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Miloslav Kalab

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DEVELOPMENT OF MICROSTRUCTURE IN SET-STYLE NONFAT YOGHURT - A REVIEW

Miloslav Kaláb, Paula Allan-Wojtas, and Beverley E. Phipps-Todd

Food Research Institute, Research Branch, Agriculture Canada,
Ottawa, Ontario, Canada K1A 0C6

Abstract

The development of microstructure in natural set-style nonfat yoghurt was studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In addition to thin-sectioning and conventional SEM described in the literature, this review illustrates gelation of milk with micrographs obtained by rotary shadowing of casein micelles and their clusters. The existence of void spaces occupied by lactic acid bacteria in yoghurt was confirmed by cold-stage SEM of uncoated specimens. The microstructure of yoghurt is affected by the preheat treatment of milk, bacterial starter cultures, total solids content, and the presence of thickening agents. The microstructure was found to be related to firmness and susceptibility to syneresis. Suggestions on the preparation of yoghurt samples for electron microscopy have been included in this review.

Introduction

Traditionally, yoghurt is a cultured (fermented) milk product made by incubating milk with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* cultures. Yoghurt (yogurt) is made with bacteria, except in Turkey, where the product is called yahaourt or yaourt⁷, jugurt or eyran⁴⁴, in which "a lactose fermentation yeast culture is also included with the lactic-acid organisms"⁷.

Initially, yoghurt was made from milk concentrated by boiling but today it is made from homogenized whole milk, partially skimmed milk, or from skim milk with or without added nonfat dry milk (NDM) solids. Technology, biochemistry, and quality appraisal of yoghurt have been reviewed in great detail^{33, 35, 36, 44}.

In North America, yoghurt has become increasingly popular in the last 20 years and in Canada alone the production of yoghurt between 1977 and 1981 was increased by 56% (from 27.504×10^6 to 42.972×10^6 litres)⁴. Although initially yoghurt had a characteristic custard-like body and texture, recent development of the product has proceeded in various directions and yoghurt texture may vary today from a semifluid stirred yoghurt to a set-style yoghurt in which added gelatin may lead to a rigid, brittle gel. The introduction of flavoured yoghurt and yoghurt with fruits or jams has been an important factor leading to the increase in the popularity of yoghurt in North America.

The development of microstructure in yoghurt has been studied only to a limited extent^{5, 22, 23, 28} in spite of the number of yoghurt varieties with different characteristics (stirred, set-style, plain, flavoured, "low-calorie", dried, frozen yoghurt, baby yoghurt etc.) on the market and in spite of the fact that some physical properties such as firmness, smoothness, ropiness, and susceptibility to syneresis are reflected by the microstructure.

The objectives of this review are to summarize recent results of microscopical studies of yoghurt and to stimulate the interest of microscopists in this milk product which is an excellent milk gel model.

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Direct inquiries to M. Kaláb.
Telephone number: 613-995-3700.

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Transmission electron microscopy; Thickening agents;
Whey proteins; Yoghurt.

Principles of yoghurt manufacture

In fluid milk, casein micelles exist as individual entities. Lactic acid produced by lactic acid bacteria (cocci and bacilli) lowers pH of the milk and destabilizes the casein micelles which become subsequently linked to each other in the form of clusters and chains. The clusters and chains are part of a protein network (matrix) in which the liquid phase of milk is immobilized and a gel is formed. The way in which casein micelles may interact depends on many variables such as the heat treatment of the milk destined for gelation, bacterial strains and the ratio of cocci to bacilli, rate of acid development and amount of lactic acid developed, temperature of incubation, total solids, presence of additives (thickening agents) etc.

Heating of milk destined for the production of yoghurt prior to the inoculation with lactic acid bacteria plays an important role in the development of the yoghurt. Grigorov¹ demonstrated that heating of milk to 85°C for 30 min led to minimum syneresis in yoghurt when compared to heating at lower or higher temperatures. Preheating the milk to higher temperatures (90° to 95°C), however, led to a decrease in the consistency of the yoghurt.^{2,3} Galesloot and Hassing⁴ suggested that milk should be heated to 90°C in a continuous-flow heater or to 85°C when heating is carried out in tanks. Thus, in general practice, milk destined for the manufacture of yoghurt is preheated to 90° to 95°C for 15-30 min⁵

Heating of milk

In a study on the effect of heating of milk upon yoghurt microstructure, Kalab et al.^{2,3} preheated freshly skimmed milk or reconstituted low-heat NDM of an undisclosed fat content to 44, 55, 65, 75, 85, and 90°C. Immediately after the desired temperature was reached, the milk was cooled to 44°C, inoculated with *L. bulgaricus* and *S. thermophilus* cultures, and incubated at 44°C until it was gelled and a pH value of 4.3 in the yoghurt was reached. The yoghurt was then cooled to 6°C.

No differences in the appearance of the casein micelles between unheated milk (i.e. milk preheated to 44°C) and milk heated to 90°C were found by the authors 30 min before gelation^{2,3}. At approximately 16 min before gelation, casein particles in unheated milk developed short projections (appendages) (Fig. 1) and within 3 min groups containing two or three particles had joined together and these groups combined into larger clusters which started to form a three-dimensional network (Fig. 2). The appendages had vanished at that stage. Casein micelle chains were robust but "seams" between the fused casein particles were clearly visible (Fig. 3a). Finer chains were formed in yoghurt made from heated milk (Fig. 3b).

Holding milk at a high temperature was reported⁶ to have an effect on the casein micelles: in raw or in high-temperature short-time pasteurized milk the micelles had relatively smooth, uninterrupted contours compared to micelles from milk which had been heated to 90°C for 10 min or was autoclaved at 121.7°C for 15 min. In these latter milk samples, filamentous appendages bridged adjacent micelles and small amounts of the filamentous material were found free in the heated milk

samples (Fig. 4). The appendages were retained until the late stages of culturing (at least 3.5 h). Although they became gradually more diffuse, their presence appeared to be sufficient to inhibit micellar contact and fusion (Fig. 5)⁵. In their search for the origin of the appendages, Davies et al.⁵ found that casein micelles heated in a protein-free milk dialyzed were free of such appendages whereas the appendages developed on casein micelles heated in the presence of whey proteins. It was established that the presence of β -lactoglobulin was the prerequisite for the development of the appendages.

These appendages could have been formed either by heat-denatured β -lactoglobulin alone or by a complex consisting of β -lactoglobulin and κ -casein; such a complex develops in heated milk⁷. The addition of N-ethylmaleimide (a thiol-blocking agent) to milk appreciably reduced the development of appendages when the milk was heated⁸. However, because both the heat denaturation and precipitation of β -lactoglobulin and the formation of the β -lactoglobulin- κ -casein complex most probably involve disulfide bonding⁹, the only conclusion made by Davies et al.⁵ was that β -lactoglobulin participated in the formation of the appendages.

The hypothesis that denatured β -lactoglobulin is bound to casein micelles in milk heated to 90°C is supported by gel electrophoresis of whey separated by centrifugation from yoghurt made from heated milk. β -Lactoglobulin was present in whey separated from yoghurt made from unheated milk but was absent in whey separated from yoghurt made from heated milk^{2,3}.

