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Changes in the free amino acid content during ripening of Idiazabal cheese: influence of starter and rennet type

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Abstract

The effect of starter and rennet type on free amino acid release during ripening of Idiazabal cheese was studied. Four batches of cheeses were manufactured depending on the rennet used, commercial calf rennet or artisanal lamb rennet, and the addition or not of starter culture. Cheese samples contained 24 individual free amino acids Leu, Glu, Val and Phe showing the highest contents during the ripening. The results indicated that the release of the free amino acids during ripening was strongly affected by starter added to the cheeses, and that this effect varied markedly with the rennet used for cheesemaking. Total amounts of free amino acids were higher for the cheeses made with commercial calf rennet than for those made with artisanal lamb rennet, regardless of starter addition. Likewise, the highest total free amino acid levels were found in the cheeses made with starter, regardless of the type of rennet used.

Keywords: Cheese ripening; Free amino acids; Starter; Rennet type

1. Introduction

Proteolysis during cheese ripening strongly affects the sensory properties of the cheese (Grappin, Rank & Olson, 1985; Law, 1987). Proteinases and peptidases released from the rennet and starter are some of the principal proteolytic enzymes acting during cheesemaking and ripening (Fox & Stepaniak, 1993).

Many studies have focused on the proteolytic activity of enzymes in rennet during cheesemaking. Several authors have reported that rennet proteinases hydrolyze caseins mostly yielding high molecular weight fractions and few free amino acids (O'Keeffe, Fox & Doly, 1978). Pitchard and Coolbear (1993) reported that the high molecular weight peptides released first by the rennet proteinases were the substrates for the proteolytic activity of the starter proteinases, leading to low molecular weight peptides and free amino acids. In many types of cheeses, particularly sheep cheeses, artisanal lamb rennets have been used (Anifantakis & Green, 1980). Nevertheless, no information on the properties and characteristics of these artisanal rennets is found in the revised literature. The use of artisanal rennets often entails problems concerning curd formation and final characteristics of the cheese, probably because the rennet activities are not standardized. Therefore, many manufacturers are replacing the artisanal lamb rennet by standardized commercial calf rennets.On the other hand, the use of recombinant chymosin as coagulant yielded cheeses with similar characteristics and quality to those of the traditional Cheddar or Gouda cow cheeses (van der Berg & de Koning, 1990; Bines, Young & Law, 1989).

Although starters are usually added to milk for acidifying cheese paste, their proteolytic activity during cheese ripening has been reported. It has been pointed out that proteinases released from the starter are capable of hydrolyzing β-casein and, to a lesser extent, α_{s1}-I-casein (Visser & Groot-Mostart, 1977). On the other hand, addition of lactic acid bacteria (LAB), as a frequently used starter produced a higher content of short-chain

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peptides and free amino acids during cheese ripening (Lee, Laleye, Simard, Munsch & Halley, 1990; Visser & Groot-Mostart, 1977). Also, due to the higher level of proteolysis caused by LAB Cheddar cheese taste intensified as ripening time increased (Broome, Krause & Hickey, 1990; Lane & Fox, 1996; McSweeney, Walsh, Fox, Cogan, Drinan & Castelo-González, 1994).

Idiazabal cheese is a typical product of the Basque Country region of Northern Spain and is manufactured from raw ovine milk according to the specifications of its Denomination of Origin Regulatory Board (Ministerio de Agricultura, Pesca y Alimentación, 1993). Traditionally, Idiazabal cheese has been manufactured with artisanal lamb rennet, although commercial calf rennets are nowadays widely used by artisanal and industrial manufacturers alike. Nevertheless, cheeses made with artisanal lamb rennets show specific sensory characteristics distinguishable from the cheeses made with commercial calf rennet. The regulatory board does not specify the type of rennet to use as coagulant for Idiazabal cheesemaking although it is particularly interested in promoting the use of lamb rennets in order to achieve a more distinct and typical product. On the other hand, Idiazabal manufacturers usually add starter cultures to milk for acidifying the cheese paste and inhibiting the growth of pathogens and other microorganisms.

The objective of this work was to study the effect of starter and rennet type on free amino acid release during ripening of Idiazabal cheese. The use of different rennet types together with the addition or not of the starter will affect the sensory characteristics of the cheese and therefore its quality.

2. Materials and methods

2.1. Cheese samples

Cheeses were made in the plant of Quesería Dorrea (Dorrea, Navarra, Spain). Bulk ewe's milk was from several local flocks of *latxa* sheep collected in May and used herein for cheesemaking. Cheeses were manufactured according to the traditional method for the production of Idiazabal cheese approved by its Denomination of Origin (Ministerio de Agricultura, Pesca y Alimentación, 1993).

