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. Physico-chemical parameters and volatilome of the fermented liver sausages were also studied. Finally, the presence of hepatitis E virus (HEV) was also assessed via real-time-RT-(q)PCR assays. Active microbial populations mainly represented by lactic acid bacteria, enterococci, coagulase-negative cocci, and eumycetes were detected. Enterobacteriaceae, Pseudomonadaceae, and sulfite-reducing anaerobes were not detected in most of the samples. *Latilactobacillus sakei* dominated in all the analyzed samples, 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 mycobiota was mainly composed by *Debaromyces hansenii* that was present in all samples at 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 discovered. The absence of HEV in all the samples attested a high level of safety. Finally, most of the VOCs detected in the analyzed fermented liver sausages belonged to six classes, as: terpenoids, aldehydes, ketones, alcohols, esters, and acids. Nitrogen compounds, sulfur compounds, phenols, hydrocarbons, lactones, furans and aromatic hydrocarbons were also identified. Several significant relationships were observed between mycobiota and VOCs.

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. 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 (Belleggia et al., 2020). 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 and on their interactions with the raw materials, other ingredients, and environment. Numerous variations in terms of processing methods and ingredients to produce fermented sausages are applied around the world and, particularly, in Southern Europe. In such a territory, the production of an ample variety of fermented meat sausages is realized, including, 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 for the valorization of their distinctive characteristics. In such a context, food product names can be awarded by "geographical indications" that include Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), Geographical Indication (GI), and Traditional Specialty Guaranteed (TGI). In Italy, the Ministry of Agricultural, Food and Forestry Policies (MiPAAF, 2021) has also focused on niche products of limited production, by publishing once per year a list of traditional Italian foods. 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 for defining procedures, ingredients, and product parameters, the production process can frequently be subjected to modifications based on the manufacturer's practice.

In the Marche Region, 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 ratio. 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 at temperature from 20 to 18°C and relative humidity from 90 to 75%. 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-brown color and their tender consistency. Such jewel of the Italian gastronomy is appreciated for its metallic (ferrous) taste conferred by liver, and its spreadability. It is noteworthy that, as reported by Estèvez et al. (2005), sausages containing liver can be considered excellent sources of dietary iron for humans, since the addition of liver produces sausages with levels of iron that are higher than other meat products or fortified foods.

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 organic 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 volatilome of the fermented liver sausages were also studied.

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, 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). Each fermented liver sausage sample consisted of at least 150 g of whole end product. Samples were collected at production plant, placed into sealed sterile bags (Whirl-Pak®, Merk Life Science, Milan, Italy), and stored under refrigeration (+4°C) until analysis. All the analyses were carried out within 3 days from sample collection.

Although all sausages were prepared based on the use of swine meat and liver, the list of other added ingredients slightly differed, depending on the producer. Therefore, the different formulations of the fermented liver sausages under study are reported in Table 1.

2.2. Physico-chemical analysis

The pH value was determined using a pH meter equipped with a HI2031 solid electrode (Hanna Instruments, Padova, Italy) inserted 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 ion chromatography analysis. Briefly, 2 ± 0.1 g of sample was weighted, added with 20 mL of water, mixed for 20 minutes (orbital mixer KS 501 Digital, IKA® Werke, Staufen, Germany), centrifuged at 800 rpm for 5 min (Rotanta 460 R, Hettich GmbH & Co. KG, Tuttlingen, Germany), filtered through a 0.45 µm syringe filter and analyzed by ion chromatography (ICS

5000 Dionex, ThermoFisher Scientific, Milan, Italy). The chromatography conditions were: Dionex IonPack CS12A 4x250 mm column and Dionex IonPack CG12A 4x50 mm precolumn (Thermo Fisher Scientific, Milan, Italy), 50 µL injection, 1 mL min^{-1} flow, 100 mA SRS and 20 mM methanesulphonic acid as mobile phase.

Lipid oxidation was monitored by determining the peroxide value (PV, mEq peroxide per kg) 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 content was 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.

A Chroma Meter CR-200 (Minolta, Osaka, Japan), equipped with a D 65 illuminant, was used to analyze the color of fermented liver sausage samples on 2 cm thick slices. Color measurements were obtained according to CIE L*a*b* system (L*, lightness; a*, redness/greenness; b*, blueness/yellowness).

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^{-1}) to 10 g of each fermented liver sausage sample, followed by homogenization for 10 min at 260 rpm into a sterile Stomacher 400 Circulator bag (Seward Limited, Worthing, UK), using a Stomacher 400 Circulator apparatus (PBI, Milan, Italy). Subsequently, the serial ten-fold dilutions were set up to determine the presence and concentration of the following microbial groups: (i) presumptive lactic acid bacteria on De Man, Rogosa and Sharpe (MRS) agar (VWR Prolabo Chemicals, Leuven, Belgium), supplemented with 250 mg L^{-1} 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, analyses were carried out 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 liver sausage sample and 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, San Diego, CA, USA) 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 with 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 sample 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, 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., 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 were performed as describe by Di Pasquale et al. (2019). Briefly, approximately 10 g of each fermented liver sausage sample was homogenized 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.

To evaluate the correctness of the HEV RNA extraction procedure, every sample was spiked with 10 µL of Mengovirus ($3x10^4$ TCID50 mL^{-1}), used as process control virus. Extraction efficiency was assessed through the recovery of Mengovirus by comparing the threshold cycle (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.

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).

2.7. Statistical analyses

The statistical analysis of microbiological and physico-chemical data was performed to determine differences among fermented liver sausage samples using 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, lactic acid, NaCl, 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 and correction for multiple comparisons was applied; a *P* value of 0.05 or lower was considered statistically significant.

Color measurement data were used to build principal component analysis (PCA) by the function dudi.pca in R environment.

Shannon indices 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 2.

The pH mean values were comprised between 4.85 ± 0.06 (samples of producer L) and 5.70 ± 0.11 (samples of producer A).

The a_w data showed the lowest mean value of 0.772 ± 0.005 (samples of producer O) and the highest mean value of 0.912 ± 0.003 (samples of producer A).

The salt (NaCl) concentration showed the lowest mean value of 1.67 ± 0.02 g 100 g^{-1} (samples of producer M) and the highest mean value of 3.88 ± 0.05 g 100 g^{-1} (samples of producer S).

Regarding the peroxide values, the lowest mean value was detected in samples of producer A with 5.0 ± 0.0 meq O_2 kg^{-1} of fat, whereas the highest mean value was detected in samples of producer O with 41.5 ± 0.7 meq O_2 kg^{-1} of fat.

The TTA mean values ranged between 18.07 ± 1.42 (samples of producer U) and 39.83 ± 1.65 mL of 0.1 N NaOH (samples of producer L).

