Microbial succession during ripening of Naples-type salami, a southern Italian fermented sausage

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Received 21 December 1999; received in revised form 17 April 2000; accepted 18 April 2000

Abstract

Studies were carried out on the microbiological and physico-chemical changes which occurred during the ripening of five batches of Naples-type salami, manufactured without starter cultures. Salami were sampled internally and externally, and the following microbial groups were studied: lactic acid bacteria, Micrococcaceae and yeasts. The results obtained indicated that lactobacilli constituted the predominant flora, both on the surface and in the interior of the pieces throughout the ripening period. Micrococcaceae and yeasts were also found in considerable number in both locations. Characterisation of 191 lactic isolates indicated that the salami microflora was dominated by homofermentative lactobacilli; approximately 63% of them could be identified as Lactobacillus sake; 40% showing the traits of a racemase negative variant of this species, once referred to Lactobacillus bavaricus. Yeast population mainly comprised Debaryomyces strains. All the colonies grown on mannitol salt and Kranep agar were catalase-positive cocci; novobiocin-resistant staphylococci were the only Micrococcaceae found. The API Staph identification system did not prove to be reliable: 82% of the isolates remained unidentified. To achieve improved characterisation, cluster analysis was subsequently performed on this group, corroborating the existence of a fairly homogeneous group representing an intermediate variety between Staphylococcus xylosus and Staphylococcus saprophyticus that was isolated during the whole ripening process. © 2000 Published by Elsevier Science Ltd.

1. Introduction

“Naples-type salami” is a popular dry fermented sausage native to the Naples area, today produced throughout Italy. It is made of coarsely minced lean pork, mixed with fat similarly cut into small pieces, with the addition of pepper, salt and skimmed milk powder. The ingredients are stuffed into natural or artificial casings, stewed for some hours at around 30°C, often by smoking, and ripened at low temperature and relative humidity for about 30 days.

The microflora of this type of meat product has already received attention in the literature. Pirone, Manganeli and Diaferia (1990) studied lactic acid bacteria (LAB) from ready-to-be-consumed salami. They tentatively identified 39 isolates as Lactobacillus sake, 13 L. curvatus, two as L. alimentarius and the remaining four strains as L. casei subsp. pseudoplantarum, L. farcininis, L. amylophilus and L. viridescens. From similar samples Pirone and Manganeli (1990) isolated 71 Micrococcaceae: 93% of the isolates could be ascribed to the genus Staphylococcus, amongst those 21 were designed S. xylosus and the remaining strains were not clearly identified with any known species. Coppola, Marconi, Rossi and Dell’Aglio (1995) performed counts of mesophilic lactobacilli, Micrococcaceae, enterococci, Enterobacteriaceae and yeasts during the whole production and ripening process, but only LAB isolated after 7 days of ripening were identified. They identified 29 isolates belonging to L. sake, 13 L. curvatus, two L. alimentarius, one L. casei subsp. tolerans and one L. bavaricus. The same authors also reported the carbohydrate fermentation pattern of these strains, revealing the great variability in the utilisation of different carbon sources by LAB. Villani, Pepe, Mauriello, Salzano, Moschetti and Coppola (1994) and Villani, Sannino et al. (1997) isolated strains of Staphylococcus xylosus in such sausages which produced inhibitory substances with
antagonistic activity against *Listeria monocytogenes*. However definitive and detailed information about species and biotypes occurring within the different stages of ripening of Naples type salami is lacking.

During the last 30 years, much attention has been focused on the use of starter cultures due to the scientific progress in understanding the desirable role of microorganisms during meat fermentation (Bacus, 1986; Palumbo & Smith, 1997). Today it is well known that inoculation of the sausage mixture with selected strains of LAB and micrococci/non pathogenic staphylococci improves the quality and safety of the final product (Metaxopoulos, Genigeorgis, Fanelli, Franti & Cosma, 1981; Nychas & Arkoudelos, 1990). It is also known that the most suitable strains have been isolated from the same habitat, since the application of dairy starters in fermented meats has proven unsuccessful (Cahalan & Genigeorgis, 1986). Naples-type salami production, especially on a small scale, is still based on the experience and the skill of local manufacturers, rather than being a process wholly based on scientific and technological know-how. In such cases, the contribution of the naturally occurring microflora is decisive.

