Molecular and physiological characterization of natural microbial communities isolated from a traditional Southern Italian processed sausage

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Abstract

The development of the natural microbial populations during traditional processing of the “Salame di Senise”, a typical little known sausage produced in the South of Italy, was investigated by using molecular and physiological techniques for taxonomic identification and technological characterization of strains.

The application of RAPD-PCR over more than 90 colonies made it possible to isolate 18 bacterial and two yeast biotypes identified by partial rDNA sequencing as belonging mainly to three species of Bacillus, three species of Lactobacillus, three species of Staphylococcus and Debaryomyces Hansenii. The physiological analyses revealed that the isolates belonging to Lactobacillus genus were the most acidifying, whereas Staphylococcus strains did not develop significant proteolytic and lipolytic activities. Interestingly, some Bacillus strains produced the highest values of proteolytic and lipolytic activities. The results for the technological properties of Bacillus strains isolated from this Southern Italian sausage, made without a selected starter, suggest that Bacillus strains, always present in meat curing, could play a role in the development of texture and organoleptic characteristics of the sausages.

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Keywords: Natural microbial community; Cured sausage; Proteolytic-lipolytic activities; Bacillus; Lactobacillus; Staphylococcus; Debaryomyces hansenii

1. Introduction

The data regarding dry cured sausages show that Germany, Italy, Spain and France rank as the biggest producers and consumers of sausage (Lücke, 1998). In Italy, the meat-based processing sector is made up of more than 3500 companies, including those turning out artisanal products which continue to hold their ground in the production of traditional Italian foods (Istituto per la Valorizzazione dei Salumi Italiani).

In recent years, the microflora of many Southern European traditional sausages have been characterized. The fermenting microflora of “Fuets” and “Chorizos”, two low-acid cured Spanish sausages, is constituted by Lactobacillus sakei, L. curvatus, L. plantarum, Staphylococcus xylosus, S. carnosus, and S. epidermidis depending on the sausage type (Aymerich, Martín, Garriga, & Hugas, 2003). Also two types of naturally fermented Greek dry sausages showed a large presence of L. sakei, L. curvatus, L. plantarum, S. saprophyticus, and S. xylosus strains, but sporadic isolates of Weissella viridescens, Enterococcus faecium, E. faecalis, Leuconostoc pseudomesenteroides, and Pediococcus sp. were also detected (Papamanoli, Tzanetakis, Litopoulos-Tzanetaki, & Kotzekidou, 2003; Samelis, Metaxopoulos, Vlassi, & Pappa, 1998; Tzanetakis, Litopoulos-Tzanetaki, & Kotzekidou, 2003).

Abbreviations: PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNA; LAB, lactic acid bacteria.

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In Italy, many fermented meat products are still made with traditional technologies without selected starters, resulting in a wide range of sausages with different flavours, consistencies, and microbiological quality (Coppola, Mauriello, Aponte, Moschetti, & Villani, 2000; Zambonelli, Papa, Romano, Suzuki, & Grazia, 1992).

The microflora present in some Southern Italian traditional fermented sausages are dominated by \textit{L. sakei} (1992). During the last 30 years, much attention has been focused on the use of starter cultures to guarantee safety and standardize product properties, including consistent flavour and colour and shorter ripening time. A wide variety of microorganisms, mainly lactic acid bacteria (LAB) and \textit{Staphylococcus} and \textit{Kocuria} spp., have already been isolated from sausage fermentations and have been selected for metabolic activities especially suited for fermentation in meat ecosystems (Coppola et al., 2000; del Carmen de la Rosa, Mohino, Mohino, & Mosso, 1990; Hugas, Garriga, Aymerich, & Monfort, 1993), improving the quality and safety of the final product (Metaxopoulos, Genigeorgis, Fanelli, Franz, & Cosma, 1981; Nychas & Arkoudelos, 1990)..

Recently, various molecular typing methods such as restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), ribotyping and PCR-derived techniques such as repetitive extragenic palindromic (REP-) and enterobacterial repetitive intergenic consensus (ERIC-) PCR have been used to distinguish between isolated bacterial biotypes. Moreover, RAPD analysis has been used to estimate the diversity among several genera of bacteria such as \textit{Lactobacillus}, \textit{Bacillus}, and \textit{Staphylococcus} isolated from many sources and 16S rDNA sequence analysis has made the taxonomic identification of biotypes feasible (Morea, Baruzzi, Cappa, & Cocconcelli, 1998; Morea, Baruzzi, & Cocconcelli, 1999; Rebecchi, Crivori, Sarra, & Cocconcelli, 1998).

