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**Original article**

# Identification of lactic acid bacteria isolated from Roncal and Idiazábal cheeses

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**Summary** — The lactic flora of two ewe's-milk cheeses regulated by Appellations of Origin, Roncal and Idiazábal cheeses, manufactured in the autonomous region of Navarre (Spain), was identified and compared. 263, 494, and 464 isolates of lactococci, lactobacilli, and leuconostocs, respectively, were isolated throughout ripening from both cheeses and presumably identified using morphological, phenotypical and biochemical methods. The distribution of lactococci species was not significantly different in the two cheeses, *Lactococcus lactis* subsp *lactis* being the main species from 10 days of ripening. *Lactobacillus casei* and *Lactobacillus plantarum* were the major lactobacilli species in both cheeses. In Roncal cheese *L casei* accounted for 53% of the isolates and *L plantarum* for 30%, whereas in Idiazábal cheese the frequency of *L casei* (39%) was slightly lower than the frequency of *L plantarum* (44%). *Leuconostoc mesenteroides* subsp *mesenteroides* and *Leuconostoc mesenteroides* subsp *dextranicum* were the predominant leuconostoc species in both cheeses, but the ratios of the two subspecies were approximately 3:1 in the Roncal cheese and closer to 1:1 in the Idiazábal cheese. The experimental results of this work are considered to be a first step to prepare indigenous starters including representative bacteria isolated from each type of cheese.

**ewe's cheese / lactic acid bacteria / ecosystem / identification / comparison**

**Résumé — Identification de bactéries lactiques isolées des fromages roncal et idiazábal.** La flore lactique du roncal et de l'idiazábal, deux fromages AOC pur brebis fabriqués dans la région de la Navarre (Espagne), a été identifiée et comparée ; 263 isolats de lactocoques, 494 de lactobacilles et 464 de leuconostocs provenant des deux fromages au cours de l'affinage ont été identifiés d'après leur morphologie et leur phénotype. La distribution des espèces de lactocoques ne différait pas significativement pour les deux fromages, *Lactococcus lactis* subsp *lactis* étant l'espèce la plus représentée. *Lactobacillus casei* et *Lactobacillus plantarum* constituaient les deux espèces de lactobacilles majoritaires dans les deux types de fromages. Dans le roncal, la proportion de *L casei* (53%) était supérieure à celle de *L plantarum* (30%), mais la tendance inverse était observée dans l'idiazabal : 39% de *L casei* contre 44% de *L plantarum*. *Leuconostoc mesenteroides* subsp *dextranicum* et subsp

*mesenteroides* constituait l'espèce majoritaire pour le roncal et l'idiazábal, mais les proportions pour les deux sous-espèces étaient d'environ 3:1 pour le roncal et 1:1 pour l'idiazábal. Les résultats expérimentaux de ce travail sont considérés comme une première étape pour concevoir des levains pour les deux fromages à partir de souches représentatives isolées de chacun des fromages.

**fromage pur brebis / bactérie lactique / écosystème / identification / comparaison**

## INTRODUCTION

Roncal and Idiazábal cheeses are both hard cheeses made from ewe's milk in Navarre (Spain). Roncal cheese was the first cheese to be awarded an Appellation of Origin in Spain, in 1981. It is produced according to the manufacturing process approved by the Appellation of Origin's Regulatory Board (Ministerio de Agricultura, Pesca y Alimentación, 1991). Idiazábal cheese has been regulated by an Appellation of Origin since 1986. Its manufacturing process is similar to that for Roncal cheese, though with certain differences in the coagulation time, coagulation temperature, and ripening time in the regulations approved by the Regulatory Board (Ministerio de Agricultura, Pesca y Alimentación, 1993).

The addition of starter is optional in both cheeses. However, most producers consider the use of starter cultures as necessary, particularly in winter, when milk acidity levels are lower (Pérez Elortondo et al, 1993).

An understanding of the microbial flora present in a cheese, the respective contributions by the different species, and growth trends during ripening is an essential basis for developing appropriate manufacturing technology. The different bacterial species in each genus, and even the different strains of individual species, have different technological, sensory, and antimicrobial attributes, and as a result may give rise to different milk breakdown products with differing effects on the final organoleptic properties of the cheese (Hegazi and Abo-Elnaga, 1990). Therefore, identification of the flora to species level is a necessary first step in preparing specific indigenous starters appropriate for each type of cheese (Requena et al, 1991, 1992).

In the past 25 years considerable attention has focused on the study of the species taking part in the ripening of ewe's-milk cheeses, namely, Manchego (Ordóñez et al, 1978), Casar de Cáceres (Poullet et al, 1993), La Serena (Fernández del Pozo et al, 1988), Serra (Macedo et al, 1993), Pecorino Romano (Deiana et al, 1984), and Feta (Tzanetakis and Litopoulou-Tzanetaki, 1992). However, the information available on the species involved in the ripening of Roncal (Ordóñez et al, 1980) and Idiazábal (Rúa et al, 1993) cheese is scarce.

