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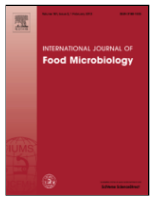

Bacterial community of industrial raw sausage packaged in modified atmosphere throughout the shelf life

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ABSTRACT

Ten lots of industrial raw sausages in modified atmosphere (CO_2 30%, O_2 70%), produced in the same plant over 7 months, were analyzed at the day after production (S samples) and at the end of shelf life (E samples), after 12 days storage at 7 °C to simulate thermal abuse. Quality of the products was generally compromised by storage at 7 °C, with only 3 E samples without alterations. During the shelf life, the pH decreased for the accumulation of acetic and lactic acids. A few biogenic amines accumulated, remaining below acceptable limits. The profile of volatile compounds got enriched with alcohols, ketones, and acids (e.g. ethanol, 2,3-butanediol, 2,3-butandione, butanoic acid) originated by bacterial metabolism. Throughout the shelf life, aerobic bacteria increased from 4.7 log to 6.6 log cfu/g, and lactic acid bacteria (LAB) from 3.7 to 8.1 log cfu/g. Staphylococci, enterobacteria, and pseudomonads passed from 3.7, 3.0, and 1.7 to 5.5, 4.8, and 3.0 log cfu/g, respectively. Dominant cultivable LAB, genotyped by RAPD-PCR, belonged to the species *Lactobacillus curvatus/graminis* and *Lactobacillus sakei*, with lower amounts of *Leuconostoc carnosum* and *Leuconostoc mesenteroides*. *Brochothrix thermosphacta* was the prevailing species among aerobic bacteria. The same biotypes ascribed to several different species where often found in E samples of diverse batches, suggesting a recurrent contamination from the plant of production. Profiling of 16S rRNA gene evidenced that microbiota of S samples clustered in two main groups where either Firmicutes or Bacteroidetes prevailed, albeit with taxa generally associated to the gastro-intestinal tract of mammals. The microbial diversity was lower in E samples than in S ones. Even though a common profile could not be identified, most E samples clustered together and were dominated by Firmicutes, with Lactobacillaceae and Listeriaceae as the most abundant families (mostly ascribed to *Lactobacillus* and *Brochothrix*, respectively). In a sole E sample Proteobacteria (especially *Serratia*) was the major phylum.

1. Introduction

Italian raw sausages are the result of the mixing of lean pork meat and fat in a ratio and at a mincing level which depends on several traditional recipes, varying in relation to the production geographical area. In addition to meat, other ingredients are used, such as spices (black pepper, fennel, chili pepper, garlic, etc.), water, and salt in variable amounts. No thermal treatment is applied during the production. At industrial level, also sugars (generally glucose or sucrose) and few acidifying/antioxidant preservatives are allowed (European Commission, 2014). Raw batters are stuffed in natural or synthetic casings with variable diameters (2–5 cm), marketed under refrigeration conditions (0–4 °C), and consumed after cooking. Raw sausages are packaged under modified atmosphere and require cooking before eating (Torrieri et al., 2011; Tremonte et al., 2005).

The shelf-life is rather short (10–15 days), mainly due to the high water activity, usually around 0.97, and to a pH of approx. 6 immediately after production (Cocolin et al., 2004). It depends on the development of negative sensorial characteristics concerning mainly odor and

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color, as well as on the production of exudates (Benson et al., 2014; Fougy et al., 2016). The progress of these defects mainly relies on microbial metabolic activity in combination with chemical and biological reactions associated to the raw materials, such as lipid oxidation and other transformations carried out by meat endogenous enzymes (Casaburi et al., 2015; Hugo and Hugo, 2015; Kamdem et al., 2007). Extending the shelf life of fresh sausage is a main challenge, since the peculiar flavor and taste and the agreeable appearance can be easily lost during storage.

The shelf life is strictly linked to the quali- and quantitative composition of the initial microbiota, the evolution of which is related to the storage conditions. At packaging, microbial population can derive from cross contamination during slaughtering and successive operations, from the industrial environments where sausages are produced, and from ingredients (mainly spices) and casings (Benson et al., 2014; Chaillou et al., 2015). Temperature is the main driver of the growth of microorganisms during storage, with refrigeration favoring psychrotrophic species. However, also packaging conditions, and in particular vacuum, ordinary or modified atmosphere packaging (MAP) are important factors affecting microbial growth (Chaillou et al., 2015). NaCl concentration, the diminution of which is an important challenge for processed meat industry, also influences the composition of microbiota and can affect spoilage (Fougy et al., 2016).

The bacteria responsible for spoilage of fresh sausage are mainly Firmicutes (in particular carnobacteria, lactobacilli, leuconostocs, and *Brochothrix thermosphacta*, but Proteobacteria (e.g. *Serratia*, *Yersinia*, *Pseudomonas*, *Acinetobacter*)), Staphylococci, micrococci, and Bacteroidetes are also recurrent and may be abundant, with detrimental effects on sensorial properties (Benson et al., 2014; Cocolin et al., 2004; Fougy et al., 2016;). Pathogenic species, such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Yersinia enterocolitica*, and *Salmonella*, may be present resulting in hygienic risks for the consumers in case of improper cooking tempertures (De Cesare et al., 2007; Giovannini et al., 2004; Joutsen et al., 2012).

Exploring the diversity of microorganisms in sausage is a relevant focus of research, since a better understanding of the role of bacterial populations in food quality and safety greatly impacts on both health, sustainability, and economy. Despite an approach based on 16S rRNA gene analysis has recently been applied to investigate the effect of salt concentration on microbiota of fresh sausage (Fougy et al., 2016), a detailed overview of the microbiota of industrial pork raw sausage during the shelf life is still missing.

The present study aimed to describe the microbial composition of industrial raw sausage at the beginning and at the end of the shelf life, combining culture-dependent methods and 16S rRNA gene profiling, to specifically trace the bacterial biotypes and to describe the whole bacterial community, respectively. Ten lots of raw sausages, produced in the same plant over a period of 7 months, were analyzed at the packaging time and at the end of shelf life, after 12 days storage in MAP at 7 °C. For all the lots, the occurrence of defects was evaluated and the pH, organic acids, biogenic amines, and prevalent volatile compounds (VOCs) were analyzed.

