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Microbiota of sliced cooked ham packaged in modified atmosphere throughout the shelf life
Microbiota of sliced cooked ham in MAP

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ABSTRACT

Fourteen lots of cooked ham in modified atmosphere packaging (CH) were analyzed within a few days from packaging (S) and at the end of the shelf-life (E), after storage at 7 °C to simulate thermal abuse. Five more lots, rejected from the market because spoiled (R), were included in the study. Quality of the products was generally compromised during the shelf life, with only 4 lots remaining unaltered. Analysis of 16S rRNA gene amplicons resulted in 801 OTUs. S samples presented a higher diversity than E and R ones. At the beginning of the shelf life, Proteobacteria and Firmicutes dominated the microbiota, with *Acinetobacter*, *Brochothrix*, *Carnobacterium*, *Lactobacillus*, *Prevotella*, *Pseudomonas*, *Psychrobacter*, *Weissella*, *Vibrio rumoiensis* occurring frequently and/or abundantly.

E and R samples were dominated by Firmicutes mostly ascribed to Lactobacillales. It is noteworthy the appearance of abundant *Leuconostoc*, negligible in S samples, in some E and R samples, while in other LAB were outnumbered by *V. rumoiensis* or *Brochothrix thermosphacta*. The microbiota of spoiled and R samples could not be clustered on the basis of specific defects (discoloration, presence of slime, sourness, and swollen packages) or supplemented additives. LAB population of S samples, averaging 2.9 $\log_{10}$(cfu/g), increased to 7.7 $\log_{10}$(cfu/g) in the E and R samples. Dominant cultivable LAB belonged to the species *Lactobacillus sakei* and *Leuconostoc carnosum*. The same biotypes ascribed to different species where often found in the corresponding S and R samples, and sometime in different batches provided from the same producer, suggesting a recurrent contamination from the plant of production. Consistently with growth of LAB, initial pH (6.26) dropped to 5.74 in E samples. Volatiles organic compound (VOCs) analysis revealed that ethanol was the major metabolite produced during the shelf life. The profile of volatile compounds got enriched with other molecules (e.g. 2-butanone, ethyl acetate, acetic acid, acetoin, butanoic acid, ethyl ester, butanoic acid, and 2,3-butanediol) mainly ascribed to microbial metabolism.

1. Introduction

Modified Atmosphere Packaging (MAP) is widely utilized to reduce the addition of exogenous preservatives and to extend the shelf life of food without altering the physical and chemical properties. However, sliced cooked ham in MAP (hereinafter referred to as CH) still represents a perishable product, very sensitive to bacterial spoilage (Borch et al., 1996; Samelis et al., 1998; Vasilopoulos et al., 2015). The

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Table 1
Spoilage assessment, pH, and viable counts on MRS and PDA of CH samples. The number of biotypes identified by RAPD-PCR, the species attribution, and the relative abundance are reported.

Sample[a]		Spoilage assessment[b]					pH	MRS																		PDA[c]						
ID code	Analysis day	Slime/Colonies	Discoloration	Swollen pack	Sour smell/taste	Overall evaluation		Log_{10}(cfu/g)	No. of biotypes	*Carnobacterium* sp.	*C. gallinarum*	*Enterococcus* sp.	*E. durans*	*E. faecalis*	*E. gilvus*	*E. malodoratus*	*L. curvatus/graminis*	*L. fuchuensis*	*L. sakei*	*Leuconostoc* sp.	*L. carnosum*	*L. mesenteroides*	*S. epidermidis*	*S. hominis*	*S. infantis*	*S. oralis*	*S. rubneri*	*W. viridescens*	Log_{10}(cfu/g)	No. of biotypes	*C. sake*	*C. curvatus*	*K. servazzii*	*Y. lipolytica*
01R	24	X		X	X	B	5.69	8.5	4												100%								1.5	1	100%			
02S	4						6.27	1.2	5		12%					12%					39%		25%	12%									
02E	30				X	B	5.58	8.6	2								21%				79%								2	1	100%			
03S	7						6.10	1.8	3								62%				38%													
03E	28				X	B	5.73	8.1	2								94%				6%								4.3	1		100%		
04R	32	X	X			B	5.52	7.4	3												100%								<1.0	-				
05S	3						6.22	3.2	3										100%															
05E	32	X			X	B	5.59	8.8	4										100%										<1.0	-				
06S	7						6.22	6	3										100%															
06E	31				X	B	5.14	8.6	2										100%										<1.0	-				
07S	5						6.19	3.8	2										96%		4%													
07E	30					G	6.00	7.8	2										8%									92%	<1.0	-				
08S	8						6.20	4	4										100%															
08E	32	X	X		X	B	5.50	7.2	5										98%									2%	<1.0	-				
09S	4						6.22	1.2	5	7%									67%						7%	7%	14%							
09E	44					G	5.60	7.7	2										100%										<1.0	-				
10S	7						6.18	1.9	3						4%				30%		66%													
10E	45	X	X		X	B	5.68	7.6	1						100%														<1.0	-				
11R	49			X	X	B	5.59	8.4	3										94%	6%									<1.0	-				
12S	11						7.09	2.9	5										13%		68%							19%						
12E	24					G	6.16	8.4	2										4%		96%								<1.0	-				
13S	2						6.93	2.2	3									2%	4%		94%													
13E	17	X			X	B	6.06	7.5	3									4%	13%		83%								<1.0	-				
14S	2						6.16	4	2				49%						51%															
14E	29	X			X	B	5.39	7.8	1										100%										<1.0	-				
15R	25	X				B	5.48	6.4	1										100%										3.9	1	100%			
16S	7						6.05	2.2	5												90%	5%	5%											
16E	28					G	6.06	6.2	2										90%			10%							<1.0	-				
17S	3						6.17	4.2	1									100%																
17E	23	X				B	5.76	6.3	1												100%								7.2	3	72%		25%	3%
18S	2						6.60	1.6	1			100%																						
18E	28				X	B	6.10	6.6	2			16%		84%															<1.0	-				
19R	21			X	X	B	5.98	7.6	2										100%										<1.0	-				

