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

LUCA COCOLIN,^{1*} MARISA MANZANO,¹ CARLO CANTONI,² AND GIUSEPPE COMI¹

Dipartimento di Scienze degli Alimenti, Facoltà di Agraria, Università degli studi di Udine, 33100 Udine,¹ and Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza degli Alimenti, Facoltà di Medicina Veterinaria, Università degli studi di Milano, 20121 Milan,² Italy

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In this study, a PCR-denaturing gradient gel electrophoresis (DGGE) protocol was used to monitor the dynamic changes in the microbial population during ripening of natural fermented sausages. The method was first optimized by using control strains from international collections, and a natural sausage fermentation was studied by PCR-DGGE and traditional methods. Total microbial DNA and RNA were extracted directly from the sausages and subjected to PCR and reverse transcription-PCR, and the amplicons obtained were analyzed by DGGE. Lactic acid bacteria (LAB) were present together with other organisms, mainly members of the family *Micrococcaceae* and meat contaminants, such as *Brochothrix thermosphacta* and *Enterococcus* sp., during the first 3 days of fermentation. After 3 days, LAB represented the main population, which was responsible for the acidification and proteolysis that determined the characteristic organoleptic profile of the Friuli Venezia Giulia fermented sausages. The PCR-DGGE protocol for studying sausage fermentation proved to be a good tool for monitoring the process in real time, and it makes technological adjustments possible when they are required.

The microbiology of fermented sausages is varied and complex. The type of microflora that develops is often closely related to the ripening technique utilized. Sausages with a short ripening time have more lactobacilli from the early stages of fermentation, and at the end of ripening an acid flavor with little aroma predominates. In contrast, sausages with longer maturation times contain higher numbers of *Micrococcaceae* in the early stages of fermentation. Members of the *Micrococcaceae* have a low rate of acidification and produce protease and lipase and thus release various aromatic substances and organic acids (11).

Manufacturing of fermented sausages has a long history in Italy, and there are a wide variety of typical preparations (32). Many typical fermented meat products are still produced with traditional technologies without selected starters. However, the use of starter cultures for sausage production is becoming increasingly necessary to guarantee safety and to standardize product properties, including consistent flavor and color and shorter ripening time.

In the Friuli Venezia Giulia region in northeast Italy, a traditional fermented sausage is produced without microbial starters; this sausage is characterized at the end of ripening by accentuated acidity, slight sourness, and an elastic semihard consistency. This product is produced from fresh pork meat and lard that are mixed with other ingredients, such as sugars, NaCl, and additives (nitrate, nitrite, and spices). According to

company guidelines, starters can be added, but this is usually done only for large-scale production.

A wide variety of microorganisms have already been isolated from sausage fermentations by traditional methods. These microorganisms are mainly lactic acid bacteria (LAB) and *Staphylococcus* and *Kocuria* spp. (9, 16).

Due to the known limitations of conventional microbiological methods, characterization of microorganisms that require selective enrichment and subculturing is problematic or impossible. Moreover, in the last decade it was shown that classical microbial techniques do not accurately detect microbial diversity (3, 17). One culture-independent method for studying the diversity of microbial communities is analysis of PCR products, generated with primers homologous to relatively conserved regions in the genome, by using denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (15, 23, 24). These approaches allow separation of DNA molecules that differ by single bases (25) and hence have the potential to provide information about variations in target genes in a bacterial population. By adjusting the primers used for amplification, both major and minor constituents of microbial communities can be characterized.

The aim of the present study was to use molecular approaches to describe the bacterial diversity during natural fermentation of Italian sausages. The PCR-amplified V1 region of the 16S rRNA gene (rDNA) was analyzed by DGGE to monitor the evolution of the predominant populations during the aging period. The 16S rRNA and rDNA profiles obtained were compared to determine the active population responsible for the changes that occurred during ripening of the sausages. For comparison purposes, LAB strains were isolated from fer-

* Corresponding author. Mailing address: Dipartimento di Scienze degli Alimenti, Via Marangoni 97, 33100 Udine, Italy. Phone: 0039/0432/590-746 or 0039/0432/590-730. Fax: 0039/0432/590-719. E-mail: luca.cocolin@dsa.uniud.it.

