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Monitoring of the microbiota of fermented sausages by culture independent rRNA-based approaches

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Running title: Fermented sausages ecology by rRNA approaches

Abstract

In Italy, fermented sausages (called "salami") are consumed in large quantities. Salami samples from a local meat factory in the area of Torino were analysed at 0, 3, 7, 30 and 45 days of ripening. Swab samples from the production environment were collected as well at the beginning of the experiment. The diversity of metabolically active microbiota occurring during the natural fermentation of salami was evaluated by using RT-PCR-DGGE coupled with RNA-based pyrosequencing of the 16S rRNA gene. A culture-dependent approach was also applied to identify and characterize isolated *Staphylococcaceae* and LAB populations. *Staphylococcus succinus*, *S. xylosus* and *Lactobacillus sakei* were the species more frequently isolated during the maturation time. Rep-PCR analysis showed that *S. succinus* and *S. xylosus* isolated from swabs and salami samples clustered together suggesting a possible contamination during the production process. RT-PCR-DGGE and rRNA based pyrosequencing showed that the metabolically active populations were dominated by *S. succinus, Lb. sakei* and *Leuconostoc carnosum*. In this specific case study, only few species belonging to *Staphylococcaceae*, *Lactobacillaceae* and *Leuconostocaceae* may be metabolically active and contribute to determine the final characteristics of the products.

Keywords: RT-PCR-DGGE, pyrosequencing, fermented meat, molecular methods

1. Introduction

In Italy there is a big variety of fermented sausages (called "salami"), consumed in large quantities. They are produced using the same main ingredients (pork meat, pork fat, sugars and salts) but with important differences in the type and quantities of spices (Cocolin et al., 2009). Microbiota development during ripening of these products has been largely

studied (Aquilanti et al., 2007; Cocolin et al., 2009; Połka et al., 2015; Villani et al., 2007). Lactic acid bacteria (LAB) and coagulase-negative *Staphylococcaceae* (CNS) are the two main groups of bacteria that are considered technologically important during fermentation and ripening of fermented meat products. An evident and strong connection is valid between the microbiota that develop during fermented sausages ripening and the sensory characteristics of the final product (Rantsiou and Cocolin, 2006).

In the last decades, a great improvement in the detection and identification of microorganisms has been achieved by the introduction of molecular biology-based methods and mostly by culture-independent approaches. When RNA is analyzed, the microbial populations that are metabolically active can be potentially highlighted. Those are the populations that contribute the most to the fermentation process (Cocolin et al. 2013). Reverse transcription RT-PCR-DGGE has been applied in dairy products to investigate their microbiota (Dolci et al., 2013; Masoud et al., 2012; Rantsiou et al., 2008). High-throughput sequencing (HTS) allows an in-depth study of the microbial diversity in food. Workflow, limits and perspectives in applying culture-independent HTS to study food microbiota have been reviewed by Ercolini (2013). In general, HTS applications include metagenome sequencing approaches, studying the microbiome i.e. the distribution of genes and functions in food, or studying the structure of food microbiota by high-throughput sequencing of amplicons of taxonomic interest (Ercolini, 2013). The culture-independent, sequencing-based approach has been successfully applied for the identification of microorganisms in meat products (De Filippis et al., 2013; Ercolini et al., 2011) and is expected to improve the sensitivity and efficiency in the evaluation of microbial diversity.

The aim of this study was to evaluate the diversity of metabolically active microbiota occurring during the natural fermentation of a traditional Piedmontese fermented sausage by using RT-PCR-DGGE coupled with RNA-based pyrosequencing of 16S rRNA gene. In addition, in order to have a better picture of the microbiota, a culture-dependent approach was

used to identify and characterize *Staphylococcaceae* and LAB population isolated during fermentation and from the meat processing plant.

2. Material and methods

2.1. Salami manufacturing and samples collection

Salami samples were manufactured in a local meat factory in the area of Torino, by traditional techniques. Two batches were prepared in different moments (A and B). The formulation used in the manufacture included pork meat, lard, salt, pepper, coriander, nutmeg, cinnamon, sugar (0.1% w/w sucrose), nitrate salt (E252). Meat batter was stuffed into bovine casings resulting in sausages of about 36 mm of diameter. Ripening was carried out in a climatic chamber: for the first week at 19 °C and 80-83 % relative humidity (RU), while from the second week till end it was carried out at 14°C, 87% RU. Three samples of the meat mixture prior to filling (0) and three salami samples obtained after 3, 7, 30, and 45 days of ripening were analyzed. In addition, swab sampling was performed in the meat processing environment inside the local factory prior to manufacturing. The sample sites chosen were: I) cutting table, II) wall of the room where the batter is prepared, III) filling tube of the stuffing machine, IV) feeding compartment of the stuffing machine. At each sampling site, a moistened (0.1% buffered peptone water plus 0.85% sodium chloride solution) sponge (Biogenetics, Milan, Italy) was rubbed vertically, horizontally, and diagonally across the sampling site (100 cm^2) delineated by a template. Samples were cooled at 4 °C and analyzed within 3 hours.

