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Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages

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Abstract

The dynamics of the microbial community responsible for the artisanal fermentation of dry sausage produced in Argentina was investigated by using classical and molecular approaches. The combined use of RAPD analysis with primers M13, XD9, RAPD1 and RAPD2 and 16S rDNA sequencing were applied to the identification and intraspecific differentiation of 100 strains of lactobacilli and *Micrococcaceae*. DGGE analysis was used to monitor the dynamic changes in population after total microbial DNA was directly extracted from sausages and subjected to PCR using V3f (GC), Bact-0124f-GC and Univ-0515r primers. The sequence analysis of 16S rDNA of the dominant species was also carried out. *Lactobacillus sakei* and *Lactobacillus plantarum* were the dominant lactic acid organisms during the fermentation while *Staphylococcus saprophyticus* represented the dominant species of *Micrococcaceae*. It was demonstrated that the ripening process of Argentinean artisanal fermented sausage is driven by a limited number of *Lactobacillus* and *Staphylococcus* strains selected from environmental microbiota by the ability to best compete under the prevailing conditions of the ecological niche. The identification of dominant communities present in this artisanal fermented sausage can help in the selection of starter cultures consisting in well adapted strains to the particular production technology.

Keywords: Fermented sausages; RAPD; DGGE; 16S rDNA; *Lactobacillus sakei*; *Lactobacillus plantarum*; *Staphylococcus saprophyticus*

1. Introduction

Fermented sausages can be defined as meat products consisting of a mixture of meat and fat particles, salt, curing agents, spices, etc., which have been stuffed into a casing, fermented (ripened) and dried. Nowadays, dry fermented sausages manufacture is a very important part of the meat industry in

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many countries. Large-scale industrial processes that relied on the use of selected starter cultures led to a low variability in the present microflora. Lactic acid bacteria (LAB) and *Micrococcaceae* are the two main groups involved in the fermentation of dry cured sausages (Lücke and Hechelmann, 1987; Coventry and Hickey, 1991). The physiological properties of bacteria isolated from meat fermentations, such as bacteriocins production (Stiles, 1996; Aymerich et al., 1998), proteolysis (Johansson et al., 1994; Fadda et al., 1999; Sanz et al., 1999), lipolysis (Johansson et al., 1994; Talon et al., 1995; Kenneally et al., 1998), nitrite and nitrate reductases (Wolf and Hammes, 1988) have been studied in detail. It is well known that LAB, in particular lactobacilli, play an important role in meat preservation and fermentation processes. Their ability to lower the pH and produce bacteriocins prevent the growth of pathogenic and spoilage microorganisms, improving the hygienic safety and storage of meat products. They also contribute to the color and texture development mainly through their acidification capacity. On the other hand cocci Gram positive (*Staphylococcus* and *Kocuria*) reduce nitrates to nitrites leading to the formation of nitrosomyoglobin which give the product its characteristic color, and also produce protease and lipase and thus release various aromatic substances and organic acids (Talon et al., 2002).

The type of microflora that develops is often closely related to the ripening technique utilized. Sausages with short ripening time have more lactobacilli from the early stages of fermentation than sausages with longer maturation time which contain higher numbers of *Micrococcaceae*. Manufacturing of fermented sausage has a long history in Argentina, this being in relationship with Italian and Spanish traditions as well as the well known quality of meat. Even when the use of starter culture has become common in the manufacture of several types of fermented products, many typical fermented sausages are still produced with traditional technologies without selected starters. In artisanal production of traditional fermented sausages it is important to use starter cultures consisting in strains isolated from local products and thus well adapted to the particular product and to the specific production technology (Papa and Grazia, 1990). In the northern region of Argentina, a traditional fermented sausage is produced without microbial starters from fresh pork meat and lard that are mixed with other ingredients, such as sugars, NaCl, and additives (nitrate, nitrite and spices).

Due to the limitations of conventional microbiological methods such as sugar fermentation profiles or other biochemical/physiological traits, the identification of the *Lactobacillus* and *Micrococcaceae* species involved in meat fermentation resulted ambiguous. In particular, strains of *Lactobacillus curvatus* and *Lactobacillus sakei* isolated from meat products are not easy to differentiate (Reuter, 1981; Kandler, 1984). Identification at intraspecies level is also an important issue since it may help to distinguish groups of strains or single strains with peculiar technological properties. Moreover, in the last decade it was shown that classical microbial techniques do not accurately detect microbial diversity in environments such as traditional fermented foods in which culture methods not only underestimated biodiversity but failed to quantify precisely some dominant taxa (Hugenholtz et al., 1998).

Recently, several molecular methods have been applied for the identification of bacteria isolated from meat products, such as SDS-PAGE of whole cell proteins (Dykes and Von Holy, 1995), restriction fragment length polymorphism analysis of the 16S rRNA gene (Sanz et al., 1998; Björkroth and Korkeala, 1996), hybridization with rRNA probes (Nissen and Dainty, 1995), specie-specific PCR (Yost and Nattress, 2000), RAPD–PCR analysis (Andrighetto et al., 2001; Berthier and Ehrlich, 1999; Rebecchi et al., 1998), PCR followed by temperature/denaturing gradient gel electrophoresis (TGGE/DGGE) (Cocolin et al., 2000, 2001). The last approaches allow separation of DNA molecules that differ by single bases (Myers et al., 1987), this having the potential to provide information about variations in target genes in a bacterial population. By adjusting the primers used for amplification, all constituents of microbial communities can be characterized.

The aim of this study was to use molecular approaches together with microbiological counts to describe bacterial diversity during natural fermentation (no addition of starter cultures) of an Argentinean dry cured sausage. The combination of these techniques allowed the identification of the dominant

bacterial strains and the variation in the community composition over the ripening period.

