Ann Microbiol (2013) 63:53–62
DOI 10.1007/s13213-012-0444-5

ORIGINAL ARTICLE

Microbial characterisation of fermented meat products from the Sicilian swine breed "Suino Nero Dei Nebrodi"

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Received: 12 January 2012 / Accepted: 28 February 2012 / Published online: 21 March 2012

Abstract Two traditional sausage products ("salsiccia" and "salame") processed from the raw meat of the Black Sicilian swine "Suino Nero dei Nebrodi" were microbiologically investigated during the manufacturing and ripening stages. Both products were dominated by lactic acid bacteria (LAB), especially rod-shaped types. The concentration of enterococci was consistent in salame. Coagulase-negative cocci increased slower than LAB. Yeasts showed an increasing trend during the ripening of both products. Enterobacteriaceae were counted at a constant level of about 10^5 CFU/g in both products, while pseudomonads diminished during ripening. Coagulase-positive staphylococci, *Listeria monocytogenes* and *Salmonella* spp. were not detected at the end of the ripening process. Characterisation of LAB at the strain and species level revealed that *Lactococcus lactis* was found only in the meat mixture, while *Lactobacillus sakei* and various enterococci persisted during the monitoring period. Some LAB strains isolated from sausages were also identified on the surface of the factory equipment. Two strains (*Lactobacillus sakei* SS106A and *Enterococcus faecalis* SS91) were characterised by their anti-*Listeria* properties due to bacteriocin-like inhibitory substance production. A multiple strain starter composed of *Lactobacillus sakei* and enterococci has been proposed to maintain the typical characteristics of the two fermented meat products microbiologically investigated in this study.

Keywords Bacteriocin-like inhibitory substances · Enterococci · Fermented sausages · Lactic acid bacteria · Traditional production · Typicality

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Introduction

Germany, Italy, Spain and France are reported to be the main sausage producing and consuming countries in the world (Lücke 1998). Italy has more than 3,500 companies that process meat-based products (Baruzzi et al. 2006), most of which are artisanal sausages, which are known as one of the typical Italian foods. Italian traditional sausages differ from region to region, primarily due to different ingredients added during processing. The standard Italian sausage production process can be described as follows. Minced pork meat is mixed with fat and other ingredients, such as salt, spices, nitrate and nitrite and starter cultures, stuffed into natural casings from the cleaned small intestine of pigs and left to ferment. When the product is partially fermented and requires cooking before consumption, it is called "salsiccia", while dried sausage is generally known as "salame". The fermentation process involves a complex system of events in which meat, ingredients (included the microorganisms added or naturally present on the raw materials), transformation process and ripening conditions are fundamental parameters that need to be controlled to obtain a final product with the desired safety and organoleptic characteristics. Artisan sausage production is often driven by autochthonous microflora, which sometimes can lead to fermentation failure; hence, the use of starter cultures isolated from local products and showing the desirable characteristics are recommended for successful sausage fermentations (Papa and Grazia 1990).

It is not an easy task to determine the contribution of the different microbial groups in the reactions associated with the ripening process of sausages, since they have different nutritional requirements. In addition, the effects of the various ingredients on microbial development must also be taken into account (Hierro et al. 1997). Basically, the microorganisms

present at the beginning of sausage fermentation include yeasts and anaerobe, psychrotrophic and mesophilic bacteria, most of which are responsible for spoilage, and some of which could even be pathogenic (Cocolin et al. 2004).

Enterobacteriaceae members are usually found in the first days of fermentation. After that, their number decreases due to acidification performed by LAB (Aymerich et al. 2003; Papamanoli et al. 2003; Drosinos et al. 2005). Fermented sausages are usually free of sulphite-reducing clostridia and coagulase-positive staphylococci (CPS) (Comi et al. 2005; Rantsiou and Cocolin 2006), but in some studies suspected colonies of *Staphylococcus aureus* were isolated from the meat mixture (Rebecchi et al. 1998; Metaxopoulos et al. 2001). With the aim of evaluating the safety aspect of the fermented sausages, several studies have investigated the presence of *Listeria monocytogenes* and *Salmonella* spp. during the different stages of the production (Aymerich et al. 2003; Comi et al. 2005; Rantsiou et al. 2005). *Listeria monocytogenes* can contaminate the fresh sausage mixture, but it is generally undetectable at the end of the fermentation process (Samelis et al. 1998; Metaxopoulos et al. 2001; Drosinos et al. 2005).

Lactic acid bacteria (LAB) of different genera and Gram-positive coagulase-negative cocci (CNC), specifically those belonging to the *Staphylococcus* and *Kocuria* genera, are considered technologically fundamental for meat preservation and fermentation processes (Hammes and Knauf 1994; Hammes and Hertel 1998). LAB are usually present at low numbers (10^2–10^3 CFU/g) in raw meat of a high hygienic quality, but they rapidly reach a dominant position during fermentation due to the anaerobic environment and to the presence of NaCl, nitrate and nitrite, all of which favour their growth despite the presence of other microbial groups. 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 carbohydrates available and the sources of meat proteins and additives (Aymerich et al. 2003).