2. Materials and methods

2.1. Sausage productions

Ten different lots of raw sausages were produced in an industrial plant (*CLA*I S.c.a., Imola, Italy) between September 2016 and March 2017, numbered from 01 to 10 in chronological order. Fresh pork meat from hot deboning of the shoulders was used. The ratio lean meat/fat was approx. 90:10. The meat was cut (about 4–5 mm) at 0 °C and mixed with NaCl 2.2%, sugars (sucrose and glucose) 1.2%, grounded black pepper 0.1%, nutmeg 0.1%, ascorbate 0.025%. The mixture was stuffed in pork casings (diameter about 3.6–3.8 cm). Sausages were packed as 500 g portions in polypropylene trays under modified atmosphere (30% CO_2, 70% O_2). The shelf life assigned to this product when stored at 0–4 °C was 12 days. Analyses were carried out the day after production (S samples) and after 12 days of storage at 7 °C (E samples), analyzing three diverse packages for each time point.

2.2. Spoilage assessment

Sausage packages were let to reach room temperature and were evaluated by 5 judges to detect the presence of main defects, such as discoloration, presence of off-flavors and off-odors (e.g. acidic, putrefactive, and rancid), production of exudates, and wrinkling of the casing. Each judge expressed the evaluation on a scale from 0 to 5 (0 = highly acceptable, 5 = highly defected). For each indicator, mean scores higher than 4 were considered as not acceptable.

2.3. Chemical analyses

The pH was measured in 5 diverse points per sample using a puncture electrode (Sension+ electrode 5233, HACH, Manchester, UK). Organic acids were determined with an HPLC method according to Montanari et al. (2014). The content of biogenic amines was determined following the HPLC method reported by Gardini et al. (2013). The limit of detection for all the amines was 3 mg/kg of sausage.

Volatile organic compounds (VOCs) were monitored by solid phase micro extraction (SPME) coupled with gas chromatography – mass spectrometry analysis (GC–MS). A Carboxen-Polydimethylsiloxane fiber (Sigma Aldrich, St. Louis, MO, USA) was exposed for 1 h at 25 °C to the headspace of a 10-mL vial containing 2 g of the sample. The analyses were performed in duplicate. The volatiles were released through thermic desorption at 240 °C in the injector of a GC–MS apparatus (7820–5975; Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-624 column (Agilent Technologies). Separation was achieved with 1.3 mL/min helium flux following a thermal gradient: 2 min isotherm at 50 °C, 6 °C/min increase to 110 °C, 10 °C/min increase to 240 °C, 4 min isotherm at 240 °C. The tentative assignment of the volatiles was based on the comparison of their retention times and mass spectra to those from the National Institute of Standards (NIST) mass spectral library (2014).

2.4. Microbiological analyses

Approx. 20 g of sausage were 10-fold diluted with saline water (8.5 g/L NaCl and 1 g/L peptone) and homogenized in a Lab Blender Stomacher (Seward Medical, London, UK) for 3 min. Decimal dilutions were spread on agar plates to enumerate and isolate bacteria able to grow on different culture media provided by BD Difco (Franklin Lake, NJ, USA). Total (aerobic and facultative anaerobic) bacteria were enumerated on Plate Count Agar (PCA), lactic acid bacteria (LAB) on Lactobacilli MRS agar (MRS), staphylococci on Baird-Parker Agar (BP), coliforms on MacConkey Agar (MAC), and pseudomonads on Cetrimide Agar Base (CET). MRS plates were incubated in anaerobic jar provided with GasPaK (BD Difco) at 30 °C for 72 h. The other plates were incubated in aerobiosis at 30 °C for 48 h.

After isolation, a representative number of colonies was randomly picked up from PCA, MRS, and BPA and purified. Instagene Matrix (Bio-Rad Laboratories, Redmond, WA) was used for extraction of PCR amplifiable DNA. From each sample and media, up to 48 colonies were clustered by RAPD-PCR using M13-RAPD primer (5′-GAGGGTGGCG-GTTCT-3′) at a similarity level of 75% using the Pearson correlation coefficient (Quartieri et al., 2016). A single strain for each biotype was

taxonomically characterized through sequencing of the V1–V3 portion of 16S rRNA gene. The couple of primer 16S-500f (5′-TGG AGA GTT TGA TCC TGG CTC AG-3′)/16S-500r (5′-TAC CGC GGC TGC TGG CAC-3′) was utilized to amplify the target region (Kolbert et al., 2004). The DNA sequences were determined by a DNA sequencing service provider (Eurofins genomics, Ebersberg, Germany). Comparisons with the reference sequences available in the GenBank database were obtained by the NCBI BLAST software (https://blast.ncbi.nlm.nih.gov).

2.5. 16S rRNA gene profiling

For each lot and time point, equal amounts of sausage from the 3 packages were pooled and homogenized before extraction. Total DNA was extracted using the DNeasy Mericon Food Kit (Qiagen, Hilden, Germany) following the manufacturer's standard protocol. The DNA was normalized to 5 ng/μL after quantification with a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). Partial 16S rRNA gene sequences were amplified using Probio_Uni/Probio_Rev primers, which target the V3 region of the 16S rRNA gene sequence and sequenced using a MiSeq (Illumina) according to Milani et al. (2013).

The fastq files were processed using a custom script based on the QIIME software suite (Caporaso et al., 2010). Paired-end reads pairs were assembled to reconstruct the complete amplicons. Quality control retained sequences with a length between 140 and 400 bp and mean sequence quality score > 20 while sequences with homopolymers >7 bp and mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥99% sequence homology using uclust (Edgar, 2010). All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the SILVA database (Quast et al., 2013). Biodiversity of the samples (alpha-diversity) were calculated with Chao1 and Shannon indexes. Similarities between samples (beta-diversity) were calculated by unweighted uniFrac (Lozupone and Knight, 2005). PCoA representations of beta-diversity were performed using QIIME.

2.6. Statistical analysis

All values, unless otherwise stated, are reported as mean ± standard deviation of the analysis of three trays for each lot and time point. Comparisons were performed with Student's *t*-test or one-way ANOVA followed by Tukey post hoc comparisons. Statistical analysis was done using SPSS Statistics 21 (IBM Corp., Armonk, NY, USA).

Principal component analysis (PCA) was used to explore the VOCs data, after excluding compounds deriving only from spices. The data matrix of VOCs, with size {40, 29}, included the 29 relative VOCs areas determined for the two replicate measurements made for each one of the 20 samples (10 samples S and 10 samples E). Before calculating the PCA model, the relative area values were pre-processed using Pareto scaling (Van den Berg et al., 2006). The number of significant principal components (PCs) was defined using the scree plot, which reports the percentage of variance explained by each PC vs. the PC number. The PCA model was calculated using PLS Toolbox software ver. 8.5 (Eigenvector Research Inc., Wenatchee, WA, USA).