[a] Samples were given an ID composed by the batch number followed by a letter: S, analyzed at the start of shelf life; E, analyzed closed to the sell-by date; R, rejected from the market. Analysis days are the days elapsed from the packaging date.
[b] Spoilage evaluated on E and R samples; B, Bad; G, Good; X, observed defect.
[c] Counts on PDA were done only for sample E and R; – no biotype.

main undesired defects caused by microbial growth include pH decrease, gas and slime production, blowing, discoloration, purge, and off-flavors formation. In some cases, the defects can result in the premature spoilage and in the reduction of shelf-life (Korkeala and Björkroth, 1997).

Manufacturing of cooked ham ends with cooking at a core temperature up to 70 °C, thus killing most of vegetative microbes. However, CH undergoes a massive bacterial proliferation toward the end of the shelf life despite the combination of hygienic precautions, preservative procedures such as chilling, micro-aerophilic conditions, and presence of NaCl and nitrites. The bacterial community in CH can reach 10^7–10^9 cfu/ g in few weeks after packaging, with a composition depending on many factors, including packaging, gas atmosphere, product composition, hygienic conditions throughout the processing line, and the temperature during both the distribution and the storage in consumer fridge (Audenaert et al., 2010; Kreyenschmidt et al., 2010; Samelis et al., 1998; Vasilopoulos et al., 2008). Previous studies reported a preferential growth of psychrotrophic lactic acid bacteria (LAB), with *Leuconostoc carnosum*, *Leuconostoc gelidum*, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Carnobacterium divergens*, *Carnobacterium maltaromaticum* as the most recurrent species, which dominated the bacterial community at the end of the shelf life (Audenaert et al., 2010; Geeraerts et al., 2017; Vasilopoulos et al., 2008). Nonetheless, information on the microbiota composition in CH is still scarce with respect to the bacterial groups other than LAB.

In order to obtain a wide description of the microbiota composition and diversity, CH samples coming from 10 producers in 6 European countries were analyzed. They presented different characteristics in terms of MAP composition, time of storage, and presence of additives. Microbiota was studied throughout the shelf life of CH by 16S rRNA gene profiling and by a culture-dependent method to specifically isolate and trace the LAB biotypes, with the perspective to select candidates for CH biopreservation. Dominant LAB were isolated, genotyped by RAPD-PCR fingerprinting, and were given a taxonomic designation by partial sequencing of 16S rRNA gene. CH samples were analyzed at the packaging time and at the end of shelf life, during which they were stored at 7 °C. The set of samples included also a group of CH that were rejected as spoiled. For all the samples, the occurrence of defects was evaluated and the prevalent volatile compounds (VOCs) were determined by solid phase micro extraction (SPME) coupled with GC–MS.

2. Materials and methods

2.1. Sample collection and experimental design

Eleven commercial CH were received from 10 producers spread over 6 European countries. The additives and the gas mixture, composed by N_2 and CO_2, are reported in Table S1. As a whole, 19 batches of production

were studied. On 14 batches, microbiological and chemical analyses were performed at the start of the shelf life (S) and at the expiration date (E). The initial measurements were generally performed 2–4 days after packaging but, because of logistic management, in some case the analyses were carried out after a week or more (n = 14, mean 5 days; Table 1). To simulate actual storage conditions, CH was kept at 7 °C until analysis at the sell-by date, on average 30 days after packaging. Five further samples, rejected from the market because spoiled (R), were analyzed immediately after delivery, on average 30 days after packaging.

2.2. Spoilage assessment

CH samples (S, E, and R) were let to reach room temperature and were evaluated in order to detect the presence of the main sensory defects, i.e. blowing of the package, slice discoloration, presence of slime or colonies, and sourness (olfactory perception). An overall evaluation was expressed, sorting samples in good and spoiled ones.

2.3. Culture-dependent enumeration and isolation of LAB and yeasts

Approx. 20 g of CH were 10-fold diluted with saline peptone 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 plates of Lactobacilli MRS agar (BD Difco, Franklin Lake, NJ, USA) and Potato Dextrose Agar supplemented with 100 mg/L chloramphenicol and 100 mg/L ampicillin (PDA; BD Difco). LAB from S, E, and R samples were enumerated and isolated after incubation in anaerobic conditions (GasPack EZ, BD Difco) at 30 °C for 72 h. Yeasts from E and R samples were enumerated and isolated on YPD, after incubation at 28 °C for 3–5 days.

2.4. Taxonomic attribution of MRS and PDA isolates

Colonies was randomly picked up and purified onto MRS and YPD agar plates, respectively. Instagene Matrix (Bio-Rad Laboratories, Redmond, WA) was used for extraction of PCR amplifiable DNA. From each sample, up to 48 putative LABs and 48 yeasts were analyzed by RAPD-PCR using M13-RAPD primer (5′-GAGGGTGGCGGTTCT-3′) (Quartieri et al., 2016) and clustered at a similarity level of 75% using the Pearson correlation coefficient. A single strain for each biotype was taxonomically characterized through sequencing of the V1–V3 portion of 16S rRNA gene, in the case of bacteria, or the internal transcribed spacer (ITS) regions ITS1, 5.8S, and ITS2 for yeasts. The couples of primer 16S-500f (5′-TGG AGA GTT TGA TCC TGG CTC AG-3′)/16S-500r (5′-TAC CGC GGC TGC TGG CAC-3′) and ITS1(5′-TCC GTA GGT GAA CCT TGC GG-3′)/ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were utilized to amplify the two target regions, respectively (Kolbert et al., 2004; White et al., 1990). 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. Chemical analysis

pH was measured at 5 diverse points per each sample using a puncture electrode (Sension+ electrode 5233, HACH, Manchester, UK). Volatile organic compounds (VOC) were monitored by solid phase micro extraction (SPME) coupled with GC–MS analysis according to Raimondi et al., 2018. 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). VOCs analysis was not quantitative, since internal standards were not used. However, the most relevant compounds were roughly extrapolated from the respective relative areas to evaluate presence or absence in the samples.

