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Culture-Dependent and -Independent Methods To Investigate the Microbial Ecology of Italian Fermented Sausages

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In this study, the microbial ecology of three naturally fermented sausages produced in northeast Italy was studied by culture-dependent and -independent methods. By plating analysis, the predominance of lactic acid bacteria populations was pointed out, as well as the importance of coagulase-negative cocci. Also in the case of one fermentation, the fecal enterocci reached significant counts, highlighting their contribution to the particular transformation process. Yeast counts were higher than the detection limit (>100 CFU/g) in only one fermented sausage. Analysis of the denaturing gradient gel electrophoresis (DGGE) patterns and sequencing of the bands allowed profiling of the microbial populations present in the sausages during fermentation. The bacterial ecology was mainly characterized by the stable presence of *Lactobacillus curvatus* and *Lactobacillus sakei*, but *Lactobacillus paracasei* was also repeatedly detected. An important piece of evidence was the presence of *Lactococcus garvieae*, which clearly contributed in two fermentations. Several species of *Staphylococcus* were also detected. Regarding other bacterial groups, *Bacillus* sp., *Ruminococcus* sp., and *Macrococcus caseolyticus* were also identified at the beginning of the transformations. In addition, yeast species belonging to *Debaryomyces hansenii*, several *Candida* species, and *Willopsis saturnus* were observed in the DGGE gels. Finally, cluster analysis of the bacterial and yeast DGGE profiles highlighted the uniqueness of the fermentation processes studied.

Fermentation is one of the oldest technologies used to preserve food for long periods, and it is a process in which microbes, meat, and technology converge. Besides the valuable protein and fat nutrients, flavor compounds are abundant and can vary, depending on raw materials, starter cultures and processing conditions. Optimization and standardization of both organoleptic and safety characteristics require detailed knowledge of the relative contribution of these three factors (33).

The microbial flora that are established and that participate in the phenomena of fermentation and ripening of sausages originate from the environment, the equipment, and the raw materials used in the manufacture of the products. It is not an easy task to determine the contribution of the different bacterial groups in the reactions associated with ripening since the microbial flora are diverse and the effects of the different ingredients must be taken into account (18).

The first studies on the ecology of fermented sausages date back to 1970 (28). From then on, several investigations established two groups of microorganisms as being the main organisms responsible for the transformations involved during fermentation and ripening of sausages. Lactic acid bacteria (LAB), in particular *Lactobacillus* spp., and gram-positive coagulase-negative cocci (CNC), specifically *Staphylococcus* and *Kocuria* spp., are considered technologically fundamental.

LAB are responsible for lactic acid production, for the

“tangy” flavor of sausages, and for the small amounts of acetic acid, ethanol, acetoin, carbon dioxide, and pyruvic acid that are produced during fermentation, depending on the starter applied, the carbohydrate substrate, and the sources of meat proteins and additives (2). Some LAB strains are also able to produce antimicrobial compounds (bacteriocins), thereby enhancing the safety of fermented sausages (20), a process often termed bioprotection or biopreservation (19, 47).

CNC participate in the development and stability of a desirable red color through nitrate reductase activity that leads to the formation of nitrosomyoglobin. Furthermore, nitrate reduction produces nitrite that can limit lipid oxidation (48). Moreover, various aromatic substances and organic acids are released from their protease and lipase activity (34).

Early in the 1990s, Muyzer et al. developed a culture-independent method that has the potential to study the microbial flora quickly and economically, termed denaturing gradient gel electrophoresis (DGGE) (35). This approach has the advantage of directly profiling microbial populations present in specific ecosystems by separating PCR products that have originated with universal primers, on the basis of the melting domain of the DNA molecules. The trend to move toward methods that avoid the use of selective cultivation and isolation of bacteria from natural samples is justified, considering the biases related to traditional culture-dependent methods. As a matter of fact, different authors described tremendous differences between isolated and naturally occurring species present in various habitats (17, 22, 23, 36).

DGGE has been widely used to study the ecology of fermented foods (3, 5, 6, 8, 30, 32, 39, 49) and to profile pathogens

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TABLE 1. PCR primers used in this study

Designation ^a	Sequence (5' → 3')	Position	Reference
P1 (F)	GCGGCGTGCCTAATAC ATGC	24–43 ^b	26
P2 (R)	TTCCCCACGCGTTACT CACC	97–116 ^b	26
NL1 (F)	GCATATCAATAAGCGGAG GAAAAG	–25–0 ^c	27
LS2 (R)	ATTCCCAACAACCTCG ACTC	203–222 ^c	5

^a A GC clamp (5'-CGCCCGCGCGCCCGCGCCGTCGCCGCCGCCGCGCCCG) (46) was added to primers P1 and NL1 when used for DGGE analysis. F, forward; R, reverse.

^b *E. coli* 16S rRNA gene nucleotide numbering (accession no. Z83204).

^c *S. cerevisiae* 26S rRNA gene nucleotide numbering (accession no. U44806).

directly in food samples (7), and its use in food microbiology was recently reviewed (14).

In this paper, we describe the application of culture-dependent and -independent methods to study the ecology of fermented sausages produced in three different plants in northeast Italy. Sausages were collected at determined time points and subjected to traditional microbiological analysis. DNA was extracted directly from the samples and amplified by PCR, using universal bacterial and yeast primers. DGGE analysis and sequencing of the resulted bands allowed fingerprinting of the microbial populations present in the three fermentations. Moreover, cluster analysis of the bacterial and yeast DGGE profiles was carried out to determine their similarity.

MATERIALS AND METHODS

Fermented sausage technology and sampling procedures. Three local meat factories in northeast Italy were selected to carry out the study. They are called plants C, L, and U in this study. For the production of the fermented sausages, traditional techniques were employed without the use of starter cultures. Plant C was characterized by extended maturation of the sausages that lasted 120 days, while plant U produced sausages with fast maturation (28 days). Plant L sold the final product after 45 days of ripening. The following ingredients were common to all three fermentations: pork meat (60 kg), lard (40 kg), sodium chloride (2.5 kg), nitrite and nitrate (200 ppm), and black pepper (70 g). For fermentation C, no sugars were added, while for fermentations L and U, 1.5 and 2.5 kg of sugars, respectively, were used. The mixture was used to stuff natural casings, and the procedure resulted in fresh sausages that were 25 cm long and 5 cm in diameter. Ripening parameters were different for the three plants considered. Plant C performed maturations at low temperatures (below 10°C) and relative humidity (RH) between 60 and 90% for the first 60 days, followed by a 60-day period with temperatures of 14°C and RH of 65 to 85%. For plants L and U, the first stage consisted of 2 days of drying with an RH of 85% at 22°C. The temperature was then decreased to 12 and 14°C, for plants L and U, respectively, at a rate of 2°C per day with an RH between 60 and 90%. Ripening was then carried out for the rest of the period at the temperatures reached in storerooms with 65 to 85% RH. Triplicate samples of the sausages were used for microbiological and molecular analyses. For plant C, samplings were performed at 0, 3, 10, 20, 30, 60, 90, and 120 days, whereas for plant L, samplings were performed at days 0, 3, 10, 20, 30, and 45. Sausages from plant U, characterized by fast ripening, were examined at 0, 3, 5, 7, 14, and 28 days.

pH measurements. Potentiometric measurements of pH were carried out with a pin electrode of a pH meter (Radiometer Copenhagen pH M82, Cecchinato, Italy) inserted directly into the sample. Three independent measurements were done on each sample. Means and standard deviations were calculated.

