MONITORING OF A STARTER CULTURE FOR FERMENTED SAUSAGES BY MOLECULAR METHODS

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Abstract The ability of a commercial starter culture to perform a sausage fermentation was evaluated by culture –dependent and –independent methods. The starter culture, as well as the sausage during fermentation, was sampled and strains of lactic acid bacteria (LAB) and coagulase negative cocci (CNC) were isolated. After identification it was determined that Lactobacillus plantarum, contained in the inoculated starter, was the main LAB representative that conducted the transformation, while *Staphylococcus xylosus*, not declared in the label of the starter culture, was able to colonize the sausages studied. Molecular characterization of isolated *Lb. plantarum* and *S. xylosus* highlighted that the commercial mix contained several strains of the same species, and their behavior during the fermentation was different. Analysis of the nucleic acids extracted directly from the sausages confirmed the performance of *Lb. plantarum*, which was present and active throughout the fermentation, and highlighted the contribution of *Lactobacillus curvatus.*

Introduction For fermented sausage production, the starter cultures are represented by mixtures of lactic acid bacteria (LAB) and coagulase-negative cocci (CNC). LAB are the main population responsible for the pH drop, followed by a second step in which CNC are neutralizing the organic acids produced by LAB, through production of peptides and aminoacids from their proteolytic activity. In addition, CNC are able to induce the release of various aromatic substances due to their capability to produce lipases (Montel *et al.,* 1996).

Recently, direct amplification of DNA and RNA by polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.,* 1993) showed very good applicability in monitoring the microbial ecology of Italian fermented sausages (Cocolin *et al.*, 2001; Rantsiou *et al.*, 2005).

In this paper we wanted to validate the use of a commercial starter for the production of fermented sausages by molecular methods. DGGE was used to analyze PCR and RT-PCR products obtained from DNA and RNA extracted directly from the sample to monitor the bacterial dynamics during transformation. Moreover, LAB and CNC were isolated both from the starter culture and during fermentation and subsequently subjected to RAPD analysis to understand if the strains inoculated were able to conduct the fermentation process considered in the study.

Materials and Methods Fermented sausages were prepared in a local meat factory using traditional techniques as previously described (Comi *et al.*, 2005) and inoculated with 20 g starter culture containing *S. carnosus* and *Lb. plantarum* (Biostart SL1-200, Wiesby GMBH Co., Niebull, Germany). The ripening was conducted for 28 days. The fermented sausages were analyzed in triplicates at 0, 3, 5, 7, 14 and 28 days.

Potentiometric measurements of pH were made using a pin electrode of a pH-meter (Radiometer Copenhagen pH M82, Cecchinato, Italy).

The starter culture (20 g), of the same batch that was used in the production followed, was resuspended in 200 ml sterile water and left at room temperature for 30 min and serial dilutions were prepared in saline/peptone water (8 g/l NaCl, 1 g/l bacteriological peptone, Oxoid, Milan, Italy) and analyzed on MRS agar (Oxoid) incubated with a double layer at 30°C for 48 h and on Mannitol Salt Agar (MSA, Oxoid) incubated at 30°C for 48 h. Fifteen colonies of LAB were isolated from MRS agar and the same number of CNC were selected from MSA agar. The fermented sausages were analyzed to monitor the dynamic changes in the populations responsible for the ripening of fermented sausages, as well as their hygienic quality. In particular, 25 g of each sample were transferred into a sterile stomacher bag and 225 ml of saline/peptone water were added and mixed for 1 min and 30 s in a Stomacher machine (PBI, Italy). Further decimal dilutions were made and the following analyses were carried out on duplicate agar plates: a) total bacterial count (TBC) on Gelisate Agar (Oxoid) incubated for 48–72 h at 30°C; b) LAB on MRS agar; c) CNC on MSA (Oxoid);

d) total *enterobacteria* and *Escherichia coli* on Coli-ID medium (Biomerieux, Rome, Italy) incubated with a double layer at 37°C for 24-48 h; e) Staphylococcus aureus on Baird Parker medium (Oxoid) with added egg yolk tellurite emulsion (Oxoid) incubated at 37°C for 24-48 h; g) yeasts and moulds on Malt Extract Agar (Oxoid) supplemented with tetracycline (1 mg/ml, Sigma) incubated at 25°C for 48-72h. For *Listeria monocytogenes* and *Salmonella spp.* the ISO/DIS methods (1990; 1991) were applied. During fermentation, a total of 70 LAB strains, from MRS plates, and a total of 70 CNC strains, from MSA plates, were randomly selected.

Isolated strains from the starter culture and the sausages were subjected to molecular identification by the use of species-specific PCR and DGGE analysis followed by 16S rRNA gene sequencing as described elsewhere (Cocolin *et al.*, 2001; Rantsiou *et al.*, 2005).

