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Characterisation of naturally fermented sausages produced in the North East of Italy

Giuseppe Comi [a], Rosalinda Urso [a], Lucilla Iacumin [a], Kalliopi Rantsiou [a], Patrizia Cattaneo [b], Carlo Cantoni [b], Luca Cocolin [a,*]

[a] Dipartimento Scienze degli Alimenti, Università degli studi di Udine, via Marangoni 97, 33100 Udine, Italy
[b] Dipartimento di Scienze e Tecnologie, Veterinarie per la Sicurezza degli Alimenti, Università degli studi di Milano, via Celoria 10, 20121, Milan, Italy

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Abstract

In the Friuli Venezia Giulia region, in the North East of Italy, a traditional fermented sausage is produced without the use of microbial starters. It is characterized at the end of the ripening period by accentuated acidity, slight sourness and elastic, semi-hard consistency. In this study, three fermentations, carried out in different seasons (winter, spring and summer) were followed analyzing the microbiological, physicochemical and sensory aspects of this product. The sausages were characterized by an important microbial activity of lactic acid bacteria and micro/staphylococci that resulted in a product with a final pH of about 5.6–5.7. An interesting aspect was the high number of fecal enterococci that can play an important role in the definition of the organoleptic profile of the final product. No *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* were ever isolated from the raw materials or the fermented sausages during the maturation, underlining the safety of this product. The final water activity of the product was 0.91–0.92. One hundred and fifty lactic acid bacteria were isolated and identified by molecular methods to understand which species were more predominant in the product. *Lactobacillus curvatus* and *Lactobacillus sakei* were the most numerous (54 and 64 strains isolated, respectively) and they were the only species common to all three fermentations. A cluster analysis of the profiles obtained from these strains after RAPD-PCR highlighted a population distribution that was fermentation-specific.

Keywords: Italian fermented sausages; Microbial ecology; Physicochemical profile; Sensorial evaluation; Molecular identification; RAPD-PCR

1. Introduction

In Europe, natural fermented sausages have a long tradition originating from Mediterranean countries during Roman times (Lucke, 1985). In Italy, there are many different types of natural fermented sausages and almost all of them are only known at local or regional level. In the Friuli Venezia Giulia region, in the North East of Italy, a traditional fermented sausage is produced without the use of microbial starters, characterized at the end of the ripening by accentuated acidity, slight sourness and elastic, semi-hard consistency. Because of these characteristics, it displays unique organoleptic and sensory profiles that make it different from other products produced in the rest of Italy. This product is obtained from fresh pork meat and lard mixed with other ingredients, such as sugars, NaCl and additives (nitrate, nitrite and spices).

A wide variety of microorganisms have already been isolated, by traditional methods, from these types of fermentations, mainly represented by lactic acid bacteria (LAB) and *Staphylococcus* and *Kocuria* spp. (del Carmen de la Rosa, Mohino, Mohino, & Mosso, 1990; Hugas, Garriga, Aymerich, & Monfort, 1993). The species

* Corresponding author. Tel.: +39 0432 590 759; fax: +39 0432 590 719.
E-mail address: lscocolin@uniud.it (L. Cocolin).

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of LAB most commonly found in meat and meat products, including fermented sausages processed with different technologies, are *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* (Hammes, Bantleon, & Min, 1990; Hugas et al., 1993; Schillinger & Lücke, 1987), with *Lb. sakei* being the species most frequently isolated. *Staphylococcus xylosus* is one of the prevalent staphylococci species found in naturally fermented sausages (Cocolin, Manzano, Aggio, Cantoni, & Comi, 2001; Coppola, Iorizzo, Saotta, Sorrentino, & Grazia, 1997). Nevertheless, *Kocuria varians* and *Kocuria kristinae* have also been identified in fermented sausages (Fischer & Schleifer, 1980).

The study of the ecology of fermented sausages is of primary importance to understand the physical and chemical changes occurring during fermentation and maturation. The microflora that develops is closely related to the ripening technique utilized. Sausages with a short ripening time have more lactobacilli right from the early stages of fermentation. In contrast, sausages with longer maturation times, contain higher numbers of *Micrococcaceae* (Demeyer, Verplaetse, & Gistelink, 1986).

LAB are responsible for lactic acid production, for the "tangy" flavour 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 used, and the sources of meat proteins and additives (Bacus, 1986; Demeyer, 1982; Thornill & Cogan, 1984). *Staphylococcus* and *Kocuria* are important for colour stabilization, decomposition of peroxides, and aroma formation due to their proteolytic and lipolytic activities (Cai, Kumai, Ogawa, Benno, & Nakase, 1999; Miralles, Flores, & Perez-Martinez, 1996; Schleifer, 1986).

Sensitive and reliable methods for the detection and identification of microorganisms involved in food fermentations are of great importance to monitor population changes during the process (Holzapfel, Geisen, & Schillinger, 1995). In the last decade, it was shown that classical microbiological techniques do not give a real view of microbial diversity (ben Omar & Ampe, 2000; Hugenholtz, Goebbel, & Pace, 1998). In an effort to improve identification of microorganisms, molecular methods are being employed nowadays. The majority of them are based on the DNA sequence of the 16S rRNA gene (rDNA) (Collins et al., 1991). This gene gives important information for the detection, identification and classification of LAB as well as other microorganisms. Protein profiling (Pot, Vandamme, & Kersters, 1994), ribotyping (Zhong, Millsap, Bialkowska-Hobrzanska, & Reid, 1998) and pulsed field gel elecrophoresis (PFGE) (Tenover et al., 1995), are applied as well, but they are either too laborious, or limited in their resolving power or even require a species-specific methodology. Recently, molecular tools, based on denaturing gradient gel electrophoresis (DGGE) analysis of PCR products obtained from the V1 region of the 16S rDNA to identify *Lactobacillus* spp. isolated from Italian fermented sausages (Cocolin, Manzano, Cantoni, & Comi, 2001), were developed.

In this paper, we describe the microbiological, physicochemical and sensory characterization of a typical Friuli Venezia Giulia natural fermented sausage. An industrial plant, where no starter cultures are used, was selected and three productions, in different seasons, were followed. Microbiological counts, measurements of the main physicochemical parameters and a profiling of the most important organoleptic characters were carried out. Moreover, 150 LAB were isolated and identified by the means of PCR-DGGE. Strains of *Lb. curvatus* and *Lb. sakei,* present in all three fermentations, were subsequently characterized by analyzing the random amplification of polymorphic DNA (RAPD) profiles obtained with the M13 primer.