The increasing interest in preserving the biodiversity of the microorganisms involved in the production of traditionally fermented food products led us to investigate the natural microbial association that brings about the curing of “Salame di Senise”.

Randomly amplified polymorphic DNA (RAPD), partial rDNA sequence analysis, and physiological assays were used to characterize the growth and the changes of dominant microbial populations during “Salame di Senise” curing. The physiological characterization of the strains contained in this typical sausage such as acid production, proteolytic and lipolytic activities and nitrate reduction was carried out to gain an insight into the role played by microbial strains belonging to different genera in meat fermentation and curing.

2. Materials and methods

2.1. Production technology and sampling

The sausages were produced by a local craft plant in Tursi according to the traditional method (without the adjunct of starter cultures). The typical protocol for production entails the addition of NaCl (28 g kg$^{-1}$), seeds of wild fennel (\textit{Foeniculum sylvestre}) harvested from wild plants (1–3 g kg$^{-1}$) and powder from the local pepper cultivar “Peperone di Senise” (8–12 g kg$^{-1}$) to the minced meat is mixed with other ingredients, such as NaCl, dried vegetables and additives (nitrate, nitrite, and spices).

In the recent past, in the heart of Basilicata pigs browsed in the Mediterranean maquis feeding off fruits and tubers of the underbrush. A particular sausage named “Pezzente della Montagna Materana” (Beggar of the Mountains of Matera) was obtained from the native breed pig, Black of Lucania, a dying species; this sausage was produced using less noble parts of pork like throat, harder muscles, stomach and residual fats from previous processings. Powder of the pepper of Senise, wild fennel, garlic and salt were added to the minced meat. Today the use of discarded parts of pork for sausage processing is no longer carried out (Sardo, Milano, & Ponzo, 2004).

“Salame di Senise” is a traditional Southern Italian dry sausage manufactured in some small-scale sausage factories throughout the Sinni Valley in the Basilicata region. The processing is based on the experience and skill of local manufacturers, rather than on scientific and technological know-how. The main characteristic of this cured sausage is the addition of powder from a locally produced pepper, named “Peperone di Senise”. The “Peperone di Senise” (\textit{Capsicum annuum} cv. Senise) is a bell-shaped, thin-skinned red pepper with a low water content and a distinctive mild taste. This vegetable grows only in the Sinni and Agri valleys and was added to the list of products with protection of geographical indications, PGI, (EC Regulation No. 1263/96). The absence of a selected starter and the addition of significant amounts of un-pasteurized spices, lead us to suppose that the contribution of naturally occurring microflora is essential to ensure safe, palatable products. Distribution of this premium food product outside the Italian borders is hampered by the difficulties inherent in controlling the fermentation and curing processes. During the last 30 years, much attention has been focused on the use of starter cultures to guarantee safety and standardize product properties, including consistent flavour and colour and shorter ripening time. A wide variety of microorganisms, mainly lactic acid bacteria (LAB) and \textit{Staphylococcus} and \textit{Kocuria} spp., have already been isolated from sausage fermentations and have been selected for metabolic activities especially suited for fermentation in meat ecosystems (Coppola et al., 2000; del Carmen de la Rosa, Mohino, Mohino, & Mosso, 1990; Hugas, Garriga, Aymerich, & Monfort, 1993), improving the quality and safety of the final product (Metaxopoulos, Genigeorgis, Fanelli, Franz, & Cosma, 1981; Nychas & Arkoudelos, 1990).

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pork meat. Potassium nitrate was also added at 0.25% to inhibit the growth of undesirable microorganisms. The mixture was kept at 10 °C for about 12 h and then pressed and stuffed into pork casings. The shaped sausages were kept at 13–15 °C and 65% RH for 25 days, after which the sausage was ready for consumption. The weight of sausages was ca. 0.3 kg with a diameter of 4.0 cm.

The sausage was sampled at two different stages of manufacturing: at day 0 of fermentation, sample T0, consisting in minced pork meat after the addition of spices and storage at 10 °C, and at the 25th day of ripening, sample T25, representing ready-to-eat sausage at the end of the curing period. Immediately after collection, samples were transferred to the laboratory in refrigerated conditions. Before grinding, the ripened sausage was cleaned with a 0.5% hypochlorite solution and the casing was aseptically removed. Twenty five g of sausage was sampled from the un-ripened meat and the inner part of the ripened sausage were cut using sterile knives, transferred to sterile plastic pouches and homogenized for 2–3 min at 6000 rpm with 225 ml of sterile diluent containing peptone (1 g L⁻¹) and NaCl (0.8%) using a blender (Lab Blender, Seward, London, UK).

