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Sardinian fermented sheep sausage: Microbial biodiversity resource for quality improvement

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SUMMARY – With the aim of improving the quality and the value of the Sardinian sheep sausage, the microflora involved in fermentation and ripening processes of this traditional foodstuff has been studied and characterized. During the fermentation phase a prominent presence of micrococci and staphylococci has been observed. The lactic acid bacteria (LAB) that were showing a slow growth rate during the first days were found as the prevalent microflora during the ripening. The presence of coliforms was observed especially during the early fermentation phase. *Salmonella* and *Listeria* spp. were never detected. Bacterial species identified were *Lactobacillus plantarum*, *Staphylococcus xylosum*, *Staphylococcus lentus*, *Kocuria varians*, *Micrococcus* spp. while yeast strains belonged to *Trichosporon* spp. *Debaryomyces hansenii* and *Candida famata*. The technological selection of autochthonous LAB and staphylococci, which is actually ongoing, will be crucial for the development of starter cultures suitable for the quality improvement of this typical Mediterranean foodstuff.

Keywords: Sheep sausage, traditional technology, autochthonous microflora, quality.

RESUME – "Saucisse fermentée de brebis de Sardaigne : Ressources en biodiversité microbienne pour l'amélioration de la qualité". Dans le but d'améliorer la qualité et la valeur de la saucisse de brebis, produite en Sardaigne, les microflores impliquées dans la fermentation ont été étudiées et caractérisées. Pendant la phase de la fermentation une présence considérable de microcoques et staphylocoques a été observée. Les résultats ont montré que les bactéries lactiques, en dépit d'un développement initial lent, se sont avérées être la microflore prédominante pendant la maturation. La présence de coliformes a été observée pendant la phase de fermentation. Les espèces bactériennes identifiées étaient *Lactobacillus plantarum*, *Staphylococcus xylosum*, *Staphylococcus lentus*, *Kocuria varians* et *Micrococcus* spp. parmi les levures les espèces *Debaryomyces hansenii*, *Candida famata* et *Trichosporon* spp. La sélection technologique de LAB et staphylocoques autochtones sera cruciale pour le développement des cultures starter pour l'amélioration de la qualité de ce produit alimentaire méditerranéen typique.

Mots-clés : Saucisse de brebis, technologie traditionnelle, microflore autochtone, qualité.

Introduction

Different projects have been recently undertaken in Sardinia with the aim to improve the global value of sheep meat-based food products. Most of the times this was achieved through the recovery of ancient and/or traditional manufacture procedures. This was the case of the ripened sheep sausages as well as the "prosciutto" (ham) or "coppa" (air-dried whole shoulder) made from sheep meat. The production of these kind of traditional foodstuffs constitutes a valuable alternative to jobs connected with meat-based manufacture and a good opportunity to improve their value possibly providing higher remunerations.

Among the stuffed meat-based fermented products manufactured in Sardinia (Italy) ripened sheep sausage (actually a kind of salami) plays a special role because of its typical features. The production technology, that is currently very similar to the traditional one, is comparable to the manufacturing process employed for pork sausages. Few differences can be identified with respect to this latter and they are mainly related to the extra-care for sheep meat selection and preparation such as the elimination of the connective tissues as well as the fat, both responsible for unpleasant taste. The technological procedures employed for sheep sausage production are as follows.

Meat preparation: different cuts of sheep's meat from healthy animals are commonly used, in particular shoulder, sirloin and legs.

Preparation of the mix: traditionally, sheep meat is minced in small pieces (approx. 1 cm diameter) and then mixed with pork fat (cut in small pieces) that soften the typical taste of ovine meat. Variable amounts of salt (2.8-3%) and spices (mostly pepper and garlic) are added. In the traditional manufacturing process, that was made without any starter culture, the fermentation process is carried out by natural microflora associated to the ingredients. In some cases vinegar or white wine, undergoing malolactic fermentation, are used to obtain a superior quality product. The mix is left to set down overnight in a fresh and dry environment. Only rarely sugar, a microbial starter and/or other additives are added.