2. Materials and methods

2.1. Sausage production and sampling procedures

Fermented sausages were prepared in a local meat factory by traditional techniques according to the following formulation: pork meat 85%, pork fat 10%, sodium chloride 2.6%, sugar (sucrose) 0.4%, starch 1.5%, binding and flavoring additives 0.9%, nitrite and nitrate 0.017%. The raw sausage material was mixed, stuffed into artificial casings and fermented for 7 days at 23 °C and relative humidity (RH) of 95% followed by a gradual reduction of temperature (from 22 to 15 °C) and RH (from 92% to 89%) during the next 7 days. Duplicate samples of sausages were collected at start of the ripening process (time 0) and at 1, 2, 4, 5, 7, 9, 11 and 14 days. The sampling was performed during one cycle of sausage production and was used for microbiological and molecular analysis.

2.2. pH measurements

Potentiometric measurements were obtained with the pin electrode of a pH meter (692 pH/Ion Meter-Metrohm, USA) that was inserted directly into the sample. Three independent measurements were obtained for each sample. The reported values are the average of three readings.

2.3. Microbiological analysis and preliminary physiological characterization of isolates

The samples were subjected to a microbiological analysis to monitor the dynamic changes in the population responsible for ripening of fermented sausages and their hygienic quality. Ten grams of each sample were transferred into sterile stomacher bag, 90 ml of saline-peptone water (NaCl, 2%; bacteriological peptone, 0.1% and Tween 80, 1%) was added and the preparation was mixed for 3 min in a stomacher machine (Stomacher Lab-Blender 400, A.J. Seward Lab. London, England). Additional decimal dilutions were prepared, and the following analysis were carried out: (i) mesophilic aerobic bacterial counts on Plate Count Agar incubated for 48 h at 30 °C; (ii) LAB and lactobacilli on MRS and acetate agar (Rogosa et al., 1951) media, respectively, incubated for 48 h at 30 °C under restricted oxygen conditions; (iii) *Micrococcaceae* on mannitol salt agar (MSA) incubated for 48 h at 30 °C; (iv) coliforms on McConkey agar incubated for 24 h at 37 °C; (v) enterococci on SF agar (Difco, USA) incubated for 72 h at 42 °C; (vi) *Staphylococcus aureus* on Baird-Parker medium (Oxoid, UK) with egg yolk tellurite emulsion (Oxoid, UK) incubated at 37 °C for 24 to 48 h and (vii) yeast and molds on Sabouraud agar (Britania, Argentina) incubated at 30 °C for 48 to 72 h. Ten colonies from MRS, Rogosa, SF and MSA plates for each sample were randomly selected, transferred to MRS and BHI (Brain hearth infusion) broth, incubated overnight at appropriate temperatures and stored at −20 °C in the same liquid media containing 30% glycerol before they were subjected to DNA extraction. Unless otherwise specified all media and ingredients were obtained from Britania, Argentina. All isolates were preliminarily characterized by means of cell morphology, Gram reaction and catalase activity.

2.4. DNA extraction from pure cultures

Two different kits for rapid DNA extraction from pure culture were used according to the protocol described by the manufacturer; Bacterial DNA Miniprep (eZNA-LABOGEN, USA) for lactobacilli and Microlysis (LABOGEN, UK) for *Micrococcaceae* strains.

2.5. Extraction of DNA from fermented sausages

At time 0, 5 and 14 days of the ripening process, duplicate 10-g samples were homogenized in a stomacher bag with 10 ml of saline-peptone water for 3 min. One milliliter of each sample was washed with 200 μl ammonia hydroxide, 200 μl absolute ethanol, 400 μl petrol ether and 20 μl SDS (10%). The samples were centrifuged for 10 min at 14,000×*g* to pellet the cells, which were resuspended in a solution containing 200 μl of 6 M urea, 200 μl absolute ethanol, 400 μl petrol ether, 80 μl SDS (10%) and 13 μl of 3 M sodium acetate. A second centrifugation for 10 min at 14,000×*g* was performed, the pellet was

resuspended in 0.6 ml TE buffer (Tris-EDTA) pH 8.0 and 100 μl lysozyme (50 mg ml^{-1}) (Sigma) was added. After 60 min of incubation at 37 °C in shaker, 35 μl of SDS (10%) and 10 μl of DNase-free RNase (10 mg ml^{-1}) were added. The tubes were incubated at 37 °C for 30 min before the addition of 10 μl of proteinase K (20 mg ml^{-1}; Sigma), this preparation being incubated for 30 min and finally 130 μl of 6 M sodium perchlorate and 500 μl phenol–chloroform–isoamyl alcohol (25:24:1; pH 6.7) were added for DNA extraction. The tubes were then centrifuged at 12,000×*g* for 5 min, the aqueous phases were collected, and the nucleic acids were precipitated with isopropanol. The DNA was obtained by centrifugation at 14,000×*g* for 15 min, washed briefly with ethanol (70%) and the pellets were dried under vacuum at room temperature. 50 μl of sterile water was added and the preparations were incubated for 30 min at 37 °C to facilitate nucleic acid solubilization.

2.6. RAPD–PCR analysis

Four different primers were singly employed (i) M13 (Huey and Hall, 1989); (ii) RAPD1, (iii) RAPD2 (Cocconcelli et al., 1995) and (iv) XD9 (Moschetti et al., 1998). The different conditions used in the amplification experiments are shown in Table 1. Amplification was performed in a GeneAmp PCR System 9600 thermocycler (Applied Biosystems), RAPD reactions were performed in a reaction volume (50 μl) containing 3 mM $MgCl_2$, buffer reaction (1×), deoxynucleoside triphosphate (200 μM each), 1 μM of each primer, DNA (10–15 ng), and *Taq* polymerase (0.1 IU; Promega, Italy). RAPD products were electrophoresed at 100 V on 2.5% agarose gel and stained with ethidium bromide.

