Microstructural Changes in Fat During the Ripening of Iranian Ultrafiltered Feta Cheese

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ABSTRACT

In this study, fat globules in Iranian ultrafiltered Feta cheese (3 to 60 d) were directly observed during the ripening period by scanning electron microscopy. According to images of ultrafiltered Feta cheese samples obtained by scanning electron microscopy, individual fat globules and aggregates of fat were easily distinguishable on d 3 and had completely disappeared within 20 d of storage. On d 20, only the fingerprints of the fat globules and pools of free fat in the casein matrix remained. After 40 d of ripening, the texture was homogeneous and no fat globules or fat voids were detected. Chemical analysis of cheese samples showed that with an increase in the ripening period, the contents of dry matter and fat decreased significantly, whereas the pH values and salt content did not indicate any significant changes.

Key words: fat, lipolysis, microstructure, ripening

INTRODUCTION

The chemical and physical changes occurring during cheese ripening cause the body of the freshly made cheese to lose its firm, tough, and curdy texture (Tunick, 2000). Storage temperature and ripening period change the physical state of fats and cheese texture (Watkinson et al., 1997; Dufour et al., 2000). During maturation, several biochemical events (primarily proteolysis, glycolysis, and lipolysis) as well as slow solubilization of some of the residual colloidal calcium phosphate change curd from a rubbery bland product to a mature cheese with a characteristic flavor, texture, and aroma (Collins et al., 2003).

Microstructure studies provide strategic information to understand and therefore to control cheese properties (Lobato-Calleros et al., 2006). Direct observation of fat globules requires some procedures that are not provided by some microscopic methods (Madadlou et al.,

2006, 2007; Karami et al., 2008). Some studies about the cheese microstructure indicate that defatting of samples through washing with a series of solvents is the main step in the preparation of samples for scanning electron microscopy analysis (Lobato-Calleros et al., 2006; Madadlou et al., 2006, 2007). By using such sample preparation procedure, only fingerprints and holes of fat remain (Lobato-Calleros et al., 2006; Madadlou et al., 2006, 2007; Karami et al., 2008). This makes it hard to fully understand the details of the sample and therefore differentiate among the different forms of fat. Furthermore, images obtained by scanning electron microscopy are black and white, although some image analysis software can provide pseudo-color and colored images (Lobato-Calleros et al., 2006). Despite that, images obtained by electron microscopy have high resolution, and therefore they can provide a detailed view of the interaction among cheese constituents (Guinee et al., 2000). Fixation, cracking in liquid nitrogen, and drying of the samples are some crucial steps for scanning electron microscopy analysis (Madadlou et al., 2006, 2007; Karami et al., 2008). Most of milk fat triglycerides are in the liquid form under room temperature (Fox et al., 1993). Thus, for the investigation of fat in the cheese matrix, it is very important to prepare the samples under low temperature.

Ultrafiltration offers an alternative way of concentrating milk before the formation and handling of the curd (Mistry and Maubios, 1993). Ultrafiltration has been successfully applied in Feta cheese making (Tamime and Kirkegaard, 1991). One of the main steps during cheese production is whey removal, which results in the concentration of the major constituents in the retentate or curd (Mistry and Maubios, 1993).

Iranian UF-Feta cheese made from bovine milk is manufactured in modern dairy factories from pasteurized milk with mesophilic starter cultures and commercial microbial rennet with a volume concentration factor of 5.1 to 5.4. Among cheese varieties in Iran, UF-Feta cheese has the highest per capita consumption. There is not enough information about the effect of lipolysis on the microstructure of fat in Iranian UF-Feta cheese throughout the ripening. The published reports

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on the texture of Feta cheeses mainly concern those made traditionally without any attention to the modern technology of ultrafiltration (Samal et al., 1993; Lalos, 1996; Pappas, 1996; Katsiari, 1997; Wium and Qvist, 1997; Wium et al., 1997; Sipahioglu et al., 1999; Madadlou et al., 2006, 2007), and none of them have reported on the microstructural properties. Therefore, the objective of this study was to evaluate possible microstructural changes in Iranian UF-Feta cheese during 60 d of ripening period with an emphasis on the direct observation of fat globules.

MATERIALS AND METHODS

Materials

Starter cultures (DM-230 and Y-502) with combination of *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis*, which are not gas-producing bacteria, were obtained from Danisco Deutschland GmbH (Alemanha, Germany). The rennet (Fromase 2200 TL Granulate ≥ 2200 international milk-clotting units/g) used was a microbial coagulant from *Rhizomucor miehei* (DSM Food Specialties, Seclin, France). Raw cow's milk, equipment, and filtration moduli were provided by Hamedan Pegah dairy company (Hamedan, Iran).