More recently, Kalab et al.^{2,4} examined casein micelles in unheated and heated milk using rotary shadowing with platinum and carbon. In milk heated to 90°C for 10 min the casein micelles had "ragged" surfaces, whereas micelles in unheated milk had smooth surfaces (Fig. 6). The same technique was used to examine changes in casein micelles developing during the gelation of milk by yoghurt starter cultures and the formation of casein micelle chains was also demonstrated (Fig. 7 and 8)

Thus, it may be concluded that casein micelles in heated milk acquire a "ragged" appearance as the result of the attachment of denatured β -lactoglobulin or a heat-induced β -lactoglobulin- κ -casein complex to their surfaces. The temporary appendages observed by Kalab et al.^{2,3} in unheated milk shortly before gelation are difficult to explain unless an assumption is made that they are different from the appendages observed on casein micelles in heated milk; in their work, Kalab et al.^{2,3} mixed the milk with a warm agar sol and the casein micelle; were fixed in a glutaraldehyde solution only after the mix had solidified. In contrast, Davies et al.⁵ first fixed the micelles and subsequently immobilized them in agar gel. When unfixed milk is mixed with warm agar, an interaction between casein micelles "ready to gel" and the agar cannot be excluded as the cause of the temporary appendages.

As the pH of the milk gradually declined below 4.3 because of the production of lactic acid by the bacterial culture, the gelation of the milk was completed and yoghurt was formed. It has already been mentioned earlier that yoghurt made from unheated milk consisted of a network considerably more robust (Fig. 3a) than yoghurt made from heated

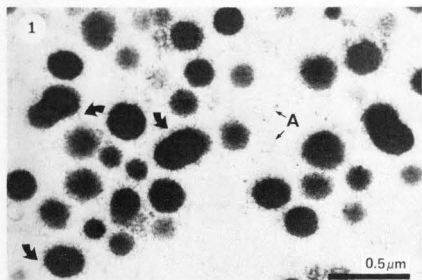


Fig. 1. A detail of casein micelle aggregation in unheated skim milk 16 min before gelation. Appendages on casein micelles (arrows) are clearly visible and are different from agar fibres (A). Reproduced by permission from Milchwissenschaft²³.

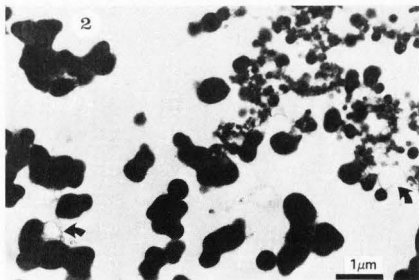


Fig. 2. Onset of casein micelle aggregation and formation of clusters (large structures in the left half of the micrograph) in unheated skim milk. Arrows point to small fat globules present in very small quantities in that skim milk.

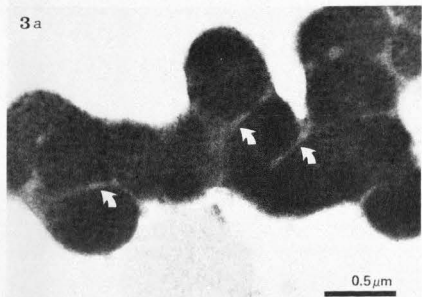
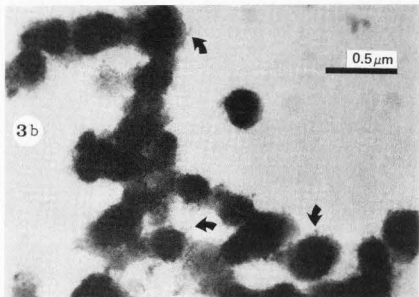


Fig. 3. Protein matrix in yoghurt made from unheated (a) and heated (b) skim milk. In (a) casein micelle clusters are tightly fused and form large aggregates, yet "seams" between the particles are visible in thin sections (arrows).



In (b) the casein micelles are linked in finer chains; appendages on the casein micelle surfaces are still present at the early stages of gelation (arrows). Micrograph (a) reproduced by permission from Milchwissenschaft²³.

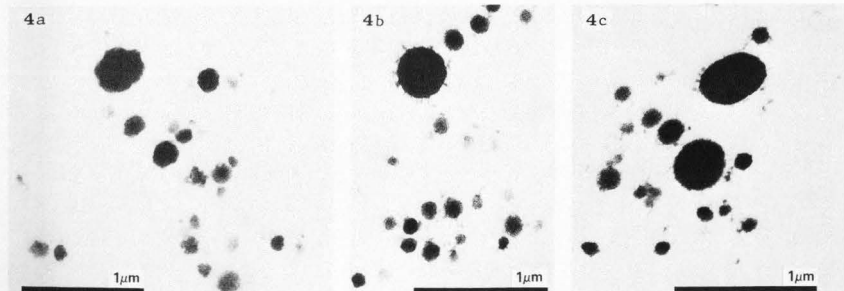


Fig. 4. Casein micelles in raw milk (a), in milk heated to 95°C for 10 min (b), and in milk auto-

claved at 121.7°C for 15 min (c). Reproduced by permission from the Journal of Dairy Research⁵.

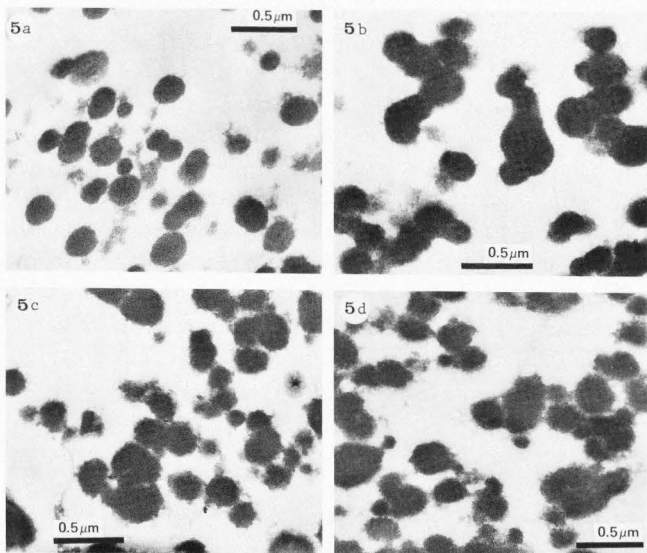


Fig. 5. Casein micelles in raw (a and b) and in heated (95°C for 10 min) (c and d) skim milk at various intervals during fermentation with *S. thermophilus* and *L. lactis* to yoghurt. Fermentation times: a: 4.5 h; b: 5 h (both samples are from raw milk); c: 3.5 h; d: 3.75 h (both samples are from heated milk). Reproduced by permission from the *Journal of Dairy Research*⁵.

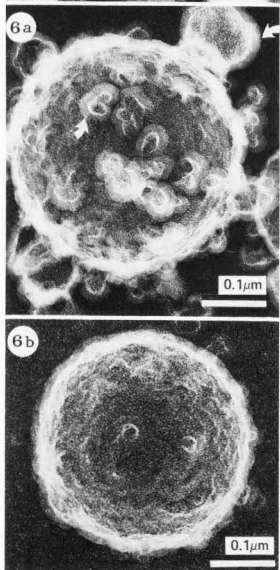


Fig. 6 (left). Details of casein micelle surfaces by rotary shadowing with platinum and carbon. a: Casein micelle in heated skim milk; halo around the appendages (arrows) is caused by carbon reinforcement of the platinum replica; b: casein micelle in unheated skim milk has a smooth surface.

