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Ibores goat's milk cheese: Microbiological and physicochemical changes throughout ripening

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Abstract – The microflora of Ibores cheese made with raw goat's milk without any starter addition was studied throughout the ripening period. The microbial counts for total lactic acid bacteria, presumptive lactococci and presumptive lactobacilli attained maxima of 9–10 log units with little or no variation throughout ripening. Leuconostocs and enterococci levels were 2–3 log units lower, also with little variation. Coliforms and staphylococci declined steadily from relatively high initial counts, the coliforms to low levels and the staphylococci disappearing completely. Moulds and yeasts were at low levels throughout ripening. Among the identified isolates, lactococci formed the prevalent group throughout ripening, followed by leuconostocs and enterococci at similar levels to each other, and then lactobacilli at low levels. The prevailing species and subspecies were *Lactococcus lactis* ssp. *lactis, Leuconostoc mesenteroides* ssp. *dextranicum, Ln. mesenteroides* ssp. *mesenteroides, Weissella paramesenteroides, Enterococcus faecium, E. faecalis, Lactobacillus casei* and *Lb. plantarum.* Small numbers of other species from each of the genera were also identified. The changes in certain physicochemical parameters during ripening were determined. By 60 days, the values of the pH, total solid content, and NaCl content were 5.18, 58.9% and 2.5%, respectively. The correlation of some of the physicochemical parameters with the log counts of the microbial groups was also established.

Goat's milk cheese / ripening / lactic acid bacteria / identification

Résumé – **Fromage des Ibores : changements microbiologiques et physico-chimiques pendant l'affinage.** La microflore de fromages de type Ibores, faits avec du lait cru de chèvre, sans addition de levain, a été suivie pendant l'affinage. Les concentrations de bactéries lactiques totales, de

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lactocoques présumés et de lactobacilles présumés atteignaient un maximum de 9 à 10 log ufc·g^{-1}, sans changement notable pendant l'affinage. Pendant la même période, les concentrations de leuconostocs et d'entérocoques étaient de 2 à 3 log plus faibles. Les populations de coliformes et de staphylocoques diminuaient régulièrement à partir de valeurs relativement élevées. Les coliformes ne disparaissaient pas totalement, contrairement aux staphylocoques. Les niveaux de moisissures et de levures étaient bas durant tout l'affinage. Parmi les isolats identifiés, les lactocoques constituaient le groupe dominant durant l'affinage, suivi des leuconostocs et des entérocoques (à des niveaux similaires) alors que les lactobacilles étaient rares. Les espèces et sous-espèces prédominantes étaient : *Lactococcus lactis* ssp. *lactis*, *Leuconostoc mesenteroides* ssp. *dextranicum*, *Ln. mesenteroides* ssp. *mesenteroides, Weissella paramesenteroides, Enterococcus faecium, E. faecalis, Lactobacillus casei* et *Lb. plantarum*. De rares isolats appartenant à d'autres espèces ont également été identifiés, pour chaque genre. Les changements de quelques paramètres physico-chimiques ont également été étudiés pendant l'affinage. Au bout de 60 jours les valeurs de pH, extrait sec et NaCl ont été de 5,18 ; 58,9 % et 2,5 % respectivement. De même, ont été établies des corrélations entre paramètres physico-chimiques et niveaux de populations des groupes microbiens.

Fromage au lait cru de chèvre / affinage / bactérie lactique / identification

1. INTRODUCTION

The production of different farm-made varieties of cheeses in Extremadura (south-west Spain) is important economically for this rural area. These types of cheese are usually made in farmhouses from ewe's and goat's milk. Ibores cheese is one significant variety of semi-hard goat's milk cheese.

Ibores cheese was awarded the Appellation of Origin in 1997. The regulations make the use of raw milk obligatory in the manufacturing process, but the use of pasteurized milk is also permitted. In the latter case specific starters prepared with autochthonous microorganisms should be used, even though this kind of starter is still non-existent on the market.

In order to avoid anomalous fermentations in raw milk cheeses, cheesemakers have begun to add non-specific commercial starter cultures to improve lactic fermentation. This could soon lead to the loss of the characteristics of this traditional cheese. It is therefore necessary to obtain new starter cultures with autochthonous microorganisms to maintain the quality of both raw and pasteurized milk cheeses.