2. Materials and methods

2.1. Fermented sausages technology and sampling procedures

Fermented sausages were prepared in a local meat factory using traditional techniques. A total of three fermentations were followed. The first started on February 21, 2003, the second on April 14, 2003, while the third on July 11, 2003. Pork meat (60 kg), lard (40 kg), a mix of sodium chloride (2.5 kg) and black pepper (70 g), sugars (1.5 kg), and nitrite/nitrate (200 ppm) were mixed and used to fill natural casings, resulting in 25 cm long and 5 cm diameter fresh sausages. The ripening was performed as follows: the first stage consisted of 2 days drying with the relative humidity (RH) of 85% and a temperature of 22 °C that was then decreased to 12 °C, with a rate of 2 °C/day with an RH between 60% and 90%. The ripening was then carried out for 21 days at 12 °C in storerooms with 65–85% RH. No smoke was applied at any stage. Meats, spices, natural casings and the fermented sausages at 0, 3, 5, 7, 14 and 28 days were analyzed. Three samples were collected and used for the analyses.

2.2. Physicochemical analysis

The determination of moisture, inorganic matter, proteins, fat and the nitrite and nitrate concentration was performed according to AOAC (2002). Water activity (A_w) was measured by Acqua Lab CX-2 (Decagon Devices, Inc. Pulman, WA). Potentiometric measurements of pH were made using a pin electrode of a pH-meter (Radiometer Copenhagen pH M82, Cecchinato, Italy) inserted directly into the sample. Three independ-

ent measurements were made on each sample. Means and standard deviations were calculated.

2.3. Microbiological analysis

Raw materials and fermented sausages were subjected to microbiological analysis to monitor the dynamic changes in the populations responsible for the ripening of fermented sausages and their hygienic quality. In particular, 25 g of each sample were transferred into a sterile stomacher bag and 225 ml of saline/peptone water (8 g/l NaCl, 1 g/l bacteriological peptone, Oxoid, Milan, Italy) were added and mixed for 1 min and 30 s in a Stomacher machine (PBI, Italy). Further decimal dilutions were made and the following analyses were carried out on duplicate agar plates: (a) total viable count on Peptone Agar (8 g/l bacteriological peptone, 15 g/l bacteriological agar, Oxoid) incubated for 48–72 h at 30 °C; (b) LAB on MRS agar (Oxoid) incubated with a double layer at 30 °C for 48 h; (c) *Micrococcaceae* on Mannitol Salt Agar (Oxoid) incubated at 30 °C for 48 h; (d) total enterobacteria and *Escherichia coli* on Coli-ID medium (Bio Merieux, France) incubated with a double layer at 37 °C for 24–48 h; (e) fecal enterococci on Kanamycin Aesculin agar (Oxoid) incubated at 42 °C for 24 h; (f) *Staphylococcus aureus* on Baird Parker medium (Oxoid) with added egg yolk tellurite emulsion (Oxoid) incubated at 37 °C for 24–48 h; (g) yeasts and moulds on Malt Extract Agar (Oxoid) supplemented with tetracycline (1 mg/ml, Sigma, Milan, Italy) incubated at 25 °C for 48–72 h; (h) *Pseudomonas* spp. on Pseudomonas CN Agar (Oxoid) incubated at 25 °C for 48 h; (i) aerobic spore formers on Plate Count Agar (Oxoid) after pasteurization at 80 °C for 10 min, incubated at 30 °C for 48 h; (l) sulphite reducing clostridia on SPS Agar (Oxoid) incubated anaerobically at 37 °C for 72 h. For *Listeria monocytogenes*, the ISO/DIS method (1990) was used, while for *Salmonella* spp. the ISO/DIS method (1991) was applied. After counting, means and standard deviations were calculated. Different microbiological analyses were performed on the basis of the sample considered. From the fermented sausages, 50 LAB strains, from MRS plates were randomly selected from each fermentation, streaked on MRS agar and stored at −20 °C in MRS broth containing 30% (v/v) glycerol before being subjected to molecular identification and characterization. Fifty percent of the strains were isolated in the first 7 days of the fermentation while the rest were selected from the 14th and 28th day of maturation.

2.4. Sensory evaluation

A group of 10 people evaluated the sensory characteristics of the sausages studied. It is important to underline that these persons were not experts and they were represented by students and employees of the Department of Food Science, University of Udine. They were asked to score different parameters of the sausages on the basis of the intensity perceived. Colour, cut surface, coherence, smell, rancidity, fat quality, acidity, juiciness, tenderness, overall flavour, after taste and overall impression were taken into consideration for the sensory evaluation. To facilitate grading, a line 10 cm long was used. The members of the panel had to mark the line at the point they felt it represented their evaluation based on the intensity perceived. The marking was then translated in grades based on the position on the line. After evaluation, means were calculated.

2.5. DNA extraction from pure cultures

Four millilitres of a 24 h culture were centrifuged at 14,000*g* for 10 min at 4 °C to pellet the cells, which were subjected to DNA extraction as suggested by Andrigetto, Zampese, and Lombardi (2001) modified by using only lysozyme (50 mg/ml, Sigma) for the bacterial cell-wall digestion.

2.6. Identification of the LAB isolates

Gram staining and catalase test were used to screen the isolates and identify the strains belonging to the LAB group. LAB were then identified by molecular methods by means of the PCR-DGGE as described by Cocolin, Manzano, Aggio, et al. (2001). Strains with the same DGGE profiles were grouped and representatives of each group were amplified using primers P1 and P4 as described by Klijn, Weerkamp, and deVos (1991), targeting 700 bp of the V1–V3 region of the 16S rDNA. After purification, products were sent to a commercial facility for sequencing (MWG Biotech, Edersberg, Germany). Sequences were aligned in GenBank using the Blast program (Altschul et al., 1997) to determine the closest known relatives of the partial 16S rDNA sequence obtained.

2.7. RAPD analysis

One hundred nanograms of the DNA extracted from isolated LAB strains were subjected to RAPD-PCR using primer M13 (5′-GAG GGT GGC GGT TCT-3′) as previously reported (Andrigetto et al., 2001). Reactions were carried out in a final volume of 50 μl containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 1 μM primer and 1.25 U *Taq*-polymerase (Applied Biosystems, Milan, Italy). The amplification cycle was as follows: 35 repetitions of 94 °C for 1 min, 38 °C for 1 min, ramp to 72 °C at 0.6 °C/s, 72 °C for 2 min. An initial denaturation at 94 °C for 5 min, and a final extension at 72 °C for 5 min, were also carried out. RAPD-PCR products were

analyzed by electrophoresis on 1.5% (w/v) agarose gels in 0.5× TBE at 120 V for 4 h. Gels were stained in 0.5× TBE buffer containing 0.5 μg/ml ethidium bromide (Sigma) for 20–30 min. Pictures of the gels were digitally captured using the BioImaging System GeneGenius (SynGene, Cambridge, UK) and the pattern analysis software package, Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium) was used for the analysis. Calculation of similarity in the profiles of bands was based on Pearson product–moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm (Vauterin & Vauterin, 1992). A coefficient of correlation of 70% was arbitrarily selected to distinguish the clusters. LAB isolates were subjected to RAPD-PCR analysis at least twice.