Lactic acid showed the lowest mean value of 0.163 ± 0.079 g 100 g^{-1} (samples of producer C) and the highest mean value of 2.279 ± 0.143 g 100 g^{-1} (samples of producer L), whereas the acetic acid showed the lowest mean value of 0.002 ± 0.003 g 100 g^{-1} (samples of producer U) and the highest mean value of 0.047 ± 0.003 g 100 g^{-1} (samples of producer H).

Color measurement results on fermented liver sausage samples are reported in Table 3.

As far as the lightness is concerned, results were comprised between 38.31 ± 3.66 (samples of producer M) and 54.88 ± 2.35 (samples of producer U). For this parameter, ANOVA results allowed to highlight a strong variability among the fermented liver sausage from the 20 producers. Moreover, the analysis of redness/greenness showed values from 10.02 ± 0.22 (samples of producer T) to 14.57 ± 0.83 (samples of producer H). For this parameter, ANOVA results showed a modest variability among producers, as no statistically significant differences were evidenced among 13 producers (F, G, I, L, M, N, O, P, Q, R, T, U,

V). Finally, the values of yellowness/blueness were comprised between 4.98 ± 0.50 (samples of producer F) and 12.37 ± 1.17 (samples of producer H). For this parameter, samples from ten producers (A, C, D, E, G, M, O, P, T, V) did not show statistically significant differences.

A high variability was observed by the PCA (Supplementary Figure 1) where 19 out of 20 samples could not be classified into separate clusters. Only samples from producer H were those well separated from the others.

3.2. Microbiological analyses

The results of the microbiological viable counts of fermented liver sausages are reported in Table 4. The counts of presumptive lactobacilli were comprised between 6.80 ± 0.30 of samples of producer Q and 8.76 ± 0.12 Log cfu g^{-1} of samples of producer I. As for enterococci, the lowest mean values belonged to samples of producers L and R with counts lower than 1.00 Log cfu g^{-1}, whereas the highest mean value was reached in samples of producer Q with 5.71 ± 0.84 Log cfu g^{-1}. The coagulase-negative cocci viable counts ranged between 3.00 ± 0.07 Log cfu g^{-1} in samples of producer P and 7.18 ± 0.30 Log cfu g^{-1} in samples of producer N. Low counts of Enterobacteriaceae and Pseudomonadaceae were detected among fermented liver sausage samples; however, the highest mean values of Enterobacteriaceae and Pseudomonadaceae counts were 4.18 ± 0.47 Log cfu^{-1} in samples of producer U and 5.10 ± 0.22 Log cfu g^{-1} in samples of producer M, respectively. Sulfite-reducing anaerobes were not detected in any sample, except for those of producer M showing average value of 2.27 ± 0.18 Log cfu g^{-1}. Finally, the counts of total eumycetes were comprised between 4.18 ± 0.54 in samples of producer H and 6.47 ± 0.19 Log cfu g^{-1} in samples of 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 of producers D, H, and S had the highest richness, whereas those of producers L, P, U, and V the lowest (Supplementary Figure 2). 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 ($P<0.05$) (Supplementary Figure 2). 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 abundance above 80% in samples of 16 producers, and 77% and 50 % in samples of four producers, respectively (Figure 1). *Staphylococcus xylosus* was observed at 15% relative abundance only in samples of two producers (S and I), at about 10% in samples of three producers (Q, V, and C), and from 1 to 7% in samples of nine producers. *Staphylococcus equorum* was found at 40% relative abundance in samples of producer B, at about 10% in samples of two producers (C and N), and from 1 to 5% in samples of nine producers. *Weissella* was observed at 18% relative abundance in samples of producer D and 5% in samples of producer H. *Leuconostoc* was observed at 5% relative abundance only in samples of 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, whereas *Macrococcus caseolyticus* and *Staphylococcus xylosus* were associated with samples belonging to cluster Q1 ($P < 0.05$) (Supplementary Table 1). Highest pH values seemed to favor the presence of *Leuconostoc*, *S. equorum* and *Brochothrix thermosphacta*. *Clostridium* was associated with samples belonging to Q2, whereas the presence of *Staphylococcus succinus* and *Macrococcus caseolyticus* was associated with samples clustered in Q2 quartile. Acetic acid showed the association with *Lactobacillus coryniformis* and *Weissella confusa*. *Lactiplantibacillus plantarum*, *Lactococcus garviae* and *Weissella* where associated with samples that displayed the highest level of NaCl (Q4 quartile). In samples belonging to the Q1 quartile, lactic acid was associated with *Staphylococcus equorum*, whereas *Lactiplanibacillus plantarum*, *Lactobacillus* and *Leuconostoc* were associated with samples belonging to Q2 quartile. Regarding the total titratable acidity, *Psychrobacter* was associated with samples that displayed the lower range for this parameter (Q1 quartile), *Carnobacterium viridans* was associated with Q2 quartile and *Enterococcus* with Q4 quartile ($P < 0.05$) (Supplementary Table 1).

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 of producers A, N, R, and U showed the lower mycobiota richness (Supplementary Figure 3). Regarding the chemical determinations, the samples that had the highest level of NaCl and total titratable acidity (belonging to quartile Q4) showed the highest microbial richness, whereas samples with highest a_w value displayed the lower richness (Supplementary Figure 3).

Twenty-six ASVs were detected in all the samples analyzed, however a few taxa dominated the mycobiota (Figure 2). In more detail, *Debaromyces hansenii* was detected in all samples with the highest relative abundance. Only samples of 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% relative abundance in samples of producer B and at 53.98% relative abundance in samples of producer O. *Penicillium* was found at various percentages from 0.11% relative abundance in sample of producer N to 42.99% relative abundance in samples of producer V. *Kurtzmaniella zeylanoides* was detected in samples of producer I (0.04 % relative abundance) and, with the highest frequency, in samples of producer P (25.71 %). Samples from producer F showed the presence of *Candida* spp. (8.33%), *Candida metapsilosis* (23.79 % relative abundance), *Yamadazyma atlantica* (6.25 % relative abundance), and *Yamadazyma triangularis* (3.55 % relative abundance). *Scopulariopsis* spp. was abundant in samples of producer G (3.45% relative abundance), whereas samples of producer H showed the presence of *Yarrowia deformans* and *Yarrowia lipolytica* at a relative abundance of 11.10% and 6.22%, respectively. *Starmerella* spp. was found in samples of producers O and Q, reaching 3.17% and 1.01% of the relative abundance, 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 quartile based on a_w, unlike *Aspergillus* and *Yamadazyma triangularis* which showed the highest frequency in the Q1 quartile. *Kurtzmaniella zeylanoides* was associated with samples belonging to Q2 quartile ($P < 0.05$) (Supplementary Table 1). *Penicillium roqueforti* was associated with samples belonging to Q4 quartile based on pH range, whereas *Galactomyces candidum* was associated with quartile Q3 and *Yarrowia deformans* with quartile Q1. Regarding acetic acid, *Debaromyces* was associated with Q1 quartile, *Starmerella* spp. with Q3 quartile, and *Geotrichum* with Q4 quartile. *Galactomyces candidum* was associated with the highest NaCl concentrations (Q4 quartile), whereas

Candida alimentaria was observed mainly in Q1 quartile. Regarding the total titratable acidity, *Candida galli* showed the association with quartile Q4 ($P < 0.05$) (Supplementary Table 1).