In this work, the desirable groups of microorganisms associated with the natural fermentation and ripening of five batches of Naples-type salami were enumerated, isolated and identified, with the aim of understanding the evolution of the different species of lactobacilli, micrococci/staphylococci and yeasts during the ripening process and to provide original isolates as a reserve of suitable strains to be used as starter cultures in sausage production.

2. Materials and methods

2.1. Sausages manufacture

Five batches of sausages were produced by a local artisanal plant in Mugnano del Cardinale (Campania, Italy) according to the traditional method (without the use of starter cultures and with a natural fermentation step at 30°C for 2 h after filling). The basic initial sausage mixture included (%w/w): lean pork 88–92, lard 3–4, NaCl 3, KNO₃ 0.02, pepper 0.2 and skimmed milk powder 4. Samples were taken immediately after filling and at 2, 7, 14, 23 and 41 days of ripening (12–14°C and 80–82% relative humidity).

2.2. Physico-chemical analysis

The potentiometric measurement of pH was made by inserting the pin electrode of a pH-meter (Beckman PH143) directly into each sample. Water activity (A_w) was measured using a “Lufft aw-wert-Messer” supplied by PBI (Milan, Italy).

2.3. Microbiological analyses

2.3.1. Microbial counts

For microbiological analysis, the casing was aseptically removed; pieces from the inner and the external part of each sample were cut using sterile knives and separately homogenised with nine parts of sterile quarter-strength Ringer’s solution in a Lab blender stomacher. Ten-fold dilutions were made in the same diluent. Mesophilic lactic acid bacteria were enumerated on Rogosa pH 5.4 (Oxoid) in anaerobic conditions and Mayeux agar (Mayeux, Sandine & Elliker, 1962) after 48 h at 30°C; Micrococccaeaceae on Mannitol Salt Agar (MSA, Oxoid) and Kranep agar (Oxoid) after 24 h at 30°C; yeasts and moulds on Malt Extract Agar (MEA, Oxoid) pH 5.4 after 4 days at 25°C.

2.3.2. Identification

Individual isolates from countable plates of Rogosa and Mayeux agar were selected at random, isolated and then maintained in MRS broth (Oxoid) plus 25% glycerol (Merk) at −20°C. Bacterial isolates were tested for Gram reaction, catalase production and cell morphology. Mesophilic lactobacilli were identified according to Shillinger and Lücke (1987) by detecting growth at 8, 15, and 45°C, gas production from glucose by hot loop test (Sperber & Swan, 1976), arginine hydrolysis (MRS without glucose and meat extract but supplemented with 0.3% arginine and research for NH₃ by addiction of Nessler’s reagent) and isomer of lactic acid by an enzymatic method (Von Krush & Lompe, 1982) using D- and L-lactate dehydrogenases (Boehringer Mannheim, Italy). The ability to ferment various carbohydrates was determined using MRS without glucose and meat extract with 2% added carbohydrate and 0.004% chlorophenol red (Schillinger & Lücke, 1987). The presence of diaminopimelic acid (Dpm) in the cell walls of isolates was determined in the total cell hydrolysates (16 h, 4 N HCl, 100°C) according to the chromatographic methods described by Harper and Davis (1979). Cocci and short rod, unable to hydrolyse arginine, producing gas and D-(-)-lactate from glucose, were classified as *Lactobacillus* and identified according to the presumptive scheme proposed by Villani, Moschetti, Blaiotta and Coppola (1997).

