Journal of Dairy Research

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The effect of high-temperature heat treatment and homogenization on the microstructure of set yogurt curd networks

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Research Article

Cite this article: Ichimura T, Kusaka M and Nakamura T (2023). The effect of high-temperature heat treatment and homogenization on the microstructure of set yogurt curd networks. *Journal of Dairy Research* **90**, 306–311. https://doi.org/10.1017/S0022029923000523

Received: 3 December 2022
Revised: 27 July 2023
Accepted: 27 July 2023
First published online: 31 August 2023

Keywords:
Electron microscopy; heat treatment; homogenization; microstructure; set yogurt

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Abstract

Set yogurt's physical characteristics are greatly affected by the homogenization and heat treatment processes. In our previous study, set yogurt treated at 130°C and with the fat particle size reduced to ≤0.6 µm had equivalent curd strength, less syneresis and smoother texture than yogurt treated at 95°C. When investigating the mechanisms underlying yogurt's physical properties, it is important to evaluate the yogurt's microstructure. We conducted electron microscopy evaluations to investigate the mechanisms of changes in yogurt's physical properties caused by 130°C heat treatment and by a reduction in the fat globule size. We prepared yogurt mixtures by combining heat treatment at 95 and 130°C and homogenization pressure at 10 + 5 and 35 + 5 MPa and then fermented the mixtures in a common yogurt starter. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used for the structural observations. Fine particles were observed on the surface of the casein micelles of the yogurt treated at 95°C, and the coalescence density between micelles was high. The surface of the yogurt treated at 130°C had few fine particles, and the coalescence density between micelles was low. The yogurt treated at 130°C with 35 + 5 MPa homogenization had low coalescence density between casein micelles, but smaller-particle-size fat globules increased the network density. Approximately 30% of the fat globules were estimated to be incorporated into the yogurt networks compared to the volume of casein micelles. We speculate that 130°C heat treatment alters the structure of whey protein on the surface of casein micelles and interferes with network formation, but reducing the size of fat globules reinforces the network as a pseudoprotein.

The curd strength of set yogurt and customer satisfaction with set yogurt are greatly affected by the yogurt's physical characteristics and texture (Aryana and Olson, 2017). The microstructure of foods generally has an important influence on the foods' physical properties, and many studies linking physical property evaluations and structural observations of foods have been reported (Pascua *et al.*, 2013; Saldana *et al.*, 2015; Joardder *et al.*, 2017). When modifications of physical properties are sought, microstructural analyses can provide useful information (Aguilera, 2005), and a microstructural evaluation can support an evaluation of physical properties. Evaluations of the microstructure of yogurt have been performed mainly with confocal laser scanning microscopy (CLSM: Bai *et al.*, 2020; Wang *et al.*, 2020; Jia *et al.*, 2022), scanning electron microscopy (SEM: Zhang *et al.*, 2015; Jia *et al.*, 2022), and transmission electron microscopy (TEM: Harte *et al.*, 2002).

Yogurt's physical characteristics are greatly affected by heat treatment processes (Xu *et al.*, 2008), and yogurt is usually pasteurized at 90°–95°C for 5–10 min (Sodini *et al.*, 2014). A low denaturation rate of whey due to low pasteurization temperatures results in a significant decrease in the viscosity and firmness of yogurt (Dannenberg and Kessler, 1988). However, the viscosity and firmness of the yogurt will also decrease significantly if the pasteurization is performed at ≥120°C (Krasaekoopt *et al.*, 2003). Milk for human consumption (i.e., drinking milk) is generally pasteurized by a high-temperature short-time or ultra-high temperature (UHT) method (Lorenzen *et al.*, 2011) and it is, therefore, usually not possible to use the same pasteurizer for drinking milk and yogurt.

The pasteurization conditions for drinking milk differ slightly from country to country, and in Japan drinking milk is pasteurized at 125°–135°C for 2–5 s (Ohkubo *et al.*, 2019). We recently developed a method of producing set yogurt by pasteurization at 125°–135°C for 2–5 s and homogenization at 30–40 + 5 MPa giving a reduction of fat globule size to ≤0.6 µm (Ichimura *et al.*, 2022). The set yogurt obtained by this method has the same firmness, less syneresis and better smoothness than yogurt prepared by general manufacturing conditions (90°–95°C, 10–20 + 5 MPa), and it was significantly preferred in a sensory evaluation.