Stuffing sausage and ripening: after stuffing the sausages into the casings, these are smoked for up to 10-15 days and then ripened for up to 20 or 30 days.

The Sardinian sheep sausage is currently a niche foodstuff product that, even if manufactured following a traditional technology, is often inadequate and too much diversified in order to obtain a qualitatively stable product with constant features. Moreover, and most importantly, the final quality of the sheep sausage is strictly dependent on the knowledge of all those factors influencing the microbial presence and contamination in the production chain and hence the safety of the product. Ovine meat, particularly deep tissues, presents a less extent of microbial contamination with respect to other meats. Microbiological studies on sheep meat revealed the presence, almost exclusive, of staphylococci while clostridia, *Escherichia coli*, *Pseudomonas*, *Enterobacter* and *Yersinia* were not found (Newton *et al.*, 1978). More recently the presence of *Lactobacillus plantarum*, *Staphylococcus xylosus* and a significant number of yeasts and micrococci was also assessed (Mangia *et al.*, 2002). Research investigations on meat-based Sardinian foodstuffs such as sheep sausages are globally few and do not offer a global picture of the microbial and chemico-physical status of these products (Mazzette *et al.*, 1996).

Deepening our knowledge on the microbial species responsible for fermentative and ripening processes in traditional Sardinian sausages can be certainly a suitable way to improve the intrinsic value and the quality of these foodstuffs.

The aim of this work was to isolate, characterize and select from a physiological, biochemical and technological point of view the natural microflora present in sheep sausages manufactured with the traditional technology. This will finally offer the possibility to make up an autochthonous and specific starter culture for this typical Mediterranean production.

Materials and methods

Sampling procedures

Mince sheep meat and sausage samples (made from this meat) were collected at different ripening times and particularly at 1, 2, 3, 5, 7, 9, 13, 18 and 23 days of ripening. Samples were collected from a factory that ordinarily employs a traditional manufacturing technology. Meat samples were collected before and after spice and wine addition. Before starting microbiological analysis, sheep sausages were cleaned with a brush to avoid microbial contamination from the outside.

pH measurements

Potentiometric measurements of the pH were made using a pin electrode of a pH-meter (KNICK Portamess 911) inserted directly into the sample.

Microbiological analysis

Aliquots of 25 g from each sample were transferred into a sterile stomacher bag and 225 ml of peptone water (Oxoid) were added and mixed for 1.5 min in a Stomacher Lab-Blender 80 (PBI).

Further, ten-fold dilutions were made and aliquots used to inoculate the following solid media in

order to detect and quantify different microbial groups: aerobic mesophilic bacteria were determined on PCA (Oxoid) incubated at 30°C for 48-72 h; lactic acid bacteria (LAB) were counted on a double layer MRS agar (Oxoid) after incubation for 48 h at 30°C; staphylococci and micrococci were quantified on Mannitol Salt Agar (Oxoid) incubated for 48 h at 30°C; BGGB (Oxoid) was used for the enumeration of total and faecal coliforms. Numbers were determined after incubation on a double layer BGGB at 37 and 44°C for 24-48 h; yeasts and moulds were enumerated on Malt Extract Agar (Oxoid) incubated at 25°C for 48-72 h; the presence of *Salmonella* spp. was tested using a Salmonella Rapid Test (Oxoid) while the presence of *Listeria* spp. was verified after enrichment in Fraser Broth Base (Oxoid) plus supplement Fraser SR 156 (Oxoid).

Identification of microbial isolates

From each counting media approximately 10 colonies were randomly selected for further identification.

The morphology, Gram-stain and catalase activity of these isolates were preliminary tested. Gram positive and catalase-positive cocci were purified on MSA and maintained on slant tubes with the same medium at 4°C. Purified strains were classified according to Kloos *et al.* (1991). They were further subjected to the following tests to differentiate micrococci and staphylococci: sensitivity to furazolidone (Von Rheinbaben and Hadlok 1981) and lysostaphin (Schleifer and Kloos 1975). Staphylococci were assayed for coagulase activity, and finally identified using API STAPH and ID 32 STAPH Systems (bioMérieux, Marcy l'Etoile, France).