Microbiological analysis. The samples were subjected to a microbiological analysis to monitor the changes in the populations responsible for ripening of fermented sausages, as well as 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 per liter; Oxoid) was added, and the mixture was treated for 1.5 min in a stomacher machine (PBI, Milan, Italy). Further decimal dilutions were made, and the following analyses

were carried out on duplicate agar plates: (i) total aerobic mesophilic flora on Gelysate agar (Oxoid) incubated for 48 to 72 h at 30°C, (ii) LAB on MRS agar (Oxoid) incubated with a double layer at 30°C for 48 h, (iii) CNC on mannitol salt agar (Oxoid) incubated at 30°C for 48 h, (iv) total enterobacteria and *Escherichia coli* on Coli-ID medium (Bio-Merieux, Marcy d'Etoile, France) incubated with a double layer at 37°C for 24 to 48 h, (v) fecal enterococci on kanamycin-esculin agar (Oxoid) incubated at 42°C for 24 h, (vi) *Staphylococcus aureus* on Baird Parker medium (Oxoid) with added egg yolk tellurite emulsion (Oxoid) incubated at 37°C for 24 to 48 h, and (vii) yeasts and molds on malt extract agar (Oxoid) supplemented with tetracycline (1 mg/ml; Sigma, Milan, Italy) incubated at 25°C for 48 to 72 h. For *Listeria monocytogenes* (24) and *Salmonella* spp. (25), the International Organization for Standardization ISO/DIS methods were performed. After counting, means and standard deviations were calculated.

Direct extraction of DNA from sausages. At each sampling point, 10-g samples, in triplicates, were homogenized in a stomacher bag with 20 ml of saline-peptone water for 1 min. After each preparation had settled for 1 min, 1 ml of supernatant was transferred into a screw-cap tube containing 0.3 g of glass beads with a diameter of 0.5 mm and centrifuged at 4°C for 10 min at 14,000 × g. The resulting pellet was treated with 1 ml of petrol ether-hexane (1:1; Sigma) for 10 min at room temperature to extract lipids. A second centrifugation was performed, as described above, and the pellet was resuspended in 150 µl of proteinase K buffer (50 mM Tris-HCl, 10 mM EDTA, pH 7.5, 0.5% [wt/vol] sodium dodecyl sulfate [SDS]). Twenty-five microliters of proteinase K (25 mg/ml; Sigma) was added, and treatment at 65°C for 1 h was performed. After this step, 150 µl of 2× breaking buffer (4% Triton X-100 [vol/vol], 2% [wt/vol] SDS, 200 mM NaCl, 20 mM Tris, pH 8, 2 mM EDTA, pH 8) was mixed in the tubes, and 300 µl of phenol-chloroform-isoamyl alcohol (25:24:1, pH 6.7) (Sigma) was added. Then, three 30-s treatments at the maximum speed, with an interval of 10 s, were performed in a bead beater (Fast Prep; Bio 101, Vista, Calif.). The tubes were centrifuged at 12,000 × g at 4°C for 10 min, and the aqueous phase was collected and mixed with 1 ml of ice-cold absolute ethanol. The DNA was precipitated at 14,000 × g at 4°C for 10 min, and the pellets were dried under vacuum at room temperature. Fifty microliters of sterile water was added, and a 30-min incubation at 45°C facilitated the nucleic acid solubilization. One microliter of DNase-free RNase (Roche Diagnostics, Milan, Italy) was added to digest the RNA by incubation at 37°C for 1 h.

PCR. The primers used in this study are described in Table 1. Amplifications were carried out in a final volume of 25 µl, containing 2 µl (50 ng total) of template DNA, 10 mM Tris HCl, pH 8.3, 50 mM KCl, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1.25 U of *Taq* polymerase (Applied Biosystem, Milan, Italy), and 0.2 µM each primer. For primers P1 and P2, 1.5 mM MgCl₂ was employed, whereas for primers NL1 and LS2, a concentration of 2 mM was used. Amplifications were carried out in a PTC-220 DNA Engine Dyad MJ Research thermalcycler (Celbio, Milan, Italy). The amplification cycle for primers P1 and P2 was characterized by an initial touchdown step in which the annealing temperature was lowered from 60 to 52°C in intervals of 2°C every 2 cycles, and 20 additional cycles were done with annealing at 50°C. Denaturation was performed at 95°C for 1 min, and extension was performed at 72°C for 1 min 30 s. For primers NL1 and LS2, reactions were run for 30 cycles of denaturation at 95°C for 60 s, annealing at 52°C for 45 s, and extension at 72°C for 60 s. For both amplification cycles, an initial denaturation at 95°C for 5 min and a final extension at 72°C for 7 min were carried out as well. Five microliters of each PCR mixture was analyzed by electrophoresis in a 0.5× Tris-borate-EDTA (TBE) agarose gel.

DGGE analysis. The Dcode Universal Mutation Detection system (Bio-Rad, Hercules, Calif.) was used for DGGE analysis. Electrophoreses were performed in an 0.8-mm-thick polyacrylamide gel (8% [wt/vol] acrylamide-bisacrylamide at 37.5:1), using a denaturant gradient increasing in the direction of the electrophoretic run. For PCR products obtained with primers P1 and P2, a denaturant gradient from 40 to 60% (100% corresponded to 7 M urea and 40% [wt/vol] formamide) was used. Amplicons produced by primers NL1 and LS2 were analyzed in a denaturant gradient from 30 to 60%. Electrophoretic runs were carried out at a constant temperature of 60°C in 1.25× Tris-acetate-EDTA (TAE) for 3.5 h at 130 V for the P1 and P2 primers and for 4 h at 120 V for the NL1 and LS2 primers. After the electrophoresis, gels were stained for 20 min in 1.25× TAE containing 1× SYBR green (final concentration; Molecular Probes, Eugene, Oreg.) and visualized under UV light. Images were digitally captured and analyzed with the BioImaging System GeneGenius (SynGene, Cambridge, United Kingdom) for the recognition of the bands present. Comparisons of DGGE fingerprints obtained from the different sausages were performed with the pattern analysis software package Gel Compare, version 4.1 (Applied Maths, Kortrijk, Belgium). Calculation of similarity in the profiles of bands was based on Pearson product-moment correlation coefficient. Dendrograms were obtained by