2.6. 16S rRNA gene profiling

Total DNA was extracted using the DNeasy Mericon Food Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The DNA was normalized to 5 ng/μL after quantification with a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA).

16S rDNA gene amplicons were amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). Gene-specific primers (PCR1_f: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; PCR1_r: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) targeting V3–V4 region containing Illumina adapter overhang nucleotide sequences were selected according to Klindworth et al. (2013). After 16S rDNA gene amplification, the multiplexing step was performed using Nextera XT Index Kit (FC-131-2001). We run 1 μL of the PCR product on a Bioanalyzer DNA 1000 chip to verify the amplicons size (~550 bp) on a Bioanalyzer (Agilent). After size verification the libraries were sequenced using a 2 × 300 pb paired-end run (MiSeq Reagent kit v3, MS-102-3003) on a MiSeq Sequencer according to manufacturer's instructions (Illumina).

Sequencing data have been demultiplexed using Illumina bcl2fastq© program. Forward and reverse ancillary reads were checked for quality and trimmed using the program prinseq-lite applying trimming from the 3′ side of both ends when average Phred33 quality in a window of 20 base pairs was lower than 30 (Schmieder and Edwards, 2011).

QIIME2 pipeline was used to perform denoising, forward and reverse reads joining and chimera removal, data filtering, taxonomic annotation, and basic statistics analysis (Caporaso et al., 2010). Sequences were imported in QIIME2 artifact format and dada2 denoising pipeline was then applied removing primer sequences and filtering chimeras basing on “consensus” method (Callahan et al., 2016). After denoising, resulting amplicon sequence variants, here referred as OTUs, have been annotated using QIIME2 Naive Bayes machine-learning classifier implemented in q2-feature-classifier plugin (Bokulich et al., 2018), using SILVA ribosomal RNA gene database (v119, Quast et al., 2013). The sequences have been submitted to European Nucleotide Archive under project number: PRJEB24662.

2.7. Statistics

Except as otherwise specified, values are means of the separate analysis of three different samples (packages). Comparison were carried out according to Student's t-test. Differences were considered statistically significant for $P < 0.05$.

Principal component analysis (PCA) was used to explore the data matrix of VOCs with size {66, 109}, including the 109 relative VOCs areas determined for the two replicate measurements made for each one of the 33 samples. Before calculating the PCA model, the relative area values were pre-processed using mean centering. The number of significant principal components (PCs) was defined using the screen 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

Fourteen CH lots were evaluated both within a few days (mean 5 days) from the date of packaging (hereinafter referred to as S samples) and at the end of a shelf-life of 23–45 days (mean 30 days), during which they were stored at 7 °C (E samples). The remaining 5 lots, rejected from market because spoiled (R samples), were analyzed on average after 30 days from the date of packaging.

All the S samples were tasty and did not present any alteration. Only

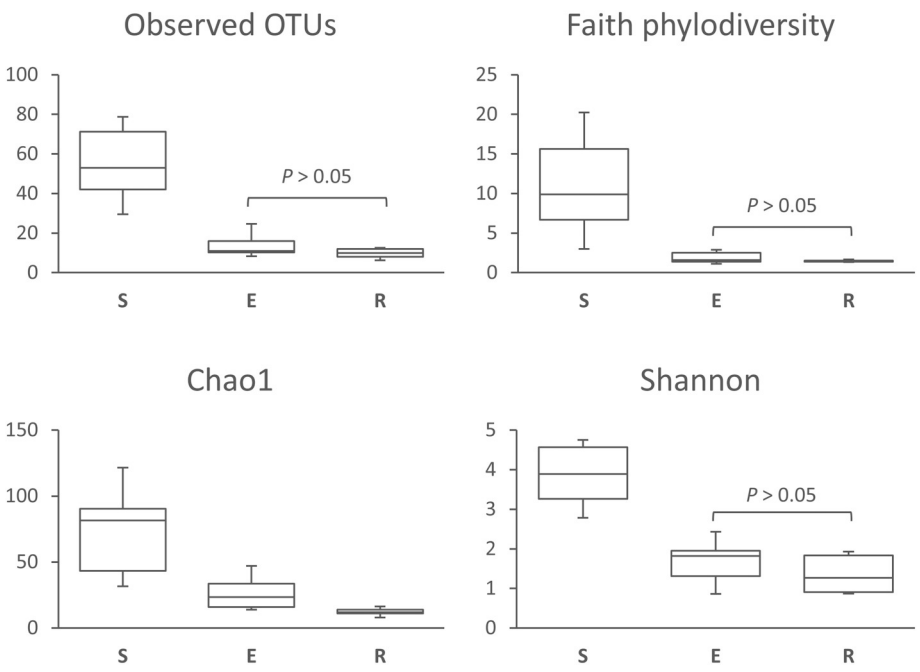

Fig. 1. Number of observed OTUs and alpha diversity metrics, calculated as Chao1, Faith phylodiversity, and Shannon indexes for S, E, and R groups of samples. Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles. Within each parameter, groups significantly differed (Kruskal-Wallis test, $P < 0.05$), unless otherwise indicated.

4 CH lots reached the use-by date in very good condition, maintaining initial properties, and were referred to as EG samples. On the other hand, 10 lots got worst during the shelf life and were referred to as EB samples (Table 1). Adverse modifications that occurred in EB samples included sour odor and flavor (6 samples), presence of slime or colonies (6 samples), and discoloration (2 samples). Similar defects, together with package swelling, were observed in all R samples.