3. Results and discussion

3.1. Spoilage assessment

The quality of the sausages was evaluated in terms of appearance, color, odor, and exudate production, at the beginning and at the end of the shelf-life. The applied temperature (7 °C) was chosen to simulate a realistic thermal abuse, being slightly beyond the temperatures of 4–5 °C recommended in many countries for the cold chain throughout production, storage, and transport of fresh meat products (European Commission, 2004a, 2004b; USDA, 2000).

All S samples had no detectable alteration, with scores of spoilage indicators ranging from 0 to 2. After 12 days storage at 7 °C, 3 E samples out of 10 were still acceptable. Conversely, storage determined a negative evolution of the organoleptic characteristics for 7 samples. Recurrent defects were off-flavors, acidic odor, exudate production, and discoloration (Fig. 1). Only the sample 08E showed the presence of wrinkled casings.

3.2. pH, organic acids, and biogenic amines

The initial pH of the fresh sausages ranged between 5.77 and 6.05 (Fig. 2a, Table S1), in agreement with results reported for other similar products (Cocolin et al., 2004; Torrieri et al., 2011). These values of pH are justified by post-mortem transformation of glycogen in lactic acid, that was present in S samples at levels comprised between 3.01 and 4.85 g/kg. After 12 days, the pH showed a higher variability, ranging from 5.12 (02E) to 5.87 (10E). This latter sample was the sole where pH did not decrease during storage. Lactic acid increased up to approx. 9 g/kg in the samples 02E and 04E, and at a lesser extent in the others, with the exception of 10E where it did not increase, accordingly with the trend of pH. Acetic acid, present in traces in the sausages immediately after production, increased on average to 0.49 g/kg, with a maximum of 0.95 mg/kg in 02E. Likewise lactic acid, accumulation of acetic acid was not detected in sample 10E.

The presence of biogenic amines after 12 days was rather low (Fig. 2b). Tyramine was the most abundant (mean value 25.7 mg/kg), followed by cadaverine (13.0 mg/kg), and histamine (9.1 mg/kg). Generally, putrescine and 2-phenylethyl-amine did not accumulate during storage. However, sample 01E was characterized by a particularly high amount of cadaverine and putrescine (38.4 and 23.6 mg/kg, respectively; outlier in Fig. 2b). In any case, the final concentration of these substance was well below their mean amounts in fermented meats (Suzzi and Gardini, 2003) and the level of concern suggested by EFSA (2011).

3.3. Culture-dependent enumerations

All the bacterial groups increased through the storage in MAP, suggesting the presence of a wide and complex community of psychrophilic bacteria (Fig. 3; Table S2). On average, aerobic bacteria were 4.7 log cfu/g at packaging (S) and reached 6.6 log cfu/g in E samples. LAB were 3.7 log cfu/g in S samples and reached 8.1 log cfu/g in E ones. Putative staphylococci, coliforms, and pseudomonads passed

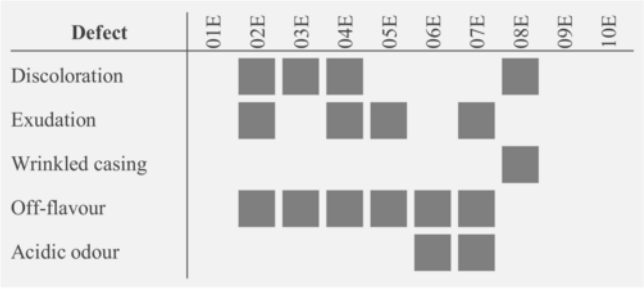

Fig. 1. Sensory defects detected in the lots of sausages (columns) at the end of the shelf life. Within each row, gray squares indicate the occurrence of the defect (mean score higher than 4) in the corresponding E lot.

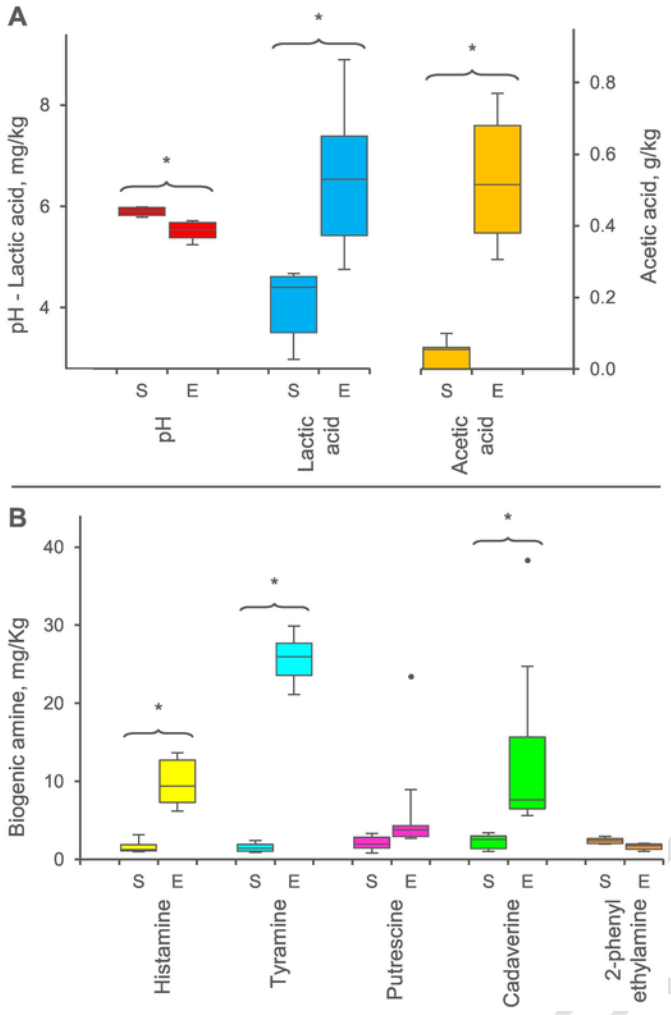

Fig. 2. Panel a: pH and concentration of lactic acid (mg/kg) and acetic acid (g/kg) in S and E sausages. Panel b: Concentration of the biogenic amines histidine, tyramine, putrescine, cadaverine, and 2-phenylethylamine (mg/kg) in S and E samples. Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles; black circles represent outliers. For each parameter, * indicates difference statistically significant between S and E samples (Student's *t*-test, $P < 0.01$).

from 3.7, 3.0, and 1.7 to 5.5, 4.8, and 3.0 log cfu/g, respectively. As a whole, the initial bacterial charge indicated good hygienic operations in both the slaughter and transformation plants.

