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AGE RELATED CHANGES IN THE MICROSTRUCTURE OF MOZZARELLA CHEESE

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Abstract

Changes that occurred in the microstructure of low-moisture, part skim Mozzarella made with a mixed starter consisting of *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* and coagulated with fermentation-produced chymosin, were examined during 50 days of ripening at 4°C. Immediately after manufacture (day 3), a homogeneous and continuous phase of amorphous paracasein represented a three-dimensional protein network in the cheese. A large number of irregularly shaped and sized microcavities were present. During 50 days of aging, an increase in the porosity of the defatted paracasein matrix was apparent. These changes were coincidental with a fourfold increase in water-soluble nitrogen (from approximately 2 to 8% of total N) and hydrolysis of approximately 50% of α_{s1}-casein. It is suggested that the confluence of adjoining microcavities that occurred progressively throughout storage may be due to proteolysis or CO_2 production by the starter culture.

Key Words: Mozzarella, cheese, structure, casein, proteolysis, fat globule, chymosin, scanning electron microscopy.

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Introduction

Freshly manufactured low-moisture, part skim (LMPS) Mozzarella cheese is texturally unsuitable for use on pizza and is usually ripened for 1-3 weeks so that appropriate textural and melting properties develop (Kindstedt, 1991). Initially, the melted cheese exhibits a tough and fibrous consistency that is accompanied by a marked separation of free water (whey) and limited release of free oil. As aging progresses, the cheese becomes softer, less fibrous and more flowable when melted, and the water phase ceases to separate from the melted cheese whereas the release of free oil increases. These changes and the factors affecting them have been assessed by this group using a number of empirical tests (Kindstedt and Rippe, 1990; Kindstedt and Kiely, 1992; Kindstedt *et al.*, 1992) and by others (Casiraghi *et al.* 1985; Masi and Addeo, 1986; Oberg *et al.*, 1991; Tunick *et al.*, 1991).

Cheese texture is largely a function of cheese microstructure (Emmons *et al.*, 1980). The microstructure of Mozzarella cheese has been investigated by transmission and scanning electron microscopy (Kalab, 1977; Taranto *et al.*, 1979; Masi and Addeo, 1986; Paquet and Kalab, 1988; Kiely *et al.*, 1992). While much of the earlier work on cheese structure investigated structural changes that occur in milk and curd during Cheddar cheese making, systematic investigations of cheese structure changes during the ripening period are surprisingly few (Ruegg *et al.*, 1980; Green *et al.*, 1981; Rousseau, 1988; Rousseau and LeGallo, 1990) and none pertinent to Mozzarella cheese were found.

Rapid and extensive proteolysis occurs in Mozzarella cheese during the ripening period (Di Matteo *et al.*, 1982; Farkye *et al.*, 1991). It has been suggested by Kiely *et al.* (1991a) that changes in functional properties (i.e., melted consistency and free oil formation) of Mozzarella cheese are associated with proteolytic destruction of the casein component of the cheese. Considering the extent of proteolysis reported to occur in ripening Mozzarella, it was postulated that alterations in the protein network that constitute the cheese matrix would be apparent. This study is an attempt to evaluate these changes.

Materials and Methods

Cheese manufacture

As part of an investigation of the impact of starter culture rod to coccus ratio on the functional properties of Mozzarella cheese (Yun *et al.*, 1992), three 170-kg vats of LMPS Mozzarella were manufactured at Cornell University using a new cheese making technique which does not use brine salting (Barbano *et al.*, 1991). Standardized (2.25% fat) and pasteurized (72°C for 16 seconds) milk was inoculated with either a 1:1, 1:10, or 10:1 mixture of *Streptococcus salivarius* ssp. *thermophilus* (Thermococcus C120, Rhone-Poulenc, Madison, WI) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Thermorod R160, Rhone-Poulenc, Madison, WI) and coagulated with pure, fermentation-produced chymosin (Chy-Max, Pfizer Inc., Milwaukee, WI) added at a rate of 0.1 ml (double strength)/kg milk. Curds were cooked to 41°C and whey was drained at pH 6.4. Following whey drainage, curd slabs were cheddared at 41-42°C (steam jacketed cheese vats) and were milled at pH 5.25, dry-salted, and stretched in a twin screw pilot scale Mozzarella mixer (Model 640, Stainless Steel Fabricating, Columbus, WI) which contained a 10% (w/w) salt solution at 57°C.

