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Physicochemical properties and secondary microflora variability in the manufacture and ripening of Idiazabal cheese

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Abstract — Secondary microflora in three batches of Idiazabal cheese were studied. In one of them the milk was cold-stored for 3 d and showed lower counts, except for psychrotrophs, *Enterobacteriaceae*, *Clostridium tyrobutyricum* and moulds. Significant differences in NaCl, dry matter and pH in raw milk were observed. Aerobic mesophilic, psychrotrophic flora and *Enterococcus* increased during coagulation, pressing and brining, while the rest of the secondary microflora was inhibited. During ripening, *Enterobacteriaceae* and *Micrococcaceae* declined in the first 2 ripening months; the *Enterococcus* were stable, except in the cheese from milk cold-stored for 3 d, for which they presented low counts and *Clostridium tyrobutyricum*, yeast and moulds showed an irregular evolution. © Inra/Elsevier, Paris.

ewe's cheese / physicochemical characteristic / secondary microflora

Résumé — Propriétés physicochimique et variabilité de la microflore secondaire au cours de la fabrication et de l'affinage du fromage idiazabal. La microflore secondaire de trois lots de fromage idiazabal a été étudiée. Le lot fabriqué avec du lait réfrigéré pendant 3 j a presenté les dénombrements les plus bas, sauf les bactéries aérobies psychrotrophes, *Enterobacteriaceae*, *Clostridium tyrobutyricum* et les moisissures. Des différences significatives dans le lait cru ont été observées en fonction des paramètres : chlorure de sodium, extrait sec et pH. La flore aérobie mésophile, aérobie psychrotrophe et les entérocoques ont augmenté pendant les phases de coagulation, pressage et salage, tandis que les autres microflores secondaires ont été inhibées. Le niveau des *Enterobacteriaceae* et des *Micrococcaceae* a chuté pendant les deux premiers mois d'affinage ; *Enterococcus* est stable (environ 6 log ufc·g^{-1}), à l'exception des fromages fabriqués à partir du lait réfrigéré pendant 3 j, où ce groupe microbien a présenté des dénombrements inférieurs à 5 log ufc·g^{-1}. *Clostridium tyrobutyricum*, les levures et les moisissures ont montré une évolution irrégulière. © Inra/Elsevier, Paris.

fromage de brebis / caractéristique physicochimique / microflore secondaire

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1. INTRODUCTION

Idiazabal cheese is produced in the Basque country and Navarre (Spain) exclusively from raw Latxa ewe's milk. Since 1986, it was regulated under a Denomination of Origin. At present it is protected by the European Union [20]. Microbiological and physicochemical characteristics of Spanish ewe's milk cheeses are well known only in some varieties such as Manchego [22, 24, 33, 39], Roncal [40], La Serena [21, 36] and Casar de Cáceres [45].

High microbiological quality in cheese production is dependent on the microbiological quality of raw material, technological parameters, potable quality water and proper hygienic processing conditions [16]. At present, the microbiological milk quality required from the European Union makes it necessary to use starters during cheesemaking. Several authors have studied the influence of raw milk microflora in cheese [19] in order to guarantee the specific characteristics of traditional products, but specific cultures are not used in many typical cheeses, such as Idiazabal. Recently, experimental designs to study the influence of raw milk flora on cheese characteristics have been reviewed [7].

Research on Idiazabal cheese has not yet revealed the full picture of all the microbiological aspects. A preliminary study on the indigenous lactic acid bacteria from Idiazabal cheese, such as *Lactococcus*, *Lactobacillus* and *Leuconostoc*, predominant microflora in this product, was undertaken in order to define a specific starter [44]. However, there are other microorganisms in cheeses that could participate in the formation of particularly sensorial characteristics [46, 48].

Cheese is quite a hostile environment and consequently very few genera of bacteria can grow or even survive in properly made cheese [23]. The secondary microflora, not well-defined in the literature, is different according to the cheese variety. Among pressed uncooked ewe's cheese, some high microorganism levels have been reported, such as *Micrococcaceae* and *Enterococcus* [25, 41, 50]. Other microorganisms, normally present in this type of products, such as *Enterobacteriaceae* and *Clostridium tyrobutyricum* are also of health and technological interest.

