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Microbial dynamics of model Fabriano-like fermented sausages as affected by starter cultures, nitrates and nitrites

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ABSTRACT

The present study promotes the valorization of Fabriano-like fermented sausages, which are central-Italy salami with an origin that dates to the early 17th century, for the possible future selection of autochthonous starter cultures to be used with respect to local traditions. To the best of the authors' knowledge, this study represents the first attempt to define the microbial dynamics in Fabriano-like fermented sausage and the effect of nitrates/nitrites and starter cultures on its natural bacterial biota. Culture and RNA-based techniques (RT-PCR-DGGE and Illumina sequencing) were used to assess the microbial ecology of model Fabriano-like fermented sausages together with the impact of starter cultures and different nitrate and nitrite concentrations. The meat batter was used to produce two batches of fermented sausages that were prepared as follows: i) without commercial starters or ii) with the use of starter cultures composed of *Pediococcus pentosaceus* and *Staphylococcus xylosus*. Each batch was further divided into three different batches with the addition of 0/0 mg kg^{-1} nitrate/nitrite, 75/60 mg kg^{-1} nitrate/nitrite and 150/125 mg kg^{-1} nitrate/nitrite to the first, second and third batch, respectively. The samples, which were produced in triplicate, were analyzed on the day of production and after 7, 21, and 42 days of ripening. *Enterobacteriaceae* counts were always higher in model Fabriano-like sausages produced without the use of starter cultures at all of the sampling times irrespective of the tested nitrate/nitrite concentrations. Lactobacilli counts were positively influenced by the starters, although this influence was not evident over time; moreover, the effect of nitrates and nitrites on the counts of lactobacilli differed over time. As a general trend, coagulase-negative cocci counts were apparently not influenced by the tested nitrate/nitrite concentrations. Regarding the effect of nitrates/nitrites on the microbial diversity revealed by RT-PCR-DGGE, the higher the concentration, the lower the presence of some genera/species such as *Pseudomonas* spp., *Serratia liquefaciens* and *Staphylococcus* spp. However, Illumina sequencing detected *Pseudomonas* spp. as a minority species after 7, 21 and 42 days of ripening irrespective of the nitrate/nitrite concentration. The presence of *Staphylococcus* species was highlighted by both RT-PCR-DGGE and Illumina sequencing at all of the stages of ripening, although its presence was massively detected in fermented sausages produced without the use of nitrates/nitrites at the end of ripening. Overall, the data collected clearly highlighted the dominance of *Lactobacillus sakei* in all of the fermented sausages during ripening (from day 7 to day 42) and irrespective of the nitrate/nitrite concentration and added starter cultures. Moreover, *Pediococcus* spp. was principally detected in model Fabriano-like fermented sausage with added starter cultures irrespective of the nitrate/nitrite concentration.

1. Introduction

Dry fermented sausages are usually produced in accordance with antique traditions that successfully achieve meat preservation. To obtain the end product, pork, beef, or veal meats are fermented, salted, dried and in certain cases, smoked. The meat is first minced and then inlaid into animal bowels; the addition of spices, herbs, and starter cultures, as well as preservatives, can also be utilized (Aquilanti et al., 2012).

Starter cultures are normally used by Italian salami producers to

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obtain standardized features of the end product. However, it is known that the wild microbiota that occurs during ripening mostly maintains a crucial role in the final characterization of the product (Bassi et al., 2015; Ferrocino et al., 2018; Montanari et al., 2016; Połka et al., 2015).

Regarding preservatives, nitrates (sodium nitrate, E251, and potassium nitrate, E252) and nitrites (sodium nitrite, E250, and potassium nitrite, E249) are usually added to the meat batter due to their antimicrobial effect, especially against pathogenic bacteria such as *Clostridium botulinum* and *Staphylococcus aureus* (Paik and Lee, 2014). The use of nitrates and nitrites in fermented sausages is allowed notwithstanding that their presence in foods may exert an adverse effect on human health. Recent epidemiological studies found evidence of a link between dietary nitrite and gastric cancers as well as an association of nitrite and nitrate from processed meat with colorectal cancers (EFSA ANS Panel, 2017). For both nitrates and nitrites, 150 mg kg^{-1} represents the maximum amount that may be added during the manufacturing of cured meat products (Commission Regulation (EU) No 1129/2011).

To produce salami, meat fermentation is the key phase that is mainly driven by lactic acid bacteria (LAB) that lowers the pH through the production of organic acids and the production of many volatile compounds. The production process is also affected by the metabolic activity of coagulase-negative cocci (CNC) that are responsible for proteolysis, lipolysis, and the decomposition of free amino acids and peroxides (Aquilanti et al., 2016). Moreover, through the metabolic activity of this microbial group, added nitrate can be reduced to nitrite and then to nitric oxide, and this contributes to the red color of the meat when reacting with myoglobin (Holck et al., 2017). Among LAB, *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* have been most frequently detected in dry sausages manufactured in Mediterranean countries (Aquilanti et al., 2016), whereas *Staphylococcus xylosus* and *Staphylococcus simulans* were generally found to dominate over the CNC group (Aquilanti et al., 2016).

Many fermented meat specialties are manufactured all over Italy according to ancient local traditions; many of these have also received the Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) or traditional product (TP) designation. These products, which are often considered by consumers as a heritage of undisputed value, must, therefore, guarantee their safety to consumers.