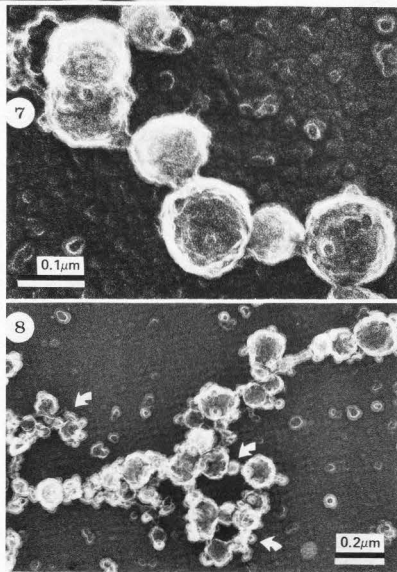


Fig. 7 (below). Detail of casein micelle chaining (rotary shadowing) during the gelation of reconstituted NDM.

Fig. 8 (right). Casein micelles in heated skim milk form chains and loops (arrows) which will develop into a three-dimensional matrix at later stages of gelation.

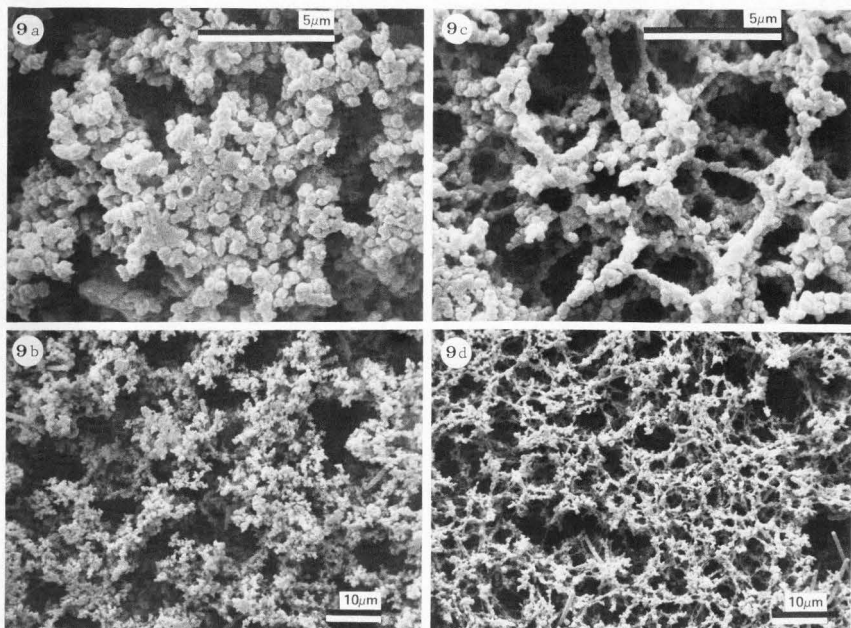


Fig. 9. SEM of yoghurt made from unheated (a and b) and heated skim milk (c and d). In yoghurt made from unheated skim milk the casein micelles are in the form of coarse clusters (a) which results in large compartments (cells) in the matrix (shown at

a lower magnification in b). In yoghurt made from heated skim milk the casein micelles are in the form of chains (c) and this results in smaller compartments in the matrix (d).

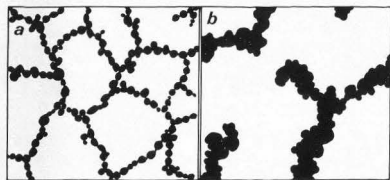


Fig. 10. A schematic diagram comparing the microstructure of yoghurt made from heated milk (a) composed of small compartments formed by single branched chains of casein micelles and the microstructure of a yoghurt made from unheated milk (b) composed of large compartments formed by clustered casein micelles. Firmer immobilization of the liquid phase is experienced with (a) than with (b).

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milk (Fig. 3b). This was also confirmed by SEM²³; details of casein micelle aggregation are shown in Figs. 9a and c and the organization of the matrices is presented at a lower magnification in Figs. 9b and d. On the basis of similar micrographs a model (Fig. 10) of the different matrices had been designed earlier¹². In the diagram in Fig. 10, the same number of casein micelles is shown in sections of a fine network (a) and a coarse network (b). The liquid phase (whey) was immobilized in the fine matrix which consisted of small compartments but the whey separated more easily from larger compartments in the coarse matrix. Susceptibility to syneresis in both yoghurts was measured by the volume of whey separated from the milk gels by centrifugation at several different centrifugal forces for 10 min^{13,14} (Fig. 11) and by draining the yoghurts for up to 1 h²⁰ (Fig. 12). It is evident from these measurements that yoghurts made from unheated milk were considerably more susceptible to the separation of whey than yoghurts made from heated milk. Tarodo de la Fuente and Alais⁴⁵ explained that heating increased solvation of casein micelles and that after heating to 90°C for 2 min

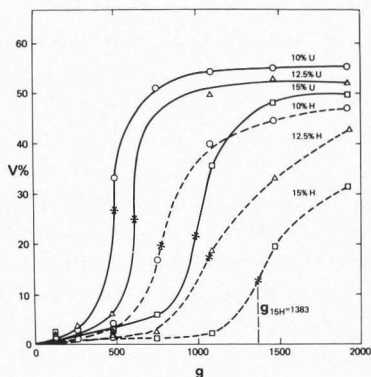


Fig. 11. Separation of whey from yoghurts (10.0, 12.5, and 15.0% total solids) at pH 4.0 with increasing centrifugal force applied for 10 min. Abscissa: Centrifugal force (g). Ordinate: Volume V (%) of the whey separated relative to the total volume of the yoghurt. Yoghurts made from unheated reconstituted nonfat dry milk are shown in solid lines (U) and yoghurts made from reconstituted nonfat dry milk preheated to 90°C for 10 min are shown in dashed lines (H). [From¹⁴ - to be published in *Milchwissenschaft*].

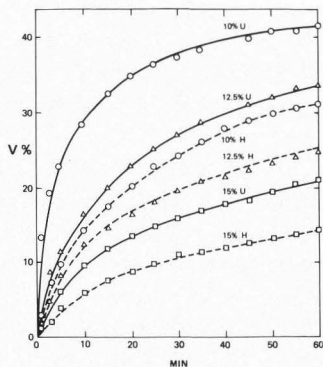


Fig. 12. Separation of whey by draining yoghurts (10.0, 12.5, and 15.0% total solids) at pH 4.0 for varying periods of time.

Abscissa: Time (min). Ordinate: Volume V (%) of the whey separated relative to the total volume of the yoghurt. Total solids contents and heat treatments (U: unheated milk; H: preheated milk) are indicated in the diagram and are the same as in Fig. 11. [From¹⁴ - to be published in *Milchwissenschaft*].

there was about twice as much non-solvent water in the micelles as in the raw milk micelles. Denatured β -lactoglobulin either alone or in the form of a complex with κ -casein in heated milk⁹ inhibited micelle fusion, which is in agreement with an earlier postulate by Knoop and Peters²⁶. The formation of a fine protein network in yoghurt made from heated milk resulted from this inhibition of micelle fusion.