The aim of this work was the study of the secondary microflora evolution in Idiazabal cheese, with special emphasis on technology (*Enterococcus*, yeast and *Micrococcaceae*), results in defects (*Enterobacteriaceae*, *Clostridium tyrobutyricum* and moulds) and the possible harm to human health (*Escherichia coli*, *Salmonella* and *Micrococcaceae*).

2. MATERIALS AND METHODS

2.1. Cheesemaking and sampling

Cheesemaking protocol as well as details about the samples of raw milk, curd and cheeses collected in this study were reported in a previous work [44]. In this study, we also consider four samples of whey collected from each batch during cheesemaking.

2.2. Microbiological analysis

Decimal dilutions of the milk and whey were prepared in 1/4-strength Ringer's solution. Curd and cheese samples (10 g) were homogenised in 90 mL of sterile 2 % (w/v) sodium citrate solution, preheated to 45 °C in a Colworth Stomacher 400 (A.J. Seward Ltd., London, UK). Microbiological counts were assayed in the following conditions: aerobic mesophilic flora (plate count agar at 30 °C for 72 h) according to AFNOR [2]; aerobic psychrotrophic flora (plate count agar at 6.5 °C for 7 d) according to the standards of AENOR [1]; *Enterobacteriaceae* on violet crystal, methyl red, bile, glucose at 37 °C for 24 h [6]; total coliforms by means of the most probable number in 2 % brilliant green-bile at 37 °C for 48 h [3]; faecal coliforms by means of the most probable number (EC medium at 43 °C for 24 h) and the presence of *E. coli* and *Salmonella* according to AFNOR [4, 5, respec-

tively]; *Enterococcus* on bile esculin azide agar at 37 °C for 48 h [30]; *Micrococcaceae* in mannitol salt agar at 37 °C for 24–48 h [25]; moulds and yeast in Sabouraud chloramphenicol agar at 20–25 °C for 3–5 d [12] and *Clostridium* in Bryant and Burkey broth with resazurin at 37 °C for 7 d by means of the most probable number [9].

2.3. Physicochemical analysis

The pH, dry matter, NaCl and water activity (a_w) were measured according to the techniques already reported [44].

2.4. Statistical analysis

Variance analysis with 95 % confidence intervals was performed on the mean parameter values over the ripening period between the three batches. Calculation of the *F*-statistic was carried out using BMDP [11] programs 2V for analysis of variance (ANOVA).

3. RESULTS

3.1. Physicochemical parameters and secondary microflora in ewe's milk

Comparison of pair batches showed significant differences for all parameters, except for faecal coliforms in batches 1 and 3 (*table I*). Microbiological counts were higher in batch 2, except for aerobic psychrotrophs, *C. tyrobutyricum* (undetected in this batch) and moulds. Lower pH and counts were observed in batch 3, except for psychrotrophs, *Enterobacteriaceae*, *C. tyrobutyricum* and moulds. Dry matter and NaCl showed higher values throughout the lactation period.

3.2. Physicochemical parameters and secondary microflora during cheesemaking

Means from the three batches presented lower counts for total coliforms and moulds

Table I. Means and standard error for pH, dry matter (%), NaCl (mg·100·mL^{-1}) and microbiological counts (log cfu·mL^{-1}) in ewe's raw milk.

Tableau I. Moyennes et écarts type des pH, extraits secs (%), NaCl (mg·100·mL^{-1}) et dénombrements des microorganismes (log ufc·mL^{-1}) dans le lait de brebis.

Variables	Batch 1	Batch 2	Batch 3	Means
pH	6.63 ± 0.01^a	6.43 ± 0.02^b	6.32 ± 0.01^c	6.46 ± 0.16
Dry matter	16.77 ± 0.03^a	16.92 ± 0.08^b	17.46 ± 0.15^c	17.05 ± 0.36
NaCl	877 ± 2^a	990 ± 3^b	1039 ± 19^c	969 ± 83
Aerobic mesophiles	7.55 ± 0.06^a	8.30 ± 0.07^b	7.04 ± 0.14^c	7.63 ± 0.63
Aerobic psychrotrophs	6.88 ± 0.09^a	7.51 ± 0.13^b	8.08 ± 0.05^c	7.49 ± 0.60
Enterobacteriaceae	5.52 ± 0.24^a	6.72 ± 0.14^b	6.39 ± 0.06^c	6.21 ± 0.62
Total coliforms	5.04 ± 0.15^a	5.91 ± 0.08^b	4.60 ± 0.14^c	5.18 ± 0.67
Faecal coliforms	3.60 ± 0.32^a	4.90 ± 0.11^b	3.48 ± 0.23^a	3.99 ± 0.79
Enterococcus	4.45 ± 0.12^a	4.82 ± 0.07^b	3.00 ± 0.31^c	4.09 ± 0.96
Micrococcaceae	4.33 ± 0.11^a	4.79 ± 0.19^b	2.87 ± 0.16^c	4.00 ± 1.00
Clostridium tyrobutyricum	0.95 ± 0.00^a	n.d.	0.45 ± 0.17^b	0.47 ± 0.35
Yeast	2.74 ± 0.12^a	3.27 ± 0.09^b	2.35 ± 0.19^c	2.79 ± 0.46
Moulds	1.02 ± 0.29^a	1.54 ± 0.08^b	2.65 ± 0.12^c	1.74 ± 0.83

