PRELIMINARY CHARACTERISATION OF LACTIC ACID BACTERIA ISOLATED FROM "SARDINIAN SAUSAGES".

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Background

Sardinian sausages are typical dry fermented products made with minced lean pork and fat, salt, garlic, pepper, cloves, added with preservatives and sometimes aromatised. In these foodstuffs, the control of microbial behaviour during processing is essential to guarantee the quality of the product by promoting the development of useful microflora and the establishment of a safe habitat. Previous research described the technology (Greco M. et al., 1997), the microbiological and physical-chemical characteristics of the end-product (Mazzette R. et al, 1994 and 1995) and the evolution of these parameters during the ripening process and defined the biochemical-metabolic properties of *Micrococcaceae* (Mazzette R. et al, 1998; Greco M. et al, 1998). During ripening, a reduction in pH (down to 5.25±0.26) and the a_w value (down to 0.895±0.02) is observed as well as the progressive development of coagulase-negative *Staphylococci* (SNC) and lactic acid bacteria (LAB), which are the dominant flora. The SNC are prevalent during the first part of the process with levels up to 10^6 ufc/g. They show considerable variability in biochemical profiles with reduced proteolytic activity (4%); most strains are lipolytic (72%) and *Staph. xylosus* is the most frequently isolated (70%). LAB are prevalent during the second part of the ripening, reaching levels up to 10^8 ufc/g.

Objectives

Lactic acid bacteria are determinant for sensorial characteristics and safety in dry fermented meat products. This is due to lactic acid, bacteriocin and inhibitory metabolite production which ensures successful fermentation and the formation of a useful habitat for *Micrococcaceae* (Papa F. et al., 1993; Torriani S. et al., 1994; Jay J.M., 1997; Grazia L. et al. 1998; Leroy F. et al., 1999). Even if microbial characterisation and selection are important steps in protecting and stardardising typical products, no research on Sardinian sausage acid lactic bacteria has been carried out. Several procedures and schemes have been proposed for lactic acid bacteria presumptive identification (Schillinger et al., 1987; Tiecco G., 1992; Torriani et al., 1994); these are specific for meat products and include several species recently described (Schillinger et al., 1987). The cornerstone of these schemes is the evaluation of fermented carbohydrates by a micromethod; this has proved to be simple and effective, combining a high percentage of correlation with conventional systems and the fast reading of the commercial methods, at low cost (Font de Valdez et al., 1993). As a follow-up of previous research, the aim of the present study is to make a preliminary characterisation and to give a first assignment of lactic acid bacteria isolates from Sardinian sausages to one of the known species by the definition of some of their biochemical and metabolic properties.

Methods

A total of 112 strains of lactic acid bacteria were isolated during the ripening of 5 experimental batches of Sardinian sausage, made without using starter cultures, in MRS Agar (De Man et al., 1960) acidified with Acetic Acid Glacial to pH 5,7 (Ottaviani F., 1992) and incubated in anaerobic jars (Oxoid) for 48 h at 32°C. Colonies were tested for Gram stain, cellular morphology, catalase, oxidase and then for: a) *Gas production:* The production of CO_2 was determined in vials with 10 ml of MRS broth, adjusted to pH 7.0 and including a Durham tube, after incubation for 48 h at 37°C. The test was considered positive in the presence of bacterial growth and gas production (Ottaviani F., 1992); b) *Growth at + 15 °C:* Bacterial growth was tested in vials with 10 ml of MRS broth (pH 7.0) after incubation for 3 days at 15°C (Schillinger U. et al., 1987). Vials were considered positive in the presence of sediment or turbidity; c) *Fermentation of carbohydrates*: gas producing species were tested by using the API 50 CHL identification system (bio-Merieux). Obligate homofermentative ("*Thermobacteria*") and facultative heterofermentative ("*Streptobacteria*") strains were tested by modifying the miniaturised method suggested by Jayne-Williams (1975) and Font de Valdez et. al. (1993). The test sugars were chosen according to Schillinger and Lucke (1987). *Preparation of the inoculum:* All isolates were inoculated into vials with 5 ml of MRS broth (pH 7.0) for 12 h at 37°C. Strains were harvested and washed twice at 2000 rpm x 10 min and once at 3000 rpm x 15 min in vials with 5 ml and with 1 ml of sterile NaCl solution respectively. A bacterial suspension with a turbidity equivalent to McFarland Standard point 3 (Densimat, bio-Merieux) was then prepared in vials containing 1 ml of sterile distilled water. *Preparation of the basal medium:* MRS broth at final pH 6.8 (without glucose and meat extract, with 0.004% chlorophenol red) was freshly prepared and distributed in tubes with a 2% Seitz-filtered carbohydrate solution. *Assay performance:* The tests were performed in covered sterile miniplates with 230 µl wells. Each well was inoculated with 200 µl of the basal medium and 15 µl of the bacterial suspension. Plates were incubated in anaerobic jars for 48 h at 37°C. Only the evidence of a clear yellow colour in the wells was considered as positive result; d) *Arginine hydrolysis:* The capacity to produce ammonia from arginine was tested in vials with 10 ml of modified MRS broth (pH 7.0) without glucose and meat extract, containing 0.3 % arginine and 0.2 % sodium citrate replacing ammonium citrate. Ammonia was detected by mixing 1:1 the broth with Nessler's reagent in white porcelain dishes after incubation for 4 days at 32°C. The evidence of a clear yellow-orange colour was considered as positive result (Ottaviani F., 1992); e) *Acidifying capacity:* the acidifying capacity was tested in vials with 10 ml of MRS broth (pH 7.0). The pH value was determined with pH-meter GLP 21 CRISON after incubation for 24 h at 32°C in the presence of bacterial growth.

