

CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM AN ITALIAN DRY FERMENTED SAUSAGE

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Abstract

Studies were carried out on the microbiological changes which occurred during the ripening of an Italian dry fermented sausage, manufactured without starter cultures. Lactic acid bacteria were studied using the commercially available system API 50 CH for the characterization of carbohydrate fermentation patterns. The results obtained indicated that lactobacilli constituted the predominant flora throughout the ripening period. Characterisation of 90 lactic isolates indicated that microflora was dominated by facultative heterofermentative lactobacilli: approximately 51,1% of them could be identified as *Lactobacillus sakei*. The API 50 CH identification system did not prove to be reliable: 10% of the isolates remained unidentified and software response for *L. sakei* subs. *sakei* reference strain was *Lactococcus lactis* subsp. *lactis*. In this way, this species could not be positively identified solely by means of the match index, but must be associated with other phenotypic or genotypic characters.

Riassunto

È stato condotto uno studio sulle modificazioni microbiologiche che si verificano durante la maturazione di un salame italiano prodotto senza colture starter. L'evoluzione dei batteri lattici è stata seguita impiegando il sistema API 50 CH per la caratterizzazione dei pattern di fermentazione dei carboidrati. I risultati ottenuti indicano che i lattobacilli costituiscono la parte principale della flora del salame durante la maturazione. La caratterizzazione dei 90 ceppi di batteri lattici isolati mostra che la microflora è dominata dai lattobacilli eterofermentanti facoltativi: il 51,1% degli isolati, infatti, è stato identificato come *Lactobacillus sakei*. Il sistema di identificazione API 50 CH non è, però, completamente affidabile: il 10% degli isolati è rimasto non identificato, inoltre la risposta del software di identificazione per *L. sakei* è stato *Lactococcus lactis* subsp. *lactis*. In questo modo, questa specie non può essere identificata impiegando solamente l'indice di corrispondenza, ma deve essere associato con altre caratteristiche fenotipiche o genotipiche.

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1. Introduction

'Filzetta' is a popular dry fermented sausage, produced in Italy. It is made of minced lean pork, mixed with fat, spices and different additives, stuffed into natural casing and ripened for two months. The shelf-life is determined by a low water activity (aw) and a pH level close to 5.0 which prevents growth of pathogenic microorganisms. Different microorganisms, derived from raw materials and the environment, naturally contaminate dry sausage mixtures. Among them, lactic acid bacteria (LAB) are responsible for the main event during dry sausage ripening. Lactic acid bacteria ferment the sugars to acid and thus lower the pH, improving the texture of the products, providing prolonged stability against the proliferation of food pathogens and producing some aromatic compounds (Montel et al., 1998; Ordóñez et al., 1999). Lactobacilli are the predominant part of the lactic acid microflora of meat and meat products (Schillinger & Lücke, 1987).

Although the use of starter cultures is increasing in large scale factories, small manufacturers are continuing to use the traditional method without starter cultures. In this way, the LAB present in these products come from the meat itself or the environment, representing the so-called 'house flora'. Different studies on the identification and characterisation of these 'house flora' have been carried out on different types of products (Schillinger and Lücke, 1987; Santos et al., 1998). Little information is available on the isolation and characterisation of LAB from Italian dry fermented sausages (Vergara et al., 1999; Parente et al., 2001; Greco et al., 2005).

The aim of this study was the isolation and characterisation of the LAB present in the traditional 'filzetta' manufactured in Northern Italy. Samples were taken at three different stages of ripening to determine the species present and the succession of these species during the process.

2. Materials and Methods

2.1 Food samples, sampling, strains and culture conditions

Sausages were produced by a local artisanal plant in Langhirano (Parma, Italy), according to the traditional method (without the use of starter cultures and with a natural fermentation step at close to +10°C for 30 days after filling). The basic initial sausage mixture included: lean pork, lard, NaCl, black pepper, lactose, garlic, and spices. Samples were taken immediately after filling, 30 days after ripening (+7°C and 70% relative humidity), and at the end of seasoning (30 days, +15°C and 75% relative humidity). For microbiological analysis, the natural casing was aseptically removed; pieces of each sample were cut using sterile knives and homogenised with nine part of sterile Ringer's solution (Oxoid, UK) in a stomacher (AES Laboratoire).

The appropriate dilutions were plated on the appropriate medium.

Enterobacteriaceae were enumerated on Violet Red Bile Glucose (VRBGA) agar (Oxoid, Italy), incubated at +32°C for 24-48h, enterococci on Kanamycin Aesculin Azide agar (Oxoid, Italy), incubated at +35°C for 24h. Malt Extract agar

(Oxoid, Italy) and *Pseudomonas* CFC agar Cetrimide, Fucidine and Cephaloridine (Oxoid, Italy), were used to enumerate yeast and mould and *Pseudomonas* spp. respectively. Yeast and mould were incubated at +25-30°C for 2-5 days respectively; *Pseudomonas* spp. were incubated at +25°C for 48h.

