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Investigation of the microbial ecology of Ciauscolo, a traditional Italian salami, by culture-dependent techniques and PCR-DGGE

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Abstract

The microbial ecology of 22 samples of commercially available Ciauscolo salami were investigated using a polyphasic approach, based on culture-dependent and -independent techniques. The viable counts of pathogen and hygiene indicator microorganisms highlighted the adequate application of good manufacturing practices, while the viable counts of the lactic acid bacteria, coagulase negative cocci, and yeasts showed dominance of the first of these microbial groups. Bacterial and fungal DNA were extracted directly from the salami and amplified by PCR, using two primer sets targeting the 16S and 28S rRNA genes, respectively. Denaturing gradient gel electrophoresis (DGGE) and sequencing of selected bands were used to investigate the microbial ecology of these Ciauscolo salami. The most frequently found bacterial species were *Lactobacillus sakei* and *Lb. curvatus*, while *Debaryomyces hansenii* was the prevalent yeast species detected. Cluster analysis of the DGGE profiles and calculation of biodiversity indices allowed the degree of microbial similarity across these salami to be determined.

Keywords: Ciauscolo; PCR-DGGE; Lactic acid bacteria; Coagulase negative cocci; Yeasts

1. Introduction

The production of typical and traditional foods provides an important opportunity for economic progress, particularly in marginal areas. This is the case of the Ciauscolo salami, which is also known as "*Ciabuscolo*" or "*Ciavuscolo*". This is a fermented sausage that has been recently proposed for Protected Geographical Indication (PGI) product classification, within the meaning of European Economic Community (EEC) Regulation No. 2081/92. Ciauscolo was traditionally produced in the mountainous hinterland of the Italian regions of Umbria and Marche (Central Italy), in the area of the Sibillini Mountains, although at present it is also produced in other areas of Central Italy. Its characteristics, and particularly its ability to be spread, are due to its high lard content, to the specific production techniques used, and to the particular conditions of its processing and ripening (Rea, Pacifici, Stocchi, Loschi, & Ceccarelli, 2003).

Ciauscolo is produced from Italian heavy-swine carcasses (weighting from 144 kg to 176 kg), using bacon as the main meat cut, which constitutes about 60% of the minced meat mix. The other cuts used in the mix include shoulder and ham; trimmings deriving from the production of other traditional sausages (Ham and Lonza) are also sometimes used. The meat is minced once or twice and mixed with salt (2.5–3.0%), black pepper (0.3–0.5%), white wine (0.5–1.0%) and crushed garlic (0.25–0.45%). A less diffuse type of Ciauscolo is characterized by the absence of garlic and the addition of liver and bloody meat, which confer on the product a dark red colour that turns to brown (Cerutti, 1999). For large-scale production, preservatives (potassium nitrate and L-ascorbic acid) are generally added to prolong the microbial stability and to

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prevent the abundant lipid component becoming rancid; starter cultures are not usually used.

Bovine and swine guts, which are characterized by homogeneous structures and good resistance, are used in both the industrial and artisan production. After needling, consisting of a slight perforation of the gut that is designed to eliminate small air bubbles, Ciauscolo is dried at room temperature or under controlled conditions, with a 65–70% relative humidity at 19–23 °C for 4–7 days. After this step, a slight smoking may also be performed by saturating the ripening chamber for a few hours with smoke produced by braziers fed with sweet wood sawdust. In the typical areas of its production, where the climate is temperate-cool (Fusari, 2000), the ripening is carried out over the whole year, although traditional production was originally carried out during the Christmas period (Bittarelli, 1985). The ripening lasts 30–80 days (never less than 15 days), depending on the seasonal variations and market demand.

The raw materials and ingredients and the production technique used are of great importance for the qualitative characteristics of fermented meat products. This is due to the strong influence of these parameters on the composition and activity of the microbial population, which is mainly composed of lactic acid bacteria (LAB), coagulase negative cocci (CNC), and to a small extent, yeasts and moulds (Osei Abunyewa, Laing, Hugo, & Viljoen, 2000).

Over the past decade, a culture-independent method that is based on PCR and denaturing gradient gel electrophoresis (DGGE) has been developed for the investigation of complex microbial communities (Muyzer & Smalla, 1998). In the present study, culture-dependent techniques and PCR-DGGE have been successfully used for the investigation of the microbial ecology of Ciauscolo. The aim was thus the assessment of the microbial hygienic quality of ready-for-sale Ciauscolo salami produced in industrial and artisan plants in its typical area of production, and the investigation of its microbial ecology, by culture-dependent and PCR-DGGE techniques. Identification of the main species of technological interest is fundamental for the selection of an autochthonous starter, the use of which should guarantee the microbiological safety of this artisan speciality, and allow control of the fermentation process, without violating the particular qualitative characteristics of the product.

2. Materials and methods

2.1. Bacterial and yeast control strains

The bacterial and yeast strains used as controls for the construction of the DGGE ladders are listed in Table 1. The lactobacilli were grown on MRS agar (Oxoid, Basingstoke, England, UK), with incubation at 30 °C for 48 h; the yeasts were grown on WLN agar (Oxoid), with incubation at 25 °C for 72 h; CNC were grown on TSA (Fluka, Sigma–Aldrich, Milan, Italy), with incubation at 37 °C for 48 h. The reference strains were obtained from: the Deutsche Sammlung von Mikrorganismen und Zellkulturen (DSMZ, Braunschweig, Germany); the National Collection of Food Bacteria (NCFB, Reading, England, UK); the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands); the National Collections of Industrial Bacteria (NCIB, Aberdeen, Scotland, UK); the Northern Regional Research Laboratory ARS Culture Collection (NRRL, Peoria, Illinois, USA); and the Industrial Yeasts Collection Dipartimento di Biologia Vegetale e Biotecnologia Agroambientale (DBVPG, Perugia, Italy).