In addition to the preceding experiments which had been carried out using fresh milk or reconstituted spray-dried NDM^{5,23}, yoghurts were also made from reconstituted roller-dried and freeze-dried NDM²³. Although the milk in the roller-dried NDM had received a severe heat treatment during production, yoghurt made from this source was of a very poor quality as far as flavour, texture, and susceptibility to syneresis were concerned. This is probably associated with the formation of fused micellar aggregates and the inability of the casein micelles in roller-dried NDM to freely disperse in water. Additional heating of the reconstituted NDM suspension had no effect on the dimensions of casein particles in that yoghurt. However, in yoghurt made from reconstituted freeze-dried NDM the differences between casein particle dimensions depending on the heating of milk were greatest of all types of NDM.

Total solids content

Dimensions of the compartments in the protein matrix of yoghurt and, thus, susceptibility to syneresis, are also affected by factors other than the preheat treatment of milk prior to gelation.

One of these factors is the total solids content in the milk. In practice, the total solids content is largely dictated by legal standards of the country concerned²⁴. It may vary from 9 to over 20%, but bacterial activity is retarded at levels exceeding 25% total solids³¹. The range most generally recommended lies between 14 and 18% according to Robinson and Tamime³⁵ or between 15.5 and 16.0% according to Kozhev et al.²⁷.

The increase in the total solids content can be achieved in several ways³⁴. By maintaining the same casein to non-casein protein ratio, there is a higher number of casein micelles per unit volume at a higher total solids content which results in smaller compartments occupied by a smaller volume of the liquid phase. This is evident in Fig. 13, where the protein matrices are shown in yoghurt made from low-heat spray-dried reconstituted NDM containing 10 to 20% total solids. In addition to smaller compartments, a higher number of casein micelles and a larger amount of whey proteins per unit volume bind a larger proportion of water. This was demonstrated by draining the different yoghurts using the procedure described by Modler et al.²⁹ (Fig. 12) and by centrifuging the yoghurts at different centrifugal forces for 10 min¹⁴ (Fig. 11). Only a part of the aqueous phase in the yoghurt may be separated from the protein matrix by centrifugation because the other part is firmly bound by the proteins. As the total solids content in the yoghurt is increased, the volume of the aqueous phase which may be separated is decreased. Matrices of low-solids yoghurts are subject to noticeable compaction whereas matrices of

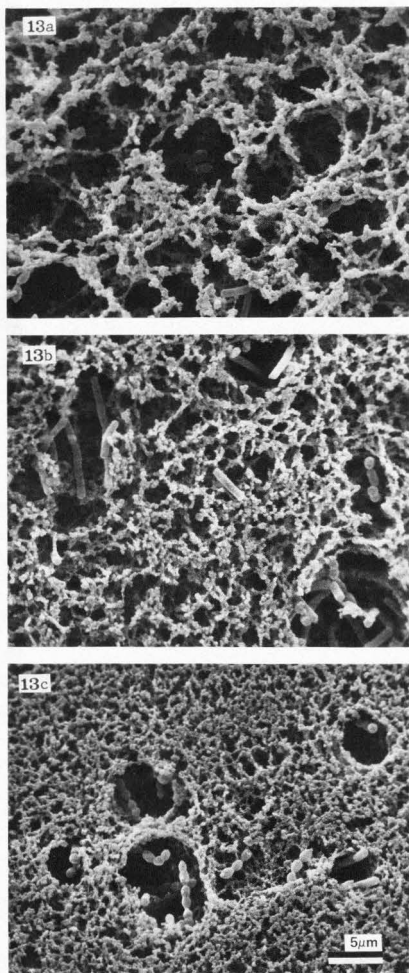


Fig. 13. Protein matrices in yoghurts made from heated reconstituted nonfat dry milk containing 10.0% (a), 15.0% (b), and 20.0% (c) total solids. The dimensions of compartments (pores) in the matrices are decreased as the total solids contents are increased. At higher (15 and 20%) total solids contents void spaces around lactic acid bacteria become more clearly evident.

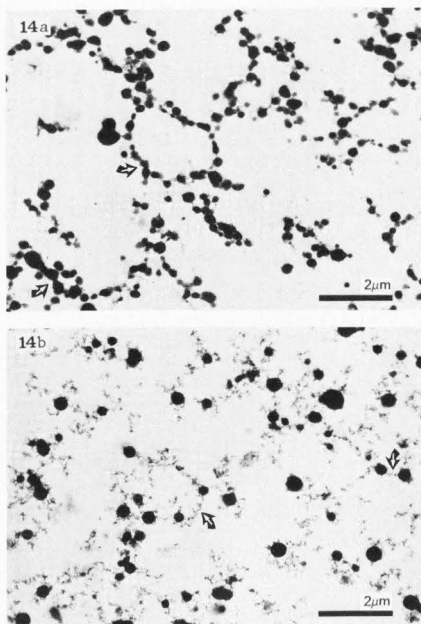


Fig. 14. Comparison of protein matrices in an unfortified yoghurt (a) and a yoghurt fortified with a whey protein concentrate (b). In unfortified yoghurt (a) the casein micelles form chains by contacting each other (arrows) whereas in the fortified yoghurt (b) the casein micelles are linked to each other with fine aggregates of whey protein (arrows). Reproduced by permission from the *Journal of Dairy Science*^{2,8}.

high-solids yoghurts are compacted to a considerably lesser extent or are not compacted at all.

The total solids content in milk may be increased by evaporation, ultrafiltration (which selectively increases only the fat and protein content), reverse osmosis, or by addition of NDM, milk protein concentrate, whey powder etc. Microstructure of yoghurt made from skim milk (3.5% total protein) fortified to 5.0% total protein was studied recently^{2,9,29}. Sodium caseinate, NDM, milk protein concentrate, and whey protein concentrates, commercially obtained by electrodialysis, ultrafiltration, and ion exchange were used as the fortification agents. Because of the different composition of the additives, the casein to non-casein protein ratio was 4.56 : 1.00 in yoghurt fortified with sodium caseinate, 2.85 : 1.00 in yoghurt fortified with NDM and milk protein concentrate, and 1.08 : 1.00 in yoghurt fortified

with whey protein concentrates.

Sodium caseinate significantly increased the dimensions of casein particles in the fortified yoghurt and formed a microstructure similar to that in yoghurt made from unheated milk. Because the addition of sodium caseinate decreased the relative non-casein protein content in the milk mixture to 18%, it may be hypothesized that the casein micelles in the milk thus fortified were insufficiently coated with denatured β -lactoglobulin or with the β -lactoglobulin- κ -casein complex and, thus, were not prevented from excessive fusion²⁶. However, the true mechanism has yet to be studied. Interestingly, susceptibility to syneresis was lowest (8%) and gel strength⁸ was highest (117.9 g) in the yoghurt fortified with sodium caseinate. NDM and milk protein concentrate reduced susceptibility to syneresis and increased gel strength as compared to unfortified yoghurt but had no significant effect on the microstructure.

