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Community dynamics of coagulase-negative staphylococci during spontaneous artisan-type meat fermentations differ between smoking and moulding treatments

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ABSTRACT

Coagulase-negative staphylococci (CNS) that are naturally present in the raw meat batter of fermented sausages or that originate from the addition of a starter culture play a role in flavour development. A wide species diversity of CNS can be present in fermented meats, but it is not fully clear yet how specific process parameters select for specific CNS by affecting their community dynamics. Therefore, the influence of smoking and moulding treatments on the CNS community dynamics in spontaneously fermented, artisan-type sausages was investigated. During the fermentation stage, the meat batter was in all cases dominated by *Staphylococcus saprophyticus*, in addition to *Lactobacillus sakei* as governing lactic acid bacterium. Following fermentation, the bacterial communities were not perturbed by the smoking treatment, since both *L. sakei* and *S. saprophyticus* remained dominant throughout the ripening stage and prevailed in the end-products. Yet, when fermentation was followed by a moulding step with *Penicillium nalgiovense*, a shift of the CNS communities towards dominance by *Staphyloccocus equorum* was seen, despite a similar evolution of *L. sakei*. This effect was possibly due to a pH rise caused by the mould, a hypothesis which was reinforced by the finding that the isolated strain *S. equorum* DBX-S-17 was more sensitive to low pH than the isolated strain *S. saprophyticus* DFL-S-12 during growth experiments in brain heart infusion (BHI). Differences in CNS communities may affect sausage flavour, due to intraspecies variations in metabolic conversions of, for instance, amino acids. The fact that 3-methylbutanal was only found in the moulded sausage, which was dominated by *S. equorum*, may be related to the finding that the isolated strain of this species was able to produce this compound in BHI medium, whereas the isolated strain of *S. saprophyticus* was not.

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1. Introduction

Traditional variations in ingredients and processing conditions have resulted in a large diversity of end-products during the production of fermented sausages. In all cases, different important microbial groups can be distinguished during fermentation and ripening, namely lactic acid bacteria (LAB), coagulase-negative staphylococci (CNS), yeasts, and/or moulds (Leroy et al., 2006; Talon et al., 2007; Ravyts et al., 2012). Especially LAB play a key role during sausage fermentation, as they cause acidification through the production of lactic acid, resulting in stable and safe end-products. For flavour formation and colour stabilization, CNS are of particular importance due to their conversion of amino acids and fatty acids into aroma compounds and their nitrate reductase and catalase activities, respectively (Leroy et al., 2006; Talon et al., 2007; Gøtterup et al., 2008; Ravyts et al., 2012). In some processes, the surface of the fermented sausage is covered by atoxic moulds, e.g. *Penicillium nalgiovense* (Sunesen and Stahnke, 2003; Sunesen et al., 2004). Moulds can contribute to the sensory properties of fermented sausages by altering both their appearance and flavour, which is achieved through lactate oxidation, proteolysis, conversion of amino acids, and lipolysis. However, this is mostly performed in less acidic Southern-European fermented sausages, while Northern-European products are almost invariably smoked (Flores, 1997; Demeyer et al., 2000; Leroy et al., 2006; Talon et al., 2007).

Nowadays, most European fermented sausages are produced with starter cultures to ensure safe and uniform end-products, generally consisting of a combination of LAB (mostly *Lactobacillus sakei* and/or *Lactobacillus curvatus*) and CNS (mostly *Staphylococcus xylosus* and/or *Staphylococcus carnosus*) (Rossi et al., 2001; Corbière Morot-Bizot et al., 2006; Leroy et al., 2006). The use of these commercially available starter cultures may however result in a loss of distinctive sensory properties found in artisan-type, spontaneously fermented sausages (Leroy et al., 2006). Artisan-type sausages are for this reason often perceived as superior by the consumer, possibly in part due to the composition and metabolic activity of the spontaneously established microbiota (Samelis et al., 1994, 1998; Papamanoli et al., 2002; Leroy et al., 2006).

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Spontaneous fermentations rely on the indigenous microbiota that are present on the raw materials and in the manufacturing environment, the so-called house microbiota (Blaiotta et al., 2004; Rantsiou et al., 2005; Leroy et al., 2006; Talon et al., 2007).

Whereas in the LAB communities (lactobacilli, pediococci, leuconostocs, and enterococci) of European fermented sausages *L. sakei* is by far the main contender, reaching 8 to 9 log colony forming units (cfu) per gramme, the outcome of the species diversity of the communities of Gram-positive, catalase-positive cocci (mostly CNS) after spontaneous fermentation is less predictable (Leroy et al., 2006; Ravyts et al., 2012). Although the initial level of CNS varies from 3 to 5 log cfu/g in spontaneously fermented sausages, their population will remain well below the one of the LAB. *S. xylosus* is frequently encountered in Southern-European fermented sausages, but *Staphylococcus saprophyticus* and/or *Staphylococcus equorum* may also be among the dominant species (Mauriello et al., 2004; Leroy et al., 2006; Marty et al., 2012; Ravyts et al., 2012). In addition, other species such as *S. carnosus*, *Staphylococcus epidermidis*, *Staphylococcus pasteuri*, *Staphylococcus sciuri*, *Staphylococcus succinus*, *Staphylococcus vitulinus*, and *Staphylococcus warneri*, may sometimes be present in meaningful numbers (Cocolin et al., 2001; Papamanoli et al., 2002; Blaiotta et al., 2004; Drosinos et al., 2005, 2007; Corbière Morot-Bizot et al., 2006; Martín et al., 2006; García Fontán et al., 2007; Ravyts et al., 2012). However, the presence and relative proportions of all above-mentioned CNS species may differ considerably, seemingly associated for a part with the type of sausage and the production region (Talon et al., 2007; Iacumin et al., 2012; Ravyts et al., 2012).

