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Original Article

Investigation on the Protein Degradation, Free Fatty Acid Content and Area Fraction of Poosti Cheese, Iranian Traditional Cheese Ripened in Skin

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ABSTRACT

Background and Objectives: In this study, the proteolysis and lipolysis of Poosti cheese produced from raw sheep milk in mountainous eastern regions of Iran were investigated during 90 days of ripening.

Materials and Methods: Sodium dodecyl sulfate polyacrylamide gel electrophoresis for proteolysis (SDS-PAGE) and gas chromatography (GC) for free fatty acids (FFAs) were applied to investigate the intensity of lipid degradation. To evaluate the Poosti cheese microstructural changes, the area fraction parameter of the scanning electron microscopy (SEM) micrographs was also calculated by the Image J software.

Results: The most alteration in protein profile was occurred in the first month of aging for high activity of the proteolytic microorganisms in this period. The amount of free fatty acids was depended on their length due to the variety of involved mechanisms. In addition, the microstructural parameter was considerably affected by the aging as a consequence of the effect of salt on the activity of raw milk and skin micro flora.

Conclusions: The decline in proteolysis rate during the last stage of aging could be correlated with the inhibitory effects of salt on the engaged microorganisms, and increase in the pore fraction of the microstructure during the first month of Poosti cheese aging could be due to casein rearrangement and gas release by the fermentative activity of microorganisms.

Keywords: Proteolysis, Lipolysis, Poosti cheese, Raw sheep milk

Introduction

Cheese production from raw sheep milk has a special place among the traditional dairy products in Iran. Lack of heat treatment during the production and ripening are the two major characteristics of this cheese. In addition to this fact that traditional cheeses are good sources of beneficial bacteria, their unique texture and flavor are appreciated by many consumers. Several features of different kinds of home-made sheep cheeses, which are produced in various regions of Iran, have been studied by many researchers (1-7).

Poosti cheese is one of the traditional cheeses, which is made from raw sheep milk in the nomadic tribes of Sangesar region, located in Semnan province (east of Iran). The main difference between Poosti cheese and other traditional types is the sheep skin, which is used for cheese preserving during the aging period (Fig. 1). Poosti cheese production consists of several stages including coagulation, whey removal (2 times), salting, packing in the prepared skin, and ripening for three months.

Ripening -a slow and complex process with sequential changes in microbiological and biochemical specifications- is the most important stage in the formation of various cheeses (8). Two dominant biochemical processes during the cheese aging are *proteolysis* and *lipolysis*. Degradation of cheese proteins by proteolytic enzymes ends with the formation of sulfur and nitrogen containing compounds, as well as textural properties changes (9). In this way, numerous researches have been conducted on the protein profiles of different kinds of

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cheeses (10-12). In addition to proteolysis, lipolysis causes multiple alterations in cheese properties by releasing free fatty acids (FFAs) from milk lipids during the ripening period, and lipoprotein lipase and raw milk microflora are responsible for these lipolytic reactions (13). Due to the importance of lipolysis, several studies have been performed to assess the FFA contents of diverse types of cheeses including Idizabel (14), Torta del Casar (15), and Roncal (16). The effect of proteolysis and lipolysis processes on the cheese texture could be assessed by evaluating the cheese's microstructural properties (17, 18).

Accordingly, the changes in protein profile, FFA contents, and microstructural properties of Poosti cheese during 90 days of ripening were investigated in the present work.

Materials and Methods

Cheese making and sampling: Raw sheep milk from a local native sheep population (Sangesar Mountains, Semnan, Iran) was clarified by thin tissue. The lamb rennet was then added to milk at room temperature (28–35ºC) and kept intact about 2 hours. For whey drainage, the curd was transferred into rectangular cotton bags, and a heavy rock (5-6 kg) was used for better compressing for ten days. During this period, salt (25%) was added and robbing was done every day. Four sheep skins were filled with the prepared cheese and packed tightly. Then they were kept in a man-made cave (Sangesar, suburbs of Semnan province, Iran) for three months at an average temperature of 4-6°C. In each stage of ripening (1, 30, 60 and 90 days), one of the filled sheep skins was selected for sampling.

