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Lipolysis and Proteolysis During the Ripening of Fresh Moroccan Goats' Milk Cheese

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Abstract: Lipolysis, primary proteolysis and secondary proteolysis during ripening of a goats' milk cheese manufactured with commercial starter (A), commercial rennet and starter culture (B) and starter culture (C) were studied. The concentration of acetic acid and total C4:0–C18:2 FFA in cheeses A, B and C, increased throughout ripening. The total FFA contents in cheese A and B was significantly higher ($P < 0.05$) from those in cheese C throughout ripening. The short chain FFAs represented approximately 13, 14 and 14% of the TFFA content in cheeses A, B and C respectively, at 5 d. The percentage of medium chain FFAs (C10:0–C14:0) representing approximately 28, 29 and 38% of total FFAs in cheeses A, B and C respectively, at 5 d. The percentages of long chain FFAs (C16:0–C18:2) represented approximately 59, 58 and 48% in cheeses A, B and C respectively, at 5 d. In Moroccan goat's cheese, intense proteolytic activity took place during ripening. In the cheeses produced with commercial rennet and starter culture, proteolysis was more intense and after a 5-day ripening period values of 7.15, 12.51 and 6.11 for TN (Total Nitrogen), water-soluble nitrogen (WSN) at pH 4.6 and non-protein nitrogen (NPN) were obtained.

Key words: Goat Milk Cheese · Lipolysis · Proteolysis · FFA

INTRODUCTION

Cheese ripening is a slow process, involving a concerted series of microbiological, biochemical and chemical reactions. Primary degradation of milk constituents by glycolysis, lipolysis and proteolysis leads to the formation of a whole range of precursors of flavor compounds. These changes are followed and/or overlapped by a series of secondary catabolic reactions, which are responsible for the unique aroma profile of a particular variety of cheese [1].

Cheese making in Africa is largely dictated by tradition. The cheese produced is generally consumed very soon after manufacture, primarily because of the poor shelf-life at ambient temperature. The composition and flavor of cheese is affected by milk composition and Mould Counts were enumerated in cheese samples by milk ripening and cooking of cheese curd cubes and process during manufacture [2].

Lipolysis is one of the major biochemical changes that occur during cheese ripening. The free fatty acids (FFA) released during lipolysis contribute, together with the volatile compounds and the proteolysis products, directly to cheese flavor [3, 4]. The lipolytic agents in cheese are lipolytic enzymes found naturally in milk (milk lipase), rennet (pregastric esterases) (PGE) and microflora [5, 6].

Proteolysis in cheese during ripening plays a vital role in the development of texture as well as flavor. Proteolysis contributes to textural changes of the cheese matrix, due to breakdown of the protein network, decrease in aw through water binding by liberated carboxyl and amino groups and increase in pH (in particular in surface mould-ripened varieties), which facilitates the release of sapid compounds during mastication [7].

Enzymes play a significant role in our life. Their existence had been associated with the history of ancient civilizations. Enzymes from plant and microorganisms have been used in brewing, baking, alcohol production, cheese, vinegar making etc. The uses of enzymes are variable ranging from just making wine or bread to producing complicated fermentation processes [8].

Goat's milk is becoming increasingly important in Morocco, especially because of the popularity of its

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products, in particular cheese. In the mountainous zones of the North of Morocco, the sectors of agriculture and the breeding being of a low productivity and the other economic sectors are developed very little. The populations concerned live under precarious conditions. In these areas, the goat's milk is usually consumed as such however it does not miss assets: dietetic properties of this milk like the strong content of casein beta and it is hypoallergenic.

Lipolysis and Proteolysis in cheese during ripening has been an active area for research in recent years and the literature on the topic has increased substantially in the last decade. The objective of this article was to study the changes which occur and the protein and lipid fractions during ripening of fresh goat cheese from northern of Morocco.

MATERIALS AND METHODS

Cheese Making and Sampling: Three batches of cheese (four cheeses of each batch) were manufactured with unpasteurized goat milk by 3 different cheesemakers, according to the traditional method. The experimental design was three blocks of batches, as follows:

Batch A: Raw-milk batch manufactured with commercial rennet (1/10,000 strength) and without added starter.

Batch B: Experimental batch manufactured with commercial rennet (1/10,000 strength) and a commercial starter composed by Lc. lactis subsp. lactis, Lc. lactis subsp. cremoris, Lc. lactis subsp. lactis var. diacetylactis strains.