3. Results

3.1. Microbiological analysis of the raw materials

The results of the microbiological analysis of the raw materials are reported in Table 1. Meat, spices and natural casings were considered. Meats used in the three productions showed always a total aerobic count between 10^4 and 10^5 and only in the last case of Fermentation 3 was the count a little higher, reaching values of 10^6 cfu/g. The same trend was also observed for the other counts, in fact *Micrococcaceae*, LAB, total enterobacteria and *E. coli* showed a higher number in the third fermentation, with respect to the first and second production followed. Meats were characterized by the presence of low numbers of *Micrococcaceae* (<100 cfu/g), 10^4 cfu/g of LAB, 10^3–10^4 cfu/g of yeasts, 10^3 cfu/g of fecal enterococci, 10^2 cfu/g of total enterobacteria and *E. coli* and 10^3 cfu/g of *Pseudomonas* spp. Moulds were present only on the meat used for the second production considered in the study. Pathogenic microorganisms *S. aureus*, *L. monocytogenes* and *Salmonella* spp. were always absent or below 100 cfu/g (in the case of *S. aureus*). Spices had a total counts of about 10^5 cfu/g in productions 1 and 3, and in the second fermentation reached values of 10^7 cfu/g. Yeasts were always absent and the moulds were detected only in the first fermentation. Also the total enterobacteria and *E. coli* were present in very low numbers: only in the first fermentation we could count 10^2 cfu/g of total enterobacteria. Aerobic spore formers were present at 10^5 cfu/g and sulphite reducing clostridia and *S. aureus* were always less than 100 cfu/g. Finally, the natural casings used in the production were characterized by a variable number of total aerobic count: in the first fermentation it was 10^4, in the second almost 10^5 and in the last it reached 10^6 cfu/g. Yeasts were present at 10^3 cfu/g and in the second production they were not detected. Moulds were always <100 cfu/g and total enterobacteria and *E. coli* were <10 cfu/g apart from the third fermentation where the total enterobacteria were 10^3 cfu/g. *Salmonella* spp. were always absent in 25 g of product.

3.2. Microbiological analysis of the fermented sausages

The results of the microbiological analysis of the fermented sausages during ripening are reported in Table 2. Between the three fermentations followed, the first two showed values that were similar, whereas the third presented counts that were a bit higher that the first two. In fermentations 1 and 2 the total bacterial count was about 10^5 cfu/g and it remained pretty stable until the 14th day of fermentation. After that day, the counts increased and the final values reached were 10^7

Table 1
Results of the microbiological analysis carried out on raw materials used for the production of the natural fermented sausages studied

Microbiological analysis	Meat						Spices						Natural casings					
	F1		F2		F3		F1		F2		F3		F1		F2		F3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total aerobic count	4.33	0.73	4.84	0.80	5.80	0.81	4.97	0.03	6.89	0.39	4.47	0.09	3.77	0.39	4.85	0.29	6.14	0.43
Micrococcaceae	<100	n.a	<100	n.a	4.58	0.56	n.p		n.p		n.p		n.p		n.p		n.p	
Lactic acid bacteria	4.10	1.57	3.76	0.47	5.77	0.77	n.p		n.p		n.p		n.p		n.p		n.p	
Yeasts	3.14	0.95	3.23	0.48	3.52	0.38	<100	n.a	<100	n.a	<100	n.a	2.95	0.42	<100	n.a	3.09	0.19
Moulds	<100	n.a	2.37	0.60	<100	n.a	2.26	0.24	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a
Fecal enterococci	2.95	0.83	3.08	0.62	2.94	1.30	n.p		n.p		n.p		n.p		n.p		n.p	
Total enterobacteria	1.74	0.75	<10	n.a	3.71	0.87	2.19	1.43	<10	n.a	<10	n.a	<10	n.a	<10	n.a	3.22	0.04
Escherichia coli	1.36	0.39	<10	n.a	2.65	0.75	<10	n.a	<10	n.a	<10	n.a	<10	n.a	<10	n.a	<10	n.a
Aerobic spore formers	n.p		n.p		n.p		5.18	0.18	4.99	0.34	4.65	0.35	n.p		n.p		n.p	
Sulfite reducing clostridia	n.p		n.p		n.p		<100	n.a	<100	n.a	<100	n.a	n.p		n.p		n.p	
Pseudomonas spp.	2.97	0.85	<100	n.a	3.10	0.35	n.p		n.p		n.p		n.p		n.p		n.p	
Staphylococcus aureus	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a	n.p		n.p		n.p	
Listeria monocytogenes	Absent[a]		Absent		Absent		n.p		n.p		n.p		n.p		n.p		n.p	
Salmonella spp.	Absent		Absent		Absent		n.p		n.p		n.p		Absent		Absent		Absent	

Values are expressed in $\log_{10}$ colony forming unit (cfu)/g.
Abbreviations: F1, fermentation 1; F2, fermentation 2; F3, fermentation 3; SD, standard deviation; n.a, not applicable; n.p, not performed.
[a] Absent in 25 g of product.

Table 2
Results of the microbiological analysis carried out on the natural fermented sausages studied