The autochthonous swine "Suino Nero dei Nebrodi", which is allowed to run free in a restricted area of the Nebrodi Mounts (Sicily, Italy), has been saved from extinction due to its meat which, when processed into several cured meat products, among which are the well-known fermented sausages (salsiccia and salame), known as niche foods, well appreciated by consumers.

This study focussed on the microbiological characterisation of salsiccia and salame originating from the meat of Suino Nero dei Nebrodi swine. The specific objectives were: to follow the evolution of several microbial groups during the fermentation process; to investigate the presence of the main pathogenic bacteria; to isolate, characterise and identify LAB at the strain level; to evaluate the bacteriocinogenic potential of LAB.

Materials and methods

Sample collection and pH measurements

The production of two fermented sausage products, salsiccia (SS) and salame (SM), produced from the meat and fat of the autochthonous swine "Suino Nero dei Nebrodi", was followed in this study (in duplicate). The products were produced at the Sicilian factory "Società cooperativa agro-zootecnica Roccaforte" (San Fratello, Messina, Italy). The two production processes differ in terms of ripening period, final shape of the product and the consumption modality: SS is ripened for 2 weeks, the meat is roughly minced, the shape of the sausage is elongate and narrow and it is cooked before consumption; SM is ripened for 6 weeks, the meat is thinly minced, the shape of the sausage is shorter and thicker than that of salsiccia and it is eaten raw. Both production processes are carried out without the addition of nitrates or nitrites, and LAB starter cultures are not used to drive the fermentation, which is naturally conducted. Samples were collected from minced meat (MM) prior to mixing with the other ingredients [3 % (w/w) NaCl, black pepper (seeds and ground), red hot pepper (ground) for both SS and SM and also wild fennel seeds for SS], just after stuffing into natural casings (ST), on a weekly basis until the end of ripening [week (W) 1–W2 for salsiccia and W1–W6 for salame].

The pH values were determined electrometrically using a pH meter (BASIC 20+; Crison Instrument S.A., Barcelona, Spain) on 10-g samples, diluted with distilled H_2O and homogenised by means of a stomacher (LAB Blender 400; Seward Medical, London, UK) at the maximum speed for 2 min. With the exception of MM, the other samples were peeled before dilution. Measurements were carried out in duplicate.

Microbiological analysis

Decimal dilutions of SS and SM samples (10 g) were carried out in Ringer's solution (Oxoid, Milan, Italy). Several microbial groups were investigated: total mesophilic count (TMC) on Plate Count Agar (PCA), incubated aerobically at 30 °C for 72 h; total psychrotrophic count (TPC) on PCA, incubated aerobically at 7 °C for 7 days; Enterobacteriaceae on Violet Red Bile Glucose Agar, incubated anaerobically at 37 °C for 24 h; enterococci on Kanamycin Aesculin Azide (KAA) agar, incubated aerobically at 37 °C for 24 h; pseudomonads on *Pseudomonas* Agar Base supplemented with 10 mg/mL cetrimide fucidin, incubated aerobically at 20 °C for 48 h; CPS and CNC on Baird Parker (BP) supplemented with RPF (Oxoid), incubated aerobically at 37 °C for 48 h; rod LAB on de Man–Rogosa–Sharpe (MRS) agar, incubated anaerobically at 30 °C for 48 h; coccus LAB on M17

agar, incubated anaerobically at 30 °C for 48 h; yeasts on Yeast Glucose Chloramphenicol agar, incubated aerobically at 25 °C till 5 days. The counts were performed in duplicate.

At the end of the ripening period, both SS and SM products were also analysed for the presence of *Salmonella* spp. and *Listeria monocytogenes*. *Salmonella* spp. detection included a (1) pre-enrichment in buffered peptone water at 37 °C for 24 h; a (2) selective enrichment of 0.1 mL of pre-enriched culture in 10 mL of Rappaport–Vassiliadis (RV) broth for 24 h at 42 °C; (3) selective isolation by streaking 10 μL of RV onto plates of Rambach agar at 37 °C for 24 h. *Listeria monocytogenes* was evaluated after a multi-step enrichment procedure: (1) primary selective enrichment in Half Fraser Broth (225 mL) inoculated with 25 g of samples, incubated at 30 °C for 24 h; (2) secondary selective enrichment in Fraser Broth (10 mL) inoculated with 0.1 mL of primary enriched culture, incubated at 37 °C for 48 h. A 10-μL sample of the secondary enriched culture was then streaked onto supplemented *Listeria* Selective Agar Base supplemented with SR140 (Oxford formulation), incubated aerobically at 37 °C for 48 h.

