilome composition as well as microbiota of fermented liver sausages. Indeed, most of the available studies are related to liver sausages or sausages containing blood to be consumed cooked, hence, the results of the present study could represent a step forward in understanding the biodiversity of fermented sausages containing swine organs.
Regarding pH, the analyzed samples had an average pH value of 5.25 ± 0.28, slightly lower than those observed by Cardinali et al. (2018) in *Fabriano* fermented sausages produced in the Marche Region, that attested between 5.76 and 5.95. The observed pH values were also lower than those reported by Di Cagno et al. (2008) for the Italian PDO sausages *Varzi*, *Brianza,* and *Piacentino*, that attested at 6.57, 5.99, and 6.62, respectively. Moreover, Pini, Aquilani, Giovannetti, Viti, & Pugliese (2020) observed pH values comprised between 5.58 and 5.85 in Italian *Cinta Senese* dry-fermented sausages. The safety of fermented sausages is strongly correlated with pH, that, at acidic values, inhibits the growth of spoilage and pathogenic microorganisms naturally occurring in the raw materials. In fermented sausages, as soon as the fermentation starts, pH progressively decreases at values that should be as low as 4.4. Then, at the end of ripening, metabolic activities of moulds and yeasts naturally occurring in the surface of salami, cause a slight increase of pH and a subsequent improvement of sensory traits (Cardinali et al., 2018; Pisacane, Callegari, Puglisi, Dallolio, & Rebecchi 2015).
As for a_w, values below 0.92 were detected in all samples. The values were in accordance with those reported by Rocchetti et al. (2021) for Italian salami which stood at 0.875 after 45 days of ripening. Moreover, a_w values detected in the analyzed fermented liver sausages were generally in accordance with those reported by Di Cagno et al. (2008) for the *Varzi*, *Brianza,* and *Piacentino* salami attesting at 0.89, 0.87, and 0.89. A_w, together with pH, represents one of the key physico-chemical parameters that stabilize the microbiological activities in fermented meat sausages. Indeed, the potential growth of some foodborne pathogens (e.g., *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, etc.) can be reduced by controlling the a_w, in addition with low pH (Oliveira, Ferreira, Magalhães, & Teixeira 2018).
Regarding organic acids, the amount of lactic acid was higher than those of acetic acid in all the samples analyzed, thus likely attesting the occurrence of homofermentative or facultative heterofermentative lactic acid bacteria.
Microbial viable counts carried out in the present study highlighted the presence of active microbial populations mainly composed by lactic acid bacteria, enterococci, coagulase-negative cocci, and eumycetes. Only samples collected from a few producers showed the presence of low levels of *Enterobacteriaceae*,

Pseudomonadaceae, or sulfite-reducing anaerobes, thus attesting the high quality of raw materials and production processes.

Regarding lactic acid bacteria, high counts were detected in all the analyzed samples. Data can be compared with those collected by Iacumin, Manzano, Stella, & Comi (2017) in *Sanganel*, a typical blood sausage produced in the Friuli Region (Italy), containing about 8.5 log cfu g^{-1} of lactic acid bacteria after 30 days of ripening. Indeed, although *Sanganel* is not produced with pork liver, its recipe foresees the use of swine organs, as lungs and kidneys, in addition with blood. The occurrence of high loads of lactic acid bacteria was also observed by Cardinali et al. (2018) in *Fabriano* fermented sausages after 45 days of ripening, with counts attesting at about 7.5 log cfu g^{-1}. Lactic acid bacteria represent the key microorganisms in meat fermentation, being them able to produce organic acids (mainly lactic and acetic acid) through catabolism of pentoses or hexoses during fermentation (Belleggia et al., 2020). In more detail, the acidification process driven by lactic acid bacteria creates a distinctive gel-like texture produced by protein denaturation. Moreover, the interaction between the myoglobin of the meat and nitrogen monoxide, that originates from the nitrate and/or nitrite in the curing salt, lead to the formation of a pleasant red color (Leroy, Geyzen, Janssens, De Vuyst, & Scholliers 2013). During fermentation, both bacterial and meat proteinases progressively hydrolyse sarcoplasmic proteins to the subsequent peptides and free amino acids that further serve as precursors for aroma formation (Todorov et al. 2017).