Although other Spanish goat's milk cheeses have been extensively studied (Majorero cheese [6], Gredos cheese [24], Armada cheese [33], Tenerife cheese [36]), there have only been some preliminary studies of Ibores cheese [22, 23]. Moreover, recent modifications of some aspects of Ibores cheese production, the improvement of hygiene, and more controlled ripening conditions, suggested to us that the microbiological and physicochemical characteristics could be quite different to those a decade ago.

As a first step to obtain autochthonous starter cultures, the aim of this work was to study the present conditions of the most representative microbiological groups throughout ripening and their relationships with certain physicochemical properties, as well as to isolate and to characterize the main culturable lactic acid microflora.

2. MATERIALS AND METHODS

2.1. Cheese manufacture and sampling

Four batches of Ibores cheese were made in the only two dairy farms which still use only raw goat's milk, from Verata, Serrana or Retinta breeds, without any addition of commercial starters. The milk was obtained by mixing refrigerated evening

and morning milk. The coagulation is carried out with calf rennet, at 27–30 ºC. Curd is cut into 4–6 mm grains and put into moulds. Cheeses, of 15–18 cm diameter, 5–9 cm height, and 750–1200 g weight, are mechanically pressed and salted in brine. The ripening time is between two and three months, at 8–12 ºC and 80% relative humidity. At the end of the ripening, the cheeses are usually finished by covering with paprika and oil.

Milk samples, and 3-, 15-, 30- and 60-d-old whole cheeses were taken from each batch and transported to the laboratory under refrigeration. Sampling and microbiological analyses were performed within the following 24 h.

2.2. Microbiological analysis

For the cheeses, after removing the rind, representative 10 g samples were homogenized with 90 mL sterile 2% (w/v) sodium citrate solution, in a Stomacher 400 (Seward Medical, London, UK). Decimal dilutions of both milk and the cheese homogenates in quarter-strength Ringer's solution were plated for the following microbial groups: lactic acid bacteria on (a) MRS agar pH 5.7 (Merck, Darmstadt, Germany) incubated under anaerobiosis at 30 ºC for 72 h; (b) presumptive lactococci on M17 agar (Merck) at 30 ºC for 72 h; (c) presumptive leuconostocs on MSE agar (Merck) at 22 ºC for 5 d; (d) presumptive lactobacilli on Rogosa agar (Merck) at 30 ºC for 72 h in anaerobiosis; and (e) enterococci on Slanetz-Bartley agar (Merck) at 44 ºC for 48 h. Coliforms were determined in Violet Red Bile Agar (Merck) at 37 ºC for 24 h. *Staphylococcus aureus* was estimated on Baird-Parker agar (Merck) at 37 ºC for 24–48 h and then confirmed by a coagulase test (BioMérieux, Marcy-l'Étoile, France). Moulds and yeasts were counted on YGC agar (Merck) at 25 ºC for 5 d. All analyses were performed in duplicate.

2.3. Physicochemical analyses

The pH was measured with a Crison 2001 pH-meter (Crison Instruments S.A., Barcelona, Spain) for the milk samples, and with a solid Crison 52-32 electrode for the cheeses. Total solids (TS) content was determined in the milk and cheeses according to the IDF standards 21B [11] and 4A [10], respectively. The NaCl content was determined according to the IDF standard 88A [12] for cheeses. Fat content was determined by the Gerber procedure in milk according to the ISO standard 2446 [14], and by the Van-Gulik method for cheeses according to the ISO standard 3433 [13].

Total nitrogen (TN), soluble nitrogen at pH 4.6 (SN), and soluble nitrogen in 12% trichloroacetic acid (TCASN) were determined by the Kjeldahl method [1].

All analyses were performed in triplicate

2.4. Isolation and identification of lactic acid bacteria

Isolation of lactic acid bacteria was carried out by picking at random 7–9 colonies per cheese sample (3, 15, 30 and 60 d) and per batch, from each of the M17, Rogosa, and MRS agar pH 5.7 plates. The isolates were purified by consecutive subculturing in MRS agar.

A total of 330 Gram-positive and catalase-negative isolates were stored at –80 ºC in MRS medium containing 30% (v/v) glycerol. Working cultures were prepared by two consecutive transfers in MRS broth at 30 ºC.

Identification of the isolates was performed according to the criteria of Bergey's manual of determinative bacteriology [9] and the methods and criteria of Sharpe [32]. We also applied the criteria of Mundt [27] and Schleifer et al. [31] for lactococci; those of Mundt [26], Manero et al. [20] and Devriese et al. [5] for enterococci; those of

Garvie [8] for leuconostocs; and those of Kandler and Weiss [17] for lactobacilli. Carbohydrate fermentation patterns of the strains were determined using the API50 CH system (BioMérieux), and homofermentative coccal-shape organisms of doubtful classification were tested using the API 20 STREP system (BioMérieux).