All media were purchased from Oxoid.

The tank used for preparing the meat mixture and the mincing machine orifice were investigated for the presence of LAB as follows: sterile cotton swabs were first streaked onto the surfaces of the factory equipments and then onto M17 and MRS agar media.

Isolation of LAB and phenotypic grouping

After growth, colonies of various shapes were randomly picked from count plates used for LAB enumeration (MRS, M17 and KAA) and transferred to the corresponding broth media. The isolates were purified by successive sub-culturing. The purity of the cultures and cell morphology were checked microscopically. Gram-positive (Gregersen KOH method) and catalase-negative (determined by transferring fresh colonies from a petri dish to a glass slide and adding 5 % H_2O_2) isolates were stored in glycerol at −80 °C until further analyses.

Rod- and coccus-shaped LAB cultures were first grouped on the basis of cell disposition, growth at 15 and 45 °C and CO_2 production from glucose. The latter test was carried out in the optimal growth media (MRS for rod LAB and M17 for coccus LAB) containing all components except citrate, whose fermentation by certain LAB may determine gas formation (Parente and Cogan 2004). M17 contained glucose in place of lactose. The assay consisted of inoculating LAB into test tubes that were then sealed with H_2O agar (2 %, w/v). The strains negative to the assay were inoculated into test tubes containing the optimal growth media prepared with a mixture of pentose carbohydrates (xylose, arabinose and ribose, 8 g/L each) in place of glucose. Coccus isolates were further sub-grouped for their growth at pH 9.6 and in presence of 6.5 % (w/v) NaCl.

DNA extraction, genotypic differentiation and identification of LAB

DNA was extracted from lysed cells (Instagene Matrix kit; Bio-Rad, Hercules, CA) as described by the manufacturer. Crude cell extracts were used as the template for PCR reactions.

Strain differentiation was performed by random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis in a 25-μL reaction mix using, separately, primers M13 (Stenlid et al. 1994), AB111 and AB106 (van den Braak et al. 2000). Amplifications were performed in a T1 Thermocycler (Biometra, Göttingen, Germany) under the conditions reported by Zapparoli et al. (1998) for primer M13 and under those reported by the reference paper for primers AB111 and AB106. PCR products were separated by electrophoresis on a 1.5 % (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) and visualised by UV transillumination after staining with SYBR® safe DNA gel stain (Molecular Probes, Eugene, OR). O' gene Ruler™ DNA ladder Mix (MMedical Srl, Milan, Italy) was used as a molecular size marker. RAPD-PCR profiles were analysed with the pattern analysis software package Gel Compar Ver. 4.1 (Applied Maths, Kortrijk, Belgium). Similarities between band profiles were calculated based on the Pearson product moment correlation coefficient. Dendrograms were obtained by means of the unweighted pair group method using the arithmetic average clustering algorithm.

Genotypic identification of LAB with different RAPD-PCR profiles was carried out by 16S rRNA gene sequencing. PCR reactions were performed as described by Weisburg et al. (1991). DNA fragments were visualised and the amplicons of about 1600 bp were purified by the QIAquick Purification kit (Qiagen S.p.A., Milan, Italy) and sequenced using the same primers employed for PCR amplification. DNA sequencing reactions were performed by PRIMM (Milan, Italy). The sequences were compared by a BLAST search in the GenBank/EMBL/DDBJ database.

Assays for antibacterial compound production

The antimicrobial activity of each LAB strain was first detected by the agar-spot deferred method (ASDM), and the strains showing positive results were subsequently tested by the well diffusion assay (WDA) (Schillinger and Lücke 1989). Both assays were performed following the modifications of Corsetti et al. (2008) using *Lactobacillus sakei* LMG2313, *Listeria innocua* 4202 and *L. monocytogenes*

ATCC 19114^T as indicator strains. Tests were carried out in triplicate.

Sensitivity of active supernatants to proteolytic enzymes was tested by treatment with proteinase K (12.5 U/mg), protease B (45 U/mg) and trypsin (10.6 U/mg) at a final concentration of 1 mg/mL in phosphate buffer (pH 7.0). All enzymes were purchased from Sigma-Aldrich (St. Louis, MO). The supernatants were incubated for 2 h at 37 °C and the remaining activity was determined by the WDA (Settanni et al. 2005).

Results

pH determination and microbiological analysis

The pH decreased from 5.71 to 5.18 in SS after 2 weeks of fermentation (Table 1). A similar drop in pH was registered also for SM during the same period and this parameter remained almost constant, ranging between 5.18 and 5.23 for the other 4 weeks of ripening.

Several microbial (pro-technological and spoilage/pathogenic) populations were investigated in the salsiccia and salame of Suino Nero dei Nebrodi (Table 1).