Sausage pH was determined by direct insertion of a solid pH meter. Water activity (aw) was measured with the aw recorder AquaLab, series 3, Model TE (Decagon Devices, Inc., Pullman, Wash.).

2.2. Microbiological analyses

The samples were serially diluted in peptonated saline solution and the appropriate dilutions plated in triplicate on different media: Plate Count Agar (PCA) was used for total aerobic bacterial count, de Man Rogosa Sharpe Agar pH 5.5 (MRS) for the counting of presumptive lactobacilli, and Mannitol Salt Agar (MSA) for the counting of staphylococci present.

In addition, a selective isolation of Bacillus cereus strains was carried out using Mannitol-Egg Yolk-Polyminix (MYP) agar. Five millilitres of pasteurized (10 min at 80 °C) samples were used for total aerobic heat resistant cell count; decimal dilutions were plated on Tryptic Soy Agar with 5 g L⁻¹ of added glucose (TSA) plates. After heat treatment, the samples were immediately placed on ice in order to prevent spore germination.

PCA, MSA, MYP and TSA media were incubated under aerobic conditions whereas MRS plates were incubated under anaerobic conditions (AnaeroGen™, Oxoid S.p.A., Garbagnate, Milano, Italy). All the plates were kept at 30 °C for 48 h.

Yeast and moulds were enumerated on Glucose Yeast Agar Base plates supplemented with 200 ppm Chloramphenicol (CGYA) to inhibit bacterial growth. Plates were kept at 25 °C from 3 to 7 days, as recommended by the International Organization Standardization (ISO 13681, 1995).

For each collection step, 15–20 bacterial colonies randomly isolated from PCA, MRS, MSA and CGYA plates, respectively, were inoculated in Brain Heart Infusion (BHI) broth for bacilli growth, in M17 broth for staphylococci, in MRS broth for lactobacilli and in Yeast Peptone Dextrose broth (YPD, Oxoid) for yeasts and incubated under the appropriate conditions; 15–20 random colonies from TSA plates were analyzed for Gram staining, cell morphology, presence of endospores and catalase reaction. All isolates, grown under the appropriate conditions, harvested by centrifugation (6000g for 5 min), suspended in the same medium containing 20% glycerol, were frozen at −80 °C and used for further characterization.

PCA, MRS, TSA and MSA media were obtained from Oxoid (Oxoid Ltd., Basingstoke, UK), whereas MYP was purchased from Merck (Merck, Darmstadt, Germany).

2.3. DNA isolation and purification

DNA for strain typing and rDNA for sequencing was obtained from staphylococci and yeasts using a synthetic resin (Gene Releaser, Bioventures, TN, USA) whereas DNA from Bacillus and Lactobacillus was extracted using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer’s instructions.

DNA quantity and quality were determined by electrophoresis with known amounts of molecular weight marker (Marker VI, Roche Diagnostics, Milan, Italy) as a standard.

2.4. Bacteria and yeast strain typing

Bacteria and yeast strain typing was performed on more than 90 randomly selected colonies of cocci, bacilli and yeasts, using the "two-step RAPD PCR" protocol, as described by Baruzzi, Morea, Matarante, and Cocconcelli (2000), including control reaction mixtures lacking DNA template in each experiment. PCR amplifications were carried out in a Thermal Cycler 9700 (Perkin–Elmer, Alameda, CA, USA). Taq polymerase and deoxynucleoside triphosphates were purchased from Sigma–Aldrich S.r.l. (Milan, Italy), gel-filtration purified oligonucleotides from Sigma–Genosys Ltd. (Cambs, UK) whereas DNA molecular weight markers were from Roche Diagnostics. The amplified fragments, separated by electrophoresis in 2.0% agarose gel, were sized using the Quantity One 4.3.1 software (Bio-Rad Laboratories S.r.l., Milan, Italy). When different isolates gave the same electrophoretic pattern, they were grouped. The ratio (P = n/t) between the number of colonies grouped for each strain (n) and total colonies analyzed (t) from the same medium for each sample represented the percentage (P) of the presence of that strain.
in total population. The product of viable cell count value and the percentage ($P$) of each isolate made it feasible to calculate the viable cell count for each strain. One isolate from each group was chosen as the representative strain and used for further analyses.

2.5. Taxonomic identification of strains

Each representative strain from RAPD-PCR groups was identified by partial amplification and sequencing of 16S rRNA genes for bacteria (Klijn, Weerkamp, & de Vos, 1995) or 28S rRNA genes for yeasts (Kurtzman & Robnett, 1997). Amplification and sequencing of 28S rRNA genes were performed using the primers NLF184/21 and NLR1126/22 listed by Van der Auwera, Chapelle, and De Wachter (1994).