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 detected in the 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 terpenoids (52), aldehydes (22), ketones (17), alcohols (16), esters (13), and acids (12). Nitrogen compounds (7), sulfur compounds (6), phenols (5), hydrocarbons (5), lactones (4), furans (2), and aromatic hydrocarbons (1) were also identified (Supplementary Table 2). In all the samples, the proportion of terpenoids prevailed over other compounds, varying between 93.4% (samples of producer B) and 39.6% (samples of producer I), with exception of the sample of producer H having the lowest percentage of terpenoids (6.9%). Samples of producers B, T, M, D, and L were characterized by the highest percentage of total terpenoids area, whereas samples of 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% and 32.2%, acids (between 1.1 and 21.3%), ketones (between 1.1 and 29.5%), hydrocarbons (between 0.9% and 7.2%) and alcohols (between 0.1 and 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, β-pinene, sabinene, delta 3-carene, and caryophyllene were found in moderate percentage in all the samples. Some terpenoids appeared only in one or two samples. After terpenoids, aldehydes were the class of compounds found at high percentage in almost all the samples. In more detail, samples of producers H, O, U, S, and R were characterized by the highest percentage of total aldehydes area, whereas samples of 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 of producers O, P,

R, S, and U. Samples of producer H were characterized for high area percentage of benzeneacetaldehyde and benzaldehyde. Ketones occurred at low percentage in all the samples, with the exception of samples of 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 for this compound were those of producer I.

Regarding acids, samples of producers H, I, A, O, and S were characterized by the highest area percentage, whereas samples of 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, and ethyl decanoate, found in 11, 7, 12 and 14 samples, respectively. Samples of producers I and H were the sample with the highest total area percentage of esters.

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

Low area percentage of hydrocarbons was found in all the sample except for samples of producer H and Q that were characterized by 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 sulfur compounds.

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

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

Nitrogenous compounds were detected at low percentage and in very low number of samples. The most representative nitrogenous compounds were 2,5-dimethyl-pyrazine, 2,6-dimethyl-pyrazine, and trimethylpyrazine, detected in samples of 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 of producers F, G, and T. Eugenol and methyleugenol characterized 11 and 9 samples.

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

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

3.6. Correlation analysis

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

In more detail, isolvaleric and propanoic acids were highly correlated with the presence of lactic acid bacteria such as *Lact. plantarum*, *L. coryniformis*, *Levilactobacillus 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

Regarding the presence of HEV RNA assessed via real-time RT-PCR in liver fermented sausages, samples with Cq above 40 and no evidence of amplification were considered negative. All reactions were run in duplicate, and no positive samples were detected. The extraction efficiency was greater than 1% for each sample and so, all the results were considered acceptable. Moreover, 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

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 showed average pH value of 5.25 ± 0.28 that was slightly lower than those observed by Cardinali et al. (2018) in *Fabriano* fermented sausages produced in the Marche Region, attesting 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. It is noteworthy that 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 in 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 the samples. The values were in accordance with those reported by Rocchetti et al. (2021b) for Italian salami which stood at 0.875 after 45 days of ripening. 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, together with low pH (Oliveira, Ferreira, Magalhães, & Teixeira 2018).

Regarding organic acids, the amount of lactic acid was higher than that of acetic acid in all the analyzed samples, thus likely attesting the occurrence of homofermentative or facultative heterofermentative lactic acid bacteria in the analyzed samples.

Of note, samples of producer S and producer T were manufactured without the addition of ascorbates. As reported by Flores & Toldra (2021), ascorbates are reducing agents that speed up the curing process of fermented sausages and prevent lipid oxidation. In the present study samples without ascorbates did not show appreciable higher peroxide values than those containing such ingredient. Such finding suggests the effectiveness of the microbiota (e.g., yeasts, molds, lactic acid bacteria, etc.) in protecting fermented sausages against fat oxidation even in the absence of ascorbates, as already proposed by many authors (Cocolin et al., 2011; Hertel, Schmidt, Fischer, Oellers, & Hammes, 1998).

Regarding color measurements, data were in average comparable with those reported by Icumin et al. (2017) in *Sanganel*, a typical blood sausage produced in the Friuli Region (Italy). Data on redness were comparable with those reported by Estèvez et al. (2005) for a traditional Finnish liver sausage that attested at about 11. Conversely, lightness and yellowness were different from those reported by Estèvez et al. (2005) that attested at about 66 and 13, respectively. As reported by Estèvez et al. (2005), the amount of fat in sausages strongly affects the final color of the product. Hence, the extreme variability of color parameters measured in the samples analyzed in the present study could partly be attributed to the different amounts of fat used by the producers (data not available) as well as to differences in the texture of sausages (data not available). Moreover, it is known that the absence of nitrates and nitrites in sausages could limit the development of color with subsequent reduction of the redness and its intensity (Estèvez et al., 2005). In the sausages analyzed in the present study, samples I, L, O, T, U, and V showed the lowest redness values. Interestingly, among those

samples, only those from producer T were prepared without the addition of nitrate or nitrite, thus suggesting the need for a more in-depth investigation taking into account the amount of nitrate or nitrite added.

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 general 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*, which contained 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 to 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, during fermentation, both lactic acid bacteria 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. 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 must 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).

It is noteworthy that fermented sausages of producer S and producer T were prepared without the addition of nitrate or nitrite. Such preservatives are usually added to the meat batter for their antimicrobial effect exerted against clostridia (e.g., *Clostridium botulinum*) (Cardinali et al., 2008). Notwithstanding, the use of nitrate or nitrite in fermented sausages is still controversial because of their adverse effect on human health, although no valuable alternatives have been found, yet (Cardinali et al., 2008). Hence, although fermented sausages produced without nitrate or nitrite can result in healthier food for nutritional aspects, the absence of such preservatives can lead to a lack of control over clostridia (Rocchetti et al., 2021a). In the present study no specific concerns regarding sulfite-reducing clostridia have emerged for samples of producer S and producer T, thus witnessing the use of high-quality raw materials.

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 activity (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). Of note, the protective action of molds can be particularly important in fermented sausages containing liver since they are commonly prone to oxidation (Xu et al., 2021).

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 in the presence of high salt concentration (up to 8%), at a temperature range comprised between 5 and 35°C, with an optimum between 25 and 35°C (Rocchetti et al., 2021b), thus explaining the ability of this lactic acid bacterium to grow in the meat batter used for the production of the analyzed sausages. In the meat batter, *L. sakei* contributes to hydrolysis of myofibrillar proteins, thus exerting 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 rancidity (Hertel et al., 1998), thus resulting particularly important in fermented liver sausages for the above-mentioned reasons regarding oxidation (Xu et al., 2021).