TPC values increased during the fermentation of both SS and SM. This increase was about 1.5 log CFU/g for SS, while almost three log cycles higher were registered for SM at the fourth week of ripening. Similar trends were displayed by TMC, although the initial values were higher than those for TPC during both production processes. TPC and TMC reached their maximum concentration at the fourth week.

CPS and CNC were counted on the same agar medium (BP) and were differentiated by the formation of a clear halo around the colonies of CPS. Undesired staphylococci were detected (approximately at 10^1 CFU/g) before (MM) and after stuffing (ST) for SS and SM, but not during ripening. On the contrary, CNC showed an increase of about three log cycles (from 3.6–3.7 $\log_{10}$ CFU/g of the beginning) at the second week in SS and at the third week in SM. In the last case, CNC remained at the same level for the remaining 3 weeks.

Among spoilage bacteria, Enterobacteriaceae and pseudomonads did not show any great changes in concentration during sausage maturation. Both groups were present at similar levels (approx. 10^5 CFU/g) in MM of SS and SM.

Rod and coccus LAB showed a similar trend and comparable concentrations during SS production, but they behaved differently during SM production. During the latter, coccus LAB stopped increasing (6.2 $\log_{10}$ CFU/g) at the second week, decreasing to 4.9 $\log_{10}$ CFU/g at the end of observation, while the concentration of rod LAB increased (7.6 $\log_{10}$ CFU/g) up to the fifth week. Interestingly, enterococci developed at levels similar to those registered for the other LAB, especially for SM, reaching a final concentration of 5.4 $\log_{10}$ CFU/g in both SS and SM.

Yeast concentration increased slightly during fermentation, not surpassing 5.7 and 5.8 $\log_{10}$ CFU/g in SS and SM, respectively, and the final level was found to be one order of

Table 1 pH and microbiological counts ($\log_{10}$ CFU/g) of salame and salsiccia made from raw meat of the swine Suino Nero dei Nebrodi during production and ripening

Samples	pH	Media									
		TPC	TMC	Enterobacteriaceae	Enterococci	Pseudomonads	Coccus LAB	Rod LAB	Yeasts	CPS	CNC
SS-MM	5.71±0.1	5.2±0.4	5.6±0.2	5.0±0.2	4.3±0.2	4.9±0.2	5.1±0.0	5.3±0.1	4.6±0.4	1.3±0.3	3.7±0.1
SS-ST	5.68±0.1	5.3±0.1	5.5±0.1	5.2±0.1	4.1±0.0	4.6±0.2	4.9±0.3	5.4±0.2	4.5±0.3	1.6±0.3	3.8±0.4
SS-W1	5.44±0.1	6.7±0.5	6.8±0.6	5.2±0.3	4.6±0.6	4.6±0.5	6.9±0.8	7.1±0.2	5.7±0.2	<1	5.8±0.2
SS-W2	5.18±0.1	6.8±0.1	7.0±0.4	4.9±0.2	5.4±0.2	4.3±0.3	6.6±0.9	6.6±0.4	5.7±0.2	<1	6.9±0.3
SM-MM	5.76±0	5.0±0.5	5.3±0.8	5.0±0.3	4.1±0.5	4.7±0.2	5.3±0.3	5.1±0.4	4.4±0.3	1.4±0.3	3.6±0.2
SM-ST	5.72±0.1	5.1±0.2	5.4±0.4	4.7±0.3	4.1±0.2	4.3±0.4	5.4±0.2	5.5±0.3	4.5±0.4	1.2±0.2	3.9±0.3
SM-W1	5.39±0.1	6.6±0.4	6.7±0.4	5.2±0.3	5.5±0.2	4.6±0.7	6.1±0.3	6.4±0.3	5.8±0.3	<1	5.3±0.1
SM-W2	5.21±0	6.2±0.6	6.9±0.4	5.1±0.4	6.4±0.5	4.0±0.4	6.2±0.2	6.4±0.2	5.5±0.1	<1	5.6±0.4
SM-W3	5.20±0.1	7.2±0.3	7.1±0.8	5.0±0.6	6.6±0.2	3.9±0.3	5.8±0.2	6.7±0.1	5.6±0.4	<1	6.6±0.2
SM-W4	5.18±0.1	7.8±0.2	8.0±0.3	5.3±0.3	5.8±0.3	3.7±0.6	5.8±0.4	7.1±0.3	5.4±0.2	<1	6.7±0.2
SM-W5	5.23±0	7.7±0.2	7.7±0.2	5.7±0.6	5.7±0.2	3.6±0.3	5.0±0.2	7.6±0.1	5.5±0.2	<1	6.6±0.4
SM-W6	5.21±0	7.2±0.2	7.5±0.3	4.9±0.3	5.4±0.3	3.7±0.6	4.9±0.4	7.4±0.1	5.2±0.7	<1	6.9±0.2

SS, Salsiccia; SM, salame; MM, minced meat; ST, stuffed meat; W, week; TPC, total psychrotrophic count; TMC, total mesophilic count; LAB, lactic acid bacteria; CPS, coagulase-positive staphylococci; CNC, coagulase-negative cocci

Data are presented as mean values ± standard deviation (SD)

magnitude lower than that of LAB in SS and two orders of magnitude lower than that in SM.

