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Bacterial diversity in typical Italian salami at different ripening stages as revealed by high-throughput sequencing of 16S rRNA amplicons

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ABSTRACT

The bacterial diversity involved in food fermentations is one of the most important factors shaping the final characteristics of traditional foods. Knowledge about this diversity can be greatly improved by the application of high-throughput sequencing technologies (HTS) coupled to the PCR amplification of the 16S rRNA subunit. Here we investigated the bacterial diversity in batches of Salame Piacentino PDO (Protected Designation of Origin), a dry fermented sausage that is typical of a regional area of Northern Italy. Salami samples from 6 different local factories were analysed at 0, 21, 49 and 63 days of ripening; raw meat at time 0 and casing samples at 21 days of ripening where also analysed, and the effect of starter addition was included in the experimental set-up. Culture-based microbiological analyses and PCR-DGGE were carried out in order to be compared with HTS results. A total of 722,196 high quality sequences were obtained after trimming, paired-reads assembly and quality screening of raw reads obtained by Illumina MiSeq sequencing of the two bacterial 16S hypervariable regions V3 and V4; manual curation of 16S database allowed a correct taxonomical classification at the species for 99.5% of these reads. Results confirmed the presence of main bacterial species involved in the fermentation of salami as assessed by PCR-DGGE, but with a greater extent of resolution and quantitative assessments that are not possible by the mere analyses of gel banding patterns. Thirty-two different *Staphylococcus* and 33 *Lactobacillus* species where identified in the salami from different producers, while the whole data set obtained accounted for 13 main families and 98 rare ones, 23 of which were present in at least 10% of the investigated samples, with casings being the major sources of the observed diversity. Multivariate analyses also showed that batches from 6 local producers tend to cluster altogether after 21 days of ripening, thus indicating that HTS has the potential for fine scale differentiation of local fermented foods.

1. Introduction

Salami are a typical example of dry sausages obtained by microbial fermentation of raw pork meat, and are among the most important and diverse local food products in Italy. This diversity is the resultant of several factors, the main ones being represented by the meat characteristics and its grinding size, the composition and activity of the microbial communities involved in the fermentation (including those eventually added as starters), the use of different spices (e.g., garlic, fennel seeds, wine) or additives, and the ripening conditions. Salame Piacentino, a typical product from the Northern Italy, is one of the 7 Italian salami that are granted with a PDO label established by the MIPAF (Italian Ministry for Agricultural and Forestry Policies) regulation (Italian regulation n.119, 24 May 2001) in accordance to the European Union regulation on the protection of geographical indications and designation of origins for agricultural products and foodstuffs (EC No 1263/96). According to the PDO guidelines, Salame Piacentino must be derived from meat of pigs reared in the Lombardia or Emilia-Romagna regions, and produced according to a number of strict guidelines establishing the weight of pigs, the % of fats in the meat, the grinding size and the use of additives, where nitrates, sugars and Na-L-ascorbate are allowed, while pepper, garlic and wine must be present within a certain range.

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In spite of this variability of conditions and richness of local products, a number of common processes characterize the microbial fermentation of raw meat responsible for the production of salami, and the main microbial groups involved in the fermentation, as well as eventual pathogens and spoilage causative agents, have been extensively studied, firstly by culture based methods (Albano et al., 1965; Campanini et al., 1993; Marchesini et al., 1992; Staib et al., 1980), and afterwards by taking advantages of molecular methods, mainly PCR-DGGE (see Cocolin et al., 2013 for a comprehensive review on the topic). At the beginning of the production process, the microbiota of the meat batter stuffed in the casings is dominated by the species present in the raw materials, with meat and casings considered a major source of *Enterobacteriaceae* and *Pseduomonas* dominating the microbial community, while lactic acid bacteria (LAB), Gram-positive catalase-positive cocci and yeasts are usually present at populations lower than 10^5 cfu g^{-1} (Paramithiotis et al., 2010). At these first stages pathogens such as *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* may also be present, but their presence is usually reduced until disappearance by the activity of the fermenting groups: the use of starter culture is indeed generally considered useful to control the presence of pathogens (Cenci-Goga et al., 2008) and to standardize the quality and safety of the final products (Cocconcelli, 2007; Cocconcelli and Fontana, 2008; Villani et al., 2007). As the fermentation proceeds, *Staphylococcaceae* and *Micrococcaceae* contribute to the proteolysis and lipolysis of meat components, while LAB are mainly responsible for the acidification, the production of lactic acid, the flavour and the production of small amounts of acetic acid, ethanol, acetoin, carbon dioxide and pyruvic acid (Francesca et al., 2013).

