ISOLATION AND CHARACTERISATION OF *MICROCOCCACEAE* BACTERIA FROM TRADITIONAL " CHORIZOS" MADE IN CASTILLA-LEÓN

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Background: Fermented sausages are defined as ground meat mixed with salt and curing agents, stuffed into casings and subjected to a fermentation process in which microorganisms play a crucial role (Lücke, 1994). Many fermented foods have developed from natural fermentation, with selecting a particular flora by means of salting, temperature control or "back–slopping".
In dry fermented sausages the curing process establish selective conditions for the microorganism by lowering the pH and impeding the nitrite sensitive microorganisms. The most important microorganisms for raw-sausage production belong to the genera *Lactobacillus* and *Staphylococus*; micrococci, yeasts and moulds are also significant (Leistner, 1995).
Chorizo is the most popular dry fermented sausage in Spain with an annual production totalling more than 80000 tonnes. Santos et al (1997) identified and characterised the acid lactic bacteria from the chorizos in the Spanish region Castilla-León. Other important microorganisms are *Micrococcaceae*. It is well known that *Micrococcus* sp and *Staphylococcus* sp reduce the nitrate to nitrite and ensure the colour development. Further more, the lipolytic and proteolytic activity may contribute to the flavour of fermented meat products (Lücke and Hechelman, 1985).

Objective: The aim of this study was to identify and to characterise the bacteria *Micrococcaceae* of the same samples used in the study mentioned above for further selection of a suitable strain to be used as a starter culture.

Methods: Samples studied for this project were "chorizos" from three zones in Castilla-León. Burgos (17-18 days of ripening), Segovia (20 days of ripening) and Salamanca (2-3 months of ripening). All the samples were taken in factories that produce chorizo without adding any starter culture. Two manufactures from each zone were also chosen, and two series of samples were taken at different stages of ripening: the early minced meat stage, the semi-ripened stage and the ripened stage. The factories were labelled factories 1 and 2, those in Segovia factories 3 and 4 and those in Salamanca, factories 5 and 6.
16 strains from each sample were randomly selected from high dilution MSA plates, purified by streaking on MSA agar and kept in broth with the following composition (casein peptone, 10gr; meat extract, 1gr; glucose, 5 gr; salt 75 gr, potassium phosphate dibasic, 3gr, distilled water 1l) for their characterisation. Only cocci Gram positive and catalase positive strains were further identified.
The *Micrococacceae* were separated in *Staphylococcus* and *Micrococcus* by using furazolidone agars and erythromycin agars (von Rheinbaben et al, 1981).
Carbohydrate fermentation. It was used the commercial method API System (Biomerieux. France) and, a modification of the miniplate method described by Jayne-Williams (1975) developed for *Miccrococcacea*.
Based on blibliography it was designed a new dichotomy key which can identify one strain to the level of species according to his carbohydrate fermentation pattern and other biochemical characteristics.
Nitrate reductase and Urease. These assays were also carried out using the miniplate method. For the first one the strains were allowed to grow in a medium containing NO_3K. The presence of NO_2 was tested using the Griess reaction. The second one was determined using the Christensen broth.
Production of acetoin. It was studied by the Voges Proskauer test.
Proteolytic activity and lipolytic activity. The strains were cultured in PCA agar plates additionated of 2% of sterile skimmed milk (Torriani, 1994). For lipolytic test it was used the trybutirin agar (Oxoid). Halos produced by the protelytic and lipolytic bacteria were measured.