Colonies from Kranep and MSA medium were initially tested for morphology, Gram-stain and catalase production in order to select only Gram-positive and catalase-positive cocci. Strains were purified by streaking on MSA and maintained in BHI agar (Oxoid) on slant tubes at 4°C. Sensitivity to furazolidone (Von Rheinbaben & Hadlock, 1981) and lysostaphin (Schleifer & Kloos, 1975) were used to separate staphylococci from micrococci. Production of pigment was observed. Staphylococci were assayed for coagulase activity using the tube test (Baird-Parker, 1974) with coagulase plasma
were performed for every isolate: Diazonium Blue B salt staining spores with 0.5% (w/v) malachite green solution; sporulation, which was eventually confirmed by temperature up to one month in case of difficulty in inducement. For strains belonging to Micrococcaceae genus were used for the data matrix (S. xylosus, S. saprophyticus, S. colhinit, S. kloosii, S. sciuri and S. lenitus); they were selected to include those likely to be present in the salami habitat. Their biochemical patterns (reference profiles) were deduced from the API Staph identification table and Kloos (1990). The results of the API Staph were coded as 0 (negative test) or 1 (positive test) and examined by the simple matching coefficient with the Corr procedure of Systat 5.2.1 (Systat, 1992). Relationships were established by cluster analysis using the average linkage method by the Cluster procedure of Systat 5.2.1 (Systat).

2.4. Statistical analysis

For strains belonging to Micrococcaceae cluster analysis was subsequently performed. Phenotypic characteristics of six species belonging to the Staphyloccocus genus were used for the data matrix (S. xylosus, S. saprophyticus, S. colhninit, S. kloosii, S. sciuri and S. lenitus); they were selected to include those likely to be present in the salami habitat. Their biochemical patterns (reference profiles) were deduced from the API Staph identification table and Kloos (1990). The results of the API Staph were coded as 0 (negative test) or 1 (positive test) and examined by the simple matching coefficient with the Corr procedure of Systat 5.2.1 (Systat, 1992). Relationships were established by cluster analysis using the average linkage method by the Cluster procedure of Systat 5.2.1 (Systat).

3. Results and discussion

Changes in microbial contents during ripening of “Naples-type salami” are represented in Table 1. Counts on Rogosa and Mayeux agar, monitored throughout the ripening process, rapidly increased during the first days, reaching a maximum at 7 days, and then stayed constant until the end of ripening. These results are in agreement with those reported for other traditional fermented sausages produced in Italy (Coppola, Giagnacovo, Iorizzo & Grazia, 1998). No significant difference was observed between the edge and the core of the product. Numbers of lactobacilli increased from 3.0×10^3 at the beginning to 5.5×10^8 CFU g^-1 at the end of ripening, showing that this group rapidly dominates the total microflora. During the first week of drying, a pH decrease from 5.81 to 5.22 (results not shown), parallel to a maximal increase in LAB counts (Table 1) was observed. Counts on Kranep and MSA were similar and did not increase beyond 10^6 and 10^7–10^8 CFU g^-1 in surface and internal samples, respectively. Aerophilic and/or facultative anaerobic Micrococcaceae, known as occurring at high levels in marine salt and fresh meat, and as not inhibited by sodium chloride, osmotic pressure and partial desiccation (Carrascosa & Cornejo, 1991), are probably favoured by the more aerobic conditions of the external part of salami. In Naples-type salami, Coppola et al. (1995) detected populations of catalase-positive cocci on MSA at least 1 log higher than 10^6 CFU g^-1 found in this study. On the other hand, some authors (Sanz, Selgas, Parejo & Ordonez, 1988; Samelis, Stavropoulos, Kokouri & Metaxopoulos, 1994) reported that bacteria grown on MSA plates during late ripening did not actually belong to Micrococcaceae, but were Gram-positive, catalase-positive spore-forming bacilli. In this study, by checking more than 50% of the colonies present on high dilutions of MSA and Kranep plates for cell morphology and catalase formation, it was confirmed that none of them belonged to other genera, apart from Staphylococcus and Micrococcus. Yeast in samples obtained from the core of the product peaked at 10^4 CFU g^-1, this value remaining almost unchanged until the end of ripening. By contrast, counts on MEA for samples from the external part of the product reached a maximum of 10^6 CFU g^-1 after three weeks and then ceased to increase. Probably, higher oxygen availability in the external part of the sample promotes a major level of growth for these microorganisms too. These results confirm the predominance of LAB in dry fermented sausages previously reported by many authors (Cahalan & Genigeorgis, 1986; Lizaro, Chasco & Beriau, 1999; Samelis et al., 1994; Sanz et al., 1988). Nevertheless, micrococi/staphilococi and yeasts, in spite of their lower number if compared to LAB, turn out to be very important to provide salami sausages with their peculiar features. The interconnection of their metabolic activities has been underlined by Liepe (1983) and is considered essential to the healthiness, structure and colour of salami.