2.5. Statistical analyses

Analyses of variance (ANOVA) on values at different ripening stages and statistical correlations using Pearson's correlation coefficient between log counts of the microbial groups in the cheese samples and the physicochemical parameters, were performed using the statistical software package SPSS version 9.0 for Windows (SPSS Inc., Chicago, USA).

3. RESULTS AND DISCUSSION

3.1. Physicochemical changes during ripening

The changes in the physicochemical properties of the Ibores cheese during ripening are given in Table I.

The pH decreased sharply after day 30 of ripening, showing a slight increase by day 60. The evolution of the pH was very similar to that observed some years ago [23] for the same type of cheese, although the values were then somewhat lower throughout ripening. Other goat's milk cheese varieties [16, 24, 25, 36] usually reach lower pH levels than those of Ibores cheese.

The cheese total solid content increased gradually, reaching 58.9% by 60 d of ripening. In other types of goat's milk cheeses, the total solid content is very variable, possibly due to the very different cheesemaking methods used [6, 18, 24, 33]. Even in the case of the Ibores cheeses analysed some years ago [23], the total solid content was much higher (65.4% at 60 d of ripening), possibly because the ripening was not performed at a controlled temperature, whereas at present it is.

With respect to the NaCl content, the levels increased gradually to 2.5% by 60 d. Values were also very close to those that have been reported for some other goat's milk cheeses [21, 24, 33], although usually goat's milk cheeses have high salt levels [16, 18, 36]. Surprisingly, the present salt content in Ibores cheese is almost 50%

Table I. Changes in physicochemical parameters of Ibores cheese throughout ripening.

Parameters	Milk	Days of ripening			
		3	15	30	60
pH	6.63 ± 0.14	5.81 ± 0.57	4.99 ± 0.28	4.98 ± 0.31	5.18 ± 0.28
TS %	14.08 ± 1.56	51.96 ± 1.32	55.28 ± 2.26	56.19 ± 2.55	58.90 ± 2.12
NaCl %	nd	1.46 ± 0.48	1.92 ± 0.47	2.19 ± 0.55	2.50 ± 0.68
Fat/TS %	37.42 ± 3.64	51.74 ± 2.50	50.72 ± 3.39	52.69 ± 3.21	52.64 ± 3.24
TN %	nd	3.19 ± 0.11	3.43 ± 0.22	3.52 ± 0.24	3.68 ± 0.26
SN/TN %	nd	11.79 ± 2.77	13.99 ± 2.64	16.59 ± 3.45	21.06 ± 2.38
TCASN/TN %	nd	4.11 ± 1.35	5.76 ± 2.53	7.19 ± 2.80	10.21 ± 2.92

Mean ± standard deviation of 4 batches. TS: total solids; TN: total nitrogen; SN: soluble nitrogen at pH 4.6; TCASN: soluble nitrogen in 12% trichloroacetic acid; nd: not determined.

lower than that observed a decade ago for the same type of cheese. The cause of this major change is probably the modification in the salting method, given that nowadays the cheesemakers use controlled brines instead of the strong salting with coarse salt applied to the cheeses years ago to prevent microbial spoilage.

The mean fat content at 60 d of ripening was 52.6%, a value very close to those reported by Juarez et al. [15] for other Spanish goat's milk cheeses with similar characteristics.

With regard to the products of proteolysis in the cheeses (SN/TN and TCASN/TN), their levels increased as the cheese aged.

The cheese's age was a significant determinant of total solid content and SN/TN ($P < 0.01$) and of pH and TCASN/TN ($P < 0.05$).

3.2. Changes in microbial group counts throughout ripening

The counts of different microbial groups in the milk and cheeses are given in Table II. During cheese ripening, the counts of lactic acid bacteria in MRS and M17 reached a maximum after 15 d ripening, decreasing only slightly by 60 d. However in Rogosa and in MSE, the counts increased slightly through to the end of ripening. The levels reached in MRS, M17 and Rogosa at different stages of cheese ripening were very close, while the counts for presumptive leuconostocs in MSE were about 2 log units below those obtained for the other lactic acid bacteria groups throughout ripening. The pH, NaCl content and total solid content did not seem to have any significant effect on the numbers of the aforementioned microbial groups during ripening, and no significant differences were found due to the age of ripening.

