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Use of molecular methods to characterize the bacterial community and to monitor different native starter cultures throughout the ripening of Galician chorizo

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ABSTRACT

The development of *Lactobacillus* and *Staphylococcus* strains used as starter cultures throughout the ripening of Galician chorizo, a traditional dry fermented sausage from the north-west of Spain, was monitored combining different molecular-based techniques. The bacterial diversity occurring in the inoculated sausages at the beginning and the end of the ripening was also studied and compared to the indigenous population in an uninoculated control batch.

Real-time PCR was used to monitor the *Lactobacillus* and *Staphylococcus* community using genus and species-specific primer to quantify the occurring microbiota. The identification of isolates at genus or species level was achieved by specific PCR and 16S rRNA gene sequencing. rep-PCR using $(GTG)_5$-PCR primer was used to characterize this bacterial community at strain level.

According to the data obtained, the strains *Lactobacillus sakei* LS131, *Staphylococcus equorum* SA25 and *Staphylococcus saprophyticus* SB12 were dominant during the ripening process, whereas the strain *Staphylococcus epidermidis* SA49, that was added in order to study its behaviour with a merely scientific purpose, did not succeed in dominating ripening, since it seemed to be outcompeted by autochthonous microbiota.

In conclusion, the combination of a quantitative method such as real-time PCR with the identification and typing techniques used in this study (genus and species-specific PCR, 16S rRNA gene sequencing and $(GTG)_5$-PCR) provided accurate and complete information about the starter cultures development, assessing their growth and survival over the ripening process.

1. Introduction

Traditional fermented products elaborated with pork are commonly produced and consumed in different countries throughout the world. Galician chorizo is a dry fermented sausage from the north-west of Spain which has a broad acceptance by consumers. As most fermented sausages, Galician chorizo consists of a mixture of pork, fat, salt and spices stuffed into a natural casing such as pig's small intestine and characterized by a bacterial fermentation process followed by a ripening period.

Dry fermented sausage manufacturing has become a very important part of the meat industry in many countries and, although many typical fermented sausages are still produced with artisanal technologies, the use of starter cultures has become common in the processing of several types of fermented products. Since indigenous meat fermentations are not able to guarantee homogeneous production, starter cultures are chosen not only to ensure safety, contribute to colour and flavour and extend shelf-life (Papamanoli et al., 2002; Rebecchi et al., 1998), but also to standardize the production, thus maintaining the typical characteristics obtained in artisanal elaborations (Coppola et al., 1997).

Meat starter cultures used in sausage elaboration generally consist of a combination of lactic acid bacteria (LAB) and *Staphylococcus* (Rebecchi et al., 1998; Rossi et al., 2001). Lactic acid bacteria produce lactic acid and bacteriocins (Lücke, 2000) and staphylococci are important because of their biochemical–metabolic properties, such as lipolytic activity and nitrate reduction, affecting positively the quality and the stability of the products (Johansson et al., 1994). There are several LAB commercially available to be used in sausage fermentations, mainly *Lactobacillus sakei*, used in Europe, and *Pediococcus acidilactici*, most popular in USA (Leroy et al., 2006). However, there are only two staphylococcal

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species developed as commercial starters for the fermentation of dry sausages, *Staphylococcus xylosus* and *Staphylococcus carnosus*, designed to be used separately or mixed, but always together with a LAB (Corbière Morot-Bizot et al., 2007). Nevertheless, commercial starter cultures are not always able to compete with the autochthonous microbiota occurring in the product, so the final properties of the product are not necessarily the result of their activity (Babić et al., 2011). Therefore, in order to select a suitable starter strain, in addition to safety, economic and other technological requirements, it should be taken into account that the strain must compete with the autochthonous microbiota occurring in the sausage, which will be coincident with at genus or even species level.

It has been reported that species identification of LAB or staphylococci from dry fermented sausages is not always possible when using classical biochemical methods, due to the variable expression of some phenotypic characters (Perl et al., 1994; Renneberg et al., 1995). In consequence, reliable selective identification and quantitation methods are essential for monitoring inoculated starter strains throughout the sausage ripening in order to assay its capacity for survival among the resident strains of the same species.

Repetitive element palindromic polymerase chain reaction (rep-PCR) fingerprinting has been recognized as a low-cost and easy-to-perform technique in which primers directed to repetitive sequences randomly distributed over bacterial genomes are used (Versalovic et al., 1994). rep-PCR using primer $(GTG)_5$-PCR is considered to be a reliable tool for classifying and typing a wide range of bacteria, including LAB (Bevilacqua et al., 2010; Gevers et al., 2001) and staphylococci (Iacumin et al., 2006; Švec et al., 2010), allowing differentiation at species, subspecies and sometimes even at strain level. However, the main drawback of these techniques is that they do not allow quantitation. In order to enumerate viable bacteria in fermented foods, traditional culture-based plate counting methods were, and still are, often used, but they are usually time consuming and selective growth media are sometimes unavailable for certain species. For that reason, the development of a molecular culture-independent enumeration method appeared to be of great importance to control and monitor either endogenous or inoculated starter cultures. Among other molecular enumeration techniques, quantitative real-time PCR can provide an accurate and sensitive method for the enumeration of bacterial species and genus directly from complex ecosystems such as food samples (Fukushima et al., 2007).