Microbiological analysis	0		3		5		7		14		28	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fermentation 1												
Total aerobic count	5.01	0.04	4.45	0.28	4.01	0.12	4.90	0.07	7.44	0.34	6.62	0.12
Micrococcaceae	3.54	0.06	4.87	0.39	5.77	0.73	5.97	1.13	6.02	0.45	6.08	0.42
Lactic acid bacteria	4.11	0.19	7.27	0.27	7.86	0.10	8.22	0.15	8.28	0.05	8.39	0.37
Yeasts	3.86	0.10	3.72	0.10	2.88	0.34	2.84	0.10	3.20	0.70	<100	n.a
Moulds	<100	n.a	<100	n.a	<100	n.a	<100	n.a	3.00	0.70	<100	n.a
Fecal enterococci	3.91	0.18	3.09	0.93	5.14	0.09	5.66	0.22	6.26	0.07	6.05	0.28
Total enterobacteria	2.91	0.78	3.44	0.37	2.98	0.24	2.69	0.68	1.69	0.82	2.09	0.54
Escherichia coli	<10	n.a	<10	n.a	<10	n.a	<10	n.a	<10	n.a	<10	n.a
Pseudomonas spp.	<100	n.a	<100	n.a	n.p		n.p		n.p		n.p	
Staphylococcus aureus	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a
Listeria monocytogenes	Absent[a]		Absent		Absent		Absent		n.p		n.p	
Salmonella spp.	Absent		Absent		Absent		Absent		n.p		n.p	
Fermentation 2												
Total aerobic count	5.09	0.20	5.41	0.22	5.88	0.19	5.33	0.34	6.90	2.06	9.11	1.47
Micrococcaceae	3.66	0.10	3.80	0.63	5.67	0.57	4.62	0.69	5.54	0.06	5.01	0.12
Lactic acid bacteria	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	n.a	<100	n.a	<100	n.a	3.53	0.68	2.32	0.28
Moulds	2.59	0.26	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a
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
Escherichia coli	<10	n.a	<10	n.a	<10	n.a	<10	n.a	<10	n.a	<10	n.a
Pseudomonas spp.	<100	n.a	<100	n.a	n.p		n.p		n.p		n.p	
Staphylococcus aureus	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a
Listeria monocytogenes	Absent		Absent		Absent		Absent		n.p		n.p	
Salmonella spp.	Absent		Absent		Absent		Absent		n.p		n.p	
Fermentation 3												
Total aerobic count	6.22	0.66	7.84	0.25	8.04	0.07	8.30	0.05	8.93	0.65	7.81	0.17
Micrococcaceae	4.85	0.14	5.82	1.00	4.80	0.44	5.78	0.18	6.09	0.40	4.57	0.47
Lactic acid bacteria	5.61	0.19	8.39	0.10	8.15	0.09	8.34	0.07	8.55	0.15	8.47	0.03
Yeasts	4.01	0.27	4.76	0.59	4.62	0.38	4.20	0.17	2.54	0.28	2.39	0.36
Moulds	2.46	0.28	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a
Fecal enterococci	4.20	0.17	5.62	0.25	5.52	0.07	5.91	0.39	5.39	0.09	5.97	0.07
Total enterobacteria	4.14	0.02	6.49	0.26	6.67	0.04	6.06	0.25	5.73	0.40	4.61	0.01
Escherichia coli	1.59	0.66	2.60	0.25	2.38	0.07	1.75	0.05	1.67	0.65	1.10	0.17
Pseudomonas spp.	4.62	1.07	<100	n.a	n.p		n.p		n.p		n.p	
Staphylococcus aureus	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a	<100	n.a
Listeria monocytogenes	Absent		Absent		Absent		Absent		n.p		n.p	
Salmonella spp.	Absent		Absent		Absent		Absent		n.p		n.p	

Values are expressed in $\log_{10}$ colony forming unit (cfu)/g.
Abbreviations: SD, standard deviation; n.a, not applicable; n.p, not performed.
[a] Absent in 25 g of product.

cfu/g in the first fermentation and 10^9 cfu/g in the second. The third fermentation presented a higher initial number, but this resulted in a final value of 10^8 cfu/g, lower than the value observed in the second fermentation. *Micrococcaceae* showed a load of 10^3–10^4 cfu/g that increased during ripening. The average number at the end of the three fermentations followed was about 10^5–10^6 cfu/g. LAB were present at the beginning of the fermentation with counts of about 10^4–10^6 cfu/g, that rapidly increased in the very first days of fermentation. At 3 days they were already 10^8–10^9 cfu/g. They remained stable throughout the fermentations and reached a final number of 10^8 cfu/g. Yeasts and moulds were present at maximum levels of about 10^3 cfu/g, and in some cases they were not detected (Table 2). Fecal enterococci, present in the mix at 10^3 cfu/g, showed in all three fermentations a gradual increase during ripening. At 28 days they were about 10^6cfu/g. Total enterobacteria showed an initial count of about 10^3 cfu/g and only in the case of the third fermentation were 10^4. During ripening these counts showed an increase that was more contained in the first fermentation and the final counts were determined to be 10^2 cfu/g while they were 10^4 cfu/g in the second and third fermentations. *E. coli* showed counts higher than 10 cfu/g only in the third fermentation, where they remained constant until the end.

Also *Pseudomonas* spp. were counted only in the third fermentation, but they were present in the mix and not anymore at 3 days of fermentation. *S. aureus* was always below 100 cfu/g and *L. monocytogenes* and *Salmonella* spp. were always absent in 25 g of product.

3.3. Physicochemical analysis of the raw materials

In the meat used in the three fermentations followed the pH was always between 5.80 and 5.90, with an A_w of about 0.98–0.99. Sodium chloride, nitrates and nitrites were present in traces.

3.4. Physicochemical analysis of the fermented sausages at the end of ripening

The results of the physicochemical analysis of the fermented sausages at the end of the ripening time, are reported in Table 3. In all the fermentations followed, the values of pH were about 5.60–5.70 in the final product. The A_w decreased gradually during ripening reaching values of 0.91–0.92, with a RH of 40–46%. The final value of the protein content in all three fermentations was around 20%, while the final fat content was 35%, 27% and 32% for the first, second and third fermentation, respectively. Carbohydrates were not detected anymore after the seventh day of maturation. The inorganic matter reached values of 4.0–4.3 at the end of the maturation and the sodium chloride, added at the beginning of the production to the value of 2.5%, increased to 3.3%. The final values for NO_3 and NO_2 were about 10 and 8 ppm, respectively.

3.5. Sensory evaluation

The main sensorial characteristics of the products, are reported in Fig. 1. As shown, the three fermentations followed produced fermented sausages that were similar in the parameters considered. All the sausages had a discrete smell, fat quality and acidity. The rancidity of the product was always very low. The scores for the cut surface and coherence, as well as for the juiciness and tenderness, were not very high. The sensorial evaluations described the sausages studied as products characterized by an acceptable acidity and, in all the cases investigated, by a very low rancidity. The overall flavour and impression was determined to be medium.