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

Whereas previous studies reported the occurrence of LAB at the end of the shelf life without delving into other microbial groups (Audenaert et al., 2010; Geeraerts et al., 2017; Vasilopoulos et al., 2008), in this study a wide snapshot of the bacterial community of CH was obtained by 16S rRNA gene profiling. A total of 415,225 quality-trimmed 16S rRNA gene sequences were obtained from 33 CH samples, on average 12,583 reads per sample, clustered into 801 bacterial OTUs (Table S2). S samples were richer in OTUs than E and R ones (the mean ± SD being 75 ± 42, 28 ± 18, and 12 ± 4, respectively). Coherently, the Alpha diversity metrics (i.e. Chao1, Shannon, and Faith indexes) pointed out that E and R groups generally presented similar richness and diversity, while the S group was significantly ($P < 0.05$) more complex (Fig. 1; Table S3). In the PCoA plot displaying Beta diversity distances (Weighted Unifrac), S samples appeared disperse, whereas a compact cluster at low PCo1 and high PCo2 values contained most R and E (EG and EB together) samples (Fig. 2).

At the beginning of the shelf-life the microbiota of S samples were distant from each other and rich in disparate bacterial taxa. S samples were dominated by Proteobacteria and Firmicutes (Fig. 3). Lactobacillaceae, mainly belonging to *Lactobacillus*, occurred abundantly in most of the samples, reaching up to 56.0% in 12S. Other families dominated the microbiota of single samples, such as Carnobacteriaceae (*Carnobacterium*) in 18S, Leuconostocaceae (*Weissella*) in 13S, Listeriaceae (*Brochothrix*) in 03S, Moraxellaceae (*Acinetobacter* and *Psychrobacter*) in 06S, Pseudomonadaceae (*Pseudomonas*) in 16S, and Vibrionaceae (*Vibrio rumoiensis*) in 16S. The genera occurring with the greatest frequency in S samples (12 to 14 samples out of 15) were *Lactobacillus*, *Pseudomonas*, *Acinetobacter*, *Brochothrix*, *Prevotella*, and *Psychrobacter*, although with low abundances in some cases. The large diversity of S samples could likely derive from DNA of dead bacteria which did not survive the cooking process. Furthermore, as already suggested (Audenaert et al., 2010), it is probable that post cooking contamination of CH occurred during slicing and/or packaging phases of manufacturing, with the bacteria occurring in the environment of the facility, entering in contact to and being packaged with virtually sterile CH.

During the shelf life, the microbiota changed toward simpler and similar communities mostly dominated by LAB, regardless the evolution into good or defected products. Firmicutes, particularly Lactobacillales, were the most abundant with LAB ascribed to the genera *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, and *Weissella* less frequently (Fig. 3). *Leuconostoc* was remarkably abundant (> 17%) in 7 samples (4 E and 3 R), but scarcely represented in others. *Vibrio rumoiensis*, generally a minor component of S microbiota, outnumbered other taxa in samples 14E and 01R. The samples 14S, 14E, and 01R, sharing *V. rumoiensis* as major component, formed a separate cluster in the PCoA plot (Fig. 2). *Bacillus* resulted a rare component of CH microbiota, found only in 12E in a notably high abundance (40.0%). Minor amounts of *Brochothrix* were identified in several samples at the end of the shelf life. Conversely, it was peculiarly abundant in 17E, and even more in 04R (65.8%).

B. thermosphacta is recognized as one of the most common spoilage bacteria responsible for deterioration of refrigerated meat products, causing defects such as sour off-flavors, discolouration, gas production, and slime production (Borch et al., 1996; Vasilopoulos et al., 2015). *V. rumoiensis* is a facultative psychrophilic bacterium exhibiting a high catalase activity, which had been first isolated from a fish product processing plant utilizing H_2O_2 as a sanitizing agent (Yumoto et al., 1999). No evidences are available so far on the association of *V. rumoiensis* with meat spoilage. It is likely that storage temperature applied in this study (7 °C) played a major role in shaping the microbiota during the shelf-life, since *B. thermosphacta* had already been described to take advantage at storage temperatures at least of 7 °C (Vasilopoulos et al., 2008). In the present study, the temperature was purposely chosen to simulate a realistic thermal abuse, slightly beyond the temperatures of 4–5 °C recommended in many countries for the cold chain throughout production, storage, and transport of fully cooked not shelf stable meat products (European Commission, 2004a; European Commission, 2004b; USDA, 2000).

Specific taxa associated to the maintenance of positive sensorial

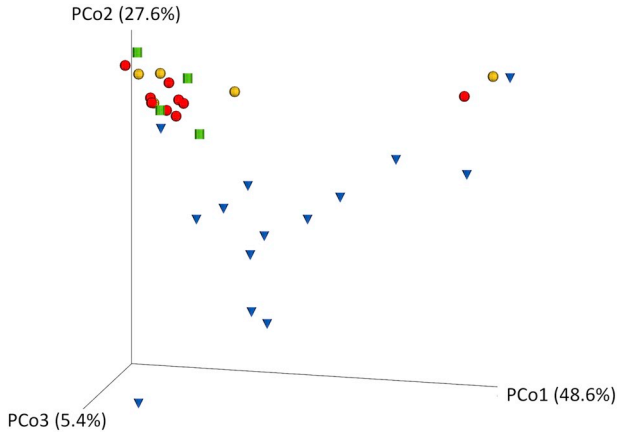

Fig. 2. PCoA plot of weighted Unifrac distances between CH microbiota. Symbols: Blue triangles, S samples; Green squares, EG samples; Red circles, EB samples; Yellow circles, R samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

characteristics in the four EG samples could not be identified, likely because of the low size of this subsample. Furthermore, the microbiota of the EG samples was similar to that of EB and R ones, suggesting the involvement of species or strain specific physiological traits that can affect evolution of sensorial properties. For instance, the presence of abundant *Leuconostoc* was shared by 2 out of 4 EG samples, but was also a feature of 3 out 5 R samples (Casaburi et al., 2015; Geeraerts et al., 2017).

