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PCR–DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages

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Abstract

Different PCR–DGGE protocols were evaluated to monitor fermentation process and to investigate bacterial communities developed in two artisanal Argentinean fermented sausages. Bacterial universal primers frequently used in PCR–denaturing gradient gel electrophoresis (DGGE) were evaluated. Lactic acid bacteria (LAB) and staphylococci species isolated from Tucumán sausages were used to determine the experimental conditions for PCR amplification and DGGE differentiation. Total microbial DNA extracted directly from both fermented sausages was subjected to DGGE analysis. PCR–DGGE results were different for each set of primers used. Primers Bact-0124f(GC)–Uni-0515r and V1f(GC)–V1r showed to be efficient to differentiate LAB and *Staphylococcus* cultures while the set V3f(GC)–Uni-0515r allowed to demonstrate the succession of different *Lactobacillus* and *Staphylococcus* species during ripening process. An intense band corresponding to *Lactobacillus sakei* was observed to be present in both samples. *Staphylococcus saprophyticus* was only observed in Tucumán sausage while a band identified as *Brochothrix thermophacta* was detected in Córdoba sausage. PCR–DGGE analysis of different 16S rDNA amplicons was able to discriminate between LAB and Gram-positive, coagulase-negative cocci, resulting an effective tool to establish the microbiota developed in artisanal dry sausages.

Keywords: 16S ribosomal DNA; Denaturing gradient gel electrophoresis; Dry sausages

1. Introduction

Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal DNA (rDNA) fragments has been frequently applied to the fingerprinting of natural bacterial populations. Species identification and population enumeration are critical in the study of microbial communities.

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Traditionally, microbial species are cultured and then characterized by their physiological and biochemical properties. This method, however, has a serious drawback because most of the bacteria cannot be readily isolated and cultured. It was estimated that only less than 1% of bacteria in the natural environment can be cultured (Amann et al., 1995). In the last few years, the development of molecular typing methods offered the possibility to advance more rapidly and efficiently on bacterial identification; moreover, direct sampling in complex matrices such as foods may avoid biases related to traditional methods. DGGE has been used to monitor microbial dynamics during production of Mexican fermented maize dough pozol (ben Omar and Ampe, 2000); Italian sausages fermentation (Cocolin et al., 2001, 2004); artisanal Sicilian cheese (Randazzo et al., 2002); buffalo Mozzarella and Stilton cheese (Ercolini et al., 2001b, 2003) and sourdough fermentation (Meroth et al., 2003). 16S rDNA fragments from different microbial species have the same length but different DNA sequences therefore the species can be identified by the band positions on the DGGE gel. DGGE allows the simultaneous analysis of multiple samples and the comparison of microbial communities based on temporal and geographical differences (Muyzer et al., 1998). Phylogenetic identification of individual members of the bacterial communities can be obtained either by excising DGGE bands from gel and their subsequent sequencing, or by the construction of the 16S rDNA clone libraries which are screened by DGGE (Muyzer et al., 1993).

Fermented dry sausages ("salame") are produced in different regions of Argentine using artisanal local techniques for their preparation including beef and/or pork meat, pork fat, salt and different spices. The meat, fat and other ingredients are mixed together and stuffed in pieces that can have different dimensions based on the type of sausage to be produced in each region. The knowledge and control of their typical in-house microbiota as well as the production processes are critical in terms of their microbiological quality and organoleptic characteristics, food safety being a top priority nowadays. Lactic acid bacteria (LAB) and staphylococci are the two main groups of bacteria technologically important in the fermentation and ripening of cured sausages. LAB are responsible for lactic acid production and for the "tangy" flavor of sausages, *Staphylococcus* and *Kocuria* are important for colour stabilization, decomposition of peroxides and protease and lipase production (Talon et al., 2002). Among LAB, *Lactobacillus sakei*, *L. curvatus* and *L. plantarum* are the most widely described species in fermented meat products (Hugas et al., 1993; Parente et al., 2001; Fontana et al., in press). However, in some slightly fermented sausages, such as Salame Felino, *Enterococcus* and *Lactobacillus* populations are balanced (Dellapina et al., 1994). Within the group of gram-positive coagulase-negative cocci isolated from meat, staphylococci are mainly isolated from dry cured and ripened products, whereas *Kocuria* spp. are dominant in freshly prepared sausages (Comi et al., 1992; Rodrguez et al., 1994). *Staphylococcus xylosus* is frequently isolated as the main *Staphylococcus* species, but other species have also been reported: *S. carnosus*, *S. simulans*, *S. saprophyticus*, *S. epidermidis*, *S. haemolyticus*, *S. warneri* and *S. equorum* (Comi et al., 1992; Hugas and Roca, 1997; Torriani et al., 1994).