Salame of Fabriano is a meat specialty that is included in the official list of traditional products published by the Italian Ministry of Agriculture and Forestry (G.U. Repubblica Italiana no. 168, 22/07/2015 Suppl. Ord. no. 43). Salame of Fabriano owes its name to the town of Fabriano located in the Marche region (central Italy). The origin of this fermented sausage presumably dates to the early 17th century and the production is traditionally carried out from December–January to March–April in accordance with the ancient tradition deeply rooted in the territory of origin. The disciplinary production of Salame of Fabriano establishes the area of production that is geographically identified by the following municipalities of the Marche region, namely, Fabriano, Arcevia Cerreto D'esi, Genga, Serra San Quirico, Sassoferrato, Matelica, Esanatoglia, Serra S'Abbondio, Frontone, Pergola, Pioraco, and Fiuminata. In the traditional production areas, the meats are obtained from 12-month-old swine that were born in mountainous areas of the Umbria and Marche regions (Luciani, 2006).

The original product consists of pork meat and 8–12% fat (cut into 0.5–1 cm cubes). The chilled meat is minced using a 2–6 mm plate and then mixed with lard, salt, saltpeter, whole peppercorns, ground pepper and white wine. The mixture is then stuffed into bovine or pork-intestine casings and ripened for 2–4 months. The end product, which is 30–35 cm long and weighs approximately 400–500 g, is characterized by a slice with low elasticity due to the neat separation between meat (a ruby red color) and the fat cubes.

To the best of the authors' knowledge, no published studies investigating the bacterial biota of Salame of Fabriano are available in the scientific literature.

Based on these premises, this study uses a polyphasic approach based on culture and RNA-based techniques (RT-PCR-DGGE and Illumina sequencing) to assess the microbial ecology of model Fabriano-like fermented sausages together with the impact of starter cultures and different nitrate and nitrite concentrations. To this aim, salami manufactures produced with or without the use of starter cultures and with different concentrations of nitrates and nitrites were analyzed in parallel, and the results were comparatively evaluated.

The present study intends to promote the valorization of such an ancient fermented sausage by providing useful information for the possible future selection of autochthonous starter cultures to be used with respect to local traditions.

2. Materials and methods

2.1. Model Fabriano-like fermented sausage production

Independent manufacturing trials were performed according to the traditional procedure to produce model Fabriano-like fermented sausages. The sausages were made from pork shoulder (80%), ham (10%) minced with a 2 mm plate and lard (10%) cut into 0.5–1 cm cubes. The other ingredients were NaCl (28 g kg^{-1}), black pepper powder and grains (3 g kg^{-1}).

The total meat batter was divided into two batches; the first batch (NS) was prepared without commercial starter cultures, whereas the second batch (S) was added with the commercial starter cultures composed of *Pediococcus pentosaceus* and *Staphylococcus xylosus* (Startec TCSD1/300, Tec-Al srl, Traversetolo, Italy) in a ratio of 75 g 100 kg^{-1}. Specifically, the two bacterial strains used as a starter were provided by the producer as a freeze-dried mixed culture that was added following the starter manufacturer's suggestions to reach a final concentration of approximately 6 log cfu g^{-1}.

For each batch (namely, NS and S), the meat batter was further divided into three different sub-batches with the addition of 0/0 mg kg^{-1} nitrate/nitrite (0/0), 75/60 mg kg^{-1} nitrate/nitrite (75/60) and 150/125 mg kg^{-1} nitrate/nitrite (150/125) to the first, second and third sub-batch, respectively.

Specifically, 12 sausages were produced for each recipe, and the desired concentration of nitrate/nitrite was obtained using Saltec Whitec-16 + Niko Sa and Nitritec 50/50 Sa (Tec-Al srl, Traversetolo, Italy). The sampling was performed at 0, 7, 21 and 42 days (T0, T7, T21, and T42); for each ripening time, the samples were collected in triplicate. In more detail, 3 sausages were produced and sampled to test $NS_{0/0}$ at T0, $NS_{0/0}$ at T7, $NS_{0/0}$ at T21, and $NS_{0/0}$ at T42. The same sampling scheme was also followed for $NS_{75/60}$, $NS_{150/125}$, $S_{0/0}$, $S_{75/60}$, and $S_{150/125}$.

Meat batter was stuffed into commercial collagen casing and subjected to fermentation at 25 °C for 8 h. Subsequently, the drying was performed under controlled conditions as follows: 24 h at 21 °C with 55% relative humidity (R.H.); 24 h at 19 °C with 60% R.H.; 24 h at 18 °C with 65% R.H.; 24 h at 16 °C with 70% R.H.; 24 h at 15 °C with 74% R.H.; and 24 h at 14 °C with 74% R.H. Finally, the ripening was conducted at 13 °C for 37 days at 74% R.H. The sampling was performed at 0, 7, 21 and 42 days (T0, T7, T21, and T42, respectively); for each ripening time, the samples were collected in triplicate.

2.2. Microbial counts

To obtain a representative sample, 5-gram slices were collected from the center and end part of each salami. The two slices were then pooled and analyzed. Hence, the 10-g samples were aseptically homogenized in 90 mL of sterile peptone water in a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy) for 2 min at 260 rpm. Homogenate decimal dilutions were then subjected to microbial counts. The presence of Enterobacteriaceae, LAB and coagulase-positive cocci (CC+) was assessed in accordance with ISO 21528-1:2004, ISO