Sheepskin container preparation: The virgin sheep or goat skin, which has no hole or rupture, was selected. The inner surface of the skin was cleaned by washing and scraping by knife. Then it was sun dried and rubbed by yogurt and salt to make it white. Actually, the sheep skins were prepared for three times, and sampling of each prepared cheese was done for three times (number of samples: 9).

Physicochemical analysis: During the ripening of Poosti cheese, its moisture (19), salt (20), fat (21), and pH (by a Knick 766 pH-meter Niels, Bohrweg, Utrecht, Netherlands) were determined.

Proteolysis

Preparation of cheese samples: Poosti cheese (5 g) was mixed with 20 mL water for 3 min at 20°C. The pH of the mixture was adjusted at 4.6, and kept at 40°C for 1 hour. Then centrifugation of the prepared samples was performed at 3000×g for 20 min at 5°C. Finally, the pellets were vortexed with 5 mL urea (7 M; Merck Chemical Ltd., Darmstadt, Germany) and stored in freezer (−18°C).

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE): Proteolysis profile of Poosti cheese during 90 days of ripening was estimated electrophoretically with SDS-PAGE according to the Laemmli method (22) using a vertical slab gel apparatus with 5% stacking and 15% resolving gel. Before loading of the samples into the gel, their proteins and peptides were extracted using the following method (23):

To 150 mg of each prepared solution, 2.5 mL SDS (7%; Sigma Chemical Co, St Louis, MO, USA) and 2.5 mL Tris (0.166 M; Sigma Chemical Co, St Louis, MO, USA)–EDTA (1 mM; Merck Chemical Ltd., Darmstadt, Germany) with pH 8 were added. After centrifugation of the samples at 5000×g at 4°C for 20 min, 1 mL of the supernatant was heated for 5 min at 95°C, and 0.2 mL β-mercaptoethanol (Sigma Chemical Co, St Louis, MO, USA) was added to it. At the last stage, 0.2 mL glycerol and 0.2 mL bromophenol blue (0.02%, Merck Chemical Ltd., Darmstadt, Germany) were added to the prepared sample. Unstained protein molecular weight marker (Fermentas, Life Sciences, USA) was also used as standard.

Electrophoresis was performed at a constant 125 V (8.0 mA) for 4 h in Tris-Glycine-SDS buffer pH 8.3 (running buffer). After electrophoresis, proteins present in the separating gel were made visible by staining using Coomassie Brilliant Blue (Merck Chemical Ltd., Darmstadt, Germany).

Analysis of Free Fatty Acids: FFA analysis of Poosti cheese during 90 days of ripening was performed by gas chromatography according to Türkoğlu (2011) (24). As described, the cheese fat was extracted by a mixture of methanol and methylene chloride (1:9), followed by evaporation of the solvent at 40°C under vacuum (25). After methylation of the extracted fats by the method of Sukhija and Palmquist (26),

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injection to the gas chromatograph (Shimadzu GC-17 AAF, V3, 230V series; Shimadzu Corporation, Kyoto, Japan) equipped with flame ionization detector (FID), and fitted with a fused silica capillary column (SP-2380, 100 m × 0.25 mm; Supelco Inc., Bellefonte, PA) was done. During this test, helium gas was used as a carrier, and the temperature of both the injector and detector was adjusted at 250°C.The temperature program of the column was set as follows: the initial temperature (40°C for 1 minute) reached to 240°C with 5°C.min^{-1} rate and then maintained for 10 minutes in the final temperature. For determining the retention time, a mixture of standard fatty acids and purified acids, namely *individual fatty acids* was used. Nonanoic acid was also selected as an internal standard.

Microstructure Analysis: Area fraction of Poosti cheese microstructure during the ripening was calculated by analyzing the SEM micrographs. For this purpose, the cheese samples were prepared by the following manner (18); the cheese cubes (1.5 × 1.5 × 1.5 mm) were fixed by immersion in gluteraldehyde (2.5% w/w; Merck Chemical Ltd., Darmstadt, Germany) for 3 hours. After 6-times washing with distilled water, the samples were dehydrated by various ethanol preparations (40, 55, 70, 85, 90 and 96%), each for 30 minutes. The prepared samples were scanned by SEM (XL Series, model XL30, Philips, Eindhoven, Netherlands), and evaluated by Image J software (National Institutes of Health, Bethesda, MD). The images were adjusted by selecting the threshold option from the *Image* menu, and then the area fraction of the samples was obtained from *Analyze Particles* option from Analyze menu of the mentioned software.

