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Characterisation of *Micrococcaceae* isolated from different varieties of chorizo

Mónica García-Varona, Eva M. Santos, Isabel Jaime, Jordi Rovira*

Departamento de Biotecnología y Ciencia de los Alimentos, Facultad de Ciencias, Universidad de Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos, Spain

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Abstract

A total of 426 strains of *Micrococcaceae* bacteria isolated from chorizo (a traditional Spanish fermented sausage) were identified. The chorizos were sampled from three regions of Castilla and León in Spain: Burgos, Segovia and Salamanca. Two factories were chosen in each region and the samples were taken at three stages of ripening. *Staphylococcus xylosus* was the most predominant species isolated (95%). Twelve strain types of *S. xylosus* were established according to their fermentation patterns, and two of them, *S. xylosus* type 2 and *S. xylosus* type 5, made up the majority of the strains of *S. xylosus* isolated (27 and 52%). Production of acetoin, nitrate reductase, urease activity, proteolytic and lipolytic activity were determined for all isolates. The percentage of strains of *S. xylosus* producing acetoin depends on the manufacturing location. In general, the proteolytic and lipolytic activities of the *S. xylosus* isolated from chorizo from Castilla and León were low and moderate; 97% of the strains showed nitrate reductase and urease activity. According to our results and to previous investigations, it seems that *S. xylosus* type 5, showing nitrate reductase and urease activity, low–moderate proteolytic and lipolytic activities and not producing acetoin would be suitable as a starter culture. Of the strains isolated in this study, 38% comply with these requirements.

Keywords: Chorizo; *Micrococcaceae*; Dry fermented sausage; Starter culture

1. Introduction

Chorizo is the most popular dry fermented sausage in Spain, with a total annual production of more than 80,000 tons. It is made of minced meat, mixed with fat, spices and different additives, stuffed into natural or artificial casing and ripened at low temperature (24–12°C) and decreasing relative humidity (from 90 to 65%).

This product has a long shelf life, which is determined by the low water activity (a_w) and a pH close to 5.0. Lactic acid bacteria produce lactic acid, which is responsible for the low pH. The pH decrease leads to coagulation of the soluble proteins giving the desired texture and the lactic acid

*Corresponding author. Tel.: +34-947-258-814; fax: +34-947-258-831.

E-mail address: jrovira@ubu.es (J. Rovira)

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bacteria are also responsible for proteolytic and lipolytic reactions (González-Fernández et al., 1997).

It is generally accepted that micrococci participate in desirable reactions occurring during the ripening of dry fermented sausages, such as colour stabilisation, decomposition of peroxides (Schleifer, 1986; Barrière et al., 1998), proteolysis and lipolysis (Cantoni et al., 1967; Sajber et al., 1971; Debevere et al., 1976; Schleifer, 1986; Selgas et al., 1988; Miralles et al., 1996). Some studies clearly show that the aroma of fermented meat products can be modulated by the presence of *Staphylococcus* spp. (Berdagué et al., 1993; Stahnke, 1994). The relative importance of *Micrococcaceae* and lactic acid bacteria in aroma development has recently been reviewed (Montel et al., 1998) and it is reported that *S. xylosus* and *S. carnosus* are able to produce esters and other important aromatic components from amino acids. These strains also prevent the formation of off-flavours, mainly by their high nitrate reductase and catalase activities. It is remarkable that, contrary to earlier concepts, a strong salami odour can be obtained by using weakly lipolytic and proteolytic strains of the two species (Montel et al., 1996). Considering such biochemical properties, *S. xylosus* is considered a suitable starter, however it should be demonstrated that enterotoxins are not produced (Rodríguez et al., 1996).

Although the tendency in large-scale factories is the addition of starter cultures, small manufacturers are continuing to use the traditional method without adding starter cultures. In this way, the *Micrococcaceae* present in these products come from the meat itself or the environment and constitute a part of the so-called 'house flora'. Only a few authors have published information concerning the different species of *Micrococcaceae* occurring in chorizo (Daporta Padín, 1988; Selgas et al., 1988), and the evolution of *Micrococcaceae* during ripening has largely been ignored.

The aim of this study was to isolate and characterise the *Micrococcaceae* present in traditional chorizo from three different regions of Castilla and León, Spain, in order to select a suitable strain to be used as a starter culture, continuing the work initiated by Santos et al. (1998) with lactic acid bacteria.