2.7. PCR amplification and DGGE analysis

PCR to investigate bacterial community by DGGE was performed with the following 16S rDNA primers: V3f (GC) (Ercolini et al., 2001), Bact 0124f (GC) (Lane, 1991), that were paired with the reverse primer Uni-0515r (Lane, 1991). Nested PCR was performed with these primers on previously generated fragments with P0–P4 primers (Klijn et al., 1991). The different conditions used in the amplification experiments for the first PCR and nested PCR are shown in Table 1. PCR products that were used as templates in nested PCR were purified with Quantum Prep PCR Klen Spin Columns (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. Amplification reactions were carried out in a 50 μl reaction volume containing 1.5 M $MgCl_2$, buffer reaction (1×), deoxynucleoside triphosphate (200 mM each), DNA (10–15 ng), *Taq* polymerase (0.03 IU) (Promega, Italy) and 0.5 μM of each primer for the first PCR and 0.1 μM for nested PCR. To determine DGGE sensitivity, different concentrations (10^2, 10^4, 10^6 and 10^8 CFU g^{-1}) of a *Streptococcus thermophilus*

Table 1
PCR primers and amplification programs used in this study

Primers	Sequence, 5′–3′	Amplification stage				Use
		Denaturing	Annealing	Extension	Cycles	
M13	GAG GGT GGC GGT TCT	94 °C, 1′	45 °C, 20″	72 °C, 2′	40	RAPD
XD9	GAA GTC GTC C	94 °C, 1′	36 °C, 20″	72 °C, 1′	45	RAPD
RAPD1	AGC AGG GTC G	94 °C, 1′	29 °C, 1′[a]	72 °C, 2′	20	
RAPD2	AGC AGC GTC G	94 °C, 30″	55 °C, 30″	72 °C, 30″	45	RAPD
V3f (GC)[1]	CC TAC GGG AGG CAG CAG					
Bact 0124f (GC)[2]	CA CGG ATC CGG ACG GGT GAG TAA CAC G	94 °C, 1′	56 °C, 30″	72 °C, 30″	35	DGGE[3]
Uni-0515r	ATC GTA TTA CCG CGG CTG CTG CTG GCA					
P0	GAG AGT TTG ATC CTG GCT CAG	94 °C, 30″	56 °C, 30″	72 °C, 1′	35	DGGE
P4	ATC TAC GCA TTT CAC CGC TAC					

(GC) [1]CC GGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G; (GC) [2]CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G; [3]The amplification stage is the same for both combinations V3f-Uni-0515r and Bact 0124f (GC)-Uni-0515r.

[a] Ramp to 72 °C: 1.5 min.

strain, a specie not generally present in sausages, were inoculated to the homogenate from sausage samples before DNA extraction. The obtained PCR products were analyzed by DGGE in the same way as that of other samples.

The PCR amplicons were separated by DGGE according to the specifications of Muyzer et al. (1993) by using the Dcode system (Bio-Rad Laboratories, Hercules, CA) with the following modifications. Polyacrylamide gels (dimensions, 160×160×1 mm) consisted of 8% (v/v) polyacrylamide (37.5:1 acrylamide/bisacrylamide) and 1× TAE (Sambrook et al., 1989). Denaturing acrylamide of 100% was defined as 7 M urea and 40% formamide. The gels were poured from the top by using a gradient maker and a pump (Econopump; Bio-Rad Laboratories, Hercules, CA) and two different gradients of 30% to 60% and 40% to 60% were used for the separation of the generated amplicons. Electrophoresis was performed for 16 h at 85 V in a 1× TAE buffer at a constant temperature of 60 °C. Gels were stained for 10 min in 1× TAE containing 1× (final concentration) SYBR Green and photographed under UV illumination.

2.8. Sequencing of DGGE bands

Small pieces of selected DGGE bands were punched from the gel with sterile pipette tips. The pieces were then each transferred into 50 μl of sterile water and incubated overnight at 4 °C to allow diffusion of the DNA. Two microliters of the eluted DNA was used for re-amplification with primer without the GC clamp, purified and sequenced (CRIBI, Universitá degli Studi, Padova, Italy).

2.9. Taxonomic identification

5′ region of 16S rDNA gene was amplified according to the primers and conditions described by Klijn et al. (1991). PCR products were purified by using Quantum Prep PCR Kleen Spin Columns (BIO-RAD). 16S rDNA sequencing were carried out by CRIBI, Universitá degli Studi, Padova (Italy). Searches in the GenBank with the BLAST program (Altschul et al., 1997) were performed to determine the closest known relatives of the partial 16S rDNA sequences obtained.

2.10. Nucleotide sequence accession numbers

Eleven sequences of the 16S rDNA determined in this study were deposited with the GenBank database under the following accession numbers: AY357581, *L. sakei* (100% identity); AY359240, *Lactobacillus plantarum*, (99% identity); AY375292, *L. curvatus* (100% identity); AY362452, *Lactobacillus pentosus* (100% identity); AY375293, *Enterococcus faecalis* (100% identity); AY375299, *Pediococcus acidilactici* (99% identity); AY375295, *Staphylococcus sciuri* (100% identity); AY375297, *Staphylococcus equorum* (99% identity); AY375298, *S. epidermidis* (100% identity); AY375296, *S. pulvereri* (99% identity); AY375294, *Staphylococcus saprophyticus* (99% identity) and AY499536, *Corynebacterium variabilis* (98% identity).

3. Results

3.1. Microbiological analysis of fermented sausages

Sausage fermentation was characterized by a rapid increase in the number of LAB which increased from an initial value of 10^4 CFU g^{-1} to 10^7 CFU g^{-1} within the first 5 days of ripening and remained stable for the rest of the fermentation period (Fig. 1). The aerobic mesophilic bacterial counts were 10^5 CFU g^{-1} at the starting time, and the highest number (10^7 CFU g^{-1}) occurred between 4 and 5 days, LAB being the dominant population at 14 days. The initial number of *Micrococcaceae* in the sausage was 10^4 CFU g^{-1}, which increased to 10^6 CFU g^{-1}after 5 days, then decreased to 10^5 CFU g^{-1} and remained quite stable in number to the end of the fermentation. Enterococci increased steadily their numbers from 10^2 CFU g^{-1} at the beginning to 10^5 CFU g^{-1} at 14 days of ripening. Total coliforms decreased after 5 days while yeast and molds were detected along the fermentation period. No presumptive *S. aureus* colonies were observed on Baird-Parker medium with egg yolk tellurite emulsion during the ripening period (data not shown). The decrease in pH from 5.9 to 5.2 and the corresponding increase in lactobacilli counts are shown in Fig. 2. Lactobacilli were present in relatively low numbers (10^3 CFU g^{-1}) at time 0 and reached the highest level (10^7 CFU g^{-1}) after 11 days of ripening and then