One hundred and ninety-four presumptive LAB were randomly isolated from Rogosa pH 5.4 and Mayeux
agon plates. With three exceptions, all the isolates were Gram-positive and catalase-negative bacteria. The three catalase-positive strains were regarded as non-lactic acid bacteria and were not tested further. Characterised. The remaining 191 lactic isolates, using the dichotomous scheme worked out by Schilling and Lücke (1987) is given in Table 2. Most strains (82.5%) were mesophile homofermentative lactobacilli (streptobacteria), confirming that meat is one of the main habitats for this group of LAB; the remainder were heterolactic lactobacilli (1.5%) and cocci (10.8%). The conventional physiological and fermentative tests applied to lactobacilli from meat products, allowed homogeneous phenotypic groups to be defined and the taxonomic position of the isolates to be found with good accuracy: only seven strains out of 191 could not be identified at species level and are reported in Table 2 as Lactobacillus spp. 34.4 and 10% of the total number of homofermentative lactobacilli could be exactly identified as L. sake and L. curvatus respectively, 40.6% of L-(+)-lactate-producing mesophilic lactobacilli corresponded with the physiological and morphological description of L. bavaricus (Stetter & Stetter, 1980). This species produces L-(+)-lactic acid from glucose and is often considered a race-competitive-defective variant of L. sake, with which shows a high genetic homology (Kandler & Weiss, 1986). Actually, the border between these two species is somewhat hard to draw and according to Kagermeier-Callaway and Lauer (1995) L. sake is the senior synonym for L. bavaricus. Therefore, L. sake and L. curvatus widely represent the dominant species of lactic acid bacteria in Naples type salami, in accordance with results obtained on similar salami produced in southern Italy (Coppola et al., 1995; Coppola et al., 1998). Leuconostocs (18 isolates) were the major group of coccal-shaped lactic acid bacteria isolated. They have been widely reported as occurring on meat or meat products but, as found in the present study, they are usually not a dominant component of the flora (Cahalan & Genigeorgis, 1986; Sanz et al., 1988). The 18 strains were classified, according to the presumptive scheme proposed by Villani, Moschetti et al. (1997), as Leuconostoc mesenteroides subsp. dextranicum.

A total of 136 cultures were isolated from MSA and Kranep plates. They were all identified as Staphylococcus species as they shared the typical characteristics of the genus: Gram-positive, catalase-positive, non-motile and susceptible to lysostaphin and furazolidone. In addition, all isolates were coagulase-negative and novobiocin-resistant. According to API Staph results, clear identification was obtained for only 34 strains: 18 were identified as S. xylosus, the coagulase-negative staphylococci (CNS) most frequently isolated from Italian fermented sausages (Comi, Citterio, Manzano, Cantoni & de Bertoldi, 1992; Coppola, Iorizzo, Saotta, Sorrentino & Grazia, 1997; Pirone & Manganelli, 1990). Other species identified were: S. saprophyticus (6), S. chromogenes (4), S. hominis (1), S. warneri (1), S. lugdunensis (1) and S. epidermidis (1). To our knowledge, the occurrence of S. saprophyticus and S. warneri in dry sausage is not often mentioned. Samelis, Metaxopoulos, Vlassi and Pappa (1998) found that S. saprophyticus represented 72% of their isolated from traditional Greek salami, S. saprophyticus and S. warneri probably come from the skin of the pork. The clear predominance of staphylococci over micrococci has been reported in almost all the recent works on the characterisation of microbial flora in meat products (Pirone & Manganelli, 1990; Samelis et al., 1998); these results can be explained by the higher resistance to NaCl and particularly the lower oxygen demands of staphylococci. Although no coagulase-positive isolate was found, two strains were classified as S. aureus according to API Staph. Moreover, S. warneri, S. lugdunensis, S. chromogenes, S. hominis and S. epidermidis are novobiocin-sensitive species, whereas every strain isolated in this study showed uninhibited growth in the presence of this antibiotic. Only 25 staphylococcal isolates (approximately 18%) could be definitively identified at species level, 76 isolates were at least reported as actually belonging to the