TABLE 1. PCR primers used in this study

Primer ^a	Sequence, 5'–3'	Position ^b	16S rRNA gene target region	Reference
338f (S) ^{c,d}	ACT CCT ACG GGA GGC AGC AG	338–357	V3	2
518r (A)	ATT ACCGCG GCT GCT GG	518–534		23
Ec1055 (S)	ATG GCT GTC GTC AGC T	1055–1070	V9	13
Ec1392 (A) ^c	ACG GGC GGT GTG TAC	1392–1406		
HDA1 (S) ^{c,d}	ACT CCT ACG GGA GGC AGC AG	338–357	V2–V3	30
HDA2 (A)	GTA TTA CCG CGG CTG CTG GCA C	539–561		
U968 (S) ^c	AAC GCG AAG AAC CTT AC	968–985	V6–V8	33
L1401 (A)	GCG TGT GTA CAA GAC CC	1401–1418		
P1 (S) ^c	GCG GCG TGC CTA ATA CAT GC	41–60	V1	19
P2 (A)	TTC CCC ACG CGT TAC TCA CC	111–130		
P3 (S) ^c	GGA ATC TTC CAC AAT GGG CG	361–380	V3	19
P4 (A)	ATC TAC GCA TTT CAC CGC TAC	385–705		

^a (S), forward primer; (A), reverse primer.

^b *E. coli* numbering.

^c A GC clamp (CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G) (28) was attached to the 5' end of the primer.

^d Primers 338f and HDA1 have the same sequence, but they were given different designations by the authors who described them.

mented sausages by traditional plating techniques and were identified by molecular methods.

MATERIALS AND METHODS

Bacterial control strains. *Lactobacillus sake* DSM 6333, *Lactobacillus casei* DSM 20011, *Lactobacillus curvatus* subsp. *curvatus* DSM 20019, *Lactobacillus brevis* DSM 20054, *Lactobacillus plantarum* DSM 20174, *Lactobacillus alimentarius* DSM 20249, *Staphylococcus xylosus* DSM 6179, *Kocuria kristinae* DSM 20032, *Kocuria varians* DSM 20033, *Staphylococcus simulans* DSM 20322, *Staphylococcus intermedius* DSM 20373, and *Staphylococcus carnosus* subsp. *carnosus* DSM 20501 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and were used for optimization of the PCR-DGGE method.

Fermented sausage technology and sampling procedures. Fermented sausages were prepared in a local meat factory by traditional techniques. Sixty kilograms of pork meat, 40 kg of lard, 2.5 kg of sodium chloride, 1.5 kg of sugars, 200 ppm of nitrite and nitrate, and 70 g of black pepper were mixed and used to fill natural casings; the procedure used resulted in fresh sausages that were 25 cm long and 5 cm in diameter. Ripening was performed as follows. The first stage consisted of 2 days of drying with a relative humidity (RH) of 85% at 22°C; the temperature was then decreased to 12°C at a rate of 2°C per day with a RH between 60 and 90%. Ripening was then carried out for 38 days at 12°C in storerooms with 65 to 85% RH. Triplicate samples of the meat mixture prior to filling and of sausages obtained at 3, 10, 20, 30, and 45 days were used for microbiological and molecular analyses.

pH measurements. Potentiometric pH measurements were obtained with the pin electrode of a pH meter (pH M82; Radiometer Copenhagen, Cecchinato, Italy) that was inserted directly into a sample. Three independent measurements were obtained for each sample. Means and standard deviations were calculated.