The aim of this study was the evaluation of different PCR–DGGE protocols for monitoring the fermentation process and the comparison of microbial communities development in two artisanal Argentinean fermented sausages "salame".

2. Materials and methods

2.1. Sausages production and sampling procedures

Fermented sausages were prepared in two artisanal meat factories from different regions of Argentine according to traditional techniques. Tucumán region sausages formulation include: pork meat 85.5%, pork fat 10%, sodium chloride 2.6%, sugar (sucrose) 0.4%, starch 1.5%, binding and flavoring additives 1.0%, 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 to 15 °C and RH to 89% during the next 7 days. Duplicate samples of sausages were collected at start of the ripening

process (time 0) and at 5 and 14 days. The sampling was performed during one cycle of sausage production and was used for microbiological and molecular analysis. Córdoba region sausages formulation include: beef 35%, pork meat 40%, pork fat 20%, sodium chloride 2.8%, sugar (sucrose) 1.5%, binding and flavoring additives 1.0%, nitrite and nitrate 0.02%. The raw sausage material was mixed, stuffed into natural casings and fermented for 10 days at 25 °C and relative humidity (RH) of 95% followed by a gradual reduction of temperature to 19 °C and RH to 89% to complete ripening process. Duplicate samples of sausages were collected at final ripening time (20 days).

2.2. Microbiological analysis and preliminary physiological characterization of isolates

Ten grams of each sample were transferred into sterile stomacher bag, 90 ml of saline-peptone water (2% of NaCl, 0.1% of bacteriological peptone and 1% of Tween 80) were added and the preparation was mixed for 3 min in a stomacher (Stomacher Lab-Blender 400, A.J. Seward Lab. London, England). Additional decimal dilutions were prepared, and the following analysis were carried out: (i) total aerobic bacterial counts on Plate Count Agar incubated for 48 h at 30 °C; (ii) LAB on MRS incubated for 48 h at 30 °C under restricted oxygen conditions in an anaerobic jar (Anaerocult® C, Microphilic gas generator; Merck, Germany), (iii) staphylococci 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 Slanetz and Bartley agar, SB (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) yeasts and moulds on Sabouraud agar incubated at 30 °C for 48 to 72 h. After counting, means and standard deviations were calculated. A total of 100 colonies were randomly selected from MRS, MSA and SB plates for each sample of Tucumán sausage and subjected to DNA extraction. In parallel the isolates were characterized by means of cell morphology, Gram reaction and catalase activity. Each colony was also transferred to MRS and BHI (Brain hearth infusion) broth, incubated overnight at appropriate temperatures and stored at −80 °C in the same liquid media containing 30% glycerol. Unless otherwise specified all media and ingredients were obtained from Britania (Argentina).

All the experiences were carried out in triplicate. The results were statistically evaluated with one way ANOVA and statistical significance was considered when $P < 0.05$.

2.3. DNA extraction

DNA extraction from pure cultures was performed used Microlysis (LABOGEN, UK) according to the protocol described by the manufacturer.