2.2. Microbiological analysis

About 10 g from each of the three salami at every sampling time were taken from the internal part and homogenized with 90 mL of Ringer's solution (Oxoid, Milano, Italy) for 2 min in a stomacher (LAB Blender 400, PBI, Italy; stomacher bags: Sto-circul-bag, PBI, Italy) at room temperature. One milliliter of the sponge buffer was also used. Decimal dilutions in quarter-strength Ringer's solution were prepared, and aliquots of 0.1 ml of the appropriate dilutions were spread in triplicate on the following media: (i) Gelatin Peptone Agar (GPA, Oxoid) for total aerobic bacteria incubated for 48 to 72 h at 30°C; (ii) De Man Rogosa and Sharpe agar (MRS, Oxoid) for LAB, incubated at 30 °C for 48 h; (iii) Mannitol Salt Agar (MSA, Oxoid) for *Staphylococcaceae* incubated at 30°C for 48 h; (iv) Violet Red Bile Agar (VRBA, Oxoid) for the *Enterobacteriaceae*, incubated at 30 °C for 24–48 h; (v) Malt Extract Agar (AMT, Oxoid) plus tetracycline (0.05 g/L, Sigma, Milan, Italy) for yeasts and moulds incubated at 25 °C for five days. Results were calculated as the means of Log colony forming units (CFU) for three independent determinations. Results from the swabs were calculated as Log CFU/cm^2.

The pH of each sample was measured in triplicate by using a digital pH meter (Waterproof pH Tester, Thermo Scientific, Nijkerk, The Netherlands). For each sampling time, mean and standard deviation were calculated.

Data obtained from viable counts of the two batches were analyzed using one-way analysis of variance (ANOVA) with time being the main factor. ANOVA analyses were performed with the SPSS 18.0 statistical software package (SPSS Inc., Cary, NC, USA). The Tukey HSD test was applied when ANOVA revealed significant differences ($P < 0.05$).

In parallel, for each sample point the presence of *Listeria monocytogenes* and *Salmonella* spp. was checked. Isolation of presumptive *L. monocytogenes* was conducted according to the ISO 11290-1 method while isolation presumptive of *Salmonella* spp. was carried out according to ISO 6579 methodology.

After measurement of the viable counts from each sample point (0, 3, 7, 30 and 45 days) 15 colonies from MRS and MSA agars were randomly isolated and purified. The purified isolates were preliminary characterized by microscopic observations and Gram, catalase and oxidase reactions. Working cultures were maintained in Brain Heart Infusion (BHI, Oxoid) or MRS broth (Oxoid) with 25% glycerol at –20 °C.

2.3. DNA extraction from pure cultures and identification by PCR-DGGE

DNA was extracted from 1 ml of an overnight culture of BHI and MRS centrifuged at 14,000 ×g for 10 min at 4 °C. The pellet was subjected to DNA extraction according to Cocolin et al. (2001). DNA was quantified by using the Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy) and it was standardized at 100 ng/μL.

For LAB isolates, the primers 518R and 338F were used (Muyzer et al. 2003) amplifying the variable V3 region of the 16S rRNA gene, giving PCR products of about 250 bp. To the forward primers, a GC clamp was added according to Muyzer et al. (1993). PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) using Bio-Rad Dcode apparatus as reported (Cocolin et al. 2001)

For *Staphylococcus* spp. the primer pair P1V1F and P2V1R (Klijn et al. 1991) was used, amplifying the variable V1 region of the 16S rRNA gene. Primer P1V1F was modified by the addition of a GC clamp as described above. PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) using Bio-Rad Dcode apparatus as previously described (Cocolin et al. 2001). PCR products (for both CNS and LAB) that migrated at the same position in the gels were grouped together.

Randomly for each group, isolates were chosen and the partial 16S ribosomal DNA gene was amplified with primers P1V1/P4V3 (Kliji et al. 1991). PCR products were then purified with

a PCR Extract Mini Kit (5PRIME, Milan, Italy) according to the manufacturer's instructions and sequenced. Sequencing was performed with a Deoxy terminator cycle sequencing kit (Perkin–Elmer Applied Biosystems) using the primer P1V1F. To determine the closest known relatives of the partial 16S rRNA gene sequences obtained, searches were performed in public data libraries (GenBank) with the Blast search program (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1997).