The amount CO_2 in the atmosphere and the presence of salt, preservatives, thickeners, and other additives did not result in any PCoA cluster of CH samples, based on microbiota diversity (data not shown). Similarly, the microbiota of EB and R samples could not be clustered on the basis of specific defects (discoloration, presence of slime, sourness, and swollen packages). The low numerosity within each category, for both additives or defects, likely weakened the possibility to form clusters with similar microbiota composition.

As a whole, 16S rRNA gene profiling provided information on major microbial groups colonizing CH and a relevant overview of sample to sample differences of composition both at the beginning and at the end of the shelf life. The diversity of the leading taxa at the end of the shelf life highlighted that phylogenetically distant genera presented a shared fitness for CH. The progress toward the dominance of a species/strain or another may reside in the strain-specific competitiveness and in the initial load, the latter associated to the steps of slicing and packaging, and to the manufacturing environment.

3.3. Culture-dependent enumeration of LAB and pH

LAB were quantified by plate count enumeration (Table 1). At the beginning of the shelf-life (S), LAB counts were distributed in a range of up to 6 magnitudes, with a mean ± SD of 2.9 ± 1.4 $\log_{10}$(cfu/g) and a median of 2.6 $\log_{10}$(cfu/g). No relationship could be established between the LAB charge in S samples and the days elapsed from the date of packaging ($R^2 = 0.019$; Fig. 4). LAB grew during the shelf-life ($P < 0.001$), reaching a charge of 7.7 ± 0.8 $\log_{10}$(cfu/g) with a mean increase of > 5 magnitudes. R samples presented similar counts compared to E ones, with a charge of 7.7 ± 0.9 $\log_{10}$(cfu/g). The charge of LAB at the end of the shelf life was similar to that of fermented foods, regardless of the outcome in terms of sensorial properties. According to 16S rRNA gene profiling, LAB were always among the main components of CH microbiota, exerting opposing roles, in spoilage or in biopreservation of the sample.

The pH of S samples was 6.26 ± 0.28 (Table 1). At the end of the shelf-life (E), the pH dropped ($P < 0.05$) to 5.74 ± 0.30. The pH of the spoiled samples (R) was 5.65 ± 0.20, similar to that detected at the end of the shelf-life ($P > 0.05$).

3.4. Identification of MRS isolates

The identification and quantification of putative LAB and the traceability of specific biotypes still rely on methods that exploit cultivation followed by taxonomic characterization, where the choice of medium and temperature inevitably shapes the spectrum of the isolates. In order to isolate LAB that may be exploited as biopreservative starters able to growth in abuse of temperature, plates were incubated at 30 °C. Approximately 1400 colonies, up to 48 from MRS plates for each sample, were typed by RAPD-PCR and clustered within biotypes presenting homogeneous 16S profile. Albeit RAPD-PCR suffers from low reproducibility and discriminatory power, it provides a quick and efficient screen on anonymous genomes at reasonable costs. In particular, it can suffer from changes in the quality of DNA, PCR components, and PCR conditions, that can affect the pattern. However, reproducible results may be obtained if care is taken to standardize the conditions used.

Sixty-two different biotypes were identified (Table S4), one to five per sample. The isolates belonged to 7 different genera (*Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, *Weissella*) and to 17 species (Table 1). Colonies of *Lactobacillus sakei* and *Leuconostoc carnosum* outnumbered those of other species: 45% of the isolates were assigned to 22 biotypes of *L. sakei*, 30% to 16 biotypes of *L. carnosum* (Table S5). Each one of the other species accounted one to three different biotypes and, although dominating the microbiota in a few samples (03E, *L. curvatus/graminis*; 07E, *W. viridescens*; 10E, *E. gilvus*; 17S, *L. fuchuensis*; 18S, *Enterococcus* sp.; 18E, *E. faecalis*), they represented a minor component (< 6% each) within all the isolates. In most of the cases, when *L. sakei* or *L. carnosum* prevailed at the beginning of the shelf life, the same species was found as the most abundant, or among the most abundant, in the corresponding E sample. At the end of the shelf-life (E + R), the pH was lower in samples colonized by *L. sakei* than in the ones dominated by *L. carnosum* (5.59 and 5.80, respectively; $P < 0.05$). Staphylococci and streptococci were found only in a few S samples, suggesting an initial contamination that was overtaken by LAB during storage.

Common RAPD-PCR profiles in different samples delivered from the same facility was observed, suggesting the presence of recurrent strains or at least of genetically similar bacteria. Often, the same biotype was found in S and E samples belonging to the same batch (Fig. 5). Most of the persisting strains were ascribed to *L. sakei* and *L. carnosum*, even if some batches were characterized by the presence of the same strains of *L. curvatus* (09), *Enterococcus gilvus* (36), and *Enterococcus* sp. (59) at the beginning and at the end of the shelf-life. In several cases, the same biotype was found in diverse batches from the same producer, including also the rejected samples. For instance, two strains of *L. carnosum* were isolated in two batches from CH produced in facility 02, both in S and E samples, and a strain of *L. carnosum* was detected in four samples provided by supplier 01 (01R, 02S, 02E, 04R).

L. sakei had already been reported in CH as a marginal or a major component of the microbiota (Audenaert et al., 2010; Samelis et al., 1998; Vasilopoulos et al., 2008), and is herein confirmed as one of the dominating bacteria in many lots from different European countries. On the other hand, *L. carnosum* commonly occurs in vacuum-packaged, sliced, cooked ham showing spoilage during the shelf life (Björkroth et al., 1998; Samelis et al., 2006). Interestingly, the same biotype of *L. sakei* and *L. carnosum*, but also of other less recurrent species, occurred both in S and E samples and/or in different batches from the same producer, in some case including also R samples. This observation is likely due to peculiar bacteria for each plant contaminating the product.