Duplicate 10 g from each sample were homogenized in a stomacher bag with 10 ml of saline-peptone water for 3 min for total DNA extraction from sausages. One milliliter of each sample was washed with 200 μl ammonia hydroxide, 200 μl absolute ethanol, 400 μl petrol ether and 20 μl 10% SDS. The samples were centrifuged for 10 min at $14{,}000 \times g$ to pellet the cells, which were resuspended in a solution containing 200 μl 6 M Urea, 200 μl absolute ethanol, 400 μl petrol ether, 80 μl 10% SDS and 13 μl 3 M sodium acetate. A second centrifugation for 10 min at $14{,}000 \times g$ was performed, the pellet was resuspended in 0.6 ml TE buffer (Tris-EDTA) pH 8.0 and 100 μl 50 mg/ml lysozyme (Sigma) was added. After 60 min of incubation at 37 °C in shaker, 35 μl of 10% SDS and 10 μl of 10 mg/ml DNase-free RNase were added. The tubes were incubated at 37 °C for 30 min before the addition of 10 μl of 20 mg/ml proteinase K (Sigma), this preparation being incubated for 30 min and finally 130 μl 6 M sodium perchlorate and 500 μl (25:24:1; pH 6.7) phenol–chloroform–isoamyl alcohol (Sigma) were added for DNA extraction. The tubes were then centrifuged at $12{,}000 \times g$ for 5 min, the aqueous phases were collected, and the nucleic acids were precipitated with isopropanol. The DNA was obtained by centrifugation at $14{,}000 \times g$ for 15 min, washed briefly with 70% ethanol and the pellets were dried under vacuum at room temperature. Fifty microliters of sterile water was added and the preparations were incubated for 30 min at 37 °C to facilitate nucleic acid solubilization.

2.4. PCR–DGGE analysis

The primers used in this study are listed in Table 1. All PCR were performed by use of PCR Master Mix 2× (Promega, Italy). PCR volumes of 50 μl contained 25 μl of PCR Master Mix, 0.5 μl of each primer (0.1 μM final concentration), 22 μl of sterile Milli-Q water, and 2 μl of 10-fold-diluted DNA solution (10 ng) and the following PCR program: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 56 °C for 30 s, and 72 °C for 1 min; and finally 7 min at 72 °C followed by cooling to 4 °C were used for all amplifications. PCR products were analyzed on a 1.5% agarose gel electrophoresis before DGGE analysis. In this study three sets of primers were used: V1f–V1r, Bact-0124f–Uni-0515r and V3f–Uni-0515r, targeting the V1, V2–V3 and V3 region of the bacterial 16S rRNA gene, respectively. A GC clamp was attached to the 5′ end of primer Bact-0124f, V3f and V1f to facilitate the resolution of PCR amplicons by DGGE. To determine DGGE sensitivity, different concentrations (10^2, 10^4, 10^6 and 10^8 CFU/g) of a *Streptococcus thermophilus* 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 as above described. DGGE was performed on a Dcode universal mutation detection system (Bio-Rad, Hercules, CA), 16S rDNA fragments were loaded onto an 8% polyacrylamide gel. In order to obtain the best discrimination between bacterial species, different denaturing gradients were assay for each PCR products obtained with the three sets of primers used. A 40% to 60% denaturing gradient (100% corresponded to 7 M urea and 40% formamide) was used for Bact-0124f(GC)–Uni-0515 and for V1f(GC)–V1r primer sets and 35–60% for V3f(GC)–Uni-0515. Electrophoresis was run in 1× TAE buffer at 60 °C, for 4 h 30 min at 120 V, after that, gels were stained in a SYBR Green solution for 15 min, visualized and photographed under UV.

2.5. Sequencing of DGGE bands and sequence analysis

Small pieces of selected DGGE bands were punched from the gel with sterile pipette tips. Each piece was then 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 were used for re-amplification with primer without the GC clamp. PCR products purified by using Wizard SV Gel and PCR Clean-Up System (Promega, Italy) and sequences were carried out by CRIBI, Universitá degli Studi, Padova (Italy).

2.6. Taxonomic identification

According to Klijn et al. (1991), 5′ region of 16S rDNA gene was amplified from pure cultures. PCR products were purified and sequenced as described before. 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. The GeneBank accession numbers for the nucleotide sequences of some of the species isolated in this study are listed in Table 3.