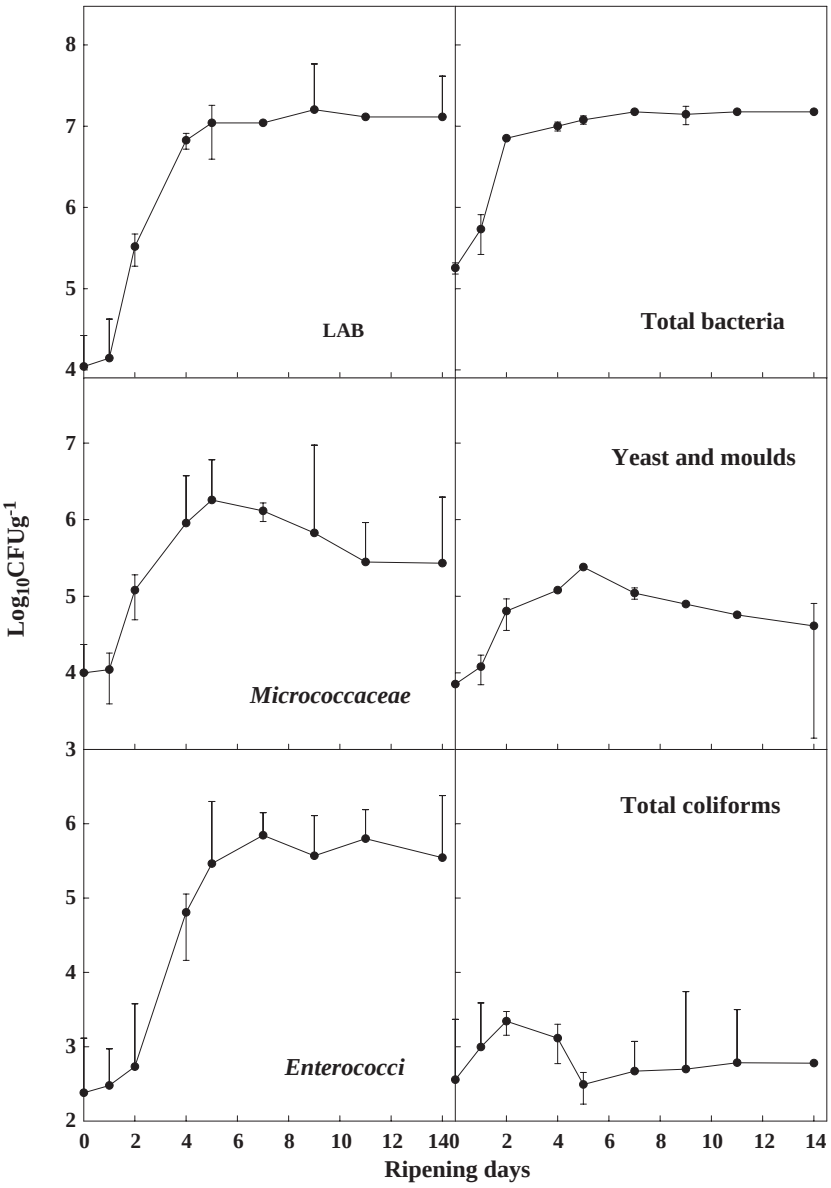

Fig. 1. Microbial population dynamics of sausages determined by classical methods during 14 days of natural fermentation.

remained stable in number. When 360 isolated colonies from MRS, Rogosa, SF and MSA were preliminarily characterized for their Gram reaction, catalase activity and cell morphology, it was possible to establish the main bacterial groups involved in the fermentation: gram positive catalase negative cocci and bacilli (LAB) and Gram positive catalase positive cocci (*Micrococcaceae*).

3.2. Population dynamics and community development during sausage fermentation

The isolated strains, 50 LAB and 50 belonging to *Micrococcaceae*, were analyzed using RAPD to identify the single strains and to monitor their growth and presence through the fermentation process. While RAPD allowed to achieve strain fingerprints, the taxo-

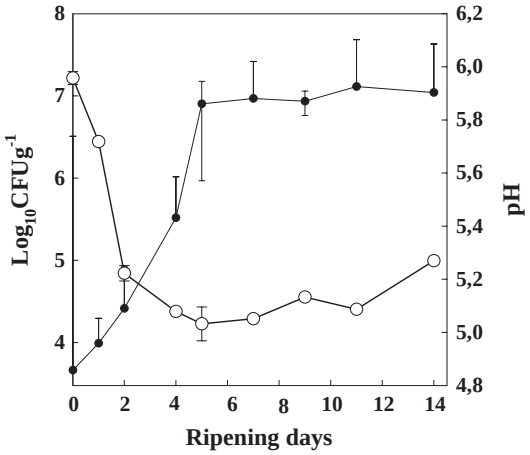

Fig. 2. Lactobacilli counts (●) and pH (○) decline during ripening.

nomical identification was achieved by sequencing the 5′ region of 16S RNA gene. Fig. 3 shows the growth dynamics of the main species of LAB isolated from MRS and Rogosa media using primer M13. Even though a high variability in the RAPD fingerprints at time 0 was observed indicating the presence of different strains in the indigenous flora of meat such as *Lactobacillus*, *Enterococcus* and *Pediococcus*, the bacterial association was dominated by three *Lactobacillus* species which were subsequently identified comparing their 16S DNA sequences as *L. sakei*, *L. plantarum* and *L. curvatus* (55%, 40% and 5% of the isolates).