Results and discussions

N. 109 strains (97.3%) resulted rod-shape and clearly Gram-positive with different cell morphology, varying from long to short and very short rods. Some strains exhibited internal granulation and chain formation was common. Only 3 homofermentative strains (2.7%) showed a coccal morphology with cells disposed in tetrads and were attributed to *Pediococcus spp.* The 75% of strains were able to grow at +15°C. N.6 rod-shape strains (5.3%) produced gas from glucose and were identified as *Lactobacillus brevis* (n.4), *Lactobacillus viridescens* (n.1) and *Leuconostoc citreum* (n.1) by API 50 CHL. By following the identification keys of Schillinger and Lucke (1987) and the biochemical patterns illustrated in Bergey's Manual of Systematic Bacteriology (1986), n.60 no gas producing strains were presumptively identified; only 4 strains (6.67%) resulted unidentifiable (Table 1). *L. sake* showed 13 different patterns and was the most prevalent species with 26 isolates (43.3%); it was differentiated from *L. curvatus* with regard to the capacity of fermenting melibiose (+/-), maltose (-/+), sucrose (+/-) and trehalose (-). *L. curvatus*, n.6 isolates (13.3%), exhibited 4 different patterns with a variable response in carbohydrates fermentation and arginine hydrolysis. *L. plantarum*, n.10 isolates (16.6%), exhibited 2 different patterns for 3 and 7 strains respectively. One of these patterns was shared with other 7 isolates. However, they were arginine (+) and unable to grow at 45°C after 3 days in MRS Broth and thus were classified as *L. carnis*, considering that in this species gas formation is frequently not detectable (Schillinger and Lucke, 1987). Further 2 strains with a different profile were classified as *L. carnis*. In all, n.9 strains (15.0%) of *L. carnis* were identified. Other 5 strains (8.33%)

resulted as *L. amylophylus*, *L. casei*, *L. delbrueckii*, *L. farciminis* and *L. sharpae*. LAB in Sardinian sausages seem to be in prevalence constituted by homofermentative mesophilic rods (84.0%) confirming that meat products are one of the main habitat for this bacteria; heterofermentative rods (13.3%) and cocci (2.7%) are less common. The low incidence of heterofermentative lactobacilli and *Leuconostoc spp.* is common and characteristic of Italian dry fermented products (Torriani S., et al., 1994; Coppola S. et al., 2000). The results agree with data reported by Schillinger and Lucke (1987); only the percentage of *L. brevis* is lower, in accord with previous research performed on Italian sausages where it was rarely isolated (Cantoni C. et al., 1994). The isolates showed biochemical and metabolic activity quite close to that of reference strains and a moderate acidifying capacity: the pH ranged between 3.85 and 5.36 at the end of the test in MRS broth with an average reduction of 2.38 units (final pH 4.62). The evidence of 29 different biochemical patterns reveals great variability in the utilisation of different carbon sources. *L. sake* was confirmed as the most frequent isolated in these foodstuffs. *L. plantarum* and *L. curvatus* appear to be the other relevant species in agreement with what has been described for other Italian dry fermented products (Comi et al., 1993; Cantoni et al., 1994 - Torriani et al., 1994; Coppola S. et al., 2000) although *L. plantarum* is prevalent.

Conclusions

The high level and metabolic activity of lactobacilli show that they are essentially involved in the ripening of Sardinian sausages and in the determination of their typical properties. Their characterisation as naturally occurring flora remains an important step in protecting this typical meat product, especially considering their possible use as starter cultures. Further research is needed to better characterise their properties, such us the determination of protelytic and lipolytic activities and lactic acid production, which influence the sensorial characteristics of the product. The micromethod confirms to be simple and useful to characterise a large number of isolates, even if difficulties were found in relation to species identification with biochemical tests. A confirmation and important aid to facilitate the systematic approach to LAB would be provide by the support of genetic methods.