2.4. Molecular typing by rep-PCR and cluster analysis

For the most abundant CNS and LAB species fingerprintings were obtained by using repetitive extragenic palindromic PCR (rep- PCR) with the primer $(GTG)_5$ according to Iacumin et al. (2006). Reactions were carried out in a final volume of 25 µL containing: 1X PCR Buffer, 1.5 mM MgCl2, 0.2 mM of each of dNTP, 2 µM primer $(GTG)_5$ and 2.5 U/µL *Taq* DNA polymerase (Sigma, Milan, Italy). The PCR reaction consisted of 30 cycles of denaturation at 90°C for 30s, annealing at 40°C for 60 s and extension at 65°C for 8 min. The initial denaturation was at 95°C for 5 min and the final extension at 65°C for 16 min. The amplified products were resolved by electrophoresis on 2% agarose-TBE at 120 V for 2h and the 1 kb DNA ladder was used as molecular weight marker (Promega). The rep-PCR profiles were normalised and cluster analysis were performed using Bionumerics software (version 6.1, Applied Maths, Sint-Martens-Latem, Belgium). The dendrograms were calculated on the basis of the Pearson's Coefficient of similarity with the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) clustering algorithm (Vauterin and Vauterin, 1992).

2.5. Total RNA extraction from salami matrix and RT-PCR-DGGE analysis

For RNA extraction, at each time point, 1 ml of the first decimal dilution prepared for the viable counts was collected and centrifuged at maximum speed for 30 s, the supernatant

was removed and 200 µl of RNA-later (Ambion, Applied Biosystems, Milan, Italy) was immediately added to the pellet and stored a -80°C. The RNA was extracted from the pellet using a MasterPureTM Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA) following the supplier's instructions. Three microliters of TURBO-DN*ase* (Ambion) was added to digest the DNA in the RNA samples, with an incubation of 3 h at 37 °C. RNA was quantified by using the Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Italy) and standardized at 300 ng/µL. Reverse Transcriptase (RT) was performed by using the universal primer 518R as described by Rantsiou et al. (2008). One µL of cDNA was amplified with primers 338F-GC and 518R. PCR products of approximately 250 bp were analyzed by DGGE (Bio-Rad Dcode apparatus). Samples were applied to 8% (w/v) polyacrylamide gels in 1 X TAE buffer. Parallel electrophoresis experiments were performed at 60 °C using gels containing a 30–60% urea–formamide denaturing gradient (100% corresponded to 7 M urea and 40% [wt/vol] formamide). The gels were run for 240 min at 200 V, stained with SYBR® Gold Nucleic Acid Gel Stain (Life Technologies, Milano), visualized under UV-transillumination and photographed by using UVIpro Platinum 1.1 Gel Software (Eppendorf). A database of fingerprints was created by using the software Bionumerics version 4.6 (Applied Maths, Sint Marten Latem, Belgium). A dendrogram of similarity was retrieved by using the Dice coefficient and Unweighted Pair Group Method using Arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin, 1992).

Selected DGGE bands were excised from the gel with sterile pipette tips and purified in water. One μL of the eluted DNA was used for the re-amplification by using the primers and the conditions described above, and the PCR products were checked by DGGE. The original PCR product was run on the gel as the control. Products that migrated as a single band and at the same position as the control were purified by using PCR Extract Mini Kit (5PRIME, Hilden, Germany) according to the manufacturer's instructions and then were sequenced as describe above.

2.6. RNA analysis by pyrosequencing

The cDNA was used to study the microbial diversity of the metabolically active populations by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene by using primers and PCR condition previously reported (Ercolini et al., 2012). PCR products were purified by Agencourt AMPure kit (Beckman Coulter, Milano, Italy), quantified using the QuantiFluor™ (Promega, Milano, Italy) and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing by using Titanium chemistry on a GS Junior platform (454 Life Sciences, Roche, Monza, Italy) according to the manufacturer's instructions.

2.7. Bioinformatics analysis

Raw reads were first filtered according to the 454 processing pipeline. Sequences were then analyzed and further filtered by using QIIME 1.8.0 software (Caporaso et al., 2010), using split_library.py and denoiser.py script (Reeder and Knight, 2010). 99% OTUs were picked against the Greengenes database 16S rRNA gene database (McDonald et al., 2012). Alpha and beta diversity were evaluated through QIIME (De Filippis et al., 2013). Beta diversity was evaluated by weighted UniFrac analysis (Lozupone and Knight, 2005) and Principal Coordinates Analysis (PCoA) was performed to show the diversity between the samples according to their specific microbiota. Weighted UniFrac distance matrices and OTU tables were used to perform Adonis, Anosim, g_test, ANOVA and distance comparison statistical tests through compare_category.py, make_distance_comparison_plots.py and group_significance.py scripts of QIIME, in order to verify the difference between the samples. A filtered OTUs table at 0.5% abundance in at least two samples was generated through QIIME.

All the sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (SRP049161).