From the same lot of bulk ewe's raw milk, four experimental vats, of 600 l each, were made, varying with the rennet used and the addition or not of starter culture: batch 1, commercial calf rennet and starter; batch 2, commercial calf rennet without starter; batch 3, artisanal lamb rennet and starter; batch 4, artisanal lamb rennet without starter. The commercial calf rennet (Bovigranel, Barcelona Spain) consisted of 430 mg of chymosin and 650 mg of pepsin dissolved in 200 ml of water and 180 ml was used per vat. The artisanal rennet was prepared from the stomachs of *latxa* lambs sacrificed during the first month of lactation, cleaned, dried, ground, salted (500 g/kg) and redissolved in water (125 g/l). This solution (600 ml) was added to vats. The composition of the artisanal rennet was 78±2% chymosin and 22±2% pepsin, determined according to the International Dairy Federation Standard 110 A (IDF, 1987). The starter was a homofermentative mixture of *Lactococcus lactis* ssp *lactis* and *Lactococcus lactis* ssp *cremoris* (Eurozime-Ezal, Paris, France). A total of 4 I.U. of the starter culture were used per vat for batches 1 and 3. Both rennet and starter were added to the vat after heating the milk at 30°C. After moulding and pressing, cheeses were brined in a saturated solution of salt (180 g/l) during 18 h. Cheeses were ripened at 8–10°C and a relative humidity of around 85% for 180 days. Around 75 cheeses (1.0–1.2 kg) were obtained per vat.

For each batch, 20 cheeses were analysed from days 1, 15, 30, 45, 60, 75, 90, 120, 150 and 180 of ripening, with entire cheeses used for analyses on each sampling day. Cheese samples were wrapped in plastic film and aluminium foil and frozen at −35°C until analysed.

2.2. Free amino acid analysis

Extraction of free amino acids from the cheese samples was carried out according to the method of Krause, Bockhardt, Neckermann, Henle and Klostermeyer (1995). Free amino acids were analysed by high performance liquid chromatography (HPLC) as phenylthiocarbamyl derivatives using methionine sulphone (Sigma Chemical, St Louis, USA) as internal standard. HPLC equipment (Waters, Milford, USA) consisted of two model M510 pumps, an Ultra WISP 715 automatic injector, a TCM temperature controlled oven, and a M996 UV/VIS diode-array detector. The analysis was performed using a dimethyloctadecylsilane column (300× 3.9 mm, pico Tag, Waters) held at 46±0.2°C. The chromatographic procedure employed non-linear gradient elution using a mobile phase of 100% solution A changing to 100% solution B in 67 min, with a flow-rate of 1.0 ml/min. Solution A was 20 ml/l methanol (HPLC grade, Scharlau, Barcelona, Spain) in 70 nM sodium acetate (Merck, Darmstadt, Germany), adjusted to pH 6.55 with acetic acid (Merck). Solution B was a mixture (v/v) of 450 mL acetonitrile (HPLC grade, Scharlau), 400 ml water (MilliQ grade) and 150 ml methanol (HPLC grade, Scharlau). A volume of 20 µl of the phenylthiocarbamylamino acids was injected into the column and detected at 254 nm.

A calibration solution of free amino acids (Sigma) containing the internal standard was analysed in quintuplicate to calculate the relative response factors. Identification of the cheese amino acids was carried out by comparing retention times for pure standards. HPLC analyses were done in duplicate.

2.3. Statistical analysis

SPSS statistical package, version 6.1 (SPSS Inc., Michigan, USA), was used for the statistical analysis. A three-way split-plot analysis of variance (ANOVA) was applied considering rennet type and addition of starter as factors, and ripening time as split-plot factor. Stepwise discriminant analysis was applied to establish those amino acids capable of discriminating the samples from different batches. Wilk's lambda criterion was used for selecting discriminant variables. Simple linear regression analyses were applied to fit the evolution of free amino acids with ripening time.

3. Results and discussion

Cheese samples contained 24 individual free amino acids for all the batches studied (Fig. 1). Leu, Glu, Val and Phe were the main free amino acids during ripening in all batches, representing around 50% of the total free amino acid mean content. Other authors found these same amino acids as the principal ones in other ripened cheeses (Lavanchy & Sieber, 1993; Pardo, Pérez, Gómez, Tardeguila, Martínez & Serrano, 1996; Ramos, Cáceres, Polo, Alonso & Juárez, 1987; Steefen, Eberhard, Bosset & Rüeg, 1993).

In order to clarify the effect of the addition of starter and rennet type on the free amino acid content in cheese, it must be stated that the cheeses contained organisms from the indigenous microflora of the milk and from the cheesemaking plant. Thus, the changes observed on the proteolysis process during cheese ripening will be a reflection of the shift in the microflora of the different batches of cheeses.