As for enterococci, the data obtained in the present study were in accordance with counts reported by Iacumin et al. (2017) for *Sanganel* that attested at about 4.8 log cfu g^{-1} after 30 days of ripening. Previous studies also reported that the loads of enterococci can depend on the type of product, indeed, other Italian salami showed values ranging from 5.2 (*Varzi* salami) and 7.3 (*Brianza* salami) log cfu g^{-1} (Pisacane et al., 2015). Interestingly, many enterococci isolated from sausages can produce bacteriocins (e.g., enterocin) with potential antimicrobial activity against pathogens and spoilage microorganisms (Hugas, Garriga, & Aymerich, 2003). It is noteworthy that enterococci have also been described as producers of biogenic amines (e.g., histamine, cadaverine, tyramine, phenylethylamine, and putrescine) in meat, thus suggesting possible threats to consumers' health that have to be further investigated (Pleva et al., 2012).

High counts of coagulase-negative cocci were detected in all the samples although a high variability was seen. The values detected in the present study were generally comparable with those detected in *Sanganel* by Iacumin et al. (2017) that reported counts attesting at about 6 log cfu g^{-1} after 30 days of ripening. Coagulase-negative cocci detected in the present study were generally higher than those reported by Belleggia et al. (2020) in *cacholeira* blood sausages produced in Portugal. In fermented sausages, coagulase-negative cocci are key microorganisms that exert proteolysis and lipolysis via enzymatic activity, thus contributing to flavor formation in the end product (Lorenzo et al., 2017). Of note, reductase activity of coagulase-negative cocci leads to nitrosomyoglobin formation with the subsequent development and stabilization of a pleasant red color (Cocolin, Dolci, & Rantsiou, 2011).

Finally, the viable counts of the eumycetes detected in the analyzed samples were generally similar to those detected in *Sanganel* after 30 days of ripening (Iacumin et al., 2017) and to those detected in *cacholeira* blood sausages (Belleggia et al., 2020), attesting at about 6 and 6.9 log cfu g^{-1}, respectively. The eumycetes group encompasses molds and yeasts that, in fermented sausages, are responsible for volatile compounds production due to their proteolytic and lipolytic activities (Cocolin et al., 2011). Moreover, molds are present on the surface of the sausage where they create micro-pores on the casing, thus facilitating the dehydration process. Besides, the homogeneous mold layer, that occurs on the surface of the fermented sausage, protects lipids from oxidation in the presence of light (Cocolin et al., 2011). Yeasts are generally detected internally and release peptides, free amino acids, and free fatty acids by lactic acid metabolization (Belleggia et al., 2020).

The metataxonomic analysis performed on the fermented liver sausages allowed major and minor taxa to be identified.

Among bacteria, all samples showed the dominance of *L. sakei*. This lactic acid bacterium represents the key species in fermented meat sausages, since it has the ability to compete with other bacteria naturally contaminating meat during the later phase of ripening and throughout storage (Ojha, Kerry, Duffy, Beresford, & Tiwari 2015). *L. sakei* is able to multiply in protein-rich matrices and at high salt concentration (up to 8%), at a temperature range comprised between 5 and 35 °C, with an optimum between 25 and 35 °C, thus explaining the ability of this lactic acid bacterium to grow in meat batter used for production of sausages (Rocchetti et al., 2021). *L. sakei* is able to use ribose contained in meat as carbon source via ATP-dependent system (Zagorec, & Champomier-Vergès, 2017). In the meat batter, *L. sakei* contributes to hydrolysis of myofibrillar proteins, thus showing a complementary activity to endogenous muscle endopeptidases (Tremonte et al., 2010). Indeed, as reviewed by Flores, & Toldrà (2011), *L. sakei* is able to produce endo and exo-peptidases (dipeptidase,