3. Results

3.1. Enumeration of the microorganisms

The results of the pH and viable counts during salami ripening are shown in Table 1. pH values increased from 5.75 ± 0.05 to 6.75 ± 0.08, while LAB and staphylococci grew significantly ($P<0.05$) after 45 days of ripening, as well as the total bacterial counts. Moulds, yeasts and enterobacteria did not show a significant development in all the sampling points ($P>0.05$) remaining constant at about 2 to 3 Log CFU/g. *Listeria* sp. and *Salmonella* sp. were never detected.

Concerning the environmental swabs (Tab. 2), LAB and staphylococci counts were around 3 Log CFU/cm^2 and only in the filling tube of the stuffing machine (III) LAB reached 5.64 Log CFU/cm^2. The other populations were found to be present only in some sampling locations (Tab. 2).

3.2. Identification and grouping of isolates from media plate

A total of 208 isolates from MSA and 169 isolates from MRS plates were identified by PCR-DGGE coupled with the 16S rRNA gene sequencing. The results are shown in Table 3 and Table 4, respectively. The majority of CNS isolates were identified as *Staphylococcus succinus* and *S. xylosus* both from the salami (42% and 46%, respectively) and from the environmental swabs (61% and 30%, respectively). Isolates identified as *S. equorum* were also found in both salami and swabs, while *S. saprophyticus* and *S. cohnii* were only isolated from the salami samples. Few isolates of *Leuconostoc carnosum*, *Enterococcus gilvus* and *Lb. pentosus* were found in both the salami and in the environmental samples.

Lactobacillus sakei was the species mainly isolated from the salami samples (83%), but no isolates belonging to this species were found in the environment and the same was observed for *Lc. mesenteroides*. *E. gilvus* and *Lb. pentosus* were found only in raw meat at time 0 and on swabs from the table. *Enterococcus faecium* was detected only on the stuffing tank. *Leuconostoc carnosum* was found in raw meat at time 0 and from the wall and *Aereococcus viridans* was only found on the wall. *Carnobacterium divergens* (two isolates) were found only in raw meat at time 0 and after 45 days of ripening.

For the most abundant CNS and LAB species, namely *S. succinus* (163 isolates), *S. xylosus* (188 isolates) and *Lb. sakei* (125 isolates) rep-PCR was carried out and the cluster analysis is presented in Figure 1 (panels A, B and C, respectively). At 70% of similarity, 12 clusters resulted from *Lb. sakei* isolates (Fig. 1A), while 3 clusters were identified for the isolates of both *Staphylococcus* species (Fig. 1B and 1C). In the case of *S. succinus* (Fig. 1B), all the isolates from the environmental swabs clustered together and in the same cluster as the majority of the isolates from the salami fermentation. A similar case was observed for *S. xylosus*, where 2 different strains from the environment clustered with those isolated during fermentation. On the other hand, no *Lb. sakei* isolates where found in the environment swabs, but several different strains of *Lb. sakei* were found in the salami samples.

3.3. RT-PCR-DGGE analysis

Representative RT-PCR-DGGE fingerprints obtained from RNA directly extracted from salami during the ripening are presented in Figure 2, while the results of the band sequencing are shown in Table 5. DGGE profiles of the two production resulted to be very similar with no differences in the fingerprints (data not shown). Fragments arising from different profiles and migrating with the same distance in DGGE gels were repeatedly sequenced giving the same results in terms of closest relative species and percentage of identity. Main bands were sequenced and *Lb. curvatus* (band A) and *S. succinus* (band D)

were present in all the fingerprints. *Lb. sakei* (band F) was only identified after 3 and 30 days of maturing. Occurrence of other taxa belonging to *Photobacterium* sp., *Pseudomonas fragi*, *Lb. algidus*, *Lc. gelidum* and *Lc. carnosum* were observed (Fig. 2, Tab. 5). A large fingerprint cluster (>70%) grouping salami isolates from days 7 to 45 was seen (Fig. 3).

3.4. Pyrosequencing results

A total of 161,275 raw reads were obtained after the 454 processing. 157,476 reads passed the filters applied through QIIME, with an average value of 4,699 reads/sample and an average length of 469 bp. The rarefaction analysis and the estimated sample coverage (Tab. 6) indicated that there was satisfactory coverage for all the samples (ESC > 99%). Also the richness of the samples varied across samples from a minimum of 65 to 131 OTUs. In Figure 4 an overview of the relevant OTUs is shown. Rare taxa were grouped as "other". *Lb. sakei* was the major OTU in A and B samples, its abundance during the fermentation time was never lower than 50%. *Lb. curvatus* occurred in most of the samples although it was never abundant. Many samples contained a low abundance of *Lactobacillus* sp., which was not possible to identify at species level. *Lc. carnosum* was always present, its abundance being never lower that 20% (during time 3, 7 and 30 days) and about 10% at time 0 and 45. Many samples contained *Leuconostoc* sp. and *Lc. mesenteroides*, but at very low level. *S. succinus* was present (15%) in sample A at 45 days, while in all other samples it was found at very low percentage (1-3%). *P. fragi*, *Photobacterium* sp. and *Brochothrix thermosphacta* were found in meat batter at day zero, and sporadically at low level in the other samples.