Microbiological analysis. 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. Twenty-five grams of each sample was transferred into a sterile stomacher bag, 225 ml of saline-peptone water (8 g of NaCl per liter, 1 g of bacteriological peptone [Oxoid, Milan, Italy] per liter) was added, and the preparation was mixed for 1.5 min in a stomacher machine (PBI, Milan, Italy). Additional decimal dilutions were prepared, and the following analyses were carried out on duplicate agar plates: (i) total aerobic mesophilic flora on peptone agar (8 g of bacteriological peptone per liter, 15 g of bacteriological agar [Oxoid] per liter) that was incubated for 48 to 72 h at 30°C; (ii) LAB on MRS agar (Oxoid) that was incubated in a double layer at 30°C for 48 h; (iii) *Micrococcaceae* on mannitol salt agar (Oxoid) that was incubated at 30°C for 48 h; (iv) total enterobacteria and *Escherichia coli* on Coli-ID medium (Biomerieux, Marcy l'Etoile, France) that was incubated in a double layer at 37°C for 24 to 48 h; (v) fecal enterococci on kanamycin esculin

agar (Oxoid) that was incubated at 42°C for 24 h; and (vi) *Staphylococcus aureus* on Baird-Parker medium (Oxoid) with egg yolk tellurite emulsion (Oxoid) that was incubated at 37°C for 24 to 48 h. After counting, means and standard deviations were calculated. Ten LAB strains from MRS plates for each sample were randomly selected, streaked on MRS agar, and stored at –20°C in MRS broth containing 30% glycerol before they were subjected to DNA extraction, PCR, and DGGE.

DNA extraction from pure cultures. A single colony, from an MRS agar plate incubated at 30°C for 24 h, was resuspended in 200 µl of sterile distilled water, and 10 µl of proteinase K (25 mg/ml; Sigma, Milan, Italy) was added. The DNA was extracted by incubation at 65°C for 1.5 h followed by treatment at 100°C for 10 min. Five microliters was transferred to a PCR mixture after centrifugation at 8,000 × g for 5 min at 4°C.

Extraction of DNA and RNA from fermented sausages. At each step of the ripening process, triplicate 10-g samples were homogenized in a stomacher bag with 10 ml of saline-peptone water for 1 min. After each preparation had settled for 1 min, two 1-ml subsamples (one for DNA extraction and one for RNA extraction) were placed in 1.5-ml screw-cap tubes containing 0.3 g of glass beads. The samples were centrifuged at 4°C for 10 min at 14,000 × g to pellet the cells, which were resuspended in 500 µl of a 10% (wt/vol) sucrose solution containing 25 µl of lysozyme (50 mg/ml; Sigma). After 30 min of incubation at 37°C, a second centrifugation for 10 min at 14,000 × g at 4°C was performed, the pellet was resuspended in 500 µl of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris [pH 8], 1 mM EDTA [pH 8]), and 25 µl of proteinase K (10 mg/ml) was added. The tubes were incubated at 65°C for 1 h before the preparations were subjected to bead beater treatment. Five hundred microliters of phenol-chloroform (5:1; pH 4.7; Sigma) for extraction of RNA or 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1; pH 6.7; Sigma) for extraction of DNA was added to each tube, and three 30-s treatments at the maximum speed, with 10-s intervals between treatments, were performed with a bead beater (Mini Bead Beater 8; Biospec Products, Inc., Bartlesville, Okla.). The tubes were then centrifuged at 12,000 × g at 4°C for 10 min, the aqueous phases were collected, and the nucleic acids were precipitated with ice-cold absolute ethanol. The DNA and RNA were collected by centrifugation at 14,000 × g and 4°C for 10 min, 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 45°C to facilitate nucleic acid solubilization. One microliter of DNase-free RNase (Roche Diagnostics, Mannheim, Germany) and 1 µl of RNase-free DNase (Roche Diagnostics) were added to digest RNA and DNA, respectively, during incubation at 37°C for 1 h. Each RNA solution was checked for the presence of residual DNA by performing PCR amplification. When positive signals were detected, the DNase treatment was repeated to eliminate all of the DNA.