LAB identification was carried out according to Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986), Bridge and Sneath (1983) and carbohydrate fermentation patterns determined using API 50 CH and API 20 Strep test galleries (API System bioMérieux, Marcy l'Etoile, France).

Yeast identification was carried out after Kurtzman and Fell (2000).

Results and discussion

In this paper the composition and the evolution of micro-organisms associated to the Sardinian traditional fermented sheep sausages were investigated at different ripening times. Table 1 is showing the evolution of the main microbial groups investigated. Total mesophilic count (TMC) revealed higher values (approx. $3E+10$ cfu/g) at the end of ripening with respect of those reported by other authors (Mazzette *et al.*, 1996). However these numbers can be considered as common for stuffed fermented products. Micrococci and staphylococci, already present in the meat (MM), showed a very rapid increase reaching final values of $1.66E+09$ cfu/g 23 days after ripening. The significant number of these micro-organisms is related to the intrinsic features meat as well as to the artisanal technology employed, that involves an extensive manual treatment of the product. Although LAB showed a slow growth rate during the first period of ripening, after the 5th day values higher than one billion of cells per gram were recorded. Our data are globally supporting other findings stating that LAB are the dominating microflora during the ripening.

Heterofermentors LAB were found absent in all the samples analysed.

The microbiological survey showed a significant initial contamination of sheep sausage with faecal coliforms reaching important values most likely due to optimal pH and a_w . This can be considered an index of the poor hygienic conditions employed during the preparation steps as well as during the whole manufacturing process.

Yeasts were always found present and their number slowly increased during the ripening period while the number of moulds remained stable. Despite suboptimal hygienic conditions *Salmonella* and *Listeria* species were never detected.

Globally 161 microbial strains belonging to the species *Lactobacillus plantarum*, *Staphylococcus xylosum*, *Staphylococcus lentus*, *Kocuria varians*, *Micrococcus* spp., *Trichosporon* spp., *Debaryomyces hansenii* and *Candida famata* were isolated. Table 2 reports the general view of the species isolated and their evolution during the ripening.

Table 1. Evolution of autochthonous microflora in sheep sausage at different ripening times

Samples	Microbial groups								pH
	TMC (cfu/g)	Lactic acid bacteria (cfu/g)	Micrococci, staphylococci (cfu/g)	Faecal coliforms (mpn/g)	Yeasts (cfu/g)	Moulds (cfu/g)	<i>Salmonella</i>	<i>Listeria</i>	
MM	4.83E+02	3.67E+02	6.73E+03	23	1.43E+03	4.00E+01	absent	absent	5.69
MMS	7.57E+02	3.53E+02	1.05E+04	43	1.73E+03	7.33E+01	absent	absent	5.66
S 1d	8.26E+03	2.93E+02	7.87E+03	150	1.28E+03	8.00E+01	absent	absent	5.63
S 2d	4.43E+04	2.33E+02	6.13E+03	150	1.57E+03	6.67E+01	absent	absent	5.68
S 3d	3.80E+04	1.80E+02	6.33E+03	43	1.35E+03	4.67E+01	absent	absent	5.66
S 5d	8.68E+06	4.85E+06	1.34E+05	43	2.08E+03	1.07E+02	absent	absent	5.69
S 7d	2.69E+09	1.40E+09	3.45E+05	93	1.12E+04	5.33E+01	absent	absent	5.76
S 9d	7.27E+08	1.30E+09	6.69E+06	240	2.02E+05	4.67E+01	absent	absent	5.69
S 13d	1.58E+10	5.52E+09	5.12E+08	93	5.41E+05	1.33E+01	absent	absent	5.61
S 18d	3.52E+10	1.47E+10	6.10E+08	43	8.13E+05	1.33E+02	absent	absent	5.59
S 23d	3.10E+10	2.23E+10	1.66E+09	150	1.45E+06	1.47E+02	absent	absent	5.73

MM=minced meat; MMS= minced meat with spices; S=sausage; d= days of ripening; TMC=total mesophilic count.