The DNA sequences were obtained using an ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit ver1.1 (PE Applied Biosystems, Inc., Foster City, CA, USA) with both the forward and reverse primers being used. The reaction products were analysed with an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). The software package of the Applied Biosystems (Sequencing Analysis ver. 3.3 and MT Navigator PPC ver. 1.0.2) was used for the analysis and comparison of DNA sequences. Taxonomic strain identification was performed, comparing the rDNA sequences of minced meat and sausage isolates with sequences present in the Basic BLAST Search, as described by Altschul et al. (1997). Starch hydrolysis, performed on autoclaved wheat flour agar [8 g L$^{-1}$ wheat flour, 15 g L$^{-1}$ agar (Oxoid)], was used to improve the identification of Bacillus subtilis and B. pumilus strains that gave the same sequence alignment. After 48 h of growth at 30°C, 1–2 ml of 8% of iodine solution were added. The strains positive for starch hydrolysis showed a clear zone around the colony.

Yeast and mould isolates were identified according to Samson, van Reenen-Hoekstra, Frisvad, and Filtenborg (2000) and Yarrow (1998), respectively.

2.6. Physiological characterization of isolated strains

The strains were characterized for physiological features of interest in sausage processing, such as acidifying, proteolytic and lipolytic activities and nitrate reduction. In addition, hemolytic and lecithinase activities were evaluated.

Acidifying activity was tested in lyophilized pork meat medium, LP (10 g L$^{-1}$ glucose, 11.5 g L$^{-1}$ lyophilized pork meat, 3.5 g L$^{-1}$ starch) after 1 and 7 days of growth at 30°C. The reported values represent the means of three independent experiments.

Proteolytic activity was assayed using the LP broth in which the lyophilized pork meat added (Liommellin-Star S.p.A., div. Mellin, Agrate Brianza, Milan, Italy) was not subjected to a hydrolyzing process. After 1 and 7 days of growth at 30°C, the proteolytic activity of strains was evaluated following the o-phthaldialdehyde method of Church, Swaisgood, Porter, and Catignani (1983) reading the absorbance at 340 nm. Lactococcus lactis subsp. lactis H (Morea et al., 1999) was used as a positive control for both acidifying and proteolytic activities.

The lipolytic activity of strains was tested in a broth containing 5 g L$^{-1}$ peptone, 3 g L$^{-1}$ yeast extract, 2 g L$^{-1}$ NaCl (Oxoid), pH 7.0 (Leuschner, Kenneally, & Arendt, 1997). This broth was supplemented with 4% (w/v) pork fat, homogenized and sterilized. After sterilization, the fat was re-emulsified by vigorously shaking. About 100 ml of medium were inoculated with 1 ml of fresh overnight cultures having an absorbance value at 600 nm of 0.6–0.9. Lipolytic activity was assayed after 1 and 7 days of incubation at 30°C, with shaking following the copper soap method originally developed for screening the free fatty acid in milk (Bulletin of IDF 265, 1991) reading the absorbance at 440 nm. Control batches consisted of broth supplemented with fat and without cells. For the quantification of free fatty acids in broth containing pork fat, a standard curve was prepared using eight solutions of palmitic acid within a concentration range of 0.125–2.000 meq L$^{-1}$.

The reported values for proteolytic and lipolytic activities were calculated as the average of three independent experiments. The value of each replicate is the average of 10 absorbance readings.

Nitrate reduction was observed after 24h of growth in Nitrate broth (Merck, Darmstadt, Germany) by adding some drops of Griess-Ilosvay’s nitrite reagent. The presence of a red colour indicated that nitrate was partially reduced to nitrite. In some cases, in which no colour developed, the zinc dust test was carried out to determine if nitrate and/or nitrite had been reduced to nitrogen or ammonia. If nitrate was still present, the medium surrounding the zinc dust turned pink.

All the strains were also tested for hemolytic activity after growth on Blood Agar plates (Merck, Darmstadt, Germany) containing 5% sheep blood and lecithinase activity after growth on nutrient agar supplemented with 8% egg yolk emulsion (Oxoid). For both analyses, the strains were considered positive if their colonies were surrounded by zones of opacity after 6 days of growth at 30°C.

3. Results

3.1. Microbial growth kinetics

The viable counts from PCA, MRS, MSA and CGYA plates are shown in Fig. 1.

After pasteurization Gram-positive aerobic rods, endowed with endospores, were found at 2.81 × 10$^6$ cfu g$^{-1}$ in sample T0 and 1.20 × 10$^6$ cfu g$^{-1}$ in sample T25.
On MYP agar plates, no rough, pink-purple presumptive \textit{B. cereus} colonies, surrounded by dense precipitate were seen. Moulds were rare at both stages of production.