Enterococci attained their maximum by 30 d, decreasing only slightly by the end of the ripening. The enterococci counts were similar to those reported for other types of raw milk cheeses [7, 18, 24], in which enterococci are usually present at relatively high levels maintained until the end of ripening. This probably reflects the resistance of the enterococci to a variety of adverse

Table II. Changes in log cfu·g^{-1} of main microbial groups during ripening of Ibores cheese.

Microbial groups	Milk	Ripening time (days)			
		3	15	30	60
Lactic acid bacteria	nd	9.05 ± 0.30	9.72 ± 0.26	9.04 ± 0.28	8.85 ± 0.48
Presumptive lactococci	5.11 ± 1.17	9.40 ± 0.46	9.40 ± 0.37	9.04 ± 0.44	9.32 ± 0.87
Presumptive lactobacilli	4.11 ± 0.72	8.89 ± 0.50	9.09 ± 0.09	9.06 ± 0.29	9.15 ± 0.92
Presumptive leuconostocs	3.78 ± 0.42	7.23 ± 0.42	7.31 ± 0.82	7.55 ± 0.28	7.61 ± 1.21
Presumptive enterococci	2.21 ± 2.11	6.01 ± 1.32	6.67 ± 0.79	7.10 ± 0.28	6.86 ± 0.74
Coliforms	4.05 ± 0.40	6.55 ± 0.06	5.97 ± 1.34	4.66 ± 1.68	3.56 ± 1.42
Coagulase positive staphylococci	2.10 ± 1.27	5.06 ± 0.60	4.46 ± 1.04	3.86 ± 0.68	< 1
Moulds and yeasts	3.34 ± 0.45	3.62 ± 0.94	4.55 ± 0.35	4.52 ± 0.81	3.66 ± 0.94

Data are the average ± standard deviation values of 4 batches; nd: not determined.

conditions such as high and low pHs, drying, high salt concentrations, and high and low temperatures [34]. These characteristics are in accordance with the significant correlations found by us between enterococci log counts and values of moisture content (negative correlation, $P < 0.05$) and NaCl content (positive correlation, $P < 0.01$).

Coliforms reached maximum counts after 3 d of ripening, decreasing thereafter gradually by about 3 log units, in agreement with the significant ($P < 0.05$) influence of the ripening time on coliform numbers. Presumably lactic acid production by lactic acid bacteria caused the coliform reduction [28], which is in accordance with the significant correlation ($P < 0.05$) between coliform log counts and pH values. However, although the decrease in pH was sharp, it did not appear to be enough to reduce the coliforms as early in the ripening as in other goat's milk cheeses [24, 25, 36].

Staphylococcus aureus numbers decreased progressively and significantly ($P < 0.01$) with time of ripening. Levels were below the detection limit of 10 cfu·g^{-1} at 60 d ripening.

The low counts of moulds and yeasts detected seem to reflect the restricted role of this group in this type of cheese.

3.3. Identification of strains isolated during ripening

The identification at the genus level of the 330 isolates recognized as lactic acid bacteria and the medium (M17, Rogosa or MRS) from which each isolate was obtained is presented in Table III.

In M17 medium, as was expected due to the usefulness of this medium for lactococci isolation, the largest percentage of isolates belonged to lactococci, followed by enterococci and leuconostocs in much lower proportions. No lactobacilli were isolated in this medium. In Rogosa medium, again lactococci appeared as the most numerous group, and leuconostocs were isolated in a larger proportion than in the M17 medium, followed by enterococci, while only very small numbers of lactobacilli were found in spite of Rogosa medium being considered selective for this group of microorganisms. In MRS medium, there was also a prevalence of lactococci, although the remaining groups were isolated in higher proportions than in the M17 and Rogosa media.

Considering the isolates as a whole, lactococci formed the prevalent group, followed by enterococci and leuconostocs (both attaining levels of about half those of

Table III. Distribution of lactic acid bacteria isolates from Ibores cheese from different media.