PCR and reverse transcription (RT)-PCR protocol. Different regions of the 16S rDNA were amplified with the primers listed in Table 1 in order to deter-

mine the primers that provided the best DGGE differentiation of the *Lactobacillus*, *Staphylococcus*, and *Kocuria* spp. involved in fermentation of sausages. Amplification was conducted in a standard reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1.25 IU of *Taq* polymerase, and each primer at a concentration of 0.2 µM; the only exception was the mixture used for primers Ec1055/Ec1392 and primers U968/L1401, in which the MgCl₂ concentration was increased to 3 mM. Two microliters of template DNA was added to each mixture. Amplifications were carried out with a Minicycler (MJ Genenco, Florence, Italy) by using a final volume of 50 µl and the cycle conditions suggested by the authors. For the P1 and P2 primers a different amplification cycle was used, consisting of an initial touchdown procedure in which the annealing temperature was decreased from 60 to 52°C at a rate of 2°C every two cycles and then 20 additional annealing cycles at 50°C. A denaturation step of 95°C for 1 min was used, and extension was performed at 72°C for 2.5 min; a final extension of 72°C for 5 min ended the amplification cycle. Five microliters of each PCR product was analyzed by electrophoresis in a 0.5× TBE agarose gel.

RT-PCR was performed with the RevertAid Moloney murine leukemia virus reverse transcriptase (MJ Genenco). One microliter (approximately 0.1 µg) of total RNA was suspended in 10 µl of DNase- and RNase-free sterile water containing 10 pmol of a primer and incubated at 70°C for 5 min. Immediately being chilled in ice, a mixture containing 25 mM Tris HCl (pH 8.3), 25 mM KCl, 2 mM MgCl₂, 5 mM dithiothreitol, each deoxynucleoside triphosphate at a concentration of 1 mM, and 20 IU of RNase inhibitor (Promega, Milan, Italy) was transferred to the reaction tube. After 5 min of incubation at 37°C, 1 µl of reverse transcriptase was added, and this was followed by incubation at 42°C for 60 min and at 70°C for 10 min to stop the reaction. Three microliters of the cDNA synthesized was used for the PCR as described previously.

DGGE analysis. The Dcode universal mutation detection system (Bio-Rad Laboratories, Richmond, Calif.) was used for a DGGE analysis of the PCR products obtained from single cultures and directly from fermented sausages. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (8% [wt/vol] acrylamide-bisacrylamide [37.5:1]) by using two different ranges of denaturants to optimize separation of the products from the population involved in fermentation. Two denaturant gradients, one from 30 to 50% and one from 40 to 60% (100% denaturant was 7 M urea plus 40% [wt/vol] formamide) increasing in the direction of electrophoresis, were used. The gels were subjected to a constant voltage of 130 V for 3.5 h at 60°C, and after electrophoresis they were stained for 20 min in 1.25× TAE containing 1× (final concentration) SYBR Green (Molecular Probes, Eugene, Oreg.) and photographed under UV illumination.

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 reamplification, and the PCR products generated with the GC-clamped primer were checked by DGGE; DNA or RNA amplified from sausage was used as a control. Only products that migrated as a single band and at the same position with respect to the control were amplified with the primer without the GC clamp, purified, and sent to a commercial sequencing facility (MWG Biotech, Ebersberg, Germany) for sequencing.

Sequence analysis. Searches in the GenBank with the BLAST program (1) were performed to determine the closest known relatives of the partial 16S rDNA sequences obtained.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences obtained from the DGGE bands are shown in Table 2.