TABLE 2. Microbial dynamics, as determined by plating, of the fermentations monitored in this study

Microbiological analysis parameter	Result (log ₁₀ CFU/g) for fermentation at day given ^a							
Fermentation C	0	3	10	20	30	60	90	120
Total aerobic count	4.46 (0.27)	4.49 (0.04)	7.03 (0.88)	6.48 (0.41)	6.97 (0.56)	7.24 (0.26)	7.11 (0.03)	7.02 (0.02)
CNC	3.23 (0.10)	4.70 (0.63)	5.37 (0.80)	5.72 (0.16)	5.09 (0.12)	3.58 (0.16)	4.30 (0.12)	5.20 (0.21)
LAB	3.17 (0.93)	3.74 (0.07)	6.37 (0.69)	7.83 (0.02)	7.58 (0.12)	7.28 (0.50)	7.87 (0.51)	8.78 (0.21)
Yeasts	2.00 (0.00)	3.12 (0.46)	5.36 (0.02)	5.90 (0.14)	4.98 (0.25)	2.50 (0.08)	3.12 (0.09)	6.73 (0.05)
Molds	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)
Fecal enterococci	2.15 (0.27)	2.40 (0.17)	5.33 (0.33)	7.18 (0.11)	6.76 (0.07)	6.81 (0.03)	3.65 (0.15)	4.12 (0.20)
Total enterobacteria	1.73 (0.16)	2.16 (0.43)	3.20 (0.42)	2.98 (0.58)	1.97 (0.09)	1.98 (0.02)	<10 (NA)	<10 (NA)
<i>E. coli</i>	1.25 (0.24)	1.43 (0.22)	<10 (NA)	<10 (NA)	<10 (NA)	<10 (NA)	<10 (NA)	<10 (NA)
<i>S. aureus</i>	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)
<i>L. monocytogenes</i>	A	A	A	A	NP	NP	NP	NP
<i>Salmonella</i> spp.	A	A	A	A	NP	NP	NP	NP
Fermentation L	0	3	10	20	30	45		
Total aerobic count	4.74 (0.44)	3.59 (0.36)	8.03 (0.56)	7.68 (0.14)	8.55 (0.30)	4.19 (0.23)		
CNC	3.11 (0.18)	3.65 (0.15)	6.83 (0.07)	6.12 (0.71)	5.86 (0.06)	4.63 (0.20)		
LAB	5.29 (0.18)	5.97 (0.27)	7.81 (0.21)	7.94 (0.12)	7.93 (0.16)	8.34 (0.14)		
Yeasts	3.55 (0.80)	2.93 (0.05)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)		
Molds	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)		
Fecal enterococci	3.18 (0.12)	4.47 (0.77)	5.95 (0.15)	4.95 (0.96)	4.00 (0.00)	5.47 (0.47)		
Total enterobacteria	1.94 (0.15)	3.59 (0.36)	4.29 (0.21)	3.80 (0.66)	4.28 (0.21)	2.56 (0.09)		
<i>E. coli</i>	1.72 (0.63)	<10 (NA)	<10 (NA)	<10 (NA)	<10 (NA)	<10 (NA)		
<i>S. aureus</i>	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)		
<i>L. monocytogenes</i>	A	A	A	A	NP	NP		
<i>Salmonella</i> spp.	A	A	A	A	NP	NP		
Fermentation U	0	3	5	7	14	28		
Total aerobic count	5.09 (0.20)	5.41 (0.22)	5.88 (0.19)	5.33 (0.34)	6.90 (0.56)	9.11 (0.47)		
CNC	3.66 (0.10)	3.80 (0.63)	5.67 (0.57)	4.62 (0.69)	5.54 (0.06)	5.01 (0.12)		
LAB	4.20 (0.17)	8.24 (0.01)	7.81 (0.10)	8.21 (0.03)	8.28 (0.16)	8.45 (0.06)		
Yeasts	3.33 (0.06)	<100 (NA)	<100 (NA)	<100 (NA)	3.53 (0.68)	2.32 (0.28)		
Molds	2.59 (0.26)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)		
Fecal enterococci	3.73 (0.38)	5.60 (0.00)	5.83 (0.22)	6.02 (0.16)	6.08 (0.17)	6.28 (0.32)		
Total enterobacteria	2.38 (0.06)	4.94 (0.21)	4.88 (0.38)	2.18 (0.15)	4.70 (0.41)	4.66 (0.50)		
<i>E. coli</i>	<10 (NA)	<10 (NA)	<10 (NA)	<10 (NA)	<10 (NA)	<10 (NA)		
<i>S. aureus</i>	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)	<100 (NA)		
<i>L. monocytogenes</i>	A	A	A	A	A	NP		
<i>Salmonella</i> spp.	A	A	A	A	A	NP		

^a Results are shown as the mean with standard deviation in parentheses. NA, not applicable; NP, not performed; A, absent in 25 g of product.

means of the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm (50). DGGE analyses were performed at least twice. Normalization of the gels was performed by using band ladders. The bacterial ladder was the result of amplification of *Lactobacillus casei* DSM 20011 by primers P1 and P2, as described by Cocolin et al. (6), which was subsequently analyzed by DGGE. It was selected for its characteristic of giving a multiband pattern. The yeast ladder was a mixture of *Hanseniaspora uvarum* DBVPG 6717, *Kluyveromyces thermotolerans* DBVPG 6480, *Saccharomyces cerevisiae* DBVPG 6173, and *Brettanomyces bruxellensis* DBVPG 6706, amplified with primers NL1 and LS2, as reported by Cocolin et al. (5). To allow normalization before cluster analysis, ladders were loaded in the first and last wells of each gel run.

Sequencing of DGGE bands and sequence analysis. Blocks of polyacrylamide gels containing selected DGGE bands were punched with sterile pipette tips. The blocks were then transferred in 50 µl of sterile water, and the DNA of the bands was allowed to diffuse overnight at 4°C. Two microliters of water, containing the eluted DNA, was used for the reamplification, and the PCR products, generated with the GC-clamped primer, were checked by DGGE with sausage amplified DNA as a control. Only products migrating as a single band and at the same position with respect to the control were amplified with the primer without the GC clamp, cloned in pGEM-T Easy vector (Promega, Milan, Italy), and sequenced by a commercial facility (MWG Biotech, Ebersberg, Germany). Searches in the GenBank database were performed with the BLAST program (1)

to determine the closest known relatives of the partial 16S rRNA sequence obtained.