After storage, the sum of PCA plus MRS counts of E samples always exceeded 7 log cfu/g, which was considered as a threshold of bacteria in meat spoilage (Koutsoumanis et al., 2008; Stoops et al., 2015). The LAB enumerated on MRS plates were the dominant group in the samples 01E-06E, reaching values of 8–9 log cfu/g, with lower counts ranging between 6.6 and 7.6 log cfu/g in the other E samples. The presence of micrococci and staphylococci was even more variable in E samples, being less or equal to 4 log cfu/g in 02E and 09E, and comprised between 6.1 and 7.0 log cfu/ml in the samples 01E and 03E–07E. Coliforms, initially ranging from 2.6 and 3.7 log cfu/g, grew up to 7.1 log cfu/g in 01E. Lower amounts were detected in the other sausages, with counts below the detection limit in 08E and 09E. Pseudomonads increased their concentration of about 1 log unit during storage and their counts never exceeded 4 log cfu/g in the E samples.

Similar results were obtained by Cocolin et al. (2004) in fresh sausages for mesophilic aerobic counts, lactic acid bacteria, staphylococci, and enterobacteria. Higher initial contamination of analogous

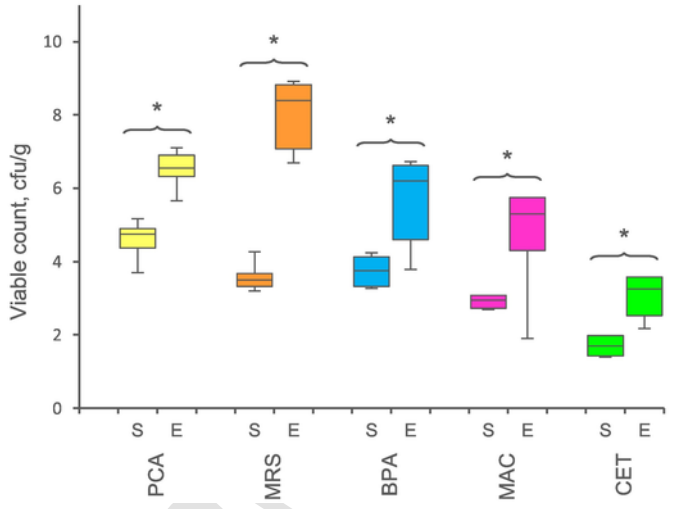

Fig. 3. Viable counts in PCA, MRS, BPA, MAC, and CET plates in S and E samples. Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles. For each amine, * indicates statistical significant difference between S and E samples (Student's *t*-test, $P < 0.01$).

products was found in previous studies (Torrieri et al., 2011; Nuvoloni et al., 2012). In contrast, lower concentrations of these microbial groups were observed when antimicrobials were used both in carcasses cleaning and sausage manufacture (Benson et al., 2014).

3.4. Identification of the isolates at the end of the shelf life

Randomly selected colonies isolated on MRS, PCA, or BPA plates at the end of the shelf life were subjected to RAPD-PCR clustering. Approximately 900 isolates were analyzed and each biotype was taxonomically characterized (Table S3).

Ten different biotypes were identified among MRS isolates, one to six per sample (Table 1). All the biotypes, with the exception of *Leuconostoc kimchii*, belonged to the species *Lactobacillus curvatus/graminis* (53% of the isolates), *Lactobacillus sakei* (20%), *Leuconosotc carnosum* (18%), and *Leuconostoc mesenteroides* (9%). In four E samples a sole LAB species was identified (3 *L. curvatus/graminis*, 1 *L. carnosum*); three more sausages were dominated (>80%) by a strain of L. *curvatus/graminis*, *L. sakei*, or L. *carnosum*, whereas the other presented a more complex population of cultivable LAB. L. *sakei* and L. *curvatus/graminis* belong to the same L. *sakei* clade, based on 16S rRNA gene phylogeny, and are the LAB most frequently associated with meat products (Zagorec and Champomier-Vergès, 2017).

Bacteria picked on PCA plates were much more heterogeneous, belonging to 14 different genera and to 26 species, 17 of which found in a sole sample. With the exception of samples 01E and 09E, where all the PCA isolates were L. *mesenteroides* and L. *carnosum*, respectively, PCA isolates from E samples were assigned to 3 to 8 diverse species. The most represented was *Brochotrix thermosphacta*, that accounted for 42% of the colonies. This dominant spoilage organism is responsible for deterioration of refrigerated meat products, harboring the genes involved in the production of malodorous compounds such as acetate, acetoin, butanediol, and fatty acids (Stanborough et al., 2017). Other major PCA isolates were LAB belonging to the species L. *mesenteroides* (15%), *L. carnosum* (13%), and *Carnobacterium divergens* (7%). L. *mesenteroides* and *L. carnosum* have been frequently isolated from several food matrices and meat goods, and have been also suggested as starters for meat biopreservation because of bacteriocin production (Wan et al., 2015). *Serratia* spp. were identified in 5 E samples and accounted for 7% of PCA isolates.

Table 1
Dominant bacteria isolated from PCA, MRS, and BPA plates. Strains were genotyped by RAPD-PCR fingerprinting and given a taxonomic designation by partial sequencing of 16S rRNA gene.

Batch	MRS					PCA						
	Lactobacillus curvatus/graminis (3)	*Lactobacillus sakei* (3)	*Leuconostoc carnosum* (1)	*Leuconostoc kimchii* (1)	*Leuconostoc mesenteroides* (2)	*Bacillus* sp. (1)	*Bacillus siamensis* (1)	*Bacillus subtilis* (1)	*Bacillus velezensis* (1)	*Bacteroides vulgatus* (1)	*Brochotrix thermosphacta* (6)	*Carnobacterium divergens* (4)
01E		96			4							
02E	36				65						10	
03E	66	21			13	5	8	12				57
04E	100										58	9
05E	80	9		2	9						53	
06E	100										89	
07E	100										52	
08E			100								83	3
09E	14		86									
10E	30	70							10	3	78	
All[a]	53	20	18	<1	9	<1	1	1	1	<1	42	7

For each medium, the percentage abundance of each species within samples is reported; in brackets, the number of different RAPD-PCR profiles.
[a] Percentage abundance calculated on the basis of total number of isolates from all samples in each medium.