F-statistic for variance analysis between batches (1, 2 and 3). Means in the same row with different letter show statistically significant differences ($P < 0.05$). n.d.: undetected.

Valeurs F du test de Fischer après l'analyse de la variance entre les trois lots. Les moyennes indiquées par des lettres différentes sont statistiquement différentes au seuil de 5 %. n.d. : non détectés.

in curd (*table II*) than in milk (*table I*). The pH curd was lower than in whey. An increment of mesophilic and psychrotrophic flora was observed up to 1-d-old cheeses in all batches (*table III*). A decline in faecal microflora was observed, except for *Enterococcus*, which showed a small increase in batches 1 and 2, and almost 4 log units in batch 3. *Micrococcaceae*, *Clostridium tyrobutyricum*, yeast and moulds were slightly lower in cheese (*table III*) than in curd (*table II*).

Table II. Means and standard error (three batches) for pH, dry matter (%), NaCl (mg·100 g^{-1}) and microbiological counts (log cfu·mL^{-1}) in ewe's curd and whey during cheesemaking.

Tableau II. Moyennes et écarts type (trois lots) des pH, extraits secs (%), NaCl (mg·100 g^{-1}) et dénombrements des microorganismes (log ufc·mL^{-1}) dans la caillé et le lactosérum de brebis pendant les fabrications.

Variables	Curd	Whey
pH	6.19 ± 0.19	6.33 ± 0.18
Dry matter	42.98 ± 0.18	8.77 ± 1.01
NaCl	305 ± 102	2231 ± 191
Aerobic mesophiles	8.20 ± 0.67	7.17 ± 0.54
Aerobic psychrotrophs	8.08 ± 0.58	7.02 ± 0.70
Enterobacteriaceae	6.49 ± 0.51	5.33 ± 0.40
Total coliforms	4.95 ± 0.76	4.42 ± 0.79
Faecal coliforms	4.34 ± 0.77	3.72 ± 1.02
Enterococcus	4.59 ± 1.31	3.50 ± 0.72
Micrococcaceae	4.60 ± 1.05	3.54 ± 0.45
Clostridium tyrobutyricum	0.64 ± 0.10	0.10 ± 0.00
Yeast	3.04 ± 0.67	2.36 ± 0.60
Moulds	1.65 ± 0.38	0.62 ± 0.00

Table III. Microbiological mean counts (log cfu·g^{-1}) and standard error in 1-d-old cheese.

Tableau III. Moyennes des dénombrements des microorganismes (log ufc·g^{-1}) et écarts type dans les fromages après un jour d'affinage.

Variables	Batch 1	Batch 2	Batch 3	Means
Aerobic mesophiles	9.35 ± 0.02^{a}	9.25 ± 0.06^{b}	9.20 ± 0.02^{b}	9.27 ± 0.08
Aerobic psychrotrophs	9.15 ± 0.03^{a}	8.94 ± 0.02^{b}	10.07 ± 0.02^{c}	9.39 ± 0.60
Enterobacteriaceae	4.81 ± 0.00^{a}	3.92 ± 0.01^{b}	3.65 ± 0.03^{c}	4.13 ± 0.61
Total coliforms	4.71 ± 0.01^{a}	3.50 ± 0.12^{b}	3.05 ± 0.01^{c}	3.75 ± 0.86
Faecal coliforms	4.71 ± 0.01^{a}	2.50 ± 0.12^{b}	2.05 ± 0.01^{c}	3.09 ± 1.42
Enterococcus	5.95 ± 0.12^{a}	5.46 ± 0.32^{b}	6.93 ± 0.02^{c}	6.11 ± 0.75
Micrococcaceae	4.94 ± 0.07^{a}	4.99 ± 0.04^{a}	3.19 ± 0.08^{b}	4.37 ± 1.03
Clostridium tyrobutyricum	0.47 ± 0.00^{a}	0.95 ± 0.00^{b}	0.31 ± 0.11^{c}	0.58 ± 0.33
Yeast	3.07 ± 0.02^{a}	3.20 ± 0.02^{b}	2.55 ± 0.03^{c}	2.94 ± 0.34
Moulds	1.38 ± 0.07^{a}	1.14 ± 0.07^{b}	2.00 ± 0.02^{c}	1.51 ± 0.44