RESULTS

Traditional microbiological analysis. The results of the plate counts obtained from the three fermentations monitored in this study are reported in Table 2. Both the microbial population changes during fermentation and the hygienic quality of the sausages were monitored. Fermentation C was characterized by a slow increase in the counts of almost all of the groups of microorganisms observed. Only the CNC showed a significant increase from 10³ to 10⁴ to 10⁵ CFU/g from zero to 3 days, as well as yeasts that increased from 10² to 10³ to 10⁴ CFU/g. The total aerobic count and counts of LAB, fecal enterococci, total enterobacteria, and *E. coli* did not increase in the first 3 days. Significant growth was observed at 10 days, when almost all of the microbial groups showed an increase

from 2 to 4 log₁₀ CFU/g. For CNC and total enterobacteria, a slightly slower increase was noticed and counts moved from 10⁴ to 10⁵ to 10⁵ to 10⁶ CFU/g and from 10² to 10³ to 10³ to 10⁴ CFU/g, respectively. At 10 days, the *E. coli* count was <10 CFU/g, and it remained below that limit. The rest of the transformation process was characterized by a slight increase in the counts of total bacteria and the establishment of the LAB population. LAB increased from 10⁵ to 10⁶ CFU/g at 10 days to 10⁷ to 10⁸ CFU/g at 20 days, counts that remained stable until the end of the process. CNC and yeasts showed a decrease during the central period of the fermentation, with an increase in the counts at the last sampling point, reaching 10⁵ to 10⁶ and 10⁶ to 10⁷ CFU/g, respectively. The same trend was observed for the fecal enterococci too, whereas total enterobacteria were undetectable starting from day 90 of the fermentation. Molds were below 100 CFU/g throughout the period observed, and *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. were absent in 25 g of product in the first 20 days of fermentation.

Fermentation L showed high counts of LAB populations right from the beginning of the fermentation. Already at day zero, the counts were 10⁵ to 10⁶ CFU/g, and the number rapidly increased to 10⁷ to 10⁸ CFU/g at 10 days. From then, the counts remained stable, with a slight increase at the end of the period, reaching 10⁸ to 10⁹ CFU/g at day 45. In terms of total bacterial count, CNC and fecal enterococci were characterized by a significant increase in the counts from 3 to 10 days, which were stable until the end of the fermentation. Yeasts were detected only at days zero and 3, while molds were always <100 CFU/g throughout the period monitored. *E. coli* was counted only at day zero, becoming undetectable from day 3. *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. were not detected in the sausages produced in plant L.

Fermentation U was characterized by very fast growth of LAB populations: in fact, from the initial count of 10⁴ CFU/g, they increased to 10⁸ to 10⁹ CFU/g already at 3 days. These counts were stable until the 28th day of fermentation. The total aerobic count was determined to be 10⁵ CFU/g at day zero, which did not change significantly until the last day of sampling. As a matter of fact, at 28 days, it reached 10⁹ CFU/g, which represented the highest count between the three fermentations monitored. CNC increased significantly between the 3rd and 5th days of transformation and remained stable after that point. As described for LAB, fecal enterococci showed a fast increase from zero to 3 days. They reached counts of 10⁵ to 10⁶ CFU/g at that moment, and from then on, a slight increase until the end of the fermentation was observed. The counts for yeasts were 10³ to 10⁴ CFU/g at day zero, and after a 2-week period, in which they were not detected, at 14 days, the yeast count was determined to be 10³ to 10⁴ CFU/g, decreasing to 10² to 10³ CFU/g at the last day of sampling. Molds were only detected at day zero. Total enterobacteria grew appreciably between zero and 3 days of fermentation. The value of 10⁴ to 10⁵ CFU/g remained stable, with the exception of day 7, when the enterobacteria decreased to 10² to 10³ CFU/g. *E. coli*, *S. aureus*, *L. monocytogenes*, and *Salmonella* were not detected.

pH measurements. The results of the pH measurements are shown in Fig. 1. Fermentation C was characterized by an initial pH of 5.52 that decreased during the first 20 days of fermenta-

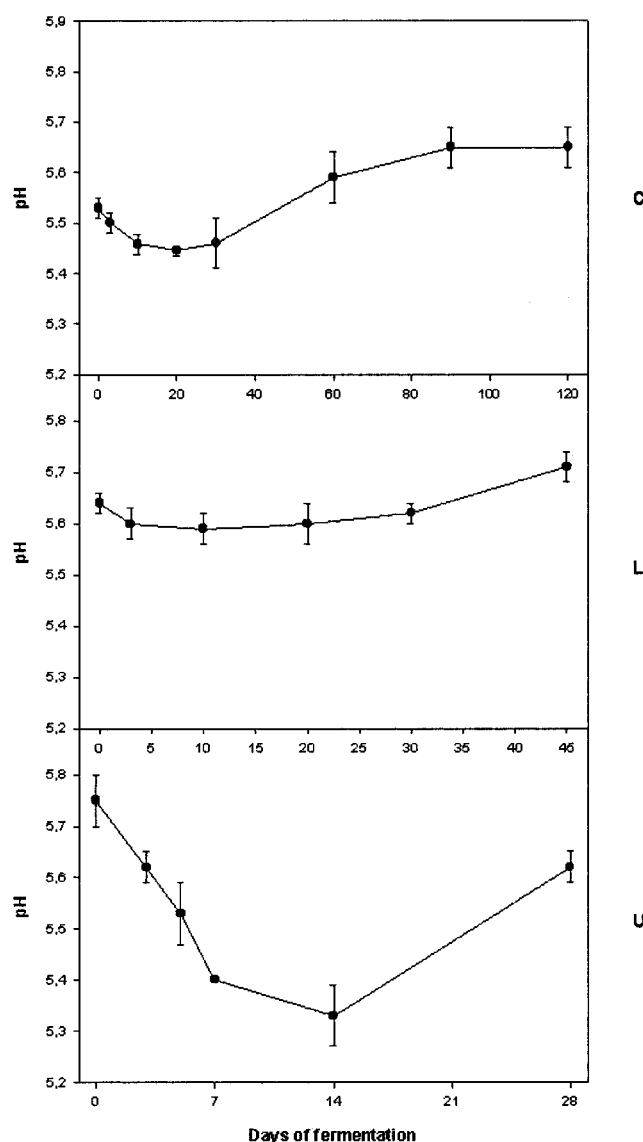


FIG. 1. pH trends of the fermentations monitored in this study. Letters (C, L, and U) on the plots indicate the fermentation code.

tation. From day 30, it started to increase, reaching a final value of about 5.65. For fermentation L, no significant decrease was observed at the beginning of the fermentation: from 5.62, pH decreased to 5.60 during the first 20 days. Only at the end of the transformation did it increase, reaching values of about 5.70. A different picture was obtained from fermentation U, in which a steep drop in the pH values was registered from day zero to day 14. From an initial pH of 5.75, it reached 5.32, which increased to 5.62, the final pH value of the sausages produced in plant U.