Stretched curd (approx. 55°C) was extruded into cylindrical stainless steel tubes (7.5 cm i.d. x 30 cm long) and cooled for 1 hour in ice water. Cheese cylinders were vacuum-packaged and shipped by overnight refrigerated express mail to the University of Vermont and stored at 4°C until analyzed.

Analysis of cheese composition

Chemical analyses were conducted in duplicate (except for moisture) on finely ground cheese samples. Cheese moisture was calculated in quadruplicate as the loss in weight of 2-3 g samples of cheese, dried for 24 hour at 100°C in a forced-air oven (Model OV-490A-2, Blue M, Blue Island, IL). Cheese calcium content was determined by ethylenediaminetetraacetic acid (EDTA) complexometric titration (Kindstedt and Kosikowski, 1985) and salt concentration by the Volhard method (Richardson, 1985). Fat content was determined by the Babcock test (Richardson, 1985) and protein (N X 6.38) by Kjeldahl determination of N (IDF, 1989). Cheese pH was determined by direct immersion of a Xerolyt electrode (Model HA405, Ingold Electrode, Wilmington, MA).

Assessment of proteolysis

A general and non-specific assessment of proteolysis was obtained by measuring the fraction of total N that was soluble in water on day 3, 29 and 50 of ripening by the method of Kuchroo and Fox (1982).

Proteolytic patterns in cheese on day 3, 29 and 50 of ripening were determined by urea-PAGE (polyacrylamide gel electrophoresis) using a modified version of the technique previously reported (Farkye *et al.*, 1991). The resolving gel in the method of Farkye *et al.* (1991) had a total monomer concentration of 12.5% (T) and a total cross-linker concentration (C) of 4%. However, up to T = 15%, pore size in polyacrylamide gels cross-linked with bisacrylamide is minimum when the cross-linker represents about 5% (by weight) of the total monomer concentration, i.e., C = 5%. Above T = 15%, achieving minimum pore size requires a proportionate increase in %C (Hames, 1990). Thus, the resolving gel in the present study had T and C equal to 12.5 and 5%, respectively. A transmission densitometer (E-C Apparatus Corporation, St. Petersburg, FL) coupled to an integrator (Hewlett Packard Model 3388, Avondale, PA) was used to quantify the percentage change in density of the primary casein bands during cheese ripening. The areas of the α_{s1}- and β-casein peaks on day 29 and 50 were compared to those on day 3 after correcting for level of protein loaded onto the gel. All other electrophoresis conditions were as reported earlier (Farkye *et al.*, 1991).

Cheese structure

Structural features of cheese samples were assessed on day 3, 29 and 50 post manufacture by scanning electron microscopy (SEM). Cheese samples, approximately 1 mm^3, were cut from sample blocks while immersed in 0.13 M sodium phosphate buffer (pH 7.3) and immediately fixed by plunging the cubes into 2.5% glutaraldehyde (same buffer) for 2 hours. The fixed cheese samples were washed four times in buffer and dehydrated in a graded ethanol series. This consisted of 15 minutes in each of a 10, 20, 35, 50, 70, 85 and 95% (v/v) ethanol solution. Samples were then frozen in liquid nitrogen and fractured. Frozen fractured samples were thawed in 100% ethanol and critical point dried with liquid carbon dioxide using a Samdri PVT-3B critical point drier (Tousimis Instruments, Rockville, MD,). The dried fractured cheese samples were mounted on copper specimen holders with fracture faces uppermost and sputter coated with gold/ palladium in a DC Sputter Coater E5100 (Polaron Instruments Inc., Doylestown, PA). Samples were examined in a JEOL 100 CX II TEMSCAN microscope equipped with a high resolution ASID scanning module operated at an accelerating voltage of 20 kV.

Results and Discussion

The original aim of this study was to determine whether the ratio of rod (*Lactobacillus delbrueckii* ssp. *bulgaricus*) to coccus (*Streptococcus salivarius* ssp. *thermophilus*) in the starter culture had an impact on the age-related changes in microstructure of Mozzarella. Structural changes in the three treatment cheeses (i.e., 1:1, 1:10 and 10:1 rod:coccus ratio) were indistinguishable over time. This is consistent with the fact that the treatment cheeses had the same composition and exhibited similar levels of hydrolysis of α_{s1}- and ß-casein throughout storage (Yun *et al.*, 1992). Consequently, the electron micrographs presented in this paper represent structural changes in LMPS Mozzarella cultured with a 1:1 rod:coccus ratio.