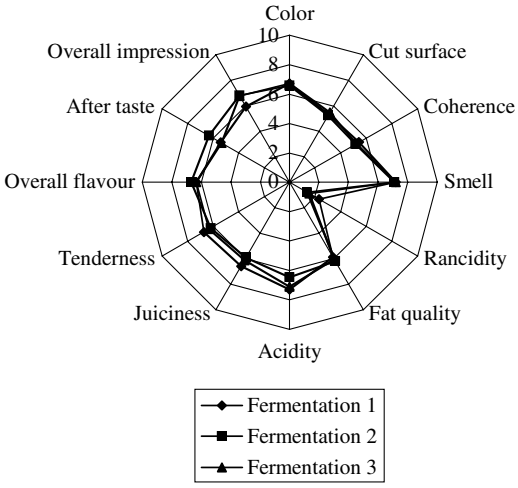

Fig. 1. Sensory evaluation of the fermented sausages considered in the study.

3.6. Molecular identification of the isolated strains

The LAB strains isolated during the production of the sausages were subjected to DNA extraction, PCR-DGGE analysis and grouping on the basis of the migration profiles. Finally, representatives of each group were sequenced. The results obtained are reported in Table 4, where the number of strains, for each fermentation, belonging to a specific species and its percentage is reported. The only species that were isolated in all three

Table 3
Physical and chemical analysis of the fermented sausages used in this study at the end of ripening

	Fermentation 1		Fermentation 2		Fermentation 3	
	Mean	SD	Mean	SD	Mean	SD
Physical analysis						
pH	5.73	0.06	5.62	0.03	5.62	0.10
A_w	0.91	0.01	0.92	0.01	0.92	0.00
RH (%)	40.83	1.04	46.33	0.58	43.50	0.50
Chemical analysis						
Protein (%)	19.13	0.23	21.67	1.15	20.50	0.50
Fat (%)	35.80	0.95	27.70	1.00	31.96	0.06
Carbohydrates (%)	Traces	n.a	Traces	n.a	Traces	n.a
Inorganic matter (%)	4.23	0.15	4.30	0.00	4.03	0.06
NaCl (%)	3.37	0.06	3.37	0.12	3.28	0.03
NO_3 (ppm)	7.17	0.47	11.00	1.73	12.00	0.00
NO_2 (ppm)	8.83	0.76	8.83	0.29	8.67	1.53

Abbreviations: SD, standard deviation; n.a, not applicable.

Table 4
Molecular identification results obtained for the strains isolated during the three fermentations followed in the study

Species	F1		F2		F3		Total number
	Number of strains	%	Number of strains	%	Number of strains	%	
Enterococcus pseudoavium	–	–	–	–	1	2.0	1
Lactococcus lactis subsp. *lactis*	–	–	–	–	1	2.0	1
Lactobacillus brevis	–	–	–	–	1	2.0	1
Lactobacillus curvatus	11	22.0	26	52.0	17	34.0	54
Lactobacillus paraplantarum	1	2.0	6	12.0	–	–	7
Lactobacillus paraplantarum/pentosus[a]	4	8.0	–	–	–	–	4
Lactobacillus plantarum	9	18.0	–	–	–	–	9
Lactobacillus sakei	21	42.0	17	34.0	26	52.0	64
Leuconostoc citreum	1	2.0	–	–	–	–	1
Leuconostoc mesenteroides	–	–	–	–	4	8.0	4
Weissella paramesenteroides/hellenica[a]	3	6.0	1	2.0	–	–	4
Total	50	100	50	100	50	100	150

Abbreviations: F1, fermentation 1; F2, fermentation 2; F3, fermentation.
[a] The sequencing results did not allow a definitive identification.

fermentations were *Lb. curvatus* and *Lb. sakei*. The total number of strains identified as *Lb. curvatus* was 54, whereas for *Lb. sakei* was 64. A low number of other species were found, as well. *Enterococcus pseudoavium*, *Lactococcus lactis*, *Lb. brevis* and *Leuconostoc mesenteroides* were present only in the third fermentation, while *Lb. plantarum*, *Lb. paraplantarum/pentosus* and *Lc. citreum* were isolated only in the first fermentation. *Lb. paraplantarum* and *Weissella paramesenteroides/hellenica* were found to be present in the first two fermentation but not in the third.

3.7. Characterization of *Lb. curvatus* and *Lb. sakei* strains by RAPD-PCR

Cluster analysis of the 54 *Lb. curvatus* isolated from the three fermentations is reported in Fig. 2. With a 70% coefficient of similarity, seven clusters were determined and two strains, I128 and I97, were unique. The most numerous were clusters V with 14 strains, followed by cluster VI (10 strains), cluster IV (9 strains) and cluster II (6 strains). Among the resultant clusters, some of them were characterized by strains isolated in one specific fermentation. Cluster I included *Lb. curvatus* from the first fermentation, whereas cluster III grouped 4 strains isolated from the third fermentation. Interesting to notice is that cluster V was formed by 14 strains, of which 12 were isolated from the second fermentation. The remaining clusters grouped strains isolated in different fermentations.

When the strains belonging to the *Lb. sakei* species were subjected to analysis, clusters reported in Fig. 3 were obtained. A total of 64 strains were included in the analysis. Twelve major clusters and eight single-strain clusters were obtained. Clusters III, VII and IX were the biggest with 10, 11 and 10 strains grouped, respectively. Of the 12 clusters identified, only clusters III and VII were almost completely formed by strains isolated from a specific fermentation. For cluster III, on a total of 10 strains, seven were isolated from the third fermentation, while for cluster VII, on a total of 11 strains, 10 came from the first fermentation. The other clusters contained strains isolated from all three fermentations considered in the study.

4. Discussion

Fermented sausages are typical products mainly produced in the Mediterranean countries. This kind of product is characterized by low acidity and the final pH is of about 5.3 to 6.2 (Aymerich, Martín, Garriga, & Hugas, 2003). Greece, Italy and Spain are the countries where more efforts have been dedicated to the study of this traditional product (Comi, Citterio, Manzano, & Cantoni, 1992; Coppola, Giagnacovo, Iorizzo, & Grazia, 1998; García-Varona, Santos, Jaime, & Rovira, 2000; Garriga et al., 1996; Hugas et al., 1993; Metaxopoulos, Samelis, & Papadelli, 2001; Parente, Griego, & Crudele, 2001; Rodríguez et al., 1994; Samelis, Maurogenakis, & Metaxopoulos, 1994; Samelis, Metaxopoulos, Vlassi, & Pappa, 1998; Santos, González-Fernández, Jaime, & Rovira, 1998). Moreover, molecular techniques have been applied either to directly profile the main populations involved in the production of Italian fermented sausages (Cocolin, Manzano, Aggio, et al., 2001) or to identify isolated LAB and nonpathogenic staphylococci from artisanal low-acid sausages produced in Spain (Aymerich et al., 2003).