The results of the ANOVA (Table 1) showed that the principal effects rennet type, addition of starter and ripening time were significant ($P \leqslant 0.05$) for the free amino acids. The interaction term between rennet type and addition of starter was significant ($P \leqslant 0.001$) for total free amino acids (TFA) and all of the individual free amino acids, except for 1H-Lys, which indicated a combined effect of these two technological factors on the proteolysis during cheese ripening. Likewise, the interaction term between ripening time and each of the other principal effects was significant ($P \leqslant 0.05$) in all cases, indicating, as expected, the influence of the ripening time on the technological factors. Table 1 shows the effect size for rennet type, addition of starter and ripening time with generally high values (< 0.7) for the free amino acids. The value of the effect size was low (< 0.5) only for 1H-Lys with respect to rennet type and this same amino acid and Tau with respect to starter addition. On the other hand, all of the individual free amino acids and TFA were significantly affected ($P \leqslant 0.001$) by the three factors simultaneously.

The effects of the rennet type and the addition of the starter on free amino acid content during cheese ripening are plotted in Figs. 2 and 3, respectively. The TFA levels were higher for the cheeses made with commercial calf rennet, regardless of starter addition (Fig. 2). Likewise, the TFA levels were higher for the cheeses made

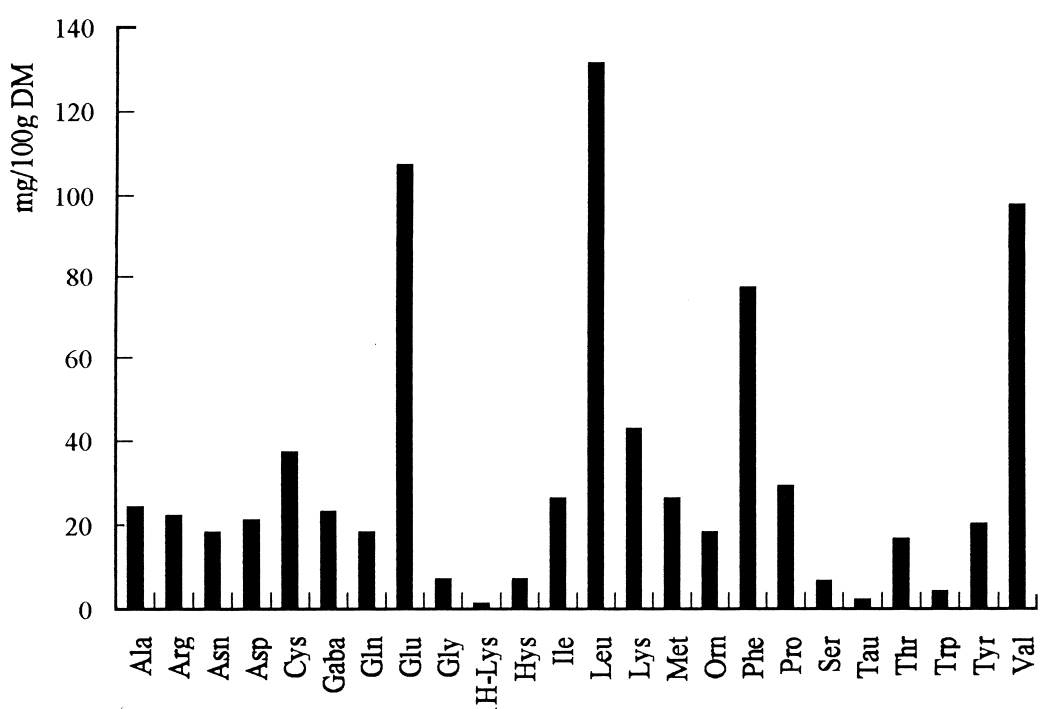

Fig. 1. Free amino acid mean content for Idiazabal cheese.

Table 1
Significance levels and size effect of the three-way analysis of variance for the effects rennet type (R), addition of starter (S) and ripening time (T)