aminopeptidase, tripeptidase, X-prolyl-dipeptidylpeptidase, and arginine aminopeptidase) that increase the concentration of free amino acids, thus positively affecting flavor development. Moreover, catalase produced by *L. sakei* exerts antioxidant activity that prevents meat rancidity (Hertel, Schmidt, Fischer, Oellers, & Hammes, 1998). *L. sakei* can also be considered a bio-protective pro-technological bacterium due to the production of bacteriocins as sakacin A, P and K, active against *L. monocytogenes* (Työppönen, Petäjä, & Mattila-Sandholm, 2003).

As for the presence of staphylococci in the analyzed samples, it is known that *S. xylosus* and *S. equorum* can exert lipolytic activity by secreting extracellular lipases that release free fatty acids and convert metmyoglobin into nitrosomyoglobin, thus contributing to color formation (Morita, Sakata, & Nagata, 1998; Xiao, Liu, Chen, Xie, & Li, 2020). *S. xylosus* has been detected as main coagulase-negative staphylococcus in different Italian salami as *Salame Napoli*, *Soppressata* (a traditional fermented meat product from the Molise region), *salsiccia sarda*, *salsiccia sotto sugna* (an artisan sausage, typically manufactured in the Basilicata region), *Salame Milano*, *Salame Mantovano*, *Salame Piacentino*, *Ciuscolo*, and *Soppressata del Vallo di Diano* (Aquilanti et al., 2016). Moreover, *S. xylosus* was also detected in fermented sausages produced in France, Greece, and Spain, thus attesting its wide adaptation to fermented sausages produced in Mediterranean countries (Aquilanti et al., 2016). To the authors' knowledge, no previous reports on the occurrence of *S. xylosus* and *S. equorum* in fermented sausages containing liver are available in the scientific literature for further comparison of data. It is noteworthy that, as reported by Sola, Barrio, & Martin (1997), swine liver is rich in iron (about 51.6 μg g^{-1}) contained in different forms, including ferritin (Lipinski et al., 2010), that is a storage protein able to capture large quantities of iron (Vermassen, Talon, & Leroy, 2016). Hence, it is likely that in such iron-rich matrix, *S. xylosus* could take advantage due to its capability to use iron from ferritin, as reported by Vermassen et al. (2016).

Based on the results of statistical analysis performed on quartile clustering, the presence of many minority species detected in the analyzed sausages seemed to be related to physico-chemical parameters. In more detail, *Leuconostoc*, *S. equorum* and *B. thermosphacta* were associated with fermented liver sausages showing the highest pH values. As reported by Cicotello et al. (2018), the growth of some *Leuconostoc* strains can be disfavored by acidic conditions. Moreover, as reported by Janssens, Myter, De Vuyst, & Leroy (2013), the increase in pH of sausages, due to lactic acid utilization by the moulds, could favour the multiplication of *S. equorum*. Finally, *B. thermosphacta* is characterized by an optimal growth at pH 6.8, whereas it is inhibited at pH below 5.5 (Mohsina et al., 2020), thus explaining the presence of this meat spoilage bacterium in high-pH samples.

L. plantarum, *L. garviae* and *Weissella,* where associated with samples that displayed the highest NaCl level. As suggested by Zhao et al. (2014), some *L. plantarum* strains can accumulate glycine betaine that is one of the most universal osmo-protectants against salt stress. Moreover, *L. garviae* has already proved to be salt resistant, having been isolated in high-salt batches of *plaa-som*, a Thai fermented fish product (Paludan-Müller, Madsen, Sophanodora, Gram, & Møller, 2002). Finally, as reported by Nath et al. (2021), a *Weissella confusa* strain showed tolerance to NaCl up to 7.5%, thus explaining the presence of this genus in the fermented liver sausages with the highest salt concentration.