Methods

Cheese Making. Cheeses were made at Hamedan Pegah dairy plant (Hamedan, Iran) according to the UF cheesemaking method proposed by the Tetra-Pak company (Bylund, 1995) adapted (with some modifications) by Hesari et al. (2006) for the production of Iranian UF-Feta cheese. After bactofugation, pasteurization ($72^{\circ}\text{C} \times 15$ s), ultrafiltration, homogenization, and second pasteurization ($80^{\circ}\text{C} \times 20$ s) stages, the retentate with a volume concentration factor of 5.4 kg of milk to 1.0 kg of retentate entered the starter tank, where by adding the starter (1.0 g for 50 kg of retentate) the pH of milk reached the 6.2 level. Then, in the filler, rennet ($\geq 2,200$ international milk-clotting units/g) was mixed with water (2 g for 100 kg of retentate) and added to each cheese container. The coagulation tunnel, which was set at 37°C for 30 min, allowed the retentate to be converted to a pre-cheese mixture. In the sealing machine, 4% (wt/wt) salt was added onto the parchment paper on the top of cheese, and then, the container was sealed by using aluminum foil. In the preripening stage (37°C), after decreasing the cheese pH to 4.80, cheese samples were transferred to a cold room ($9 \pm 1^{\circ}\text{C}$) for cooling and ripening for 3 to 60 d, after which microstructural analysis was performed. Three separate

batches following the above procedure were considered for the production of each treatment.

Physicochemical Analysis. A Knick 766 calimatic pH meter (Niels Bohrweg, Utrecht, the Netherlands) was used for measuring the pH of cheese samples. The cheese samples were analyzed in triplicate for moisture by heating to a constant weight using Sartorius moisture analyzer (Sartorius Ltd., Epsom, UK) and for fat according to the British Standards Institution method (1995). Salt content was determined according to the procedure described by Kirk and Sawyer (1991).

Microstructure. Cheese samples were prepared in triplicate for scanning electron microscopy analysis after 3, 20, 40, and 60 d of ripening following a method described by Drake et al. (1996) with some modifications suggested by Madadlou et al. (2006, 2007) applied for the evaluation of textural characteristics of low-fat Iranian White cheese. Using a sharp razor, cheese blocks were cut in 5- to 6-mm³ cubes and immersed in 2.5% (wt/wt) glutaraldehyde fixative (Merck, Darmstadt, Germany) for 3 h. To avoid possible changes in the fat microstructure or the likely loss of fat from the cheese matrix, the washing and dehydration stages applied by Drake et al. (1996) were not used in this study. Samples were refrigerated until used for scanning electron microscopy analysis. During scanning electron microscopy analysis, samples were freeze-fractured in liquid nitrogen (Sipahioglu et al., 1999) to approximately 1-mm pieces, and these pieces were then mounted on aluminum stubs with silver paint, dried to critical point, and coated with gold for 300 s in a sputter-coater (Type SCD 005, BalTec Inc., Balzers, Switzerland). Samples were viewed with a scanning electron microscope (XL Series, model XL30, Philips, Eindhoven, the Netherlands) operated at 20.0 kV. Images were recorded at 250, 500, 1,000, 2,500, and 5,000 $\times$ magnification levels.

Statistical Analysis. Experimental cheese production was repeated in triplicate and the resultant data (dry matter percentage, fat percentage, salt percentage, and pH) were statistically analyzed using SAS statistical software (Version 8.2, SAS institute Inc., Cary, NC). A multifactor ANOVA using the least significant difference test ($P < 0.05$) was applied to test the effect of ripening stages (3, 20, 40, and 60 d) on the physicochemical characteristics of the samples.

RESULTS AND DISCUSSION

Physicochemical Characteristics

In addition to age, the major compositional factors affecting cheese properties are the pH, moisture, fat, and salt content (Gunasekaran and Ak, 2003). Changes

Table 1. Changes of pH value and, fat, salt, and DM content of Iranian UF-Feta cheese during different ripening periods

Ripening period (d)	pH	Fat	Salt	DM
3	4.36 ± 0.03 ^a	15.9 ± 0.1 ^a	4.2 ± 0.01 ^a	36.1 ± 0.1 ^a
20	4.37 ± 0.02 ^a	15.4 ± 0.1 ^b	4.2 ± 0.02 ^a	35.8 ± 0.1 ^b
40	4.40 ± 0.03 ^a	15.2 ± 0.1 ^c	4.2 ± 0.02 ^a	35.5 ± 0.1 ^c
60	4.42 ± 0.04 ^a	15.0 ± 0.1 ^d	4.2 ± 0.02 ^a	35.2 ± 0.1 ^d