The objects of the present study were to identify the lactic acid bacteria species (lactococci, lactobacilli, and leuconostocs) present in Roncal and Idiazábal cheese and to compare the ecosystems of these two cheeses.

## MATERIALS AND METHODS

### Isolation of strains

Six batches of Idiazábal cheese and six batches of Roncal cheese were studied. The cheeses were made from raw ewe's milk following the regulations approved by the Regulatory Boards of the Appellations of Origin for Idiazábal cheese (Ministerio de Agricultura, Pesca y Alimentación, 1993) and Roncal cheese (Ministerio de Agricultura, Pesca y Alimentación, 1991), respectively. A freeze-dried starter (Ezal, Texel,

Dangé Saint Romain, France) was used at a level of 1 U/100 L. It contained a combination of *Lactoccus lactis* subsp *lactis* and *Lactococcus lactis* subsp *cremoris*.

In each batch analyses were performed on the cheeses after 2, 10, 20, 30, 60, 90, 120 and 150 days of ripening. Samples were transported to the laboratory at 4 °C and analysed the same day. Sampling was performed as prescribed by the International Commission on Microbiological Specifications for Foods (ICMSF, 1982). Lactococci were isolated on M17 agar (Biokar Diagnostics, Beauvais, France) at 32 °C for 48 h; lactobacilli were isolated on Rogosa Agar (Difco Laboratories, Detroit, MI, USA) under anaerobic conditions at 32 °C for 48 h; and leuconostocs were isolated on MSE agar (Biokar Diagnostics) at 26 °C for 72 h. In each case, eight colonies from each sample (64 per batch and group) were chosen at random and purified in the same media used for isolation. Strains were stored for up to 30 days at 4 °C in Elliker Broth (Difco), except for the lactobacilli, which were stored at that same temperature in MRS broth (Difco).

### Identification of strains

Identification of lactic acid bacteria to genus level was carried out following the criteria of Sharpe (1979) using morphological, phenotypical and biochemical methods. These methods are considered to have a discriminatory power which includes genera, species, and subspecies (Curk et al, 1994). Identification of lactococci, lactobacilli and leuconostocs was carried out according to Bergey´s Manual of Systematic Bacteriology (1986) and Hernández-Haba and Dubón (1992). The biochemical tests employed have been described by Sharpe (1962), Harrigan and MacCance (1979), Gireaud-Galzy (1985), and MacFaddin (1990). Not all the strains of each species fully conformed to all the physiological characteristics of the type strain, and strains were assigned to a species when the results of no more than two physiological tests differed from the type strain. The ability of strains to grow at 45 °C and/or at a saline concentration of 6.5% was conclusive in differentiating strains belonging to the genus *Lactococcus* from those belonging to the genus *Enterococcus* according to the classification of Schleifer and Kilpper-Bälz (1984) and Schleifer et al (1985).

### Statistical treatment of data

Statistical tests were applied using the SPSS-X Statistical Package (SPSS, Inc, 1988). Since the attributes were qualitative in nature, $\chi^2$ test was applied. The variable considered was the overall species distribution after studying the species distribution at each ripening stage. To ensure that the test results were sufficiently reliable, rows and columns were pooled or discarded when the number of entries with theoretical frequencies lower than 5 made up more than 20% of the data matrix.

## RESULTS AND DISCUSSION

### Identification of lactococci

Total counts on M17 medium were higher than 8.1 $\log_{10}$ cfu/g in all cheeses. 300 streptococcal isolates were identified. 37 strains were assigned to the genus *Enterococcus* and 263 (87.7%) to the genus *Lactococcus*. The other isolates purified did not belong to this microbial group and the proportion of isolates which were not identified as lactococci or enterococci did not significantly vary neither during ripening nor between both cheeses. The high enterococci levels can be ascribed to poor hygiene during milking, transport, or manufacture and to the ability of these bacteria to adapt to adverse conditions: high saline concentrations (6.5%) and high temperatures (45 °C), as reported by other workers for similar cheeses (Suárez et al, 1983; Ordóñez et al, 1988).