The safety of the sausages for consumption was confirmed by the absence of *Salmonella* spp. and *Listeria monocytogenes*, which were investigated only at the end of the ripening period.

Isolation and grouping of LAB

Colonies which developed in the generic media used for LAB growth were isolated after visual inspection in order to collect, at least once, samples of all colony morphologies detected in the countable plates. A total of 201 pure cultures were propagated in the broth media corresponding to those used for counts, with the exception of enterococci, which were cultivated in M17 broth, under the same incubation conditions. Of the 201 isolates, 165 cultures were further characterised as Gram-positive and catalase-negative. After microscopic inspection, 102 cultures were assessed as presumptive LAB rods and 63 as coccus-shaped.

All of these 165 isolates were tested for growth temperature and CO_2 production from glucose, whereas only cocci were also evaluated for growth at pH 9.6 and in presence of 6.5 % NaCl (w/v). The phenotypic characterisation allowed the separation of the cultures into four groups (Table 2), three for cocci and one for rods. The most numerous group was group IV, which included all rod-shaped isolates that represented almost two-thirds of the presumptive LAB collected from both SS and SM productions. Group IV bacteria did not generate CO_2 from glucose. However, the unequivocal determination of the fermentative metabolism of this putative LAB needed the evaluation of their growth in the presence of pentose sugars, which demonstrated their facultative heterofermentative metabolism.

Strain typing and identification of LAB

About 30 % of the isolates of each phenotypic group were selected from the samples collected throughout the sausage production process and subjected to RAPD analysis. The 50 isolates chosen were divided into seven main clusters (Fig. 1) representing the four phenotypic groups: four clusters for group I and one cluster each for groups II, III and IV.

One isolate representative of each RAPD group was subjected to genotypic identification. The BLAST search for the 16S rRNA gene sequences analysed revealed a percentage of identity with sequences available in the NCBI database of at least 97 % for all seven strains (Table 3). All strains were confirmed to belong to the group of LAB, with six species belonging to three genera (*Enterococcus*, *Lactobacillus* and *Lactococcus*) identified. Phenotypic group I included *Enterococcus casseliflavus*, *E. hirae* and *E. faecalis*, while groups II, III and IV comprised a single species each, *Enterococcus devriesei*, *Lactococcus lactis* and *Lactobacillus sakei*, respectively.

The speciographic distribution of LAB among salame and salsiccia of Suino Nero dei Nebrodi during the production and ripening processes is also reported in Table 3. Although representing barely one-third of the LAB population collected in this study, enterococci constituted the more heterogeneous group. *Enterococcus casseliflavus* and *Lactococcus lactis* were the only two LAB species (three strains) found in the minced meat and in the mixture soon after stuffing of both SS and SM products. During ripening, *L. lactis* was no more detected, while *E. casseliflavus* was present until the fourth week in SM. The species *E. hirae* appeared from the first week of observation in both products, whereas *E. devriesei* and *E. faecalis* appeared in the third week and were therefore only found in SM samples, where, together with *E. hirae*, they persisted until the sixth week. The species dominant throughout the ripening process, in terms of number, was *Lactobacillus sakei*, which like *E. hirae*, was identified from the first week onward in both sausage products.

The presumptive LAB colonies ($n=11$) obtained by analysis of cotton swabs streaked onto the surfaces of the tank and mincing machine orifice were first checked for being Gram positive and catalase negative and then subjected to RAPD-PCR; the resulting profiles were compared to those

Table 2 Phenotypic grouping of LAB isolates collected during the production and ripening processes of salame and salsiccia of Suino Nero dei Nebrodi

Characters	Clusters			
	I ($n=56$)	II ($n=2$)	III ($n=5$)	IV ($n=102$)
Morphology	Coccus	Coccus	Coccus	Rod
Growth				
15 °C	+	+	+	+
45 °C	+	–	–	–
pH 9.6	+	+	+	n.d.
6.5 % NaCl	+	+	–	n.d.
CO_2 from glucose	–	–	–	–
Growth in presence of pentose carbohydrates	n.d.	n.d.	n.d.	+