Table 1
Bacterial and yeast reference strains used for the construction of the DGGE identification ladders

Ladder	Species	Strain
I	*Lactobacillus paracasei* ssp. *paracasei*	DSMZ 5622, DSMZ 2649
	Lactobacillus casei	NCIB 4114, NRRL 1922
	Lactobacillus pentosus	DSMZ 20199, DSMZ 20314
	Lactococcus lactis ssp. *lactis*	DSMZ 20481, DSMZ 20729
	Lactobacillus parabuchneri	DSMZ 5708, DSMZ 5707
	Lactobacillus buchneri	DSMZ 20057, DSMZ5987
	Lactobacillus brevis	DSMZ 20556, DSMZ 2647
	Pediococcus acidilactici	DSMZ 20238, DSMZ 20284
	Lactobacillus helveticus	NCFB 2712, DSMZ 20075
	Lactobacillus farciminis	DSMZ 20184, DSMZ 20180
	Lactobacillus curvatus ssp. *curvatus*	DSMZ 20019, NRRL B-4562
	Lactobacillus plantarum	DSMZ 20174, DSMZ 2601
	Lactobacillus sakei	DSMZ 20017, DSMZ 6333
II	*Candida stellata*	DBVPG 3877, CBS 157
	Debaryomyces hansenii	DSMZ 70238, DSMZ 70244
	Rhodotorula mucilaginosa	DSMZ 70404, DSMZ70403
	Saccharomyces cerevisiae	CBS 1171, CBS 4054
	Yarrowia lipolytica	DSMZ 70561, DSMZ 70562
III	*Kokuria kristinae*	DSMZ 20032, DSMZ 20321
	Kokuria varians	DSMZ 20033, DSMZ 20319
	Staphylococcus carnosus ssp. *carnosus*	DSMZ 20501, DSMZ 4600
	Staphylococcus simulans	DSMZ 20322, DSMZ 20323

2.2. Salami technology and sampling procedures

The Ciauscolo salmi were sampled from 14 artisan and eight industrial production plants located in different geographical areas of the Marche region (Central Italy) (Fig. 1). The ready-for-sale products were sampled at the end of the ripening process (from 15 to 45 days), and they immediately underwent microbiological analyses before being stored at −20 °C until the DNA extraction and PCR-DGGE analyses. For each product, detailed information about the raw materials, ingredients, manufacturing process and ripening conditions were recorded.

2.3. Microbiological analysis

The samples were subjected to culture-dependent assays to monitor the microbial populations involved in the ripen-

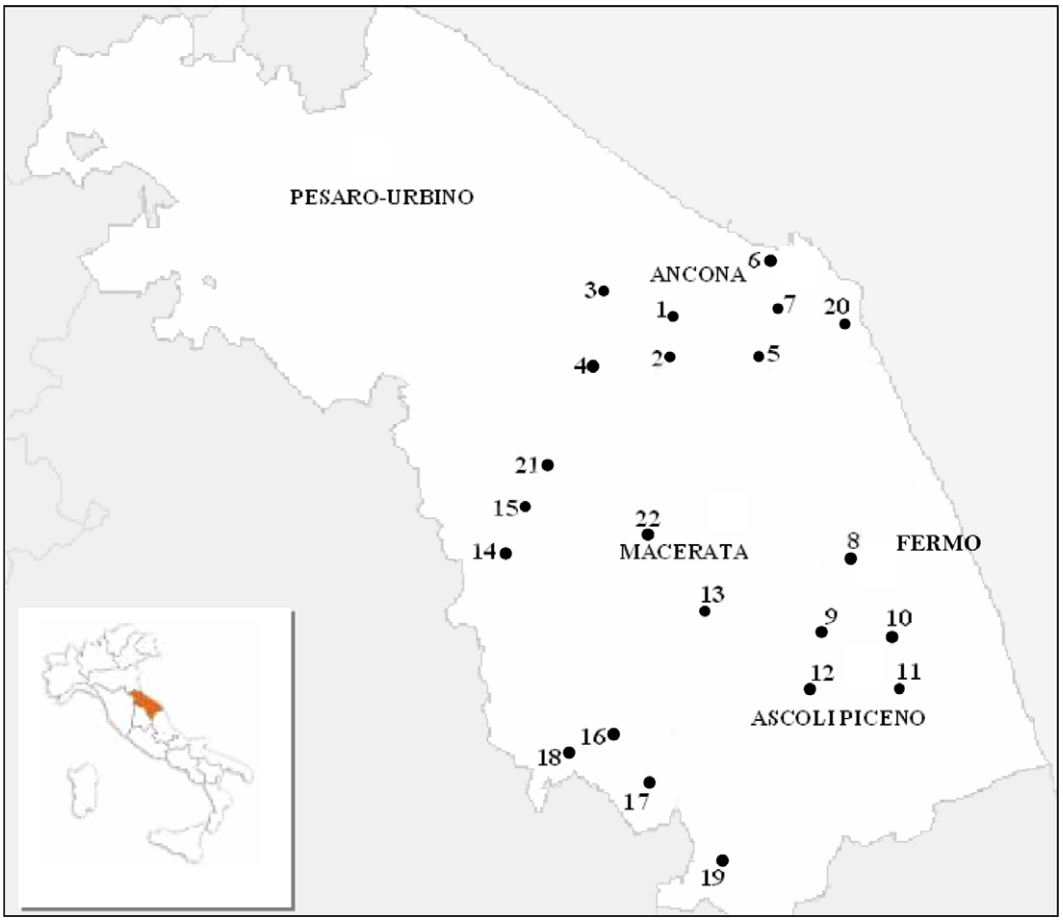

Fig. 1. Location of the 14 industrial and eight artisan plants selected for the sampling of the Ciauscolo.

ing, along with any contaminating micro-organisms. Twenty-five grams of each sample were homogenised in 225 mL of saline-peptone water (8 g/L NaCl, 1 g/L bacteriological peptone) in a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 260 rpm. Serial dilutions were prepared and viable counts of the following micro-organisms were determined in a laboratory using validated methods according to issued standards (UNI CEI EN ISO/IEC 17025): (i) total coliforms and *Escherichia coli* on Coli-ID medium (Biomerieux, Marcy l'Etoile, France), incubated at 37 °C for 24–48 h; (ii) mesophilic LAB on MRS agar (Oxoid) with 300 μg/mL cyclohexamide, incubated at 30 °C for 48 h; (iii) CNC on Baird Parker Medium supplemented with Egg Yolk Tellurite (Oxoid), incubated at 37 °C for 36 h; *Staphylococcus aureus* on BP Medium with RPF supplement (Oxoid), incubated at 37 °C for 24–48 h to test the production of coagulase; and (iv) yeasts on WLN agar (Oxoid) with 250 μg/mL chloramphenicol, incubated at 25 °C for 72 h.