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). 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 (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 with 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 favor 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 samples with high pH.

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 activity (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. 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 fermented liver sausages characterized by high a_w.

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 into fermented sausages, thus causing a decrease in lactic acid and an increase in pH. 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, 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, and 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 of volatiles 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

sausages 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 found benzaldehyde and benzacetaldehyde in numerous fermented sausages (Bis-Souza et al., 2019).

As aldehydes, alcohols are also considered as markers of secondary oxidation of fatty acids. The highest percentage area of ethanol was found in samples of producer I, where wine was added as ingredient. As also reported by other authors, in different sausages from the Mediterranean area, wine can be added to directly influence the aroma profile of the final product. In more detail, Coloretti et al., (2014) found that the total alcohols were higher in sausages containing wine where ethanol was the most relevant compound of this chemical class, as also found in the present research. Also, Montanari et al. (2016) found that ethanol represents the main alcohol occurring in Italian salami. Moreover, in the present study, different sausages (samples R, S, Q, and H) showed high relative percentage of alcohols, although no wine or other liqueurs were present in their recipes. This fact can be attributed to microbial metabolism or enzymatic activities occurring during ripening of fermented sausages (Montanari et al., 2021). As also reported by Sulejmani et al. (2019), primary alcohols generally originate from the corresponding aldehydes produced from fatty acids and from amino acid metabolism, whereas secondary alcohols are obtained from the enzymatic reduction of methyl ketones.

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 identified compounds. Both acetoin and 2-butanone give important aroma notes commonly associated to meat products because of their 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 with *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 of 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, thus 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

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 ripening of fermented liver sausages.

In view of obtaining a geographical indication status (e.g., PGI) for the fermented liver sausages of the Marche Region, the data obtained could serve as reference in drawing up a production disciplinary. Such disciplinary should be drawn up based on the microbiological and technological peculiarities of the products manufactured by each producer in order to provide common reference values for creating 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 (A-V) 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 (A-V) 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 (A-V) 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.

Fig. 1.

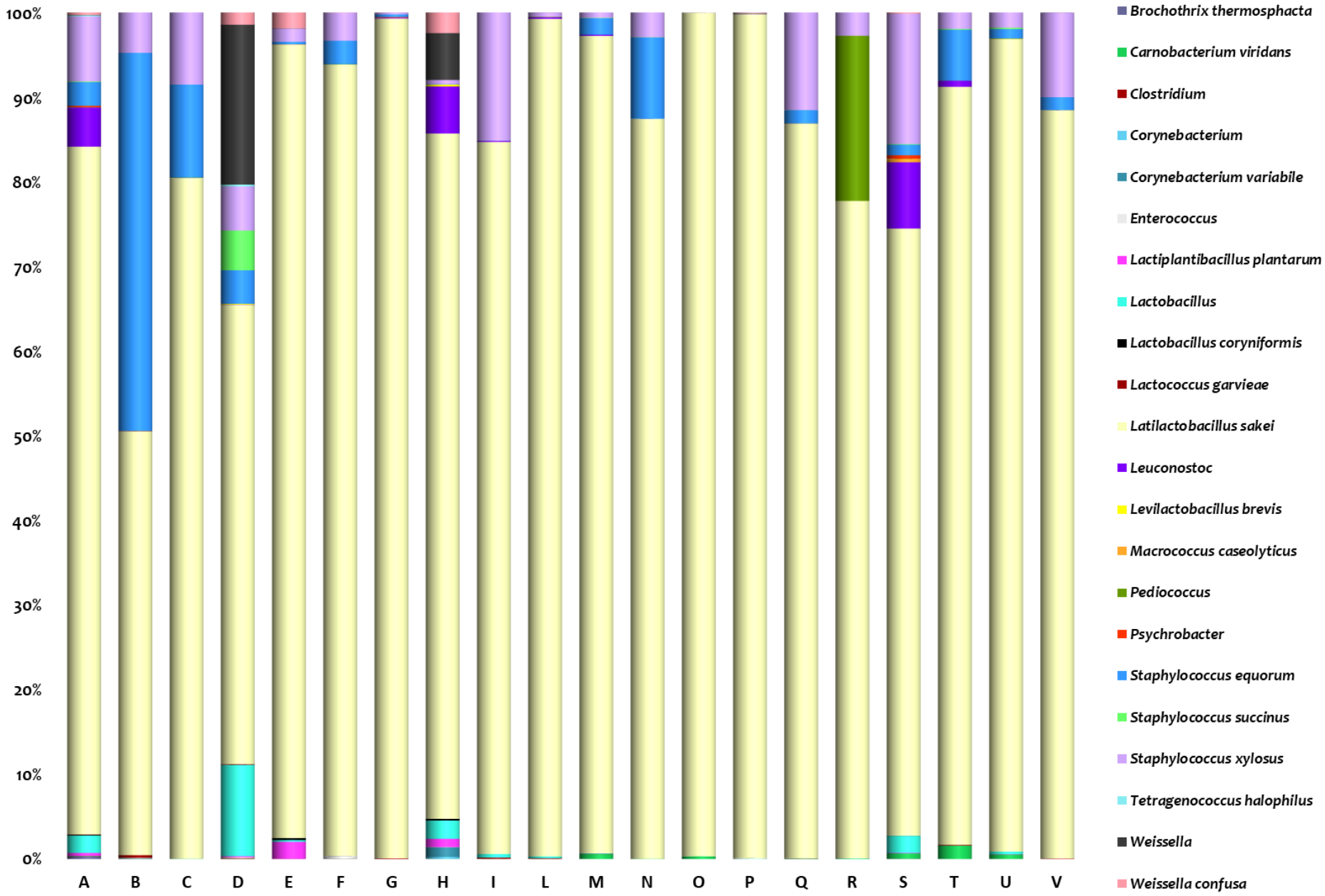

Fig. 2.