The number of isolates is reported in parenthesis

n.d., Not determined

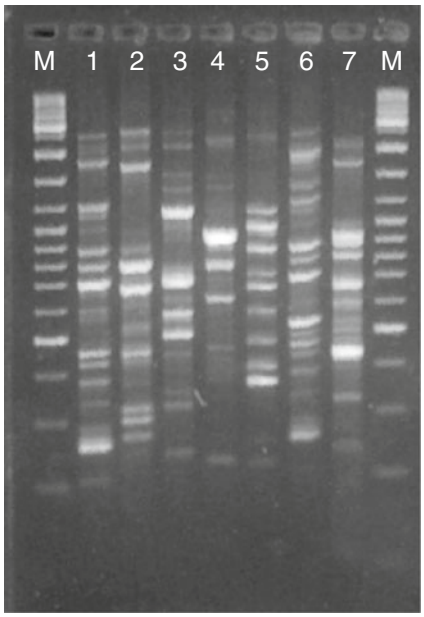

Fig. 1 Random amplified polymorphic DNA-PCR profiles (obtained with primer M13) of lactic acid bacteria (LAB) isolates collected during the production and ripening processes of salame and salsiccia of Suino Nero dei Nebrodi. Lanes: M, molecular marker; 1-7, strains SS19B, SS54, SS127A, SS33B, SS91, SS11A, SS106A

of the LAB previously characterised. Profile 4, belonging to *E. hirae* (Fig. 1, Table 3), was recognised for the isolates from both pieces of factory equipment, while profile 7 of *Lactobacillus sakei* was found for two isolates from the mincing machine orifice. Furthermore, two isolates grown on MRS, both taken from the mincing machine orifice and sharing the same RAPD profile (results not shown), could not be recognised as one of the LAB found in SS and SM samples. One of the two isolates was analysed by 16S rRNA gene sequence and identified as *Brochotrix thermosphacta*, although it tested negative for catalase activity.

Bacteriocin-like inhibitory substance production

The seven different LAB strains were investigated for their antimicrobial potential. LAB were initially tested by means of the ASDM against three indicator strains with a high sensitivity to bacteriocins (Corsetti et al. 2008; Hartnett et al. 2002). In this step, the possible inhibitory effect of the organic acids and of hydrogen peroxide was not excluded, since the overlay containing the indicator strains was in direct contact with colonies of the producer strains. Only *Lactobacillus sakei* SS106A and *Enterococcus faecalis* SS91 produced a clear halo of inhibition against listerias. The active supernatants were subsequently treated with catalase, neutralised, sterilised by filtration and tested by the WDA against the same three indicators. Both strains retained their antimicrobial activity, showing the following clear zones around the wells: *Lactobacillus sakei* SS106A, 13 (± 1) mm against *Listeria innocua* 4202, 12 (± 1) mm against *L. monocytogenes* ATCC 19114^T; *E. faecalis* SS91, 12 (± 1.5) mm against *L. innocua* 4202, 11 (± 1) mm against *L. monocytogenes* ATCC 19114^T. The antibacterial compounds were inactivated by proteolytic enzymes, proving their proteinaceous nature, a general characteristic of bacteriocins (Jack et al. 1995; Tagg et al. 1976). These substances have not yet been characterised for amino acid and gene sequences and are therefore referred to here as bacteriocin-like inhibitory substances (BLIS).

Discussion

With the aim of investigating the microbiological profile during the fermentation process of two products, “salsiccia” and “salame”, of the raw meat of the autochthonous swine breed “Suino Nero dei Nebrodi”, from the Sicilian area of

Table 3 Species of LAB isolated during the production and ripening processes of salame and salsiccia from raw meat of the swine Suino Nero dei Nebrodi

Species	Strain	RAPD profile	Identification			Isolation source	
			bp	% Homology	Accession no.	Salsiccia	Salame
Enterococcus casseliflavus	SS19B	1	1,493	99	JQ411240	MM, ST, W1, W2	MM, ST, W1, W2, W3, W4
	SS54	2	1,496	99	JQ411241	MM, ST, W1, W2	MM, ST, W1, W2, W3, W4
E. devriesei	SS127A	3	1,488	99	JQ411242		W3, W4, W5, W6
E. hirae	SS33B	4	1,504	99	JQ411243	W1, W2	W1, W2, W3, W4, W5, W6
E. faecalis	SS91	5	1,495	99	JQ411244		W3, W4, W5, W6
Lactococcus lactis	SS11A	6	1,464	98	JQ411245	MM, ST	MM, ST
Lactobacillus sakei	SS106A	7	1,506	99	JQ411246	W1, W2	W1, W2, W3, W4, W5, W6

W, week; RAPD, random amplified polymorphic DNA

Nebrodi Mounts, we analysed the two sausage types during the ripening stage,, which lasted 2 weeks for salsiccia and 6 weeks for salame. Both sausage types are produced by traditional techniques without the addition of nitrates or nitrites and starter cultures.

Soon after meat mincing, the samples were characterised by pH values ranging from 5.71 to 5.76. After 15 days, the pH had decreased to 5.18–5.21, and then it remained almost constant for salame. These evolution in pH is typic of sausage products, both at the second week (Kaban and Kaya 2009) and after 6 weeks (Aquilanti et al. 2007; Sawitzki et al. 2008) of fermentation. Although the "shelf-stable" meat products (e.g. sausages) should be characterised by a pH of <5.2 (Ambrosiadis et al. 2003), the final value of several similar products has been found to be >5.3 (Aymerich et al. 2003; Barbut 2006; Urso et al. 2006).