BPA plates allowed the selection of a number of LAB belonging to the species *Carnobacterium gallinarum* (24%), *Enterococcus faecalis* (23%), and *C. divergens* (8%). *C. divergens* is one of the most prevalent *Carnobacterium* species in food derived from animals and it is capable to aerobically respire likely using a functional respiratory chain (Iskandar et al., 2017). *Carnobacterium* was not isolated on MRS anaerobic plates but was found in PCA and BPA plates, also because of its preference for slightly high pH and the sensitivity to acetate (Leisner et al., 2007). In four E samples, no staphylococci were isolated, likely being outnumbered by LAB. Major taxa of staphylococci were *Staphylococcus equorum* (15%), *Staphylococcus warneri* (12%), and *Macrococcus caseolyticus* (11%). A sole sample (10E) contained also a relevant amount of *Staphylococcus sciuri*.

S. equorum, and *S. warneri* are common component of the microbiota in the fermented and ready-to-eat meat products (Fijałkowski et al., 2016). Similar results were reported for Italian fresh sausages, in which LAB dominate the final microbiota (represented by the species L. *mesenteroides*, *L. curvatus/graminis*, and L. *sakei*) together with *Staphylococcus xylosus* and *B. thermosphacta* (Cocolin et al., 2004). Other microbial groups have been described in similar products, such as the Gram negative bacteria *Actinobacter*, *Moraxella*, *Psychrobacter*, *Flavobacterium*, *Shewanella*, several Enterobacteriaceae, as well as the Gram positive genera *Bacillus*, *Pediococcus*, *Weissella*, (Benson et al., 2014; Nychas and Skandamis, 2005; Stanbridge and Davies, 1998).

Although identical RAPD-PCR biotypes may correspond to different strains, the presence of common RAPD-PCR profiles in different E samples was observed (Fig. 4), suggesting the presence of recurrent strains or at least of genetically similar bacteria. The persisting biotypes were LAB, *B. thermosphacta*, *M. caseolyticus*, *Serratia* spp. and *S. equorum*. For instance, the biotype L. *curvatus/graminis* 24 was identified in 7 out of 10 samples, *B. thermosphacta* 9 in 6 out of 10. Some biotypes were identified in samples noncontiguous in terms of temporal sequence of production. The contamination of the raw meat likely occurs during the manufacturing phases, with the bacteria contaminating the environment of the facilities entering in contact to the meat mixture. Taking into account the plausible presence of the same strains over >6 months, it is possible to hypothesize the occurrence of a promiscuous microbiota able to survive in the plant, despite the strong sanitization procedures, and in the meat. This is consistent with the wide distribution of LAB species in the environment, which goes far beyond the attribution of each species to specific habitats (Rossi et al., 2016).

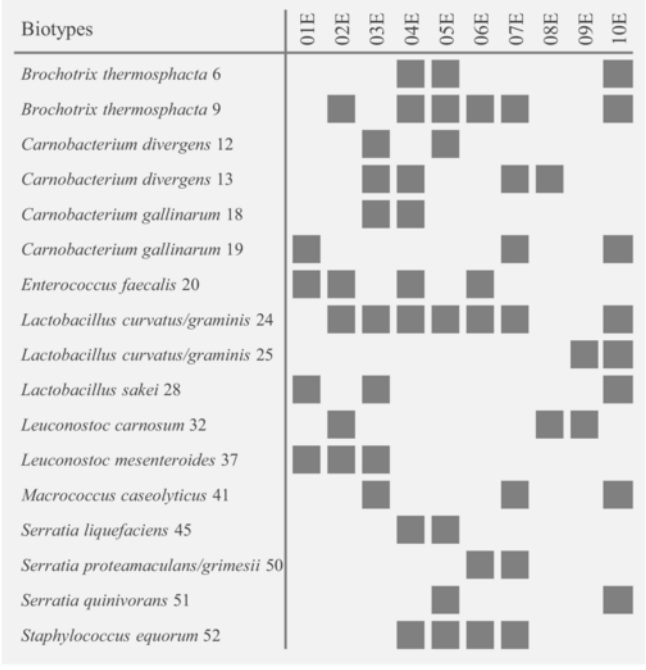

Fig. 4. Recurrent bacterial RAPD-PCR biotypes occurring at the end of the shelf life across different lots. Within each row, gray squares indicate the presence of the biotype in the corresponding lots.

3.5. Analysis of bacterial population by 16S rRNA gene profiling

A total of 792,402 quality-trimmed 16S rRNA gene sequences were obtained from 20 samples (on average 39,620 reads per sample). The sequencing depth of generally >4 log provided a wide overview of the richness of sausage microbiota. 2041 OTUs net of singletons (i.e. the OTU detected as a single read within the whole set of samples) were identified (558±214 OTUs per sample; Table S4). Alpha diversity was estimated by Chao and Shannon indices, analyzing the OTUs (Table S5). S group was more rich and diverse than E one, according to Chao (787.5 vs. 390.6; $P < 0.05$) and Shannon metrics (6.30 vs 3.16; $P < 0.05$), with the exception of sample 10E that presented a bacterial community much richer than other E samples based on Chao index. Beta diversity, calculated with the Unifrac method, was utilized to compare the bacterial communities in S and E sausages based on their phylogenetic relationship. Excluding sausage 10E, the S and E samples formed two separate clusters along PC1in the plot of coordinates of PCoA (Fig. 5).

Immediately after production, bacteria were generally ascribable to two diverse microbiota. Samples 01, 03, 04, 05, 06, and 07 were dominated by Firmicutes, always representing >50% of the reads in terms of relative abundance (51.6–65.8%), followed by Actinobacteria (9.5–11.9%), generally referred to *Bifidobacterium* (7.7–10.3%), and Bacteroidetes (4.1–6.8%, Fig. 6). Accordingly, this set of samples converged in the same region within the PCoA plot, distant from other S samples (Fig. 5). Proteobacteria ranged between 4.8 and 27.6%, and Erysipelotrychaceae were the most abundant family (31.1–42.7%), with OTUs mostly assigned to the genera *Allobaculum* (17.4–24.8%) and *Faecalibaculum* (9.4–12.3%).