2. Material and methods

2.1. Sampling

The chorizos studied in this project came from three regions of Castilla and León: Burgos, Segovia and Salamanca. The chorizos from Burgos (35 mm in diameter) were fermented for 24–48 h at 23°C and 90% relative humidity (RH) and then ripened at 12–18°C for 17–18 days at lower RH (65–80%). The chorizos from Segovia (45 mm in diameter) were fermented for 30 h at 25°C and 90% RH and then ripened for 20 days at 14–18°C and 70% RH. Finally, the chorizos from the Salamanca region (80 mm in diameter) were ripened for 2–3 months at 10–12°C and 80–90% RH without an initial fermentation period at higher temperatures. Two manufacturers from each region were chosen, and two series of samples were taken at three different stages of ripening: the early minced meat stage, the semi-ripened stage and the ripened stage. The factories chosen in Burgos were labelled 1 and 2, those in Segovia 3 and 4 and those in Salamanca 5 and 6.

Sixteen representative strains from each sample were selected for characterisation from high dilution Mannitol Salt Agar (MSA) plates (Cultimed, Barcelona, Spain). They were purified by streaking onto MSA, and kept in a broth suitable for *Micrococcaceae* (MB) with the following composition: peptone casein, 10 g (Cultimed); meat extract, 1 g (Biokar Diagnostics, Beauvais, France); glucose, 5 g (Panreac, Barcelona, Spain); NaCl, 75 g (Probus, Barcelona, Spain); phosphate dibasic, 3 g (Probus); distilled water, 1 l. Only Gram- and catalase-positive cocci, a total of 426 strains from the 496 isolated, were further identified. The strains were preserved frozen at −80°C in MB with 20% (w/v) glycerol (Panreac).

2.2. Strain characterisation

The distinction between the genera *Staphylococcus* and *Kocuria*, formerly *Micrococcus* (Stackebrandt et al., 1995), was studied by means of erythromycin and furazolidone agar plates and a lysostaphin test (Kloos et al., 1974; von Rheinbaben and Hadlok, 1981). Fermentation of carbohydrates and the presence of the enzymes nitrate reductase and urease

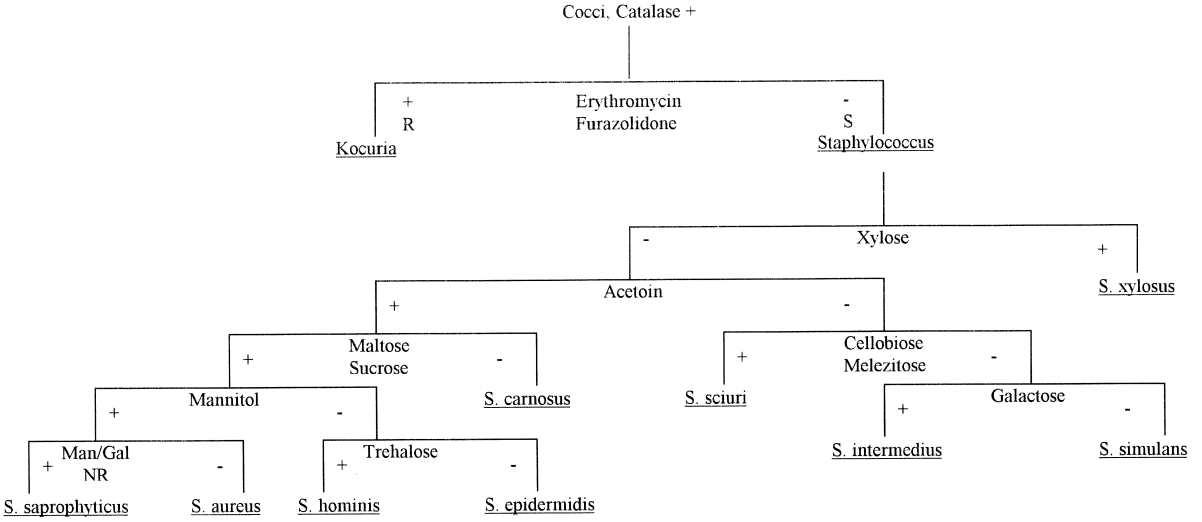

Fig. 1. Scheme for rapid identification of *Micrococacceae* isolated from chorizo. Man, mannose; Gal, galactose; NR, nitrate reductase; R, resistant; S, sensible; +, positive reaction; −, negative reaction.

were determined using a modification of the miniplate method described by Jayne-Williams (1976), but with Methyl Red as indicator. Production of H_2O_2 was determined as described by Whittenbury (1964). Production of acetoin was determined by the Voges-Proskauer test (Reuter, 1970). Proteolytic activity was studied according to Torriani et al. (1994) in PCA plates (Biokar Diagnostics), with 10% (v/v) sterile skimmed milk (Oxoid, Basingstoke, UK) added. Lipolytic activity was studied in tributyrin agar plates (Oxoid). Halos formed by proteolytic and lipolytic strains were measured in millimetres from the edge of the colony.

The classification of the different *Micrococcaceae* species isolated was performed by means of a dichotomic key defined according to the sugar fermentation pattern, the production of acetoin and the presence of nitrate reductase, following the current bibliography, as shown in Fig. 1.