Amino acids	R		S		T		RS		RT		ST		RST	
	Sig[b]	Effect size	Sig	Effect size	Sig	Effect size	Sig	Effect size	Sig	Effect size	Sig	Effect size	Sig	Effect size
Ala	***	0.958	***	0.973	***	0.990	***	0.746	***	0.809	***	0.721	***	0.734
Arg	***	0.635	***	0.980	***	0.993	***	0.948	*	0.364	***	0.952	***	0.927
Asn	***	0.989	***	0.998	***	0.999	***	0.995	***	0.998	***	0.998	***	0.997
Asp	***	0.912	***	0.995	***	0.999	***	0.937	***	0.917	***	0.984	***	0.847
Cys	***	0.943	***	0.849	***	0.993	***	0.631	***	0.996	***	0.993	***	0.994
Gaba	***	0.878	***	0.960	***	0.989	***	0.994	***	0.850	***	0.962	***	0.900
Gln	***	0.939	***	0.998	***	0.995	***	0.987	***	0.965	***	0.995	***	0.972
Glu	***	0.862	***	0.991	***	0.997	***	0.890	***	0.886	***	0.965	***	0.948
Gly	***	0.782	***	0.672	***	0.996	***	0.971	***	0.918	***	0.902	***	0.964
1H-Lys	*	0.113	***	0.468	***	0.723	NS	0.003	***	0.698	***	0.694	***	0.657
Hys	***	0.987	***	0.992	***	0.996	***	0.986	***	0.958	***	0.944	***	0.967
Ile	***	0.978	***	0.990	***	0.995	***	0.966	***	0.840	***	0.982	***	0.868
Leu	***	0.985	***	0.993	***	0.999	***	0.919	***	0.967	***	0.982	***	0.917
Lys	***	0.974	***	0.994	***	0.998	***	0.850	***	0.893	***	0.986	***	0.785
Met	***	0.963	***	0.964	***	0.994	***	0.875	***	0.852	***	0.931	***	0.775
Orn	***	0.775	***	0.989	***	0.995	***	0.975	***	0.748	***	0.968	***	0.977
Phe	***	0.987	***	0.989	***	0.998	***	0.949	***	0.954	***	0.979	***	0.800
Pro	***	0.979	***	0.987	***	0.992	***	0.917	***	0.944	***	0.937	***	0.775
Ser	***	0.946	***	0.967	***	0.985	***	0.956	***	0.940	***	0.953	***	0.912
Tau	*	0.132	***	0.704	***	0.940	***	0.790	***	0.886	***	0.768	***	0.878
Thr	***	0.974	***	0.977	***	0.987	***	0.974	***	0.826	***	0.816	***	0.863
Trp	***	0.965	***	0.980	***	0.990	***	0.866	***	0.914	***	0.943	***	0.819
Tyr	***	0.930	***	0.974	***	0.988	***	0.840	***	0.825	***	0.915	***	0.533
Val	***	0.989	***	0.988	***	0.999	***	0.967	***	0.967	***	0.925	***	0.728
TFA[a]	***	0.979	***	0.995	***	0.999	***	0.761	***	0.891	***	0.978	***	0.902

[a] TFA = total free aminoacids.
[b] ***$P \leqslant 0.001$, ** $P \leqslant 0.01$, * $P \leqslant 0.05$; NS: not significant.

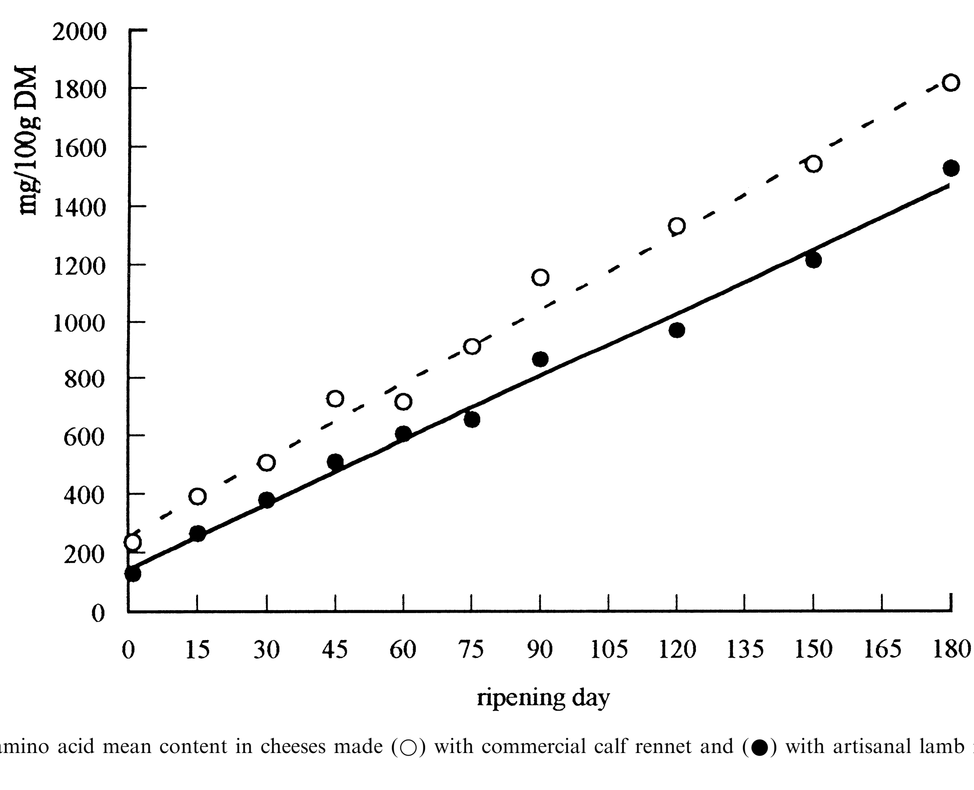

Fig. 2. Total free amino acid mean content in cheeses made (○) with commercial calf rennet and (●) with artisanal lamb rennet during cheese ripening.