^{a-d}Means in each column with the same superscript are not significantly different ($P < 0.05$).

in pH, fat, salt, and dry matter contents on the series of UF-Feta cheeses throughout ripening (3, 20, 40, and 60 d) are shown in Table 1. With an increase in the ripening period, DM and fat contents significantly decreased ($P < 0.05$), whereas pH and salt content did not exhibit any significant changes. The pH of a 3-d-old cheese and that of a 60-d-old cheese were similar (4.36 to 4.42). Such pH values are necessary for a mature Feta cheese to maintain its good quality during storage (Anifantakis, 1991). Lactose permeability to the UF membrane is very high, and trace amounts of this compound remain in the retentate phase (Mistry and Maubios, 1993). Therefore, the lactic acid formation from residual lactose in UF-Feta cheese in this study was low and did not have any influence on the pH level during the ripening period. Also, some of the lactic acid bacteria convert lactic acid into other secondary metabolites (Collins et al., 2003). In low-pH cheeses (such as UF-Feta cheese) submicelles are split into casein aggregates in the form of nonlinear strands (Lawrence et al., 1987).

With an increase in the ripening period, a slight decrease was observed in the fat and DM contents of the cheese samples due to the decomposition of fat into FFA and finally to volatile flavorful compounds (Collins et al., 2003). The effect of fat content on the cheese microstructure and texture has been widely investigated (Sipahioglu et al., 1999; Guinee et al., 2000; Lobato-Calleros et al., 2006; Madadlou et al., 2006, 2007). The texture of high-fat cheeses is generally more acceptable than that of low-fat cheeses (Madadlou et al., 2006, 2007). Microstructure comparison of a regular-fat Feta cheese with a low-fat Iranian white cheese indicated that the latter had a more compact protein matrix (Madadlou et al., 2006, 2007). Therefore, decomposition of fat and lowering of its plasticizing effect (Madadlou et al., 2007) result in a compact texture. This is associated with the harder texture even when the moisture content is also high (Gunasekaran and Ak, 2003). Moisture content is inversely correlated with DM content (Fox, 1993), and therefore, it is expected that greater moisture content will result in the production of a softer cheese product (Gunasekaran and Ak, 2003). In addition, the fat, salt, and DM contents of

matured cheeses met the specifications for first-quality Feta cheese as described by Food Chemicals Codex (Codex Alimentarius, 2003) and those of the Iranian standard regulations for UF-Feta cheese.

Microstructure

Day of Production (d 3). Figure 1, panels A and B, shows the porous structure of the casein network, where fat globules of a 3-d-old cheese sample are surrounded by this network. Based on the scanning electron microscopy images obtained here, a mean diameter of about $5.0 \pm 1.8 \mu\text{m}$ was obtained for the fat globules using a microstructure distance measurement software (Manual microstructure distance measurement, Nahamin Pardazane Asia Co., University of Mashhad, Iran), which was in agreement with the results of Rousseau and Le Gallo (1990). In a dairy product, when fat content is high, the number of large globules becomes abundant because of partial coalescence and aggregation of fat (Ding and Gunasekaran, 1998; Gunasekaran and Ding, 1999). Because of concentration of fat by ultrafiltration, it seems that the contact surface among fat globules increases, resulting in the aggregation followed by the fusion of fat globules with each other (partial coalescence; McKenna, 2003). In such cases (i.e., when the fat globules are intact in protein matrix), the plasticizing effect of fat and water limits the junctions among casein chains (Gunasekaran and Ak, 2003; Madadlou et al., 2007). Scanning electron microscopy images of UF cheese revealed that when whey proteins denature, the protein matrix becomes compact and fewer voids are formed (Singh and Waungana, 2001). Homogenization results in an increase in the number of fat globules. Therefore, because of the increased surface area and loss in the membrane stability, some milk proteins are adsorbed on the dispersed fat globules and form the fat aggregates (McKenna, 2003). When cheese milk is homogenized, the fat globules become part of the protein matrix due to the incorporation of casein submicelles into a new fat globule membrane (Gunasekaran and Ak, 2003; Madadlou et al., 2007). Figure 1, panels A and B, indicates that fat globules are much larger than the pores of casein network, and