The aim of the present study was to evaluate to which degree two different processing conditions, i.e. smoking and moulding, affect the species diversity, community dynamics, and metabolite production kinetics of the microbiota of artisan-type, spontaneously fermented Belgian sausages, which were prepared from the same meat and ingredients, and manufactured at the same time in the same production environment.

2. Materials and methods

2.1. Sausage manufacturing and sampling procedures

Two types of spontaneously fermented sausages, originating from the same raw materials and ingredients and produced at the same time, were obtained from a small-scale butcher in the Belgian Ardennes. For both types, fermentation was identical, but subsequent processing methods differed. Whereas one sausage type was smoked after fermentation, the other was moulded. No starter culture was used in the production of these fermented sausages, except for the surface inoculation with *P. nalgiovense* in the moulded sausages (M-Ek-72, Chr. Hansen, Hørsholm, Denmark). To verify reproducibility, each type of sausage was produced at the same time in duplicate, with each duplicate corresponding with a number of sausages to allow sampling over time. The batter for the sausages consisted in all cases (wt/wt) of 60.9% lean pork, 20.3% beef, 13.5% pork back fat, 2.2% curing salt, 1.7% soy-based binding agent (Lianta Italiana; Evlier, Merksem, Belgium), 0.4% sugar, 0.7% spices, 0.1% white pepper, 0.1% black pepper, and 0.2% glutamate-containing taste enhancer (SMAK aromatic; Van Hees, Eupen, Belgium). Relative humidity was not controlled in this empiric process due to the artisan setup and only a limited temperature control was applied. The recorded average room temperature during the fermentation process was 20 °C for day 1, 26 °C for day 2, and 20 °C for days 3 and 4. The fermentation was followed by a ripening phase at 15 °C, which lasted for 24 days. After fermentation and prior to ripening, either a smoking step with an oak fire was carried out or an inoculation of the sausage surface, by dipping the sausages into the rehydrated mould culture, was performed. Throughout the production process of each sausage type, three samples of 150 g were taken from the central part of each duplicate sausage for each sausage type at specific time points, namely at the start of the fermentation and after 1, 2, 3, 4, and 28 days. For the smoked sausage trial, samples for the measurement of microbial counts were also available on day 14.

2.2. Measurement of technological parameters

The pH of the sausage samples was recorded using a DY-P10 pH metre (Sartorius AG, Göttingen, Germany), equipped with an insertion pH probe (VWR International, Darmstadt, Germany). The water activity (a_w) was measured at 25 °C with a Hydropalm 23 a_w metre (Rotronic; New York, NY, USA). For both pH and a_w, three independent measurements were performed per sample.

2.3. Enumeration and isolation of microorganisms

Twenty-five grammes of sausage sample were aseptically added to 225 ml of maximum recovery diluents [sterile solution of 0.85% (wt/vol) NaCl (VWR) and 0.1% (wt/vol) bacteriological pepton (Oxoid, Basingstoke, Hampshire, UK)]. This mixture was homogenized at low speed for 2 min in a stomacher (Stomacher 400; Seward, Worthington, UK). For culture-independent analysis, 50 ml of the homogenized samples was centrifuged and the supernatants were collected to obtain pellets (Janssens et al., 2012), which were stored at −20 °C until further use. For culture-dependent analysis, appropriate decimal dilutions in saline (0.85%, wt/vol, NaCl) were spread on different selective agar media. For the enumeration (cfu per gramme) of the aerobic total cell count and the counts of LAB, CNS, enterobacteria, enterococci, and yeasts, plate count agar (PCA, Oxoid), de Man-Rogosa-Sharpe agar (MRS agar, Oxoid), mannitol salt-phenol red-agar (MSA, VWR International), violet red-bile-glucose agar (VRBG agar, Oxoid), kanamycin-aesculin-azide agar (KAA, Oxoid), and malt extract agar (MEA, Oxoid) were used, respectively. All agar media were incubated at 30 °C for 48 h, except for KAA and VRBG agar that were incubated at 42 °C and 37 °C, respectively, reflecting optimal growth temperatures and incubation times for a maximal recovery of the targeted microorganisms. To analyse the LAB and CNS communities, at least 20% of the colonies from MRS agar and MSA, respectively, were randomly selected and picked up from appropriate dilutions, typically corresponding with about 6 to 10 colonies per plate. Colonies picked up from MRS agar were transferred to brain heart infusion (BHI) medium (Oxoid); colonies from MSA were grown in BHI medium supplemented with 7.5% (wt/vol) of NaCl. Next, 1.5 ml of overnight cultures incubated at 30 °C, was transferred into cryovials, containing their respective media supplemented with 25% (wt/vol) of glycerol, and stored at −80 °C until further use.

2.4. Identification of bacterial isolates through $(GTG)_5$-PCR fingerprinting

Identification of bacterial isolates was done through $(GTG)_5$-PCR fingerprinting, as described previously (Janssens et al., 2012). Briefly, cell pellets obtained from centrifuged BHI cultures of isolates from MRS agar and MSA, grown overnight at 30 °C, were subjected to DNA extraction with the Nucleospin 96 tissue kit (Macherey Nagel GmbH, Düren, Germany), according to the manufacturer's instructions. Subsequent $(GTG)_5$-PCR fingerprinting of the DNA and image analysis of the fingerprints were performed as described previously (Ravyts et al., 2008; Janssens et al., 2012). For numerical analysis of the $(GTG)_5$-PCR fingerprints, BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium) was used. For further identification and verification of the species assigned to a $(GTG)_5$-PCR cluster, sequencing of the 16S rRNA gene or the *rpoB* gene of DNA of representative isolates of each cluster was performed, as described previously (Ravyts et al., 2008).

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