Table 1

<table>
<thead>
<tr>
<th>Days of ripening</th>
<th>Rogosa agar</th>
<th>Mayeux agar</th>
<th>Mannitol salt agar</th>
<th>Kranep agar</th>
<th>Malt agar</th>
</tr>
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<tbody>
<tr>
<td>Core</td>
<td>Edge</td>
<td>Core</td>
<td>Edge</td>
<td>Core</td>
<td>Edge</td>
</tr>
<tr>
<td>0</td>
<td>3.0±0.2×10^3</td>
<td>3.0±0.2×10^3</td>
<td>&lt;10^2</td>
<td>1.9±0.1×10^2</td>
<td>1.9±0.1×10^2</td>
</tr>
<tr>
<td>2</td>
<td>6.9±0.2×10^4</td>
<td>1.0±0.6×10^4</td>
<td>1.6±0.1×10^4</td>
<td>2.0±0.1×10^1</td>
<td>2.2±0.9×10^1</td>
</tr>
<tr>
<td>7</td>
<td>8.5±0.6×10^4</td>
<td>1.4±0.1×10^4</td>
<td>3.3±0.5×10^4</td>
<td>1.4±0.1×10^4</td>
<td>4.0±0.1×10^1</td>
</tr>
<tr>
<td>14</td>
<td>8.4±0.5×10^4</td>
<td>3.0±0.2×10^4</td>
<td>1.2±0.1×10^4</td>
<td>2.3±0.1×10^1</td>
<td>6.4±0.3×10^1</td>
</tr>
<tr>
<td>23</td>
<td>2.6±0.6×10^5</td>
<td>3.2±0.7×10^5</td>
<td>5.8±0.2×10^5</td>
<td>4.0±0.5×10^4</td>
<td>1.5±0.1×10^1</td>
</tr>
<tr>
<td>41</td>
<td>5.5±0.3×10^6</td>
<td>5.5±0.3×10^6</td>
<td>1.1±0.0×10^6</td>
<td>1.1±0.0×10^4</td>
<td>0.8±0.2×10^1</td>
</tr>
</tbody>
</table>

a Each number is the mean of five different batches: standard deviation.
b Samples were separately obtained from the edge and the core of the product.
c Not detected.
genus *Staphylococcus*, while 35 isolates could not be clearly identified.

To attempt to achieve additional identifications and improve characterisation, cluster analysis was subsequently performed with the 20 characters of the API Staph strip.

Simplified dendrograms for each sampling time during ripening are depicted in Figs. 1–5. The cophenetic correlation value was 0.70, showing that the dendrograms depicted relationships between strains reasonably well. There is the evident formation of a main cluster that often included the species *S. xylosus* or *S. saprophyticus*. The reference profile of *S. sciuri*, *S. cohnii*, *S. kloosii* and *S. lentus* usually formed a separate cluster.

In Fig. 1, the largest cluster included the reference strain of *S. saprophyticus*, staphylococci identified at genus level and one strain identified from API Staph software as *S. epidermidis*, despite its a novobiocin-resistance. Like the reference profile of *S. saprophyticus*, each strain in the cluster produced urease and was unable to develop acid from raffinose, xylose and α-methyl-d-glucoside; on the other hand very few strains were able to ferment mannitol and N-acetyl-glucosamine.

In the same dendrogram six strains clustered with the reference strain of *S. xylosus* at 72.1% of similarity. When the substrate fermentation patterns of the wild strains were compared with those described by the API Staph identification system for *S. xylosus*, it was evident that there were significant differences: no strain produced acetoin, while *S. xylosus* is described as positive to the Voges–Proskauer test in 67% of cases. Moreover a significant number of strains was unable to ferment maltose, lactose and to hydrolyse arginine. The unidentified strain S7 that clustered with the reference strain of *S. xylosus* at 85.7% is unable to ferment glucose, but its biochemical properties fits fairly well with that of *S. xylosus*. *S. xylosus* strains, unable to produce acid from glucose have been isolated by Rodríguez, Nuñez, Córdoba, Sanabria, Bermúdez and Asensio (1994) in Iberian ham throughout the ripening process.