therefore this fact is not in agreement with the theory of van Vliet and Dentener-Kikkert (1982) indicating the noninteracting filler nature of fat globules. Instead, as proposed by Lopez et al. (2007), fat globules are the breakers of casein network. Figure 1, panels C and D, shows the organization of fat globules as aggregates. Because of the negative charge on the milk fat globule membrane (MFGM) at the pH of renneting (6.2 in the current study), electrostatic repulsions among fat globules can be induced (Lopez et al., 2007). Despite that, aggregates of fat globules are formed due to the adsorption of milk proteins on the dispersed milk fat globules (Gunasekaran and Ak, 2003; McKenna, 2003). As indicated, after 3 d of ripening, fat globules have interaction and cross-links with the casein matrix. Interactions between casein matrix and fat phase can change the textural and rheological properties of cheese due to the plasticizing effect of fat (Madadlou et al., 2007). As reported by Lopez et al. (2007) for Emmentaler cheese, fat globules in this study also existed as individual and aggregates of fat globules (Figure 1, panels A, B, C, and D). When fat globules aggregate, their mean diameter becomes greater and they intensify their role as the breaker of texture (Lopez et al., 2007). According to Guinee et al. (2000), disruption of MFGM during processing (e.g., homogenization) leads to the accumulation/aggregation of fat globules. When fat globules become aggregated because of increasing the contact time among them, their coalescence also increases and, as a result, the mean diameter of fat globules also increases (Lopez et al., 2007). The increase in the diameter of fat globules due to the aggregation, as indicated in Figures 1C and 1D, can therefore be related to the above-mentioned phenomenon by Lopez et al. (2007). On the other hand, with an increase in the ripening period, lipolysis leads to the disappearance of some fat globules causing a loss in the plasticity of cheese samples. Retention of whey proteins in UF cheeses also leads to entrapment of water in the cheese matrix because of the hydrophilic properties of whey proteins (Hinrichs, 2001). This phenomenon can partially compensate the loss in the plasticity of cheese matrix due to the disappearance of fat globules. Although high moisture content in UF cheeses leads to some textural defects (Hinrichs, 2001), the trapped moisture can enhance the growth of cheese microflora and increase the activity of enzymatic/biochemical reactions such as proteolysis and lipolysis around the fat (Fox et al., 1993). Starters used in the production of Iranian UF-Feta cheese are composed of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*. The lipolytic activity of these bacteria has already been reported (Fox et al., 2000; Kondyli et al., 2002; Collins et al., 2003; Hannon et al., 2006). Hesari et al. (2006) indicated that when

no starter was added to Iranian UF-Feta cheese, the level of free fatty acids decreased significantly. Lactic acid bacteria (LAB) as starter in UF-Feta cheeses are the main lipolytic components. Heat-stable lipases from psychrophilic bacteria such as *Pseudomonades* are other sources of lipolytic activity in the cheeses (Collins et al., 2003) because they can tolerate even the UHT process (Fox, 1993). Although nonstarter LAB have shown some lipolytic activities, they are not at all active in pasteurized products (Collins et al., 2003).

Middle of Ripening (d 20 and 40). As Figure 2, panels A, B, C, and D, shows, after 20 d of ripening, fat is present in the casein matrix of UF-Feta cheese as nonglobular fat, which is also called free fat and is not protected by MFGM (Evers, 2004). The term free fat has different meanings according to how it is measured (Evers, 2004). Two methods that have been used are warming the milk and centrifuging to reveal a layer of molten fat on the top of the milk, and extraction with organic solvents, which is unable to extract fat from natural, intact fat globules (Evers, 2004). Free fat is the fat that is not protected by MFGM and therefore is highly susceptible to oiling or removal (Lopez et al., 2007). The change in the shape of fat globules shows that after 20 d of ripening some milk fat globules were disrupted. The reason for disappearance of fat globules is the lipolysis of damaged fat globules that occurs after homogenization and adding of LAB as starter (Collins et al., 2003) to UF-Feta cheese. The other mechanism is temperature-dependent rupture of MFGM due to crystallization of triacylglycerols and phospholipids at low temperature (Collins et al., 2003; $9 \pm 1^\circ\text{C}$) during ripening period of UF-Feta cheese. After 20 d of ripening, because of proteolysis reactions (Hesari et al., 2006), some of the free water is absorbed by the protein matrix; as a result, the contact surface between fat and protein increases. Due to the fermentation (proteolysis, lipolysis, glycolysis, or a combination of these) occurring during the ripening, some holes are formed in the casein matrix (Figure 2, panel D).