DNA extraction from cultures was performed as suggested by Andrigetto *et al.* (2001) modified by using only lysozyme (50 mg/ml, Sigma, Milan, Italy) for the bacterial cell-wall digestion, while DNA and RNA were extracted from the sausage sample employing the protocol developed by Cocolin *et al.* (2004).

Amplification of the nucleic acids extracted from the sausages was performed by using primers P1 (5'- GCG GCG TGC CTA ATA CAT GC -3'), and P2 (5'- TTC CCC ACG CGT TAC TCA CC -3') (Klijn *et al.,* 2001) as previously described (Cocolin *et al.*, 2004). For DGGE analysis a denaturant gradient from 40–60% was employed. Gels were subjected to a constant voltage of 130V for 3.5 h at 60°C. After staining in 1X SYBR Green, final concentration (Molecular Probes, Eugene, OR) they were visualized under UV light, digitally captured and analyzed by using the BioImaging System GeneGenius (SynGene, Cambridge, United Kingdom) for the recognition of the bands present.

One hundred ng of the DNA extracted from isolated LAB and CNC strains were subjected to RAPD-PCR using primer M13 (5'- GAG GGT GGC GGT TCT –3') as previously reported (Andrighetto *et al.*, 2001). The pattern analysis software package, Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium) was used for the analysis. Isolated strains were subjected to RAPD-PCR analysis at least twice.

Results LAB populations reached counts of about 107 colony forming unit (cfu)/g already at 3 days. On day 7, the maximum count of 108–109 cfu/ml was reached and remained stable until the end of the period followed. Yeasts were characterized by an increase of 3 to 4 orders of magnitude: from less than 100 cfu/g at day zero, they increased to 103–104 cfu/g at 3 days. TBC and enterobacteria showed an increase during the first days of fermentation, as well. Moulds were <100 cfu/g throughout the fermentation, and *S. aureus. L. monocytogenes* and *Salmonella spp.* were always absent in 25 g of the product in all the samplings performed. The pH was characterized by a steep drop from day zero to day 5, with a decrease of about 0.5 units. From day 5, it started to increase and at the end of the fermentation reached final values of about 5.7–5.8.

LAB dynamics were characterized already from day zero by a strong influence of *Lb. plantarum*. Concerning the *Staphylococcus spp.* ecology, it was characterized by a population switch between day zero and day 3. At zero days different species were isolated, with *S. pasteuri* being the main population present. At day 3, a strong presence of *S. xylosus* was observed, and this picture was repeated also at the other sampling points.

Lb. plantarum and *S. xylosus* were subjected to RAPD analysis with primer M13. A coefficient of similarity of 70% was selected for *Lb. plantarum*, while 80% was used for the *Staphylococcus spp.* A total of 64 *Lb. plantarum* strains (Figure 1A) were grouped in three clusters and only strain L3, isolated from the starter culture constituted a single-strain cluster. Cluster II grouped almost all of the isolates, while cluster I and cluster III included only 10 and 3 strains, respectively. A bigger biodiversity within the strains identified as *S. xylosus* was observed when cluster analysis was performed (Figure 1B). The 57 strains were grouped in 8 clusters and 8 single-strain clusters. The number of the strains grouped was always limited apart from cluster IV, which included 27 strains. Clusters II and VII were both formed by 7 strains, whereas the rest of the clusters had only 2 strains.

The profiles obtained by direct PCR-DGGE and RT-PCR-DGGE analyses are presented in Figure 2. The main differences were detected at zero days. At DNA level, two bands, A and B (lane 2) were detected and identified as *Staphylococcus sciuri/pulvereri* and *Staphylococcus equorum/succinicus,* respectively, while from the RNA two bands resulted to be represented by *Lb. plantarum* (band J) and *S. xylosus* (band K). Band L was sequenced and identified as *Bacillus spp.* After day

zero, the DGGE profiles became very similar at both DNA and RNA level. Two bands were repeatedly cut and sequenced and they always resulted as *Lb.plantarum* and *Lb. curvatus*. Moreover, another band was present in the RNA profile at day 3 (band M, lane 13) After sequencing, it was defined to be *Lb. sakei.* The DGGE profiles showed also other bands (C and D, Figure 2, lane 3), which resulted to be heteroduplex after amplification and DGGE analysis of the cut bands.

Discussion Only a few studies have exploited molecular methods to assess the capability of selected strains to carry out sausage fermentations. Garriga *et al.* (1996) studied the performance of several lactobacilli strains isolated from naturally fermented sausages as starter cultures, and their capability to dominate was determined by plasmid fingerprint. Moreover, Di Maria *et al.* (2002) used pulsed-field gel electrophoresis (PFGE) and RAPD-PCR to monitor *S. xylosus* DSM20266 added as starter during fermentation and ripening of "soppressata molisana", a typical Italian sausage.