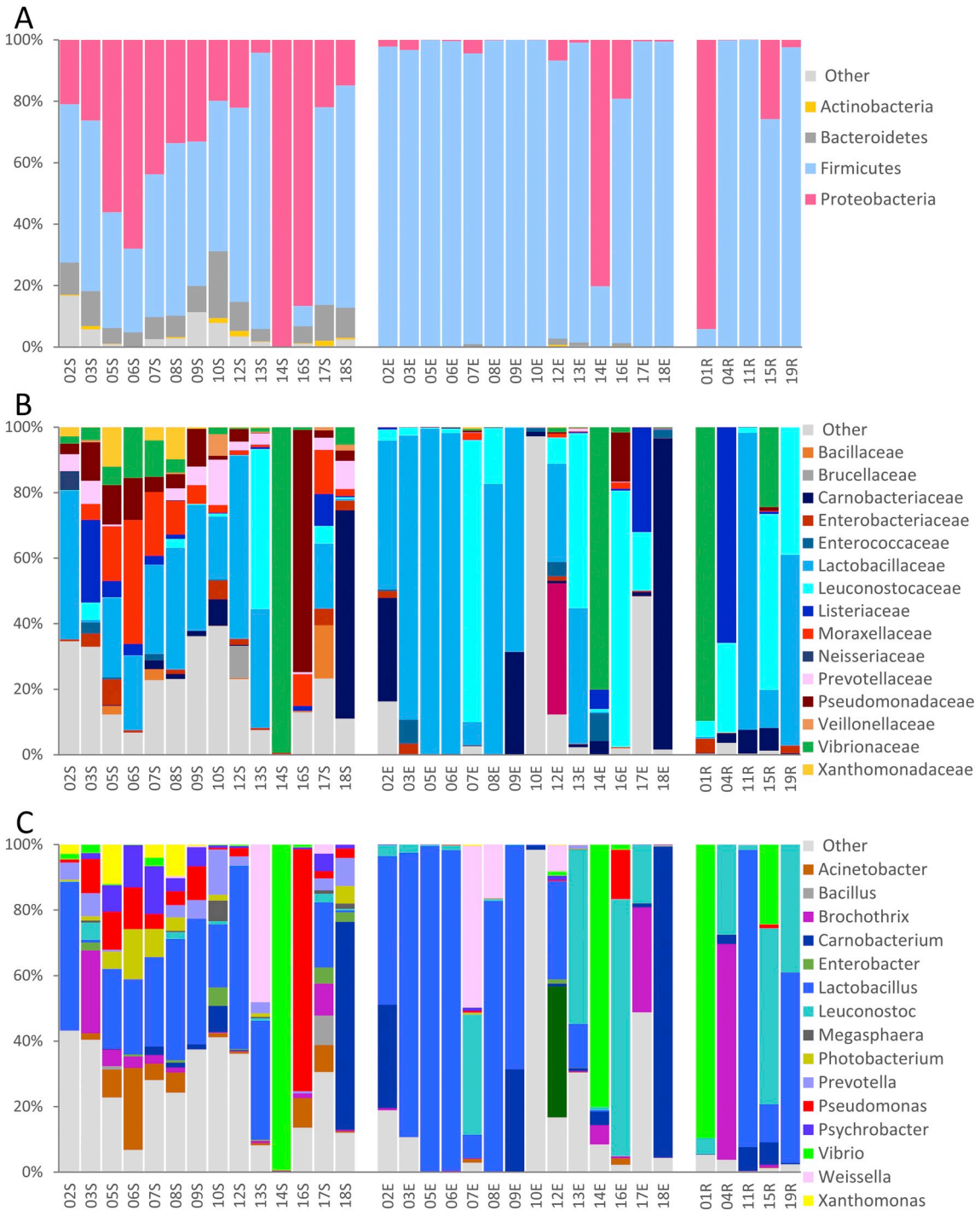

Fig. 3. Relative abundance of the main phyla (panel A), families (panel B), and genera (panel C) in CH samples. The plots report only the phyla, families, and genera occurring at least once with abundance higher than 5%, all the others being grouped as 'others'.

3.5. Occurrence of yeasts

Yeasts were plate enumerated and clustered by RAPD-PCR in E and R samples, then characterized by ITS partial sequencing (Table S4). They were isolated from 4 out of 14 E samples, and from 2 out of 5 R ones (Table 1). The fungal charge was generally low (3.6 ± 1.9 $\log_{10}$(cfu/g), mean ± SD), with a single outlier contaminated by 7.2 $\log_{10}$(cfu/g) cultivable yeasts (17E). This was the sole sample that resulted in isolation of more than a single species (*Candida sake* 72%, *Kazachstania servazzii* 25%, *Yarrovia lipolytica* 3%). *C. sake* seemed to take an advantage growing on CH packed in MAP, since it was the sole isolate in samples 01R, 02E, 14E, and 15R. Sample 03E was contaminated only by *Cryptococcus curvatus*. The role of yeasts in CH spoilage deserves further investigation. Previous studies reported significant increase of yeasts during refrigerated storage of cooked ham, but especially under aerobic conditions (Samelis et al., 1998; Samelis et al., 2000), but did not delve in the taxonomic identification of the contaminant yeasts.