In this study, three traditional fermented sausage productions were followed. Microbiological analyses were performed on the raw materials and during the ripening of the sausages. They were designed both to define the

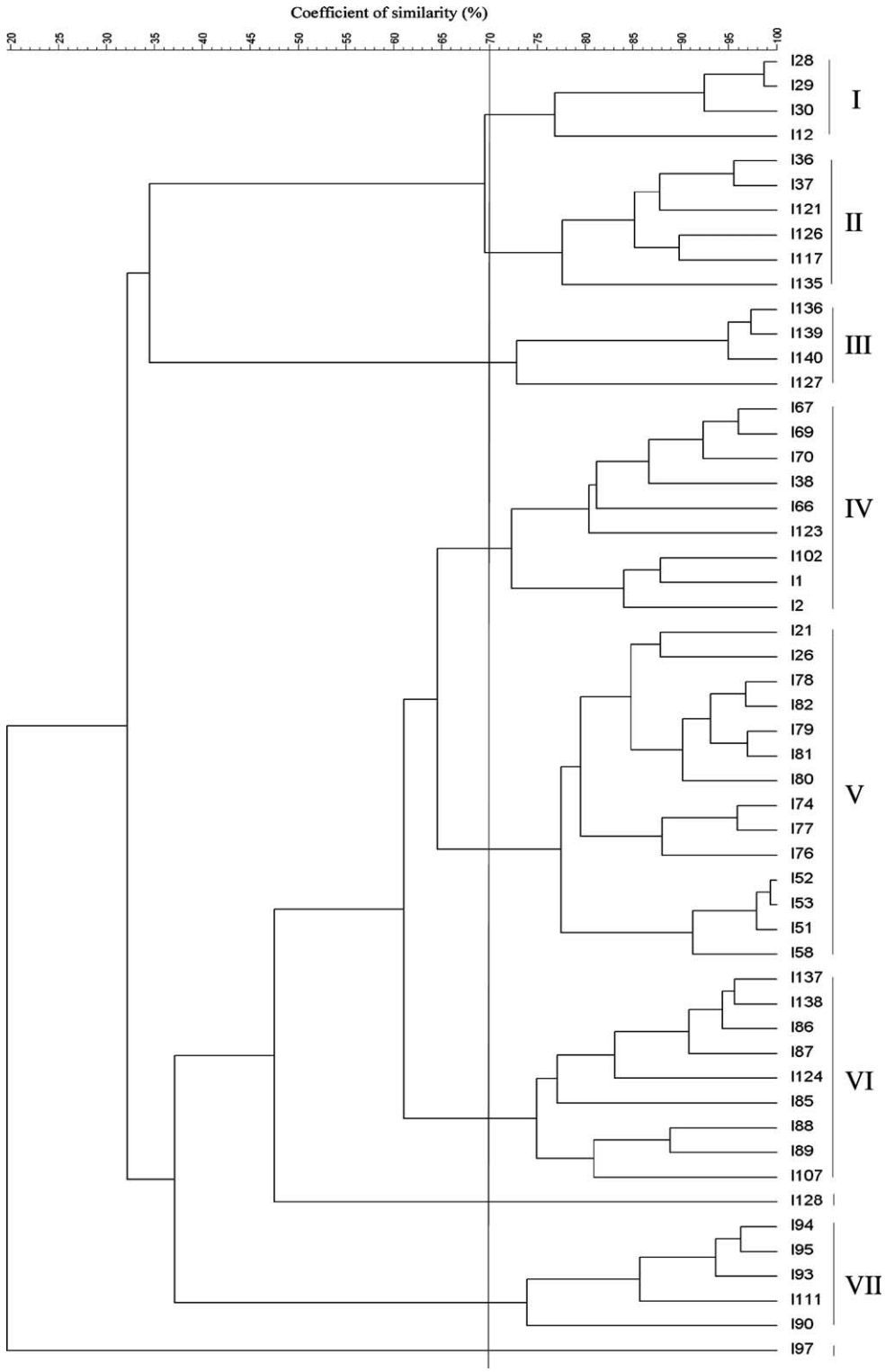

Fig. 2. Cluster analysis of the profiles obtained by RAPD-PCR from the *Lactobacillus curvatus* strains isolated from the naturally fermented sausages followed in the study. Strains isolated during the first fermentation were numbered from 1 to 50, while for strains of the second and the third fermentation, numbers from 51 to 100 and from 101 to 150 were used, respectively. Identified clusters are indicated with roman letters.

safety aspect of the product and to follow the fermentation process carried out by LAB and micro/staphylococci strains.

The results of the microbiological analysis on the raw materials underlined the good hygienic quality of the meat, spices and natural casings used in the three pro-