To defined the best conditions for strain typing RAPD1, RAPD2 and XD9 primers were used on DNA from a subset of 30 strains, representing the two species: *L. plantarum* (17) and *L. sakei* (13) and the comparison of profiles obtained are shown in Fig. 4. Primer RAPD1 and RAPD2 were more discriminative than XD9 determining the presence of 7 different biotypes for each dominant specie indicating that there is a considerable polymorphism among the *L. plantarum* and *L. sakei* strains. The same RAPD protocol was applied to the SF medium isolates and were identified as *Enterococcus faecium*, *E. faecalis* and *P. acidilactici* by means of their 16S DNA sequences (data not shown).

To establish a picture of the *Micrococcaceae* community development, isolates from MSA medium were analyzed using the same set of primers as for LAB. A discrete number of bands were generated with primers M13, RAPD1 and RAPD2 that allowed the biotyping of isolates. The same informative patterns were obtained with primer XD9 but a lesser number of bands were obtained when compared with the other three primers (data not shown). RAPD patterns with RAPD1 primer for this bacterial group (Fig. 5) show a high variability at the first stage of the sausage fermentation (time 0) as occurred with LAB community allowing the differentiation of strains, which were subsequently identified as *S. sciuri*, *S. equorum*, *S. epidermidis*, *S. pulvereri* and *S. saprophyticus* by the analysis of 5′ region of 16S DNA. A

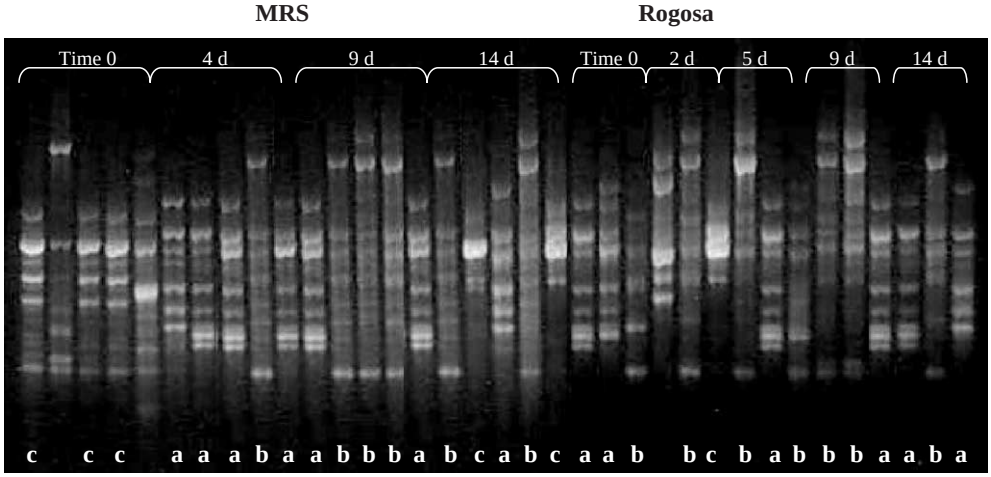

Fig. 3. RAPD fingerprints obtained with primer M13 of LAB isolated from MRS and Rogosa medium at different fermentation days. Three dominant profiles were found. a, b, and c identified as *L. sakei*, *L. plantarum*, and *L. curvatus*, respectively.

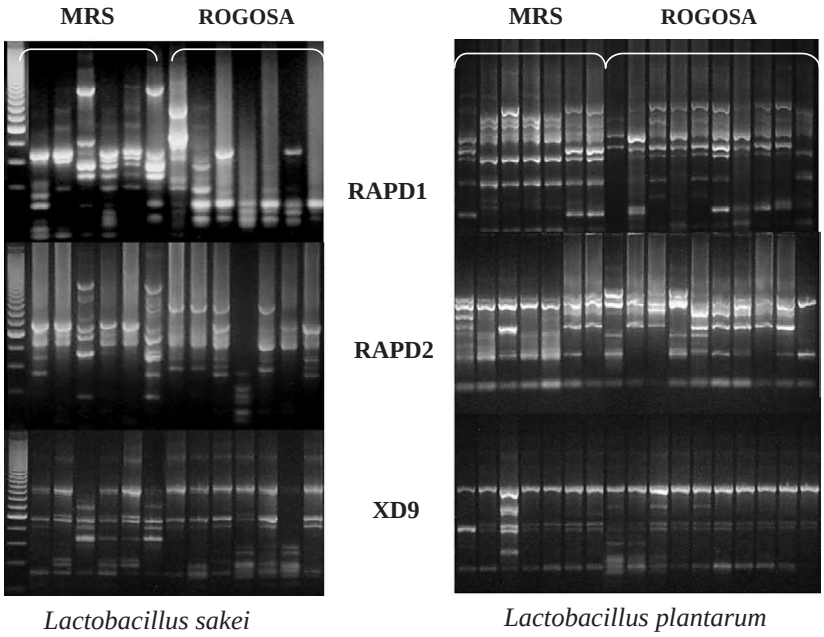

Fig. 4. Comparison of RAPD profiles obtained with primers RAPD1, RAPD2 and XD9 from purified DNA of 13 *L. sakei* strains and 17 *L. plantarum* strains isolated from MRS and Rogosa medium.

strong selection of the microflora after 5 days occurred and two different strains of *S. saprophyticus* become dominant in the population which were still present at the end of the production process.