Table 1
Primers used in this study

Primer	Primer sequence (5′–3′)	Reference
Bact-0124GCf	CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G GGA CGG GTG AGT AAC ACG	Lane, 1991
Uni-0515r	ATC GTA TTA CCG CGG CTG CTG CTG GCA	Lane, 1991
V3GCf	CC GGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG	Ercolini et al., 2001a,b
V1GC f	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G GCG GCG TGC CTA ATA CAT GC	Klijn et al., 1991
V1r	TTC CCC ACG CGTTAC TCA	Klijn et al., 1991
P0	GAG AGT TTG ATC CTG GCT	Klijn et al., 1991
P4	ATC TAC GCA TTT CAC CGC	Klijn et al., 1991

3. Results

3.1. Microbiological analysis of fermented sausages

Table 2 shows the results obtained by traditional enumeration of microorganisms from sausages of Tucumán and Córdoba regions at the end of ripening process. Number of total bacterial and LAB counts were observed to be highly similar (10^7 CFU/g), while enterococci and staphylococci populations from Córdoba sausage were observed to be 2 and 1 log cycles lower, respectively. Absence of *S. aureus* was observed in Baird Parker medium while the number of total coliforms and yeasts and moulds reached 10^2 CFU/g and 10^3 CFU/g, respectively. Samples from Tucumán region from time 0 to 5 days showed that LAB and *Staphylococcaceae* counts changed from 10^4 CFU/g to 10^7 CFU/g and 10^6 CFU/g, respectively. LAB populations remained stable up to the end of ripening while a 1 log decrease in final counts of *Staphylococcaceae* was observed at 14 days (data not shown).

3.2. Differentiation of bacterial strains by DGGE analysis

A total of 100 colonies isolated from MRS, MSA and SB media during ripening of Tucumán sausage were analyzed by PCR–DGGE in order to establish a strain reference database. Primary differentiation and grouping of the strains was performed using Bact-0124f(GC)–Uni-0515r primer set to amplify V2–V3 regions of 16S rRNA gene. Analysis of amplicons (~400 bp) on a DGGE gel with a denaturing gradient from 40% to 60% showed the same DGGE mobility for many strains; however 12 different profiles were distinguished (Fig. 1). By means of sequencing and alignment of the 16S rDNA PCR product generated with primer pair P0–P4, the strains with different DGGE profiles were identified as *L. sakei*, *L. plantarun*, *L. pentosus*, *L. curvatus*, *S. saprophyticus*, *S. equorum*, *Pediococcus acidilactici*, *Enterococcus faecalis*, *E. flavescens*, *E. faecium*, *E. mundtii* and *E. durans.* The relative identification obtained by alignment in GenBank and accession numbers for the submitted sequences are reported in Table 3. 60% of the isolated strains with identical DGGE patterns were identified as *L. sakei*. The same informative pattern was obtained with set V1f(GC)–V1r that targets V1 region of bacterial 16S rRNA gene (~100 bp) but some single strains showed profiles formed by too many bands (Fig. 2).

Table 2
Microbial counts at the end of ripening process determined by plating

Organisms	$\log_{10}$ CFU/g			
	Tucumán		Córdoba sausage	
	14 days		20 days	
	Mean	S.D.	Mean	S.D.
Total bacteria	7.2	0.01	7.5	0.05
LAB	7.1	0.00	7.1	0.06
Staphylococcaceae	5.4	0.05	4.0	0.10
Yeasts and molds	4.6	0.07	3.8	0.01
Fecal enterococci	5.5	0.21	3.5	0.08
Total coliforms	2.8	0.28	2.8	0.25
S. aureus	0.0	N.A.	0.0	N.A.

Abbreviation: N.A., not applicable; S.D., standard deviation.

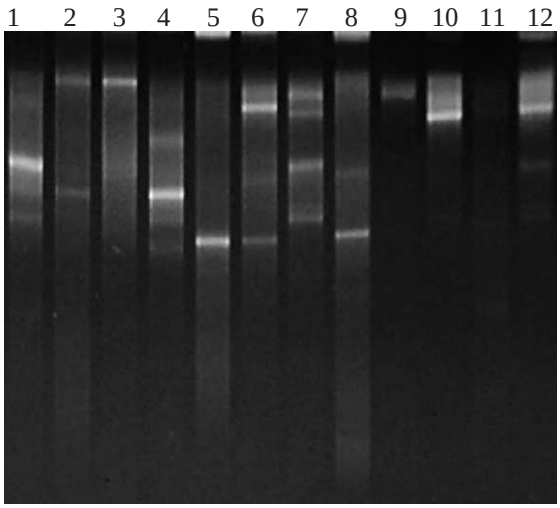

Fig. 1. DGGE profile of PCR products originated with Bact-0124f(GC)–Univ-0515r primers from the strains isolated in this study. Lane 1, *L. sakei*; lane 2, *L. plantarum*; lane 3, *L. pentosus*; lane 4, *L. curvatus*; lane 5, *S. saprophyticus*; lane 6, *S. equorum*; lane 7, *P. acidilactici*; lane 8, *E. faecalis*; lane 9, *E. flavescent*; lane 10, *E. faecium*; lane 11, *E. mundtii*; lane 12, *E. durans*.