RESULTS

Enumeration of microorganisms and pH curve. Sausage fermentation was characterized by a rapid increase in the number of LAB, which increased from an initial value of 10⁴ CFU/g to 10⁸ to 10⁹ CFU/g within the first 10 days of ripening and remained stable for the rest of the fermentation (Fig. 1). This large increase in LAB abundance correlated with the decrease in pH values in the first stages of the maturation, and the pH reached after 10 days of fermentation, pH 5.46, was the lowest pH during the process. An increase in the pH to 5.56 at the end of the period monitored was explained by the proteolytic activity of the microorganisms involved in the fermentation. The

TABLE 2. Sequence information for the DGGE bands obtained by analyzing the fermented sausage microbial community

Band ^a	Closest relative	% Identity ^b	Accession no.
1	<i>Staphylococcus xylosus</i>	98.0	AF318151
2	<i>Staphylococcus intermedius</i>	97.8	AF318152
3	<i>Staphylococcus carnosus</i>	98.0	AF318153
4	<i>Staphylococcus lentus</i>	98.0	AF318154
5	<i>Staphylococcus pulvereri</i>	95.7	AF318155
6	<i>Staphylococcus intermedius</i>	100	AF318156
7	<i>Lactobacillus plantarum</i>	100	AF318157
8	<i>Lactobacillus curvatus</i>	100	AF318158
9	<i>Lactobacillus sake</i>	98.6	AF318159
10	<i>Brochothrix thermosphacta</i>	98.4	AF318160
11	<i>Enterococcus</i> sp.	100	AF318161
12	<i>Staphylococcus xylosus</i>	98.0	AF318162
13	<i>Staphylococcus intermedius</i>	97.9	AF318163
14	<i>Lactobacillus mesenteroides</i>	96.6	AF318164
15	<i>Staphylococcus pulvereri</i>	94.2	AF318165
16	<i>Brevibacillus</i> sp.	100	AF318166
17	<i>Lactobacillus curvatus</i>	95.3	AF318167
18	<i>Lactobacillus sake</i>	94.3	AF318168
19	<i>Brochothrix thermosphacta</i>	98.3	AF318169
20	<i>Enterococcus</i> sp.	100	AF318170

^a The numbers correspond to the band numbers in Fig. 4 and 5.

^b Percentage of identical nucleotides in the sequence obtained from the DGGE band and the sequence of the closest relative found in the GenBank database.

initial number of members of the *Micrococcaceae* in the meat was 10⁴ CFU/g, which increased to 10⁶ CFU/g after 20 days of fermentation and then started to decrease. The final number was 10⁴ CFU/g at 45 days. The total bacteria were 10⁵ CFU/g, and the highest number (10⁸ CFU/g) occurred at 20 days; the number decreased at the next sampling point. Fecal enterococci increased steadily to 10⁴ CFU/g at 20 days and then decreased to 10² CFU/g after 35 days of fermentation. Total enterobacteria and *E. coli* decreased rapidly, and the decrease was correlated with the decrease in pH; after 10 days of maturation no suspected colonies were detected on the Coli-ID plates. No presumptive *S. aureus* colonies were observed during the ripening period.

Optimization of PCR-DGGE. All of the primers described in Table 1, which targeted different regions of the 16S rDNA, were used successfully with DNA extracted from the strains used, but only the P1/P2 primer set gave PCR products that allowed differentiation by DGGE. The DGGE profiles obtained for the control strains are shown in Fig. 2. For almost all of the strains profiles consisting of more than one band were obtained in the DGGE analysis. The patterns were reproducible and characteristic for each species tested, indicating that there was interspecies sequence divergence. For several strains DGGE bands contained two bands that migrated very close together (Fig. 2A, lanes 3 to 5), perhaps due to incomplete extension of the same template due to the GC clamp (27).

The 40 to 60% denaturant gradient allowed differentiation of members of the *Micrococcaceae*, distinguishing *Kocuria* strains from *Staphylococcus* strains (Fig. 2B, lanes 7 to 12), while the 30 to 50% denaturant gradient allowed differentiation of all the *Lactobacillus* spp. tested (Fig. 2A, lanes 1 to 6).

