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Species diversity and metabolic impact of the microbiota are low in spontaneously acidified Belgian sausages with an added starter culture of *Staphylococcus carnosus*

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ABSTRACT

Quality of fermented sausages is affected by acidifying lactic acid bacteria (LAB) and colour- and flavour-promoting coagulase-negative staphylococci (CNS), whether or not used as starter culture. Artisan fermented sausages are often perceived as superior to industrial variants, partially because of the specific microbiota due to spontaneous acidification, which may be considered as an artisan characteristic. Therefore, two kinds of spontaneously acidified Belgian sausages were prepared (Belgian-type salami and Boulogne sausage), but with addition of a *Staphylococcus carnosus* culture. The Belgian-type salami was made from pork and beef, whereas the Boulogne sausage contained pork and horse meat. In all cases, *Lactobacillus sakei* was the dominant LAB species present on the raw materials and during fermentation, whereas enterococci remained present in the background. Enterobacteriaceae vanished after fermentation. The CNS species diversity on the raw materials was large and differed between the pork, beef, and horse meat. Nevertheless, this species diversity was annihilated during fermentation by the added *S. carnosus* culture. The volatiles fraction was mainly composed of aldehydes that originated from lipid oxidation and spices-derived compounds. Aromatic compounds that are typically associated to CNS activity, such as end-products from the metabolism of branched-chain amino acids, were not present in the Belgian-type salami and only marginally present in the Boulogne sausage. In conclusion, spontaneous acidification of Belgian-type fermented sausages leads to dominance of *L. sakei* and is no guarantee for bacterial contribution to the aroma profile when *S. carnosus* is added as a starter culture.

1. Introduction

Fermented sausages are produced mainly from minced meat (often pork meat), fat, spices, and curing salt; these ingredients are stuffed into casings and subjected to microbial fermentation whether or not initiated by a starter culture (Campbell-Platt and Cook, 1995; Leroy et al., 2006; Talon et al., 2007). In general, this fermentation process is governed by lactic acid bacteria (LAB), coagulase-negative staphylococci (CNS), yeasts, and/or moulds, which all grow spontaneously. LAB are responsible for acidification of the meat and a concomitant decrease in pH and protein coagulation, which contributes to microbial safety and firmness, respectively, through the production of mainly lactic acid (Leroy et al., 2006). CNS are important for the formation and stability of colour and flavour (Talon et al., 2007; Gøtterup et al., 2008). Nowadays, sausage fermentation has become mostly an industrial process, controlled by the use of starter cultures (Leroy et al., 2006). However, fermented sausage production processes based on spontaneous fermentation remain in use, because of their artisan connotation. In the absence of a starter culture, the indigenous microbiota that is present on the raw materials and in the manufacturing environment (the so-called house microbiota) is responsible for the fermentation process (Blaiotta et al., 2004; Rantsiou et al., 2005a; Leroy et al., 2006; Talon et al., 2007).

Recently, molecular methods have revealed a great species diversity among the LAB and CNS communities present in naturally fermented sausages. Among the different LAB species, mostly *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus* are encountered in fermented sausages (Ammor et al., 2005; Comi et al., 2005; Rantsiou et al., 2005b; Benito et al., 2007; Drosinos et al., 2007). In several

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European-style, spontaneously fermented sausages, *L. sakei* represents 90–100% of the LAB communities (Aymerich et al., 2003, 2006; Ammor et al., 2005; García Fontán et al., 2007). In others, *L. curvatus* is the dominant species, as is the case for some Italian and Greek sausages (Comi et al., 2005; Rantsiou et al., 2005b; Drosinos et al., 2005). Most often, *L. curvatus* is subdominant when *L. sakei* is present (Papamanoli et al., 2003; Rantsiou et al., 2005a; Benito et al., 2007). Next to lactobacilli and pediococci, also *Leuconostoc* spp. and enterococci can be members of the LAB communities isolated from traditional sausages (Parente et al., 2001; Papamanoli et al., 2003; Ammor et al., 2005; García Fontán et al., 2007). In industrialized processes, mainly *L. sakei* (Europe) and *P. acidilactici* (USA) are used as starter cultures that are commercially available (Leroy et al., 2006).

Whereas *Staphylococcus xylosus*, *Staphylococcus saprophyticus*, and *Staphylococcus equorum* are the most abundant CNS species present in naturally fermented sausages, other species such as *Staphylococcus warneri*, *Staphylococcus pasteuri*, *Staphylococcus vitulinus*, *Staphylococcus epidermidis*, *Staphylococcus sciuri*, *Staphylococcus carnosus*, and *Staphylococcus succinus* have been found too (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). In industrialized processes, *S. xylosus* and *S. carnosus* are applied as starter cultures that are commercially available (Cocolin et al., 2006b; Corbière Morot-Bizot et al., 2007). The latter species is only marginally present in sausages of an artisan nature, which may raise questions concerning its appropriateness as starter culture (Ravyts et al., 2010). Moreover, its performance is likely to be influenced by the applied process technology and could lead to suboptimal end-products. This could in turn lead to the loss of desirable sensory properties, in particular with respect to artisan quality (Leroy et al., 2006; Talon et al., 2007).