Table 2. Number of species isolated from sheep meat and sausage at different ripening times

Isolated species	MM	MMS	Ripening times									Frequency
			S 1d	S 2d	S 3d	S 5d	S 7d	S 9d	S 13d	S 18d	S 23d	
<i>Lactobacillus plantarum</i>	1	2	1	3	1	1	8	11	12	5	1	29.00%
<i>Lactobacillus</i> spp.	0	2	4	0	1	3	3	2	1	3	1	12.42%
<i>Staphylococcus xylosus</i>	0	0	1	1	1	2	2	4	3	3	3	12.42%
<i>Staphylococcus lentus</i>	0	1	0	0	0	0	0	0	0	0	0	0.62%
<i>Staphylococcus</i> spp.	1	2	1	0	2	1	2	3	3	2	1	11.18%
<i>Micrococcus</i> spp.	0	1	1	1	2	1	2	0	0	0	0	4.97%
<i>Kocuria varians</i>	1	0	0	0	0	0	0	0	0	0	0	0.62%
<i>Debaryomyces hansenii</i>	1	1	0	1	2	3	3	4	4	7	5	19.25%
<i>Candida famata</i>	2	3	2	0	1	2	0	0	0	0	0	6.21%
<i>Trichosporon</i> spp.	1	2	1	1	1	0	0	0	0	0	0	3.73%

MM=minced meat; MMS= minced meat with spices; S=sausage; d= days of ripening.

The identified LAB were only represented by species belonging to a single genus in contrast to what found for Sardinian pork sausages (Greco *et al.*, 2005). The number of *L. plantarum* increased until the 13th day then declined in the following period. *L. plantarum* is the most represented LAB in all the stuffed foodstuffs fermented by natural microflora (Zambonelli *et al.*, 1992). Optimal growth for *L. plantarum* occurs at temperature higher than 30°C hence the fermentation rate is substantially influenced by this parameter. To this regard temperature of about 25-30°C were recorded during the first phases of drying and smoking of the sheep sausage, most likely maximizing the *L. plantarum* presence.

Strains belonging to the *Micrococcus* genus have been recovered during the first days of ripening (4.97% frequency); being these micro-organisms obligate aerobes, after this period their number decreased most likely because of a gaseous phase progressively lacking oxygen. During this phase micrococci are replaced by staphylococci that predominated during the last part of the ripening. The most abundant specie was *Staphylococcus xylosus* with the 12.42% of recovery frequency; this is confirming what reported by other studies investigating the microbiological features of Italian stuffed products (Zambonelli *et al.*, 1992; Aymerich *et al.*, 2003). *Staphylococcus lentus* and *Kocuria varians* have been recovered in low number (0.62%). The slow growth rate showed in the beginning by LAB and staphylococci put in evidence the need for adding glucose or lactose in order to support fermentative processes and inhibit undesirable organisms such as *faecal coliforms*. *Debaryomyces hansenii* has been the most recovered species (19.25%, possibly because it is more halotolerant than

other yeasts) confirming previous findings (Dalton *et al.*, 1984). Because of its invariable presence this species is commonly considered as the typical yeast of stuffed products (Entel, 1961). *Trichosporon* spp. that was found highly present in the slaughters has been recovered with a 3.73% of frequency during the first phase of ripening.

This study allowed to identify the micro-organisms characterizing fermentative and ripening processes of the Sardinian sheep sausage. This can be considered as a suitable baseline for the choice of the microbial species that could be employed to make up an autochthonous starter culture for the manufacturing process. By the way it is essential to deepen our knowledge on strain metabolism, on their ability to carry out fermentative and ripening processes as well as their suitability for quality improvement and the maintenance of the typical features of sheep sausage.

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