Fermented sausages are often traditional products with a great diversity in production methods and organoleptic characteristics among different countries and different regions of the same country. Extensive studies have been performed on the microbiological characterisation of traditional sausages (Fontana et al. 2005; Drosinos et al. 2007; Benito et al. 2008; Di Cagno et al. 2008) and a wide variety of microorganisms have been reported. It is generally agreed upon that the main microbial groups positively involved in these productions are LAB, especially lactobacilli, and CNC (*Staphylococcus* and *Kocuria*), the ones that are often numerically more important at the end of the fermentation (Aymerich et al. 2003; Mauriello et al. 2004).

The microbiological results obtained in this work show that LAB were the most numerous microorganisms present during both production processes, i.e. manufacturing and ripening, investigated. Rod LAB concentrations were, on average, slightly higher than those of coccus-shaped LAB, and the concentrations of the latter were almost comparable with those of enterococci. Raw meat is generally contaminated by LAB, with values of 3.5–4.0 $\log_{10}$ CFU/g commonly found in minced meat; however, during the fermentation process LAB concentrations may increase up to 8.5–9.0 $\log_{10}$ CFU/g (Bonomo et al. 2011). For enterococci, the concentration in pork meat may reach levels of 10^4 CFU/g (Teuber et al. 1996) as a consequence of intestinal or environmental contamination (Giraffa 2002), and contrasting results are reported in literature on the presence and persistence of these LAB. In some studies, a reduction in the numbers of enterococci were observed after 20 days of fermentation, following an initial increase to 10^5 CFU/g, leading to a final count of about 10^2 CFU/g (Papamanoli et al. 2003; Cocolin et al. 2004). However, levels of enterococci in the range of about 10^5–10^7 CFU/g at the end of the fermentation period have been also reported (Rebecchi et al. 1998; Rantsiou et al. 2005; Di Cagno et al. 2008).

Thanks to the generation of lactic acid, LAB cause a decrease in the pH, making the environment hostile for the development of several unwanted (spoilage and/or pathogenic) microorganisms. This aspect is crucial to maintain the hygienic safety of these meat products (Lücke 2000; Työppönen et al. 2003). However, in our study, we observed a decrease in the concentrations of rod LAB in salame towards the end of the fermentation period, while coccus LAB remained the predominant microbial group, although the levels detected were lower than those reported for other Italian salame products analysed at the same time of ripening (Di Cagno et al. 2008). The same trend was found for the concentrations of LAB in salsiccia, which were between 1 and 2 orders of magnitude lower than those found for salsiccia produced in Basilicata region (Southern Italy) and ripened for a couple of weeks.

In this work, the growth of LAB may have contributed towards keeping the undesired microbial groups at low levels (Drosinos et al. 2005), even though Enterobacteriaceae were found at an almost constant level at 10^5 CFU/g throughout the ripening of both production processes. Similar concentrations of Enterobacteriaceae were registered during the ripening of sausages produced in northern Italy (Comi et al. 2005). In case of pseudomonads, a progressive decrement was registered, which is typical during sausage production (Drosinos et al. 2005).

It is worth noting that a too rapid development of LAB may result in an intense acidification of the meat matrix determining the inhibition of CNC, characterised by a slower growth (Papamanoli et al. 2003), as confirmed by our data showing the highest CNC counts (6.9 $\log_{10}$ CFU/g) at the end of the monitoring period. Other authors have reported values comparable to our results reported here in terms of these microbial groups, such as Coppola et al. (1997), who noted concentrations of about 6 $\log_{10}$ CFU/g for *Micrococcaceae* after the fourth week of ripening of fermented sausages, and levels in the range 10^6–10^7 CFU/g are commonly reported for salami-type products ripened for more than 6 weeks (Di Cagno et al. 2008). In fact, the longer the ripening period of sausages, the higher the number of CNC (Iacumin et al. 2006). Among the pro-technological microorganisms, yeasts were detected during the entire period of observation, showing concentrations similar to those reported previously for sausage of southern Italy (Coppola et al. 2000; Gardini et al. 2001).

The hygienic safety of the salame and salsiccia of the Suino Nero dei Nebrodi was evidenced by the absence of CPS, *Salmonella* spp. and *Listeria monocytogenes*. This aspect is fundamental for the traditional production of local products that are carried out without the addition of starter cultures and nitrates or nitrites. Although the final products are characterised by a strong typicality, which links several organoleptic features to the production area, when naturally processed, sausages may be subjected to large fluctuations in their final characteristics, particularly sensorial, but also

hygienic. On the other hand, the use of commercially available starters, which may be necessary to guarantee consumer safety, may determine and impoverish flavour and aroma (Gardini et al. 2001). From this perspective, the addition of starter cultures, mainly LAB selected from the traditional production, could represent a defining strategy to produce fermented meat products with microbiological characteristics that are stable over time and to minimise the variability of the sensorial/aromatic aspects. With this in mind, LAB were isolated, grouped, differentiated and identified in order to evaluate their composition at the species and strain level, in view of their selection to develop a starter culture to be used in the production of salame and salsiccia from Suino Nero dei Nebrodi raw meat.