The minced meat had a pH of 5.5 and an $a_w$ of 0.96 whereas after ripening these values decreased to 4.6 and 0.82, respectively.

### 3.2. Typing and taxonomic identification of strains

The application of the “two-step RAPD-PCR” protocol over more than 90 colonies produced PCR fragments ranging from 300 to 2500 bp in size. In the minced meat, the isolated colonies from PCA, MRS, MSA and CGYA plates gave 14 different electrophoretic patterns whereas the microflora of the ripened sausage was made up of 12 biotypes.

The comparison of 26 electrophoretic RAPD-PCR patterns of isolates from minced meat and sausage made it possible to recognize 18 bacterial biotypes and two yeast biotypes; six of them were present in both T0 and T25 samples.

The strains analyzed in this work are listed in Table 1. The taxonomic position of all bacterial biotypes was achieved by means of sequence analysis of at least 500 bp of the 5′ region of the 16S rRNA gene; sequence analysis of a 600 bp fragment of 28S rRNA gene was carried out for the taxonomic identification of yeasts. The percentage of similarity between the rRNA gene sequences of sausage isolates and those in the database ranged from 95% to 99%. The starch hydrolysis assay confirmed that the positive strains belonged to \textit{B. subtilis}, whereas the negative ones were \textit{B. pumilus}.

### 3.3. Microbial Composition of minced meat and ripened sausage

Strain composition of bacterial populations enumerated on different media from each sample was obtained from viable cell counts, RAPD analysis and rDNA sequencing. The viable cell counts on MRS agar plates were always composed of lactobacilli as well as those on CGYA plates only of yeasts. The viable cell counts from PCA plates were mainly composed of \textit{Bacillus} strains. From MSA plates both bacilli and staphylococci were recovered.

In minced meat (T0), the similar viable cell counts between PCA (3.7 × 10^3 cfu g$^{-1}$) and MSA (2.74 × 10^4 cfu g$^{-1}$) plates were due to a high presence of \textit{Bacillus} strains (4.30 × 10^5 cfu g$^{-1}$) enumerated on both media. They represented the dominant population made up of seven \textit{Bacillus} strains belonging to \textit{B. subtilis}, \textit{B. pumilus} and \textit{B. amyloliquefaciens} species; when a biotype was present on both media, its highest value was considered to represent its viable cell count.

Viable cell counts on MSA plates were 2.74 × 10^3 cfu g$^{-1}$; fingerprinting and rDNA sequence analyses showed that both \textit{Bacillus} and \textit{Staphylococcus} strains were isolated from this medium; the same analyses showed that two \textit{Staphylococcus} strains belonging to \textit{S. succinim} and \textit{S. equorum} species accounted for 6.80 × 10^6 cfu g$^{-1}$.

In T0 sample lactobacilli were recovered in a low amount (7.57 × 10^3 cfu g$^{-1}$) with two \textit{Lactobacillus} strains, belonging to \textit{L. sakei} and \textit{L. curvatus} species. Some colonies of \textit{Acinetobacter johnsonii}, \textit{Psychrobacter glacinicola} and \textit{P. faecalis}, isolated from PCA and MSA plates, were also found in this sample. The yeast population was made up of only one strain of \textit{Debaryomyces Hansenii}.

### Table 1

Microbial strains identified in T0 and T25 samples as described in Section 2

<table>
<thead>
<tr>
<th>Item No.</th>
<th>Strain</th>
<th>Species</th>
<th>Isolated in</th>
</tr>
</thead>
<tbody>
<tr>
<td>701</td>
<td>tr 12</td>
<td>\textit{Acinetobacter johnsonii}</td>
<td>T0</td>
</tr>
<tr>
<td>702</td>
<td>tr 28</td>
<td>\textit{Lactobacillus curvatus}</td>
<td>T0 and T25</td>
</tr>
<tr>
<td>703</td>
<td>tr 44</td>
<td>\textit{Bacillus pumilus}</td>
<td>T0</td>
</tr>
<tr>
<td>704</td>
<td>tr 47</td>
<td>\textit{Psychrobacter glacinicola}</td>
<td>T0</td>
</tr>
<tr>
<td>705</td>
<td>tr 50</td>
<td>\textit{B. subtilis}</td>
<td>T0 and T25</td>
</tr>
<tr>
<td>706</td>
<td>tr 51</td>
<td>\textit{B. pumilus}</td>
<td>T0</td>
</tr>
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<td>T0 and T25</td>
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<tr>
<td>709</td>
<td>tr 56</td>
<td>\textit{B. amyloliquefaciens}</td>
<td>T0</td>
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<td>tr 58</td>
<td>\textit{P. faecalis}</td>
<td>T0</td>
</tr>
<tr>
<td>711</td>
<td>tr 59</td>
<td>\textit{Staphylococcus succinim}</td>
<td>T0 and T25</td>
</tr>
<tr>
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<td>tr 60</td>
<td>\textit{B. subtilis}</td>
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<tr>
<td>721</td>
<td>E2</td>
<td>\textit{Debaryomyces Hansenii}</td>
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</tr>
<tr>
<td>722</td>
<td>B8</td>
<td>\textit{D. hansenii}</td>
<td>T25</td>
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</tbody>
</table>