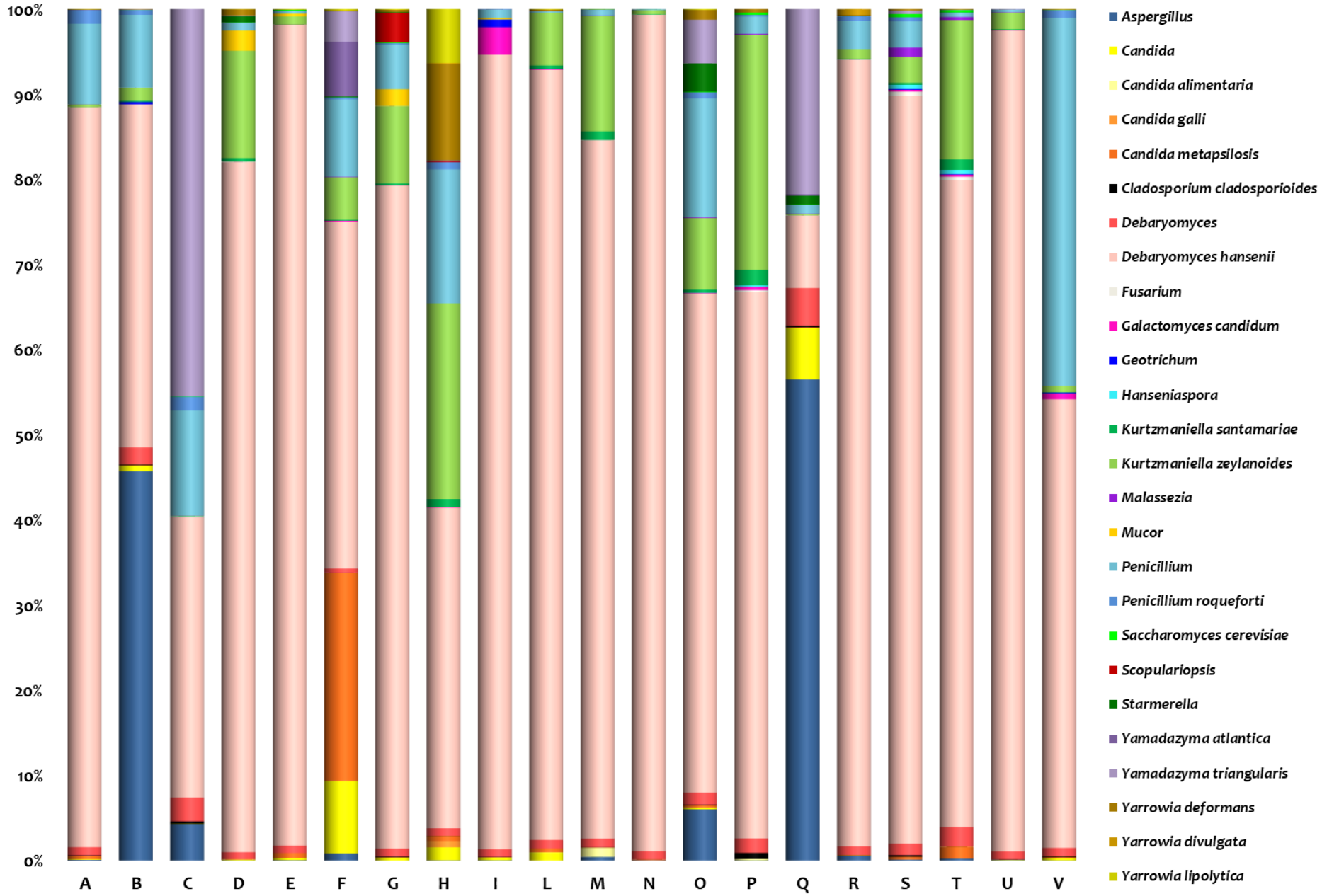

Fig. 3.

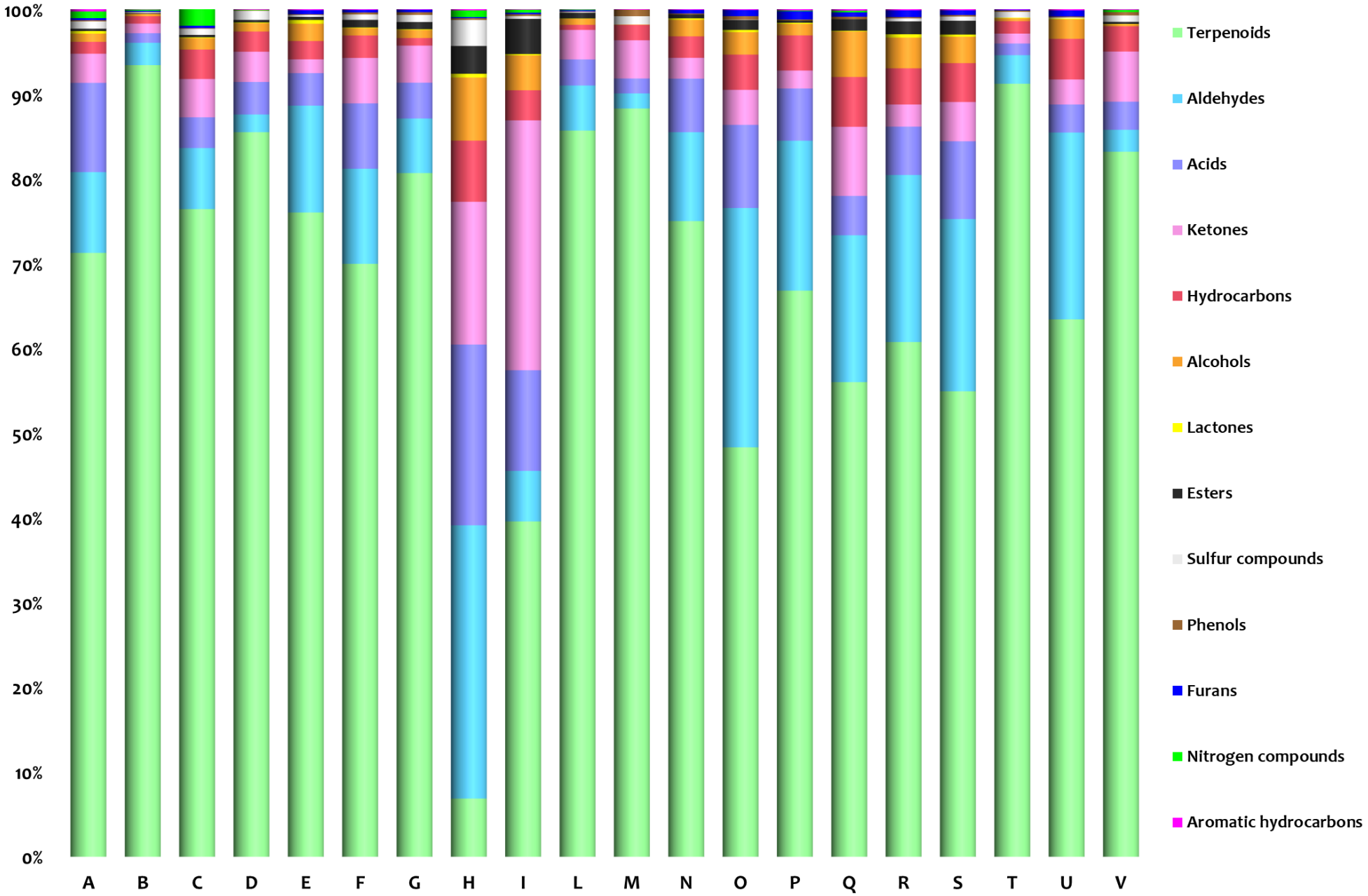

Fig. 4.