To highlight the significant differences in the viable counts within CNC, yeasts and LAB, a one-way analysis of variance (ANOVA) was carried out, along with the Tukey Kramer honestly significant difference (HSD), using the Statistica software package (version 5.1, StatSoft Inc., Tulsa, Oklahoma, USA). The following variables were considered: VAR1, sample, VAR2, CNC viable counts; VAR3, yeast viable counts; VAR4, LAB viable counts.

2.4. Direct extraction of bacterial and fungal DNA

Bacterial DNA was extracted directly from the salami samples using the method proposed by Fontana, Vignolo, and Cocconcelli (2005), which was modified as follows: after the addition of phenol–chloroform–isoamyl alcohol (25:24:1) and collection of the aqueous phases, the nucleic acids were further purified by the addition of 900 μL chloroform–isoamyl alcohol (24:1). The extraction of fungal DNA from the salami samples was carried out as previously described by Rantsiou et al. (2005).

2.5. PCR protocols

Four microlitres of the bacterial DNA extract, which corresponded to about 80 ng of DNA, were used for the PCR assays, in a total volume of 50 μL that contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 0.01% Tween-20, 0.2 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 U Taq DNA polymerase (Euro-Clone, Milan, Italy).

One microlitre of the fungal DNA extract was used for the PCR assays, in a total volume of 25 μL that contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 0.01% Tween-20, 0.5 μM of each primer, 1.6 mM $MgCl_2$, 0.2 mM dNTPs and 0.625 U Taq DNA polymerase (EuroClone).

The thermal cycling for the different amplification reactions was optimised as follows: (i) for the V1 region of the 16S rRNA gene: initial denaturation at 95 °C for 4 min, followed by a touchdown step (10 cycles) with denaturation at 95 °C for 1 min, annealing at temperatures lowered from 60 °C to 50 °C in intervals of 1 °C every cycle, and elongation at 72 °C for 2.5 min. Twenty additional cycles followed for annealing at 50 °C, and a final extension at 72 °C for 30 min ended the amplification cycle; (ii) for the V3 region of the 16S rRNA gene: initial denaturation at 95 °C for 4 min, followed by a touchdown step (20 cycles) with denaturation at 95 °C for 1 min, annealing at temperatures lowered from 65 °C to 55 °C in intervals of 0.5 °C every cycle, and elongation at 72 °C for 3 min. Ten additional cycles followed for annealing at 55 °C, and a final extension at 72 °C for 30 min ended the amplification reaction (the last of these to eliminate artifactual bands) (Janse, Bok, & Zwart, 2004); (iii) for the 28S rRNA gene: initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 57 °C for 1 min, and 72 °C for 1 min, and final elongation at 72 °C for 30 min.

To recondition all of the PCR products (Thompson, Marcelino, & Polz, 2002), the amplification mixtures were diluted 10-fold into fresh reaction mixture and cycled three times under the conditions specified. All of the PCR assays were carried out in a Gene Amp PCR System 9700 (Applied Biosystem, Foster City, California, USA). The target genes, and their sequences and the positions of the primers are listed in Table 3.

The amplicons were analysed on 1.5% (w/v) agarose gels (Euroclone) using a GeneRuler™ DNA Ladder Mix (Fermentas International, Burlington, Canada) as molecular weight standard. The gels were run at 6 V/cm (constant voltage) in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) and stained with 0.5 μg/mL ethidium bromide. The digital images were viewed and captured using the Image Master VDS (Amersham Biosciences, Piscataway, New Jersey, USA), equipped with the Multi-Analyst system (Bio-Rad Laboratories, Hercules, California, USA).

2.6. DGGE protocols

The PCR products were analyzed by DGGE using the DCode apparatus (Bio-Rad). The PCR products were electrophoresed in a 0.8 mm polyacrilamide gel (8% [w/v]) acrilamide–bisacrilamide (37.5:1), as described by Cocolin, Manzano, Cantoni, and Comi (2001), using two denaturing gradients: one from 30% to 50% and the other from 40% to 60%. The amplicons obtained with the U1–U2 and 338f-518r primers were electrophoresed for 4.5 h, while those obtained with the P1–P2 primers were electrophoresed for 3.5 h. The gels were stained for 20 min in 1.25× TAE buffer (50 mM Tris–HCl, 25 mM acetic acid, 1.25 mM EDTA, pH 8.0) containing ethidium bromide solution, rinsed in distillate water, and photographed under UV illumination. Two replicate runs were always performed for each PCR product.

2.7. Construction of the DGGE ladders

For rapid identification of the DGGE bands, three identification ladders were prepared by mixing equal amounts (5 μL) of the PCR products obtained from the DNA extracted from reference strains belonging to five species of LAB (ladder *I*), five species of yeast (ladder *II*), and four species of CNC (ladder *III*). The choice of the reference strains used in the construction of these ladders was performed through preliminary assays conducted on a larger number of different species (Table 1). For each species, two strains were tested to verify the species-specific migration positions. Only the strains which produced PCR products that migrated as single bands were selected for the construction of the three identification ladders.