Among the factors that are responsible for the properties that characterize a local food product such as the Salame Piacentino PDO, the ecological composition of bacterial communities that are present and active in the different ripening stages is clearly one of the most important: a fine scale characterization of such communities is thus very important in order to better understand and describe the features of such products, and may also lead in the future to the identification of strains that are specific for a given local product. PCR-DGGE has been for a long time the most applied technique for microbial ecology studies; its advantages include the relatively low cost of instrumentation, the possibility of processing several samples altogether (tens on a single gel) and the relative ease of use. As outlined by Cocolin et al. (2013), its application in food microbial ecology has been very important not only to confirm and expand previous knowledge obtained through traditional microbiological methods, but also to achieve a more complete investigation of the microbial ecology involved in food fermentation. Microbial community screening by means of PCR-DGGE suffers however from a number of drawbacks, the main ones being represented by low resolution power (tens of dominant bands that can be analysed per sample), background noises and difficulties in extrapolating quantitative data by the analysis of band intensities.

Recent advancements in high-throughput sequencing technologies (HTS) are revolutionizing our assessment and comprehension of microbial communities: the amplification of 16S rRNA regions coupled to the HTS generation of multimillion reads have the full potential for obtaining a complete coverage of microbial communities, and for investigating at deeper level the complex interactions between the species present in a given community. Microbial ecologists have recently started to apply HTS for the investigation of the microbial ecology of fermented foods: example include cheese (Ercolini et al., 2012; Masoud et al., 2011), wine (Bokulich et al., 2012b), beer (Bokulich et al., 2012a; Jung et al., 2012), seafoods (Kiyohara et al., 2011; Koyanagi et al., 2011; Roh et al., 2010) and fermented soybean (Nam et al., 2012a; Nam et al., 2012b), while as far as we know no reports on fermented dry sausages have been published yet. Apart from two examples (Bokulich et al., 2012a; Bokulich et al., 2012b), all of these works have used 454 sequencing technology, which allows higher reads length but with a lower throughput as compared to llumina. Recent evidence have however shown that analyses of shorter reads already allows a good taxonomical analyses of reads (Vasileiadis et al., 2012), while the most recent releases of the technology have increased both throughput and read length, providing several thousands of high quality reads covering two hypervariable 16S rRNA regions when tens of samples are analysed altogether in a single sequencing run.

The main aim of the present work was to achieve a fine scale characterization of the bacterial diversity of Salame Piacentino PDO batches at different ripening stages. Samples were retrieved from 6 local producers, and analysed at different ripening stages until complete maturation at 63 days. Raw meat at time 0 and casing samples at 21 days of ripening where included in the analyses, and the effect of starter addition was also included in the experimental set-up. Culture-based microbiological analyses and PCR-DGGE were carried out in order to compare the results with HTS analyses conducted by Illumina MiSeq analyses of the two bacterial 16S hypervariable regions V3 and V4. Our main hypotheses was that the application of HTS to food microbial ecology allows gaining a complete picture of the bacterial species involved in the fermentation of this local product, and identifying species that cannot detected with classical microbiological and molecular methods.

2. Materials and methods

2.1. Salami manufacturing and sampling procedures

Manufacturing and ripening of salami was carried out from May 2012 in 6 different factories (labelled from A to F) located in the Province of Piacenza, Northern Italy. All factories followed the procedural guidelines for Salame Piacentino PDO indicated by MiPAF (Italian Ministry for Agricultural and Forestry Policies) in accordance to the European Union regulation on the protection of geographical indications and designation of origins for agricultural products and foodstuffs (EC No 1263/96). The meat utilized for salami production was derived from pigs weighting between 154 and 176 kg, reared in livestocks located in Lombardia or Emilia-Romagna region, and it contained a % of fats between 10 and 30%, and grinded with a minimum sieve of 10 mm; the additives used were (ranges or maximum doses per 100 kg of meat): NaCl (1.5–3.5 kg), KNO_3 (max 15 g), white or black pepper (30–50 g), garlic (5–20 g) steeped in wine (0.1–0.5 L), sugars (max 1.5 kg), Na-L-ascorbate (max 200 g). Meat was stuffed into natural pig intestine casings, and ripened at a temperature between 12 and 19 °C, humidity between 70 and 90%, for 63 days.

Two batches of salami were produced in each factory: in factories A, B, D and F one batch was with a starter culture addition and the second without; in factory E two different starter cultures were compared; finally in factory C no starter culture was added but one batch was derived from meat slaughtered in the factory and not in external slaughterhouses as in all other factories.

Sampling was carried out at time zero on meat mixture prior to stuffing, and on salami samples after 0, 21, 49 and 63 days of ripening. For HGS analyses, casings samples at 21 days from each producer were also included. Water activity (a_w) was determined on day 49 and day 63 samples at 25 °C with an AquaLab 4 TE a_w metre (Decagon Devices, Inc., Pullman, WA).