3.6. Volatiles organic compounds (VOCs)

Although the aroma of fresh cooked and sliced CH is mainly due to

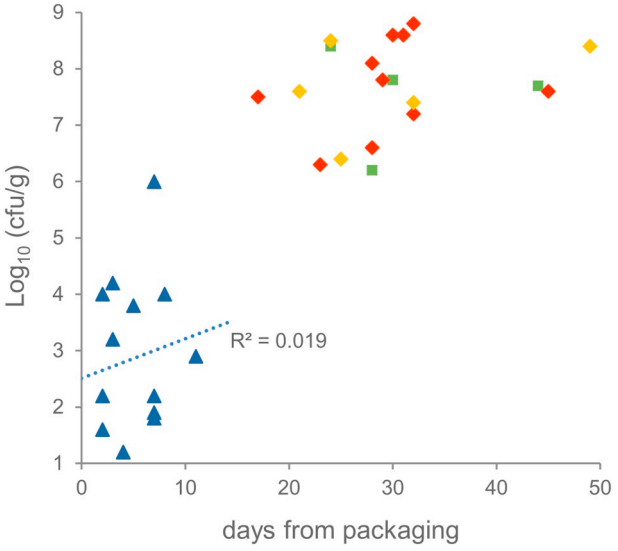

Fig. 4. Viable LAB in CH samples, enumerated on MRS agar plates throughout the shelf life. Symbols: Blue triangles, S samples; Green squares, EG samples; Red diamonds, EB samples; Yellow diamonds, R samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

products of thermally induced lipid oxidation (e.g. straight chain fatty acids, aldehydes, ketones and alcohols) and to the spices added to the product (Casaburi et al., 2015; Estévez et al., 2003; Holm et al., 2013; Rivas-Cañedo et al., 2009), during storage a number of VOCs are surely produced by metabolic activity of microbes. The head space of CH samples presented a total of 109 volatile compounds (Table S6), including organic acids, ethanol and other alcohols, ketones, fatty aldehydes, esters, phenols and a variety of aromatic compounds, furans, linear and branched aliphatic hydrocarbons, terpenoids, and sulfides.

S samples, that did not exhibit any sensory defect, were characterized by terpenoids coming from spices and by diverse compounds presumably deriving from lipid oxidation (e.g. C6–C10 aldehydes and fatty acids). However, S samples also presented putative bacterial metabolites (e.g. ethanol, acetic acid, 2-butanone and acetoin), the presence of which may be the result of an early onset bacterial metabolism, that may have started in the few days between the packaging and the inoculum.

At the end of the shelf-life, most of the samples presented some defect. Their VOCs profile was characterized by ethanol and a variety of bacterial metabolites. In particular, acetic and butanoic acids were produced abundantly during the storage, and were likely the responsible for the significant pH reduction observed in E samples. Ethanol, acetic, and butanoic acids are among the main VOCs occurring in spoiling meat (Casaburi et al., 2015). Ethanol originates from sugar fermentation, while acetic and butanoic acids can derive from bacterial catabolism of carbohydrates and/or free amino acids. Both the acids can be associated to the presence of LAB (e.g. *Carnobacterium* spp., *Leuconostoc* spp., *L. curvatus*, *L. sakei*, and other lactobacilli), *B. thermosphacta*, *Pseudomonas*, and other bacteria (Casaburi et al., 2015). Other volatiles characterizing the profile of E and R samples (e.g. 2-butanone, acetoin, 3-methy-butanal and 3-methyl-butanol) could be similarly attributed to the metabolism of LAB (mainly *Carnobacterium* spp. and *Lactobacillus* spp.), *Enterobacteriaceae*, *Pseudomonas*, and *Brochothrix*, the branched aldehydes and alcohols being likely generated from branched aminoacids by spoilage bacteria (Beck et al., 2004; Ercolini et al., 2009). On the other hand, the esters (e.g. ethyl acetate, isoamyl acetate, ethyl butanoate) are reported to be produced by *Pseudomonas* spp. (e.g. *P. fragi*) (Argyri et al., 2015; Ercolini et al., 2009; Ercolini et al., 2010). 2-ethyl-1-hexanol, which appeared with similar frequencies in S and E samples, is reported as a natural odorous molecule occurring in fresh meat (Rivas-Cañedo et al., 2009), although it is recognized as a microbial metabolite as well, produced by LAB (e.g. *Carnobacterium*) and *Pseudomonas* (Ercolini et al., 2009; Casaburi et al., 2011).

The Principal Component Analysis (PCA) model confirmed ethanol as a major VOC produced during the shelf life (Fig. 6). PC1 accounted for approx. 44% of data variance and essentially described the variation of ethanol content. In the PCA plot, the samples were separated in two

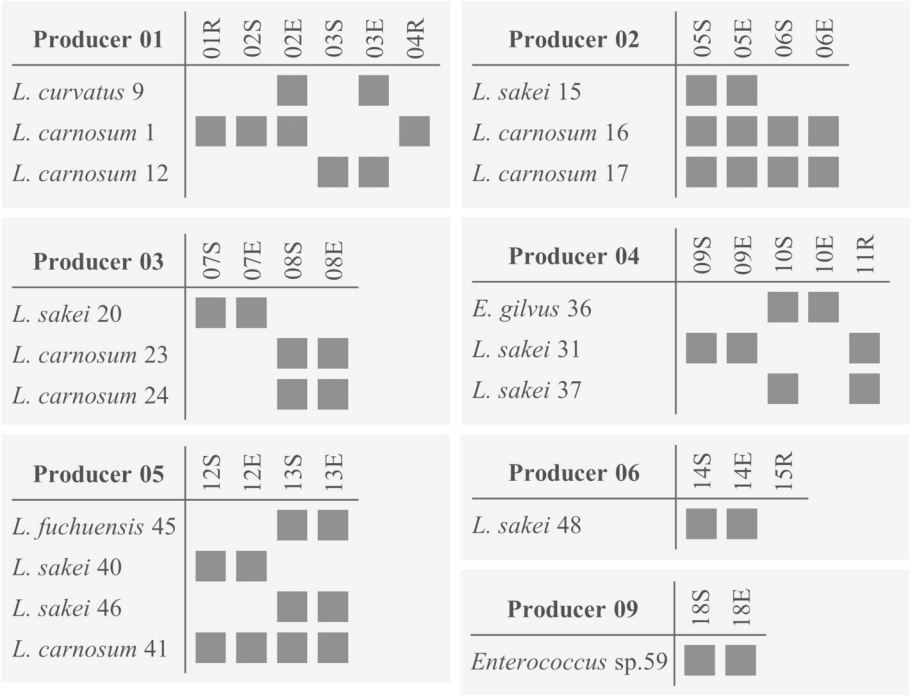

Fig. 5. LAB biotypes recurrently found at the beginning and at the end of the shelf life in the same CH lot and/or in different lots from the same producer. For each producer, LAB identified in more than a sample are reported (the grey box corresponds to the occurrence of the LAB biotype).