2.8. Detection limits of the PCR-DGGE assays

To verify the detection limit of each PCR-DGGE protocol used, aliquots (1 mL) of serial 10-fold dilutions of pure cultures of *Lactobacillus plantarum* DSMZ 20174 (from 10^8 to 0 cfu/mL), *Lb. sakei* DSMZ 6333 (from 10^5 to 0 cfu/mL), and *Debaryomyces hansenii* DSMZ 70238 (from 10^6 to 0 cfu/mL) were inoculated into 9 mL of 3% meat extract (Oxoid) broth (w/v). The DNA was extracted from 1 mL of each spiked sample, amplified by PCR with the relevant primer set, and run in DGGE under the conditions previously described. The viable cells were counted on the appropriate solid culture media. The detection limit of each PCR-DGGE assay was defined as the lowest spike (cfu/mL) yielding a positive signal on the gels.

2.9. Sequencing of DGGE bands

The DGGE bands to be sequenced were excised from the gels with sterile scalpels. The DNA was eluted with 50 μL sterile distilled water and incubated overnight at 4 °C, as suggested by Cocolin et al. (2001). One microlitre of the DNA eluted from each DGGE band was used for amplification and checked by DGGE. Only the products that migrated as single bands and at the original positions with respect to the controls were amplified (with the forward primer without the GC clamp), further purified with the GFX-PCR-DNA and Gel Band purification kit (Amersham Health, Buckinghamshire, UK), and sent to M-Medical/MWG Biotech (Milan, Italy) for sequencing. The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the basic BLAST search tools (Altschul et al., 1997).

2.10. Cluster analysis and calculation of the diversity indices

The bands were assigned to the gel profiles, and the corresponding bands in independent profiles were matched using each DGGE band as the measured unit of diversity. Only the bands which gave a positive result after sequencing or which migrated at a same position of the sequenced bands were considered.

To determine the similarities of the microbial populations present in the salami samples, pairwise comparisons of the DGGE patterns obtained were performed, and a matrix was constructed, based on the presence or absence of each band. The matrix was computed by using the unweighted pair group method with arithmetic mean (UPGMA) (Sokal & Michener, 1958), the Dice similarity coefficient (Dice, 1945), and the NTSYS programme (version 1.8) (Rohlf, 1993). The Dice coefficient is a term-based similarity measure (0–1) whereby the similarity measure is defined as twice the number of terms (DGGE bands) common to the compared entities (DGGE profiles) divided by the total number of terms in both tested entities.

The microbial complexity of each sample was expressed by various indices of biodiversity, calculated from the DGGE patterns: (i) S (species diversity), which corresponds to the number of bands in a DGGE profile; (ii) I (simple index), which was calculated using the formula $I_i = n/n_M$, where i = an index number for each band present in a DGGE profile; n = the number of DGGE bands in a given DGGE profile; n_M = the number of bands in the DGGE profile with the highest number of bands (Ercolini, Mauriello, Blaiotta, Moschetti, & Coppola, 2004); (iii) H (Shannon–Weaver index), which was calculated using the formula $H_i = -\sum p_i \ln(p_i)$, where i = an index number for each band present in a DGGE profile; p_i = frequency of a given band i, which is calculated by dividing the number of DGGE profiles which contain the band i by the total number of DGGE profiles considered (Shannon & Weaver, 1963); (iv) D (Simpson's index), which was calculated using the formula $D_i = \sum(p_i^2)$, where i and p_i are the same as those in the Shannon–Weaver index (Simpson, 1949).

3. Results and discussion

Ciauscolo is a traditional Italian fermented sausage that is characterized by a short ripening period and a high ability for its spreading. Notwithstanding its particular sensory characteristics that render this product unique in the national context, very little is known about its manufacturing technique and its microbial ecology. Accordingly, the present study was designed to determine the composition of the microbial populations of ready-for-sale Ciauscolo from 22 different production plants that were selected on the basis of their distribution within the original area of production (Beccaceci et al., 2006).

3.1. Microbial viable counts

The results of the conventional microbiological analyses are given in Table 2. The LAB populations ranged from 6.77 to 8.65 log cfu/g, while CNC and yeasts ranged from <2.0 to 6.60 log cfu/g and 2.70 to 5.95 log cfu/g, respectively. In all except for three samples (Table 2), the total coliforms and *E. coli* reached viable counts <10 log cfu/g, while *S. aureus* was never detected in the Baird Parker medium.

The statistical analysis of LAB, CNC and yeast viable counts highlighted a great variability between the 22 fermentations, thus suggesting that the composition of the microbial population of Ciauscolo is strongly related to the manufacturing techniques used, as well as to the environmental diversity that characterizes different geographical areas. Notwithstanding this heterogeneity, in all of the fermentations, the microbiological analyses highlighted the dominance of LAB over CNC and yeasts, thus confirming the pivotal role of LAB in the ripening process of fermented sausages (Coppola, Giagnacovo, Iorizzo, & Grazia, 1998; Rantsiou et al., 2005).

3.2. Construction and validation of the DGGE identification ladders

Eight out of 13 LAB reference strains belonging to different species showed complex DGGE profiles, which included two or more bands. Ladder *I* was prepared by

Table 2
Microbial viable counts determined by plating

Sample	log cfu/g				
	Coliforms	*E. coli*	CNC	Yeasts	LAB
1	<10	<10	6.34 b	5.28 h	7.89 g
2	1.78	<10	5.04 d	3.81 r	8.08 f
3	2.04	1.00	6.60 a	5.08 l	8.18 d
4	<10	<10	6.00 bc	4.71 n	8.48 b
5	<10	<10	4.94 de	4.92 m	7.78 i
6	<10	<10	3.48 i	5.84 b	6.77 q
7	<10	<10	3.00 l	4.34 o	7.76 i
8	<10	<10	4.92 de	4.11 q	7.43 m
9	<10	<10	4.76 ef	5.11 i	8.08 f
10	<10	<10	5.15 d	3.45 t	8.26 c
11	<10	<10	3.70 hi	5.43 f	8.11 e
12	<10	<10	4.65 ef	5.95 a	8.11 e
13	1.00	<10	4.52 efg	5.59 e	8.64 a
14	<10	<10	3.00 l	5.57 e	8.65 a
15	<10	<10	3.90 h	4.15 p	8.18 d
16	2.43	1.60	3.48 i	3.80 r	7.49 l
17	<10	<10	5.82 c	3.56 s	7.32 n
18	<10	<10	4.94 de	5.64 d	7.20 o
19	<10	<10	3.60 hi	5.74 c	6.94 p
20	<10	<10	<100 m	2.70 u	6.92 p
21	<10	<10	4.36 fg	5.36 g	7.76 i
22	<10	<10	3.90 h	5.26 h	7.51 h

Within each data set referred to LAB, CNC, and yeast viable counts, the values labelled with the same letter(s) are not significantly different ($P < 0.05$).