F-statistic for variance analysis between batches (1, 2 and 3). Means in the same row with different letters show statistically significant differences ($P < 0.05$).

Valeurs F du test de Fischer après l'analyse de la variance entre les trois lots. Les moyennes indiquées par des lettres différentes sont statistiquement différentes au seuil de 5 %.

3.3. Ripening physicochemical properties and secondary microflora evolution

In 1-d-old cheese, pH (4.94–5.29), dry matter percentage (56.77–60.47) and NaCl expressed by mg NaCl·100 g^{-1} (131–215) were higher from batches 1 to 3. Cheese pH in batches 1 and 2 were similar between d 1 and 15 while in batch 3 a small decline was detected (0.10). A small pH increment at the intermediate times (5.13–5.30) and a reduction at the end of ripening (5.01–5.15) was observed. All cheeses showed an increase of the dry matter and NaCl and a reduction of the water activity during ripening [44].

Trends for aerobic mesophilic flora were similar in all batches (*figure 1*). An important reduction (1 log unit) occurred the first 2 months and a lower decline (1.5 log unit) at the end of ripening, particularly in batches 1 and 2. Aerobic psychrotrophic flora showed the same evolution with counts 0.5 log lower than mesophiles. From very similar values on d 1, mesophilic and psychrotrophic bacteria fell to lower levels in batch 3. Significant differences in all samples were observed over the entire ripening period.

Enterobacteriaceae were no longer detectable after 60 d of ripening. Trends for total and faecal coliforms were similar to those for *Enterobacteriaceae*. *Escherichia coli* has been confirmed in milk, curd and cheese in all batches. The percentage was higher in cheese than in milk or curd. Of a total of 161 isolates from the three batches, 50 % were identified as *E. coli*. *Salmonella* was not detected in all analysed samples. Other faecal groups such as *Enterococcus* showed a stable evolution during ripening for batches 1 and 2, while in batch 3 a great decline (4 log units) was observed (*figure 2*).

Micrococcaceae underwent a substantial decrease from 15 (10^5 cfu·g^{-1}) to 360 (10 cfu·g^{-1}) ripening days in batches 1 and 2.

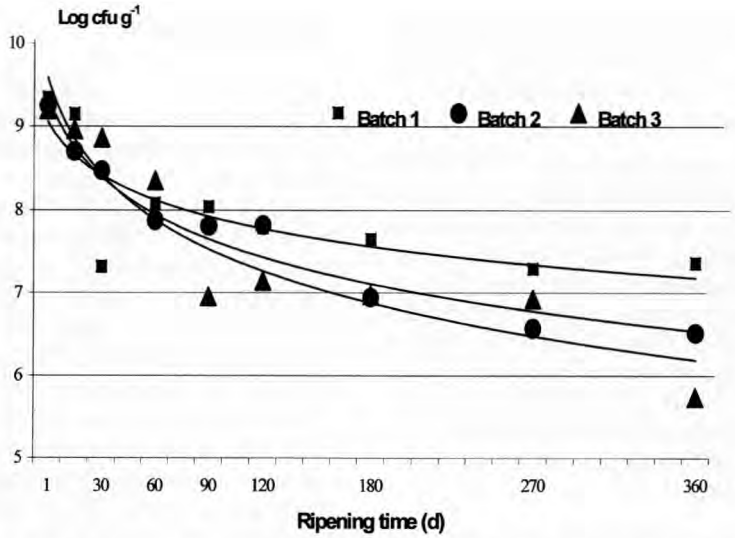

Figure 1. Trends for aerobic mesophiles microflora during ripening of ewe's cheese (data points represent the means of four replications).