* Institute of Sciences of Food Production Bacterial collection accession number.
After 25 days of ripening (T25), three *B. subtilis* strains were isolated; one of them, *B. subtilis* tr50, recovered from MSA plates at 1.60 x 10^5 cfu g^-1, accounted for more than 80% of total *Bacillus* population (1.97 x 10^5 cfu g^-1), obtained summing *Bacillus* populations from PCA and MSA plates.

*B. subtilis* tr50 and *Staphylococcus* strains were enumerated together on MSA plates (2.36 x 10^6 cfu g^-1); staphylococci accounted for 2.20 x 10^6 cfu g^-1. Together with the previously isolated staphylococci strains, a new strain of *S. saprophyticus* was recovered.

The *Lactobacillus* community was the dominant population (1.09 x 10^7 cfu g^-1); this population was enriched by three new strains belonging to *L. sakei*, *L. casei* and *L. curvatus* species.

A new strain of *D. hansenii* dominated the yeast population.

The PCA counts decreased from T0 to T25. In the T25 sample *Bacillus* strains, that grew better than all other strains on PCA plates, hid other colonies belonging to *Lactobacillus* and *Staphylococcus* strains. This behaviour could explain the lower count in the T25 sample on PCA plates compared with MSA and MRS plates.

Sample pasteurization is usually done to improve isolation of heat resistant and spore-forming bacteria when they are present in complex microflora. The presence of a *Bacillus* population count in T0 and T25 samples higher than that found on TSA plates indicates that presumably about the 10% of *Bacillus* cells were present as endospores when sampling was done. Probably a large number of *Bacillus* cells were in the vegetative form both in T0 and T25 samples.

Among the 20 different isolates characterized for their electrophoretic RAPD-PCR patterns, six were isolated at the beginning and the end of ripening: in Fig. 2, the growth dynamics of these strains is shown.

Among rare moulds (less than 100 cfu g^-1) isolated from both samples, mostly *Aspergillus niger* and *Rhizopus stolonifer* were found.

### 3.4. Physiological characterization of isolated strains

All isolated microorganisms were tested for physiological features of interest in meat processing such as acidifying, lipolytic, proteolytic activities and nitrate reduction.

The strains belonging to *A. johnsonii*, *P. glucincola* and *P. faecalis*, species showed no significant activities and therefore they are not any further mentioned.

The strains showed pH values ranging from 4.6 to 6.2 after 1 day of growth in LP broth, whereas after 7 days pH values ranged from 3.6 to 5.8. *Lactobacillus* strains gave pH values lower than other isolates; the lowest value being for *L. lactis* subsp. lactisH control strain.

All strains were analysed for their ability to degrade pork proteins and fats at the beginning (reading at 24 h) and during ripening (reading at 7 days). The strains were considered positive for proteolytic activity when absorbance values at 340 nm were >0.28 considering that the negative control sample (un-inoculated broth) gave OD_{340} value of 0.24.

The strains were considered positive for lipolytic activity when free fatty acids gave a value >0.125 meq L^-1 palmitic acid as below this value readings were not reproducible. In Table 2, the values of acidifying, proteolytic and lipolytic activities of all *Bacillus*, *Lactobacillus*, *Staphylococcus* and *Debaryomyces* strains are reported.

Nitrate reduction evaluated for all bacterial strains showed that all *Lactobacillus* and *B. pumilus* were negative whereas all *Staphylococcus* and *B. subtilis* were positive.

The development of red colour from *B. subtilis* tr54 cells was partially covered by slime production.

All strains were further characterized for hemolytic and lecithinase activities. Hemolytic activity was developed only by four *Bacillus* strains (tr44, tr50, trf22 and tr56). The strains tr44, tr50, and tr56 were the only ones that developed a halo surrounding the colony after growth on nutrient broth supplemented with egg yolk emulsion indicating also a lecithinase activity.