Three subspecies of *Lactococcus lactis* (*L lactis*) were identified: *L lactis* subsp *lactis*, subsp *cremoris* and subsp *lactis* biovar *diacetylactis* (table I). The distribution of these species was not significantly different in the two types of cheese. *L lactis* subsp *lactis* was the predominant species. Significant differences in the proportions of *L lactis* subsp *lactis* and *L lactis* subsp *cremoris* arose over time. However, *L lactis* subsp *cremoris* levels were twice those of

**Table I.** Lactococci species isolated from Roncal and Idiazábal cheeses throughout ripening.
*Espèces de lactocoques isolées des fromages roncal et idiazábal au cours de l'affinage.*

| | *Cheese type* | | | |
|---|---|---|---|---|
| | *Roncal* | | *Idiazábal* | |
| *Species* | *No*[a] | *%*[b] | *No* | *%* |
| *Lactococcus lactis* subsp *lactis* | 63 | 70.8 | 135 | 77.6 |
| *Lactococcus lactis* subsp *cremoris* | 25 | 28.1 | 35 | 20.1 |
| *Lactococcus lactis* subsp *lactis* biovar *diacetylactis* | 1 | 1.1 | 4 | 2.3 |

[a] Number of isolates from six batches at eight ripening stages. b Proportion of subspecies by cheese type.
[a] *Nombre d'isolats à huit stades d'affinage pour six fabrications.* [b] *Proportion des sous-espèces par type de fromage.*

*L lactis* subsp *lactis* at the end of brining (day 2), *L lactis* subsp *lactis* predominated in the cheese samples from 10 days of ripening. *L lactis* subsp *lactis* and *L lactis* subsp *cremoris* are the two most common species in mesophilic starter cultures used in the dairy industry and are responsible for initial acidification of the milk, thereby contributing to curd formation and separation of the whey. Besides addition of strains of *L lactis* subsp *lactis* to milk has been reported to accelerate declines in the levels of enterobacteria, coliforms, and faecal coliforms (Medina et al, 1991).

*L lactis* subsp *lactis* biovar *diacetylactis* was present in low proportions that in no case exceeded 2.5%. This species produces $CO_2$, diacetyl, and acetoin and contributes directly to aroma and eye formation in cheese (Colman et al, 1992).

In an earlier study, *L lactis* subsp *lactis* was reported to be the sole representative of the genus *Lactococcus* in Roncal cheese (Ordóñez et al, 1980). This discrepancy in the findings may be ascribable to the smaller number of isolates (80) identified in that study, lowering the likelihood of detecting the minor species present. *L lactis* subsp *lactis* has been reported to be the predominant species in other cheeses similar to those considered in the present study, namely, Manchego (Martinez Moreno, 1976), Gamonedo (González de Llano et al, 1992), and La Serena (Fernández del Pozo et al, 1988). One possible explanation for the failure to record the presence of *L lactis* subsp *cremoris* in other similar cheeses may be that these cheeses were not examined at the end of brining.

## Identification of lactobacilli

Total counts on Rogosa agar were of the order of 8 $\log_{10}$ cfu/g in cheeses. A total of 494 lactobacilli isolates were identified, including five different species: *L brevis, L plantarum, L casei, L acidophilus and L delbrueckii* subsp *lactis*. It was not possible to identify subspecies of *L casei* with the identification procedures employed. All the species remained detectable in the samples throughout the entire ripening period. *L casei* and *L plantarum* were the major species in both Roncal and Idiazábal cheeses (table II). However, there were significant differences in the proportion of *L plantarum* and *L casei* in the two cheeses.

Acid production by *L casei* and *L plantarum* is similar (Suárez et al, 1983). It is reasonable to suppose that both *L casei* and *L plantarum* contribute extensively to the organoleptic characteristics of cheeses as a result of their proteolytic and lipolytic extra-

**Table II.** Lactobacilli species isolated from Roncal and Idiazábal cheeses throughout ripening. *Espèces de lactobacilles isolées des fromages roncal et idiazábal au cours de l'affinage.*

| | *Cheese type* | | | |
|---|---|---|---|---|
| | *Roncal* | | *Idiazábal* | |
| *Species* | *No*[a] | *%*[b] | *No* | *%* |
| *Lactobacillus brevis* | 37 | 15.0 | 33 | 13.4 |
| *Lactobacillus plantarum* | 74 | 30.0 | 110 | 44.5 |
| *Lactobacillus casei* 130 | 130 | 52.6 | 97 | 39.3 |
| *Lactobacillus acidophilus* | 5 | 2.0 | 5 | 2.0 |
| *Lactobacillus delbrueckiii* subsp *lactis* | 1 | 0.4 | 2 | 0.8 |

[a] Number of isolates from six batches at eight ripening stages. [b] Proportion of subspecies by cheese type.

[a] *Nombre d'isolats à huit stades d'affinage pour six fabrications.* [b] *Proportion de sous-espèces par type de fromage.*

cellular activity (Suárez et al, 1984) and the production of diacetyl (Núñez, 1976a; Rocabayera, 1991). The ability of *L plantarum* to produce oxygen peroxide and bacteriocins may accelerate the elimination of enterobacteria and coliforms (Rocabayera, 1991).