Figure 1. Évolution de la flore aérobie mésophile pendant l'affinage des fromages de brebis (les points représentent la moyenne de quatre répétitions).

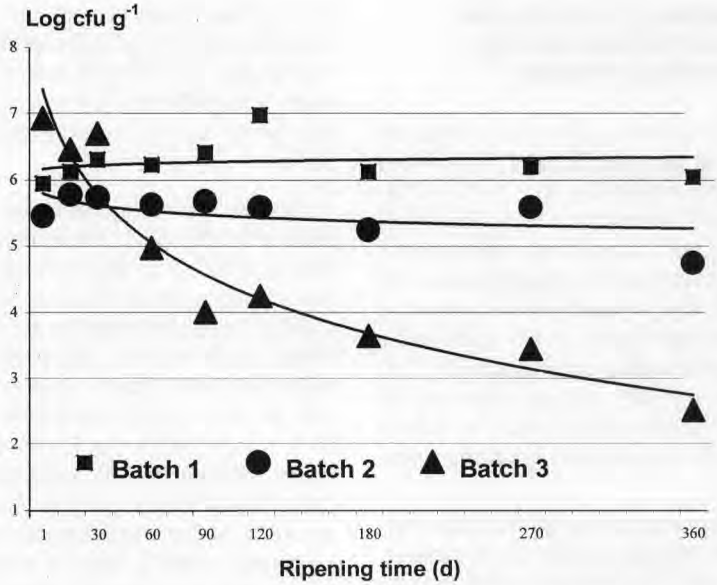

Figure 2. Trends for *Enterococcus* during ripening of ewe's cheese (data points represent the means of four replications).

Figure 2. Évolution de *Enterococcus* pendant l'affinage des fromages de brebis (les points représentent la moyenne de quatre répétitions).

Counts in batch 3 declined the first 15 d (10^3 cfu·g^{-1} on d 1 and 10 cfu·g^{-1} on d 15) and the evolution was stable during the rest of ripening.

Yeasts were not detectable at 6 months in batch 3 and at 9 months in batch 1. In batch 2, a level of 10^4 cfu·g^{-1} maintained constant during ripening. Moulds showed an irregular evolution in all batches (*figure 3*). In most ripening times, counts from batch 1 were lower. From 270 d to the end of ripening, the mould numbers were higher in batch 3. Cheeses from batch 3 were pervaded by blue moulds and the resultant manifestation of this quality defect increased with ripening.

Maximum counts for *C. tyrobutyricum* were slightly higher than 1 log unit during the ripening period. These levels were not sufficient to produce technological problems.

4. DISCUSSION

Physicochemical characteristics and microflora counts' variability from ewe's milk agree with those reported by other authors who studied changes in milk throughout ewes' lactation period [8, 21, 26, 42, 43, 45]. Higher psychrotrophic microflora in batch 3 could be explained by the different cold-stored time [14, 29]. It is well-known that high psychrotroph levels could produce rancid off-flavours in advanced cheese-ripening time [17, 31, 38].

Results observed during coagulation prove that most microorganisms were incremented with manufacture temperature and physical retention of the microorganisms in the curd during whey drainage. *Micrococcaceae* levels detected do not present the risk of enterotoxin production [8]. Coliform levels were not sufficient to produce technological problems.

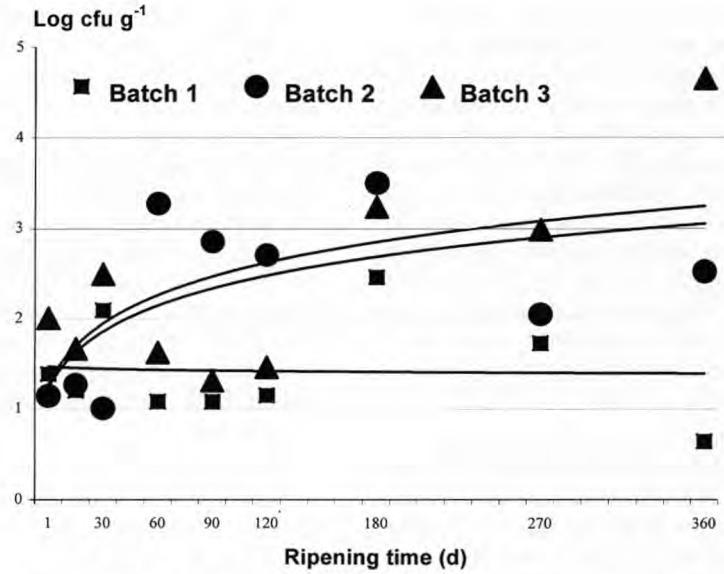

Figure 3. Trends for moulds during ripening of ewe's cheese (data points represent the means of four replications).