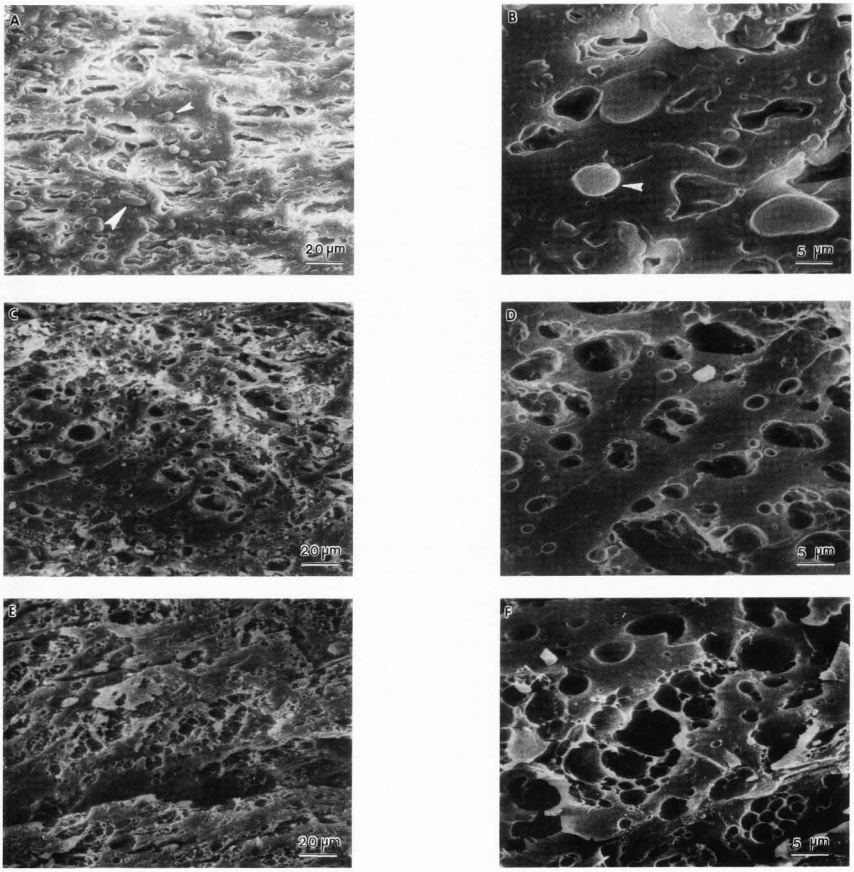

Figure 1. Scanning electron micrographs of LMPS Mozzarella during ripening at 4°C at two (A, C, and E at low; and B, D, and F at high) magnifications. A and B, C and D, and E and F are representative of curd structure after 3, 29 and 50 days, respectively.

The chemical composition of the LMPS Mozzarella cheese (n = 1) used for structural analysis is shown in Table 1 and was within the legal limits for LMPS Mozzarella (USFDA, 1989). The morphological features of cheese samples (fractured in cross-section) during the ripening period are shown in Fig. 1. Three days

Table 1. Composition of LMPS Mozzarella used for structural analysis (n = 1).

Fat	20.75%
Moisture	46.00%
Protein	28.36%
NaCl	1.35%
Ca	0.79%
pH	5.20

after manufacture (Fig. 1A and 1B), a relatively dense, homogeneous and continuous phase of amorphous paracasein, constituting a three-dimensional network, was present and was similar to that in LMPS Mozzarella reported earlier by Taranto and Yang (1981). A large number of microcavities of irregular dimensions were dispersed randomly throughout the paracasein matrix (Fig. 1A). A number of unextracted fat globules were present (arrowheads Figs. 1A, 1B). These resulted from the inadvertent post-fixing of day 3 samples in osmium tetroxide.

The origin of these microcavities is questionable. Some may represent the sites originally occupied by the extracted fat, but since these samples have been dehydrated, some may represent areas originally occupied by bulk phase water or whey. The dimensions of some of these cavities are within the diameter range for milk fat globules, i.e. 0.1-10 μm (Walstra and Jenness, 1984). Microcavities larger than this may be occupancy sites of aggregated fat globules. Hall and Creamer (1972) reported that increasing cooking and cheddaring temperatures during Cheddar manufacture increases fat globule aggregation. It is likely that the high temperatures encountered during the manufacture of LMPS Mozzarella in this study (cook, 41°C; stretch, 57°C) would facilitate either directly (via increased rupture and coalescence of milk fat globules) or indirectly (via increased deformability of the paracasein matrix) the formation of irregularly shaped microcavities. Taranto and Yang (1981) claimed that individual fat globules do not aggregate in LMPS Mozzarella. This is surprising since cavities with diameter greater than 10 μm can be discerned from their micrographs. At higher magnification, the irregular geometry of these cavities is more apparent (Fig. 1B).