The effects of heat treatment and homogenization conditions on the physical characteristics of yogurt have been examined by several research groups. When the milk used for yogurt is

heated, the heat-denatured beta-lactoglobulin (β-Lg) and alpha-lactalbumin (α-La) bind to the κ-casein of casein micelles (Elfagm and Wheelock, 1978). The binding of casein micelles and whey by heat treatment increases the firmness of the yogurt and changes the isoelectric point to the high pH side (Donato *et al.*, 2007). The denaturation rate of whey by heat treatment has an important effect on the firmness of yogurt (Dannenberg and Kessler, 1988), but heat treatment at temperatures >120°C reduces the firmness of yogurt regardless of the whey denaturation rate (Savello and Dargan, 1995). In addition, the mechanisms involved in the reduction of yogurt firmness have not been known.

After homogenization, casein micelles, β-Lg, and α-La locate to the surface of the yogurt's fat globules (Hayes *et al.*, 2005). The amount of casein micelles present on the fat globule surface increases as the homogenization temperature and pressure increase (Cano-Ruiz and Richter, 1997). Homogenization using ultra-high pressure (>100 MPa) improves the syneresis and physical properties of yogurt (Serra *et al.*, 2008), but there are few data regarding the effects of 30- to 90-MPa homogenization.

The mechanisms underlying changes that occur in the curd structure when 130°C heat treatment and homogenization pressure that is higher than conventional conditions (such as 35 + 5 MPa) are used are not yet known. Our CLSM observations confirmed that the structure of the yogurt changed (Ichimura *et al.*, 2022). Regarding the effect that fat globules have of reinforcing the curd network, we speculated that it is possible that the fat globules may adhere to or otherwise be included in the network. If the mechanisms that underlie the changes in physical properties that occur with an increase of the heat treatment temperature from 95 to 130°C and a reduction of the fat globule size can be clarified, useful knowledge for producing set yogurt with a good texture will be obtained. We thus conducted the present study to investigate the mechanisms of changes in the physical properties of set yogurt by identifying the precise structural changes that are the results of several heat treatment and homogenization conditions. We used SEM and TEM to observe the microstructure of the yogurt samples and to identify the details of the network structure.

Materials and methods

Set yogurt preparation

Culture LB81, which contains *Lactobacillus delbrueckii* subsp *bulgaricus* 2038 and *Streptococcus thermophiles* 1131, was used in this study. The preparation of the yogurt starter was as described by Ichimura *et al.* (2022). The yogurt mixture was obtained by mixing raw milk, skim milk powder and water; it contained 3.0% (wt/wt) fat, 9.5% (wt/wt) SNF (solids-not-fat) and 3.6% (w/w) protein. The raw milk and skim milk powder were supplied by Meiji Co. (Tokyo) facilities. The yogurt mixture was pre-warmed at 75°C. Prior to the heat treatment described below, each mixture was homogenized (model H20, Sanwa Engineering, Hyogo, Japan) at 10 (first stage) + 5 MPa (second stage) or 35 + 5 MPa. The yogurt mixtures were then pasteurized at 95°C for 5 min or at 130°C for 2 s. Heat treatment at 95°C for 5 min was conducted as vat heat treatment. The heat treatment at 130°C for 2 s (experimental preparation conditions) was conducted with an indirect plate exchange system (Powerpoint International, Saitama, Japan). In this study, we defined the control preparation conditions as heat treatment at 95°C for 5 min and homogenization at 10 and 5 MPa. The yogurt mixtures' inoculation, fermentation, and sample cooling were carried out as described by Ichimura *et al.* (2022). Detailed methodologies are given in the online Supplementary File, including a process flow chart at Supplementary Fig. S1.