Whey protein concentrates considerably altered the microstructure of the fortified yoghurts as compared to unfortified yoghurt (Fig. 14). Instead of casein micelles tightly fused into compact chains common to yoghurt with a natural casein to non-casein protein ratio, the casein micelles were linked to each other at relatively long distances with finely flocculated whey proteins (Fig. 15). Gel strength of the fortified yoghurts was approximately the same (77-79 g) as that of yoghurt fortified with NDM when the whey protein concentrate used was obtained by ultrafiltration. The gel strength was lower (50-55 g) with whey protein concentrates obtained by methods in which electro-dialysis and ion exchange were part of the process.

Thickening agents

It is not always feasible to increase the total solids content of yoghurt in order to improve texture, increase firmness, and reduce susceptibility to syneresis. There may be various reasons against such a step, for example the concern for the joule (calorie) value, the lactose content, retardation of the bacterial action and hence prolonged coagulation time, titratable acidity etc.⁴⁴. There is a wide variety of thickening agents available to facilitate the immobilization of the liquid phase in yoghurt; gelatin, pregelatinized starch, cellulose derivatives, alginates, and various gums have been used commercially. Effects of a great number of thickening agents on the quality of yoghurt were studied by Radema and Van Dijk³². Some thickening agents were found to have a tendency to decrease the rate of the lactic acid production. Kalab et al.²² examined the microstructure of yoghurt as related to the presence of gelatin, carrageenan, and pregelatinized starch. At a 0.5% concentration, gelatin did not significantly affect gel strength²² but considerably reduced syneresis to less than 2.5%²⁹. However, yoghurts containing this concentration of 225 Bloom gelatin were indistinguishable by SEM and TEM from yoghurts not containing any additive. No change in the microstructure of the protein matrix was observed even with the gelatin concentration increased to 10% when the yoghurt resembled a gelatin gel rather than a typical yoghurt. This was probably because neither of the electron microscopical techniques used was suitable to detect collagen fibres

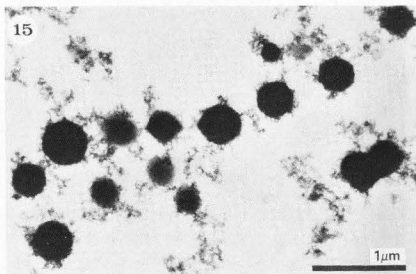


Fig. 15. Detail of a casein micelle chain in yoghurt fortified with a whey protein concentrate. The links between the casein micelles are provided by fine aggregates of whey protein. Reproduced by permission from the *Journal of Dairy Science*²⁸.

of gelatin in the milk gel. Although the authors referred to an earlier report²¹ showing a gelatin gel composed of thin sheets, such sheets represented typical artefacts formed by slow freezing of the gel whereby large ice crystals developed, and the gelatin was compressed into the form of thin sheets between the ice crystals. After freeze-drying and sublimation of the ice, these sheets were visualized by SEM.

Carrageenan and pregelatinized starch were reported²² to considerably change the microstructure of yoghurt. Both additives led to the clustering of casein micelles as was demonstrated in thin sections of embedded specimens; the density of the casein micelle clusters was higher in the presence of carrageenan. Interactions with carrageenan, which led to the aggregation of casein micelles, were studied by Snoeren⁴⁰ and Hood and Allen¹⁷. Although short fibres with free terminals and sheets were reported by SEM²² to be present in yoghurt containing pregelatinized starch, it is highly probable that the initial microstructure of the specimens had been altered during preparatory steps such as freeze-drying and that the structures found were actually artefacts. Several authors^{3, 20} pointed to critical-point drying as a technique not suitable to dry food samples containing pregelatinized starch. The only proper electron microscopic technique to visualize gelatinized starch is freeze-fracturing followed by replication with platinum and carbon². Hence it follows that results obtained by examining the effects of thickening agents on the microstructure of yoghurt should be revised.

Lactic acid bacteria

In yoghurt, the milk is gelled by a combined action of cocci and bacilli which digest lactose and convert it into lactic acid, thus lowering pH of the milk which leads to its gelation. In addition, lactic acid bacteria cause a significant degree of proteolysis in yoghurt⁴⁴, which leads to changes in the physical structure of the product²⁵, although these bacteria are usually considered to be only weakly proteolytic⁶.

Yoghurt starter bacteria, *L. bulgaricus* and *S. thermophilus*, are thermophilic, homofermentative lactic acid bacteria whose characteristics were summarized by Tamime and Deeth⁴⁴. The ratio of *L. bulgaricus* to *S. thermophilus* determines flavour and body characteristics of the ripened yoghurt⁴⁵. In yoghurt manufacture, ratios of 1:1 to 1:3 have been used. However, Tamime and Deeth⁴⁴

noted that the term "ratio" as it appears in the literature, is somewhat vague because it may refer to the colony-to-colony, clump-to-clump, chain-to-chain, or cell-to-cell ratios of *Lactobacillus*-to-*Streptococcus*. According to the starter manufacturers, a ratio of 1:1 means 5 to 10 cells of *S. thermophilus* to one cell of *L. bulgaricus*⁴³.

SEM examination at low magnifications of smooth

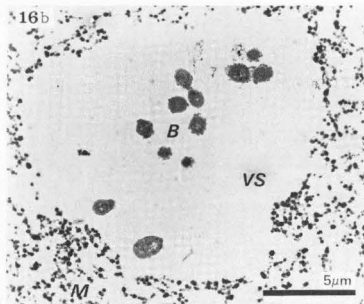
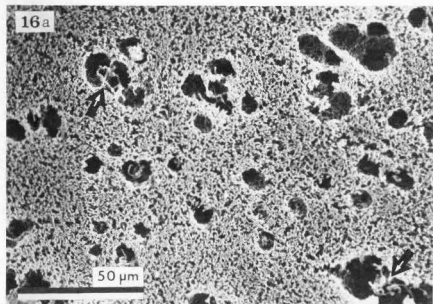


Fig. 16. Void spaces occupied by lactic acid bacteria in a set-style nonfat yoghurt containing 20% total solids. Most void spaces shown in the SEM micrograph (a) contain streptococci (arrows). Void spaces around lactic acid bacteria can also be observed in thin sections (b). B = bacteria, VS = void space, M = protein matrix.

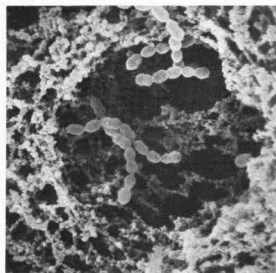
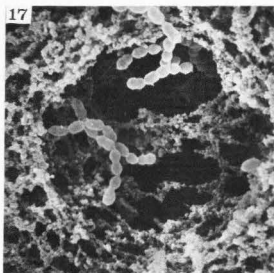


Fig. 17. A pair of stereo micrographs showing details of a void space and its contents in set-style yoghurt. Casein micelle chains surrounding the void space are oriented as if subjected to some stress. This is a cross section of an elongated void space.

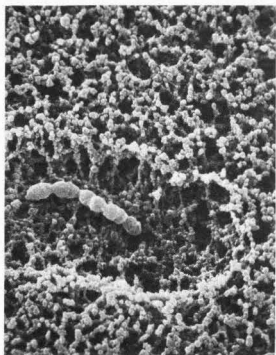
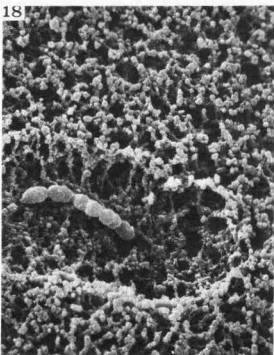


Fig. 18. A pair of stereo micrographs showing the orientation of casein micelle chains in a longitudinal section of a void space occupied by a chain of streptococci.