Genus			Medium					
			M17		Rogosa		MRS pH 5.7	
	Total	%	No of isolates	%	No of isolates	%	No of isolates	%
Lactococcus	157	45.6	66	64.1	48	48	43	33.9
Enterococcus	71	21.5	22	21.3	12	12	37	29.1
Leuconostoc-Weissella	73	22.1	15	14.6	31	31	27	21.3
Lactobacillus	29	8.8	0	0.0	9	9	20	15.7

the lactococci), with lactobacilli, even in Rogosa medium, being the minority group. This last finding shows either the low selectivity of this medium for lactobacilli or the sparse numbers of these microorganisms in Ibores cheese. This could facilitate the isolation of the other most abundant genera. The scarcity of lactobacilli was initially unexpected because lactobacilli had been detected as the predominant group (nearly 50%) in the previous study of Ibores cheese [22]. This prevalence of lactobacilli had also been found in other goat's milk cheeses [18, 33, 36], in which, as the ripening progressed, lactobacilli outnumbered lactococci. This probably reflects a combined effect of high salt concentration and low pH, which lactobacilli tolerate better than lactococci [2]. Nowadays, as we mentioned above, Ibores cheeses are manufactured with lower salt concentrations and have somewhat higher pHs than before, this fact probably being the main cause for the changes in the two types of lactic acid bacteria.

The distribution throughout ripening of the identified species is given in Table IV. Lactococci were isolated throughout ripening as the major group. Nearly all were identified as *Lactococcus lactis* ssp. *lactis*;

Table IV. Changes in distribution of species of lactic acid bacteria during ripening of Ibores cheese.

Species	Days of ripening				
	3	15	30	60	Total
Lactococcus	46[a]	42	36	33	157
L. lactis ssp. *lactis*	34	42	24	26	126
L. lactis ssp. *lactis*[b]	12	0	10	7	29
L. lactis ssp. *cremoris*	0	0	1	0	1
L. raffinolactis	0	0	1	0	1
Enterococcus	24	26	14	7	71
E. faecium	13	17	7	5	42
E. faecalis	10	9	6	2	27
E. avium	1	0	1	0	2
Leuconostoc-Weissella	4	26	31	12	73
L. mesenteroides ssp. *dextranicum*	2	17	16	6	41
L. mesenteroides ssp. *mesenteroides*	0	3	6	2	11
W. paramesenteroides	2	6	9	4	21
Lactobacillus	0	7	3	19	29
L. casei	0	4	1	13	18
L. plantarum	0	2	1	4	7
L. brevis	0	0	1	1	2
L. curvatus	0	1	0	0	1
L. coryniformis	0	0	0	1	1

a: number of isolates; b: growth in 6.5% NaCl.

only one isolate was characterized as *Lc. lactis* ssp. *cremoris*, and one was assigned to *Lc. raffinolactis.* Among isolates with the *Lactococcus* phenotype, 18.5% grew in the presence of 6.5% NaCl. This characteristic, considered to be typical of enterococci, has also been described for lactococci [5, 27], and indeed many wild strains of lactococci isolated from cheeses show this property [3, 29, 30]. The isolation of *Lc. lactis* ssp. *lactis* as almost the sole representative of the genus *Lactococcus* is reported frequently for raw milk cheeses [18, 19, 22, 30, 33]. It is also in agreement with the observations of Weerkamp et al. [35] on the predominance of *Lc. lactis* ssp. *lactis* among lactococci isolated from raw milk and naturally fermented products.

The most frequently detected species of the genus *Enterococcus* was *E. faecium*, especially during the early ripening, followed by *E. faecalis* and *E. avium* which were much less abundant. Indeed, *E. faecium* and *E. faecalis* are generally the commonest species of enterococci isolated in raw milk cheeses but their proportions vary depending on the cheese variety [6, 18, 22, 30, 33].

The dextran producer *Leuconostoc mesenteroides* ssp. *dextranicum* accounted for more than 56% of the leuconostoc-weissella isolates, followed by *Weissella paramesenteroides* with 28.8% and, to a lesser extent, *Ln. mesenteroides* ssp. *mesenteroides*.

Lactobacilli were detected only after 15 d of ripening. *Lactobacillus casei* was the main isolate, while *Lb. plantarum* was found in lower proportions. Two isolates were identified as *Lb. brevis*, one isolate as *Lb. curvatus,* and another as *Lb. coryniformis*. Although both *Lb. casei* and *Lb. plantarum* are usually isolated as the main lactobacilli in cheeses, it is the latter which predominates in other goat's milk cheeses, including the Ibores cheese studied some years ago [18, 22, 36]. This probably reflects the greater tolerance of *Lb. plantarum* to the salt and low pHs [4] which are common conditions in those cheeses.

The technological and genetic characterization of these lactic acid bacteria isolates from Ibores cheeses is now at an advanced stage (unpublished results). The following step will be to choose some of these isolates as candidates to be used as autochthonous starters.

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