3.3. Optimization of PCR-DGGE and fermented sausages DGGE profiles

PCR with primers P0–P4 amplified a 700 bp fragments from 5′ region of 16S rDNA gene and were used for the nested PCR with two primers combinations. Primer Bact-0124f (GC)-Univ-0515r was used to amplified 400 bp fragments of V2–V3 region and V3f (GC)-Univ-0515r to obtain 200 bp fragments of V3 region of 16S rDNA gene. The first combination and the application of a 30% to 60% denaturant gradient allowed differentiation of some members of lactic acid bacteria (LAB) (Fig 6a, lanes 1 to 5) and two members of *Micrococcaceae* family (Fig. 6a, lanes 6 and 7) isolated during the

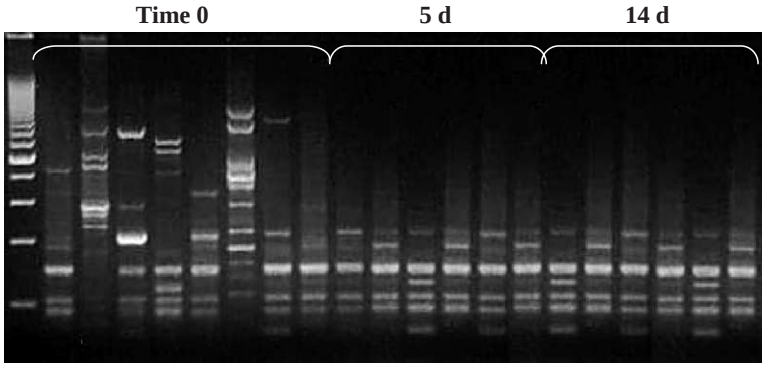

Fig. 5. RAPD fingerprints using primers RAPD1 for *Staphylococcus* populations during sausage fermentation. At time 0 several *Staphylococcus* species were identified but at 5 and 14 days only two strains identified as *S. saprophyticus* were isolated.

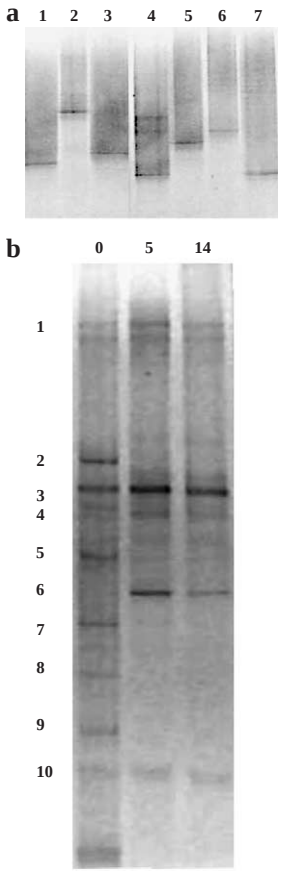

Fig. 6. DGGE analysis of PCR-amplified 16S rDNA fragments from culture pure: (a) *L. sakei* (lane 1), *L. plantarum* (lane 2), *L. pentosus* (lane 3), *E. faecalis* (lane 4), *E. faecium* (lane 5), *S. equorum* (lane 6), *S. saprophytcus* (lane 7). (b) DGGE profiles of the DNA amplicons obtained directly from fermented sausages. Profiles obtained at zero time and after 5 and 14 days of fermentation are shown. Bands indicated by numbers were identified as (1) *L. plantarum*, (2) *P. acidilactici*, (3) *L. sakei*, (4) *L. curvatus*, (5) *S. equorum*, (6) *S. saprophyticus* and (10) *C. variabilis*.

fermentation process giving a band that was reproducible and characteristic for each specie tested. The DNA extracted from each fermented sausage samples (time 0, 5 and 14 days) were used in PCR to obtain the V2–V3 and V3 region products that were analyzed by DGGE; two denaturant gradients proved to allow differentiation of the populations involved in fermentation.

The best DGGE patterns of the microbial community from fermented sausages were obtained with a 40% to 60% of denaturant gradient for the both primer combinations. The DGGE profiles obtained for the V3 region are shown in Fig. 6b: a high diversity was observed at the beginning of the fermentation process, when multiple bands were detected. Bands 2, 7, 8 and 9 were obtained only at time 0 and disappeared after 5 days, while bands 1, 3, 4 and 5 remained throughout fermentation showing different intensity during the process and only one band appear after 5 days and remained until the end. Bands 1 to 6 were identified by comparing their relative position of migration in the acrylamide gel with the DGGE profiles of the control strains as *L. plantarum*, *P. acidilactici*, *L. sakei*, *L. curvatus*, *S. equorum* and *S. saprophyticus*. Band 10 was excised from acrylamide gel, re-amplified to sequence and identified as *C. variabilis*. With the conditions applied in the DGGE protocol, the detection limit for individual members in the mixed populations was determined to be 10^4 CFU g^{-1} (data not shown).

4. Discussion

The use of combined techniques of traditional microbiological methods and molecular characterization allowed the study of the complex microbial associations involved in the production of an artisanal Argentinean fermented sausage and the microbial population development during the 14 days of ripening. Microbiological analysis used to study the growth kinetics of different bacterial groups in the fermented sausage confirmed the relevant role played by lactobacilli and staphylococci in the ripening process as was demonstrated by Hammes et al. (1990), Rebecchi et al. (1998) and Cocolin et al. (2001). The rapid increase of LAB numbers will assure an adequate decrease of the pH, this having a strong influence in the final texture and the hygienic quality of the product, while the growth of *Micrococcaceae* community guarantee the color formation and contribute to the flavor development (Talon et al., 2002).

The molecular techniques are shown to be efficient tools for the study of the complex associations developed in food fermentations and for the selection and characterization of dominant populations. The data obtained by RAPD fingerprinting techniques showed the strong selection effect of the stringent conditions

present in fermented sausages on the indigenous microflora. The selective pressure arising during the process, mainly the reduction in water potential, increase in ionic strength and decrease in pH and the competitive properties of sausage bacterial strains, led to a progressive reduction of bacterial populations such as *Enterobacteriaceae*, mold and yeasts.

For RAPD experiments the primers were chosen because of their high G+C content (approximately 70%) and they have been previously applied on other microorganisms. The analysis of LAB community begun with the use of M13 primer which allowed a preliminary selection between the isolated strains, indicating that this primer is able to discriminate this group only at species level. This primer was also used previously by Torriani et al. (1999) and Andrighetto et al. (2001) for differentiation of *Lactobacillus* species. A significant heterogeneity was observed within the species *L. plantarum* and *L. sakei* applying primers RAPD1, RAPD2 and XD9. Similar application of the RAPD1 and RAPD2 primers was also reported by Cocconcelli et al. (1997), Morea et al. (1999) and Baruzzi et al. (2000), when RAPD analysis was used to evaluate the growth dynamics of *Lactobacillus* strains in Ricotta forte cheese, Mozzarella cheese and whey fermentation.