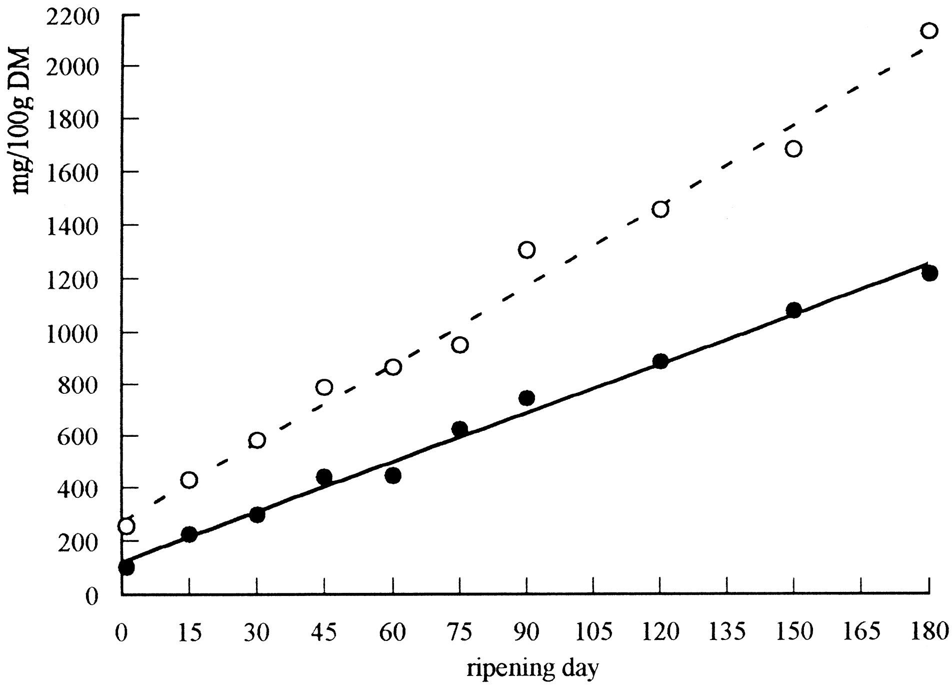

Fig. 3. Total free amino acid mean content in cheeses made with addition of (○) starter and (●) without starter during cheese ripening.

with starter, regardless of the type of rennet used (Fig. 3). Similar behaviour was found for most of the individual free amino acids studied. The effect of these two technological factors on Ser and Tau were close to the effect on TFA but their increase during ripening was rather different. So, the content of Tau decreased with cheese ripening and Ser presented a maximum around day 60. On the other hand, higher contents of Asn and Gly were found in artisanal lamb rennet cheeses at the end of the ripening period studied. Gly also showed higher contents in the cheeses made without starter at the end of the ripening. Nevertheless, other free amino acids, such as Cys and 1H-Lys, did not show a clear pattern for the effect of rennet type and addition of starter during cheese ripening.

As the results of the ANOVA have shown, a combined effect of rennet type and addition of starter on the proteolysis process was significant during cheese ripening. Stepwise discriminant analysis selected 12 amino acids for discriminating among the four cheese batches made with different rennet types, with or without starter (batches 1, 2, 3 and 4). Table 2 shows the standardized coefficients of these 12 amino acids for the three discriminant canonical functions obtained. The amino acids Lys and Ala contributed the most to canonical function 1 while Pro and Tyr contributed mainly to canonical function 2, and Asp and Lys to canonical function 3. Fig. 4 plots cheese sample distribution in the two-dimensional coordinate system defined by canonical functions 1 and 2. As can be observed, the four batches of cheeses were correctly classified according to the rennet type and addition or not of the starter (100% of the total cases). The distances between the group centroids corresponding to batches without starter culture (batches 2 and 4) were lower than the distance corresponding to batches with starter culture (batches 1 and 3). On the other hand, the value of function 1 for the batch made with commercial calf rennet and starter (batch 1) was much higher (6.36), in absolute value, than for the other three batches. The same occurred for the value of function 2 for the batch elaborated with artisanal lamb rennet and starter (batch 3) (4.02) compared to the other batches. These results indicated that the release of the free amino acids during ripening was strongly affected by the starter added to the cheeses, and

Table 2
Amino acids selected for discriminating among the four batches of cheeses and their standardized canonical discriminant function coefficients

	Functions		
	1	2	3
Ala	3.185	−0.627	0.750
Asp	−2.238	3.503	−4.035
Gaba	−1.097	0.910	1.629
Gln	0.240	2.129	0.707
Lys	−4.766	−1.738	−0.867
Met	2.130	−2.300	3.575
Phe	2.672	−2.869	0.038
pro	0.662	5.723	0.436
Tau	1.092	0.389	0.026
Thr	1.336	−0.649	−0.213
Tyr	1.856	−4.534	1.229
Val	−3.017	1.554	−2.600

that this effect depended markedly on the rennet type used for cheesemaking.