3.3. Direct analysis of fermented sausages by DGGE

The best results from the direct analysis of microbial community developed in Tucumán region sausage by DGGE were obtained amplifying V3 region of 16S rRNA gene using primer V3f(GC)–Uni-0515r set.

Table 3
Strains and DGGE bands identified in this study by means of 16S rDNA sequencing

Closest relatives	% ID	Accession no.
L. sakei	100	AY499536
L. curvatus	100	AY865645
L. plantarum	99	AY499536
L. pentosus	100	AY362452
E. faecalis	100	AY865649
E. durans	99	AY865650
E. flavescens	100	AY865651
E. mundtii	100	AY946202
S. saprophyticus	100	AY865647
S. equorum	99	AY865648
P. acidilactici	100	AY865646
Corinebacterium variabilis[a]	98	AY499536
Brochrothrix thermophacta[a]	96	AY946201

ID represents the identity with the sequences in the GenBank databases.
[a] DGGE band of total bacteria community DNA.

When a denaturing gradient of 35–60% was applied to a 200 bp PCR product, a high microbial diversity at the beginning of the fermented process was observed, this being evidenced by the presence of multiple bands (Fig. 3). Bands 5, 7 and 8 were obtained only at time 0

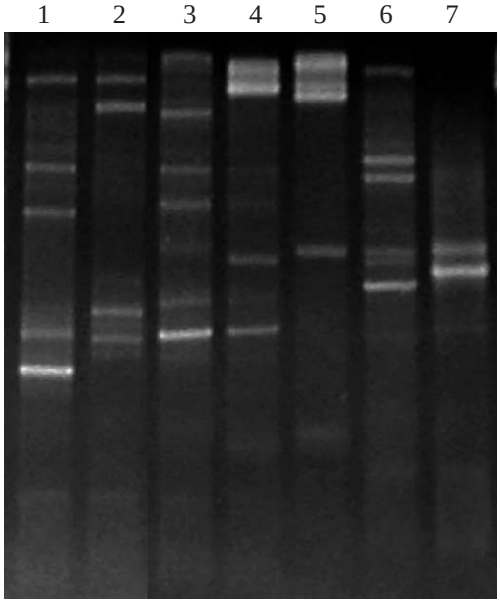

Fig. 2. DGGE fingerprints obtained using V1f(GC)–V1r primer set: lane 1, *S. saprophyticus*; lane 2, *S. equorum*; lane 3, *L. sakei*; lane 4, *L. plantarum*; lane 5, *L. curvatus*; lane 6; *E. faecium*; lane 7, *E. faecalis.*

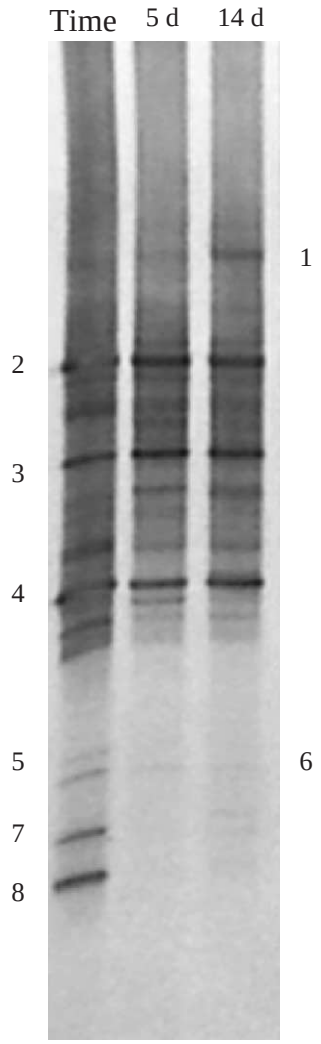

Fig. 3. DGGE profiles of the bacterial community from DNA directly extracted from Tucumán sausage at time 0, 5 and 14 days. Bands indicated by numbers were excised and, after re-amplification, subjected to sequencing.