With regard to XD9 primer, it was also used for typing experiments in the species *S. thermophilus* (Moschetti et al., 1998), although as reported by Torriani et al. (2001) no amplification was obtained with XD9 primer for the differentiation of *Lactobacillus* species. The RAPD analysis of the *Micrococcaceae* community developed during fermentation using the same set of primers resulted in strain-specific patterns even with primer M13. The applied RAPD protocol for this bacterial group was observed to be highly discriminative. The RAPD protocol here applied consisting in the use of a set of 4 primers allowed the design of a work sequence and showed to be effective for the study of the LAB and *Micrococcaceae* population dynamics in meat fermentation and for the selection and characterization of dominant population.

PCR-DGGE clearly has potential in the analysis of microbial systems from different fermented food in which many microorganisms are difficult to cultivate or are thought to be non-culturable. DGGE has recently been used to monitor microbial dynamics during production of Mexican fermented maize dough pozol (ben Omar and Ampe, 2000); during wine and Italian sausages fermentation (Cocolin et al., 2000; 2001); in the production of artisanal Sicilian cheese (Randazzo et al., 2002) and sourdough fermentation (Meroth et al., 2003). The primer combinations Bact-0124-GCf-Univ-0515r and V3f (GC)-Univ-0515r were considered suitable for obtaining good differentiation among the *Lactobacillus* and *Staphylococcus* species characterized in this work.

These strains were used as reference according to the migration of their 16S V3 region in the denaturing gradient gel when the microbial community from fermented sausage samples obtained at time 0, 5 and 14 days was analyzed by DGGE. By applying this method to the natural fermentation of sausages we were able to determine a strong LAB activity, represented by *L. sakei* which was the main organism responsible for the physical and organoleptic changes that occurred during fermentation of the studied sausages. Only two *Staphylococcus* strains were detected by DGGE, *S. equorum* and *S. saprophyticus*, who was not present at time 0 but appear at 5 days and remained during the rest of the fermentation process. These results are in agreement with the data obtained by microbiological, RAPD and 16S rDNA analyses. Even when *S. saprophyticus* is considered an opportunist pathogen it was observed to be part of the coagulase negative community generally found in fermented sausages (Gardini et al., 2003, Samelis et al., 1998). The used protocol for detecting the microbial changes during natural fermentation of sausages consisted in the application of a 30% to 60% denaturant gradient which demonstrated to be optimal for differentiation of *Lactobacillus*, while did not allow a good band resolution for *Staphylococcus* species. Moreover, the DGGE analysis revealed the presence of an additional species not identified by using culture dependent microbiological procedures. In fact, in all the analyzed samples (0, 5 and 14 days) a band not corresponding to anyone of the species previously identified by means of 16S rDNA sequencing was detected. The sequence of this DNA fragment revealed the presence in sausage of *C. variabilis*, whose role in the fermentation is not clear.

In conclusion, it was demonstrated in this work that the ripening process of Argentinean artisanal

fermented sausage is driven by a limited number of strains of lactobacilli and staphylococci selected from environmental contamination by the ability to best compete under the prevailing conditions of the ecological niche. The increasing numbers of large-scale industrial process are causing a continuous loss of microbial biodiversity, however the presence of small traditional meat plants in our country allows the study of natural microflora to prevent the extinction of unknown microbial genotypes.

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References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Andrighetto, C., Knijff, E., Lombardi, A., Torriani, S., Vancanneyt, M., Kersters, K., Swings, J., Dellaglio, F., 2001. Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. Journal of Dairy Research 68 (2), 303–316.

Aymerich, M.T., Hugas, M., Monfort, J.M., 1998. Review: bacteriocinogenic lactic acid bacteria associated with meat products. Food Science and Technology International 4, 141–158.

Baruzzi, F., Morea, M., Matarante, A., Cocconcelli, P.S., 2000. Changes in the *Lactobacillus* community during Ricotta forte cheese natural fermentation. Journal of Applied Microbiology 89, 807–814.

ben Omar, N., Ampe, F., 2000. Microbial community dynamics during production of the Mexican fermented maize dough pozol. Applied and Environmental Microbiology 66, 3664–3673.

Berthier, F., Ehrlich, S.D., 1999. Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design of PCR primers for its detection using randomly amplified polymorphic DNA. International Journal of Systematic Bacteriology 49, 997–1007.

Björkroth, J., Korkeala, H., 1996. rRNA gene restriction patterns as a characterization tool for Lactobacillus sake strains producing ropy slime. International Journal of Food Microbiology 30, 293–302.

Cocconcelli, P.S., Porro, D., Galandini, S., Senini, L., 1995. Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. Letters in Applied Microbiology 21, 376–379.

Cocconcelli, P.S., Parisi, M.G., Senini, L., Bottazzi, V., 1997. Use of RAPD and 16S rDNA sequencing for the study of *Lactobacillus* population in natural whey culture. Letters in Applied Microbiology 25, 376–379.

Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiology Letters 189, 81–87.

Cocolin, L., Manzano, M., Cantón, C., Comi, G., 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Applied and Environmental Microbiology 67, 5113–5121.

Coventry, J., Hickey, M.W., 1991. Growth characteristics of meat starter cultures. Meat Science 30, 41–48.

Dykes, G.A., Von Holy, A., 1995. Differentiation of *Lact. sake* and *Lact. curvatus* isolated from naturally fermented Greek dry salami by SDS-PAGE of whole-cell proteins. Journal of Applied Bacteriology 79, 480–482.

Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001. Behavior of variable V3 region from 16S rDNA of lactic acid bacteria in denaturing gradient gel electrophoresis. Current Microbiology 42, 199–202.

Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.C., Oliver, G., Toldrá, F., 1999. Hydrolysis of pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus* sake. Applied and Environmental Microbiology 65, 578–584.

Gardini, F., Tofalo, R., Suzzi, G., 2003. A survey of antibiotic resistance in *Micrococcaceae* isolated from Italian dry fermented sausages. Journal of Food Protection 66, 937–945.