In order to know the combined effect of the addition of starter to the cheeses made with commercial calf rennet or artisanal lamb rennet, the changes in TFA and discriminant free amino acid contents during ripening were studied. Fig. 5 depicts the changes in TFA content during ripening for the four batches of cheeses. The

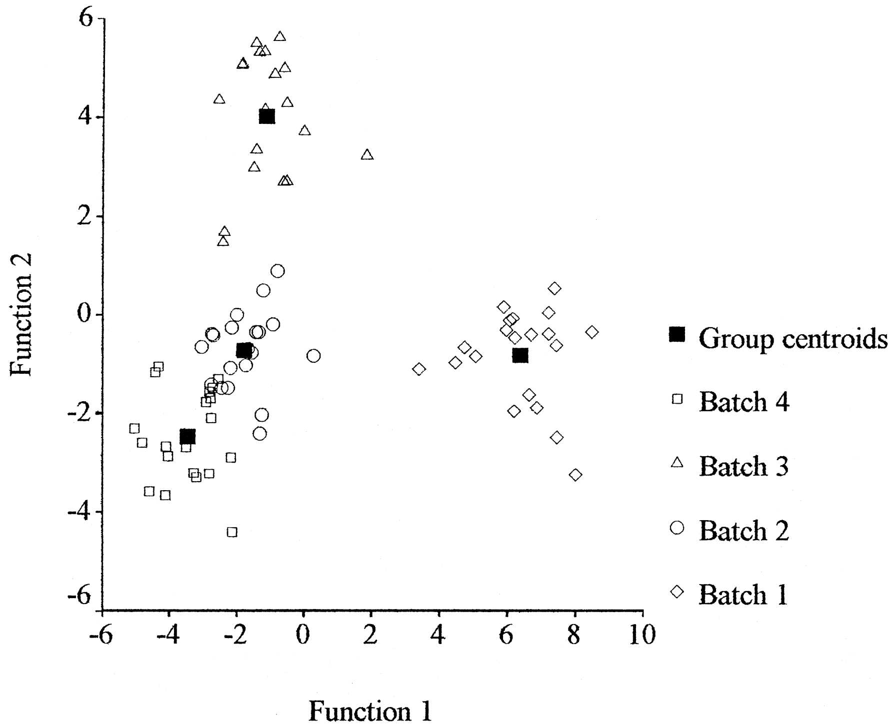

Fig. 4. Cheese sample distribution in the two-dimensional coordinate system defined by canonical discriminant functions 1 and 2. Batch 1: commercial calf rennet and starter; batch 2: commercial calf rennet without starter; batch 3: artisanal lamb rennet with starter; batch 4: artisanal lamb rennet without starter.

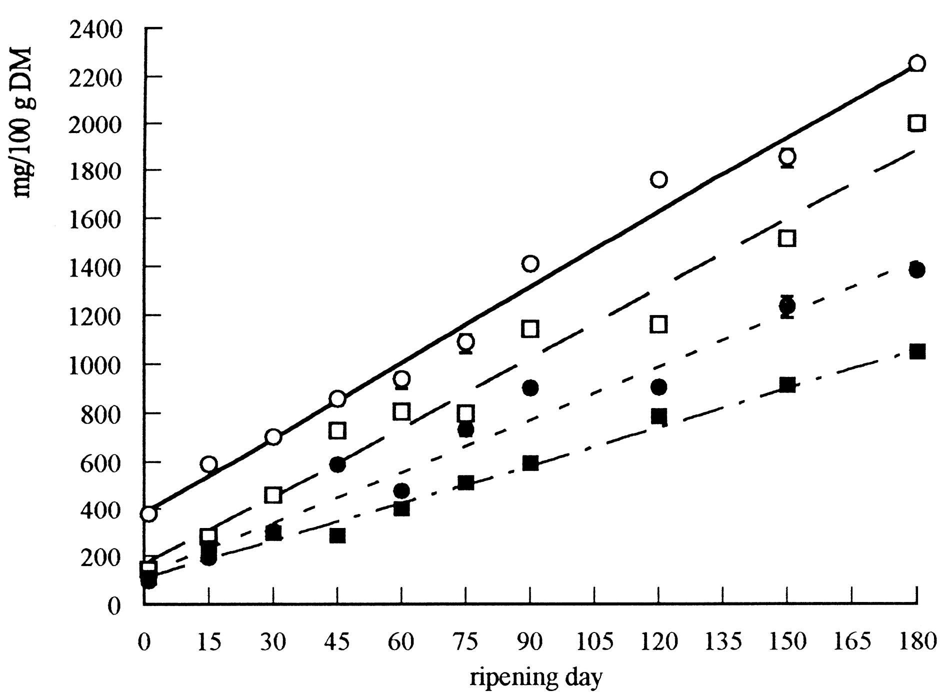

Fig. 5. Total free amino acid content in cheeses made with commercial calf rennet (○) with starter (batch 1) and (●) without starter (batch 2), and in cheeses made with artisanal lamb rennet (□) with starter (batch 3) and (■) without starter (batch 4).