3. Results and discussion

All of the 426 strains identified in this study belonged to the genus *Staphylococcus*. This result is in agreement with Hugas and Roca (1997), who only found *Staphylococcus* among the flora of several fermented sausages from Catalonia. Other authors have isolated much higher percentages of *Staphylococcus* than *Kocuria* when studying the flora from Spanish and Italian fermented sausages (Daporta Padín, 1988; Pirone and Manganelli, 1990; Comi et al., 1992). The different species isolated are shown in Table 1. *Staphylococcus hominis*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis* and *Staphylococcus xylosus* have already been isolated from chorizo by Daporta Padín (1988) and *Staphylococcus aureus* has already been found in Italian and French sausages (Delarras and Laban, 1981; Simonetti and Cantoni, 1983). However, *S. intermedius* has seldom been reported in meat products (Arkoudelos et al., 1997).

Staphylococcus xylosus was the predominant species (94.6%) from all isolated strains. According

Table 1
Strains of *Staphylococcus* spp. isolated. Percentages are indicated in parentheses

Species	No. of strains (%)
S. xylosus	403 (94.6)
S. intermedius	9 (2.1)
S. saprophyticus	6 (1.4)
S. hominis	5 (1.1)
S. epidermidis	2 (0.5)
S. aureus	1 (0.2)

to the fermentation carbohydrates pattern of each *S. xylosus* strain, 12 strain types were established. Strains type 2 and type 5 constitute the majority of the strains of *S. xylosus* isolated from Castilla y León (Table 2).

Table 3 shows the distribution of the 12 strain types for the six factories. It was observed that each factory showed a different distribution of strain types. A specific strain type from each factory was also found (strain types 12, 7, 11, 10, 8 and 9 from

Table 2
Fermentation pattern, urease nitrate reductase, and acetoin production by different *S. xylosus* types[a]

	Type											
	1	2	3	4	5	6	7	8	9	10	11	12
No. of strains	11	110	26	23	210	14	1	1	1	2	1	3
% of each type	2.7	27.3	6.5	5.7	52.1	3.5	0.25	0.25	0.25	0.5	0.25	0.7
Arabinose	+	+	+	−	+	+	+	+	−	+	+	−
Cellobiose	−	−	+	−	−	−	+	−	+	−	+	−
Fructose	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	−	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	−	−	−	+	+	+	+
Mannitol	+	−	+	+	+	+	+	−	+	−	−	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+
Melobiose	−	−	−	−	−	−	−	−	−	−	−	−
Melezitose	−	−	−	−	−	−	−	−	−	−	−	−
Rafinose	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	−	+	+	+	+	+	−	+	+	−	+	−
Xylose	+	+	+	+	+	+	+	+	+	+	+	+
Urease	100	98.2	92.3	100	97.1	92.8	100	100	100	100	100	100
Nitrate reductase	100	95.4	92.3	100	97.6	100	100	100	100	100	100	100
Acetoin production	0	32.7	23	60.8	14.3	7.1	0	0	50	0	0	0

[a] Values in percentages. +, Positive reaction; −, negative reaction.

Table 3
Percentage of the different *S. xylosus* types at each stage of ripening[a]

S. xylosus	Burgos						Segovia						Salamanca					
	Factory 1			Factory 2			Factory 3			Factory 4			Factory 5			Factory 6		
	M	S	R	M	S	R	M	S	R	M	S	R	M	S	R	M	S	R
Type 1	22.2	–	–	–	4.4	7	–	–	–	–	–	10	–	–	–	–	–	–
Type 2	7.4	–	35.7	6.7	13	–	88.5	63	37	23	38.8	15	23.1	31.3	34.6	13	20	25
Type 3	–	–	–	26.7	–	19	–	–	3	–	3.2	5	15.4	–	3.8	13	4	17
Type 4	–	–	–	3.3	–	–	–	3	1	–	–	–	–	31.3	19.2	22	–	25
Type 5	59.3	100	50	60	69.6	67	7.7	34	50	61.5	54.8	65	38.5	31.3	38.5	52	72	25
Type 6	7.4	–	–	–	13	7	–	–	–	15.5	–	–	23.1	6.3	–	–	4	–
Type 7	–	–	–	3.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Type 8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.8	–	–	–
Type 9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8
Type 10	–	–	–	–	–	–	–	–	–	–	3.2	5	–	–	–	–	–	–
Type 11	–	–	–	–	–	–	3.8	–	–	–	–	–	–	–	–	–	–	–
Type 12	3.7	–	14.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] M, minced meat; S, semi-ripened chorizo; R, ripened chorizo.

factories 1 to 6, respectively). However, there are similarities between the factories of Burgos and Segovia. As can be seen from Table 3, in the four factories from these regions, strain type 5 occurs with a higher frequency, contrary to the factories from Salamanca where this strain type is not predominant. In addition, 52.2% of the strains not belonging to *S. xylosus* were isolated from the chorizos of Salamanca. As mentioned before, Santos et al. (1998) also isolated lactic acid bacteria (LAB) from the same chorizos. It is remarkable that they also found a broader variety of LAB types in the factories from Salamanca. Salamanca is the only region that uses meat from Iberian pigs. This could be the explanation for the broader variety of 'house flora' found in chorizos from this region.