LAB were enumerated on MRS agar (Oxoid, Italy), plates were anaerobically incubated in AnaeroGen Compact (Oxoid, Italy) at +30°C for 48h. Coagulase-positive staphylococci and *Staphylococci/Kocuria* were plated on Baird Parker with RPF (Rabbit Plasma Fibrinogen) (Biomérieux, Italy) and incubated at +37°C for 24-48h.

The determination of pH was carried out by homogenisation with distilled water (1/10 weight/volume). Measurement was carried out with a pH meter (PHM 82 STANDARD Phmeter, Radiometer, Copenhagen) with a combined electrode (Crison 52-32). Water activity (aw) was measured using a water activity meter (Aqualab, Decagon devices, USA).

2.2 Identification

Individual isolates from countable MRS agar plates were randomly-picked, representatives from all morphologically distinct colonies, and were sub-cultured and purified using MRS agar five times. Pure strains, as judged by microscopic observations for homogeneity of cellular morphology, were maintained in MRS broth (Oxoid), plus 40% glycerol (Carlo Erba, Italy) at -20°C. Eleven type strains of lactic acid bacteria were sub-cultured on MRS agar, along with the 90 food isolates. Bacterial isolates were tested for Gram reaction, catalase production and cell morphology.

The type strains used in this study and their sources are illustrated in Table 1.

2.3 API 50 CH and API Staph fermentation assay

Overnight cultures of lactobacilli isolates and type strains grown in 15 ml MRS broth (Oxoid) at 30°C were centrifuged at 9800 x *g* for 10 min. The pellets were suspended in API 50 CHL medium (API system, BioMérieux, France). Using sterile PSIPette, homogenized suspension of the cells in the medium, with subsequent vortex mixing, were transferred into each of the 50 wells of the API 50 CH strips. All wells were overlaid with sterile paraffin oil to effect anaerobiosis. Strips were moistened and covered as recommended by the manufacturer and incubated at 30°C. Changes in colour from violet were monitored after 1, 2 and 7 days. Results for each strip were graded from 1 to 5, where 5 denoted a complete change to yellow and 1 no change at all. The first strip served as a control well. Aesculin hydrolysis (revealed by a change to a darker colour or black) was represented by a positive sign (+), while a negative sign (-) represented no change. Grades of fermentation results 3, 4 and 5 were interpreted as positive whereas 1 and 2 were negative (-). The resulting patterns were analysed with API WEB (BioMerieux). This computer program is commercially available and discriminates between species on the basis of a pattern-matching principle, but it does not take the basic morphological and physiological issues into account.

3. Results and Discussion

Figure 1 shows the changes in pH and aw during the ripening of the sausages. At the end of the fermentation, a slight decrease in the pH was observed, whereas in subsequent ripening stages, there was an increase. On the contrary, aw decreased during the entire maturation phase.

Changes in microbial contents during ripening of dry sausages are represented in table 2. Numbers of lactobacilli increased from $2.60 \log_{10}$ CFU/g in the stuffed batter to $6.89 \log_{10}$ CFU/g in the final product, showing that this group rapidly dominates the total microflora. These results are in agreement with those reported for other traditional fermented sausages produced in Italy (Coppola et al., 2000). During the first period of drying, a pH decrease from 5.70 to 5.15 (figure 1), parallel to a maximal increase in LAB counts (table 2) was observed. As it is well known, the final product is characterized by the colonization on the sausages surface of moulds, which oxidise lactate with consequent increase of pH. In all samples, *Micrococcaceae* showed a lower population of LAB (about 1 to 4 log units). The behaviour of *Micrococcaceae* reflected their poor competitiveness in presence of actively growing aciduric bacteria (Samelis et al., 1998). Coagulase negative staphylococci counts were lower than LAB counts, and they did not increase beyond $2.23 \log_{10}$ CFU/g. The low pH value reduces the *Enterobacteriaceae* counts, which became undetectable both in the semi-ripened sample and in the final product. The behaviour of *Pseudomonas* spp. is similar: their number decreases until $3.11 \log_{10}$ CFU/g in the final product, probably due to the lower aw value. Enterococci were not detectable at the start, but they were present in the semi-ripened sample and increased slightly up to 3 logs. Yeasts and moulds were detected only in the ripened sample.

