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High-throughput assessment of bacterial ecology in hog, cow and ovine casings used in sausages production

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ABSTRACT

Natural casings derived from different intestine portions have been used for centuries in the production of fresh and dry-fermented sausages. Here we analysed by means of culture-dependent methods and Illumina high-throughput sequencing of 16S rRNA amplicons the bacterial ecology of hog, cow and ovine casings at different stages of their preparation for sausages production. Several strains of *Staphylococcus*, *Lactobacillus*, *Bifidobacterium*, *Vagococcus* and *Clostridium* were counted, isolated and characterised at phylogenetic level. High-throughput sequencing analyses revealed a high bacterial diversity, which differed strongly between casings of different animal species. The technological processes involved in the preparation for casing had also a strong impact on the casings bacterial ecology, with a significant reduction of undesired microorganisms, and an increase in the proportion of lactobacilli and staphylococci.

Natural casings were demonstrated to be complex ecological environments, whose role as microbiological inoculants in the production of sausages should not be underestimated.

1. Introduction

Raw and dry-fermented sausages have been produced for centuries stuffed inside natural casings, which are a portion of animal intestines derived from slaughtering and stripped off mucous and muscular layers (Bakker et al., 1999). Albeit the use of manufactured collagen casings has become popular because of improved mechanical properties, standardization of final products and pathogen control (Harper et al., 2012), natural casings still represent a large share of the global casing market, and they are an essential component of many traditional meat products. Casings can be derived not only from the small and large intestines of sheep, goats and hogs, but also from cattle and horses: many sausages are indeed identified by the casings used in their production.

The industrial preparation of natural casings to be used for sausages production relies on a number of steps: after removal, intestinal tracts are washed, scraped and treated to remove soluble components. The different anatomical structures, namely the stomach, the small and large intestines, the appendix and the rectum are separated, and each portion is cleaned. The intestines are then detached from adhering mesenteric (connective and fatty) tissue, and the intestinal content is manually removed. The empty casings are then flushed with water and manually or mechanically de-slimed from their internal layer, leaving a connective tissue, mostly constituted by collagen, that provides some unique properties for processing in terms of tenderness and permeability (Wu and Chi, 2010). Casings are then inflated for grading according to size, and preserved by salting and curing and/or drying to prevent growth of bacteria, and stored in a cool place, preferably under storage temperatures not exceeding +15 °C. Just before being filled with the sausage mix, natural casings are soaked in water to remove the salt and promote the elasticity of the casing wall. Sometimes lactic acid or acetic acid (2–3% to the water) is also added to improve the hygienic conditions (Heinz and Hautzinger, 2007).

Small, large (middle) and rectum intestines are processed to casings for fresh and dry sausages production. Small intestines of sheep, goats and hogs (also called round) are small diameter natural casings, tender with thin wall, considered edible and mostly eaten with the fresh sausages, hot dogs or dried fermented sausage such as chorizos. The sheep casings combine tenderness with sufficient strength to withstand the filling, cooking and smoking operations. Finally, small intestines of cows are used for stuffing sausages such as lyoner, liver and blood sausages and dried fermented beef products, and they are used for all types of sausages in Muslim countries (Nakyinsige et al., 2012).

The middle portion of the hog's large intestine, called “middle” or “Crespone” (70–80 mm diameter), is used for Italian dry sausage production, but also for liver sausages. This casing is often used reversed, therefore the internal slimy cover is situated outside. These casings are relatively strong and tough and not eaten with the sausage, but are

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usually peeled off before consuming the sausages. The terminal part of the pig large intestines, the hog or swine bung has a diameter of 60–70 mm, is used for liver sausages, cervelas and, in Italy for a typical dry fermented sausage (*Salame Gentile*). This casing as well is relatively strong and tough and is characterised by having very thick walls and a lot of fat within the membrane layers that does not allow seeing the meat mix through the casing. These features result in a low permeability and allow a long drying time without over hardening giving a particular flavour to the sausage. The beef caecum intestine is used for precooked–cooked sausages and large raw fermented Italian products such as the *Coppa* or *Capocollo* sausages.

Because of their intestinal source, natural casings are, by their nature, matrixes rich in bacteria, with total loads that can vary between 10^4 and 10^7 cfu g^{-1} (Bakker et al., 1999). Undesired and/or pathogenic species are often present: for this reason salting or other sanitary treatments such as the use of nisine, ozone or gamma-irradiation have been adopted or proposed to reduce the microbial loads (Benli et al., 2008; Byun et al., 2001). On the other hand, casings are also sources of microorganisms that are involved in the fermentation, and that play important roles in shaping the final characteristics of fresh and dry-fermented sausages. A study on the production of French dry fermented sausages identified how the industry processing units and the natural casings are a significant source of coagulase negative staphylococci (CNS) involved in the ripening process (Leroy et al., 2010), while a recent study carried out with Illumina high-throughput sequencing of the total bacterial community of typical Italian dry-fermented sausages indeed identified how still at 21 days from the beginning of the fermentation, the highest diversity of bacterial species was found as expected in the casings as compared to the fermenting meat (Połka et al., 2015).

The present work was carried out with the aim of assessing the bacterial ecology of natural casings from different animal species (hog, ovine and cow) used in the production of sausages. Culture-based methods and high-throughput molecular methods were applied in order to gain a detailed description of the bacterial species present in the casings. In order to assess how the technological steps involved in the casings preparation can influence the dynamics of bacterial communities, samples from different production stages were analysed.