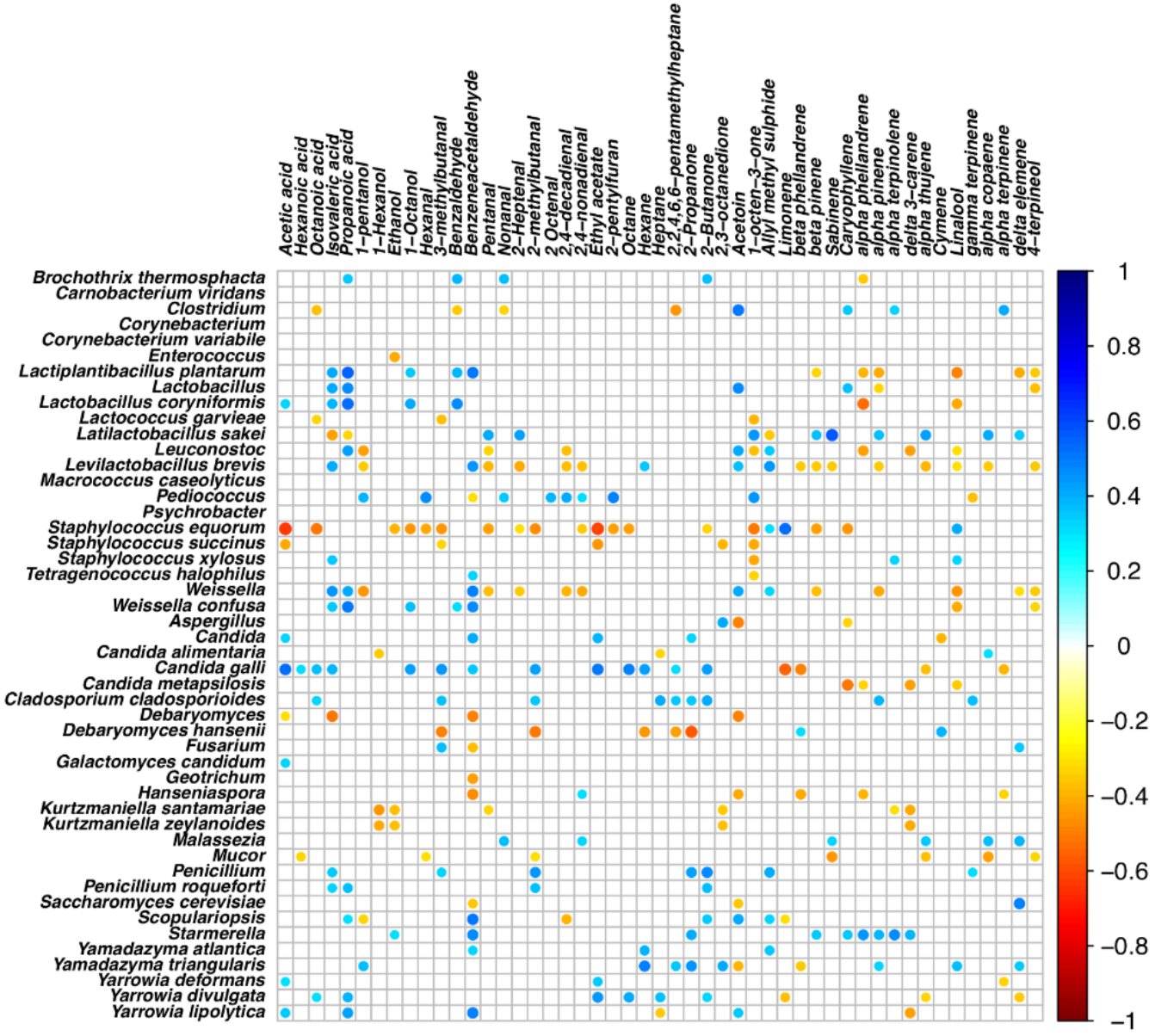

Table 1. List of ingredients for each producer of fermented liver sausages.

Ingredients		**Producers**																			
		A	B	C	D	E	F	G	H	I	L	M	N	O	P	Q	R	S	T	U	V
Pork meat (including fat)		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Additional pork fat		•	•	•			•														
Pork liver	15%																•				
	20%					•		•										•	•		•
	25%	•	•	•			•		•	•	•	•	•	•	•	•					
	33%				•															•	
Salt		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Pepper					•	•		•			•	•	•	•			•	•	•		
Garlic						•						•	•		•			•	•	•	
Chili pepper						•									•				•		
Orange peel						•							•						•		
Wine										•											
Liqueur (*Mistrà*)													•								
Milk powder								•													•
Fructose							•														
Dextrose		•	•		•		•	•	•	•	•			•		•	•			•	•
Lactose																					•
Sucrose		•	•		•				•	•	•				•	•	•			•	•
E300		•	•	•	•			•	•	•	•	•			•	•	•			•	•
E301		•					•	•			•										•
E250								•			•				•						•
E252		•	•	•	•	•	•	•	•	•	•	•	•	•	•		•			•	•

• presence

Table 2. Physico-chemical parameters of fermented liver sausages.