As shown in Figure 3, panels A and B, after 40 d of ripening, individual fat globules or their aggregates have completely disappeared and no fingerprints of fat were detected. In this period of ripening, it seems that fat is in the form of pools of fat or free fat within voids inside the casein matrix (Lopez et al., 2007). During and after this ripening period, because of junctions between the protein matrix and disappearance of fat (McKenna, 2003), the texture of cheese samples was uniform as a compact structure of casein (Figure 3, panels A and B). These results indicated an inconsistent state and change in the configuration of fat during the ripening period. Because no pressing was conducted in the production of UF-Feta cheese, some atmospheric gases can

Figure 1. Scanning electron micrographs of Iranian UF-Feta cheese after 3 d of ripening. A, B) Images with individual fat globules; C, D) images with aggregates of fat. (Magnification level is given below the images as a multiple of x at the left side and as a bar at the right side).

Figure 2. Scanning electron micrographs of Iranian UF-Feta cheese after 20 d of ripening. A, B, C) Fingerprints of fat globules; D) holes in the casein matrix that resulted from fermentation. (Magnification level is below the images as a multiple of x at the left side and as a bar at the right side.)

Figure 3. Scanning electron micrographs of Iranian UF-Feta cheese after 40 d of ripening. (Magnification level is below the images as a multiple of x at the left side and as a bar at the right side.)

be imprisoned in the cheese matrix or carbon dioxide can be produced from fermentation reactions during storage in the cold room (Fox et al., 1993). Figure 4, panel A, indicates such a gas microbubble in the texture of UF-Feta cheese, which was, however, infrequent.

Expiration Date (d 60). After 60 d of ripening period, casein aggregates were observed clearly in the cheese matrix (Figure 4, panel B). When the pH value change from 5.4 to 4.6 (as occurred in our study), the casein submicelles progressively dissociated into smaller aggregates and eventually into nonlinear strands rendering cheese from a springy texture at high pH (5.3 to 5.4) to a noncohesive texture at pH below 4.8 (Lawrence et al., 1987). Also, according to Hesari et al. (2006), adequate proteolysis occurred during the ripening of Iranian UF-Feta cheese (until 60 d). Thus, in the absence of fat globules that were the breakers of texture, dissociated proteins (peptides and polypeptides) can create junctions between each other and rearrangement of the cheese texture takes place (McKenna, 2003). In some studies of cheese microstructure using

scanning electron microscopy, fat was removed during sample preparation (washing and dehydration) by use of solvents (always acetone or ethanol); thus, what was remained in the images were only the fingerprints of the fat globules (Lobato-Calleros et al., 2006; Madadlou et al., 2006, 2007). Therefore, no interaction can be considered between fat and cheese matrix or among the different states of fat. Different configurations of fat globules during ripening period could be directly observed in the current study according to the results of scanning electron microscopy data.

In the production of Iranian UF-Feta cheese, a heavy homogenization was applied (50 to 70 bar) resulting in an increase in the number and, thereby, the surface area of fat globules (Madadlou et al., 2007). Homogenization can also disrupt MFGM and accelerate the lipolysis of nonprotected free fats by lipolytic enzymes (Deeth, 2006). About 1% of milk lipids is composed of phospholipids (Banks, 1991), and therefore, after curd formation no whey separation occurs in the UF-Feta cheese production. About 1.6 g of phospholipids per kg

Figure 4. Scanning electron micrographs of Iranian UF-Feta cheese after 60 d of ripening. A) Gas microbubbles in the cheese matrix; B) casein aggregates in the cheese matrix. (Magnification level is below the images as a multiple of x at the left side and as a bar at the right side.)

of cheese is found in UF-Feta cheese that can probably be used as a source of carbon for LAB toward the production of flavorful compounds (Collins et al., 2003). The role of phospholipids in the ripening of cheese is not yet known, which is a challenge for dairy scientists (Deeth, 2006). Therefore, the flavorful compounds such as FFA and other volatile compounds could be produced through lipolysis followed by the disruption of fat globules during ripening (Fox, 1993).

CONCLUSIONS

Based on the scanning electron microscopy data, this study characterized the changes in the microstructure of fat globules during the ripening stages of Iranian UF-Feta cheese. Fat showed a major effect on the microstructure and texture of Iranian UF-Feta cheese. After 3 d of ripening, individual and aggregates of fat globules were easily detected, whereas after 20 d, only the fingerprints of fat were observed. After 40 d of ripening, no detectable fat was found, the texture was homogeneous, and aggregates of casein were the only recognized components. The main states of fat in the UF-Feta cheese were 1) fat globules surrounded by the MFGM and 2) aggregates of fat globules. The results of this study offer a better understanding of the development of the structure of fat in the UF-Feta cheese and direct assessment of its behavior, which may be important for better comprehension of its functional and sensory properties.

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