disappearing after 5 days, while bands 1, 2, 3, 4 and 6 remained throughout fermentation showing different intensity during the process. Bands 1, 2 and 3 were identified by comparing their relative position of migration in the acrylamide gel with the DGGE profiles of the control strains as *L. plantarum*, *L. sakei*, and *S. saprophyticus*, respectively (data not shown) and reconfirmed by sequencing. The bands 4 and 6 were excised from acrylamide gel, re-amplified to sequence and identified as an uncultured bacterium and *Corynebacterium variabilis*, respectively. The other less

intense bands were identified as microorganisms that were present as natural contaminants of the meat and were not taken into account. 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 (data not shown). When DGGE profiles from the two Argentinean sausages were compared at the end of ripening time (Fig. 4) one common band corresponding to *L. sakei* was observed (band a). Nevertheless a different final microbiota on each sample was detected. Sausages from Tucumán region (line 1) showed other two intense bands (b and c) which were identified as *S. saprophyticus* and uncultured bacteria, respectively, while band d was only detected in sausage from Córdoba region (line 2), and was identified by sequencing after re-amplification as *Brochothrix thermophacta*. Lane 3 and lane 4 show the migration for *L. sakei*, and *S. saprophyticus* used as reference strains.

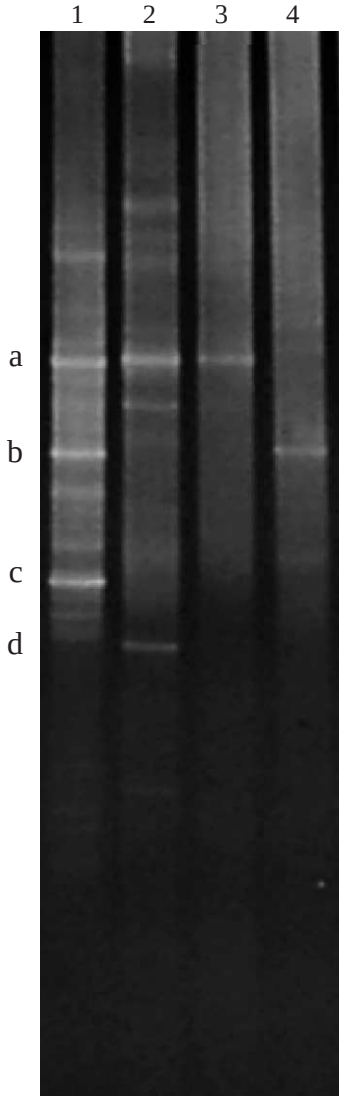

Fig. 4. DGGE profiles of the bacterial community from DNA directly extracted from Tucumán sausage (lane 1) and Córdoba sausage (lane 2) at the end of ripening process (14 and 20 days, respectively). Lanes 4 and 5 corresponding to DGGE patterns of pure culture from *L. sakei* and *S. saprophyticus*, respectively. Bands indicated by letters are identified as (a) *L. sakei*; (b) *S. saprophyticus*; (c) uncultured bacteria and (d) *B. thermophacta*.

4. Discussion

Microbiological analysis used to study the growth kinetics of different bacterial groups in fermented sausages produced in two different Argentinean regions confirmed the relevant role played by lactic acid bacteria (LAB) and staphylococci in the ripening process as was demonstrated by Hammes et al. (1990), Rebecchi et al. (1998) and Cocolin et al. (2001). Comparing the final counts obtained for both samples, no significant changes were observed in the numbers of LAB while significant differences ($P<0.05$) were found in *Staphylococcus* and *Enterococcus* populations, these counts being higher in Tucumán region sausages. This result would suggest that the type and number of microorganisms developed is often closely related to the applied house-making techniques (ingredient composition, fermentation and maturation parameters) as well as the ecological diversity present in different regions.