Batch C: Experimental batch manufactured with commercial starter composed by Lc. lactis subsp. lactis, Lc. lactis subsp. cremoris, Lc. lactis subsp. lactis var. diacetylactis strains and without added rennet.

For the free fatty acid and proteins analysis, samples of cheeses at 1 and 5 d were taken from each batch.

Free Fatty Acid Analysis: Lipid extraction was carried out on acidified cheese (10 g) slurry, using ethyl ether, followed by methylation with 20% of sodium hydroxide in methanol [9]. Two extractions were carried out per sample.

Gas chromatography was performed on a Varian model 3800 GC instrument fitted with an automatic sampler (CP Wax 52CB) for multiple injections. Free fatty acid (FFA) were analyzed on a fused silica column (30 m x 0.32 mm x 0.5 μm) equipped with a PTV injector, with helium as the carrier gas (split ratio 1:20). Oven temperature was held at 60 °C for 2 min, then raised to 180 °C at a rate of 5°C/min and held at this temperature for 60 min. Other details were as described by De la Fuente *et al*. [9]. The FFA determinations were done in duplicate for each sample.

Nitrogen Fractions: Total Nitrogen (TN) was measured using the Kjeldahl method; Water Soluble Nitrogen at pH 4.6 (WSN) and Non-Protein Nitrogen (NPN) in 12% trichloroacetic acid were determined according to Ardö [10] and then quantified using Kjeldahl method [11]. All analyses were performed in triplicate.

Electrophoretic Analysis: The degradation of caseins was studied using PAGE, 0.5g of the cheese was homogenized in 0.1M Tris-HCl, pH 7.2; 3 minutes of grinding is performed using a high-speed mill (Ultra-Turrax). The mixture is then acidified to pH 4.4 using a solution of hydrochloric acid 1 N. After a few minutes of stirring, the precipitated proteins were recovered by centrifugation (10 000 g). The pellet is dissolved in 5 ml of urea 9 M containing 1% of α-mercapto-ethanol [12].

Gel Electrophoresis: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a vertical slab gel apparatus as described by Laemmli [13]. The samples homogenized in 0.2 M NaCl were centrifuged during 20 min at 8 000 x g and the pellet was separated from the supernatant; then both fractions were denaturated by boiling in an equal volume of SDS buffer (2% SDS in 0.0625 M Tris-HC1 10% glycerol 5% 3- mercapto-ethanol pH 7) and run on a 12% acrylamide gel in 0.4 M Tris-HCl buffer at pH 8.8, with constant current set at 20 mA/gel. The gels were stained with Coomassie blue, destained in acetic acid-methanol and scanned.

Statistical Analysis: Data were subjected to one-way analysis of variance (ANOVA) using Statistical Software. Post hoc testing was carried out using the Tukey test. A significant level of 0.05 was used for all statistical tests.

RESULTS AND DISCUSSION

FFA Profile: Table 1 shows the concentration of total free fatty acids in fresh goat's cheeses.

Table 1: Changes in free fatty acids (mg kg/1)[A] during ripening of fresh Moroccan goat's cheeses

	1 d			5 d		
	A	B	C	A	B	C
C2 :0	120±9[a]	125±11[a]	75±10[b]	135±12[a]	145±15[a]	95±10[b]
C4 :0	145±18[a]	150±13[a]	55±8[b]	210±9[a]	225±11[a]	101±6[b]
C6 :0	155±6[a]	165±10[a]	101±4[b]	192±10[ab]	210±5[a]	185±7[b]
C8 : 0	85±2[a]	82±4[ab]	75±3[b]	115±6[a]	125±8[a]	92±3[b]
Total C2 :0-C8 :0	475±12[b]	522±10[a]	306±8[c]	652±10[b]	705±12[a]	473±7[c]
C10 :0	325±4[b]	355±6[a]	185±1[c]	425±5[b]	450±10[a]	225±3[c]
C12 :0	105±5[a]	95±5[a]	45±1[b]	210±6[a]	235±8[a]	75±4[b]
C14 :0	501±10[a]	495±6[a]	225±2[b]	775±11[a]	735±10[a]	515±7[b]
Total C10 :0-C14 :0	931±18[a]	945±12[a]	455±8[b]	1410±13[a]	1420±10[a]	1288±9[b]
C16 :0	475±18[b]	575±10[a]	315±3[c]	685±12[b]	715±17[a]	512±5[c]
C18 :0	712±13[a]	675±19[b]	312±6[c]	925±15[a]	895±22[a]	525±6[b]
C18 :1	745±10[a]	735±7[a]	265±1[b]	1025±19[a]	1015±12[a]	485±11[b]
C18 :2	135±3[a]	125±1[b]	62±2[c]	325±8[a]	302±6[b]	94±3[c]
Total C16 :0-C18 :2	1767±20[b]	2110±32[a]	954±15[c]	2960±11[a]	2927±11[a]	1616±11[a]

[a,b] Means of pairs in the same row with different superscripts are significantly different ($P < 0.05$).