Table 1. LAB isolated from Sardinian sausages: metabolic characteristics and presumptive identification.

			Fermented carbohydrates																
Presumptive identification	**N° of strains**	**N° (%) of isolated species**	CEL	GAL	LAT	MLZ	MEL	MAL	MAN	RAF	RHA	RIB	SAC	TRE	XIL	FRU	SOR	+15°C	ARG
	1		+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	+	+
	3		+	+	+	-	+	+	-	-	-	+	+	+	-	+	-	+	+
	3		+	+	-	-	+	-	-	-	-	+	+	+	-	+	-	+	+
	1		+	+	-	-	-	-	-	-	-	+	+	+	-	+	-	+	+
	1		-	+	+	-	+	+	-	-	-	+	+	+	-	+	+	+	+
	1		-	+	+	-	+	+	-	-	-	+	+	+	-	+	-	+	+
L. sake	1	**26 (43.3)**	-	+	+	-	+	-	-	+	-	+	+	+	-	+	+	+	+
	1		-	+	+	-	+	-	-	-	-	+	+	+	-	+	+	+	+
	8		-	+	+	-	+	-	-	-	-	+	+	+	-	+	-	+	+
	1		+	+	-	-	-	-	-	-	-	+	+	+	-	+	-	+	+
	1		-	-	-	-	+	-	-	-	-	+	+	+	-	+	-	+	+
	3		-	+	-	-	+	-	-	-	-	+	+	+	-	+	-	+	+
	1		+	+	+	-	+	-	-	-	-	+	+	+	-	+	-	+	+
L. plantarum	3	**10 (16.6)**	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-
	7		+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-
L. carnis	7	**9 (15.0)**	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+
	2		-	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+
	2		-	+	-	-	-	+	-	-	-	+	+	-	-	+	-	+	-
L. curvatus	1	**6 (13.3)**	-	+	+	-	-	-	-	-	-	+	-	-	-	+	-	+	-
	1		-	+	+	-	-	-	-	-	-	+	+	-	-	+	-	+	+
	2		-	+	+	-	-	-	-	-	-	+	-	-	-	+	+	+	-
L. casei	1		+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-
L. farciminis	1		-	+	+	-	+	+	-	-	-	-	-	+	-	+	-	+	+
L. sharpeae	1		-	+	+	-	-	+	-	-	-	-	-	+	-	+	-	+	-
L. delbrueckii	1		-	-	-	-	-	+	-	d	-	+	-	+	-	-	-	-	n.d
L. amilophylus	1		-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	+	n.d
	1		-	+	-	-	-	+	-	-	-	+	+	+	-	+	-	-	-
not identified	1	**4 (6.67)**	+	+	-	-	+	-	+	-	-	+	+	+	-	+	+	+	+
	2		+	+	+	-	+	+	-	-	-	+	+	+	d	-	+	-	n.d

Pertinent literature

Bergey's Manual of Systematic Bacteriology (1986), William and Wilkins – Baltimore; Cantoni C. et al. (1994): *Ind. Alim.*, **23** (4), 377-379; Coppola S. et al. (2000): *Meat Science*, **56**, 321-329 ; Font de Valdez G. et al. (1993): *Microbiol. Alimen.* Nutr., **11**, 215-219; Grazia L. et al. (1998): *Ind. Alim.*, **37** (7), 852-855; Greco M. et al. (1997) *Sardegna Agricoltura*, **28**, 43-46; Greco M. et al. (1999) *Atti SISVet*, **53**, 331-332; Jay J.M. (1997): "Modern Food Microbiology", Chapman and Hall; Jayne-Williams D.J. (1975): *J. Appl. Bact.*, **38**, 305-309; Leroy F. et al. (1999): *Appl. Environ Microbio.l*, **115** (3), 974-981 ; Mazzette R. et al. (1994) *Atti SISVet*, **48**, 767-771; Mazzette R. et al. (1995) *Atti SISVet*, **49**, 451-452; Mazzette R. et al. (1998) *Atti SISVet*, **52**, 391-392; Mazzette R. et al. (1999) *Atti SISVet*, **53**, 329-330; Ottaviani. F. (1992): "L'analisi microbiologica dei prodotti lattiero-caseari", Ed. Tecniche Nuove – Milano; Papa F. et al. (1993): *Ind. Alim.*, **32** (3), 258-261; Schillinger U. et al. (1987): *Food Microbiol.*, (4), 199-208; Tiecco G. F. (1999): " Microbiologia degli Alimenti di Origine Animale", Edagricole-Bologna; Torriani S. et al. (1994): *Industria delle Conserve*, **69**, (1), 3-9.