Statistical Analysis: All results were the average of three separate experiments. One way ANOVA test for evaluating the effect of ripening period on different characteristics of Poosti cheese, using SAS statistical software (version 8.2). All assessments were based on a significance level of $p<0.05$, and all experiments were designed by a completely randomized design.

Results

Physicochemical Parameters of Poosti Cheese: The data obtained for pH, moisture, salt, and fat content of Poosti cheese during 1, 30, 60, and 90 days of ripening were normal and are shown in Table 1. As shown, the pH value does not change significantly during 60 days of aging, and then drops at 90 days. During the ripening, the moisture content of Poosti cheese was decreased but salt content and fat percentage were increased.

Fig 1. The schematic of sheepskin container for Poosti cheese preserving.

Table 1. Physicochemical properties of Poosti cheese during the ripening

Physicochemical characteristics	Ripening period (days)			
	1	30	60	90
pH	5.53±0.01^{a}	5.56±0.02^{a}	5.60±0.05^{a}	5.27±0.01^{b}
Moisture content (%, w/w)	39.17±3.7^{a}	27.53±0.7^{b}	20.41±2.5^{c}	12.94±0.4^{d}
Salt (%, w/w)	3.52±0.05^{d}	5.549±0.1^{c}	6.76±0.06^{b}	7.73±0.08^{a}
Fat (%, w/w)	24.76±0.3^{d}	25.66±0.1^{c}	26.23±0.4^{b}	27.16±0.2^{a}

$^{a-d}$ Means within the same row with different superscripts differ significantly ($p < 0.05$).
(significant level: α= 0.01)

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Proteolysis Assessment of Poosti Cheese: The electrophoretic pattern of Poosti cheese samples at 1 (fresh), 30, 60 and 90 days of ripening is shown in Fig.2. A sharp decrease in the intensity of casein bands during the first month of the ripening is clearly observed. From days 30 to 90, slight alterations occurred in the intensity of protein bands.

Lipolysis Assessment of Poosti Cheese: The data for FFA profile of Poosti cheese at the beginning (day 1) and the end (day 90) of the ripening were normal and were shown in Fig. 3. Comparison of the short chain FA ($C_{4:0}$-$C_{10:0}$) contents between days 1 and 90 of storage showed an increasing trend in their levels. During the aging, the amount of medium chain fatty acids ($C_{12:0}$-$C_{16:0}$) does not changed significantly but the long chain fatty acids were decreased.

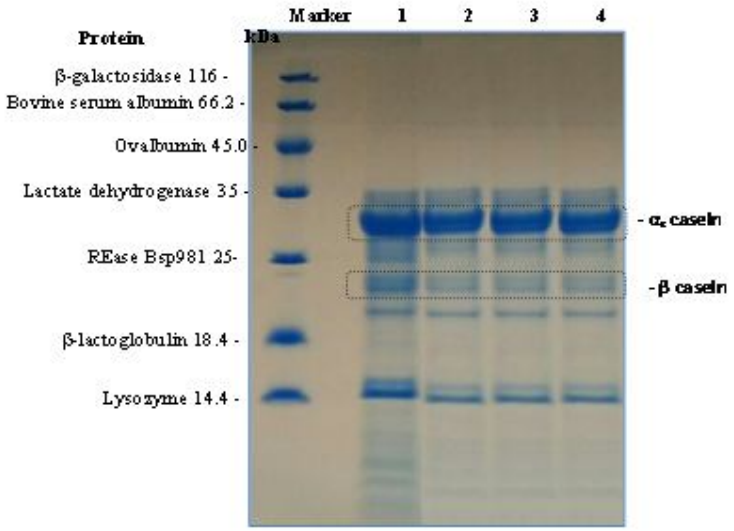

Fig. 2. Electrophoretic profile of Poosti cheese after 1 (lane 1), 30 (lane 2), 60 (lane 3), and 90 (lane 4) days of ripening in 15% resolving SDS-PAGE.