Table 3
PCR primers used in this study

Target region	Primer	Sequence (5′ → 3′)	Position	T_a (°C)	Reference
V1 16Sr RNA gene	P1 (F)[c]	GCGGCGTGCCTAATACATGC	41–60[a]	60 → 50	Klijn et al., 1991
	P2 (R)	TTCCCCACGCGTTACTCACC	111–130[a]		
28Sr RNA gene	U1 (F)[d]	GTGAAATTGTTGAAAGGGAA	403–422[b]	57	Sandhu et al., 1995
	U2 (R)	GACTCCTTGGTCCGTGTT	645–662[b]		

(F) forward primer; (R) reverse primer.
[a] *Escherichia coli* numbering.
[b] *Saccharomyces cerevisiae* numbering.
[c] A GC clamp (Sheffield et al., 1989) was attached to the 5′ end of the primer.
[d] A GC clamp (Walter et al., 2001) was attached to the 5′ end of the primer.

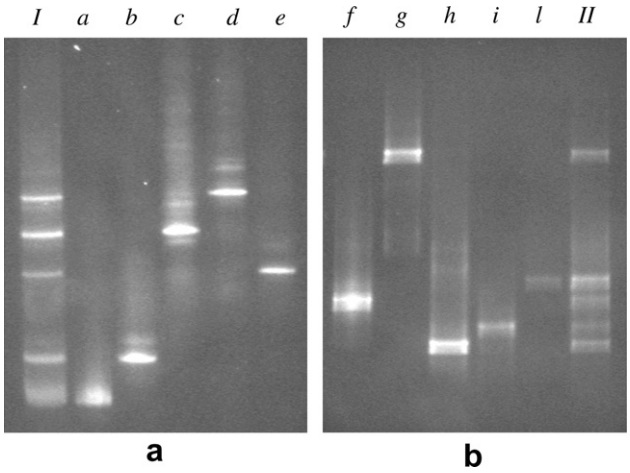

Fig. 2. DGGE identification ladder *I* (a) and *II* (b). Lanes *a* to *e*, bands in ladder *I*: *a*, *Lactobacillus helveticus* NCFB 2712; *b*, *Lactobacillus farciminis* DSMZ 20184; *c*, *Lactobacillus curvatus* ssp. *curvatus* DSMZ 20019; *d*, *Lactobacillus plantarum* DSMZ 20154; *e*, *Lactobacillus sakei* DSMZ 20017. Lanes *f* to *l*, bands in ladder *II*: *f*, *Yarrowia lipolytica* DSMZ 70561; *g*, *Debaryomyces hansenii* DSMZ 70238; *h*, *Rhodotorula mucilaginosa* DSMZ 70404; *i*, *Candida stellata* DBVPG 3877; *l*, *Saccharomyces cerevisiae* CBS 1171.

using one strain for each of the five species that showed DGGE profiles with single bands (Fig. 2a).

Unfortunately, all of the CNC reference strains assayed produced DGGE profiles consisting of multiple bands (data not shown), which in some cases co-migrated. This finding prevented the construction of a reliable ladder for the preliminary identification of CNC under the conditions used.

The visualization of complex DGGE profiles from a few of our bacterial pure cultures could be ascribed to the presence of multiple copies of the 16S rRNA gene, which are characterized by heterogeneous sequences (Dahllof, Baillie, & Kjelleberg, 2000). Thus, it has been demonstrated that different copies of the small-subunit rRNA gene within the same genome can differ by up to 6.5% (Wang & Wang, 1997).

Finally, profiles consisting of two bands that migrated very close together were seen for all of the 10 yeast reference strains assayed. Accordingly, one reference strain for each of the five species tested was selected for the construction of ladder *II* (Fig. 2b).

3.3. PCR-DGGE detection limit

Knowledge of the detection limit of PCR-DGGE assays is fundamental for the effective evaluation of the microbial ecology of fermented foods. Accordingly, the number of microbial cells that could be detected by the two PCR-DGGE protocols used was determined using exponential-phase cultures of control strains which were separately inoculated into sterilized meat extract broth. The choice of this medium arose from the need to reproduce to as great an extent as possible the physico-chemical characteristics of the food matrix under study, while avoiding overestimations due to the spiking of sausage samples harbouring resident LAB populations.

Since different detection limits can be obtained when strains belonging to different species are assayed, due to differences in the genome size, the G + C content, the organization and number of the rRNA genes, and the number of genomes within the same cell (Farrelly, Rainey, & Stackebrandt, 1995), two reference strains ascribed to *Lb. plantarum* and *Lb. sakei* were used. These strains differed in genome size (1884661 bp for *Lb. sakei* [GenBank accession number CR936503], and 3308274 bp for *Lb. plantarum* [GenBank accession number AL935263]), G + C content (41% for *Lb. sakei*, and 44% for *Lb. plantarum*), and number of *rrn* operons (7 for *Lb. sakei*, and 5 for *Lb. plantarum*).

As a result, our protocols yielded visible bands when the meat extract broths were spiked with 10^5 cfu/mL of *Lb. plantarum* DSMZ 20174, 10^3 cfu/mL of *Lb. sakei* DSMZ 6333, and 10^2 cfu/mL of *D. hansenii* DSMZ 70238.