### 4. Discussion

A number of studies have been recently published on the microbial characterization of traditional Italian dry sausages (Blaiotta et al., 2004; Cahlan & Genigeorgis, 1986; Cocolin et al., 2004; Coppola et al., 1998; Coppola et al., 1997; Coppola et al., 2000; Gardini et al., 2001; Parente et al., 2001; Rebecchi et al., 1998).

In this study, four different microbial populations, lactobacilli, staphylococci, bacilli and yeasts were
Lactobacillus lactis  
D. hansenii  
B8  
Debaryomyces hansenii  
L. casei  
trf 61  
B. subtilis  
trf22  
B. subtilis  
trf51  
B. subtilis  
tr56  
B. amyloliquefaciens  
trf109  
Staphylococcus equorum  
trf102  
S. saprophyticus  
tr59  
S. succinus  
trf62  
Lactobacillus sakei  
trf 51  
L. sakei  
trf 53  
L. curvatus  
trf 61  
L. casei  
E2  
Debaryomyces hansenii  
B8  
D. hansenii  
H  
Lactococcus lactis subsp. lactis

counted and analyzed to understand their role in the fermentation and ripening of “Salame di Senise”, a typical Southern Italian dry sausage. Among the traditional Italian dry sausages, this product is the only one made by adding powder of “Peperone di Senise”, the only Italian PGI pepper cultivar (EC Regulation No. 1263/96). The isolates belonged to L. sakei, L. curvatus and L. casei species; the strains L. curvatus trf28 and L. sakei trf51 were found at the beginning and the end of ripening. The main role of lactobacilli could be the acidification of the fresh sausage, among the physiological features analyzed, indispensable for correct ripening and control of undesirable microorganisms (Geisen, Lu¨cke, & Kroeckel, 1992; Lu¨cke, 2000), though natural lactobacilli are also considered important for aroma and control of undesirable microorganisms (Geisen, 1995; Leistner, 1986; Lu¨cke, 1985). Our results revealed that lactobacilli were the dominant microflora at the end of ripening in accordance with data obtained by other researchers (Coppola et al., 1998; Coppola et al., 2000; Rebecchi et al., 1998). The isolates belonged to L. sakei, L. curvatus and L. casei species; the strains L. curvatus trf28 and L. sakei trf51 were found at the beginning and the end of ripening. The main role of lactobacilli could be the acidification of the fresh sausage, among the physiological features analyzed, indispensable for correct ripening and control of undesirable microorganisms (Geisen, Lu¨cke, & Kroeckel, 1992; Lu¨cke, 2000), though natural lactobacilli are also considered important for aroma compound production (Coppola et al., 1998).

The strains belonging to S. equorum, S. saprophyticus and S. succinus species showed low proteolytic and lipolytic activities but were able to reduce nitrate. The role of these isolates was not completely revealed by the analyses carried out, even though other authors have found variable percentages of positive strains for proteolytic and lipolytic activities, in the production of ethyl esters of carboxylic acids, acetoin and compounds from lipid and protein degradation (Coppola et al., 1997; Lu¨cke, 2000; Papamanoli et al., 2003; Talon, Chasta`gnac, Vergnais, Montel, & Berdague, 1998). In a recent study (Mauriello, Casaburi, Blaiotta, & Villani, 2004) different proteolytic and lipolytic activities were found in coagulate negative staphylococci belonging to S. xylosus, S. saprophyticus, S. equorum and S. succinus isolated from other fermented sausages of Southern Italy. The low number of isolated strains and the different methods used to study physiological activities could explain the partial agreement with results of preceding studies on the same topic.

Among the yeast genera usually found in sausages, i.e., Candida, Pichia, Rhodotorula, Hansenula and Torulopsis (synonym of Candida), D. hansenii is the most frequently isolated species (Gardini et al., 2001). Yeasts have been mostly involved in colour and flavour development (Flores, Durá, Aurora, & Toldrá, 2004; Jessen, 1995; Leistner, 1986; Lu¨cke, 1985). D. hansenii isolates did not show lipolytic and proteolytic activities, as other D. hansenii strains from other sausages produced in Basilicata region (Gardini et al., 2001). Bacillus spp. strains are widespread in meat and meat products (Asplund, Nurmi, Hill, & Hirn, 1988; Rodel & Lu¨cke, 1990) and in traditional European sausages (Encinas, Sanz-Gomez, García-López, García-Armento, & Otero, 1996; Encinas, Sanz, García-López, & Otero, 1999; Palumbo, Rivenburgh, Smith, & Kissinger, 1975) but their actual role in meat fermentation had never been evaluated. The Bacillus strains isolated from “Salame di Senise” were included in a study carried out on the risk related to the consumption of cured meat products containing Bacillus spp. other than B. cereus (Matarante, Baruzzi, Cocconcelli, & Morea, 2004). The Bacillus strains from “Salame di Senise”, previously referred to “Salame Lucano”, showed both the absence of B. cereus