2. Materials and methods

2.1. Sampling procedures

Thirteen natural casings from three different animal species were kindly provided by Albro Company (Borghetto di Borbera, AL, Italy) and analysed in the study. Eight samples were derived from hogs: five from the hog bung, used in the production of the typical Italian dry sausage *Salame Gentile*, and three from the hog middle, which is used instead in the production of *Salame Crespone*. Three samples were derived from the ovine small intestine, typically used for stuffing fresh sausages. Finally, two samples were derived from cow caecum, used for stuffing *Coppa*. Concerning the production stage, three samples were derived from the beginning step of the processing in the casing factory, one from the intermediate, seven from the final, while two were ready-to-use casings already processed desalted by washing with water/vinegar and finally rinsed with water. Samples at the beginning of the process are raw casings, removed of the intestinal content, and immersed in dry salt. At the intermediate stage, the casings have been flushed with water and subsequently de-slimed by using casing-cleaning machines (passed between a set of rollers or strippers to remove mucosa and other unnecessary layers of the intestinal wall, the slime, leaving only the sub-mucosa): these intermediate samples were thus de-salted. After this intermediate step, the slimed intestines are graded and salted with addition of dry salt (NaCl) until oversaturation (final processing).

A description of all the casing samples is reported in Table 1, together with the labels used all throughout this work, with the first letter indicating the animal (H = hog, C = cow, O = ovine), the second the intestine portion (b = bung, m = middle, c = caecum, s = small), the third the process stage (b = begin, i = intermediate, f = final, r = ready to use). When two batches from the sample type were analysed, they were identified by a final A or B.

Table 1
Labels and descriptions of the 13 natural casing samples analysed.

Sample	Source	Position	Process	Salt
HbbA	Hog	Bung	Begin	Yes
HbbB	Hog	Bung	Begin	Yes
HbfA	Hog	Bung	Final	Yes
HbfB	Hog	Bung	Final	Yes
HmfA	Hog	Middle	Final	Yes
HmfB	Hog	Middle	Final	Yes
Hmr	Hog	Middle	Ready	No
Hbr	Hog	Bung	Ready	No
CcfA	Cow	Caecum	Final	Yes
CcfB	Cow	Caecum	Final	Yes
Osb	Ovine	Small	Begin	Yes
Osi	Ovine	Small	Intermediate	No
Osf	Ovine	Small	Final	Yes

Casings were aseptically sampled, and brought to the lab within a couple of hours. Culture-based microbiological analyses were immediately carried out on fresh samples, while aliquots for high-throughput molecular analyses were stored at −20 °C for a few days before being subjected to DNA extraction. All analyses were carried out on triplicates.

2.2. Microbiological analyses

Each 10 g sample was mixed with 90 mL of saline/peptone–water (NaCl, 8 g L^{-1}; bacteriological peptone, Oxoid, Milan, Italy, 1 g L^{-1}) and homogenized using a Stomacher machine (400 Circulator; International PBI, Milan, Italy) at 260 rpm for 1.5 min. One millilitre aliquot of the homogenate was serially diluted in 9 mL of 0.1% sterile peptone water and used for microbiological analyses according to ISO methods.

One-hundred μL of the sample suspension was plated on duplicate agar plates: total viable counts on Plate Count Agar (Oxoid) incubated at 30 °C for 72 h; lactobacilli on MRS agar (Merck, Darmstadt, Germany) anaerobically (GasPak, BBL, Cockeysville, Md., U.S.A.) incubated at 30 °C for 48 h (ISO 15214); micrococci and staphylococci on Baird Parker Agar (BP, Oxoid) with added egg yolk tellurite emulsion (Oxoid) incubated at 37 °C for 48 h (ISO 6888-1); bifidobacteria on TOS proprionate agar medium supplemented with lithium mupirocin (TOS-Mup) (Merk, Darmstadt, Germany) anaerobically (Anaerocult, Merk, Darmstadt, Germany) incubated at 37 °C for 48 h (ISO 29981); total coliforms on Violet Red Bile Agar (VRBA, Oxoid) incubated at 37 °C for 24 h; *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA, Oxoid) incubated at 37 °C for 24 h; *Escherichia coli* on VRBA supplemented with 100 μg/mL of 4-methylumbelliferyl-β-D-glucuronide (MUG, Oxoid) incubated at 44 °C for 24 h; yeasts and moulds on Rosa Bengala (Oxoid) supplemented with chloramphenicol (100 mg/L, Sigma, Milan, Italy) incubated at 25 °C for 5 days. To detect *Listeria monocytogenes*, *Salmonella* spp., *Clostridium perfringens* and *Bacillus cereus*, the procedures validated by ISO 11290-1:2005, ISO 6579:2008, ISO 7937:2005 and ISO 7932:2005 were respectively applied. Approximately the 10% of the colonies grown respectively on MRS, Baird Parker (BP), or TOS-Mup agar on the two highest dilutions were randomly selected and purified by streaking onto the respective medium. Colonies belonging to the *Staphylococcus* genus were screened by catalase and oxidase testing (oxidase detection strips, oxoid). The isolates were stored at −20 °C in specific broth plus 30% (v/v) sterile glycerol until further analysis.

Microbiological log count data were analysed in R by ANOVA followed by Tukey's HSD test for comparison of means.