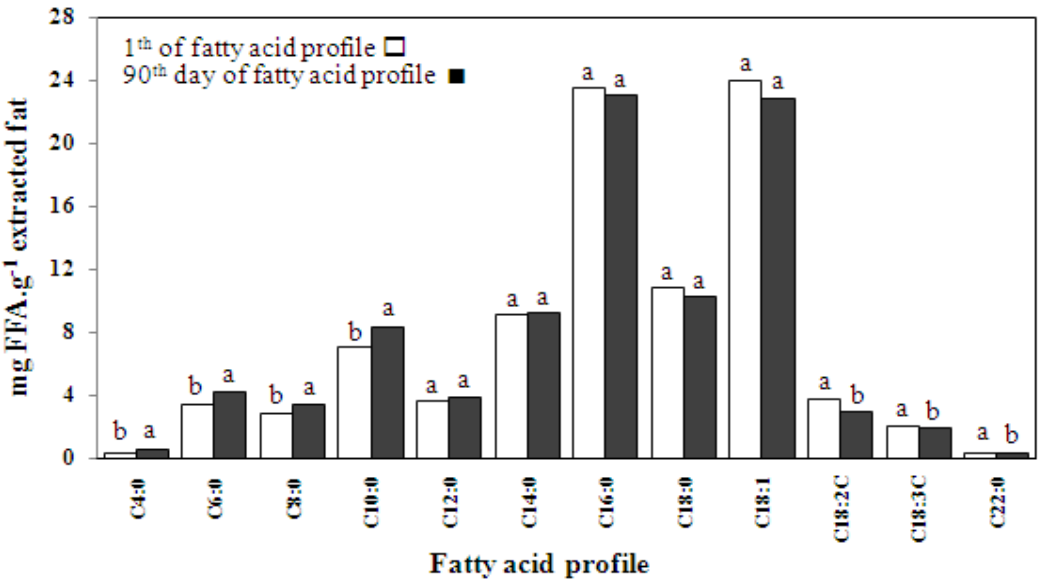

Fig. 3: Free fatty acids content of Poosti cheese after 1 (☐) and 90 (■) days of ripening; different letters (a,b) in each free fatty acid differ significantly (p<0.05).

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Microstructure Assessment of Poosti Cheese: SEM micrographs of Poosti cheese and their red-gray scales after 1, 30, and 90 days of ripening are shown in Fig. 4. It is clear that the density of cheese protein matrix increases in the first month of ripening and does not change significantly until the end of ripening (Fig. 4a-c). According to Fig. 4 (d,e), the portion of red patches increases in the first 30 days of ripening and then became constant (Fig. 3e,f). The pore fraction (%) of SEM micrographs with 4000 magnification of Poosti cheese during 90 days of aging (calculated by Image J software) showed a considerable increase from 55.3±7% to 72.6±4% during the first month of aging and then, no considerable change was observed in the mentioned parameter (pore fraction at day 90=77.4 %). During 30 days, a raise in the pore fraction value led to the development of homogenous aggregation in casein matrix (Fig. 4.b,e).