The amorphous and compact nature of the paracasein matrix is similar to that observed by Taranto and Yang (1981) and by Kalab (1977) in curd granule junctions of Mozzarella (type unspecified). Freeze etch replicas of Cheddar and Gouda (Hall and Creamer, 1972) and Meshanger (de Jong, 1978) cheeses examined at high magnification indicate that the amorphous protein matrix contains readily discernible structural units.

By day 29 of ripening, there appeared to be an increase in the porosity of the defatted paracasein matrix (Fig. 1C). These microcavities ranged in diameter from less than 1 μm to greater than 20 μm. When viewed at higher magnification (Fig. 1D), it appears that the larger cavities may be originating from the confluence of smaller cavities. By day 50 (Fig. 1E and 1F), union of these microcavities had progressed to a point at which microcavities originally separate and discrete now communicated. The age-related confluence of microcavities that confine milk fat may provide part explanation for the age related increase in free oil formation in LMPS Mozzarella (Kiely *et al.*, 1991b). The micrographs obtained in the present study indicate that fat globules or aggregates thereof are separated by an intervening paracasein matrix. Thus, it is likely that proteolytic destruction of this protein barrier will facilitate coalescence of nearby fat globules on heating. Indeed, Paquet and Kalab (1988) provide evidence that neighbouring fat globules agglomerate after collapse of the paracasein matrix during heating of stirred curd and stretched Mozzarella. Consequently, a proteolytically weakened paracasein matrix (as in aged Mozzarella) will likely possess a lowered ability to physically contain fat during heating.

Alterations in the paracasein matrix during cheese ripening may, in part, be attributed to proteolysis. Figure 2 shows a large increase in the levels of water soluble nitrogen (WSN) by day 50 of ripening, from just over 2% of total N at day 3 to almost 8% of total N by day 50. Similar increases in WSN over a 2-week period in commercial LMPS Mozzarella coagulated with *Endothia parasitica* protease have been reported by Farkye et al. (1991).

In addition to whey proteins, the water-soluble fraction contains hydrolysed casein fragments and free amino acids (Christensen *et al.*, 1991). Casein hydrolytic fragments result from the action of a variety of proteolytic agents, foremost among which is coagulant. The gel electrophoretogram indicates that the coagulant (chymosin) was active (Fig. 3). The intensity of the band corresponding to β-casein remained virtually unchanged throughout storage (Figs. 3 and 4). Coincidental with this was a minimal increase in the intensity of the γ-caseins. Thus, the electrophoretic data suggest that the endogenous milk protease, i.e., plasmin, was relatively inactive. The minor band migrating just ahead of β-casein may be the more acidic peptide, β-I casein. The latter primarily represents fragment 1-189 of β-casein and is produced (in solution) by the action of rennet on β-casein (Creamer, 1976).

In contrast, α_{s1}-casein, which is considered to be the primary structural element in Cheddar and other varieties (de Jong, 1977; Lawrence *et al.*, 1987), underwent significant hydrolysis during the storage period (Figs. 3 and 4). The intensity of the band corresponding to α_{s1}-casein decreased 50% by day 50 of ripening. The faster moving $\alpha_{s1\text{-}I}$ peptide results from the action of rennet on α_{s1}-casein (Marcos *et al.*, 1979). Thus, it appears that the observed structural alterations resulting from proteolysis, are related to the hydrolysis of α_{s1}-casein by chymosin.