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In the dendrogram of Fig. 2, a large majority of strains (22), isolated after 2 days of ripening, were grouped in a single main cluster at 71.5% similarity with the reference strain of *S. xylosus*. This cluster also
included strains classified as *S. aureus* and *S. saprophyticus* by the API Staph system. Like *S. xylosus*, strains belonging to this cluster were all able to ferment lactose, trehalose, saccharose and to produce urease. No strain utilised xylitol, D-melibiose, raffinose or a-methyl-D-glucoside. By contrast, few strains produced acid from D-mannose and N-acetyl-glucosamine, reduced nitrate and formed acetoin, while a high proportion of strains (70%) were able to hydrolyse arginine. According to the API Staph identification system the strains S62 and S53 would be assigned to the species *S. xylosus* or any known species, respectively; their biochemical profiles are absolutely identical, the sole difference being once again the inability of S53 to use glucose. The rest of the strains isolated at 7 days of ripening were roughly divided into two clusters without including any reference strain.

The dendrogram reported in Fig. 4 is characterised by the presence of a main cluster containing 22 isolates and the reference strain of *S. saprophyticus*. Strains in the cluster differed from the reference profile for the same characters reported for the *S. saprophyticus* cluster described in Fig. 1. The smallest cluster in this dendrogram consisted of only four strains joining *S. xylosus*.
None of these strains fermented trehalose or N-acetyl-
glucosamine, in contrast with the reference culture that 
was positive in both these tests. Moreover, they shared 
the same behaviour described for other clusters includ-
ing *S. xylosus*: no strain utilised sodium pyruvate but all 
were positive for arginine dihydrolase.

The last dendrogram (Fig. 5), including strains iso-
lated at the end of commercial ripening, shows a large 
cluster of atypical strains (23) not well related to the 
reference species included in this analysis. Strains 
grouped in this cluster showed a high level of similarity: 
all were able to ferment maltose, lactose and sucrose, 
but not xylitol, rafinose, mannose or \( \alpha \)-methyl-
gluco-
samide; only a minority of strains reduced nitrate, hydro-
lysed arginine or produced acetoin, and fewer strains 
were capable of using melibiose, N-acetyl-glucosamine, 
xylose or producing urease.

In conclusion, clustering carried out with a reduced 
number of tests helped to assign unidentified or mis-
identified strains, as well as strains identified just at 
genus level, by including them in defined groups. Cluster 
analysis corroborated the existence of a fairly 
homogeneous group of novobiocin-resistant, coagulase-
negative staphylococci, that reasonably represents an 
intermediate variety of *S. xylosus* and *S. saprophyticus*. 
These two species are actually quite similar phenotypi-
cally and genotypically and according to Kloos (1990) 
could be assigned, on the basis of their genomic rela-
tionships, to the single species *Staphylococcus sapro-
phyticus*. When data relative to all the staphylococcal 
strains isolated during this work were collected in a sin-
gle dendrogram, 116 strains (82% of the total amount) 
conveyed in a single cluster at 94.4% similarity with 
*S. saprophyticus* and 12 strains clustered at 88.9% with 
*S. xylosus* (data not shown). In fact, among the 136 iso-
lates, only 18 with comparable metabolic profile could 
be isolated from samples corresponding to all the dif-
ferent phases of salami ripening, which were just refer-
able to *Staphylococcus* spp. by the API Staph, while 
strains clearly showing the reference profiles of *S.
saprophyticus or S. xylosus could be more occasionally isolated. The role of S. saprophyticus in traditional meat fermentations needs to be clarified. This organism, probably acquired from pork skin, is widely present among staphylococci in fermented sausages (Samelis et al., 1998). So far, it has been regarded as a contaminating organism with opportunistic pathogenicity, unlike its close relative S. xylosus included in commercial starter culture preparations. At present, the effect of S. saprophyticus on taste and aroma of Naples-type salami is being investigated in our laboratory, in order to evaluate the suitability of carefully selected strains to be used as starter cultures.


References