The aim of this study was to monitor the development of *Staphylococcus* and *Lactobacillus* species used as starter cultures throughout the ripening of Galician chorizo combining different molecular-based techniques. The bacterial diversity occurring in the sausage at the beginning of the ripening and in the final product was also studied, using molecular methods to characterize the indigenous bacterial population in the control batch with no addition of starter culture, as well as the bacterial composition occurring in each inoculated batch. The combination of a quantitative method such as real-time PCR with the identification and typing techniques used in this study, such as genus and species-specific PCR, 16S rRNA gene sequencing and $(GTG)_5$-PCR, provided accurate and complete information about the starter cultures development, assessing their growth and survival over the ripening process.

2. Materials and methods

2.1. Sausage production

Four different batches of Galician chorizo were manufactured in triplicate according to traditional techniques, three of them with addition of different starter strains, chosen from a set of strains previously isolated in our laboratory from a traditional Galician fermented sausage and subsequently identified by 16S rRNA gene sequencing and technologically characterized (Cachaldora, 2011; García Fontán, 2004; García Fontán et al., 2007a,b). It is necessary to point out that, due to health and legal restrictions, *Staphylococcus epidermidis* cannot be properly considered nor used as starter culture in sausage production. However, due to its abundance in the artisanal sausages and also to its metabolic performances, it was inoculated in the experimental sausages in the present work to study its behaviour with a merely scientific purpose. The batches were named as follows: (i) CNT batch, control without starter culture, (ii) EQU batch, with *L. sakei* LS131 + *Staphylococcus equorum* SA25 starter culture, (iii) EPI batch, with *L. sakei* LS131 + *Staphyloccoccus epidermidis* SA49 culture, (iv) SAP batch, with *L. sakei* LS131 + *Staphylococcus saprophyticus* SB12 starter culture. The *L. sakei* strain was added in an amount of 10^6 CFU/g of mix, while the amount of the *Staphylococcus* strain was 10^7 CFU/g of mix. Galician chorizo formulation includes lean pork (80%), pork back fat (20%), sweet paprika (22 g/kg), NaCl (15 g/kg), garlic (4 g/kg), spicy paprika (1 g/kg) and water (40 mL/kg). The lean pork and the pork back fat were ground through a 10 mm diameter mincing plate and vacuum mixed together with the other ingredients for 3 min. The mix was maintained at 4 °C for 24 h and then stuffed into natural casings obtained from pig's small intestine. The sausages were fermented for 9 days at 6 °C and 80% relative humidity and then transferred into a drying-ripening chamber where they were kept for 21 more days at 12 °C and 75% relative humidity. Samples at day 0 (mix before inoculation) and after 2, 5, 9, 14, 21 and 30 days of ripening were taken for subsequent analysis.

2.2. Sample preparation

For each replicate of each batch, 10 g of sample taken from the mix before inoculation or from the sausage (after discarding the casing and the outer layer (2 mm) in contact with the casing) at the different ripening times was aseptically added to 40 mL of a sterile solution of 0.1% peptone water (Oxoid, Basingstoke, UK). This mixture was homogenized in a Masticator Classic (IUL Instruments, Barcelona, Spain) blender for 2 min at room temperature. For real-time PCR analysis, one mL of the homogenized samples was centrifuged at 7500 rpm for 10 min to obtain cell pellets for total DNA extraction, which were stored at −20 °C until further use. In order to obtain the isolates for the other assays, serial decimal dilutions in peptone water were prepared from homogenized samples from each replicate of mix before inoculation and of each sausage batch at the end of the ripening, and spread on Manitol Salt Agar (MSA) (Oxoid, Basingstoke, UK) and de Man, Rogosa, Sharpe (MRS) (Oxoid) media. After incubation and plate counting, a total of 320 colonies (32 colonies from MSA and 32 from MRS plates of the highest dilution that yielded growth from each of the 5 samples above mentioned, that is the mix before inoculation and the 4 different final products), were randomly selected, purified, grown overnight in Brain Heart Infusion (BHI) (Oxoid) and MRS broth respectively, and stored in 25% of glycerol at −20 °C until being subjected to DNA extraction.

2.3. Genomic DNA extraction

Genomic DNA was purified from cell pellets obtained from homogenized samples and from overnight cultures obtained from MSA and MRS isolates using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for Gram-positive bacteria with modifications. One μL of 10 mg/mL lysostaphin solution (Sigma–Aldrich, St Louis, USA) was added to the enzymatic lysis buffer used for cell pellets and MSA isolates. Five μL