In some studies nitrate reductase is the first criterion in the selection of strains to be used as a starter culture. The nitrate reductase activity does not only control the amount of residual nitrite. The reduction of nitrate is also related to the development of the appropriate quality of the product, since it prevents off-flavours (Miralles et al., 1996; Montel et al., 1996). As can be seen from Table 3, the majority of the strains isolated show nitrate reductase activity (97%).

The percentages of strains able to produce acetoin varied between regions. Among the strains isolated from Burgos, none were able to produce acetoin. The highest percentage of strains able to produce acetoin was found among the strains isolated from Segovia (35%) followed by Salamanca (31.3%). In general, it was observed that, for the strains isolated from minced meat, the percentage of strains able to produce acetoin was lower in those isolated from the semi-ripened meat, and the highest percentage was found among strains isolated from the ripened meat. Axelsson (1998) found that, in lactic acid bacteria isolated from fermented sausages, the production of acetoin is stimulated by low pH and low sugar concentration, conditions that could be found at the end of the ripening process. Some fermented products inoculated with strains able to produce acetoin have been characterised as having a dairy-product odour (Montel et al., 1996).

Four groups were established in relation to the

Table 4
Percentage of each proteolytic and lipolytic group at each stage of ripening in Burgos (factories 1 and 2), Segovia (factories 3 and 4) and Salamanca (factories 5 and 6)

	Stage of ripening	P1[a]	P2	P3	P4	L1	L2	L3	L4
Factory 1	M[b]	77.8	22.2	–	–	47.7	33.3	19	–
	S	75	25	–	–	50	37.5	12.5	–
	R	83.4	16.6	–	–	36.4	54.6	9	–
Factory 2	M	100	–	–	–	37.5	41.6	16.6	4.3
	S	100	–	–	–	13.3	66.7	20	–
	R	57.1	28.6	14.3	–	63.6	18.2	18.2	–
Factory 3	M	50	50	–	–	61.5	38.5	–	–
	S	50	46.6	3.4	–	–	79.3	20.7	–
	R	38.5	61.5	–	–	3.6	78.7	17.7	–
Factory 4	M	100	–	–	–	26.7	40	33.3	–
	S	69.6	30.4	–	–	22.2	55.6	22.2	–
	R	36.3	36.3	18.4	9	33.3	58.4	8.3	–
Factory 5	M	100	–	–	–	77.7	22.3	–	–
	S	71.4	28.6	–	–	25	62.5	12.5	–
	R	75	25	–	–	21.7	43.5	34.8	–
Factory 6	M	91.7	8.3	–	–	31.8	68.2	–	–
	S	100	–	–	–	52.2	39.1	8.7	–
	R	50	50	–	–	27.3	72.7	–	–

[a] P1 and L1, halo 0.1–2 mm; P2 and L2, halo 2–4 mm; P3 and L3, halo 4–6 mm; P4 and L4, halo 6–8 mm.
[b] M, minced meat; S, semi-ripened chorizo; R, ripened chorizo.

screening of proteolytic and lipolytic activity. Of the *S. xylosus* strains, 68% were shown to possess proteases, most of them with low proteolytic activity (Table 4). The highest percentage of proteolytic bacteria was found among strains isolated from Segovia. In general, it appears that a slightly higher proteolytic activity is seen among strains isolated from ripened chorizo.

More than 90% of the *S. xylosus* isolated (93.7%) was shown to possess lipases, most of them with moderate lipolytic activity (Table 4). The highest percentage of lipolytic bacteria was found among strains isolated from Segovia and, in general, higher lipolytic activity was found among strains isolated from the semi-ripened and ripened chorizo.

The use of starter cultures has become essential to ensure growth of the desired flora during the manufacture of dry fermented sausage in order to obtain a product with the appropriate technological and sensory properties. The taste and aromatic components come from the ingredients, but they also result from carbohydrate, protein and lipid degradation and, above all, from amino acids, fatty acids and nucleotides (Montel et al., 1998).

Staphylococcus xylosus type 5 was isolated from all products and from all stages of the process examined, showing its importance from a microecological point of view. Only strains with similar characteristics, i.e low proteolytic and lipolytic activity, not able to produce acetoin but with nitrate reductase and urease activity, should be chosen as starter cultures.

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