Regarding *Psychrobacter,* associated with samples that showed the lowest values of total titratable acidity, this genus of psychrotrophic cocco-bacilli is able to grow in habitats with low acidity, thus resulting inhibited by environments containing organic acids (Mounier, Coton, Irlinger, Landaud, & Bonnarme, 2017).

Among eumycetes, *D. hansenii*, that was the dominant genus in all the samples, represents one of the key yeast genera in fermented sausages, being constantly detected in Italian as well as other European salami (Aponte, Pepe, & Blaiotta, 2010; Mangia, Garau, Murgia, Bennani, & Deiana, 2014). *D. hansenii* is a halotolerant yeast that contributes to the stabilization of the red color of meat in fermented sausages due to its ability to degrade peroxides. It also contributes to aroma formation due to its proteolytic and lipolytic activities (Murgia et al., 2019). Indeed, as reported by Cano-García, Rivera-Jiménez, Belloch, & Flores (2014), some strains of *D. hansenii* caused an increase in volatile compounds as esters, acids, branched alcohols, and aldehydes in fermented sausages, thus affecting the volatile profile of the final product. Interestingly, *D. hansenii* has also been shown to be able to counteract the development of ochratoxigenic moulds in dry cured meat, suggesting its potential protective role in fermented sausages. As reported by Bonaïti, Leclercq-Perlat, Latrille, & Corrieu (2004), the growth of *D. hansenii* in food matrices is favored by high levels of relative humidity, thus explaining its association with high a_w fermented liver sausages.

In the analyzed sausages, *Aspergillus* and *Penicillium* were sporadically found. Such genera represent the two most detected molds in fermented sausages (Grazia, Romano, Bagni, Roggiani, & Guglielmi, 1986). Because of their mycelia, molds are able to deeply penetrate in fermented sausages, thus causing a decrease in lactic

acid and an increase in pH. Moreover, beside their antioxidative effect, molds produce enzymes for the degradation of the lipid- and protein-matter (Sunesen, & Stahnke, 2003). The activity of molds also facilitates the peeling of the end product (Sunesen, & Stahnke, 2003).

Among the minority fungal taxa detected in the analyzed samples, *Candida* was also found in a few samples. Species of *Candida* have already been detected by Belleggia et al. (2020) in *cacholeira* blood sausages and by Staib et al. (1980) in boiled sausages. Moreover, species of *Candida* have also been found by Giarratana et al. (2014) in *nduja di Spilinga*, a spreadable PGI Italian salami, and by Gardini et al. (2001) in *salsiccia sotto sugna*.

The SPME-GC/MS analysis identified the major and minor volatile components in the analyzed sausages.

The predominant volatiles were terpenes, which are crucial in defining the flavor profile of this type of fermented sausages. The terpenes could derive from the spices included in the formulation of the studied sausages such as pepper, garlic, chili, which are rich in terpenes or from terpenes presumably coming from animal feed, as also observed by other authors (Sulejami, & Demiri, 2020). In particular, limonene was the major terpenoid identified in almost all the samples. Significant amounts of α-pinene, α-thujene, β-pinene, cymene, caryophyllene, sabinene, delta-carene were also found. Similar results were also found in other dry-cured sausages (Bis-Souza et al., 2019) and Turkish fermented sausages (Sulejami, & Demiri, 2020) where the most abundant terpene was limonene.

Aldehydes constituted the second largest group isolated from the samples. As also found by other authors, this group of volatiles is one of the most important in fermented sausages (Dominguez, Agregán, & Lorenzo, 2016). Usually, aldehydes are better indicators of lipid oxidation than other volatile compounds. Among the volatiles detected in this study, hexanal, which presence is related to lipid oxidation of fatty acids (Montanari et al. 2018), was detected in all the samples. In almost half of the samples, hexanal was the most conspicuous aldehyde. The amount of hexanal can be decisive in defining the flavor profile of fermented salami as hexanal has herbaceous and fresh notes at low levels and a strong rancid smell at high concentrations (Dominguez et al, 2019).