LAB were phenotypically divided into four groups, and seven strains belonging to six species of three LAB genera (*Enterococcus*, *Lactobacillus* and *Lactococcus*) were recognised. Thus, the total LAB biodiversity found to be associated with the products analysed is low when compared to that revealed by other Italian sausage products (Parente et al. 2001; Urso et al. 2006; Aquilanti et al. 2007; Di Cagno et al. 2008). This finding may have a multiple explanations: the carcasses were slaughtered in the same place; sausage productions were investigated in one factory; the microbiota defined as autochthonous for sausages is not only present in the raw materials, but are also derived from the factory environment which plays an important role in the process of colonisation (Chevallier et al. 2006).

Lactobacillus sakei is the only lactobacillus identified in this study. This species is reported as typical in sausage fermentation (Chaillou et al. 2009) and has been found in several Italian sausages (Parente et al. 2001; Urso et al. 2006; Aquilanti et al. 2007; Di Cagno et al. 2008). In our study, it was detected from the first week of fermentation onward, but it was not found in minced meat or soon after stuffing. The analysis of the mincing machine orifice revealed the presence of the same *Lactobacillus sakei* strain found to be dominant among the rod LAB during the entire production process of both salame and salsiccia. Thus, the meat on the surface of the equipment may have been slightly contaminated during the mincing process (not detectable at the beginning of sausage preparation) with this specific *L. sakei* strain and when the conditions internal to the casings became favourable the strain increased in concentration.

Lactococcus lactis was only found after meat mincing and stuffing, but was not detected later during ripening. A similar result was reported by Aquilanti et al. (2007), who recorded the presence of *L. lactis* up to the third day of fermentation of Ciauscolo salami. On the contrary, Cenci-Goga et al. (2008) inoculated two strains of this species during the production of Salame Nostrano which persisted at high numbers for the 21 days of ripening.

A relatively higher biodiversity (five strains of four species) was found within the group of enterococci. In the past, enterococci were considered to be spoilage agents for processed meats (Gordon and Amad 1991), but some species, such as *E. faecalis* and, especially, *E. faecium*, both found at high numbers during meat fermentation, may contribute, together with lactobacilli, to the transformation process (Hugas et al. 2003). In our study, the four species identified were *E. casseliflavus*, *E. devriesei*, *E. hirae* and *E. faecalis*. Together with *E. faecalis*, *E. casseliflavus* is generally detected in cured meat products (Hugas et al. 2003; Danilović et al. 2011), while the other two species have not been reported as being typically present during sausage fermentation, although *E. devriesei* has been recently described in bovine samples (Svec et al. 2005) and *E. hirae* is of poultry origin, both meats used in some type of traditional sausage production (Zambonelli et al. 1992). *E. hirae* was also found to be present on the surface of both pieces of factory equipment analysed, where two isolates of the same strain which did not show catalase activity were identified as *Brochothrix thermosphacta*. This finding is not surprising, since cells of this species are catalase positive when grown in the optimal media at the optimal temperature (20–25 °C), but if cultivated at higher temperature or in other media they may lose their catalase activity (Holley 2000). This species is included in the microbial group responsible for meat spoilage (Borch et al. 1996) and, for this reason, it is undesirable during meat transformation.

In order to investigate the potential of the LAB identified in this study in view of their application as starter and protective cultures, all strains were characterised for antimicrobial compound production. *Lactobacillus sakei* SS106A and *E. faecalis* SS91 were found to be BLIS producers showing anti-*Listeria* properties. Several strains of these species have been found to produce bacteriocins (Gálvez et al. 1989; Mortvedt et al. 1991; Holck et al. 1992; Tichaczek et al. 1992; Nilsen et al. 2003), and this character is of paramount importance in strategies of the biopreservation of fermented sausages. Furthermore, the production of antimicrobial substances may confer a competitive advantage over non-bacteriocin-producing strains (Franciosi et al. 2009) and may warrant a longer persistence of the producing strains themselves (Settanni et al. 2005, 2011).

In conclusion, the fermented meat products obtained from Suino Nero dei Nebrodi swine are characterised by a high hygienic safety, also thanks to the presence of bacteriocinogenic LAB strains. We propose that the LAB isolated and characterised in this study be used in a multiple starter culture composed of *Lactobacillus sakei* and enterococci.

Acknowledgements The authors are grateful to the student Antonio Vieni (University of Palermo) for his help with microbiological analysis.

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