Identification of the LAB isolated during fermentation. A total of 192 strains of gram-positive, catalase-negative, rod-shaped bacteria belonging to *Lactobacillus* spp. were identified

FIG. 1. Microbial population dynamics, as determined by classical methods, and pH curve during natural fermentation of sausages.

by PCR-DGGE. After DNA extraction and amplification with primers targeting the V1 region of the 16S rDNA, all of the strains gave the PCR product of the expected size, which was then analyzed by DGGE. Surprisingly, only two DGGE profiles were detected, leading to the identification of all of the isolates as *L. sake* and *L. curvatus*. Figure 3 shows the trends for the two populations during the ripening period. As shown, in the first stages of fermentation *L. sake* was the main LAB present, and only at the end of maturation did *L. curvatus* become the predominant organism.

Fermented sausage DGGE profiles. Total DNA and RNA were extracted from each fermented sausage sample independently, and they were used in PCR and RT-PCR to obtain the V1 region product that was analyzed by DGGE. No differences in the fingerprints were obtained when replicates obtained at the same sampling time were analyzed (data not shown). The patterns obtained by analyzing the PCR products and the RT-PCR products are shown in Fig. 4 and 5. For both PCR and RT-PCR products, two denaturant gradients were used to maximize differentiation of the populations involved in fer-

FIG. 2. DGGE profiles of the PCR products obtained from the control strains. (A) 30 to 50% denaturant gradient; (B) 40 to 60% denaturant gradient. Lanes 1, *L. brevis* DSM 20054; lanes 2, *L. casei* DSM 20011; lanes 3, *L. alimentarius* DSM 20249; lanes 4, *L. plantarum* DSM 20174; lanes 5, *L. curvatus* subsp. *curvatus* DSM 20019; lanes 6, *L. sake* DSM 6333; lanes 7, *S. intermedius* DSM 20373; lanes 8, *S. carnosus* subsp. *carnosus* DSM 20501; lanes 9, *S. simulans* DSM 20322; lanes 10, *S. xylophilus* DSM 6179; lanes 11, *K. kristinae* DSM 20032; lanes 12, *K. varians* DSM 20033.

mentation. When the DNA amplicons were analyzed, diversity was found only at the very beginning of maturation, when multiple bands were detected. Bands 2 to 6 (Fig. 4A) were obtained only with the meat mixture and disappeared after 3 days. Only band 1 remained throughout fermentation, al-

though it was a very weak band. After the third day, an intense band appeared in the gel; this band migrated in the *Lactobacillus* sp. spreading region, revealing the increase in the number of LAB present in the meat mixture. When the products were analyzed with a gel containing the 30 to 50% denaturant

FIG. 3. *L. sake* and *L. curvatus* trends during ripening of naturally fermented sausages.

gradient (Fig. 4B), it was possible to determine the presence of different lactobacilli, represented by three species at zero time and by just two species after the third day of maturation. Bands 1 to 9 (Fig. 4) were excised from the acrylamide gel and reamplified with primers P1 and P2. After a DGGE analysis to confirm their relative positions with respect to the original PCR product obtained from the DNA extracted directly from the sausages, they were sent to MWG Biotech for sequencing.

The results obtained after alignment are shown in Table 2. Bands 1 to 6 belonged to *Staphylococcus* species, whereas bands 7 to 9 were identified as *L. plantarum*, *L. curvatus*, and *L. sake*, respectively. Only bands 1, 8, and 9 were present throughout the fermentation. It is probable that for bands 2 to 7 the PCR products were generated from intact DNA of non-viable cells. When the results obtained for DNA and RNA were compared, only a few differences were detected at the beginning of the fermentation (Fig. 5). Different bands were obtained with the meat mixture, and some were present until the third day. As described above for the DNA amplicons, for the RNA PCR products one intense band appeared in the gel on the third day which remained stable until the end of the maturation period (Fig. 5A). The *Lactobacillus* population was again represented by just two species: *L. sake*, which was present by itself until the 10th day of fermentation; and *L. curvatus*, which appeared during maturation after day 10 (Fig. 5B). Bands of interest were also excised from the gel in which the RNA amplicons were analyzed, and the results obtained from sequencing are shown in Table 2. Bands 12, 13, 15, 17, and 18 corresponded to bands 1, 2, 5, 8, and 9 from DNA amplification, respectively, which indicated that they did not originate from dead cells. The other bands were identified as microorganisms that were present as natural contaminants of the meat used for sausage production and did not have technological importance.