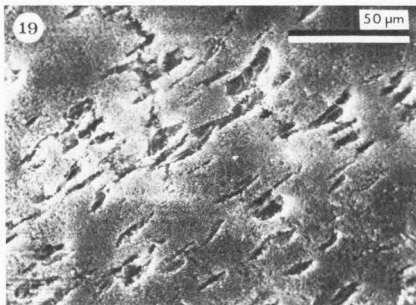


Fig. 19. Cold-stage SEM of uncoated yoghurt revealing the presence of void spaces occupied by lactic acid bacteria. Absence of a conductive metal layer led to some charging artefacts (lines and excessively light areas) but in general, the void spaces are clearly visible.

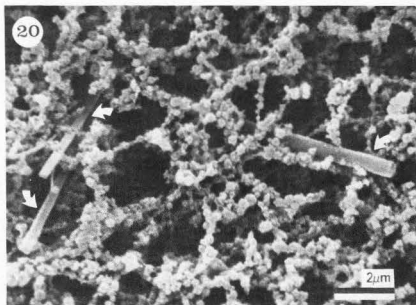


Fig. 20. Dead lactobacilli (arrows) are tightly embedded in the protein matrix of a yoghurt made by culturing the milk with viable streptococci. No void spaces are formed around dead bacteria.

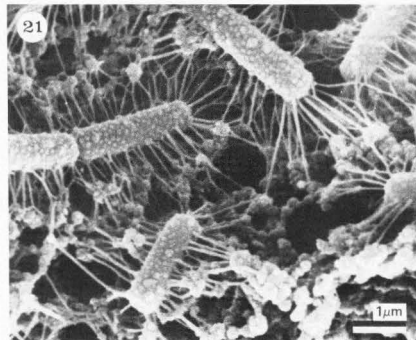


Fig. 21. Mucogenic (slime-producing) lactobacilli in a set-style yoghurt, demonstrating the presence of an exocellular polysaccharide (filaments). [The yoghurt sample was provided by Dr. A.Y. Tamime].

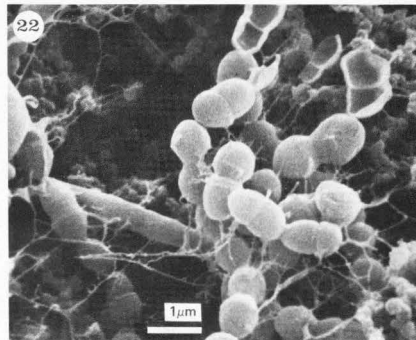


Fig. 22. Mucogenic (slime-producing) streptococci in a set-style yoghurt, demonstrating the presence of an exocellular polysaccharide (filaments).

fractures obtained by freeze-fracturing reveals numerous cavities (void spaces) in the protein matrix (Fig. 16). The presence of lactic acid bacteria in such void spaces becomes apparent as the magnification is increased (Figs. 17 and 18). The void spaces around the bacteria were attributed to bacterial action^{18, 25}, although C.J. Thomas (personal communication) suspected that they may be artefacts arising from differences in the shrinkage of the protein matrix and the bacterial clusters during the preparation of the yoghurt samples for electron microscopy. A hypothesis that the void spaces are caused by proteolytic enzymes produced by the bacteria²⁵ was tested in several kinds

of experiments: (a) One of the strains in the mixed starter culture used to make the yoghurt (cocci or bacilli) was killed by physical (radiation) or chemical (formaldehyde) means. The dead bacteria were incorporated in the milk at a concentration 1,000-fold higher than that of the viable strain; the reason for this difference was that the viable bacteria propagated in the milk whereas the dead bacteria did not. (b) Cold-stage SEM was used to examine yoghurt unaffected by fixation, dehydration, and drying and the micrographs were compared with those obtained by conventional SEM of glutaraldehyde-fixed, freeze-fractured, and dried yoghurt samples. (c) Viable bacterial cultures differing in the

amount of protease produced (i.e. so-called protease-positive and protease-negative strains) were used to make yoghurt and their effects on the microstructure of the protein matrix were studied by conventional SEM.

Cold-stage SEM of freeze-fractured yoghurt not coated with gold clearly demonstrated the existence of void spaces with bacteria in the protein matrix; this is evident (Fig. 19) in spite of the poor quality of the micrograph caused by the examination of the frozen yoghurt samples without coating them with gold which means that conductivity depended on the ions present in the yoghurt²⁰. However, this approach had the advantage in that the matrix was examined during freeze-etching as the ice present in it gradually sublimed off and exposed the underlying structures.

SEM of yoghurts containing viable and dead bacteria showed void spaces only around viable bacteria whereas dead bacteria were embedded tightly in the protein matrix (Fig. 20). Dead bacteria, however, did not retain their initial shapes and were subjected to changes, particularly shrinkage.

Protease-positive bacteria formed void spaces but protease-negative bacteria did not; however, the studies on the relationship between the dimensions of the void spaces and the production of proteolytic enzymes by the bacteria have not yet been concluded.

Another possibility for the development of the void spaces was mentioned by N.F. Olson (personal communication) who suggested that casein micelles which are closest to the bacteria are coagulated by lactic acid first, because its concentration is highest in the vicinity of the bacteria which produce it. As the coagulation proceeds outwards, the freshly formed gel may separate from the bacteria and lead to the development of void spaces.

There is evidence in some yoghurts that the casein matrix surrounding the void spaces was subjected to stress. Casein micelle chains are stretched and oriented in one direction, which is particularly clearly visible in pairs of stereo micrographs (Figs. 17 and 18), and which may support the hypothesis by Olson.

In general, higher total solids in yoghurt make it easier to detect the void spaces. In some yoghurts the number and dimensions of the void spaces may reach significant proportions affecting the integrity of the matrix.

Bacteria contribute to the microstructure of yoghurt in an additional manner. Many lactic acid bacteria possess the ability to produce extracellular polysaccharides, particularly when grown in the presence of sucrose¹. So-called slime-producing, mucogenic, or "ropy" lactic acid bacteria cultures were developed in the Netherlands¹⁰ for use in yoghurt. By SEM, such cultures are characterized by the presence of filaments which attach the bacteria (bacilli in Fig. 21 and cocci in Fig. 22) to each other and to the protein matrix. As the exocellular polysaccharides have some of the properties of the thickening agents, the use of mucogenic cultures makes it possible to prepare viscous yoghurt without the use of exogenous additives.

Electron microscopy

Examination of fixed and dried samples by conventional SEM^{18,19,22,23,28} and TEM of thin sections of yoghurt samples embedded in a resin^{9,19,22,23,28} were used most frequently to study the microstructure of this product. In this review, micrographs obtained by rotary shadowing of casein micelles and their clusters at the early stages of gelation are also presented. For this purpose, casein micelles fixed in a glutaraldehyde solution were attached to freshly cleaved mica sheets pretreated with poly-L-lysine, dehydrated in a graded ethanol series, critical-point dried, and shadowed with platinum and carbon at a 45° angle while the samples were rotated. The replicas of the casein micelles or their clusters thus obtained were cleaned in a 3% sodium hypochlorite solution, washed with water, placed on 400-mesh grids, and examined in an electron microscope operated at 60 kV. Intermediate negatives were made using 35-mm 135-ASA film²⁴.