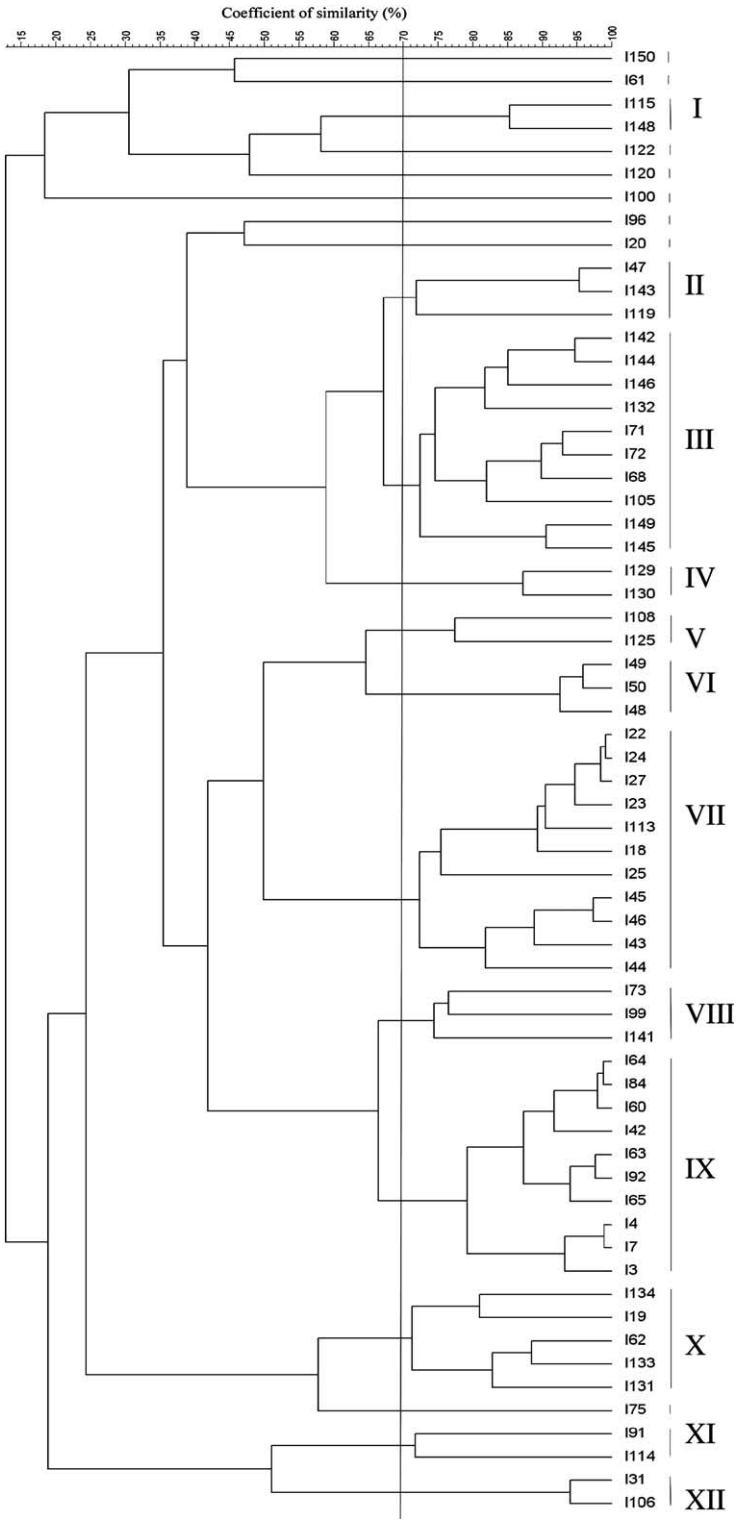

Fig. 3. Cluster analysis of the profiles obtained by RAPD-PCR from the *Lactobacillus sakei* strains isolated from the naturally fermented sausages followed in the study. Strains isolated during the first fermentation were numbered from 1 to 50, while for strains of the second and the third fermentation, numbers from 51 to 100 and from 101 to 150 were used, respectively. Identified clusters are indicated with roman letters.

ductions followed as no pathogens were detected. The fermented sausages analyzed in the study showed the same aspects of safety found in the raw materials, in fact no *L. monocytogenes*, *Salmonella* spp. or *S. aureus* were found during maturation. *E. coli* was not detected (<10 cfu/g) in the first two fermentations and only in the third

it was found to be 10^2 cfu/g throughout the period followed. No significant differences in the microbial trends were observed in the three fermentations followed. Only in the third batch, higher numbers in the counts of the meat used for the production were detected. Probably the difference found is explained by the period in which the fermentation was carried out. Since it started the 11th of July, temperatures were more difficult to control and microbial growth was facilitated. These initial differences did not affect the final product and final counts were similar to the ones determined for the previous batches. Only the *E. coli* counts were higher with respect to the <10 cfu/g of the first and second fermentation. In all the fermentations considered in the study, the fecal enterococci reached a value of 10^6 cfu/g at the end of the fermentation, becoming an important population influencing the final organoleptic characteristics of the product. Since, they are able to produce ammonia and other amines, they possibly contributed to the final flavour of the product.

The final pH of the sausages was 5.62–5.73, typical of low acidity sausages, and this was the result of the classical trend of microbial growth in the fermented sausages, where LAB are increasing in numbers at the very beginning of the fermentations, producing acids and a decrease in the pH, followed in the phases of maturation by the activity of micro/staphylococci that are able to neutralize the acids produced. A_w showed a constant decrease during the maturation, and protein, fat, inorganic matter and NaCl content increased during the ripening because of the effect of dehydration. Nitrates and nitrites decreased in concentration because of the growth of reducing bacteria.

The results of the sensory evaluation using a panel of 10 persons (Fig. 1) show that the three products had very similar organoleptic and sensory characteristics. Low acidity, good fat quality with very low rancidity, good smell, but low coherence of the slice with bad cut surface were the main qualities detected by the panellists.

One hundred and fifty strains of LAB were isolated and identified by molecular methods. Strains of *Lb. curvatus* and *Lb. sakei* were isolated from all three fermentations. The results obtained here are in agreement with previous studies, which underlined how *Lb. curvatus* and *Lb. sakei* are the most adapted species to the fermented sausages environment (Aymerich et al., 2003; Parente et al., 2001; Samelis et al., 1994; Papamanoli et al., 2003). They are the responsible for the acidification of the sausages in the initial phases of the fermentation process. Moreover, studies on the proteolytic activity showed their capability to hydrolyse meat protein thereby participating also to the flavour formation process (Fadda et al., 1999). Defining and understanding LAB dynamics, as determined by species successions, as well as LAB ecology, as determined by species interactions at each time point and throughout fermentation, is crucial since they are the parameters that will have a great impact on the organoleptic and sensorial characteristics of the final product.

RAPD-PCR and cluster analysis of the profiles obtained of *Lb. curvatus* and *Lb. sakei* strains revealed some clusters (I, II and V for *Lb. curvatus* and III, VII and IX for *Lb. sakei*), that grouped strains isolated from one specific fermentation, underlining their differences with the strains of the other fermentations. It is highly probable that strains grouped in the fermentation-specific clusters came from the ingredients used in the productions. As an example, the meat used in the first production could carry certain strains that established during fermentation, but that were different from the second and third batch considered in the study. *Lb. sakei* showed a higher degree of heterogeneity confirmed by the number of strains that did not group in the cluster analysis. It is, moreover, possible that the slight differences in the sensory characteristics of the products are due, at least partially, to the presence of different *Lb. sakei* and *Lb. curvatus* strains, as determined by RAPD-PCR cluster analysis, and to the different *Lactobacillus* ecology in the three batches considered in this study.

In conclusion, the microbiological and physicochemical aspects of the Friuli Venezia Giulia sausages studied in this paper are not significantly different from other naturally fermented sausages previously described. However, their organoleptic profile, characterized by an acceptable acidity and low rancidity, is distinctive for this product. This can be explained by admitting that the strains isolated in this study, although they belong to the species commonly regarded as responsible for sausage fermentations, they possess specific physiological and technological characteristics that make this traditional product unique.

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References

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

Andrigetto, C., Zampese, L., & Lombardi, A. (2001). RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy). *Letters in Applied Microbiology, 33*, 26–30.

AOAC (2002). *Official methods of Analysis* (17th ed.). Washington, DC: Association of Official Analytical Chemists.

Aymerich, T., Martín, B., Garriga, M., & Hugas, M. (2003). Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. *Applied and Environmental Microbiology, 69*, 4583–4594.