DGGE profiles. The bacterial and yeast DGGE profiles of the three fermentations are shown in Fig. 2 and 3, respectively, and the results of the band sequencing are reported in Tables 3 and 4. No differences were detected in the DGGE profiles when replicates obtained at the same sampling point were analyzed (data not shown). To make sure that no biases were introduced by the cloning step on PCR-amplified bands, rep-

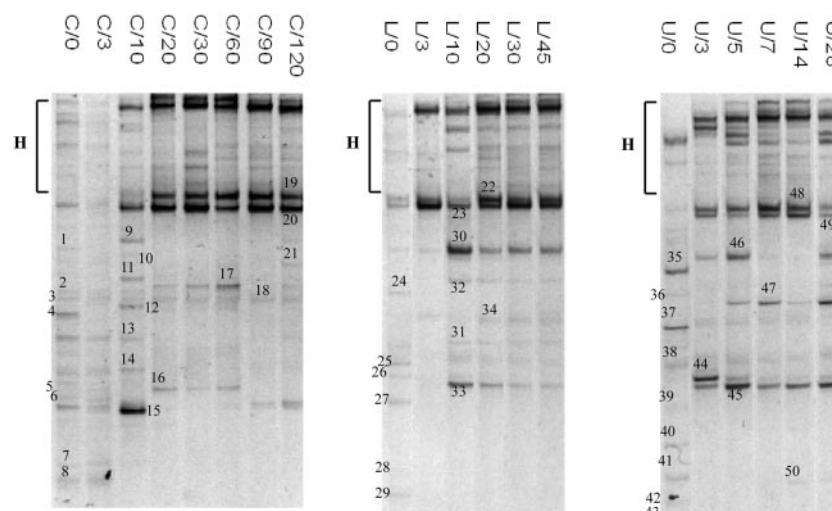


FIG. 2. Bacterial DGGE profiles. Lane designations indicate the fermentation and the day of sampling. The gels shown are not normalized. Bands indicated by numbers were excised and after reamplification subjected to sequencing. H, heteroduplex bands.

representative clones were selected and subjected to PCR-DGGE analysis with sausage amplified DNA as a control. We always obtained a comigration between the original band cut from the DGGE gel and the band amplified from the clones, thereby confirming the validity of the approach used. Direct cloning of the cut bands could not be used because it failed in the case of faint bands (data not shown). Concerning the bacterial ecology as determined by DGGE, fermentation C was characterized by

a complex picture during the first 10 days. Members of the LAB group (*L. sakei* and *Lactobacillus curvatus*), species of *Staphylococcus* (*S. xylosus*, *S. sciuri* or *S. pulvereri*, and *S. equorum* or *S. succinus*), *Macroccoccus caseolyticus*, and *Bacillus* sp. were identified. Moreover, one unidentified bacterium, with the highest homology in the BLAST search to bacterium Ellin5218, was detected. This picture did not change significantly at 3 days, and only at day 10 did new bands show up in

TABLE 3. Sequencing results from the bands cut from the bacterial DGGE gels

Band(s) ^a	Size (bp)	Closest relative	% Identity	Source ^b
1	113	<i>Lactobacillus sakei</i>	99.1	AB124845
2	91	<i>Staphylococcus xylosus</i>	98.9	AY126259
3, 18, 24, 36	113	<i>Lactobacillus curvatus</i>	99.6	AY204894
4	75	Bacterium Ellin5218	100	AY234569
5, 25, 38	91	<i>Staphylococcus sciuri</i> or <i>S. pulvereri</i> ^c	98.9	AY126231 or AY126216
6	91	<i>Staphylococcus equorum</i> or <i>S. succinus</i>	98.2	AF527483 or AY126240
7, 41	93	<i>Macroccoccus caseolyticus</i>	96.7	AY126157
8	91	<i>Bacillus</i> sp.	98.8	AY461682
9	91	<i>Staphylococcus xylosus</i>	98.6	AY126259
10, 30, 46	93	<i>Lactococcus garvieae</i>	99.6	AY438044
11	91	<i>Staphylococcus xylosus</i>	97.9	AY126259
12, 47	91	<i>Staphylococcus xylosus</i>	98.9	AY126259
13, 37	91	<i>Staphylococcus equorum</i> or <i>S. succinus</i> ^c	98.7	AF527483 or AY126240
14	91	<i>Staphylococcus xylosus</i>	99.0	AY126259
15, 33, 45	91	<i>Staphylococcus equorum</i> or <i>S. succinus</i>	98.9	AF527483 or AY126240
16, 26, 44	111	<i>Lactobacillus paracasei</i>	99.0	AY204894
17, 32	111	<i>Lactobacillus curvatus</i>	99.1	AY204894
19, 22, 48	111	<i>Lactobacillus curvatus</i>	97.2	AY204894
20, 23, 49	113	<i>Lactobacillus sakei</i>	99.5	AB124845
21, 35	91	<i>Brochothrix thermosphacta</i>	98.9	M58798
27, 39	93	<i>Staphylococcus intermedius</i>	100	D83369
28	91	<i>Bacillus</i> sp.	98.8	AY461682
29	106	<i>Ruminococcus</i> sp.	100	AY442822
31	114	<i>Lactobacillus sakei</i>	98.2	AB124845
34	93	<i>Lactococcus garvieae</i>	98.9	AY438044
41, 50	93	<i>Macroccoccus caseolyticus</i>	98.9	AY126157
42	95	<i>Macroccoccus caseolyticus</i>	97.7	AY126157
43	91	<i>Bacillus pumilis</i>	98.8	X60637

^a Bands are numbered as indicated on the DGGE gels shown in Fig. 2.

^b Accession number of the sequence of the closest relative found by BLAST search.

^c The V1 regions were 100% identical, therefore avoiding identification.

TABLE 4. Sequencing results of the bands cut from the yeast-DGGE gels

Band(s) ^a	Size (bp)	Closest relative	% Identity	Source ^b
1	241	<i>Candida tropicalis</i>	97.5	AF267497
2, 13, 15	246	<i>Mayaca fluviatilis</i>	86.5	AF293855
3	249	<i>Debaryomyces hansenii</i>	99.3	AF485980
4	249	<i>Debaryomyces hansenii</i>	99.3	AF485980
5	242	<i>Candida krisii</i>	100	AJ539355
6	248	<i>Willopsis saturnus</i>	99.6	AJ507804
7, 10	247	<i>Aerobasidium pullulans</i>	100	AY185811
8	248	<i>Willopsis saturnus</i>	100	AJ507804
9	248	<i>Candida fermentati</i>	100	AY187283
10	248	<i>Willopsis saturnus</i>	99.8	AJ507804
12	203	<i>Entyloma dahliae</i>	100	AY272033
14	242	<i>Candida sake</i> or <i>C. austromarina</i> ^c	98.9	AJ549822 or U62310

^a Bands are numbered as indicated on the DGGE gels shown in Fig. 3.