Embedding of casein micelles in a resin was usually preceded by their fixation and immobilization by either mixing them with a warm agar sol^{9,23} or by encapsulating them in agar gel tubes^{16,37}. The solidified samples were postfixed in a 2% OsO₄ solution in a 0.05 M veronal-acetate buffer (pH 7.75)²³ or in a 0.2 M cacodylate-HCl buffer (pH 7.2)⁹.

Samples (<1 mm³) of finished yoghurt were fixed and postfixed in the same way as immobilized casein micelles, embedded in a Spurr's low-viscosity medium⁴¹ or Araldite⁹, sectioned, and stained with uranyl acetate and lead citrate^{15,34} for electron microscopy.

The incubated yoghurt mix was sampled before gelation at regular 3 to 4 min intervals²³.

Embedding of immobilized casein micelles and of small yoghurt samples is easy as the porous matrices are rapidly impregnated with the resin monomer. However, Schmidt³⁹ warned that so-called appendages (projections, "hairs", "spikes" etc.) on casein micelles may be artefacts arising from the preparatory steps. The appendages observed in casein micelle thin sections were not found when similar samples were freeze-fractured and replicated with platinum and carbon; instead of threads, an increased number of free particles of submicellar dimensions was observed³⁹. Concerning the ragged surface of the casein micelle in Fig. 6a, D.G. Schmidt (personal communication) suggested that at least a part of the "spikes" may be due to precipitated calcium phosphate.

Freeze-fracturing followed by replication with platinum and carbon is better suited to examine the progress of gelation of milk in greater detail and can also be used to elucidate interactions of casein micelles^{2,39} with additives such as gelatin, starch, alginates etc. and fortifying agents such as whey proteins. However, this method has yet to be employed in such studies. One reason for not using it to its potential may be that, unless quality, and thus expensive equipment is used both to rapidly freeze the specimens and to fracture and replicate them, the artefacts arising from the inappropriate execution of the technique may be a deterrent. It is evident from the previous discussion that the onset of gelation and interactions with additives need to be studied in greater detail.

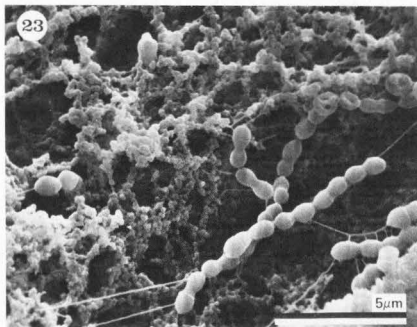


Fig. 23 (left). SEM of a dry-fractured yoghurt sample showing a complex topography (as compared to freeze-fractured samples) which is difficult to photograph and interpret.

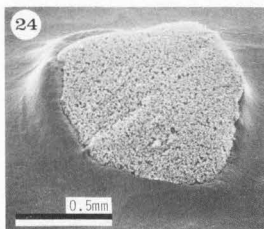


Fig. 24 (right). Low-magnification SEM of a freeze-fractured yoghurt particle mounted on an SEM stub using a conductive silver cement. The silver cement was painted on the walls of the particle close to the fractured plane to provide an uninterrupted conductive path for electrons during SEM.

Conventional SEM of the gelled yoghurt is easy to carry out provided that certain rules are followed. As was mentioned earlier, fixed samples may be freeze-fractured either with the aqueous phase present or replaced with absolute alcohol²⁰. In the former case, the fragments are freeze-dried whereas in the latter case they are melted in absolute alcohol and subsequently critical-point dried. This procedure has no harmful effects on the yoghurt matrix.

Freeze-fracturing leads to images superior to those obtained by dry-fracturing. Smooth fracture planes obtained by freeze-fracturing are suitable for studies of the porosity of the matrix, linkages of casein micelles in clusters and chains, distribution of void spaces etc. Dry-fractured particles, on the other hand, are characterized by ragged surfaces of a complex topography which are difficult to both photograph and interpret (Fig. 23) as the structure elements are not viewed in the same plane.

Mounting of the fragments on SEM stubs has to be done carefully to provide a base for a conductive path for the electrons after the particles are coated with carbon and gold. In practice it means that the particles should have relatively small fracture planes (<1 mm²) and be low (≈0.5 mm). It is advisable to position the particles on top of a droplet of a conductive cement of a proper consistency (free solvent should not penetrate the matrix) with the fractured plane facing up and to paint the sides of the particle with the cement as close to the fracture plane as possible (Fig. 24).

Coating of the fragments with carbon prior to gold coating improves the final SEM image. The angles (a minimum of two) at which coating with gold is carried out by the evaporation technique should be properly selected. Coating at an angle too acute (low) fails to provide enough gold to form an uninterrupted conductive path on the casein micelles and clusters. Also the amount of gold used is important as the large total surface of the protein matrix requires more gold to be evaporated than does coating of compact surfaces such as fractured cheese. The presence of a small number of nitrogen molecules was used²⁰ to induce collisions with the gold atoms and their deflections;

this resulted in their deposition on the protein matrix from various directions making the coating uniform. Sputter coating of yoghurt samples in the authors' laboratory failed to produce better images than evaporative coating.

The porous matrix of yoghurt is susceptible to electron beam damage and, consequently, focussing of the electron beam should be carried out rapidly.

Cold-stage SEM of uncoated samples produces inferior images and is justified only under specific circumstances. However, continuous scanning of the freeze-fractured sample makes it possible to study the gradual emergence of the protein matrix from the aqueous phase as the ice sublimates off; needless to say this technique contributes to the contamination of the microscope.

Conclusion

Yoghurt has become an important milk product which is made in a great variety of styles differing in texture, flavour, and joule (calorie) content. Microstructure and related properties such as susceptibility to syneresis and firmness depend on the heat treatment of milk, total solids contents, bacterial starter cultures, conditions of culturing, presence of additives, and other factors. A wide field is open to studies of the microstructure in set-style as well as stirred and frozen yoghurts. Electron microscopical techniques used should not be limited to thin-sectioning and conventional SEM but should also include freeze-fracturing and freeze-etching followed by replication with platinum and carbon. In addition to points already raised in this review, it would be interesting to study the role of fat globules in yoghurts made from whole unhomogenized and from homogenized milk, particularly in view of the hypothesis postulated by van Vliet and Dentener-Kikkert⁴⁶ that a fat globule membrane reacting with milk proteins will increase the strength of the resulting milk gel as opposed to a nonreactive membrane.

It is evident that electron microscopy can play an important role in the study of the gelation of milk and in anticipating and explaining some physical properties of newly developed yoghurt. The objective of this review was to stimulate such studies.

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Discussion with Reviewers

M. Rüegg: You have observed that acid coagulation of casein micelles leads to the formation of clusters and chains. In the case of rennet coagulation, the predominant form of aggregation of the casein particles seems to be the chain formation. Both theoretical considerations and experimental observations indicate that linear chains are formed with the highest probability, followed by branching. Do you think that cluster formation is typical for acid coagulation?