Through principal-coordinate analysis with a weighted UniFrac distance matrices it was possible to show that meat samples at time 0 separated well all salami samples on the basis of the microbiota (Fig. 5). Adonis and Anosim statistical tests run through compare_categories.py script of QIIME, showed no significant difference between batches A

and B ($P>0.05$). However, according to statistical tests the samples were significantly different during the ripening time ($P < 0.001$).

Results from make_distance_comparison_plots.py script of QIIME showed that samples at 3 and 7 days versus meat batter were significant as the Bonferroni corrected p-value was 0.001, and the same observation was made for samples at 7 and 45 days (data not shown). ANOVA and g_test run through group_significance.py script of QIIME showed that *Lb. sakei*, *S. succinus*, *Lc. carnosum*, *P. fragi* and *Photobacterium* sp. abundance was significantly different during time.

4. Discussion

The aim of this study was to investigate the microbiota involved in the production of a Piedmontese traditional salami by RNA based approaches, complemented with culture-dependent analysis for LAB and CNS.

Concerning the pH, it was found to be relatively high, with an increase at the end of ripening. This is a typical characteristic of Mediterranean salami that have a pH of 5.3–6.2, compared to Northern products that have a pH below 5 (Talon et al., 2007). Normally, Italian salami are not smoked, produced at low temperatures, with a slower curing process and nitrate used instead of nitrite. Mould growth is often observed on their surface and starter cultures are not added in most traditional product (Blaiotta et al., 2004).

The results of the viable counts revealed a dominance of the LAB population from the beginning till the end of the ripening time in agreement with previous studies (Aquilanti et al., 2007; Cocolin et al., 2009). The LAB population reached the highest count from the third day till the end (above 8 Log CFU/g). Concerning CNS, they were of about 3 Log CFU/g at the beginning of the process and they increased to 7 Log CFU/g after 45 days. The increased

loads in both LAB and CNS population from 3 to 30 days of ripening confirmed that these are the bacteria that are likely to play a major role in the maturing of the salami.

The results of the identification of the isolates by PCR-DGGE approach showed that the CNS populations were formed by *S. xylosus* (46 %) and *S. succinus* (42%). *S. xylosus* is a frequent species isolated from salami samples, however other species of staphylococci, such as *S. succinus, S. equorum* and *S. saprophyticus,* can play a role in sausages naturally fermented (Iacumin et al., 2006; Janssens et al., 2014; Mauriello et al., 2004) . Interestingly, strains involved in the fermentation process were isolated from the environment, highlighting a possible link between between processing plant and sausage microbiota as previously indicated (Coton et al., 2010; Leroy et al., 2010). Identification of LAB isolates showed the predominance of *Lb. sakei*, however this species was never detected from the environment.

The metabolically active species diversity developing during ripening of salami was assessed in this study by RT-PCR-DGGE and RNA based pyrosequencing analysis. DGGE has been for a long time the most frequently applied technique for microbial ecology studies, as it provides a complete picture of the microbiota in the food matrix with a low cost and in a short time. In our study we found that the distribution of the salami samples were similar by using the DGGE and the pyrosequencing technique. UPGMA cluster analysis of DGGE fingerprints confirms the beta diversity results. Meat at time 0 had a different microbiota composition in terms of bands and OTUs richness. *Photobacterium* sp. were found in both DGGE and HTS analysis; members of this genus are not often isolated from food environments, but they have been recently associated to the spoilage of atlantic cod stored in modified atmosphere packaging (Hovda et al., 2007) and in vacuum packed meat (Ercolini et al., 2010). On the other hand in our samples we found several meat spoilage bacteria, namely *C. divergens*, *B. thermosphacta* and *P. fragi*. These are recognized as meat spoilage factors producing off-odour and off-flavour (Casaburi et al., 2014). *B. thermosphacta* and *P. fragi* were present at very high percentage in raw meat samples at time 0, while they were outcompeted by LAB during ripening.

Microbial identification by DGGE showed the presence of bands identified as *Lb. gelidum*, *Lactococcus piscium* and *Lb. algidus* that were not found in the HTS analyses. On the contrary *Lb. sakei* was found to be the dominant OTU from the beginning and was not found in the DGGE profile at time 0 and 30 days. Unexpectedly, the band identified as *Lb. sakei* was absent in the direct DGGE analysis at time 0, 7 and 45 days despite their presence according to the pyrosequencing data. This could be due to PCR-bias in the food matrix where different species are present at high levels and interfering with the specific binding of the primers applied. Such results indicate the need for the simultaneous use of both techniques for studying populations in traditional fermented products.