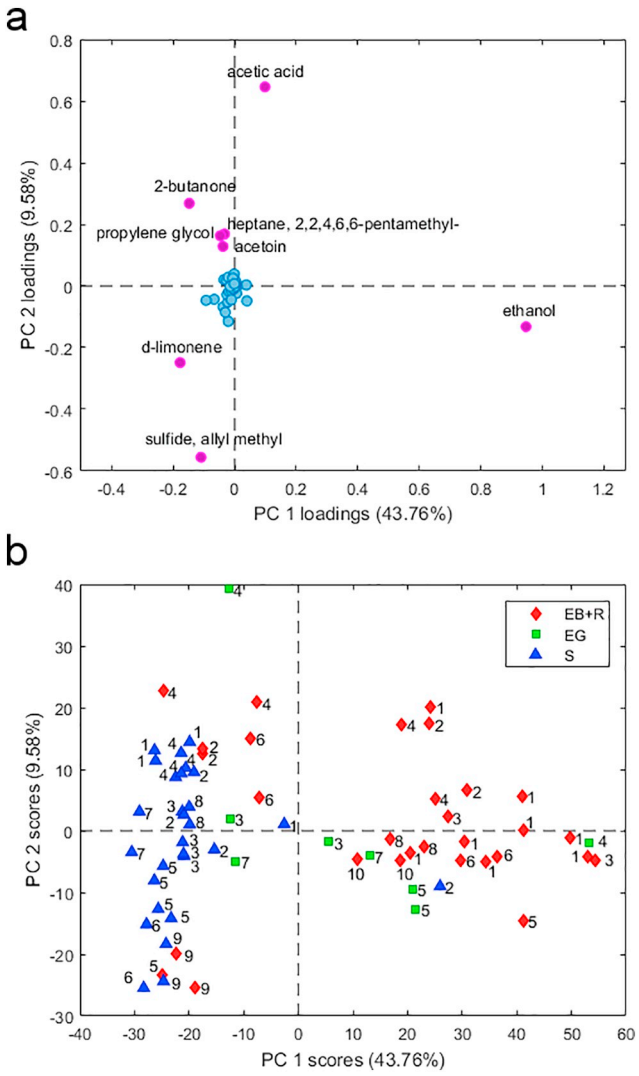

Fig. 6. PCA plot (PC1 vs PC2) of the VOCs profiles of CH samples. Panel a: loading plot of the volatile molecules. Panel b: score plot of CH samples. In the same score plot, the numbers reported in the labels are referred to the different producers.

clusters along PC1 (Fig. 6). The cluster on the left side had low ethanol and was mostly constituted by S samples, while the one on the right side presented a high ethanol content and mostly included E and R samples. PC2 accounted for approx. 10% of data variance, being positively driven by acetic acid, 2-butanone, pentamethyl heptane, propylene glycol, and acetoin, and negatively by allyl-methyl sulfide and limonene. Acetic acid, 2-butanone, and acetoin can be produced at least in part by microbial metabolism, while both the molecules showing a negative contribution to PC2 (limonene and allyl-methyl sulfide) derived from spices. Interestingly, the samples that presented good sensory properties at the end of the shelf life were generally characterized by lower levels of ethanol and located at negative values of PC2, suggesting that spices seem to favor a positive sensory perception of CH at the end of the shelf life. With few exceptions, S samples from different batches of the same producers resulted similar to each other, although in general a greater variability was observed at the end of the shelf-life.

The same PCA model was also employed to investigate the possible effect on VOCs profile of the amount CO2 in the atmosphere and of the presence of salt, preservatives, thickeners, and other additives. However, it was not possible to identify any sample clustering on the basis of additives and of CO2 amount, coherently with the PCoA results discussed in Section 3.2. Similarly, the evaluation of PCA scores did not show the presence of clusters based on specific defects (discoloration, presence of slime, sourness, and swollen packages).

4. Conclusions

The market of ready-to-eat food products is growing, with consumption of CH that has been increasing rapidly and has further growth potentials. Microbiology of CH is a relevant issue, since it has a bearing on both safety and quality of the product. Whereas the outbreaks have been rare, premature spoilage of CH can occur, and at least some sensorial defects generally affect the product, approaching to the end of the shelf life. A comprehensive overview of CH microbial communities was required. Disclosure of taxa potentially associated to spoilage is relevant to open perspectives toward the development of strategies aimed to control and lead microbiota evolution, in order to manage CH safety risk and extend the shelf life of the product.

This paper provides new knowledge on evolution of CH microbiota, that appeared quite variable within the same sets of S, E, or R samples. Analysis of microbiota at the end of the shelf life of good and spoiled samples did not unveil any specific taxon that could be surely associated to spoilage, indicating that a negative evolution can be due to strain specificity, also within the same species, or even to subdominant bacterial groups. Consistently, it was not possible to associate specific patterns of VOCs to peculiar microbial groups or taxa. A great diversity in initial microbiota was described, but also the microbiota at the end of the shelf life previously ascribed to LAB revealed a much greater complexity and variability.

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