The molecular techniques are shown to be efficient tools for the study of the complex bacterial associations developed in food fermentations and for the selection and characterization of dominant populations. PCR–DGGE has been only recently introduced to study microbial fermentations in food and food-related ecosystems. Different bacterial species have differences in base pair composition within the variable regions of the 16S rDNA, which makes it possible to distinguish them by this technique. In fact, each species is theoretically supposed to yield a different DGGE profile after

the amplification of variable regions of the 16S rDNA (Ercolini, 2004). This paper exploited the potential of the DGGE technique to separate DNA molecules with few base pair changes in the sequence. The protocol applied here use universal primers selected from those available to analyze different variable regions of the 16S rDNA in order to obtain the best differentiation among bacterial communities developed during sausages fermentation. Primers set Bact-0124f(GC)–Uni-0515r were considered suitable for a good differentiation among lactic acid bacteria and *Staphylococcus* without band co-migration for different species. Even when the same information was obtained analyzing V1 variable region of 16S rRNA gene using V1f(GC)–V1r set, DGGE profile for some single strains showed too many bands. These results were in agreement with those of Cocolin et al. (2001) who monitored the changes in bacterial population during fermentation of Italian sausage by PCR–DGGE analysis of V1 variable region. The presence of multiple copies of the 16S rDNA genes with sequence microheterogeneity represent a problem in the study of community diversity using DGGE due that single species can display a DGGE profile characterized by multiple bands, which overestimates the community diversity detected by DGGE (Nübel et al., 1996).

Analysis of V3 amplicons by DGGE allowed monitoring the bacterial communities developed in Tucumán samples during fermentation process. A strong LAB occurrence, represented by *L. sakei* was determined and *S. saprophyticus* was also detected in all samples, these organisms are probably responsible for the physical and organoleptic changes that occurred during ripening. DGGE mobility of V3 regions of 16S rDNA was also used to differentiate *Micrococcaceae* from fermented sausages by comparison of the migration of the amplicons from the isolates with PCR products from reference strains (Cocolin et al., 2001). Blaiotta et al. (2003) recently used the same strategy for the identification of staphylococci from fermented sausages. However, cases of co-migration were observed when amplified variable V3 region of the 16S rDNA was used to differentiate and identify lactic acid bacteria isolated from food (Ercolini et al., 2001a). In general, bacterial identification to the specie level is not possible for all genera, specially if only partial 16rDNA sequences such as the hypervariable V3 region are analyzed (Vandamme et al., 1996).

The use of universal primers can cause problems related to masking effects due to bacteria present in high numbers as occurs in the intestine ecosystem (Walter et al., 2001). In contrast, in food systems as fermented sausages, in which the dominant populations are restricted to two main bacterial groups (LAB and *Staphylococcus*), the suitability of the universal primers used was based on the discriminatory efficiency of the 16S bacterial rDNA variable regions that allowed species differentiation from these dominant groups.

In order to establish the detection limit of the PCR–DGGE different concentrations (10^2, 10^4, 10^6 and 10^8 CFU/g) of a *S. thermophilus* strain (specie not generally present in sausages) were inoculated to the homogenate from sausage samples before DNA extraction and then subjected to PCR–DGGE being detected from 10^4 CFU/g (data not shown). This results are in agreement with Cocolin et al. (2001) who reported that in mixed populations, individual members can be identified by PCR–DGGE when the concentrations are higher than 10^4 CFU/g. The detection limit depends on the species and perhaps even the strain considered. Moreover, the number and the concentration of the other members of the microbial community, along with the nature of the food matrix, all represent variables influencing the detection limit of DGGE by affecting both the efficiency of DNA extraction and the PCR amplification due to the possible competition among templates (Ercolini, 2004).

DGGE fingerprints from Córdoba sausages at end of ripening only showed two intense bands, one corresponding to *L. sakei* from LAB community in which counts reached 10^7 CFU/g (Table 2) and a second band identified as *B. thermosphacta* that would have been present in high numbers in this fermented sausage. However, members from *Staphylococcus* group were not able to be detected by DGGE in accordance with the low microbiological numbers found (Table 2).

In this work we confirm the suitability of PCR–DGGE analysis to discriminate bacterial populations present in sausage ecosystems, which can be achieved simply by a singly PCR using available universal primers.

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