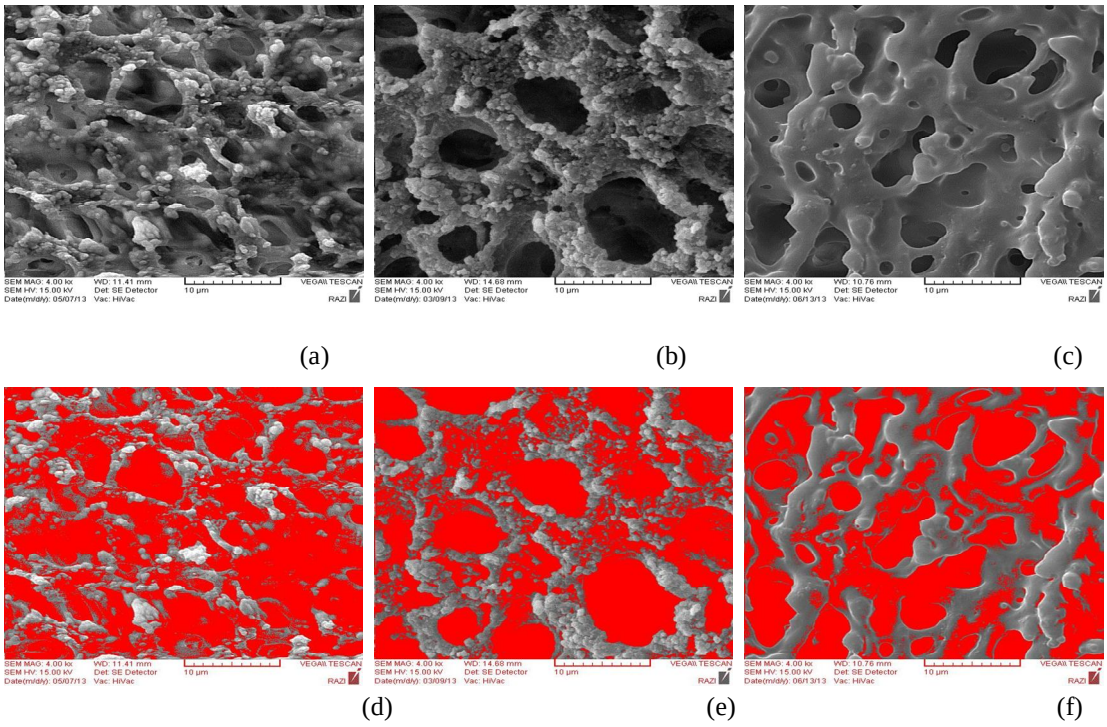

Fig 4. SEM and red-gray scale micrographs of Poosti cheese at 1 (a,d), 30 (b,e), and 90 (c,f) days of ripening with 4000 magnification.

Discussion

Physicochemical Parameters of Poosti Cheese: From 1 to 60 days, the constancy of pH is the result of the balance between fermentation and proteolytic activity of the microorganisms present in the raw milk and the sheep skin container (27). During the last month of ripening, dominancy of the lactic acid bacteria due to the inhibitory effect of salt on other types of microorganism is responsible for the pH decrease (4). The presence of different kinds of carbohydrate metabolizing enzymes such as hexokinase (HK), glucokinase (GK), and phosphofructokinase (PFK) in the sheepskin tissue plays an important role in the production of acids, as well (28). The reduction in moisture content of Poosti cheese could be attributed to the salt diffusion into the texture (29) and movement of water from the curd (which has high concentration of the medium) to the environment (which has no water). In addition, an increasing trend of salt content during the ripening was due to the diffusion of NaCl molecules into the cheese matrix (30). The reduction in fat percentage in the aging period could be related to decrease in the moisture content (4).

Proteolysis Assessment of Poosti Cheese: Various proteolytic factors including enzymes (rennet and

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plasmin) and proteolytic microorganisms of raw milk have crucial influence on the degradation of casein fractions, especially α_S- and β-caseins. It is obvious that the degradation of major fractions of casein (α_S and β) by rennet has occurred during the cheese production. Fox and Law (1991) showed that α_S-casein's bacterial lysis throughout the ripening period took place at higher level than that of β-casein (31). In addition, the proteolytic activities of the sheepskin microflora such as Proteus vulgaris, and a species of Achromobacter (32) intensified this phenomenon. Slight alterations in the intensity of protein bands from days 30 to 90, was due to the inhibitory effect of salt on proteolytic factors. Whereas, the inhibitory effect of a certain extent of salt on plasmin activity (33) and proteolytic microorganisms has been reported previously (28). Growth suppression of *Enterococcus faecalis* and *Streptococcus parauberis* at the last stage of Lighvan cheese aging in brine was also illustrated (28). A similar pattern of proteolysis process was also shown in Xinotyri traditional cheese, which has slight degradation of casein until the 22th day of ripening, and then became stable (34). Aminifar (2012) also reported comparable results in proteolysis of Lighvan cheese (3).