The aim of the present study was to evaluate the impact of spontaneous acidification on the species diversity, community dynamics, and metabolite production kinetics of the microbiota of Belgian fermented sausages through a multiphasic approach. This should indicate whether the absence of a LAB starter culture, perhaps resulting in a less intense acidification, will permit the development of a more diverse microbiota, matching artisan processes. In this context, prime interests were the impact of the microbial load of the raw materials used regarding LAB and CNS species diversity upon spontaneous acidification and if spontaneous acidification affects the CNS species diversity, despite the addition of a *S. carnosus* starter culture for colour formation, or if spontaneous acidification and flavour formation is affected by the CNS starter culture.

2. Materials and methods

2.1. Sausage manufacturing and sampling procedures

Two spontaneously acidified sausage fermentations were carried out in a small Belgian meat company, namely for the production of Belgian-type salami and Boulogne sausages. A commercial *S. carnosus* starter culture (Texel M72, Danisco, France) was used in both cases in a concentration of 6.5 log colony forming units (cfu) per gram to promote colour development while nitrite was used as curing agent (dixit the production manager). No LAB starter culture was added, contributing to the traditional character of these fermentations. The batter for the first Belgian-type salami fermentation consisted of 40.0% pork meat, 24.0% pork back fat, 30.0% beef meat, 2.6% curing salt (consisting of NaCl and nitrite), 1.2% dextrose, 1.0% milk powder, 0.8% lactose, and 0.4% seasonings (all % in wt/wt). The fermentation was carried out at 24 °C and at a relative humidity (RH) of 90–95% for the first 50 h, followed by 24 h at 21 °C and 90% RH. During the last phase of the fermentation, the temperature was lowered to 20 °C for 12 h at 90% RH. The fermentation was followed by a ripening phase, which lasted for two weeks, at 14 °C and 80–85% RH. Boulogne sausages were prepared according to the same manufacturing procedure, except that beef meat was replaced by horse meat in the batter and that the ripening phase was prolonged with one week.

Throughout the fermentation, three sausage samples of 150 g were taken at specific time points, namely at the start of the fermentation, after 1, 2, and 5 days, and after 1 and 2 weeks for the fermentation of the Belgian-type salami. For the Boulogne sausage fermentation, no samples were taken at day 2 and additional samples were taken after 3 weeks of ripening. Also, in both cases, the raw materials were sampled, *i.e.* the pork meat, the pork fat, and the beef and horse meat. Finally, environmental samples were collected by swabbing the surface, corresponding with 25 cm^2, of the storage trays for the meat, the mixer, and the cutter.

2.2. pH and water activity measurements

The pH of the meat samples was recorded using a DY-P10 pH meter (Sartorius AG, Göttingen, Germany) equipped with an insertion pH probe (VWR International, Darmstadt, Germany). The water activity (a_w) was measured with a Hydropalm 23 a_w meter (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 g of sample taken from the raw meat or from the central part of each sausage was aseptically added to 225 ml of maximum recovery diluents [sterile solution of 0.85% (wt/vol) NaCl (VWR) and 0.1% bacteriological pepton (Oxoid, Basingstoke, Hampshire, UK)]. This mixture was homogenized in a stomacher (Stomacher 400; Seward, Worthington, UK) at low speed for 2 min. For culture-independent analysis, fifty ml of the homogenized samples were centrifuged at 95×*g* and 4 °C for 15 min to remove debris. The supernatants were collected and centrifuged at 8000×*g* and 4 °C for 15 min to obtain cell pellets for total DNA extraction, 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 gram] of the aerobic total cell, LAB, CNS, enterobacteria, enterococci, and yeast counts, plate count agar (PCA; Oxoid), de Man-Rogosa-Sharpe agar (MRS; Oxoid), mannitol salt-phenol red-agar (MSA; Merck, Darmstadt, Germany), violet red bile glucose agar (VRBG; 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 were randomly selected and picked up from the appropriate dilutions, respectively. Within an incubation period of 48 h, LAB and CNS were not able to grow on MSA and MRS agar, respectively. Colonies picked up from MRS agar were transferred to BHI medium; colonies from MSA were grown in brain heart infusion (BHI) medium supplemented with 7.5% (wt/vol) of NaCl. Next, 1.5 ml of the overnight cultures, purified through plating, was transferred into cryovials, containing the appropriate medium supplemented with 25% (wt/vol) of glycerol. They were stored at −80 °C until further use.