TFA content increased progressively with ripening time according to a linear model for all of the batches. Similar observations have been reported by other authors (Barcina, Ibáñez & Ordóñez, 1995; Lane & Fox, 1996). At the end of the ripening period, the batch made with commercial calf rennet with starter (batch 1) showed the highest TFA content, followed by the batch elaborated with artisanal lamb rennet with starter (batch 3), the batch elaborated with commercial calf rennet (batch 2), and the batch elaborated with artisanal lamb rennet

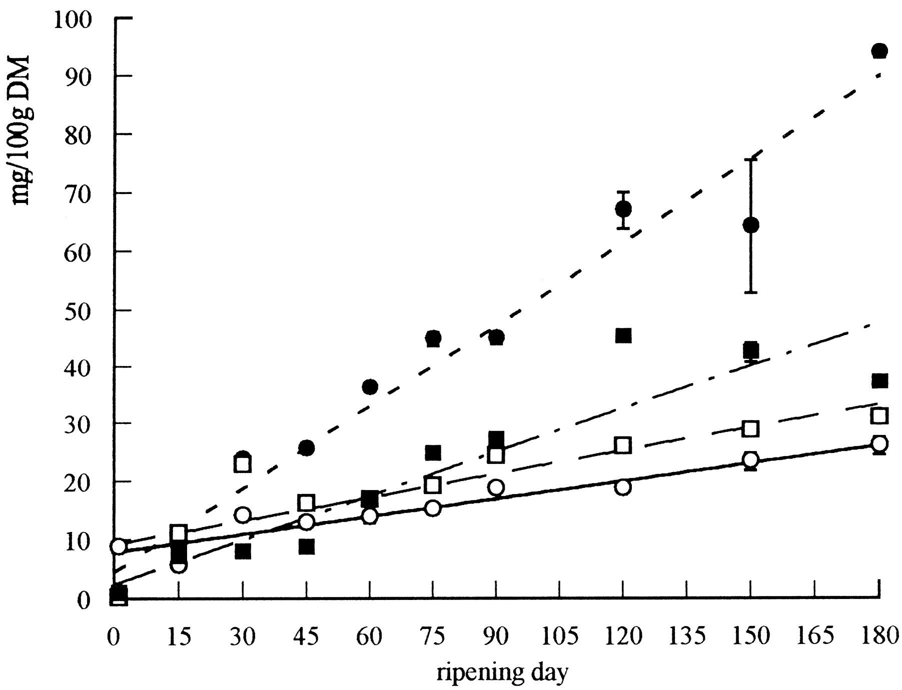

Fig. 6. Gaba content in cheeses made with commercial calf rennet (○) with starter (batch 1) and (●) without starter (batch 2), and in cheeses made with artisanal lamb rennet (□) with starter (batch 3) and (■) without starter (batch 4).

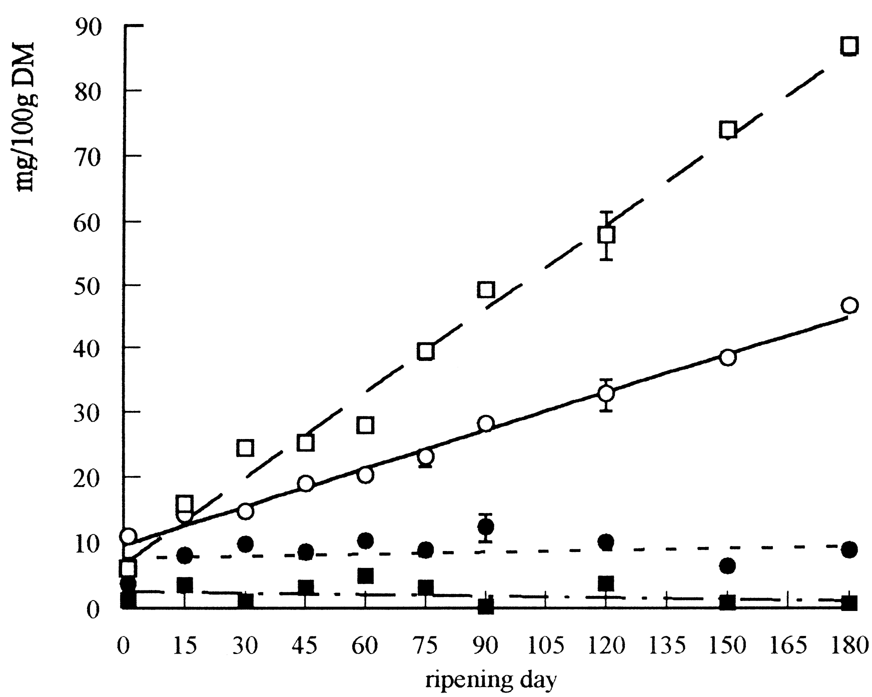

Fig. 7. Gln content in cheeses made with commercial calf rennet (○) with starter (batch 1) and (●) without starter (batch 2), and in cheeses made with artisanal lamb rennet (□) with starter (batch 3) and (■) without starter (batch 4).