The microbial trends showed a fast increase of the LAB populations, which provoked a drop in the pH within the first five days of fermentation. This fact seemed to inhibit the growth of CNC, in fact only at day 5 their counts started to increase significantly. The other populations monitored showed a first increase in the counts, followed by a constant diminution of the numbers.

Concerning the commercial starter culture used in this study, apart from *Lb. plantarum* and *S. carnosus* that were declared in the label, a third population was present, represented by *S. xylosus.* All the LAB strains isolated from the starter belonged to *Lb. plantarum* as declared, while the 15 strains of CNC were identified as *S. carnosus* (9 isolates) and *S. xylosus* (6 isolates). *S. xylosus* was the main species of *Staphylococcus spp*. isolated from the sausage throughout the period followed, whereas *S. carnosus*, was detected at a significantly lower frequency. New information became available when the results of the genetic characterization of the two main LAB and CNC populations, respectively *Lb. plantarum* and *S. xylosus,* were obtained. The cluster analysis highlighted how within the species contained in the starter culture it was possible to distinguish strains that grouped in different clusters and were characterized by different RAPD profiles. For *Lb. plantarum*, it was possible to distinguish three distinct profiles but only one was able to conduct the fermentation. A different picture was observed in the case of *S. xylosus*. The six isolates from the starter culture were all grouped in cluster IV, apart from strain S5 that was unique. It is interesting to notice that in cluster IV the strains from the starter culture grouped with strains isolated mainly at 14 and 28 days, thereby highlighting their predominance only in the latter stages of the fermentation. It is speculated that the different behavior of *Lb. plantarum* and *S. xylosus* is due to the production procedure. In particular, it should be noted that, in the sausage mix, according to the recipe, white wine is added. The ethanol of the wine represents a stress factor, especially for *S. xylosus*, that results in slower growth for this organism compared to *Lb. plantarum*. Direct DNA and RNA DGGE analysis revealed the contribution of another LAB species, *Lb. curvatus*, in the fermentation. It is possible that *Lb. curvatus* was outnumbered by *Lb. plantarum* in the sausages and could not be isolated by plating on MRS. Lastly, *Lb. sakei* was detected by direct analysis but, based on the intensity and presence of the respective bands, its impact on the fermentation can be considered marginal compared to *Lb. curvatus* and *Lb. plantarum.*

Conclusions The application of molecular methods allowed the study in detail of the dynamics of the strains contained in the starter culture used in the production. It was determined that the starter, declared in the label to contain *Lb. plantarum* and *S. carnosus,* had also *S. xylosus.* Moreover, the starter culture was formed by a mixture of strains and especially for *Lb. plantarum* different RAPD-types were determined. Among them only one was able to perform the fermentation process. The application of direct methods allowed the understanding of the contribution of *Lb. curvatus*, only marginally isolated from the plates, but present throughout the fermentation at both DNA and RNA level. The ability of the starter culture *S. xylosus* strains to carry out only the second half of the fermentation is suggesting a need for a change in the production procedures used in the processing plant.