^b Accession number of the sequence of the closest relative found by BLAST search.

^c The D1-D2 loop regions were 100% identical, therefore avoiding identification.

the DGGE gels. Different signals belonged to *S. xylosus* (Fig. 2, bands 9, 11, 12, and 14), while the remaining signals were identified as *S. equorum* or *S. succinus*. Interestingly, band 10 (Fig. 2) was determined to be *Lactococcus garvieae*. From day 20, the ecology of sausages produced in plant C was dominated by LAB members. *L. sakei*, *L. curvatus*, and *Lactobacillus paracasei* were the main populations detected from days 20 to 60. After 90 days of ripening, only *L. paracasei* was not detected anymore, and *S. equorum* or *S. succinus* showed up again in the gels. This situation remained stable until day 120. However a new band (Fig. 2, band 21) belonging to *Brochothrix thermosphacta* appeared at the last sampling point. Fermentation L was characterized, from day zero, by the presence of the specific doublet for *L. curvatus* and *L. sakei*, placed in the upper part of the gel, which was always present throughout the fermentation. At day zero, *S. sciuri* or *S. pulvereri*, *L. paracasei*, *S. intermedius*, *Bacillus* sp., and *Ruminococcus* sp. were detected. Moreover, a faint band identified as *Lactococcus garvieae* was present as well. This species was detected as well at all of the following sampling points. From day 10, the picture of the ecology did not change; in fact, the DGGE patterns were characterized by the same bands belonging to *L. sakei*, *L. curvatus*, *Lactococcus garvieae*, and *S. equorum* or *S. succinus*. Regarding fermentation U, a rich DGGE profile was obtained at day zero. *B. thermosphacta*, *L. curvatus*, *S. equorum* or *S. succinus*, *S. sciuri* or *S. pulvereri*, *M. caseolyticus*, and *Bacillus pumilis* were identified. This situation dramatically changed at 3 days, where *L. sakei*, *L. curvatus*, *Lactococcus garvieae*, *L. paracasei*, and *S. equorum* or *S. succinus* took over the fermentation process. The ecology showed slight changes as the transformation proceeded. A band belonging to *S. xylosus* (Fig. 2, band 47) appeared in the gel, and it was detected until day 28, while *L. paracasei* disappeared after day 5. At the last sampling points of fermentation U, *M. caseolyticus* and *Lactococcus garvieae* were detected again. In all three fermentations, different bands were visible in the upper part of the DGGE gels. They were all determined to be heteroduplexed by sequencing (data not shown).

The yeast ecology, as presented in Fig. 3, was considerably more simple than the bacterial ecology. In fermentation C, the main yeast detected was *Debaryomyces hansenii*, which was present right from the beginning of the fermentation. From

day 10, its band was very intense until the end, and only at 30 days of fermentation did it decrease in intensity, allowing the detection of *Candida tropicalis* (Fig. 3, band 1). During fermentation C, another band was stably present in the DGGE gels. Band 2, absent only at days 10 and 20, was identified as *Mayaca fluviatilis*, although the identity retrieved from the GenBank database was too low to guarantee a correct identification. Fermentation L presented the highest number of bands within the fermentations monitored. *Candida krisii* and *Willopsis saturnus* were present from day zero, and *Aerobasidium pullulans* and *Candida fermentati* were detected at 3 and 30 days of fermentation and at days 10 and 20, respectively. In the last sampling point, a faint *Entyloma dahliae* band was detected. As described for fermentation C, also in this case, a band identified as *M. fluviatilis* was present. For fermentation U, the yeast ecology result was the simplest. Only two bands, corresponding to *Candida sakei* or *Candida austromarina* and *M. fluviatilis* were present throughout the fermentation.

Cluster analysis of the DGGE profiles. The results of the cluster analysis of the bacterial and yeast DGGE profiles of the three fermentations are reported in Fig. 4A and B, respectively. As shown, the bacterial ecology was determined to be fermentation specific, since samples coming from the same fermentation, collected at different time points, were almost always grouped in the same cluster. Fermentations L and C were more similar to each other than to fermentation U. As a

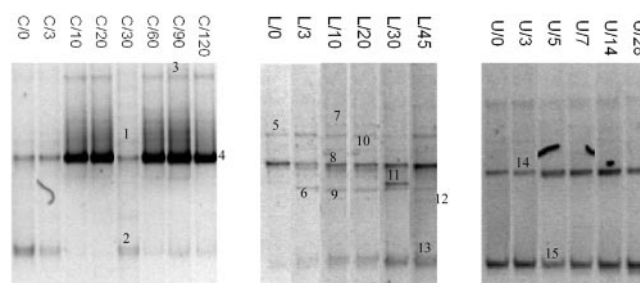


FIG. 3. Yeast DGGE profiles. Lane designations indicate the fermentation and the time of sampling. The gels shown are not normalized. Bands indicated by numbers were excised and after reamplification subjected to sequencing.

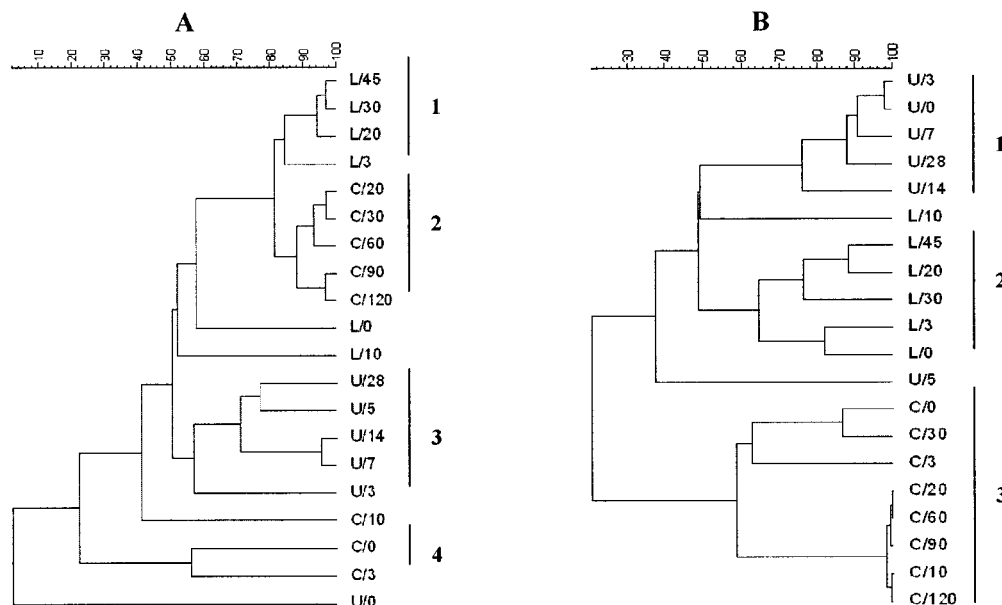


FIG. 4. Cluster analysis of the profiles obtained by bacterial (A) and yeast (B) DGGE analysis. Dendrograms were obtained with the UPGMA clustering algorithm. Samples are indicated by the fermentation letter and the day of analysis. Identified clusters are indicated by numerals to the right of each panel.

