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Molecular and technological characterization of *Staphylococcus xylosus* isolated from naturally fermented Italian sausages by RAPD, Rep-PCR and Sau-PCR analysis

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Abstract

Coagulase-negative cocci (CNC) are important microorganisms in fermented sausages because they release lipases and proteases that are able to free short-chain fatty acids and peptides and aminoacids, respectively, that are responsible for the aroma of fermented sausage. The purpose of this study was to characterize *Staphylococcus xylosus* strains isolated from naturally fermented sausages, produced in three different processing plants in the Friuli Venezia Giulia region in the Northeast of Italy. Two hundred and forty-nine strains of *S. xylosus* were identified by species-specific PCR and subjected to molecular and technological characterization. RAPD-PCR with primer M13, Rep-PCR using primer $(GTG)_5$ and Sau-PCR with primer SAG_1 were used for the molecular analysis, while the capability of the strains to grow at different temperatures and in the presence of NaCl and their lipolytic and proteolytic activity were tested in order to define the technological characteristics. The results obtained allowed us to differentiate strains coming from different plants, thereby admitting the presence of strains that are plant-specific.
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Keywords: Fermented sausages; Characterization; *Staphylococcus xylosus*; RAPD; rep-PCR; Sau-PCR; Cluster analysis

1. Introduction

Manufacturing of fermented sausages has a long history in Italy, and there is a wide variety of typical preparations (Riva, Ferlin, & Pompei, 1988; Zambonelli, Papa, Romano, Suzzi, & Grazia, 1992). However, the use of starter cultures for sausage production is becoming increasingly necessary to guarantee food safety and to standardize product properties, including consistent flavor and color and shorter ripening time.

In the fermentation of sausages, the main transformations that are leading to the final products, have to be connected with the activity of two microbial groups: lactic acid bacteria (LAB) and micro/staphylococci (del Carmen de la Rosa, Mohino, Mohino, & Mossa, 1990; Hugas, Garriga, Aymerich, & Monfort, 1993). The LAB are responsible for lactic acid production, for the "tangy" flavour of sausages and for the small amounts of acetic acid, ethanol, acetoin, carbon dioxide and pyruvic acid that are produced during fermentation (Bacus, 1986; Demeyer, 1982; Thornill & Cogan, 1984). Sausages with longer maturation times, contain higher numbers of *Micrococcaceae* (Demeyer, Verplaetse, & Gistelink, 1986). *Staphylococcus* spp. are important for color stabilization, through nitrate reductase activity, decomposition of peroxides and aroma formation due to their proteolytic and lipolytic activities (Cai, Kumai, Ogawa, Benno, & Nakase, 1999; Comi et al., 2000; Miralles, Flores, & Perez-Martinez, 1996; Schleifer, 1986). In fact, micro/staphylococci are able to release lipases that are able to free short-chain fatty acids that are responsible for the aroma of the fermented sausage (Montel, Reitz, Talon, Berdague, & Rousset, 1996).

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The application of molecular methods (PCR-DGGE), to follow the microbial dynamic changes during the fermentation of sausages, highlighted that even at three days of maturation these species are the most prevalent in the sausages, influencing the DGGE profiles obtained directly from the sausage. This situation is constant until the end of the fermentation (Cocolin, Manzano, Cantoni, & Comi, 2001). Sensitive and reliable methods for their detection and identification are of great importance to monitor population changes during food fermentation by natural micro flora, as well as when promoted by the addition of starter and/or protective cultures under controlled conditions (Holzapfel, Geisen, & Schillinger, 1995). The biochemical profiles are not always able to unequivocally identify some *Staphylococcus*, moreover, the use of substrates that change color due to the activity of the microorganisms, introduces a subjective aspect to the evaluation (Quere, Deschamps, & Urdaci, 1997). For a better identification, in the last 10 years there has been increasing interest in the use of molecular methods. The majority are based on the DNA sequence of the 16S rRNA gene (Collins et al., 1991), from which it is possible to obtain important information for the detection, identification and classification of *Staphylococcus*.