*L brevis* was the third most important species, at around 14%. The heterofermentative metabolism of *L brevis* may contribute to eye formation in cheese, which is desirable to a certain extent in Roncal and Idiazábal cheese.

The proportions of *L acidophilus* and *L delbrueckii* subsp *lactis* were low and similar in both cheeses. *L acidophilus* has also been reported in low proportions in other cheeses (Macedo et al, 1993; Poullet et al, 1993).

These findings agree with an earlier report on the predominance of *L casei* in Roncal cheese (Ordóñez et al, 1980), though they recorded *L casei* and *L plantarum* as the only lactobacillus species during ageing. *L plantarum* and *L casei* are the two most frequent lactobacillus species in many cheeses similar to those considered in this study, namely, Manchego (Núñez, 1976a; Ordóñez et al, 1978), La Serena (Fernández del Pozo et al, 1988), Serra (Macedo et al, 1993), Cabrales (Núñez, 1978), Mahón (Suárez et al, 1983,1984) and Los Ibores (Mas and González-Crespo, 1992).

## Identification of leuconostocs

Total counts on MSE medium were between 6.5 and 7 $\log_{10}$ cfu/g in cheeses. A total of 464 *Leuconostoc* isolates were identified. The relationship between *Leuconostoc* isolates and all isolates did not vary along the ripening or between both cheeses. Three species in this genus were isolated: *Lc mesenteroides* subsp *mesenteroides*, *Lc mesenteroides* subsp *dextranicum*, *Lc lactis* and *Lc paramesenteroides* (table III). The species distribution in the two cheeses was significantly different. No species was typical of any stage of ripening, and all the species remained detectable in the samples throughout the entire ripening period.

*Lc mesenteroides* subsp *mesenteroides* and *Lc mesenteroides* subsp *dextranicum* together with the lactococci, produce diacetyl, acetic acid, and ethanol, thus contributing to aroma formation in cheese. On the other hand, they have sometimes been associated with flavour defects (Milliere et al,

**Table III.** Leuconostoc species isolated from Roncal and Idiazábal cheeses throughout ripening. *Espèces de leuconostoc isolées des fromages roncal et idiazábal au cours de l'affinage.*

| | *Cheese type* | | | |
|---|---|---|---|---|
| | *Roncal* | | *Idiazábal* | |
| *Species* | *No*[a] | *%*[b] | *No* | *%* |
| *Leuconostoc mesenteroides* subsp *mesenteroides* | 184 | 71.3 | 92 | 44.7 |
| *Leuconostoc mesenteroides* subsp *dextranicum* | 66 | 25.6 | 94 | 45.6 |
| *Leuconostoc lactis* | 5 | 1.9 | 6 | 2.9 |
| *Leuconostoc paramesenteroides* | 3 | 1.2 | 14 | 6.8 |

[a] Number of isolates from six batches at eight ripening stages. [b]Proportion of subspecies by cheese type.

[a] *Nombre d'isolats à huit stades d'affinage pour six fabrications.* [b]*Proportion de sous-espèces par type de fromage.*

1989). The $CO_2$ generated by their metabolism may also contribute to the formation of small eyes in the cheese (Ordóñez et al, 1980; Rocabayera, 1991; Narvhus et al, 1993), though to a large extent this will depend on the type of pressing employed. Additionally, $CO_2$ inhibits the growth of contaminating moulds that are $CO_2$-sensitive (Vedamuthu, 1994).

Only two additional minor species were found in the two cheeses, *Lc lactis* and *Lc paramesenteroides*, which together contributed less than 10% of the total.

These results differ from the findings reported by other researchers. In Roncal cheese, Ordóñez et al (1980) identified 30 leuconostoc strains, 10 strains of *Lc mesenteroides* subsp *dextranicum* and 20 strains of *Lc lactis*. Low selectivity of the isolation medium, even though it is considered specific for isolation of this bacteria genus, together with the high number of atypical species may give rise to errors and these may be two possible explanation for the differing species distribution reported by different investigators. Besides these explanations, leuconostoc distributions may well be specific to each type of cheese, which may account for the highly variable results reported in the literature for similar cheeses (Fernández del Pozo et al, 1988; Tzanetakis and Litopoulou-Tzanetaki, 1992; Poullet et al, 1993) and even for a single cheese, eg, Manchego cheese (Núñez, 1976b; Ordóñez et al, 1978; Ramos et al, 1981).

Though the role of leuconostocs in determining the organoleptic properties of cheeses has not yet been fully elucidated, several teams of investigators have proposed including this genus in starter cultures (Martley and Crow, 1993; Massa et al, 1994).

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