Lipolysis Assessment of Poosti Cheese: An increase in short chain FA ($C_{4:0}$-$C_{10:0}$) contents during the aging, could be considered as a result of the lipolytic activity of raw milk lipase and lipoprotein lipase, and the lipase released from the microbial population of raw milk and sheep skin (35). Among these factors, raw milk lipase has a significant role due to its effect on hydrolysis of most of the butyric, caproic, and caprylic fatty acids in Sn-3 position of ewe milk triglycerides (36). Among the diverse microbial species, existing in raw milk, lactic acid bacteria (especially Lactococcus and Lactobacillus spp. for their esterolytic/lipolytic enzymes), Pseudomonas, Acinetobacter and Flavobacterium have higher lipolytic activity (37). Moreover, the high production of these volatile fatty acids as a result of lipolysis could be considered as a key factor in Poosti cheese aroma (3). Parallel results for this group of fatty acids in raw sheep and goat milk cheese were also observed by various researchers (38-40)

The constancy in the amount of medium chain fatty acids ($C_{12:0}$-$C_{16:0}$) throughout the aging could be related to the inhibitory effects of salt and pH on the microbial population and their lipolytic activities (41). As illustrated, when the pH decreases from the optimum (7-8.5) or the salt concentration increases up to 20g.100 mL^{-1}, the lipase activity of lactic acid bacteria diminishes (42). In addition, this stable situation in these groups of fatty acids could be attributed to the equilibrium between fatty acids production and their catabolism (42), as several volatile components such as methyl ketones, esters, and secondary alcohols are the well-known products of medium chain fatty acids' catabolism pathway (43).

Evaluation of long-chain fatty acids ($C_{18:0}$, $C_{18:1c}$, $C_{18:2c}$, $C_{18:3c}$, $C_{22:0}$) indicated that the biodegradation process of fatty acids was prevailed on their release. This differs in some other traditional cheeses (3, 34), which might be due to the microbial activity of the sheep skin used for cheese preserving during 90 days of ripening.

Microstructure Assessment of Poosti Cheese: Increase in cheese density could be the result of water loss from the matrix to the environment due to hypertonic condition in the presence of salt (44-46). Besides, protein degradation and its rearrangement during this period -as a result of proteolysis- could be another determining factor in the formation of this structure (47). Increase in the portion of red patches in the first 30 days of ripening was due to the formation of voidages in the protein structure caused by the fermentation activity of raw milk and skin microflora (24). The negligible changes in the red-gray patches after 30 days (Fig. 3e,f) can be attributed to the inhibitory effect of salt on proteolytic reactions, and the equilibrium between water and salt movements (18).

Increase in the pore fraction (%) of SEM micrographs during the first month of aging could be considered as a result of pore formation in the casein matrix. The gases released from the fermentative activity of raw milk and skin microorganisms accompanied by the casein breakdown by proteolytic enzymes take part in this process (48). Formation of homogenous aggregation in first month of ripening was due to enhancing in casein linkage for increased hydrophobic interactions (18). Aggregation causes the casein bind to gather, and therefore, the summation of the surface area decreases. Diffusion of salt ions to the cheese matrix plays an important role in this way. Diffusion of NaCl into the protein matrix produces chloride anions in the casein network, which could act

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as a kosmotropic ion in the cheese matrix and promote hydrophobic interactions that ultimately increase casein linkage (46). The constancy in the mentioned parameter from day 30 to day 90 could be related to the decrease in the rate of proteolytic reactions and the thermodynamical balance between water and NaCl movement. These results are also in agreement with the data obtained from electrophoresis assay.

Conclusions: This study was performed to assess the proteolysis and lipolysis of Poosti cheese (raw traditional raw sheep cheese), and characterize the microstructural parameter during 90 days of ripening. The data obtained from electrophoresis indicated that degradation of casein fractions during the first stages of Poosti cheese ripening was occurred with higher intensity than in the other stages.

The decline in proteolysis rate during the final stage of aging could be correlated with the inhibitory effects of salt on engaged microorganisms. The comparison of FFAs days 1 and 90 showed different patterns for short, medium, and long chain FFAs as a result of diversity in the mechanisms interfered in their production and degradation. Additionally, the area fraction of the microstructure was increased during the first month of Poosti cheese aging due to casein rearrangement and gas release by fermentative activity of the microorganisms.

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