3.4. DGGE analysis

Total bacterial and yeast DNA were separately extracted and amplified with two primer sets, targeting the 16S (V1 region) and 28S rRNA genes, respectively. The LAB and yeast DGGE profiles obtained are shown in Figs. 3 and 4, respectively. No differences were seen in

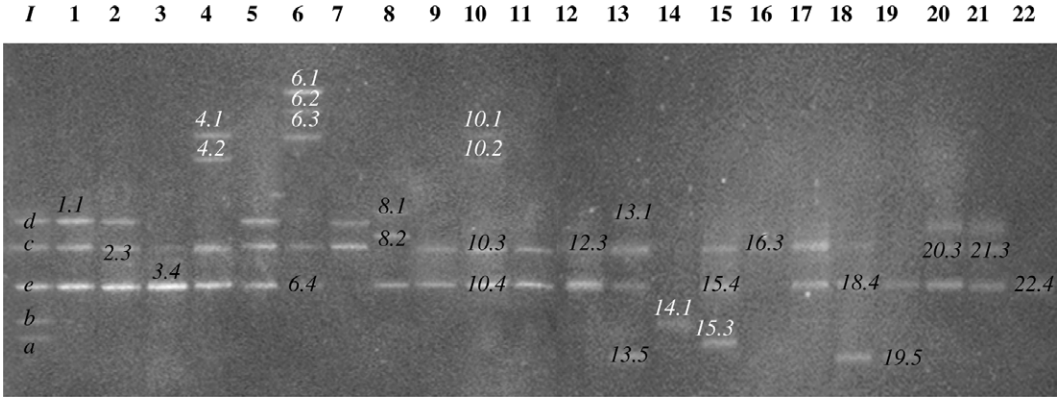

Fig. 3. LAB DGGE profiles of the 16S rRNA gene amplicons obtained from the 22 samples of Ciauscolo under study (lanes 1–22). The bands selected for sequencing are labelled with numerals (in white, those identified as heteroduplex or chimeric molecules; in black those belonging to LAB species). Lane *I*-DGGE identification ladder. Bands *a* to *e*, bands in ladder *I*: *a*, *Lactobacillus helveticus* NCFB 2712; *b*, *Lactobacillus farciminis* DSMZ 20184; *c*, *Lactobacillus curvatus* ssp. *curvatus* DSMZ 20019; *d*, *Lactobacillus plantarum* DSMZ 20154; *e*, *Lactobacillus sakei* DSMZ 20017.

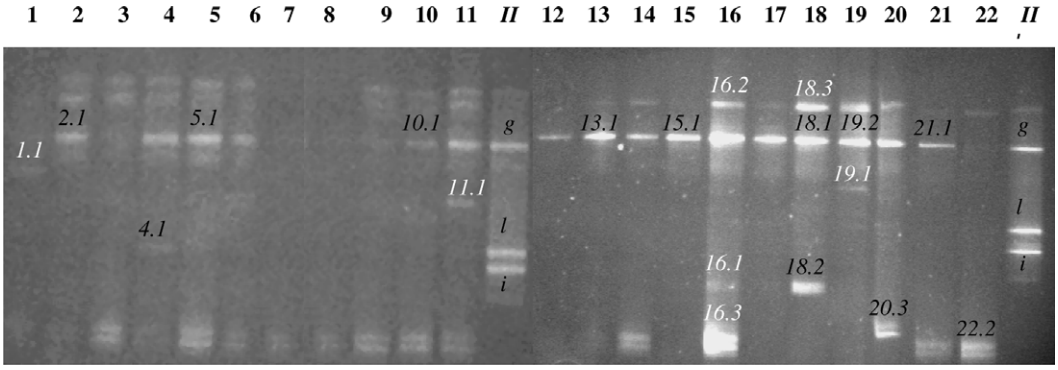

Fig. 4. Yeast DGGE profiles of the 28S rRNA gene amplicons obtained from the 22 Ciauscolo samples under study (lanes 1 to 22). The bands selected for sequencing are labelled with numerals (in white, those identified as heteroduplex or chimeric molecules; in black those belonging to yeast species). Lane *II*-DGGE identification ladder. Bands *f* to *l*, bands in ladder *II*: *f*, *Yarrowia lipolytica* DSMZ 70561; *g*, *Debaryomyces hansenii* DSMZ 70238; *h*, *Rhodotorula mucilaginosa* DSMZ 70404; *i*, *Candida stellata* DBVPG 3877; *l*, *Saccharomyces cerevisiae* CBS 1171.

Table 4
Sequencing results from the bands cut from the bacterial DGGE gels

Band(s)[a]	Closest relative	% Identity[b]	*E* value	Accession No.[c]
1.1, 8.1, 13.1	*Lactobacillus plantarum*	100	2e−22	DQ239699.1
8.2	*Pediococcus acidilactici*	100	7e−23	DQ211656.1
2.3, 10.3, 12.3, 16.3, 20.3, 21.3	*Lactobacillus curvatus*	96	0.004	AY230231.1
3.4, 6.4, 10.4, 15.4, 18.4, 22.4	*Lactobacillus sakei*	96	0.004	AY204898.1
13.5, 19.5	*Lactococcus lactis* ssp. *lactis*	94	5e−05	AE006288.1

[a] Bands are numbered as indicated on the DGGE gels shown in Fig. 3.
[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.
[c] Accession number of sequence of the closest relative found by BLAST search.

the DGGE profiles when amplicons obtained from the same samples were analyzed (data not shown).

The bands were first identified by comparing their relative positions of migration in the acrilamide gels with the DGGE profiles of the ladder strains. Preliminary assignment into species was further confirmed by the sequencing of the DNA bands excised from the denaturing gels. The identification of the bacterial and yeast DGGE bands is shown in Tables 4 and 5, respectively.