Hammes, W.P., Bantleon, A., Min, S., 1990. Lactic acid bacteria in meat fermentation. FEMS Microbiology Reviews 87, 165–174.

Huey, B., Hall, J., 1989. Hypervariable DNA fingerprinting *Escherichia coli*: minisatellite probe from bacteriophage M13. Journal of Bacteriology 171, 2528–2532.

Hugenholtz, P., Goebbel, B.M., Pace, N.R., 1998. Impact of culture independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology 180, 4765–4774.

Johansson, G., Berdague, J.L., Larsson, M., Tran, N., Borch, E., 1994. Lipolysis, proteolysis and formation of volatile components during ripening of a fermented sausage with *Pediococcus pentosaceus* and *Staphylococcus xylosus* as starter cultures. Meat Science 38, 203–218.

Kandler, O., 1984. Current taxonomy of lactobacilli. In: Nash, C.H., Underkofler, L.A. (Eds.), Development in Industrial Microbiology, vol. 25. Victor Graphics, Baltimore, pp. 109–123.

Kenneally, P.M., Leuschner, R.G., Arendt, E.K., 1998. Evaluation of the lipolytic activity of starter cultured for meat fermentation purposes. Journal of Applied Microbiology 84, 839–846.

Klijn, N., Weerkamp, A.H., de Vos, W.M., 1991. Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes. Applied and Environmental Microbiology 57, 3390–3393.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematic. John Wiley & Sons, Chichester, United Kingdom, pp. 115–175.

Lücke, F.K., Hechelmann, H., 1987. Starter cultures for dry sausages and raw ham. Composition and effect. Fleischwirtschaft 67, 307–314.

Meroth, C., Walter, J., Hertel, C., Brendt, M., Hammes, W., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 475–482.

Morea, M., Baruzzi, F., Cappa, F., Cocconcelli, P.S., 1999. Molecular and physiological characterization of dominant bacterial population in traditional mozzarella cheese processing. Journal of Applied Microbiology 87, 574–582.

Moschetti, G., Blaiotta, G., Aponte, P., Catzeddu, P., Villani, F., Deiana, P., Coppola, S., 1998. Random amplified polymorphic DNA and amplified ribosomal DNA spacer polymorphism: powerful methods to differentiate *Streptococcus thermophilus* strains. Journal of Applied Microbiology 85, 25–36.

Muyzer, G., de Waal, E.C., Uitterlinden, G.A., 1993. Profiling of complex populations by denaturing gradient gel electrophoresis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695–700.

Myers, R.M., Maniatis, T., Lerman, L.S., 1987. Detection and localization of single base changes by denaturing gel electrophoresis. Methods in Enzymology 155, 501–527.

Nissen, H., Dainty, R., 1995. Comparison of the use of rRNA probes and conventional methods in identifying strains of *Lactobacillus sake* and *L. curvatus* isolated from meat. International Journal of Food Microbiology 25, 311–325.

Papa, F., Grazia, L., 1990. Produzione ed utilizzazione di coltore starter fresche nell'industria dei salumi. Industrie Alimentari XXIX, 537–540.

Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W., Vaughan, E., 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Applied and Environmental Microbiology 68, 1882–1892.

Rebecchi, A., Crivori, S., Sarra, P.G., Cocconcelli, P.S., 1998. Physiological and molecular techniques for the study of bacterial communities development in sausages fermentation. Journal of Applied Microbiology 84, 1043–1049.

Reuter, G., 1981. Psychotropic lactobacilli in meat products. In: Roberts, T.A., Hobbs, G., Christian, J.H.B., Skovgaard, N. (Eds.), Psychotropic Microorganisms in Spoilage and Pathogenicity. London Academic Press, pp. 253–258.

Rogosa, M., Mitchell, J.A., Wisemann, R.F., 1951. A selective medium for the isolation of oral and faecal lactobacilli. Journal of Bacteriology 62, 132–133.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Samelis, J., Metaxopoulos, J., Vlassi, M., Pappa, A., 1998. Stability and safety of traditional Greek salami—a microbiological ecology study. International Journal of Food Microbiology 44, 69–82.

Sanz, Y., Hernandez, M., Ferrus, M.A., Hernandez, J., 1998. Characterization of Lactobacillus sake isolates from dry-cured sausages by restriction fragment length polymorphism analysis of the 16S rRNA gene. Journal of Applied Microbiology 84, 600–606.

Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M.C., Oliver, O., Toldrá, F., 1999. Hydrolysis of muscle myofibrillar proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. International Journal of Food Microbiology 53, 115–125.

Stiles, M.E., 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70, 331–345.

Talon, R., Dublet, N., Montel, M.C., Cantonnet, M., 1995. Purification and characterization of extracellular *Staphylococcus warneri* lipase. Current Microbiology 30, 11–16.

Talon, R., Leroy-Sétrin, S., Fadda, S., 2002. Bacterial starters involved in the quality of fermented meat products. In: Toldrá, F. (Ed.), Research Advances in the Quality of Meat and Meat Products. Research Sign Post, India, pp. 175–191.

Torriani, S., Zapparoli, G., Dellaglio, F., 1999. Use of PCR-based methods for rapid differentiation of *Lactobacillus delbrueckii* sbsp. *bulgaricus* and *L. delbrueckii* sbsp. *lactis*. Applied and Environmental Microbiology 56, 4351–4356.

Torriani, S., Clementi, F., Vancanneyt, M., Hoste, B., Dellaglio, F., Kersters, K., 2001. Differentiation of *Lactobacillus plantarum*, *Lact. pentosus* and *Lact. paraplantarum* species by RAPD-PCR and AFLP. Systematic and Applied Microbiology 24, 554–560.

Wolf, G., Hammes, W.P., 1988. Effect of haematin on the activities of nitrate reductase and catalase in lactobacilli. Archives of Microbiology 149, 220–224.

Yost, C.K., Nattress, F.M., 2000. The use of multiplex PCR reactions to characterize populations of lactic acid bacteria associated with meat spoilage. Letters in Applied Microbiology 31, 129–133.