Samples 08S, 09S, and 10S clustered together in PCoA plot. Bacteroidetes was the most abundant phylum (45.5–57.5%), followed by Firmicutes (approx. 29%), with minor amounts of Proteobacteria (4.1–10.5%) and Verrucomicrobia (3.7–4.9%). Bacteroidaceae together with Bacteroidales S24–7 group formed a large part of bacterial community (35.8–45.3%), with *Bacteroides* as major genus (21.1–26.2%). Minor

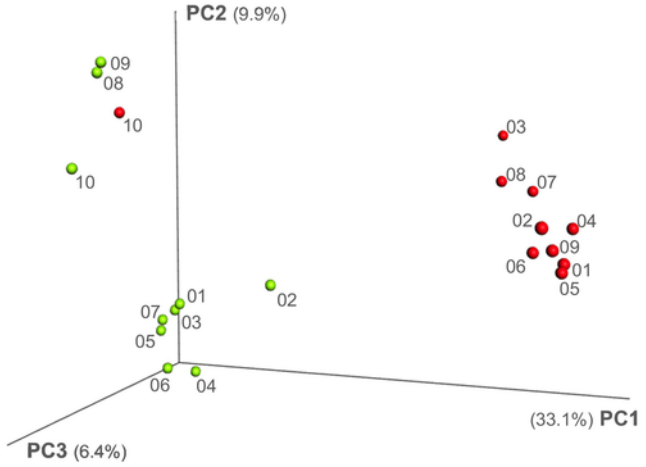

Fig. 5. PCoA plot of weighted Unifrac distances between the microbiota of ten sausage lots at the beginning and at the end of the shelf life. Symbols: green circles, S samples; red circles, E samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

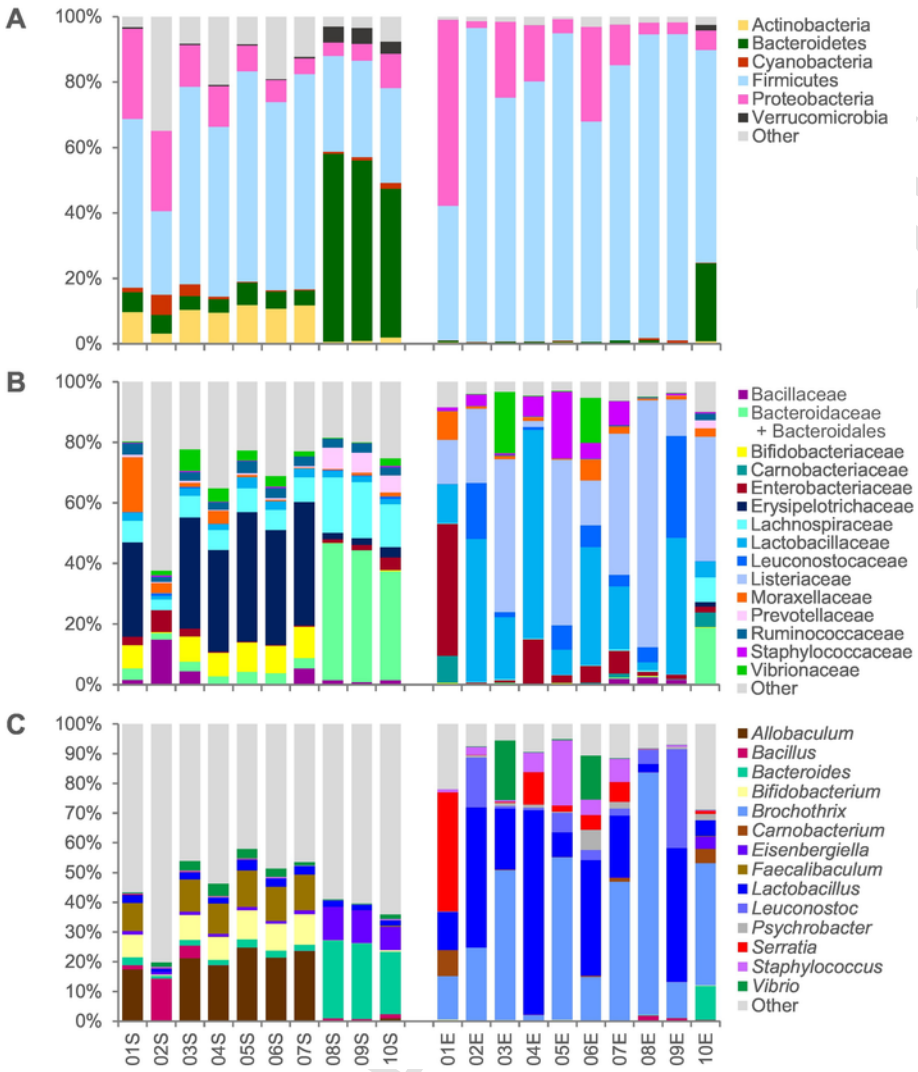

Fig. 6. Relative abundance of the main phyla (panel A), families (panel B), and taxa (panel C) in sausage samples. The plots report only the phyla, orders, families, and taxa occurring at least once with abundance higher than 4, 4, and 6%, respectively, all the others being grouped as "others".

families were Lachnospiaceae (14.2–18.5%), mostly ascribed to the genus *Eisenbergiella* (7.8–11.2%), Prevotellaceae (5.6–7.1%), and Verrucomicrobiaceae assigned to the genus *Akkermansia* (3.7–4.9%).

Microbiota of 02S was peculiar (Fig. 5; Fig. 6), with the lowest amount of Firmicutes (25.5%) and a relevant presence of Proteobacteria (24.6%). More than a half of the reads were not assigned to a family. Noteworthy, the presence of Bacillaceae was high (14.8%) and, at a lesser extent, of Enterobacteriaceae (7.2%). Interesting but hard to explicate is the fact that lots sharing similar S microbiota profiles were noncontiguous in terms of temporal sequence of production. Albeit different, all the S microbial communities were mostly associated with bacteria of the gastro-intestinal tract of mammals and it is presumable that contamination of sausages occurred during slaughter.

Storage at 7 °C for 12 days provided a competitive advantage to Firmicutes that dominated at the end of the shelf life (on average 78.8%). Only in sample 01E, Proteobacteria, mostly belonging to the genus *Serratia*, represented the major phylum (56.9%), whereas they ranged between 3.5 and 29.0% in the other E samples. Other phyla encompassed always $<2\%$ of the reads, with the exception of Bacteroidetes of 10E (23.8%). An overview of microbiota of E samples at family level, indicated the absence of a common trend, albeit in PCoA they clustered together, with the exception of the sample 10E. In 03E, 05E, 07E, 08E, and 10E Listeriaceae mostly referred to *Brochothrix* took over (41.1–81.5%). Conversely, in 01E, 02E, and 09E *Brochothrix* ranged between 12.1 and 24.5%, and only in 04E it was a minor component of microbiota (1.9%). Lactobacillaceae (mostly *Lactobacillus*) outnumbered the other families in 02E, 04E, 06E, and 09E ($>38\%$), whereas in the other E samples, they ranged between 2.7 and 20.8%. As a whole, in 8 out of 10 E samples the sum of Lactobacillaceae and Listeriaceae represented percentages variable from 50 to 75%, indicating that storage at 7 °C in this modified atmosphere (30% CO_2, 70% O_2) provided a competitive advantage to lactobacilli and *Brochothrix*. Leuconostocaceae represented a major family in 02E and 09E (18.5 and 33.7%, respectively). Samples 03E and 06E were characterized by the presence of relevant amounts of Vibrionaceae (14.9 and 20.3%, respectively) mainly ascribed to *Vibrio*. Moraxellaceae were higher in 01E and 06E (9.5 and 7.0%, respectively) than in the other E samples (on average 1.3%; $P < 0.05$). They were associated to the presence of *Acinetobacter* in 01E and *Psychrobacter* in 06E, both generally negligible in the other E samples. Staphylococcaceae (generally *Staphylococcus*) were particularly abundant only in 05E (22.0%), accounting for 1% or less in the other E samples.