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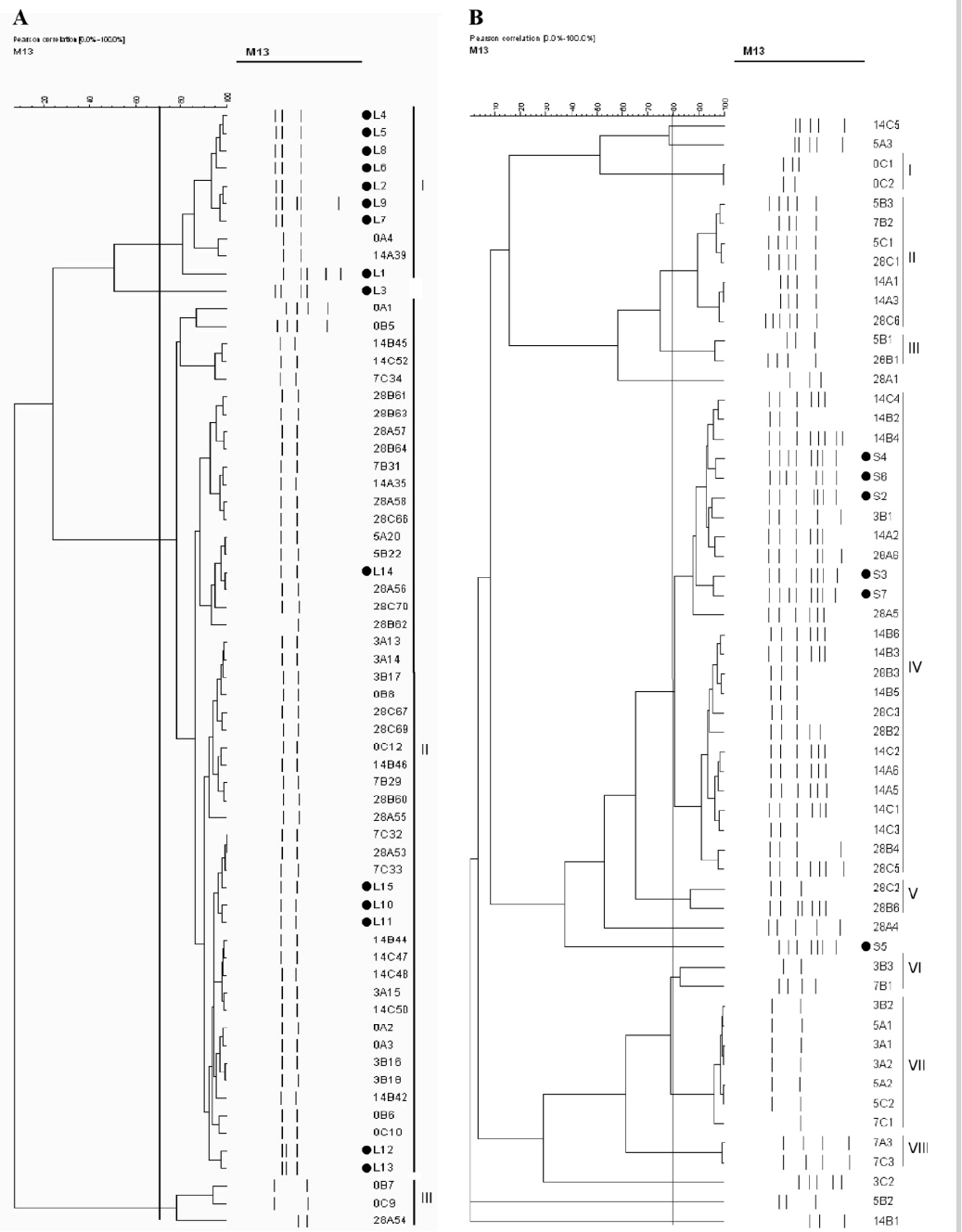

Figure 1. Cluster analysis of the profiles obtained from the *Lactobacillus plantarum* (Panel A) and *Staphylococcus xylosus* (Panel B) strains isolated during the fermentation of the sausages. Strains isolated from the starter culture are indicated with a letter L and S, respectively, and marked with a black dot. Isolates from fermented sausages are identified by code, in which the first number represents the day of isolation, the letter the code of the sample and second number, the progressive number of isolation. Identified clusters are indicated with roman numerals.

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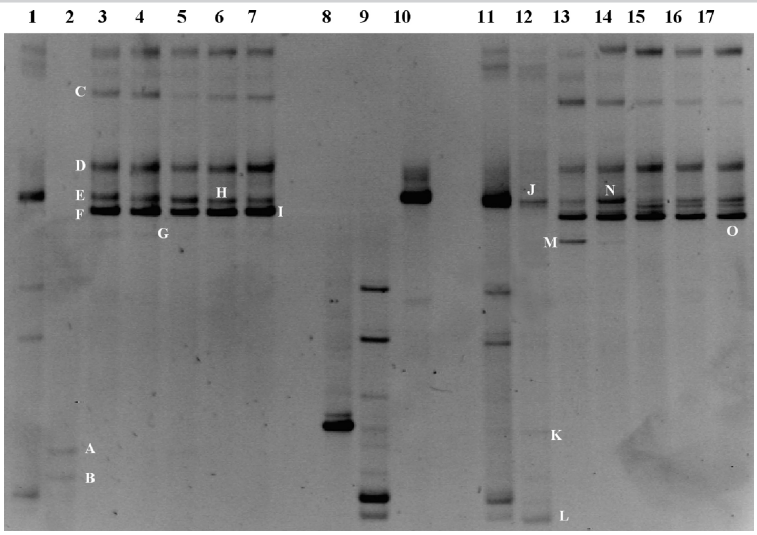

Figure 2. Bacterial DGGE profiles of the DNA (lanes 1 to 7) and RNA (lanes 11 to 17) extracted directly from the starter culture and the fermented sausages. Lanes 1 and 11, starter culture envelope; lanes 2 and 12, day zero; lanes 3 and 13, 3 days of fermentation; lanes 4 and 14, 5 days of fermentation; lanes 5 and 15, 7 days of fermentation; lanes 6 and 16, 14 days of fermentation; lanes 7 and 17, 28 days of fermentation. Band in lanes 8, 9 and 10 were produced from *S. xylosus* S4, *S. carnosus* S10 and *Lb. plantarum* L12, respectively, all isolated from the starter culture and hereby representing migration controls. Bands indicated by letters were excised and after reamplification, subjected to sequencing.