[A] Mean values (±SD) of four cheese-making trials.

The concentration of acetic acid and total C4:0–C18:2 FFA in cheeses A, B and C, increased throughout ripening, showing the significant effect of the ripening stage on cheese lipolysis (Table 1). Acetic acid contributes greatly to the final flavor of cheese and is the major volatile acid extracted with FFAs. It is not produced from lipolysis by lipases but from several biochemical pathways. It is formed during the early stages of ripening and is probably a product of citrate or lactate fermentation or of amino acid catabolism by bacteria [3, 14].

In general, the total FFA content found in the cheeses of the present study was significantly lower than those reported for industrial type Feta cheese or Teleme cheese from mixture of ewes/goats milk [15-17].

This variation may be due to differences in processing between the factories of origin and, perhaps, differences in the initial level of lipolysis in the milk used in cheese manufacture. Sousa *et al*. [7] reported that the initial degree of lipolysis in goat's milk influenced lipolysis levels in fresh cheeses.

The total FFA contents in cheese A and B was significantly higher ($P < 0.05$) from those in cheese C throughout ripening. This may be due to the higher lipolytic activity of the rennet used in cheese A and B, since the rennet is one of the major lipolytic agents in cheese ripening [5].

The percentage of this FFA group, including acetic acid (C2:0–C8:0) increased significantly from 1 to 5 d of ripening. The short chain FFAs represented approximately 13, 14 and 14% of the TFFA content in cheeses A, B and C respectively, at 5 d. The percentages of C2:0–C8:0 FFA reported by other authors is contradictory, ranging from 34% [18] up to 58% [15, 16]. The percentage of medium chain FFAs (C10:0–C14:0) representing approximately 28, 29 and 38% of total FFAs in cheeses A, B and C respectively, at 5 d. The percentages of long chain FFAs (C16:0–C18:2) represented approximately 59, 58 and 48% in cheeses A, B and C respectively, at 5 d.

The lipolytic agents in cheese are lipolytic enzymes found naturally in milk (milk lipase), rennet (pregastric esterases) (PGE) and microflora [5, 6]. The contribution of milk lipase to cheese lipolysis depends on the heating of cheese milk, since pasteurization reduces its activity. The contribution of rennet depends on the rennet type. Commercial calf and bovine rennets that are commonly used in the majority of cheese varieties have high lipolytic activities due to their contents of PGE.

Lipases are water soluble enzymes which have the ability to hydrolyze triacylglycerols to release free fatty acids and glycerol. Lipases constitute a major group of biocatalysts that have immense biotechnology applications. Lipases have been isolated and purified from fungi, yeast, bacteria, plant and animal sources. Of all these, bacterial lipases are more economical and stable [19].

Table 2: Changes throughout ripening in nitrogen fractions made of fresh Moroccan goat's cheeses batches, expressed as grams per 100 g of total [A]

	1 d			5 d		
	TN	WSN	NPN	TN	WSN	NPN
A	4.55±0.83[a]	9.25±0.66[a]	4.02±0.51[a]	5.12±0.72[a]	10.95±1.50[a]	5.72±0.85[a]
B	5.75±0.79[a]	10.25±1.03[a]	4.85±0.23[a]	7.15±0.58[b]	12.51±0.77[a]	6.11±0.91[a]
C	3.85±0.33[a]	6.55±0.59[b]	2.85±0.44[b]	4.85±0.64[a]	7.95±1.10[b]	4.15±0.95[a]

[a,b] Means of pairs in the same row with different superscripts are significantly different (P < 0.05).

[A] Mean values (±SD) of four cheese-making trials.