However, whether hydrolysis of α_{s1}-casein alone produces such geometrically proper, spherical microcavities is unclear. The general morphology and apparent confluence of adjoining microcavities may be suggestive of gas formation. Indeed, gas holes have been visually observed in excessively aged Mozzarella (>1 year) made with the same starter bacteria (Kiely, unpublished observation). In addition to producing peptidases, *Streptococcus salivarius* ssp. *thermophilus* produces CO_2 which acts as a stimulant for the more proteolytic *Lactobacillus delbrueckii* ssp. *bulgaricus* (Robinson and Tamime, 1990). Thus, the increased openness of the protein matrix may have a twofold etiology. Proteolytic

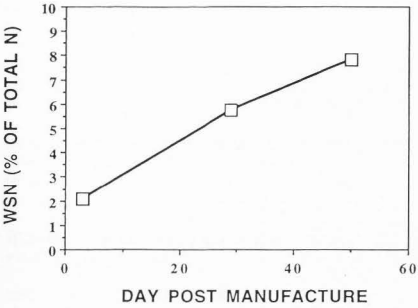

Figure 2. Development of water soluble nitrogen (WSN) in LMPS Mozzarella (n = 1) during 50 days of storage at 4°C.

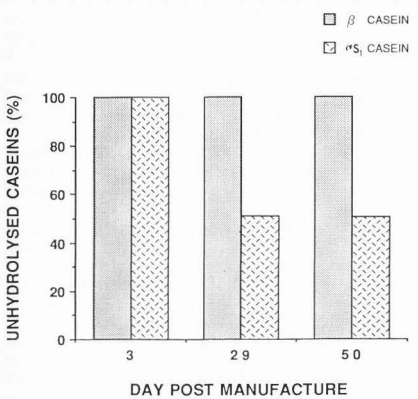

Figure 4. Amount of unhydrolysed α_{s1}- and β-casein in LMPS Mozzarella (n = 1) during 50 days of storage at 4°C (expressed as % of day 3).

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destruction of the paracasein matrix separating two adjoining microcavities, coincidental with increased pressure from CO_2 production, would facilitate their union. Void spaces routinely observed by scanning electron microscopy in defatted cheese samples are usually termed fat globule vacuoles. While this may be true of many of the observed cavities, it is clear from the present work that the time-dependent increase in porosity of the defatted paracasein matrix may be related to factors already discussed.

In conclusion, the present study showed an age-related increase in porosity in the defatted paracasein matrix of LMPS Mozzarella. This was coincidental with

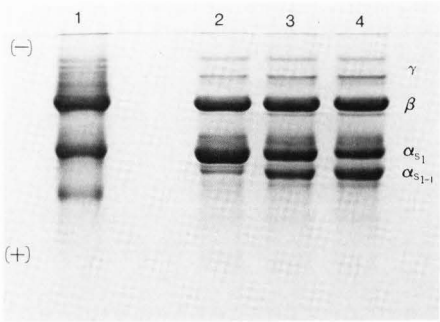

Figure 3. Electrophoretogram of LMPS Mozzarella cheese caseins during refrigerated storage. Lanes 2, 3 and 4 show hydrolytic patterns after 3, 29 and 50 days of storage at 4°C. Lane 1 contains α_{s1}-casein and β-casein markers.

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an increase in general proteolysis and specifically with hydrolysis of 50% of α_{s1}-casein. Such structural and proteolytic alterations will likely contribute to the significant changes in functional properties that occur during the ripening period. In particular, communication of fat globule vacuoles due to proteolysis, may compromise the ability of aged Mozzarella cheese to physically hold fat when heated.

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Discussion with Reviewers

H.D. Goff: Have you investigated the rheological properties of the cheese at different temperatures as a function of the ripening process, particularly the first 3 weeks, to demonstrate a correlation between the hydrolysis of α_{s1}- casein and flow behavior during melting or melting temperature?

Authors: With respect to melting temperature and it's effect on rheological properties, we investigated the effect of melting temperature on apparent viscosity and the formation of free oil during early studies that led to the development of the helical viscometry and free oil test procedures. Much of this data has been reported only in preliminary form (Rippe and Kindstedt, 1988a; Rippe and Kindstedt, 1988b) and much is unpublished; however, it can be said that: **1**) melting temperature strongly affected flow properties, and **2**) the effect of melting temperature differed with cheese age. The variable effect of melting temperature with cheese age probably was indeed related to the hydrolysis of casein, however we did not measure proteolysis in those studies.

As regards temperature during the ripening process, we have reported that changes in the apparent viscosity and free oil of Mozzarella cheese during aging were accelerated significantly when storage temperature was increased by 10°C (Kiely *et al.*, 1992b; Larose *et al.*, 1992). These accelerated functional changes at higher storage temperature were probably associated with increased hydrolysis of casein, however, we have not yet established this definitively.

C.J. Oberg: Appearance of larger cavities may only be due to where the sample was taken from. How many samples were studied and did they all show this same pattern of change between day 3, day 29, and day 50?