Authors: Chains as the predominant form of casein micelle aggregation in yoghurt are found in all samples made from preheated milk; preheating of milk is always practised in yoghurt manufacture. In milk that had not been preheated and was gelled by a yoghurt starter culture, however, the predominant form of micelle aggregation is clustering; there is sufficient electron microscopical support for this statement although no theoretical considerations have yet been made on this type of casein micelle aggregation.

A.Y. Tamime: The authors have suggested in their discussion that the protein/protein interaction takes place only between β -lactoglobulin and κ -casein; I wonder, however, whether α -lactalbumin is also involved in such interactions.

Authors: We have found no references which would indicate that α -lactalbumin is involved in the formation of "spikes" on casein micelles in heated milk.

D.N. Holcomb: What artefacts, if any, result if casein micelles are postfixed with OsO₄ without first being immobilized in agar?

Authors: Postfixation of casein micelles with OsO₄ is a logical step in preparing casein micelles for

embedding in a resin, be it executed by immobilizing them in agar or centrifuging and embedding them in the form of a pellet. Shimmín and Hill⁹ even used postfixation with OsO₄, in the case of casein micelles destined for negative staining and metal shadowing. When casein micelles are immobilized in agar, it is easier to separate the OsO₄ solution from the agar gel particles than from casein micelles freely dispersed in the postfixing solution. The question thus can be reformulated as to whether any artefacts develop in casein micelles fixed and postfixing only after they had been immobilized in agar gel. This is an interesting question and will be answered by practical experiments.

D.N. Holcomb: The authors identify agar fibres in Fig. 1. Should these also be visible in Fig. 2?

Authors: Fig. 1 was obtained at a high magnification where agar fibres are clearly distinguished. They are not evident in Fig. 2 because of the considerably lower magnification.

M. Rüegg: This review is concerned with nonfat yoghurts only. The residual fat content of skimmed milk is usually smaller than 0.05%. Nevertheless, this small percentage corresponds to at least 10⁶ to 10⁹ fat globules per 1 g of milk or yoghurt. Did you observe these fat globules or were they removed during the preparation procedure?

Authors: We did not observe fat globules in nonfat milk yoghurts although no attempts were made to remove them from the samples on purpose. It is possible, however, that the fat globules were removed during the preparation of the samples for SEM. This preparation consisted of fixing the samples in a glutaraldehyde solution, dehydrating them in a graded alcohol series, and freeze-fracturing. The resulting fragments were melted in absolute alcohol and were subsequently critical-point dried from carbon dioxide. The fat globules were, thus, exposed to absolute alcohol and carbon dioxide, both to be known lipophilic solvents. It is probable that the fat was extracted during those steps. Otherwise, there would be approximately 0.01 to 1 fat globule within the field of vision in the scanning electron microscope used at a 1,200X magnification considering your assumption that there were 10⁶ to 10⁹ fat globules in 1 g of yoghurt.

M. Rüegg: The interface between the coagulated milk and the walls of the container plays an important role for the syneresis of yoghurt. Certain surfaces retard and others promote the shrinkage of the casein network. Has this phenomenon been studied using electron microscopy? Which preparation technique could be recommended for such a study?

Authors: It is regrettable that no reference has been made concerning this phenomenon. In their book, Rašić and Krumann³³ compare the advantages and disadvantages of various packaging materials. So, for example, glass prevents gas diffusion and does not interact with the product. Polyvinyl chloride (PVC) and polyvinylidene chloride (PVDC) have a relatively low permeability to water vapour, oxygen, nitrogen, and carbon dioxide, whereas polystyrene and polyethylene demonstrate a high permeability for the above gases. Waxed carton is susceptible to discolouration by some fruits in fruit yoghurts, indicating a selective absorption of some substances from the yoghurt. However, no

relationship between the packaging materials and syneresis in yoghurt has been mentioned. We consulted commercial yoghurt manufacturers in Canada to answer your question. One of them assumed that if differences in syneresis are noted in yoghurt transported in various packaging materials, such differences can result from differences in the way vibrations are transmitted to the yoghurt during transportation. The other manufacturer reported no differences in syneresis among yoghurts made in stainless steel pots and tanks, in glass bottles, and in different types of plastic containers provided that other variables such as the type of culture used, product formula, and processing conditions were the same.

Interactions between yoghurt and the packaging materials could be studied by immersing test strips of uniform dimensions in the milk and by SEM of the material adhering to the strips in the finished yoghurt.

F.L. Davies: In experiments to determine the cause of void spaces surrounding bacteria, the authors report that proteinase-positive bacteria (lactobacilli?) formed void spaces whereas proteinase-negative variants did not. It should be remembered that *S. thermophilus* has little or no proteolytic activity, depending, in yoghurt, upon *L. bulgaricus* to effect the initial breakdown of proteins. Examination of figures 9b, 9d, 13a, 13b, 13c, 17, and 18 suggests that void spaces are equally (if no more) evident around the non-proteolytic streptococci as around the strongly proteolytic lactobacilli. This argues against proteinase activity as the cause of void spaces and perhaps adds weight to Olson's hypothesis concerning local concentrations of lactic acid.

D.G. Schmidt: In view of the results presented, which clearly show a relationship between the voids around bacteria and proteolytic activity, I consider Olson's hypothesis inferior to the proteolysis hypothesis unless a definite answer can be given to the question "Why should the gel separate from the bacteria?"

M. Rüegg: The readers should be informed whether the void spaces shown in Fig. 19 were formed after the sublimation of ice or whether they correspond to gas bubbles.

Authors: At present we are unable to draw any definite conclusion concerning the origin of void spaces around lactic acid bacteria in yoghurt, because the evidence presented is insufficient to support any hypothesis. The suggestion to more closely examine the nature of the void spaces (fluid or gas) is important, particularly in view of the work by Driessen et al.⁴⁸; these authors showed that carbon dioxide produced by *S. thermophilus* is needed by *L. bulgaricus* for optimal lactic acid production and growth. The relatively rapid changes in the images of uncoated freeze-fractured yoghurt samples in the scanning electron microscope do not make it possible to comment on the nature of the void spaces shown in Fig. 19. The experiments need to be repeated and extended. Replication with platinum and carbon will also be used.

D.N. Holcomb: What SEM conditions (beam voltage, working distance, etc.) did the authors use for cold-stage SEM? Would use of a so-called "charge neutralizer" (e.g., C.K. Crawford: Charge

neutralization using very low energy ions. Scanning Electron Microsc. 1979; 11: 31-46) help to improve the quality of the cold-stage SEM micrographs?

Authors: Accelerating voltage was 10 kV, beam current was 50 to 70 μ A, and the working distance was 7.1 mm. The charge neutralizer might help improve the quality of the micrographs, particularly those taken at a low magnification, but we have no practical experience with this SEM accessory.

Additional References

48. Driessen FM, Kingma F, Stadhouders J. (1982). Evidence that *Lactobacillus bulgaricus* in yoghurt is stimulated by carbon dioxide produced by *Streptococcus thermophilus*. Neth. Milk Dairy J. 36(2), 135-144.
49. Shimmin PD, Hill RD. (1965). Further studies on the internal structure of the casein micelles of milk. Austral. J. Dairy Technol. 20, 119-122.