Bacus, J. N. (1986). Fermented meat and poultry products. In A. M. D. Pearson (Ed.), *Advances in meat and poultry microbiology* (pp. 123–164). London, United Kingdom: Macmillan.

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

Cai, Y., Kumai, S., Ogawa, M., Benno, Y., & Nakase, T. (1999). Characterization and identification of *Pediococcus* species isolated from forage crops and their application for silage preparation. *Applied and Environmental Microbiology, 65*, 2901–2906.

Cocolin, L., Manzano, M., Aggio, D., Cantoni, C., & Comi, G. (2001). A novel polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) for the identification of *Micrococcaceae* strains involved in meat fermentations. Its application to naturally fermented Italian sausages. *Meat Science, 57*, 59–64.

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

Collins, M. D., Rodriguez, U., Ash, C., Aguirre, M., Farrow, J. E., Martinezmurcia, A., et al. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse-transcriptase sequencing of 16S ribosomal-RNA. *FEMS-Microbiology Letters, 77*, 5–12.

Comi, G., Citterio, B., Manzano, M., & Cantoni, C. (1992). Evaluation and characterization of *Micrococcaceae* strains in Italian dry fermented sausages. *Fleischwirtschaft, 72*, 1679–1683.

Coppola, R., Giagnacovo, B., Iorizzo, M., & Grazia, L. (1998). Characterization of lactobacilli involved in the ripening of sopressata molisana, a typical southern Italy fermented sausage. *Food Microbiology, 15*, 347–353.

Coppola, R., Iorizzo, M., Saotta, R., Sorrentino, E., & Grazia, L. (1997). Characterization of micrococci and staphylococci isolated from soppressata molisana, a Southern Italy fermented sausage. *Food Microbiology, 14*, 47–53.

del Carmen de la Rosa, M., Mohino, M. R., Mohino, M., & Mosso, M. A. (1990). Characteristics of micrococci and staphylococci isolated from semi-preserved meat products. *Food Microbiology, 7*, 207–215.

Demeyer, D. I. (1982). Stoichiometry of dry sausage fermentation. *Antonie Leeuwenhoek, 48*, 414–416.

Demeyer, D. I., Verplaetse, A., & Gistelink, M. (1986). Fermentation of meat: an integrated process. *Belgian Journal of Food Chemistry and Biotechnology, 41*, 131–140.

Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., & Toldrà, F. (1999). Hydrolisis of pork muscle sarcoplasmatic proteins by *Lsctobacillus curvatus* and *Lactobacillus sakei*. *Applied and Environmental Microbiology, 65*, 578–584.

Fischer, U., & Schleifer, K. H. (1980). Vorkommen von staphylokokken un mikrokokken in Rohwurst. *Fleischwirtschaft, 60*, 1046–1049.

García-Varona, M., Santos, E. M., Jaime, I., & Rovira, J. (2000). Characterisation of *Micrococcaceae* isolated from different varieties of chorizo. *International Journal of Food Microbiology, 54*, 189–195.

Garriga, M., Hugas, M., Gou, P., Aymerich, M. T., Arnau, J., & Monfort, J. M. (1996). Technological and sensorial evaluation of *Lactobacillus* strains as starter cultures in fermented sausages. *International Journal of Food Microbiology, 32*, 173–183.

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

Holzapfel, W. H., Geisen, R., & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology, 24*, 343–362.

Hugas, M., Garriga, M., Aymerich, T., & Monfort, J. M. (1993). Biochemical characterization of lactobacilli from dry fermented sausages. *International Journal of Food Microbiology, 18*, 107–113.

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

International Organization for Standardization (1990). Microbiology – General guidance on methods for the detection of *Listeria monocytogenes*. Draft International Standard ISO/DIS 11290.

International Organization for Standardization (1991). Microbiology – General guidance on methods for the detection of *Salmonella*. Draft International Standard ISO/DIS 6579.

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

Lucke, F. K. (1985). Fermented sausages. In B. J. B. Wood (Ed.), *Microbiology of fermented foods*. New York, USA: Elsevier Applied Science.

Metaxopoulos, J., Samelis, J., & Papadelli, M. (2001). Technological and microbiological evaluation of traditional processes as modified for the industrial manufacturing of dry fermented sausage in Greece. *Italian Journal of Food Science, 1*, 3–18.

Miralles, M. C., Flores, J., & Perez-Martinez, G. (1996). Biochemical tests for the selection of *Staphylococcus* strains as potential meat starter cultures. *Food Microbiology, 13*, 227–236.

Parente, E., Griego, S., & Crudele, M. A. (2001). Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). *Journal of Applied Microbiology, 90*, 943–952.

Pot, B., Vandamme, P., & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In M. Goodfellow & A. G. O'Donnell (Eds.), *Chemical methods in prokaryotic systematics* (pp. 493–521). Chichester, USA: Wiley.

Rodríguez, M., Núñez, F., Córdoba, J. J., Sanabria, C., Bermúdez, E., & Asensio, M. A. (1994). Characterization of *Staphylococcus* spp. and *Micrococcus* spp. isolated from Iberian ham throughout the ripening process. *International Journal of Food Microbiology, 24*, 329–335.

Samelis, J., Maurogenakis, F., & Metaxopoulos, J. (1994). Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. *International Journal of Food Microbiology, 23*, 179–196.

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

Santos, E. M., González-Fernández, C., Jaime, I., & Rovira, J. (1998). Comparative study of lactic acid bacteria house flora isolated in different varieties of "chorizo". *International Journal of Food Microbiology, 39*, 123–128.

Schillinger, U., & Lücke, F. K. (1987). Identification of Lactobacilli from meat and meat products. *Food Microbiology, 4*, 199–208.

Schleifer, K. H. (1986). Gram positive cocci. In P. H. A. Sneath, N. S. Mair, & J. G. Holt (Eds.). *Bergey's manual of systematic bacteriology* (Vol. 2, pp. 999–1003). Baltimore, Md: Williams & Wilkins.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology, 33*, 2233–2239.

Thornill, P. J., & Cogan, T. M. (1984). Use of gas-liquid chromatography to determine the end-products of growth of lactic acid bacteria. *Applied and Environmental Microbiology, 47*, 1250–1254.

Vauterin, L., & Vauterin, P. (1992). Computer-aided objective comparison of electrophoretic patterns for grouping and identification of microorganisms. *European Microbiology, 1*, 37–41.

Zhong, W., Millsap, K., Bialkowska-Hobrzanska, H., & Reid, G. (1998). Differentiation of *Lactobacillus* species by molecular typing. *Applied and Environmental Microbiology, 64*, 2418–2423.