In a recent study (Połka et al., 2015) on typical Italian dry fermented sausages reported the presence of 32 different *Staphylococcaceae* and 33 *Lactobacillaceae* identified by using DNA based HTS coupled with DGGE technique. Our evidence showed that only few species belonging to those genera may be metabolically active and can really contribute to determine the final characteristics of the products.

In conclusion, RNA-based analysis can significantly increase the ability to identify the impact of the microbial population on organoleptic characteristics of typical food products.

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Tables

Table 1. pH value and Log CFU/g of LAB, CNS, *Enterobacteriaceae*, moulds, yeasts and total aerobic bacteria of sausages samples at 0, 3, 7, 30 and 45 days of maturation. Different letters in the same row indicate significant differences for each media among times ($P< 0.05$). n.d. not detected.

Salami		Fermentation days				
		0	3	7	30	45
pH		6.02 ± 0.08	5.75 ± 0.05	5.85 ± 0.06	6.20 ± 0.06	6.75 ± 0.08
Log_{10} CFU/g						
	LAB (MRS)	4.29 ± 0.90 [a]	8.07 ± 0.22 [b]	8.04 ± 0.19 [b]	8.28 ± 0.39 [b]	7.84 ± 0.30 [b]
	Staphylococcaceae (MSA)	3.79 ± 0.34 [a]	4.42 ± 0.37 [ab]	5.01 ± 0.11 [b]	5.43 ± 1.60 [b]	7.01 ± 0.55 [c]
	Enterobacteriaceae (VRBGA)	2.19 ± 0.93 [a]	2.06 ± 0.58 [a]	1.59 ± 0.30 [a]	1.98 ± 0.92 [a]	1.74 ± 0.57 [a]
	MOULDS (AMT)	2.08 ± 0.40 [a]	2.03 ± 0.33 [a]	2.42 ± 1.01 [a]	2.02 ± 0.31 [a]	3.19 ± 1.22 [a]
	YEASTS (AMT)	3.16 ± 0.22 [ab]	2.98 0.03 [ab]	3.29 ± 0.42 [ab]	2.62 ± 0.54 [a]	4.18 ± 1.67 [b]
	TOTAL BACTERIA (GPA)	5.21 ± 0.41 [a]	6.93± 0.94 [b]	7.67 ± 0.09 [ab]	6.91 ± 0.19 [b]	7.90 ± 0.26 [b]
	Listeria monocytogenes (OXFORD)	n.d.	n.d.	n.d.	n.d.	n.d.
	Salmonella sp. (XLD)	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2. Viable counts in the environmental swabs: I (table), II (wall), III (filling tube of the stuffing machine), IV (feeding compartment of the stuffing machine).

Log_{10} CFU/cm^2					
LAB	CNS	Enterobacteria	Total bacteria	Moulds	Yeasts

I	2.99	3.42	2.11	5.60	0.70	1.20
II	3.84	2.34	< 1	3.90	1.18	1.28
III	5.64	3.50	< 1	5.78	< 1	2.30
IV	2.63	2.56	< 1	4.00	< 1	1.18

Table 3. Identification of the CNS isolates. The number of isolates for each species and at every sampling point is shown. I, II, III, VI are the environmental swabs. Underlined species were subjected to rep-PCR.

CNS isolates												
Species	Fermentation time (days)						Environmental swabs					
	0	3	7	30	45	Tot	I	II	III	VI	Tot	Tot
Staphylococcus succinus	16	18	11	11	7	63	10	8	7	12	37	100
Staphylococcus xylosus	9	9	14	18	20	70	5	5	8	0	18	88
Staphylococcus equorum	4	1	3	1	0	9	0	0	0	3	3	12
Staphylococcus saprophyticus	1	0	2	0	0	3	0	0	0	0	0	3
Staphylococcus cohnii	0	2	0	0	3	5	0	0	0	0	0	5
Tot	30	30	30	30	30	150	15	15	13	15	58	208

Table 4. Identification of the LAB isolates. The number of isolates for each species and at every sampling point is shown. I, II, III, VI are the environmental swabs. Underlined species were subjected to rep-PCR.