matter of fact, two clusters grouping at four time points of fermentation L and five time points of fermentation C had a similarity of about 80% (Fig. 4A, clusters 1 and 2). The samples from fermentation U were more diverse than those from fermentations C and L, showing a similarity of about 60% (Fig. 4A, cluster 3). Samples taken from the first days of fermentation (days zero and 3 and in fermentation C also at day 10) formed single clusters, apart from samples taken at days zero and 3 during fermentation C, which were grouped in one cluster with a similarity of 60%. In Fig. 4B, the cluster analysis of the yeast DGGE is presented. In this case, a fermentation-specific distribution of the samples was obtained, creating clusters containing only samples from the same fermentation (Fig. 4B, clusters 1, 2, and 3). Already with a coefficient of discrimination of about 50%, the three fermentations could be separated, while only a sample analyzed at day 10 from fermentation L and a sample analyzed at day 5 from fermentation U were found to be highly diverse from the rest and did not belong to any cluster.

DISCUSSION

Fermented sausages are a group of traditional products with great diversity in production methods and organoleptic characteristics between different countries and different regions of the same country. In Europe they have a long history, originating from Mediterranean countries during Roman times (28). Extensive studies have been performed on the characterization of traditional sausages produced in Greece, Italy, and Spain (4, 9, 10, 15, 16, 21, 31, 38, 41-44). A wide variety of microorganisms have been isolated from sausage fermentations by traditional methods. They belong to LAB and *Staphylococcus* and *Kocuria* spp. (12, 21). Among LAB, *L. sakei*, *L. curvatus*, and *Lactobacillus plantarum* are the species most

frequently isolated in acid-fermented meat products (2). However, in some cases, the contribution of enterococci seems to be important (13). *S. xylosum*, *S. carnosus*, and *S. saprophyticus* are the CNC representatives involved in the fermentation of naturally fermented sausages (2, 9, 11, 29, 37).

The study of the ecology of the naturally fermented sausages has been carried out so far by traditional microbiological methods, based on plate counts, isolation, and biochemical identification. This approach has been repeatedly criticized because only easily culturable microorganisms can be detected, while members that need elective enrichments or that are in a particular physiological condition (in a sublethal or injured state) are lost. Only in a few cases and in the last 5 years has the development of molecular techniques been taken into account and exploited to study the microbial compositions of fermented sausages. Of particular interest are the direct culture-independent methods that are able to profile microbial populations avoiding the biases described above. In 2001, we published a paper on the development of a direct PCR-DGGE analysis to profile bacterial populations in Italian fermented sausages (6). The main evidence found was the great impact of LAB populations, which were stably present at both the DNA and RNA levels throughout the fermentation, possibly accounting for important transformations and determination of the organoleptic profile of these products. Concerning the CNC, after the first days of fermentation, only *S. xylosum* was detected.

In this paper, we wanted to compare the ecologies of three traditional fermented sausages produced in three plants of the northeastern part of Italy, as determined by traditional and molecular methods. The plants were placed in different locations of the Friuli-Venezia-Giulia region, and they were selected based on the different technologies used for the production and considering the fact that no use of starter cultures

takes place. Plant C produces sausages with long maturation times (120 days), and fermentation is performed at low temperatures. In plant L, the sausages are sold after 45 days, while in plant U, fermentations end just after 28 days. The final products of the three plants have different organoleptic profiles. While sausages from plant U are characterized by an acid taste, the ones obtained from plant C are more aromatic. The products from plant L are slightly acid, with an appreciable aroma. To study the ecology of the naturally fermented sausages considered, a multiphasic approach was used, characterized by a simultaneous use of culture-dependent and -independent methods. Samples were analyzed on agar plates, and counts were determined after incubation. DNA was directly extracted from the sausages, amplified with universal bacterial and yeast primers, and after DGGE analysis of the amplicons obtained as well as sequencing of the bands present in the gels, the profiling of the ecology was revealed. Moreover, the fingerprints obtained by DGGE were subjected to cluster analysis to understand how different the three fermentations were.

The only significant difference in the microbial dynamics as determined by plating analysis was the diverse growth of the LAB in three sausages studied. These differences in the LAB trend become clear if the recipes used for the production and the fermentation conditions are taken into account. Fermentation U was characterized by the highest addition of fermentable sugars (about 2.5%) and a temperature of fermentation of 22°C, thereby allowing fast growth of the LAB populations right from the beginning. In fermentation L, the same temperature conditions were used, but most probably the sugar content (1.5%) did not support fast growth as described for fermentation U. In contrast, the slow growth of LAB in fermentation C must be the result of the low temperatures used in the fermentation process and the absence of added sugars. Nevertheless, for all of the fermentations, LAB reached about the same final number at the last sampling point (10^8 to 10^9 CFU/g). The LAB dynamics directly determined the pH values of the sausages studied. In fact, fermentation U was characterized by the highest jump in pH values at the onset of the fermentation (Fig. 1), while the pH drop for fermentations C and L was smoother and followed the increase in the LAB counts. It is interesting that in all of the fermentations considered in the study, the fecal enterococci reached a value of at least 10^6 CFU/g during fermentation, becoming an important population that possibly influences the final organoleptic characteristics of the product. Regarding yeasts, only for fermentation C was a significant increase observed. Concerning the safety aspect of the sausages studied, *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. were never detected. Moreover, *E. coli* was only counted in the very first days of fermentations C and L. This information points out that good manufacturing practices are implemented and followed in the three plants considered in this research.

It is widely recognized that the main sources of microorganisms in a food fermentation, if starters are not employed, are the ingredients used (in the case of sausage fermentation, meat, salt, and spices) and the production environment. Thus, we can conclude that differences in the microbial species isolated in the three fermentations are due to the diverse distributions of these microbial species in the ingredients and the production environments of the three plants. The species nat-

urally present are subject to a selection process that takes place during fermentation and are eventually defining the dynamics of the fermentations and the final characteristics of the products. In this study, only species able to grow at relatively low temperatures (22 to 12°C for fermentations L and U and 14 to 10°C for fermentation C) and salt resistant were able to colonize the product. Mainly LAB and CNC were responsible for the transformations, but contributions from yeasts and enterococci should be considered as well. The microbial ecologies described in this study are in agreement with the results previously described by other authors. LAB are mainly responsible for the acidification that occurs during the first days of fermentation, while CNC and yeasts participate in the proteolysis and lipolysis processes that correlate with the final characteristics of the product.