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acidifying activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proteolytic activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lipolytic activity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1d</td>
<td>7d</td>
<td>1d</td>
</tr>
<tr>
<td>tr56</td>
<td>5.8 ± 0.07</td>
<td>5.4 ± 0.01</td>
<td>0.44 ± 0.03</td>
</tr>
<tr>
<td>tr44</td>
<td>5.4 ± 0.03</td>
<td>5.4 ± 0.03</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>tr51</td>
<td>5.7 ± 0.05</td>
<td>5.2 ± 0.05</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>tr50</td>
<td>5.8 ± 0.05</td>
<td>5.5 ± 0.06</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>tr53</td>
<td>5.7 ± 0.07</td>
<td>5.5 ± 0.05</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>tr54</td>
<td>5.7 ± 0.04</td>
<td>5.5 ± 0.02</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>trf22</td>
<td>5.7 ± 0.09</td>
<td>5.5 ± 0.05</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>trf09</td>
<td>5.5 ± 0.10</td>
<td>5.2 ± 0.06</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>trf02</td>
<td>5.5 ± 0.07</td>
<td>5.1 ± 0.04</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>tr59</td>
<td>5.6 ± 0.06</td>
<td>5.2 ± 0.03</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>trf62</td>
<td>4.6 ± 0.05</td>
<td>3.6 ± 0.06</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>trf 51</td>
<td>5.7 ± 0.08</td>
<td>3.7 ± 0.03</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>trf 53</td>
<td>5.9 ± 0.08</td>
<td>3.7 ± 0.03</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>trf 61</td>
<td>4.8 ± 0.09</td>
<td>3.7 ± 0.03</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>E2</td>
<td>6.2 ± 0.07</td>
<td>5.8 ± 0.04</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>B8</td>
<td>5.9 ± 0.06</td>
<td>5.7 ± 0.05</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>H</td>
<td>3.9 ± 0.09</td>
<td>3.3 ± 0.06</td>
<td>0.32 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means of three replicates ± standard deviation.

<sup>a</sup> pH values after growth at 30 °C in LP broth.
<sup>b</sup> Proteolytic activity according to o-phthaldialdehyde absorbance values at 340 nm.
<sup>c</sup> Lipolytic activity expressed as meq L<sup>-1</sup> palmitic acid.
<sup>d</sup> Proteolytic positive and lipolytic negative control strain.
enterotoxin/enzyme encoding genes and of any toxin production.

All “Salame di Senise” Bacillus strains showed interesting features linked to meat processing. The proteolytic activity of Bacillus strains, analyzed in a broth containing lyophilized pork meat, showed interesting values mostly after 7 days of growth.

B. subtilis tr53, tr50 and trf22, and B. pumilus tr44 showed a high lipase activity on pork fats, releasing high amounts of fatty acids. The strains tr44 and tr50 also showed lecithinase activity and hemolytic activity that were not linked to known virulence factors (Matarante et al., 2004). To our knowledge, this paper reports the first study in which Bacillus strains from meat products were characterized by their technological properties. The results obtained for these Bacillus strains lead to the conclusion that they could contribute to the development of texture, flavour and taste in “Salame di Senise”.

The study of autochthonous strains endowed with important technological features is useful to identify isolates that contribute the peculiar traits of typical sausages obtained by traditional means of manufacturing. This work can be considered a preliminary study in developing a native starter that could be an important tool to standardize and scale up the “Salame di Senise” processing, making it feasible to overcome the economic difficulties of small-scale production. Further studies should be carried out to characterize the role of these strains in the development of aroma compounds, paying particular attention to sensory active molecules derived from the typical spice, PGI “Peperone di Senise”, as it is the main spice used for this sausage, and this vegetable is cultured in the same area of sausage production and is not used in other similar sausages.

5. Conclusions

The high amounts of bacilli recovered at the end of sausage processing and their properties, usually associated with organoleptic characteristics of sausages indicate their potential importance in production of “Salame di Senise”.

The results obtained from growth kinetics and physiological characterization of strains suggest that natural microflora containing strains belonging to L. curvatus, L. sakei, S. succinus, S. equorum, and also B. subtilis are involved in “Salame di Senise” ripening differently from industrial sausages obtained with selected starter mixtures not including B. subtilis strains.

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