Also, nonanal, 2-octenal, pentanal, 3-methyl-butanal, 2-methyl-butanal, and branched aldehydes, mainly correlated to proteolysis and amino acid degradation (Purrinos, Franco, Carballo, & Lorenzo, 2012), contributed to the final volatile aroma of the analyzed fermented sausages. In particular, nonanal and hexanal are considered as markers of secondary oxidation of fatty acids (Carvalho et al., 2020). Furthermore, almost all the samples were characterized for relative high percentage of benzeneacetaldehyde and benzaldehyde, cycloaldehydes derived from Streker degradation of amino acids (Lorenzo, & Carballo, 2015). Also, other authors (Bis-Souza et al., 2019) found benzaldehyde and benzacetaldehyde in numerous fermented sausages.

As aldehydes, alcohols are also considered as markers of secondary oxidation of fatty acids. The highest area percentage of ethanol was found in samples from producer I, where wine was added as ingredient in the sausage production recipe.

Ketones constituted the third largest group of volatiles isolated from fermented sausages. Among ketones, acetoin, 2-butanone, 2-propanone 2,3 butanedione (diacetyl), and 2, 3 octanedione were the main compounds identified. Both acetoin and 2-butanone give important aroma notes commonly associated to meat products because of its peculiar intense odor. Furthermore, volatiles such as diacetyl, acetoin, and 2-phenylethanol contribute to the typical final flavor of the salami.

Twelve different acids were identified in the fermented sausages. The main acids were acetic acid, isovaleric, hexanoic and propanoic acids, probably produced by citrate or lactate fermentation by bacteria. In particular, isovaleric and propanoic acids were correlated to *L. plantarum*, *Lactobacillus* spp., *L. coryniformis*, *Levl. brevis*, *Leuconostoc* spp., *Weissella* spp., *Weissella confusa*. Esters, such as ethyl acetate, ethyl hexanoate, ethyl decanoate, were another group of volatile compounds derived from bacterial metabolism. The origin of ester compounds in traditional fermented sausages can be due to different microbial groups including lactic acid bacteria, coagulase-negative cocci, yeasts, and molds (Karwowska, Kononiuk, Borrajo, & Lorenzo, 2021). By plotting the correlation between VOCs and mycobiota composition, the ethyl acetate was correlated with *Candida galli* and *Yarrovia divulgata*. Samples from producers H and I were characterized by a high percentage of esters contributing to the fruity aromatic notes which are commonly associated with high consumers' acceptance of traditional dry sausages (Rzepkowska, Zielińska, Ołdak, & Kołożyn Krajewska, 2017). Furthermore, different correlations were found between *Candida galli*, *Starmerella*, *Yarrowia divulgata*, *Yarrowia lipolytica* and different acids, aldheydes and ketones, highlighting that their presence could contribute to the aroma profile definition of these fermented products.

Regarding the detection of HEV in the analyzed fermented sausages, as reported by Di Cola, Fantilli, Pisano, & Ré, (2021), such virus is usually detected in food of animal origin, such as meat, sausages and pate of pigs

and wild boars. It is noteworthy that, in Italy, an active circulation of HEV is constantly observed in domestic pigs and wild boar, although the threat of HEV seems still low when compared with other European countries (Di Profio et al., 2019). Although no fermented liver sausage sample showed the presence of HEV in the present study, continued genomic surveillance of HEV in animal reservoir (e.g., swine and wild boar) is suggested (Lo Presti et al., 2020).