FIG. 4. DGGE profiles of the DNA amplicons obtained directly from fermented sausages. Profiles obtained at zero time and after 3, 10, 20, 30, and 45 days of fermentation are shown. (A) 40 to 60% denaturant gradient; (B) 30 to 50% denaturant gradient. Bands indicated by numbers were excised and after reamplification subjected to sequencing.

FIG. 5. DGGE profiles of the RNA amplicons obtained directly from fermented sausages. Profiles obtained at zero time and after 3, 10, 20, 30, and 45 days of fermentation are shown. (A) 40 to 60% denaturant gradient; (B) 30 to 50% denaturant gradient. Bands indicated by numbers were excised and after reamplification subjected to sequencing.

DISCUSSION

A molecular approach to monitor the dynamic changes in the main populations involved in fermentation of Italian sausages was used. This approach exploited the potential of PCR to amplify, with suitable primers, regions conserved within the domain *Eubacteria*, as well as the discriminatory power of DGGE to differentiate DNA molecules on the basis of differences in their sequences (20). Fermentation of sausages is a well-known microbial process, and ecological studies during ripening date back to the 1970s (22). These studies, based on traditional methods, described the changes in populations during ripening. LAB are the main population involved in the decrease in pH. Also involved are representatives of the *Micrococcaceae*, which neutralize the organic acids from LAB activity, produce peptides and amino acids due to their proteolytic activity, and induce the release of various aromatic substances related to their ability to produce lipases (10).

In the last few years the possibility of using molecular approaches and direct sampling of the DNA and/or RNA in complex microbial systems has opened up areas of research that were already being studied but were not completely understood because of the biases related to the traditional methods. With traditional techniques only easily culturable organisms are counted, and often microorganisms for which selective enrichment and subculturing is problematic or impossible cannot be characterized.

In this paper we describe a PCR-DGGE protocol for detecting the microbial changes during natural fermentation of sausages. The first step was optimization of the method by using standard cultures obtained from international collections to determine the experimental conditions for amplification by PCR and differentiation by DGGE. Different sets of primers were selected from those available and used for the PCR-DGGE analysis. Only primers P1 and P2 (19) were considered suitable for obtaining good differentiation among *Lactobacillus*, *Staphylococcus*, and *Kocuria* spp. without band comigration for different species. In mixed populations, individual members were identified by PCR-DGGE when the concentrations were more than 10^4 CFU/g, which allowed detection of species at a threshold level during fermentation (data not shown). Results were obtained with two different denaturant gradients in the DGGE gels. A 30 to 50% denaturant gradient was optimal for differentiation of *Lactobacillus* spp., whereas gels with a 40 to 60% denaturant gradient could be used to distinguish the *Staphylococcus* and *Kocuria* spp.

The method was used to monitor the population dynamics during natural fermentation of sausages. Both DNA and RNA were sampled directly in order to determine the levels of expression of the 16S rDNA of the most prominent bacteria, which may reflect their contributions to the fermentation process. Gels were visually inspected to identify the bands representing the populations involved in the fermentation. To cir-