Although the PCR-DGGE protocol based on the use of primers P1 and P2 has been described as being suitable for the detection and differentiation of *Lactobacillus*, *Staphylococcus* and *Kocuria* spp. (Cocolin et al., 2001), surprisingly no CNC species could be identified. This could be due to the masking effects of the DNA extracted from the more abundant LAB populations, and therefore to competition among the templates during the amplification steps (Ercolini, 2004). This is supported by the viable counts that

Table 5
Sequencing results from the bands cut from the yeast DGGE gels

Band(s)[a]	Closest relative	% Identity[b]	*E* value	Accession No.[c]
2.1, 5.1, 10.1, 13.1, 15.1, 18.1, 19.2, 21.1	*Debaryomyces hansenii*	100	7e−96	AY497693.1
4.1		93	0.19	AY497693.1
18.2	*Candida psychrophila*	100	6e−11	AY040651.1
20.3	*Saccharomyces barnettii*	99	3e−63	AY048164.1
22.2	*Penicillium hirsutum*	96	8e−79	U15466.1

[a] Bands are numbered as indicated on the DGGE gels shown in Fig. 4.
[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.
[c] Accession number of sequence of the closest relative found by BLAST search.

clearly showed the prevalence of LAB over CNC. Several LAB species were found, namely: *L. sakei*, *Lb. curvatus*, *Lb. plantarum*, *Pedicococcus acidilactici*, *L. lactis* spp. *lactis*, (Fig. 3, Table 4). Although the identities retrieved from the GenBank database for *Lb. curvatus* and *Lb. sakei* were too low (96%) to allow reliable assignment into species, sequence alignments were supported by the co-migration between the bands cut from the DGGE gels and those of the corresponding ladder strains, DSMZ 20019 and DSMZ 20017, respectively. Moreover, an identity of 94% was obtained for *L. lactis* spp. *lactis*; as for *Lb. curvatus* and *Lb. sakei*, the BLAST alignments were verified by comparison of the migration positions of these bands and those produced by the two reference strains, DSMZ 20481 and DSMZ 20729 (data not shown). Seven bands (labelled with white numbers in Fig. 3) that were visualized in the upper part of the DGGE gels failed in the sequencing, and were therefore identified as heteroduplex or chimeric amplicons.

The production of heteroduplex and chimeric sequences between heterogeneous rRNA gene copies during the PCR assay may also contribute significantly to the occurrence of complex DGGE profiles (Thompson et al., 2002). The production of multiple bands from single strains can be particularly problematic, both in the construction of suitable identification ladders and, to a greater extent, in the evaluation of complex ecosystems, because of overestimations of microbial biodiversity.

For the bacterial species distribution in the 22 productions investigated, *Lb. sakei* and *Lb. curvatus* were the most frequently detected. This is in agreement with data reported for other fermented sausages produced in Italy (Cocolin et al., 2001; Coppola et al., 1998) and in other European countries (Aymerich, Martin, Garriga, & Hugas, 2003; Rantsiou et al., 2005). As has been previously suggested (Parente, Grieco, & Crudele, 2001), the prevalence of these two psychrotropic species can be ascribed to the low temperatures used for the drying and ripening.

The less frequent detection of *Lb. plantarum* and *P. acidilactici* with respect to *Lb. sakei* and *Lb. curvatus* in these Ciauscolo samples confirmed the lower competitiveness of these former two species in fermented meat products, as has been previously described (Coppola, Mauriello, Aponte, Moschetti, & Villani, 2000; Doßmann, Hammes, Klostermaier, & Vogel, 1998).

For the yeast ecology (Fig. 4, Table 5), simpler DGGE profiles were seen, potentially due to the fungistatic effects of garlic (Olesen & Stahnke, 2000), which is in abundant use in the manufacture of Ciauscolo. *D. hansenii* was the most frequently detected species, while *Candida psychrophila*, and *Saccharomyces barnettii* were occasionally found.

The documented predominance of *Debaryomyces* spp. in pork-based products (Rantsiou & Cocolin, 2006) has been related to the high tolerance of these yeasts to salt, and to their ability to grow at low temperatures and to metabolize organic acids (Osei Abunyewa et al., 2000). Furthermore, the presence of *D. hansenii* is known to have a positive effect on the development of a characteristic yeast flavour and on the stabilization of the reddening reaction (Rantsiou & Cocolin, 2006).

A DGGE band that showed the closest relative in the GenkBank DNA database of *Penicillium hirsutum* was also detected. Although the identity (96%) of this mould species was too low to guarantee reliable assignment into species, its presence in the large majority of our samples could be explained by the addition of crushed garlic to the meat batter, seeing that *P. hirsutum* is known to be the causal agent of garlic blue mould (D'Ercole, 1972).

3.5. Calculation of the diversity indices

The microbial diversity of each sample was calculated using four diversity indices, namely *S*, *I*, *H* and *D*, based on richness (the number of sequenced bands in a given DGGE profile) and frequency (*p*) (occurrence of a given DGGE band in the 22 profiles analyzed). *S* simply reflects the number of sequenced bands in a given DGGE profile; *I* expresses the degree of complexity of a given DGGE profile, compared with the greatest biodiversity detected; *H* and *D* are two general diversity indices: the highest values for *H* are expected in samples with the highest number of sequenced DGGE bands and with similar frequencies for each band, whereas *D* is smaller when the frequencies of the sequenced bands are similar, and it is higher when one band predominates (Sneath & Sokal, 1973).

Table 6 gives the values obtained for *S*, *I*, *H* and *D* for each sample, and the mean values for each index.