These results confirm the predominance of LAB in dry fermented sausages, as previously reported by many authors (Coppola et al., 2000; Lizaso et al., 1999; Samelis et al., 1994). Ninety presumptive LAB were randomly isolated from MRS agar plates. All the isolates were Gram-positive and catalase-negative bacteria. Most strains (61.1%) were facultative heterofermentative lactobacilli, confirming that meat is one of the main habitats for this group of LAB; the remaining were obligate heterofermenter lactobacilli (17.8%). The evolution of the different species isolated during the ripening process is shown in table 3. *Lb. sakei* is confirmed as main species in meat product, as reported by other researchers (Santos et al., 1998; Greco et al., 2005). *L. fermentum*, an obligate heterofermenter, decreased during ripening process, whilst *L. brevis*, which have frequently been described in other Italian dry fermented products (Coppola et al., 2000), were rarely isolated. *Leuconostocs* (9 isolates) were the major group of coccal-shaped lactic acid bacteria isolated, and they slightly increased during the ripening. They have been widely reported as occurring on meat or in meat products but, as found in the present study, they are usually not a dominant component of the flora (Sanz et al., 1988). The isolated strains were classified as *Leuconostoc mesenteroides*.

The commercially available system used in this study for the identification of LAB, based on physiological test associated with carbohydrate fermentation patterns was not able to allocate 19 strains out of 90 isolates (21.1%). Moreover, the identification procedure, based on API WEB, resulted in an incorrect identification

of reference strain *L. sakei* subs. *sakei* DSM 20017^T because this pattern was not present in the database. The software' response for the cited reference strain was *Lactococcus lactis* subsp. *lactis*. In this way, this species could not be positively identified solely by means of the match index, but must be associated with other phenotypic or genotypic characters. In particular, microscopic observation of cellular morphology together with the scheme proposed by Schillinger & Lücke (1987) allowed the correct identification of both reference strain *L. sakei* and the field strains obtained from sausages. This aspect is particularly important, considering that *L. sakei* widely represent the dominant species of lactic acid bacteria in the investigated sausages, in accordance with the results obtained on similar salami produced in Italy (Coppola et al., 1998 and 2000; Andrighetto et al., 2001).

Characterizing LAB as a naturally occurring microflora in sausages is an important step which could help to identify and protect the product. Further research is needed to characterize the properties which have an influence on the sensorial characteristics of the product, such as the determination of lactic acid production, proteolytic and lipolytic activity, and the production of inhibitory substances. Phenotypic characterization based on sugar fermentation pattern may not always provide sufficient basis for the reliable identification of LAB, as reported by other researchers (Nigatu, 2000; Corsetti et al., 2001; Muyana et al., 2003) although it is a useful tool for presumptive classification. Commercially available systems based on carbohydrate fermentation should be combined with conventional phenotypic properties other than carbohydrate fermentation or with genotypic techniques.

Acknowledgment

The research is part of the project "Assessment and improvement of safety of traditional dry sausages from producers to consumers" (N° QLK1-CT-2002-02240), funded by European Union, in the framework of the programme "Quality of Life and Management of Living Resources" in the Fifth Framework Programme.

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Table 1: Reference strains of lactic acid bacteria and their source.

Strain	DSM number
<i>Lactobacillus curvatus</i>	20010
<i>Lactobacillus sakei</i> subsp. <i>sakei</i>	20017 ^T
<i>Lactobacillus fermentum</i>	20052 ^T
<i>Lactobacillus brevis</i>	20054 ^T
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	20072 ^T
<i>Lactobacillus plantarum</i>	20174 ^T
<i>Pediococcus acidilactici</i>	20284 ^T
<i>Pediococcus pentosaceus</i>	20336 ^T
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	20343 ^T
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>	20481 ^T
<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>	20484 ^T

Table 2: Microbial changes during ripening of dry sausages (\log_{10} CFU/g).

Samples	Enterobacteriaceae	Staph./ Kocuria	Staph. Coag +	Pseudomonas	LAB	Enterococci	Yeast/ Mould
z	3.18	1.60	<1	6.48	2.60	<1	<1
m	<1	1.17	<1	3.87	5.85	2.30	<1
f	<1	2.23	<1	3.11	6.89	3.08	2.60

z: stuffed batter

m: product after drying

f: ripened sample

Table 3: Distribution of lactic acid bacteria during ripening.

Species	Number of isolates at			Total isolates
	z	m	f	
Lactic acid bacteria	30	30	30	90
<i>Lb. sakei</i>	14	17	15	46
<i>Lb. fermentum</i>	7	4	3	14
<i>Lb. brevis</i>	1	0	1	2
<i>Ln. mesenteroides</i>	2	3	4	9
Not identified	6	6	7	19

z: stuffed batter

m: product after drying

f: ripened sample

Figure 1: Changes in water activity and pH during ripening.