Producer	pH	a_w	NaCl (%)	Peroxide value (meq O2 kg fat $^{-1}$)	TTA (mL of 0.1 N NaOH)	Lactic acid (g 100 g^{-1})	Acetic acid (g 100 g^{-1})
A	5.70 ± 0.11 ^{a}	0.912 ± 0.003 ^{a}	2.75 ± 0.02 ^{fgh}	5.0 ± 0.0 ^{j}	18.43 ± 1.68 ^{g}	0.879 ± 0.035 ^{cdefg}	0.023 ± 0.013 ^{abcdefg}
B	5.53 ± 0.04 ^{ab}	0.772 ± 0.002 ^{f}	2.64 ± 0.01 ^{fgh}	18.0 ± 0.0 ^{e}	19.20 ± 2.72 ^{g}	0.442 ± 0.022 ^{ghij}	0.002 ± 0.001 ^{fg}
C	5.58 ± 0.04 ^{ab}	0.806 ± 0.002 ^{def}	3.39 ± 0.06 ^{bc}	7.0 ± 0.0 ^{j}	25.85 ± 1.49 ^{de}	0.163 ± 0.079 ^{j}	0.035 ± 0.006 ^{abcd}
D	5.04 ± 0.29 ^{fghi}	0.888 ± 0.055 ^{ab}	2.68 ± 0.05 ^{fgh}	6.0 ± 0.0 ^{j}	25.38 ± 2.20 ^{de}	1.089 ± 0.062 ^{bcde}	0.027 ± 0.003 ^{abcde}
E	5.28 ± 0.02 ^{cde}	0.907 ± 0.003 ^{ab}	2.80 ± 0.05 ^{efg}	22.5 ± 0.7 ^{c}	21.55 ± 1.88 ^{fg}	0.828 ± 0.052 ^{defg}	0.019 ± 0.006 ^{cdefg}
F	5.16 ± 0.02 ^{efg}	0.812 ± 0.007 ^{cdef}	3.17 ± 0.02 ^{cde}	13.5 ± 0.7 ^{h}	27.67 ± 1.01 ^{d}	0.615 ± 0.088 ^{fghi}	0.029 ± 0.004 ^{abcde}
G	4.97 ± 0.03 ^{ghi}	0.849 ± 0.004 ^{bcde}	3.22 ± 0.05 ^{cd}	7.5 ± 0.7 ^{j}	21.58 ± 1.02 ^{fg}	1.328 ± 0.260 ^{b}	0.026 ± 0.011 ^{abcdef}
H	5.50 ± 0.09 ^{ab}	0.819 ± 0.006 ^{cdef}	3.46 ± 0.08 ^{bc}	17.0 ± 0.0 ^{ef}	31.35 ± 2.46 ^{c}	0.863 ± 0.075 ^{cdefg}	0.047 ± 0.003 ^{a}
I	5.40 ± 0.09 ^{bcd}	0.901 ± 0.005 ^{ab}	3.15 ± 0.51 ^{cde}	16.5 ± 0.7 ^{efg}	25.88 ± 1.33 ^{de}	0.770 ± 0.139 ^{efgh}	0.041 ± 0.008 ^{abc}
L	4.85 ± 0.06 ^{i}	0.859 ± 0.013 ^{abcd}	2.41 ± 0.07 ^{hi}	21.0 ± 1.4 ^{cd}	39.83 ± 1.65 ^{a}	2.279 ± 0.143 ^{a}	0.014 ± 0.009 ^{defg}
M	5.47 ± 0.07 ^{bc}	0.853 ± 0.024 ^{bcde}	1.67 ± 0.02 ^{j}	6.5 ± 0.7 ^{j}	27.33 ± 1.75 ^{de}	0.732 ± 0.256 ^{efghi}	0.034 ± 0.008 ^{abcd}
N	5.56 ± 0.13 ^{ab}	0.871 ± 0.006 ^{abc}	3.38 ± 0.06 ^{bc}	31.0 ± 1.4 ^{b}	18.18 ± 0.97 ^{g}	0.359 ± 0.028 ^{hij}	0.020 ± 0.004 ^{bcdefg}
O	4.92 ± 0.05 ^{hi}	0.772 ± 0.005 ^{f}	2.62 ± 0.05 ^{fgh}	41.5 ± 0.7 ^{a}	27.57 ± 2.49 ^{d}	0.974 ± 0.157 ^{bcdef}	n.d.
P	4.94 ± 0.02 ^{ghi}	0.853 ± 0.008 ^{bcde}	2.09 ± 0.06 ^{i}	18.5 ± 0.7 ^{de}	37.15 ± 1.44 ^{ab}	1.239 ± 0.155 ^{bcd}	0.021 ± 0.006 ^{bcdefg}
Q	5.12 ± 0.08 ^{efgh}	0.807 ± 0.041 ^{def}	2.50 ± 0.07 ^{gh}	10.5 ± 0.7 ^{i}	34.43 ± 0.71 ^{bc}	0.990 ± 0.098 ^{bcdef}	0.019 ± 0.021 ^{cdefg}
R	4.96 ± 0.10 ^{ghi}	0.889 ± 0.009 ^{ab}	2.46 ± 0.03 ^{ghi}	30.5 ± 0.7 ^{b}	25.73 ± 0.71 ^{de}	1.289 ± 0.121 ^{bc}	0.012 ± 0.013 ^{defg}
S	5.51 ± 0.10 ^{ab}	0.794 ± 0.032 ^{ef}	3.88 ± 0.05 ^{a}	14.0 ± 0.0 ^{gh}	25.03 ± 2.86 ^{def}	0.877 ± 0.143 ^{cdefg}	0.044 ± 0.003 ^{ab}
T	5.47 ± 0.10 ^{bc}	0.824 ± 0.017 ^{cdef}	3.67 ± 0.06 ^{ab}	14.5 ± 0.7 ^{fgh}	23.90 ± 0.94 ^{ef}	0.658 ± 0.028 ^{fghi}	0.007 ± 0.003 ^{efg}
U	5.23 ± 0.14 ^{def}	0.886 ± 0.011 ^{ab}	2.97 ± 0.04 ^{def}	32.0 ± 0.0 ^{b}	18.07 ± 1.42 ^{g}	0.312 ± 0.190 ^{ij}	0.002 ± 0.003 ^{fg}
V	4.91 ± 0.12 ^{hi}	0.871 ± 0.007 ^{abc}	3.71 ± 0.08 ^{ab}	14.0 ± 0.0 ^{gh}	27.08 ± 0.80 ^{de}	1.323 ± 0.213 ^{b}	0.042 ± 0.005 ^{abc}
Overall mean	**5.25 ± 0.28**	**0.847 ± 0.046**	**2.93 ± 0.57**	**17.4 ± 10.0**	**26.06 ± 6.13**	**0.908 ± 0.474**	**0.023 ± 0.016**

Values are expressed as means ± standard deviation. Within each column, means followed by different letters are significantly different ($P < 0.05$).
n.d. not detectable

Table 3. Color parameters of fermented liver sausages.

Producer	L*	a*	b*
A	49.56 ± 2.82 ^{abc}	12.53 ± 0.54 ^{bcd}	7.89 ± 0.82 ^{bcd}
B	39.67 ± 1.68 ^{ghi}	12.36 ± 0.92 ^{bcde}	5.65 ± 1.03 ^{efg}
C	39.38 ± 1.78 ^{hi}	12.68 ± 0.68 ^{bc}	7.18 ± 1.08 ^{bcdef}
D	47.69 ± 0.98 ^{bcde}	13.32 ± 0.42 ^{ab}	8.83 ± 0.65 ^{b}
E	49.67 ± 2.76 ^{bcdef}	13.25 ± 1.10 ^{ab}	8.51 ± 0.54 ^{b}
F	41.30 ± 1.11 ^{fghi}	11.52 ± 0.91 ^{bcdef}	4.98 ± 0.50 ^{g}
G	45.12 ± 1.53 ^{bcdefg}	11.60 ± 1.27 ^{bcdef}	7.69 ± 1.58 ^{bcd}
H	42.78 ± 1.59 ^{efghi}	14.57 ± 0.83 ^{a}	12.37 ± 1.17 ^{a}
I	44.39 ± 1.84 ^{cdefgh}	10.10 ± 0.62 ^{f}	6.55 ± 0.99 ^{cdefg}
L	48.51 ± 0.78 ^{bcd}	10.47 ± 0.75 ^{f}	5.37 ± 0.46 ^{fg}
M	38.31 ± 3.66 ^{i}	11.27 ± 2.08 ^{cdef}	7.40 ± 1.07 ^{bcde}
N	48.70 ± 4.11 ^{bcd}	11.62 ± 0.63 ^{bcdef}	5.32 ± 0.96 ^{fg}
O	42.60 ± 4.05 ^{efghi}	10.15 ± 0.55 ^{f}	8.17 ± 0.94 ^{bc}
P	50.38 ± 1.28 ^{ab}	10.73 ± 0.48 ^{def}	8.53 ± 1.06 ^{b}
Q	43.29 ± 6.25 ^{defghi}	10.61 ± 0.67 ^{ef}	5.71 ± 1.37 ^{efg}
R	50.19 ± 3.07 ^{ab}	11.40 ± 1.09 ^{cdef}	5.54 ± 0.35 ^{efg}
S	39.44 ± 2.52 ^{ghi}	12.51 ± 0.51 ^{bcd}	5.33 ± 0.58 ^{fg}
T	44.30 ± 1.31 ^{cdefgh}	10.02 ± 0.22 ^{f}	9.06 ± 0.54 ^{b}
U	54.88 ± 2.35 ^{a}	10.29 ± 0.99 ^{f}	6.21 ± 0.96 ^{defg}
V	48.51 ± 2.09 ^{bcd}	10.04 ± 0.16 ^{f}	8.87 ± 0.58 ^{b}
Overall mean	**45.28 ± 5.05**	**11.55 ± 1.50**	**7.26 ± 2.00**