Authors: Usually 3-4 samples per treatment cheese were examined by SEM on day 3, 29 and 50 and all of the sampled cheeses showed this general pattern of change in structure during the ripening period.

C.J. Oberg: The proteases and peptidases of the starter bacteria also play a significant role in aging proteolysis of Mozzarella cheese. Was this taken into account as part of the explanation for the increased proteolysis over time?

Authors: Starter bacteria indeed contribute to proteolysis in Mozzarella cheese. In a separate study (Chu *et al.*, 1992), starter-free and rennet-free Mozzarella cheeses were employed to investigate the independent effects of starter bacteria and coagulant on proteolysis. It was found that although starter bacteria contributed significantly to the formation of soluble nitrogen during aging, starter proteases did not hydrolyze intact caseins. In contrast, intact caseins were hydrolyzed into smaller peptides and amino acids by the starter. A full report of this research has been submitted for publication (J. Dairy Sci.).

C.J. Oberg: Was the proteolysis of the starter culture measured? Since there is great variation in proteolysis among Mozzarella starter cultures, the culture may play a significant role. The explanation for increased porosity with age focuses only on the milk clotting enzyme when numerous studies have shown that culture proteolysis also plays a significant role.

Authors: Proteolysis by the starter culture was not measured in this study. However, as indicated earlier, studies of rennet-free and starter-free cheeses (Chu *et al.*, 1992) showed that the bacterial strains used in the present investigation did not hydrolyze intact caseins during aging of Mozzarella cheese, whereas coagulant actively hydrolyzed intact caseins. In short, the coagulant attacked the protein matrix directly, whereas the starter acted on the peptides formed by the coagulant. Consequently, we believe that coagulant plays a greater role in the alteration of curd structure during aging than does the starter culture.

C.J. Oberg: Where are the bacteria in these micrographs? They certainly should be evident in the higher magnification micrographs, particularly at day 3 and day 29.

Authors: Bacteria (presumably lactic acid bacteria) were routinely visualized; the micrographs presented in this paper focus on the paracasein matrix.

C.J. Oberg: If the vacuoles were caused by CO_2 production from the cocci, I would expect them to be much larger. Were large vacuoles (visible without microscopy) observed, particularly in the 50 day cheese?

Authors: No, we did not observe large vacuoles (visible without microscopy) in the cheese at or before 50 days of ripening. Although *Streptococcus thermophilus* can produce CO_2, this obviously occurs at a rate insufficient for "eye" formation.

M. Rosenberg: Could it be that the large number of "pores" in the 50 days old cheese was the result of the development of gas forming bacteria (not related to the starter)?

Authors: Yes, heterofermentative bacteria or clostridia are also capable of producing gas.

M. Kalab: Omission of postfixation using osmium tetroxide was apparently intentional and the objective was to remove fat from the samples destined for scanning electron microscopy. However, omitting to extract the fat using *n*-hexane or chloroform would result in its incomplete removal by liquid CO_2 from the samples. Please explain.
Authors: The majority of published methods that relate to defatting of cheese samples destined for electron microscopy usually accomplish fat removal by treatment with hexane or chloroform. However, from past experience with LMPS Mozzarella, we have found no evidence of residual fat in cheeses (not post fixed) treated with an ethanol series and liquid CO_2. Apparently, these treatments remove the fat from LMPS Mozzarella. This did not hold true for other cheese types.

M. Rosenberg: Can the authors explain why they did not conduct post fixation in osmium tetroxide?
Authors: Post-fixing in osmium tetroxide was not conducted as fixation of the lipid component in the cheese was not one of our objectives.

M. Rosenberg: I would suggest that a strong statement about the unclear source of the structural features after 50 days of ripening be added. It seems to me that there are not enough data to allow any speculation regarding the presented structural details of the 50 days old cheese.
Authors: Concerns about the "unclear source of the structural features after 50 days of ripening" are certainly valid. While we readily acknowledge that the present study is not definitive by any means, we do feel that our lack of certainty concerning the development of structure during 50 days of ripening has been adequately addressed in the text. Our claims are of a speculative nature and stem largely from the present and previous studies where we have shown that proteolysis of casein is one of the primary biochemical events that occurs during the ripening of LMPS Mozzarella cheese. Considering that α_{s1}-casein probably represents approximately 40% of the protein matrix (based on it's relative content in milk), then it's hydrolysis by approximately 50% (as in this study) should be discernible by electron microscopy.

Additional References

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