Figure 3. Évolution des moisissures pendant l'affinage des fromages de brebis (les points représentent la moyenne de quatre répétitions).

During the first 15 ripening days, the pH differences observed between batches were probably due to lactose metabolism. While most lactose was metabolised during cheesemaking in batches 1 and 2, the small decline in batch 3 could have been the result of higher soluble compound retention in these cheeses. The pH increment at intermediate ripening times could account for the lactic acid metabolism and ammonia production by a microorganism such as yeast, a phenomenon reported in soft cheeses with surface flora [37]. Dry matter, NaCl and water activity evolution were the result of the diffusion phenomena and to the fact that samples were taken from the cheese centre.

The variability in the physicochemical properties influenced the microbiological development. Comparable trends for aerobic mesophilic and psychrotrophic flora have been reported in other ewe's milk cheeses [21, 24, 40, 45].

Enterobacteriaceae has an importance to public health and some species have technological interest due to lactose degradation that produce CO_2, which is responsible for early blowing and the formation of eyeholes [34, 52]. The decline to undetectable levels has been described by other researchers in pressed ewe's milk cheeses [39, 45]. Results for *E. coli* are in agreement with those reported by Tornadijo et al. [52], who did not isolate it from milk, curd or 1-week-old goat's cheese but found it as the predominant organism in 2-week-old cheese. This confirms the results of other authors who have shown that *E. coli* was one of the most resistant species within *Enterobacteriaceae* during ripening of cheeses [47]. Generally, *Salmonella* is not detected in long-time ripened pressed cheeses with low pH, as in our case [18].

The increase of *Enterococcus* during cheesemaking proved their contribution to curd acidification. High numbers have also

been detected in other ewe's cheeses [21, 45]. Some authors have reported glycolytic activities similar to those of lactic acid bacteria and a great capacity to resist adverse conditions: great tolerance to temperature, salt and acidity [35, 50, 53]. One remarkable difference of some *Enterococcus* strains compared to lactic acid bacteria are their strong exopeptidase activity, especially chymotrypsin-type activity [51]. Some of them could be of technological interest in cheese ripening, due to their ability to metabolise citrate and also because of their proteolytic and lipolytic activities [13, 15, 32].

Micrococcaceae observed in our work were different to those reported for Urbasa cheese [28], where this group was undetectable after 90 d. The less NaCl concentration observed [44] and the aerobic metabolism of these bacteria explain the low level detected. A beneficial role of these bacteria has been related to proteolytic, lipolytic and esterolytic activities during cheese ripening, producing metabolites such as diacetyl, acetate and methanethiol [10]. It has been suggested that micrococci could improve the flavour during ripening [41], but a proper strain should be selected for this use [10].

Although some yeast can cause spoilage and development of off-flavours in cheese, the limited occurrence of most of them are crucial for the development of a full flavour in some types of cheese [49]. Differences in mould counts were sufficient to cause perceptible differences in sensorial quality. The highest opening texture in the cheeses from batch 3 could have favoured the development of this accident. The antagonistic effect of moulds could explain the microbiological decline in this batch, *Enterococcus* included [27].

5. CONCLUSION

Microbiological counts in ewe's raw milk show significant variability. Cold milk stored for a long time (3 d) could result in higher psychrotrophic and mould counts. During cheesemaking, pH decline, manufacture temperatures and microbial antagonisms increase *Enterococcus* in detriment to other secondary microflora. *Enterococcus* during ripening shows the highest level, which suggests they have a potential major role in the ripening process. It will be interesting to carry out further studies of these groups. The low counts and aerobic metabolism of *Micrococcaceae* suggest less activity during ripening. Yeast counts are not high and some of them could be important for the development of a full flavour of this product.

The presence of certain microorganisms in cheeses can be considered as result of hazard contamination. In a previous work, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Lactobacillus* were included in the preparation of a starter to ensure the predominance of these bacteria during the ripening of Idiazábal cheese [44]. In order to guarantee typical stable characteristics of traditional cheeses, it will also be necessary to consider some secondary microflora to design specific starters that reduce the microbiological variability of ewe's raw milk.

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