LAB isolates												
Species	Fermentation time (days)						Environmental swabs					
	0	3	7	30	45	Tot	I	II	III	VI	Tot	Tot
Lactobacillus sakei	15	28	29	29	24	125	0	0	0	0	0	125
Leuconostoc mesenteroides	6	2	0	0	2	10	0	0	0	0	0	10
Leuconostoc carnosum	3	0	0	0	2	5	0	1	0	0	1	6
Enterococcus gilvus	3	0	0	0	0	3	12	0	0	0	12	15
Lactobacillus curvatus	1	0	1	1	0	3	0	0	0	0	0	3
Lactobacillus pentosus	1	0	0	0	0	1	3	0	0	0	3	4
Aerococcus viridans	0	0	0	0	0	0	0	0	1	0	1	1
Enterococcus faecium	0	0	0	0	0	0	0	0	0	2	2	2

Carnobacterium divergens	1	0	0	0	2	3	0	0	0	0	0	3
Tot.	30	30	30	30	30	150	15	1	1	2	19	169

Table 5. Microbial species identification after sequencing of the variable V3 region of the 16S rRNA gene purified from RT-PCR-DGGE profiles obtained from RNA directly extracted from salami samples.

Banda	**Closest relative**	**Closest relative Accession No.**	**Identity (%)**
A	*Lactobacillus curvatus*	KJ921832	100
B	*Leuconostoc carnosum*	NR102781	99
C	*Pseudomonas fragi*	KJ589477	99
D	*Staphylococcus succinus*	KJ524461	100
E	*Leuconostoc gelidum*	KF577567	99
F	*Lactobacillus sakei*	KJ812206	100
G	*Lactococcus piscium*	JN226415	99
H	*Lactobacillus algidus*	NR028617	100
I	*Photobacterium sp*.	KJ718943	100

[a] bands from letters A to I are indicated in Figure 2.

Table 6. Number of sequences analyzed, observed diversity and estimated sample coverage (Good's coverage) for 16S rRNA amplicons analyzed.

Sample_ID	**Reads**	**OTUs**	**goods_coverage**	**chao1**	**Shannon index**

A_0	4785	95,67	99,49	113,17	3,29
B_0	3920	65,33	99,34	101,92	2,63
A_3	4076	82,33	99,37	111,90	3,15
B_3	4806	79,33	99,40	97,31	2,97
A_7	4610	82,33	99,53	102,81	3,31
B_7	4421	88,00	99,42	111,34	2,93
A_30	4911	84,67	99,44	104,92	3,37
B_30	5227	94,00	99,56	114,90	3,19
A_45	5498	131,00	99,74	144,35	3,90
B_45	4738	118,33	99,56	136,56	3,44

Figures

Figure 1. Cluster analysis of the rep-PCR fingerprints at 70% of similarity. A, *Lactobacillus sakei* isolates; B, *Staphylococcus succinus* isolates; C, *Staphylococcus xylosus* isolates. Isolates from the environmental swabs are included in squares.

1

2

Figure 2. Representative RT-PCR–DGGE fingerprints of the 16S V3 amplicons from microbial rRNA directly extracted from salami samples, numbers indicate the days of ripening, while letters placed at the left of each band indicate the sequenced fragments reported in Table 5.

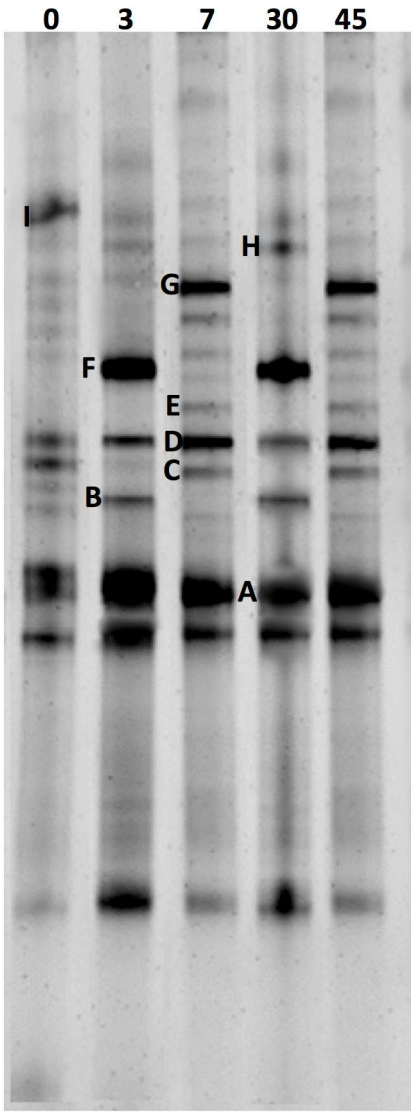

Figure 3. Dendrogram of similarity generated by the digitized RT-PCR-DGGE fingerprints. A combined data matrix of all the fingerprints (three replicates for each time point) was obtained and the dendrogram of

similarity was obtained by using the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin, 1992). Sampling time (days) is reported in the first column, while replicate number and batch are indicated in the second and third column, respectively.

