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

Anna Greppi [a,1], Ilario Ferrocino [a,1], Antonietta La Storia [b], Kalliopi Rantsiou [a], Danilo Ercolini [b], Luca Cocolin [a,*]

[a] Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy
[b] Dipartimento di Agraria, Università degli Studi di Napoli Federico II, via Università 100, 80055 Portici, Italy

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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 analyzed at 0, 3, 7, 30 and 45 days of ripening. Swab samples from the production environment were also collected 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*, *Staphylococcus xylosus* and *Lactobacillus sakei* were the species most 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 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 a few species belonging to Staphylococcaceae, Lactobacillaceae and Leuconostocaceae may be metabolically active and contribute to determine the final characteristics of the products.

1. Introduction

In Italy, there is a huge variety of fermented sausages (called "salami"), which is 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 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.

* Corresponding author. Tel.: +39 011 6708553; fax: +39 011 6708549.
E-mail address: lucasimone.cocolin@unito.it (L. Cocolin).
[1] These authors contributed equally to the manuscript.

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2. Materials and methods

2.1. Salami manufacturing and sample collection

Salami samples were manufactured in a local meat factory in the area of Torino, using traditional techniques. Two batches were prepared at different times (A and B). The formulation used in the manufacture included pork meat, lard, salt, pepper, coriander, nutmeg, cinnamon, sugar (0.1% w/w sucrose) and nitrate salt (E252). Meat batter was stuffed into bovine casings resulting in sausages of about 36 mm in 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, and (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 h.

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 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 Anonymous (1996) method while isolation presumptive of *Salmonella* spp. was carried out according to Anonymous (2002) 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 preliminarily 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 338 F were used (Muyzer et al., 1993) 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 previously 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 DGGE as previously described (Cocolin et al., 2001). PCR products (for both CNS and LAB) that migrated to 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 (Klijn 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).

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.