C. divergens and *Lactobacillus graminis* among LAB, and *Serratia* sp., *Buttiauxella brennerae*, and *Yersinia molleretti* among Proteobacteria

were reported to dominate the microbiota of fresh sausages at the end of shelf life (Benson et al., 2014). Although the approach was similar to the one herein presented, the sausages contained preservatives and were stored for 80 days. A study considering the effects of the reduction of salt from 2 to 1.5% in raw pork sausages identified a core community composed of L. *sakei*, *Lactococcus piscium*, *C. divergens*, *Carnobacterium maltaromaticum*, *Serratia proteamaculans* and *B. thermosphacta*, although the study was not carried out with industrial samples (Fougy et al., 2016).

3.6. Volatile organic compounds (VOCs)

A total of 68 VOCs were recognized in the head space of sausage samples (Fig. S1): organic acids (acetic, butanoic, hexanoic, and tetradecanoic acids); ethanol and other aliphatic or aromatic alcohols; esters (e.g. ethyl butanoate); aldehydes, mostly linear and saturated C5–C14 ones; ketones (2-butanone and 2,3-butanedione); sulfur compounds (benzothiazol and sulfides); aliphatic hydrocarbons; phenylpropens and other aromatic compounds; mono- and bi-cyclic terpenoids.

The profile of S samples mainly presented molecules deriving from the spices and the flavoring ingredients added to the product. Few other compounds characterized S samples, mainly aldehydes. Of the main microbial metabolites generally occurring in spoiled meat (Casaburi et al., 2015), only ethanol and butanone were found in S samples, although in a minority of them, while acetic and butanoic acids, butanoic acid ethyl ester, and 2,3-butanediol were never detected. These data were in good accordance with HPLC analysis of acetic acid that revealed the absence or significantly lower levels of this metabolite in S than in E samples. Terpenoids, phenylpropens, and benzothiazole persisted at the end of the shelf life, dominating the VOCs profile of E samples as well. The main source of benzothiazole seems to be the ingredients of sausage, since the molecule was found already in S samples and in the mixture of spices as well (data not shown), although it may also be generated by degradation of thiamine or sulfur containing free amino acids (Corral et al., 2016).

The volatile profile of the majority of E samples was characterized by acetic acid, 2,3-butanediol, and pentanal, undetected in the initial samples. Other compounds (e.g. 2,3-butanedione, 3-methyl-butanol, butanoic acid and its ethyl ester, hexanol, hexanoic acid) appeared in E samples only in few cases or were found more frequently than in S samples (e.g. ethanol and butanone).

Although VOCs analysis was not quantitative, since internal standards were not used, the abundance of the most relevant compounds was roughly extrapolated from their peak areas and utilized to calculate a Principal Component Analysis (PCA) model. In order to better estimate the differences between S and E samples due to microbial metabolism, all the VOCs clearly originating from spices were excluded from the PCA model. The PC1 vs. PC2 score plot showed the presence of a compact cluster in the lower left-hand side of the plot encompassing, with a sole exception, all the S samples (Fig. 7a). Conversely, the most part of E samples was distributed along the two PCs, which accounted for about 53% of total data variance. The corresponding PC1 vs. PC2 loading plot showed that PC1 essentially described an overall increase in E samples of acetic acid, pentanal, ethanol, 2,3-butanedione, squalene, and 2,3-butanediol, while PC2 described the difference in the development of these metabolites for different groups of E samples (Fig. 7a). While the E samples lying at positive values of PC2 showed a major increase of pentanal and 2,3-butanedione, the E samples with higher amounts of acetic acid, squalene, and ethanol were found at negative PC2 values. Overall, the score and loading plots showed two orthogonal paths: a former one, for samples batches 01E, 03E, 06E, 07E, 09E, and 10E, which is related to the development of