Results and discussion: A total of 426 bacteria could be included in the *Micrococcaceae* family and all of them belong to the genus *Staphylococcus*.
Recently it has been known that on the microbial composition of sausages, the presence of Staphylococci greatly exceeds those of Micrococci right from the beginning of fermentation (Comi et al, 1992). The result of the bacteria identification was: *S. xylosus* 94.6%, *S intermedius* 2.1%, *S. saprophyticus* 1.4%, *S. hominis* 1.1%, *S epidermis* 0.5%, *S. aureus* 0.2%. These results are very similar to the ones obtained by Hugas et al (1997) when studying the flora of fermented sausages coming from Catalonia. In fact, they found that the *Staphylococcus xylosus* was the only specie of *Micrococcaceae* present in the studied products.
According to carbohydrate fermentation pattern we could describe 12 *Staphylococcus* types, but one of them (*Staphylococcus* type 5) stands for the majority of the Staphylococci found in five of the six factories. This type 5 is able to ferment the following carbohydrates: arabinose, fructose, galactose, mannitol, mannose, xylose, glucose, maltose, lactose, sucrose, trealose and it is not able to ferment the melibiose, melezitose, raffinose, celobiose. It is able to produce nitrites from nitrates and it shows urease activity. The percentage of *Staphylococcus xylosus* type 5 able to produce acetoin is 16.2%.

Only at factory 3 the majority specie is the *Staphylococcus xylosus* type 2 that stands for the 60.6 % of all the strains isolated in the factory. This type of *Staphylococus* is able to ferment the same carbohydrates than the *Staphylococcus* type 5, it also has nitrate reductase and urease enzymes, but it is not able to ferment the mannitol. A 25.2% of the strains type 2 are able to produce acetoin.
Both *Staphylococcus* type 2 and *Staphylococcus* type 5 are found in the highest proportion in the semiripened sausage but they are the majority spices all over the process. This is an evidence of their ability to persist all over the ripening process.
An important difference between the flora isolated isolated from the rest of the factories and factory 3 is the big difference in percentage of strains able to produce acetyl methyl carbinol. This ability seems to be quite different at every zone. The lowest percentage of strains able to produce acetoin was found in Burgos followed by Salamanca. Segovia was the zone with the higher percentage. Anyway, this percentage was low-moderate (0-34%) excepting factory 3 where it reached 43.7%. Such a high amount of acetoin could give a butter flavour to the product. The highest percentages of strains producing acetoin were found in the strains isolated during the last two steps of the process, especially in the final product (20.8% and 30.9%).
Almost all the strains isolated in the region have nitrate reductase activity (94.4%) and urease activity (96.7%), in the case of *Staphylococccus xylosus* those percentages increase to 97% and 97.2%, respectively.
It was studied he protelolytic and lipolytic activity of all the strains isolated at the different stages of ripening in every factory. However a strong correlation between these activities and stage of ripening has not been detected. Lipolysis showed a little tendency to increase during the ripening, and so the number of bacteria with lipolytic activity seems to be slightly higher between the strains isolated from sausages at the semi-ripened or final stage than those isolated from the meat at the early minced stage. The number of bacteria with proteolytic activity is higher at the early minced stage, but those isolated in the ripened sausage show bigger halos than those isolated in the first stage.
Montel (1996) found the highest dry-cured odour with the inoculation of strains of *Staphylococcus xylosus* and *Staphylococcus carnosus* which had low proteolytic and lipolytic activities and did not produce acetoin.

Conclusions: It is clear that the *Staphylococcus xylosus* is the majority specie of *Micrococcaceae* bacteria in chorizo from Castilla-León. The importance of the inclusion of that *Staphylococcus* in the formulation of a starter is being more considerated everyday. Almost all the *Staphylococcus* isolated have the nitrate reductase enzyme (97%) that has been shown as an important technological property. Although we have studied the evolution of the flora all over the process of sausages manufacture we have not found a correlation between the different types of *Staphylococcus xylosus* and stage of ripening. There are not big differences between zones, so we can affirm that the house flora in the region behaves in a similar way, excepting factory 3.
12 different types of *Staphylococcus xylosus* were found, but two of them (*Staphylococcus xylosus* type5 and type 2) seem to be the more important from an ecological point of view. Both have a low proteolytic activity and a moderate-low lipolityc activity. The type 5 is found in a higher percentage at every stage. Stankhe (1994) showed that *Staphylococcus xylosus* can produce some esters that play an important role in the development of a fermented meat product flavour, so further studies about production of esters by *Staphylococcus xyloxus* type 5 would be desirable.

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