Table 6
The indexes of biodiversity calculated for each sample

Sample	Bacteria				Fungi			
	S	*I*	*H*	*D*	*S*	*I*	*H*	*D*
1	3	0.75	0.73	1.41	0	0.00	0.00	0.00
2	3	0.75	0.73	1.41	1	0.33	0.20	0.60
3	1	0.25	0.13	0.75	2	0.67	0.51	0.95
4	2	0.50	0.36	1.27	3	1.00	0.65	0.95
5	3	0.75	0.73	1.41	2	0.67	0.51	0.95
6	2	0.50	0.36	1.27	2	0.67	0.51	0.95
7	2	0.50	0.60	0.66	1	0.33	0.31	0.35
8	3	0.75	0.63	0.88	1	0.33	0.31	0.35
9	2	0.50	0.36	1.27	1	0.33	0.31	0.35
10	2	0.50	0.36	1.27	2	0.67	0.51	0.95
11	2	0.50	0.36	1.27	2	0.67	0.51	0.95
12	2	0.50	0.36	1.27	1	0.33	0.20	0.60
13	4	1.00	1.00	1.43	1	0.33	0.20	0.60
14	0	0.00	0.00	0.00	2	0.67	0.51	0.95
15	2	0.50	0.36	1.27	1	0.33	0.20	0.60
16	1	0.25	0.23	0.53	2	0.67	0.51	0.95
17	2	0.50	0.36	1.27	1	0.33	0.20	0.60
18	2	0.50	0.40	0.76	2	0.67	0.34	0.60
19	2	0.50	0.40	0.76	1	0.33	0.20	0.60
20	3	0.75	0.73	1.41	2	0.67	0.34	0.60
21	3	0.75	0.73	1.41	2	0.67	0.51	0.95
22	1	0.25	0.13	0.75	1	0.33	0.31	0.35
Average	2.14	0.53	0.45	1.08	1.50	0.50	0.36	0.67

For the bacterial ecology, the highest values for all of the four indices were obtained for sample 13, which is characterized by the highest LAB diversity and the presence of species occurring with different frequencies. When the results of the viable counts were comparatively evaluated, the high LAB diversity in sample 13 was also shown to be coupled to a clear dominance of this bacterial group over the overall microbial community, since significantly higher LAB loads were counted.

A generally lower diversity was seen when the yeast and mould ecologies were evaluated, as revealed by the mean values calculated for each index. A high homogeneity of the diversity indices among the samples was also seen, thus confirming the secondary role of yeasts and moulds in the ripening process of fermented meat products, as also revealed by the viable counts.

3.6. Cluster analysis

Cluster analyses based on the DGGE profiles was aimed at determining how the 22 fermentations share different levels of similarity, when the bacterial and yeast ecologies were evaluated. The UPGMA trees obtained are given in Fig. 5. For the bacterial and yeast profiles, one and two main clusters were defined at a similarity of 75% (cluster I) and 65% (cluster II and III), respectively. By considering the origin of the samples, both the bacterial and fungal ecologies seem not to be influenced by the plant location, since a high degree of microbial similarity was seen for salami collected from different geographical areas. On the other hand, samples collected from relatively narrow geographical areas differed in the composition of their microbial populations, and therefore they group into different clusters.

The absence of a correlation between the area of production and the microbial complexity of our samples apparently denies the existence of a typology of Ciauscolo, which is manufactured in a circumscribed mountainous geographical area and is characterized by particular traits that can be ascribed to the natural drying and aging in a homogeneous climate environment (Rea et al., 2003). These findings could arise from the current use of ripening rooms in both the industrial and artisan plants, which on the one hand guarantees a standardisation of the maturation process, but on the other has repercussions on the link of the product to the territory.

When a distance data matrix was prepared by combining the DGGE patterns obtained in the present study and the manufacturing parameters used in each plant (Beccaceci et al., 2006), no correlations between the clustering of the samples and the production scale (industrial or artisan) or the manufacturing technology were ever seen (data not shown). This further evidence can be explained by the standardisation of the production technology at both the

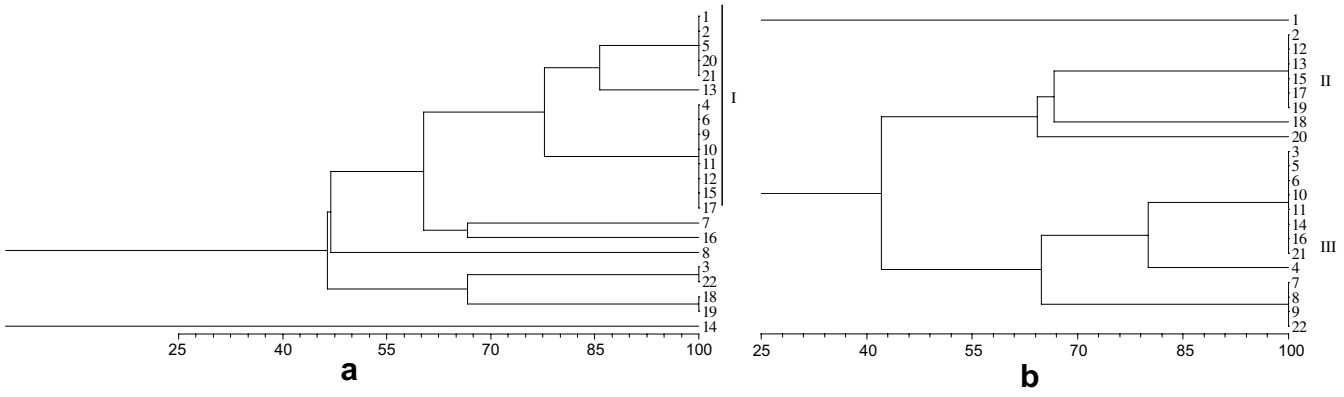

Fig. 5. Cluster analysis of the profiles obtained by the bacterial (a) and yeast (b) DGGE profiles. The dendrograms were obtained with the UPGMA clustering algorithm. The clusters identified (at a similarity threshold of 70%) are indicated by numerals to the right of each panel.

artisan and industrial level, to guarantee the high hygienic quality of the product and an optimization of the production process, while respecting the local traditions.

In conclusion, as expected, the combination of culture-dependant assays and PCR-DGGE analyses has allowed the microbial ecology of this traditional Ciauscolo salami to be profiled. This approach also allowed the identification of the microbial species that are involved in the fermentation and ripening of this traditional product, and to determine the relative distributions of these species between the 22 salami analyzed. On the basis of our overall results, no correlations between the area of production and the microbial complexity were found, probably due to the standardization of the manufacturing processes at both artisan and industrial level.

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