Means ± standard deviations of triplicate independent measurements are shown.
Within each column, means followed by different letters are significantly different ($P < 0.05$).

L* value describes the lightness; a* value describes the redness/greenness; b* describes the blueness/yellowness.

Table 4. Results of viable counting of bacteria and eumycetes in fermented liver sausages.

Producer	Presumptive lactobacilli	Enterococci	Coagulase-negative cocci	Enterobacteriaceae	Pseudomonadaceae	Sulfite-reducing anaerobes	Total eumycetes
A	8.07 ± 0.21 ^{abcd}	3.58 ± 0.23 ^{ef}	7.07 ± 0.42 ^{ab}	< 1.00	1.06 ± 0.91 ^{e}	< 2.00	6.21 ± 0.77 ^{a}
B	7.81 ± 0.15 ^{bcde}	4.87 ± 0.43 ^{abc}	6.52 ± 0.81 ^{abcd}	< 1.00	< 1.00	< 2.00	4.82 ± 0.29 ^{efgh}
C	6.94 ± 0.14 ^{f}	3.90 ± 1.10 ^{cdef}	6.39 ± 0.58 ^{abcd}	< 1.00	< 1.00	< 2.00	4.18 ± 0.54 ^{h}
D	8.21 ± 0.63 ^{abc}	5.36 ± 0.46 ^{a}	4.47 ± 0.22 ^{fg}	2.40 ± 1.51 ^{b}	1.77 ± 0.82 ^{de}	< 2.00	5.26 ± 0.63 ^{bcdef}
E	8.40 ± 0.18 ^{ab}	4.00 ± 0.09 ^{bcde}	5.36 ± 1.16 ^{def}	1.03 ± 0.52 ^{c}	< 1.00	< 2.00	5.79 ± 0.18 ^{abc}
F	8.21 ± 0.62 ^{abc}	5.76 ± 0.12 ^{a}	5.11 ± 1.15 ^{ef}	< 1.00	< 1.00	< 2.00	5.16 ± 0.12 ^{cdefg}
G	7.97 ± 0.50 ^{bcde}	3.60 ± 0.83 ^{def}	4.25 ± 0.87 ^{fg}	< 1.00	< 1.00	< 2.00	4.29 ± 0.60 ^{gh}
H	8.09 ± 0.19 ^{abcd}	4.81 ± 0.13 ^{abc}	5.97 ± 0.05 ^{bcde}	< 1.00	< 1.00	< 2.00	5.14 ± 0.05 ^{cdefg}
I	8.76 ± 0.12 ^{a}	3.52 ± 0.58 ^{ef}	6.50 ± 0.42 ^{abcd}	< 1.00	< 1.00	< 2.00	5.76 ± 0.44 ^{abcd}
L	8.22 ± 0.45 ^{abc}	< 1.00	3.82 ± 0.89 ^{gh}	< 1.00	< 1.00	< 2.00	6.36 ± 0.34 ^{a}
M	8.29 ± 0.29 ^{abc}	4.75 ± 0.25 ^{abcd}	6.58 ± 0.09 ^{abc}	< 1.00	5.10 ± 0.22 ^{a}	2.27 ± 0.18	5.62 ± 0.59 ^{abcde}
N	8.16 ± 0.08 ^{abc}	5.10 ± 0.07 ^{ab}	7.18 ± 0.30 ^{a}	< 1.00	< 1.00	< 2.00	6.47 ± 0.19 ^{a}
O	7.56 ± 0.85 ^{cdef}	1.86 ± 1.44 ^{g}	4.20 ± 0.45 ^{fg}	< 1.00	< 1.00	< 2.00	4.35 ± 0.43 ^{fgh}
P	8.38 ± 0.13 ^{ab}	3.39 ± 0.16 ^{ef}	3.00 ± 0.07 ^{h}	< 1.00	< 1.00	< 2.00	5.16 ± 0.27 ^{cdefg}
Q	6.80 ± 0.30 ^{q}	5.71 ± 0.84 ^{a}	7.18 ± 0.19 ^{a}	< 1.00	< 1.00	< 2.00	5.57 ± 0.63 ^{abcde}
R	8.04 ± 0.05 ^{abcd}	< 1.00	6.65 ± 0.06 ^{abc}	< 1.00	2.78 ± 0.12 ^{bc}	< 2.00	6.13 ± 0.10 ^{ab}
S	7.28 ± 0.08 ^{ef}	5.52 ± 0.26 ^{a}	6.98 ± 0.15 ^{ab}	< 1.00	< 1.00	< 2.00	5.12 ± 0.68 ^{cdefg}
T	7.38 ± 0.43 ^{def}	5.04 ± 0.17 ^{abc}	5.78 ± 0.40 ^{cde}	< 1.00	2.04 ± 0.74 ^{cd}	< 2.00	4.86 ± 0.40 ^{defgh}
U	8.05 ± 0.31 ^{abcd}	4.82 ± 0.11 ^{abc}	6.42 ± 0.04 ^{abcd}	4.18 ± 0.47 ^{a}	3.68 ± 0.13 ^{b}	< 2.00	6.42 ± 0.14 ^{a}
V	8.08 ± 0.15 ^{abcd}	2.73 ± 0.28 ^{fg}	6.41 ± 0.12 ^{abcd}	< 1.00	< 1.00	< 2.00	5.69 ± 0.18 ^{abcde}
Overall mean	**7.94 ± 0.59**	**3.95 ± 1.65**	**5.79 ± 1.32**	**< 1.00**	**< 1.00**	**< 2.00**	**5.42 ± 0.80**

Values are expressed as Log cfu g^{-1} ± standard deviation.
Within each column, means followed by different letters are significantly different ($P < 0.05$).

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3