5. Conclusion

As reported by Franciosa et al. (2021), metataxonomic analysis represents a robust tool to study and understand the microbial diversity occurring during food fermentation, thus allowing the link between microbial population and sensory characteristics of the end product to be investigated. In the present study, the dominance of *L. sakei* and *D. hansenii* was ascertained. This result is particularly of interest since the analyzed fermented sausages were independently manufactured by 20 producers located in all the 5 provinces of the Marche Region. Moreover, although based on the use of swine meat, lard, and liver, the recipes applied in the production of the analyzed sausages slightly differed in liver amounts and other added ingredients. Hence, the disclosure of a stable microbial population in the analyzed samples could contribute to define common traits characterizing these unique fermented sausages. Another interesting finding is represented by the associations discovered between some microbial taxa (e.g., *Leuconostoc* spp., *S. equorum*, *B. thermosphacta, Weissella* spp*., Psychrobacter* spp*., Aspergillus* spp*., Penicillium* spp*., Candida* spp., etc.) and some physico-chemical parameters, thus prompting the need for further studies to better disclose such phenomenon. The absence of HEV in all the samples represents another important outcome of this study since it attests a high level of safety of the organs used to produce the sausages under investigation. Finally, a complex volatilome associated with the ingredients and the microbial activities has been detected in the sausages for the first time. Further studies are needed to disclose the dynamics of microbial populations and volatile compounds during the production of fermented liver sausages.
In view of obtaining a geographical indication status (e.g., PGI) for fermented liver sausages of the Marche Region, the data obtained could serve as reference in drawing up a production disciplinary. Such disciplinary should take into account the microbiological and technological peculiarities of the products manufactured by each producer as well as provide common reference values in order to create uniform and recognizable products. Indeed, as recently reported by Milano, & Cazella (2021), geographical indication labels can be great allies in enabling productive systems anchored in singular environmental and social resources.

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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FIGURE CAPTIONS

Figure 1. Incidence of the major taxonomic groups detected by 16S amplicon target sequencing. Only ASVs with an incidence above 0.2% in at least 2 samples are shown. Abundances of ASVs in the 3 sausage samples for each producer were averaged.

Figure 2. Incidence of the major taxonomic groups detected by 26S amplicon target sequencing. Only ASVs with an incidence above 0.2% in at least 2 samples are shown. Abundances of ASVs in the 3 sausage samples for each producer were averaged.

Figure 3. Profiles of volatile organic compounds (VOCs) in twenty producers of fermented liver sausages. Abundances of VOCs in the 3 sausage samples for each producer were averaged.

Figure 4. Spearman's rank correlation matrix of significant relationships between ASVs. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colors of the scale bar denote the nature of the correlation, with 1 indicating a positive correlation (dark blue) and 2 indicating a negative correlation (dark red) between microbial genera and metabolites. Only significant correlations ($P<0.05$) are shown.

Supplementary Figure 1. Boxplots to describe α-diversity measures (Shannon index) of microbiota as a function of batch or according to water activity, pH, acetic acid, NaCl, lactic acid and total titratable acidity quartiles. Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively. Significant association $P < 0.05$ (Kruskal–Wallis nonparametric test with Bonferroni corrections) are also displayed.

Supplementary Figure 2. Boxplots to describe α-diversity measures (Shannon index) of mycobiota as a function of batch or according to water activity, pH, acetic acid, NaCl, lactic acid and total titratable acidity quartiles. Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively. Significant association $P < 0.05$ (Kruskal–Wallis nonparametric test with Bonferroni corrections) are also displayed.

Supplementary Table 1. Significant association $P < 0.05$ (Kruskal–Wallis nonparametric test with Bonferroni corrections) between microbiota and mycobiota according to water activity, pH, acetic acid, NaCl, lactic acid and total titratable acidity quartiles. Sample codes and value ranges for each determination are also displayed.

Supplementary Table 2. Profiles of volatile organic compounds (VOCs) in liver fermented sausages of Southern Italy (Results are reported as A%=Area Peak Compound/Area Peak Total Compounds) × 100 (A% ± SD)