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Bacterial community of industrial raw sausage packaged in modified atmosphere throughout the shelf life

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ABSTRACT

Ten lots of industrial raw sausages in modified atmosphere (CO_2 30%, O_2 70%), produced in the same plant over 7 months, were analyzed at the day after production (S samples) and at the end of shelf life (E samples), after 12 days storage at 7 °C to simulate thermal abuse. Quality of the products was generally compromised by storage at 7 °C, with only 3 E samples without alterations. During the shelf life, the pH decreased for the accumulation of acetic and lactic acids. A few biogenic amines accumulated, remaining below acceptable limits. The profile of volatile compounds got enriched with alcohols, ketones, and acids (e.g. ethanol, 2,3-butanediol, 2,3-butandione, butanoic acid) originated by bacterial metabolism. Throughout the shelf life, aerobic bacteria increased from 4.7 log to 6.6 log cfu/g, and lactic acid bacteria (LAB) from 3.7 to 8.1 log cfu/g. Staphylococci, enterobacteria, and pseudomonads passed from 3.7, 3.0, and 1.7 to 5.5, 4.8, and 3.0 log cfu/g, respectively. Dominant cultivable LAB, genotyped by RAPD-PCR, belonged to the species *Lactobacillus curvatus/graminis* and *Lactobacillus sakei*, with lower amounts of *Leuconostoc carnosum* and *Leuconostoc mesenteroides*. *Brochothrix thermosphacta* was the prevailing species among aerobic bacteria. The same biotypes ascribed to several different species where often found in E samples of diverse batches, suggesting a recurrent contamination from the plant of production. Profiling of 16S rRNA gene evidenced that microbiota of S samples clustered in two main groups where either Firmicutes or Bacteroidetes prevailed, albeit with taxa generally associated to the gastro-intestinal tract of mammals. The microbial diversity was lower in E samples than in S ones. Even though a common profile could not be identified, most E samples clustered together and were dominated by Firmicutes, with Lactobacillaceae and Listeriaceae as the most abundant families (mostly ascribed to *Lactobacillus* and *Brochothrix*, respectively). In a sole E sample Proteobacteria (especially *Serratia*) was the major phylum.

1. Introduction

Italian raw sausages are the result of the mixing of lean pork meat and fat in a ratio and at a mincing level which depends on several traditional recipes, varying in relation to the production geographical area. In addition to meat, other ingredients are used, such as spices (black pepper, fennel, chili pepper, garlic, etc.), water, and salt in variable amounts. No thermal treatment is applied during the production. At industrial level, also sugars (generally glucose or sucrose) and few acidifying/antioxidant preservatives are allowed (European Commission, 2014). Raw batters are stuffed in natural or synthetic casings with variable diameters (2–5 cm), marketed under refrigeration conditions (0–4 °C), and consumed after cooking. Raw sausages are packaged under modified atmosphere and require cooking before eating (Torrieri et al., 2011; Tremonte et al., 2005).

The shelf-life is rather short (10–15 days), mainly due to the high water activity, usually around 0.97, and to a pH of approx. 6 immediately after production (Cocolin et al., 2004). It depends on the development of negative sensorial characteristics concerning mainly odor and color, as well as on the production of exudates (Benson et al., 2014; Fougy et al., 2016). The progress of these defects mainly relies on microbial metabolic activity in combination with chemical and biological reactions associated to the raw materials, such as lipid oxidation and other transformations carried out by meat endogenous enzymes

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(Casaburi et al., 2015; Hugo and Hugo, 2015; Kamdem et al., 2007). Extending the shelf life of fresh sausage is a main challenge, since the peculiar flavor and taste and the agreeable appearance can be easily lost during storage.

The shelf life is strictly linked to the quali- and quantitative composition of the initial microbiota, the evolution of which is related to the storage conditions. At packaging, microbial population can derive from cross contamination during slaughtering and successive operations, from the industrial environments where sausages are produced, and from ingredients (mainly spices) and casings (Benson et al., 2014; Chaillou et al., 2015). Temperature is the main driver of the growth of microorganisms during storage, with refrigeration favoring psychrotrophic species. However, also packaging conditions, and in particular vacuum, ordinary or modified atmosphere packaging (MAP) are important factors affecting microbial growth (Chaillou et al., 2015). NaCl concentration, the diminution of which is an important challenge for processed meat industry, also influences the composition of microbiota and can affect spoilage (Fougy et al., 2016).

The bacteria responsible for spoilage of fresh sausage are mainly Firmicutes (in particular carnobacteria, lactobacilli, leuconostocs, and *Brochothrix thermosphacta*, but Proteobacteria (e.g. *Serratia*, *Yersinia*, *Pseudomonas*, *Acinetobacter*)), Staphylococci, micrococci, and Bacteroidetes are also recurrent and may be abundant, with detrimental effects on sensorial properties (Benson et al., 2014; Cocolin et al., 2004; Fougy et al., 2016;). Pathogenic species, such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Yersinia enterocolitica*, and *Salmonella*, may be present resulting in hygienic risks for the consumers in case of improper cooking tempertures (De Cesare et al., 2007; Giovannini et al., 2004; Joutsen et al., 2012).