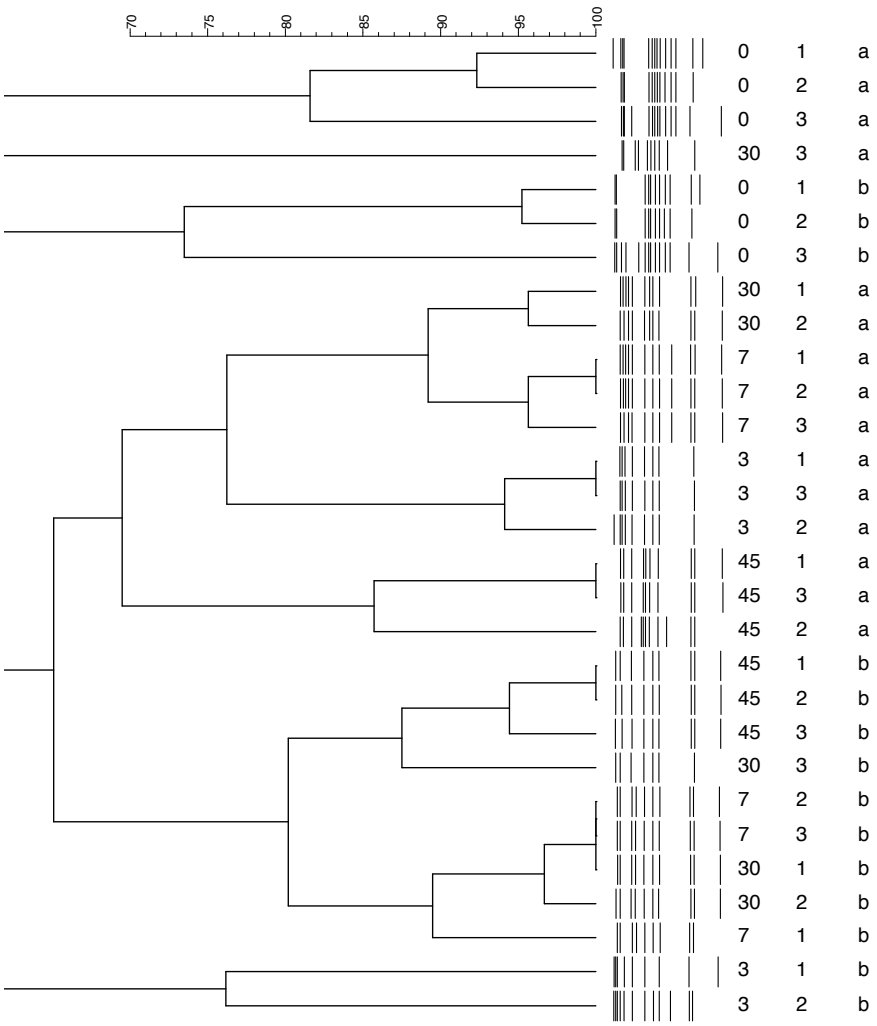

Figure 4. Incidence of the major taxonomic groups detected by pyrosequencing. Only OTUs with an incidence above 0.5% in at least two samples are shown. Samples are labeled according to the time (0, 7, 30, 45 days) and batch (A and B).

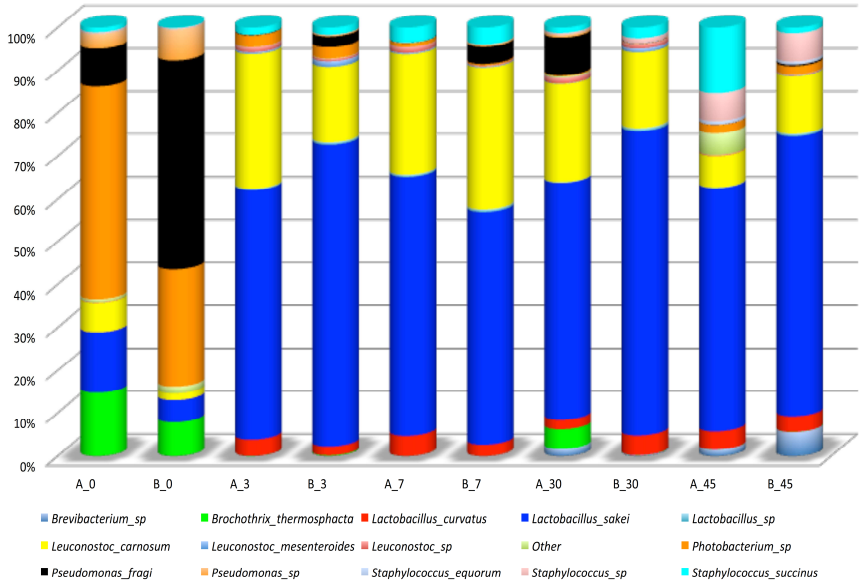

Figure 5. Principal Coordinates Analysis of Weighted UniFrac distances for 16S rRNA gene sequence data. Samples are color-coded by time.