When the sausages were subjected to direct analysis by PCR-DGGE, important information became available. A general consideration is that the main differences detected in the ecology were not represented by the species of microorganisms identified by band sequencing but by their relative distribution between the fermentations. Considering the dynamic changes highlighted by the DGGE profiles, fermentation C presented wider biodiversity than fermentations L and U. In these two cases, already at day 3, intense bands were visible, underlining the predominance of specific microbial populations represented by *L. sakei*, *L. curvatus*, *Lactococcus garvieae*, and *S. equorum* or *S. succinus*. In fermentation C, complex DGGE profiles were observed until day 20, when species of LAB took over the fermentation. As reported in Tables 2 and 3, several DGGE bands belonged to the same bacterial or yeast species, confirming the presence of multiple copies of the rRNA genes, as previously described also by other authors (6, 30, 39). The bacterial ecology (Fig. 2 and Table 3) was mainly characterized by the strong presence of LAB populations. In all three fermentations, a stable signal from the beginning of the period studied was visible for *L. curvatus* and *L. sakei*, which remained constant throughout the transformation. Fermentation C was characterized by strong signal only after day 10, while for fermentations L and U, the signal was already appreciable after 3 days. This fact can be explained by considering once more the technological parameters used. In fact, in fermentation C, no sugars were added and low temperatures (<10°C) were used in the first part of the fermentation. Contribution by *L. paracasei* was found in fermentation C, where its specific band was visible from day 20 to day 60. Another LAB representative, *Lactococcus garvieae*, was determined to have an important impact on fermentations L and U. *Lactococcus garvieae* has been described as a common fish pathogen (40), but it is also frequently isolated from dairy products. Strains of this species are able to produce antimicrobial substances (51). Another important contribution to bacterial ecology was given by *Staphylococcus* species. *S. equorum* or *S. succinus* was present in all of the transformations considered. *S. xyloso* was mainly found in fermentation U; its specific band was present from day 5. It was not revealed in fermentation L, while in fermentation C, multiple bands belonging to *S. xyloso* were recognized only at day 10. As previously described (6), the main biodiversity is found at the beginning of the fermentation. As the ripening proceeds, the better adapted populations (LAB and CNC) outnumber the rest of the microflora. This domi-

nance explains the less complex DGGE profile at the end of the fermentation. *Bacillus* spp., *Ruminococcus* sp., *S. intermedius*, and *M. caseolyticus* were detected at day zero. However, a band belonging to *M. caseolyticus* was present at the end of fermentation too for plant U. No *L. plantarum*, an LAB commonly associated with fermented meat products, was detected in the fermentations considered in the study, thereby confirming the results already reported in a previous study (6), in which dead *L. plantarum* cells were detected only at day zero.

The yeast ecology results are characterized by members of the genera *Candida*, *Debaryomyces*, and *Willopsis*. Fermentation C was the only one that showed a specific band for *D. hansenii*, described as proteolytic yeast during fermented sausage production (45), right from the beginning of the fermentation, and it was not detected in fermentations L and U. *C. krisii* and *W. saturnus* for fermentation L and *C. sake* or *C. austromarina* for fermentation U were the main species detected. It should be pointed out that a common band was detected in all of the fermentations monitored. After sequencing, the band was identified as *M. fluvialis*, but the low identity retrieved (86.5%) suggests that the band cut from the gel belongs to a yeast species not yet identified or to a yeast member for which the 26S rRNA gene (D1-D2 loop) has not been deposited yet in GenBank. It should be underlined that growth of yeasts was observed only in fermentation C, where a species highly adapted to fermented sausages was identified. For fermentations L and U, no significant increase was detected. The species found in these two cases were present in the ingredients used for production, but they did not contribute to the fermentation.

Cluster analysis of the DGGE profiles highlighted how the three fermentations shared different level of similarities, when bacterial and yeast ecologies were considered. From the point of view of bacterial profiles, the results for fermentations L and C were very similar (>80%), while fermentation U was more distinct. Samples analyzed in the early stages of fermentations were more diverse, and they formed single clusters. Concerning the yeast ecology, the fermentations considered here showed a specific yeast pattern. On the basis of these results, the finding can be sustained that both bacterial and yeast ecologies were characteristic of each fermentation, thereby allowing their differentiation.

The application of culture-dependent and -independent methods highlighted the stable presence of the LAB members and the contribution to the bacterial ecology of *Staphylococcus* species. It was also observed that LAB DGGE band intensities did not correlate with LAB concentrations obtained by plating on MRS agar. Surprisingly, no bands belonging to fecal enterococci were detected in the DGGE gels, although their counts were above the detection limit of the method, determined to be 10^3 to 10^4 CFU/g when the predominant populations are above 10^8 CFU/g (6), in all of the fermentations studied. This evidence can be explained by considering masking effects by DNA belonging to the major populations present, but most probably is the result of poor amplification of *Enterococcus* spp. obtained by the protocol applied. Efforts to increase the intensity of the PCR amplicons produced from enterococcal DNA were carried out. Different primers, $MgCl_2$ concentrations, and annealing temperatures were tested without success (data not shown). We admit that the use of V1

region does not allow the study of enterococci by the DGGE analysis protocol described here. A possible solution to the problem could be the use of different primers targeting other regions of the 16S rRNA gene. However, a previous study described that only the V1 region allows DGGE differentiation between LAB and CNC, without band comigration (6). In the case of yeast ecology, also when plate counts were below the detection limit, yeasts species were detected by DGGE.

The application of molecular methods allowed identification of strains belonging to *Lactococcus garvieae* and *M. caseolyticus* that are commonly isolated from dairy products. This is particularly important in the case of *Lactococcus garvieae*, since it was isolated in all three fermentations and was stably present throughout fermentation L, thereby deserving consideration from the point of view of its contribution to the final product. Finally, the possibility of applying cluster analysis to the DGGE profiles underlined how the three fermentations could be considered different processes from the point of view of the microbial ecology. Distinct clusters, containing samples from one specific fermentation only, were identified for both bacterial and yeast DGGE gels. In this study, the diverse technological procedures and ingredients used for the production resulted in distinct microbial ecologies that were mainly characterized by a different relative distribution of the same species within each fermentation, rather than the presence of different microorganisms. In our opinion, it is essential to underline, once more, how the use of multiphasic approaches to study food fermentations improves our understanding of these processes. Plating and DGGE analysis should not be considered separately; in fact, the combination of the results obtained allows us to carefully profile microbial dynamics. The methods described here allow a detailed depiction of the fermentation process, representing important tools to use to study the microbial ecology of sausages during transformation. They permit the identification of specific populations which can be selected for their particular characteristics and subsequently be used as starter cultures to improve the sensory profile and sanitary quality.

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