Exploring the diversity of microorganisms in sausage is a relevant focus of research, since a better understanding of the role of bacterial populations in food quality and safety greatly impacts on both health, sustainability, and economy. Despite an approach based on 16S rRNA gene analysis has recently been applied to investigate the effect of salt concentration on microbiota of fresh sausage (Fougy et al., 2016), a detailed overview of the microbiota of industrial pork raw sausage during the shelf life is still missing.

The present study aimed to describe the microbial composition of industrial raw sausage at the beginning and at the end of the shelf life, combining culture-dependent methods and 16S rRNA gene profiling, to specifically trace the bacterial biotypes and to describe the whole bacterial community, respectively. Ten lots of raw sausages, produced in the same plant over a period of 7 months, were analyzed at the packaging time and at the end of shelf life, after 12 days storage in MAP at 7 °C. For all the lots, the occurrence of defects was evaluated and the pH, organic acids, biogenic amines, and prevalent volatile compounds were analyzed.

2. Materials and methods

2.1. Sausage productions

Ten different lots of raw sausages were produced in an industrial plant (*CLA*I S.c.a., Imola, Italy) between September 2016 and March 2017, numbered from 01 to 10 in chronological order. Fresh pork meat from hot deboning of the shoulders was used. The ratio lean meat/fat was approx. 90:10. The meat was cut (about 4–5 mm) at 0 °C and mixed with NaCl 2.2%, sugars (sucrose and glucose) 1.2%, grounded black pepper 0.1%, nutmeg 0.1%, ascorbate 0.025%. The mixture was stuffed in pork casings (diameter about 3.6–3.8 cm). Sausages were packed as 500 g portions in polypropylene trays under modified atmosphere (30% CO_2, 70% O_2). The shelf life assigned to this product when stored at 0–4 °C was 12 days. Analyses were carried out the day after production (S samples) and after 12 days of storage at 7 °C (E samples), analyzing three diverse packages for each time point.

2.2. Spoilage assessment

Sausage packages were let to reach room temperature and were evaluated by 5 judges to detect the presence of main defects, such as discoloration, presence of off-flavors and off-odors (e.g. acidic, putrefactive, and rancid), production of exudates, and wrinkling of the casing. Each judge expressed the evaluation on a scale from 0 to 5 (0 = highly acceptable, 5 = highly defected). For each indicator, mean scores higher than 4 were considered as not acceptable.

2.3. Chemical analyses

The pH was measured in 5 diverse points per sample using a puncture electrode (Sension+ electrode 5233, HACH, Manchester, UK). Organic acids were determined with an HPLC method according to Montanari et al. (2014). The content of biogenic amines was determined following the HPLC method reported by Gardini et al. (2013). The limit of detection for all the amines was 3 mg/kg of sausage.

Volatile organic compounds (VOCs) were monitored by solid phase micro extraction (SPME) coupled with gas chromatography – mass spectrometry analysis (GC–MS). A Carboxen-Polydimethylsiloxane fiber (Sigma Aldrich, St. Louis, MO, USA) was exposed for 1 h at 25 °C to the headspace of a 10-mL vial containing 2 g of the sample. The analyses were performed in duplicate. The volatiles were released through thermic desorption at 240 °C in the injector of a GC–MS apparatus (7820–5975; Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-624 column (Agilent Technologies). Separation was achieved with 1.3 mL/min helium flux following a thermal gradient: 2 min isotherm at 50 °C, 6 °C/min increase to 110 °C, 10 °C/min increase to 240 °C, 4 min isotherm at 240 °C. 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).

2.4. Microbiological analyses

Approx. 20 g of sausage were 10-fold diluted with saline 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 agar plates to enumerate and isolate bacteria able to grow on different culture media provided by BD Difco (Franklin Lake, NJ, USA). Total (aerobic and facultative anaerobic) bacteria were enumerated on Plate Count Agar (PCA), lactic acid bacteria (LAB) on Lactobacilli MRS agar (MRS), staphylococci on Baird-Parker Agar (BPA), coliforms on MacConkey Agar (MAC), and pseudomonads on Cetrimide Agar Base (CET). MRS plates were incubated in anaerobic jar provided with GasPaK (BD Difco) at 30 °C for 72 h. The other plates were incubated in aerobiosis at 30 °C for 48 h.

After isolation, a representative number of colonies was randomly picked up from PCA, MRS, and BPA and purified. Instagene Matrix (Bio-Rad Laboratories, Redmond, WA) was used for extraction of PCR amplifiable DNA. From each sample and media, up to 48 colonies were clustered by RAPD-PCR using M13-RAPD primer (5′-GAGGGTGGCGG TTCT-3′) at a similarity level of 75% using the Pearson correlation coefficient (Quartieri et al., 2016). A single strain for each biotype was taxonomically characterized through sequencing of the V1–V3 portion of 16S rRNA gene. The couple of primers 16S-500f (5′-TGG AGA GTT TGA TCC TGG CTC AG-3′)/16S-500r (5′-TAC CGC GGC TGC TGG CAC-3′) was utilized to amplify the target region (Kolbert et al., 2004). 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).

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