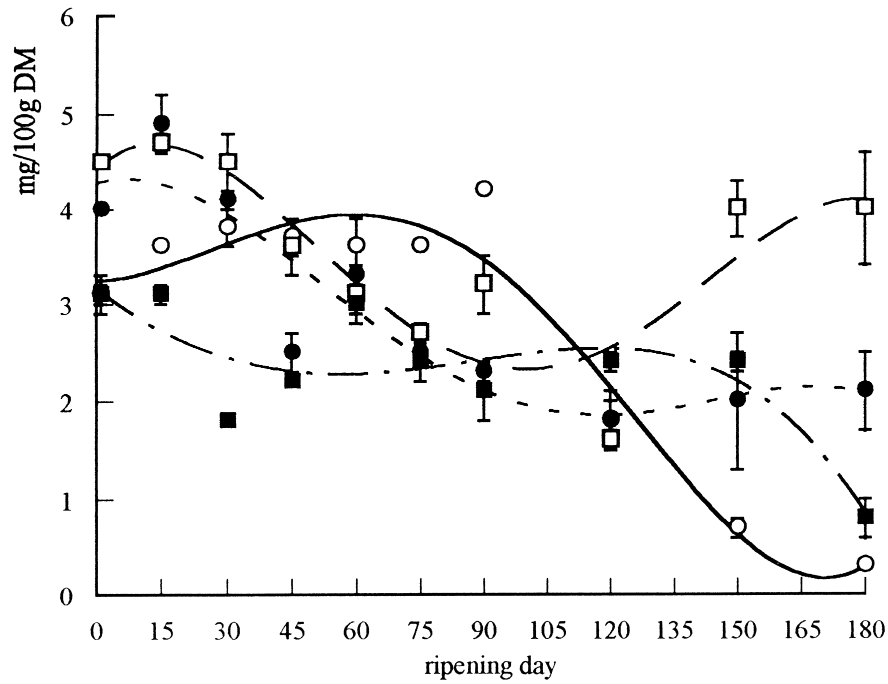

Fig. 8. Tau content in cheeses made with commercial calf rennet (○) with starter (batch 1) and (●) without starter (batch 2), and in cheeses made with artisanal lamb rennet (□) with starter (batch 3) and (■) without starter (batch 4).

(batch 4), respectively (Fig. 5). As expected, these results suggest that the effect of the starter enzymes on release of free amino acids during ripening was more important than the effect of the rennet enzymes. It can also be inferred that the effect of the starter enzymes was added to the effect of the rennet enzymes on the release of free amino acids during cheese ripening.

The changes in the contents of the discriminant amino acids Ala, Asp, Lys, Met, Phe, Pro, Thr, Tyr and Val with ripening time were very similar to that of TFA in the four batches of cheeses. Nevertheless, the patterns of behaviour of Gaba, Gln and Tau were rather different. The content of Gaba increased linearly with ripening time for all of the batches but at the end of the ripening period its content in the batch made with artisanal lamb rennet and starter (batch 3) was the lowest (Fig. 6). Also, the batch elaborated with artisanal lamb rennet (batch 4) had a higher content of Gaba than the batch elaborated with commercial calf rennet (batch 2). The content of Gln increased linearly with ripening time only in the batches made with starter (batches 1 and 3) (Fig. 7). Otherwise, as occurred for Gaba, the batch elaborated with artisanal lamb rennet and starter (batch 3) had a higher content of Gln than the batch made with commercial calf rennet and starter (batch 1). On the other hand, the Gln content in the batches elaborated without starter (batches 2 and 4) remained practically unchanged (Fig. 7). Tau was the only free amino acid which decreased during cheese ripening in the four batches, following an oscillating change (Fig. 8). Nevertheless, as Tau is not a protein amino acid, its evolution with ripening does not reflect proteolysis in cheese.

4. Conclusions

The free amino acid content of the Idiazabal cheese was considerably affected by the rennet type used and the addition, or not, of the starter culture. The free amino acid contents were higher when the cheeses were made with commercial calf rennet and when starter culture was added for cheesemaking. On the other hand, the effect of the starter enzymes on release of free amino acids during ripening was more important than the effect of the rennet enzymes. It can also be inferred that the effect of the starter enzymes was added to the effect of the rennet enzymes on the release of free amino acids during cheese ripening.

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