S. xylosus is frequently isolated as the main *Staphylococcus* species involved in fermented sausage production, although others have been reported: *S. carnosus*, *S. simulans*, *S. saprophyticus*, *S. epidermidis*, *S. haemolyticus*, *S. warneri* and *S. equorum* (Cocolin et al., 2001; Cocolin, Manzano, Aggio, Cantoni, & Comi, 2001; Comi, Citterio, Manzano, & Cantoni, 1992; Coppola, Iorizzo, Saotta, Sorrentino, & Grazia, 1997; Fischer & Schleifer, 1980; García-Varona, Santos, Jaime, & Rovira, 2000; Hugas & Roca, 1997; Pirone & Manganelli, 1990; Seager, Banks, Blackburn, & Board, 1986; Simonetti & Cantoni, 1983; Torriani, Di Bucchianico, Pattarini, Zabeo, & Dellaglio, 1994).

The purpose of this study was to use molecular methods for the characterization of *S. xylosus* isolated during the fermentation of traditional Friuli sausages. This study was performed to acquire more information about the isolates and in particular to see whether or not they can be differentiated depending on their specific provenance. Strains were characterized by determining their growth at different temperatures and in presence of NaCl and their proteolytic and lipolytic power. Moreover after DNA extraction, Random Amplified Polymorphic DNA (RAPD)-PCR, Repetitive Extragenic Palindrome (Rep)-PCR and Sau-PCR were applied to detect differences in the genome of the strains tested.

2. Materials and methods

2.1. Fermented sausages technology and sampling procedures

Three local meat factories in the North East of Italy were selected for the study. They were called plant *C*, *L* and *U*. For the production of the fermented sausages traditional techniques were employed without the use of starter cultures. Plant *C* was characterized by a slow fermentation of the sausages that lasted at least 120 days, while plant *L* was producing sausages with fast maturation (28 days). Plant *L* was selling the final product after 45 days of ripening. Common ingredients were 60 kg of pork meat, 40 kg of lard, 2.5 kg of sodium chloride, 200 ppm of nitrite and nitrate, and 70 g of black pepper. For fermentation at plant *C* no sugars were added, while for fermentation at plants *L* and U 1.5 kg and 2.5 kg of sugars were employed, respectively. This mixture was stuffed into natural casings, which resulted in fresh sausages that were 25 cm long and 5 cm in diameter. Ripening parameters were different for the three plants. Plant *C* performed maturation at low temperatures (below 10 °C) and relative humidity (RH) between 60% and 90% for the first 60 days, followed by 2 months at temperatures of 14 °C and RH from 65% to 85%. For plant *L* and *U*, the first stage consisted of 2 days with a RH of 85% at 22 °C. The temperature was then decreased to 12 and 14 °C, for plant *L* and *U*, respectively, at a rate of 2 °C per day with a RH between 60 and 90%. Ripening was then carried out for the rest of the period at this final temperature, in storerooms with RH from 65% to 85%. Triplicate samples of the sausages were used for microbiological and molecular analyses. For plant *C*, samplings were performed at 0, 3, 10, 20, 30, 60, 90 and 120 days, whereas for plant *L* samplings at 0, 3, 10, 20, 30 and 45 days were selected. Sausages from plant *U*, characterized by fast ripening, were examined at 0, 3, 5, 7, 14 and 28 days (Rantsiou et al., 2005).

2.2. CNC isolation

A total of 617 CNC strains were isolated from the plants used in this study. They were isolated from MSA plates (Oxoid, Basingstoke, Hampshire, UK) incubated at 30 °C for 48 h. Randomly selected colonies (gram positive cocci, catalase positive) were streaked on BHI agar and stored at −80 °C in BHI broth supplemented with 30% glycerol before being subjected to molecular identification. A variable number of 20 to 30 colonies were isolated at each sampling point, for each of the fermentations followed.

2.3. Technological characterization

A total of 249 *S. xylosus* isolates were subjected to technolgical characterization. The proteolytic activity was studied according to Torriani et al. (1994) in PCA plates (Oxoid), with 10% (v/v) sterile skimmed milk (Oxoid). Lipolytic activity was studied in trybutirin agar plates (Oxoid). The clearance zone, after incubation for 5 days, was taken as a positive result and the results were confirmed by giving successive transfers on the same substrate. The ability of the isolates to grow at different temperatures was checked at 12, 18 and 22 °C. A cell suspension was prepared in BHI broth (Oxoid) and used as inoculum (10%, v/v). Growth was followed by optical density ($OD_{600\ nm}$)

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