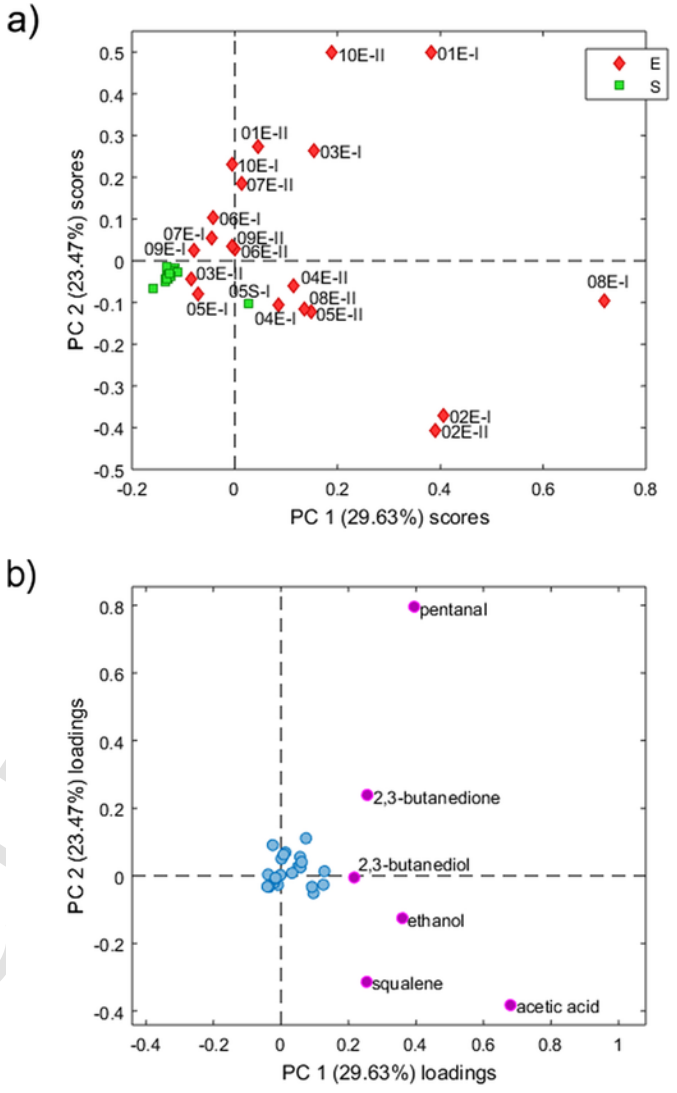

Fig. 7. PCA plot (PC1 vs PC2) of the VOCs profiles in S and E sausage samples. Panel a: score plot of sausage samples. Numerals I and II are the identifiers of duplicate analysis. Panel b: loading plot of the volatile molecules. The compounds with the highest loading value in PC1/PC2 are labelled.

pentanal and 2,3-butanedione, and a second one, for samples 02E, 04E, 05E, and 08E, which can be ascribed to the increase of acetic acid, squalene and ethanol.

Most of the molecules which appeared in E samples are attributable to the onset of bacterial metabolism, consistently with the catabolism of sugars, amino acids and/or lipids by the bacterial taxa dominating the microbiota at the end of the shelf life, such as LAB, *B. thermosphacta*, and *Enterobacteriaceae* (Argyri et al., 2015; Casaburi et al., 2011, 2015; Ercolini et al., 2009).

4. Conclusions

A combination of molecular analysis and culture-based approaches have been successfully applied to characterize microbial diversity of raw sausages in MAP, both at packaging and at the end of the shelf life. The 16S rRNA gene profiling revealed that initial microbiota mainly consisted of bacteria associated to the gut of slaughtered pigs. At the end of the shelf life, albeit the presence of oxygen on the modified atmosphere, facultative anaerobes such as *Brochothrix* and microaerophilic LAB prevailed over other taxa.

The analysis and comparison of ten different industrial lots produced by the same factory in a 7-month period was the peculiar feature of this work and allowed to focalize the attention on the variability of the spoilage pattern associated to an industrial environment. In spite of the complexity characterizing the microbiota, some general traits have been evidenced such as the key role played in many lots by LAB (in particular by L. *sakei*) and/or *B. thermosphacta*. In general, the results obtained using both cultural and not cultural method converged, at least in the product at the end of shelf life. 16S rRNA gene profiling resulted suitable to study and compare the diversity of microbial population involved in sausage degradation in industrially obtained product.

The product maintained a high level of safety over the shelf life, both in microbiological terms and based on biogenic amines production. Conversely, MAP storage at 7 °C seemed too challenging for a proper evolution of sausages that in most of the cases presented one or more defects at the end of the shelf life. Within the biotypes identified in the E samples, several were found in different E lots. The isolation and identification of cultivable strains highlighted the presence of a core microbiota recurrent in the different batches that overtook at the end of the shelf life, suggesting a cross contamination of the meat within the production facility. As a whole, disclosing dynamics of microbial composition of raw sausage in MAP can help to develop new strategies to reduce food spoilage and optimize specific preservative processes.

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The initial pH of the fresh sausages ranged between 5.77 and 6.05 (Fig. 2a, Table S1), in agreement with results reported for other similar products (Cocolin et al., 2004; Torrieri et al., 2011). These values of pH are justified by post-mortem transformation of glycogen in lactic acid, that was present in S samples at levels comprised between 3.01 and 4.85 g/kg. After 12 days, the pH showed a higher variability, ranging from 5.12 (02E) to 5.87 (10E). This latter sample was the sole where pH did not decrease during storage. Lactic acid increased up to approx. 9 g/kg in the samples 02E and 04E, and at a lesser extent in the others, with the exception of 10E where it did not increase, accordingly with the trend of pH. Acetic acid, present in traces in the sausages immediately after production, increased on average to 0.49 g/kg, with a maximum of 0.95 mg/kg in 02E. Likewise lactic acid, accumulation of acetic acid was not detected in sample 10E.

All the bacterial groups increased through the storage in MAP, suggesting the presence of a wide and complex community of psychrophilic bacteria (Fig. 3; Table S2). On average, aerobic bacteria were 4.7 log cfu/g at packaging (S) and reached 6.6 log cfu/g in E samples. LAB were 3.7 log cfu/g in S samples and reached 8.1 log cfu/g in E ones. Putative staphylococci, coliforms, and pseudomonads passed from 3.7, 3.0, and 1.7 to 5.5, 4.8, and 3.0 log cfu/g, respectively. As a whole, the initial bacterial charge indicated good hygienic operations in both the slaughter and transformation plants.

Randomly selected colonies isolated on MRS, PCA, or BPA plates at the end of the shelf life were subjected to RAPD-PCR clustering. Approximately 900 isolates were analyzed and each biotype was taxonomically characterized (Table S3).

A total of 792,402 quality-trimmed 16S rRNA gene sequences were obtained from 20 samples (on average 39,620 reads per sample). The sequencing depth of generally >4 log provided a wide overview of the richness of sausage microbiota. 2041 OTUs net of singletons (i.e. the OTU detected as a single read within the whole set of samples) were identified (558 ± 214 OTUs per sample; Table S4). Alpha diversity was estimated by Chao and Shannon indices, analyzing the OTUs (Table S5). S group was more rich and diverse than E one, according to Chao (787.5 vs. 390.6; $P < 0.05$) and Shannon metrics (6.30 vs 3.16; $P < 0.05$), with the exception of sample 10E that presented a bacterial community much richer than other E samples based on Chao index. Beta diversity, calculated with the Unifrac method, was utilized to compare the bacterial communities in S and E sausages based on their phylogenetic relationship. Excluding sausage 10E, the S and E samples formed two separate clusters along PC1in the plot of coordinates of PCoA (Fig. 5).

A total of 68 VOCs were recognized in the head space of sausage samples (Fig. S1): organic acids (acetic, butanoic, hexanoic, and tetradecanoic acids); ethanol and other aliphatic or aromatic alcohols; esters (e.g. ethyl butanoate); aldehydes, mostly linear and saturated C5–C14 ones; ketones (2-butanone and 2,3-butanedione); sulfur compounds (benzothiazol and sulfides); aliphatic hydrocarbons; phenylpropens and other aromatic compounds; mono- and bi-cyclic terpenoids.