cumvent the biases inherent in subjective interpretation, the presence of the bands was confirmed by direct sequencing. When the results obtained from both traditional plating and DGGE were analyzed, it became evident that the fermentation was characterized by strong LAB activity. In DNA and RNA DGGE gels, multiple bands were visible for the first 3 days of fermentation, when different species, most of which were related to *Staphylococcus* spp., were identified. From the 10th day of maturation only the LAB bands were present. The main difference detected by sampling RNA rather than DNA was the presence of natural meat contaminants, such as *Brochothrix thermosphacta*, *Enterococcus* sp., *Leuconostoc mesenteroides*, and *Brevibacillus* sp., which were not present after the third day. *Staphylococcus* species, recognized as proteolytic agents due to their ability to produce proteases, were found only in the meat mixture before sausages were filled and after 3 days. The only *Staphylococcus* species represented in the DGGE gel after 3 days was *S. xylosus*, which produced a specific band in the gel until the end of fermentation. It is important to emphasize that a corresponding *S. xylosus* band was not found when the RNA amplicons were analyzed, since band 12 was present only at zero time and 3 days and then disappeared. This could be explained by the large quantity of LAB RNA, which restricted amplification of RNA from different species present.

The presence of multiple copies of the rRNA operon, as described previously for other microorganisms (8, 18, 26), made evaluation of the profiles obtained from single cultures difficult, but it did not affect interpretation of the fingerprints obtained from the total DNA and RNA extracted directly from sausages during fermentation.

The profiles obtained by DGGE agreed with the results obtained by traditional methods. The LAB population was the largest population during fermentation. The LAB strains isolated at the different steps of fermentation were all identified by PCR-DGGE as *L. sake* and *L. curvatus*. These results were in complete agreement with the profiles obtained when both DNA and RNA amplicons were analyzed, where the bands identified as *Lactobacillus* belonged to the two species mentioned above. When the DNA was sampled, a single band referred to *L. plantarum* was found only at zero time. The PCR product was probably generated from dead cells, since no *L. plantarum* cells were isolated at zero time and no specific signal was detected in the RNA amplicons. Moreover, the specific band obtained from DNA disappeared after the first sampling. *S. xylosus* might have been the only *Staphylococcus* species present, as previously described by other authors (5, 7), justifying the band present in the gels in which DNA was analyzed.

The characteristic increase in pH that follows the initial decrease due to acid production by LAB is usually caused by proteolytic activity attributed mainly to endogenous muscle cathepsins in the initial phase (29) and to the ability of staphylococci to produce proteases in the second stage (6). In our opinion, in this study the increase in pH after 10 days of ripening could not be explained by the *Micrococcaceae* activity because of the low number of cells present but could be explained by attributing an extracellular proteinase activity to LAB. As previously described (12), *L. sake* and *L. curvatus* are able to use muscle sarcoplasmatic proteins as substrates, which results in peptide production that could play a role in the

increase in the pH. *L. sake* and *L. curvatus* are the only two species isolated from sausages that remained stable throughout the fermentation, and they were responsible for the proteolytic activity that resulted in a final pH of 5.56.

The DGGE approach was first used in environmental ecology studies, such as sea sediments (23), hot springs (13, 31), or wastewater treatment plants (14), and in studies of the populations present in the rumen (21) or gastrointestinal contents (30, 33). Only in the last year was the same approach used to study microbial systems such as food fermentation, in which many microorganisms are difficult to cultivate or are thought to be nonculturable. DGGE has been used to monitor the microbial dynamics during production of the Mexican fermented maize dough pozol (2, 3) and to monitor the dynamic changes during wine fermentation (4). By applying the method to the natural fermentation of sausages, we were able to determine that LAB, represented by *L. sake* and *L. curvatus*, were the main organisms responsible for the physical and organoleptic changes that occurred during fermentation of the sausages tested. *Micrococcaceae* strains had restricted importance during production compared to LAB. Their high levels and acid production made the LAB the only active population, as determined by both DNA and RNA DGGE analyses, in transformation of fermented sausages from Friuli Venezia Giulia, making them potential starter cultures for this kind of production. Moreover, the ability to monitor the population by PCR-DGGE could provide real time information concerning the state of fermentation. Since the results are available 8 h after sampling, immediate technological adjustments can be made when they are required.

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