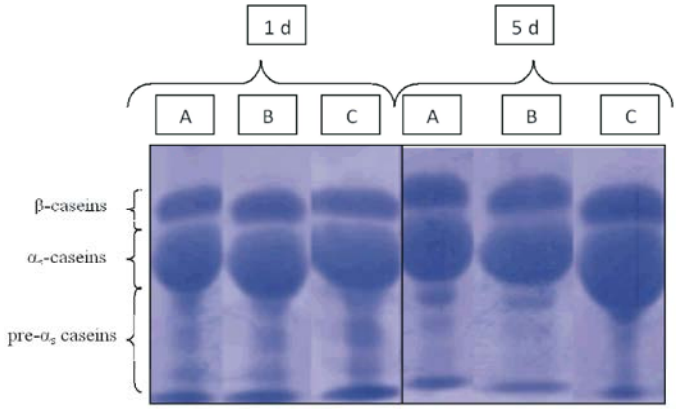

Fig. 1: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the water-insoluble extract of fresh Moroccan goat's cheeses throughout ripening. a: 1 d. b: 5 d.

Proteolytic Parameters:

Secondary Proteolysis: changes in the nitrogen components.

Table 2 shows the average values for the different nitrogen fractions at the beginning and end of ripening.

The proportion of WSN has been regarded traditionally as a ''ripening index'' for cheese because it reflects the extent of proteolysis. So it is an indicator of casein hydrolysis brought about by the action of coagulant enzymes and milk proteases present at the beginning of ripening. Throughout ripening WSN was significantly higher ($p<0.05$) in cheeses B manufactured with Rennet and starter culture compared with cheeses C produced only with starter culture. These high levels of WSN during the early stages of ripening was also reported by other authors for different varieties of goat's' milk cheese [20, 21]. The NPN?? (containing mainly small peptides of 2 and 20 residues and free amino acids) was also significantly higher ($p<0.05$) after 5 days of ripening in cheeses A (5.72 g/100g) and B (6.11 g/100g). Lactic acid bacteria and other enzymes are principal agents for the production of NPN in cheese [22]. The different nitrogen fraction contents in cheese B was significantly higher ($P < 0.05$) from those in cheese A and C throughout ripening. The intense proteolysis produced in cheeses manufactured with commercial rennet and starter culture caused partly by a broader specificity of proteinases of commercial rennet and the action of the enzymes of the starter culture. During ripening period the difference in levels of protein might be accounted for the addition of starter, moisture and/or titratable acidity [23].

Prosekov *et al*.[24] studied the proteolytic activity of lactic acid microorganisms of different taxonomic groups (Lactococcus, Lactobacillus and Leuconostoc) on skim and milk with rennet. It was shown that when milk was cultivated all twelve strains of Lactococcus lactis decompose soluble proteins existing in the environment. It was found that the rennet presence in medium leads to an increase of soluble peptides and slight increase of amino acids.

During ripening proteolysis in cheese is catalyzed by enzymes from (i) coagulant (e.g.. chymosin. pepsin. microbial or plant acid proteinases). (ii) milk (plasmin and perhaps cathepsin D and other somatic cell proteinases). (iii) Enzymes from the starter. (iv) Non starter. or (v) secondary cultures (e.g.. P. camemberti. P. roqueforti. Propionibacterium sp.. B. linens and other coryneforms) and (vi) exogenous proteinases or peptidases or both used to accelerate ripening [7].

Primary Proteolysis: Casein SDS-PAGE of the pH 4.6 insoluble fractions of fresh Moroccan goat's cheeses at 1 and 5 days of ripening were shown in Fig. 1.

We have established three areas of electrophoretic bands from greater to smaller molecular weight: β-caseins (β-CN). α_s-caseins (α_s-CN) and pre-α_s caseins (pre-α_s-CN). The residual coagulant in curd causes degradation of caseins with a specific action on α_s -caseins and a less extensive action on β –caseins [25].

No major changes were observed in the relative percentage of β-caseins throughout ripening. Levels of α_s-caseins dropped between day 1 and 5 for three types of cheese decreasing slightly thereafter. This decrease was slightly more marked in cheese A and B.

In the fresh Moroccan goat's cheeses, the concentration of acetic acid and total C4:0–C18:2 FFA in cheeses A. B and C. increased throughout ripening showing the significant effect of the ripening stage on cheese lipolysis. The total FFA contents in cheese made with commercial rennet and starter culture was significantly higher from those in cheese made without starter throughout ripening. Throughout ripening, TN and WSN were significantly higher in cheeses B manufactured with rennet and starter culture compared with cheeses C produced only with starter culture.

We can conclude that the use of commercial rennet and starter culture as coagulant in traditional goats' cheese led to higher lipolytic and proteolytic activity.

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