Particle-size distribution

We determined the distribution of the sizes of the fat particles of the yogurt mixture by a laser diffraction scattering method with a laser diffraction particle size analyzer (SALD2200, Shimadzu, Kyoto, Japan) as follows. The yogurt mixture was added into the circulating cell of the apparatus containing deionized water. Triplicate measurements were taken at 25°C, and the volume-weighted mean diameters ($d_{4,3}$) were recorded. Assuming that the distribution of fat globules follows a normal distribution, we calculated the volume of fat globules smaller than 'x' μm (F_x) contained in 100 g of yogurt mixture by using the mean value and standard deviation of the fat globules. The lower cumulative distribution of the fat globules of 'x' μm (F_x) was estimated with the use of the standard normal distribution table. (F_x) was approximated with the following equation:

$$(F_x) = [\text{fat}\,(\%) / \text{s.g.}_{\text{fat}}] \times P(\text{x}, \mu, \sigma)$$

where (F_x) is the volume of the fat globules smaller than x μm contained in 100 g of yogurt mixture, s.g._{fat} is the specific gravity of the milk fat, and $P(\text{x}, \mu, \sigma)$ is the lower cumulative distribution. For further detail see online Supplementary Fig. S2.

Physical characterization of the yogurt

The physical characteristics of the yogurt were the curd hardness, curd firmness, penetration angle and the degree of syneresis. The curd hardness and curd firmness of yogurt represent the yogurt's tolerance to vibration during transport. The penetration angle represents the smoothness of the yogurt. The degree of syneresis is an index of syneresis to the surface. The measurements of the curd hardness, curd firmness, penetration angle, and the degree of syneresis of the yogurt samples were carried out as described by Ichimura *et al.* (2022).

Scanning electron microscopy

SEM observations were performed to evaluate the set yogurt's microscopic networks. Yogurt samples were cut to 3 mm × 3 mm × 5 mm cubes and then soaked in a glutaraldehyde solution (2% final concentration) for 1 h. The cubes were washed twice with deionized water and soaked in osmium tetroxide solution (1% final concentration) for 90 min. The cubes were then washed twice with deionized water and dehydrated by soaking in 30, 50, 60, 70, 80, 90, and 99% ethanol (vol/vol) for 10, 10, 10, 10, 15, 15, and 15 min, respectively. The cubes were then soaked in 100% ethanol for 15 min, two times. The cubes were then soaked in isoamyl acetate for 20 min, two times, and then critical-point dried with the use of liquefied carbon dioxide (JCPD-5; JEOL, Tokyo) and coated with a layer of sublimated osmium plasma coater (NL-OPC80N; JEOL). The osmium-coated cubes were then viewed by SEM (JSM-6700F; JEOL).

Transmission electron microscopy

TEM observations were performed to evaluate the sizes of the fat globules in the networks of the set yogurt made by 130°C heat treatment mix. The yogurt samples for the TEM examination

https://doi.org/10.1017/S0022029923000523 Published online by Cambridge University Press

were treated in the same way as the samples for SEM up to the point of being soaked in osmium tetroxide solution. After the samples were soaked in that solution, they were dyed in an EM Stainer (Nissin EM Co., Tokyo) for 15 min. The samples were then dehydrated by soaking in 30, 50, 60, 70, 80, 90, and 99% ethanol (vol/vol) for 10, 10, 10, 10, 15, 15, and 15 min, respectively. The cubes were soaked in 100% ethanol for 15 min, two times. Next, 43.4 g of Quetol 651, 20.3 g of NSA, 48.5 g MNA, and 1.5 ml of DMP-30 (all from Nissin EM Co.) were mixed to create an epoxy resin solution for embedding. The dehydrated samples were soaked in propylene oxide solution, then a propylene oxide solution:epoxy resin solution = 1:1 mixture, a propylene oxide solution:epoxy resin solution = 1:3 mixture, and the epoxy resin solution for 60 min, respectively. The samples were then soaked in epoxy resin solution overnight with shaking. Afterward, the samples were poured into gelatin capsules with the resin solution. The resin solution with the samples was heated at 60°C for two nights, with the use of a thermostat. After the resin was cured, the samples were sectioned at 100-nm-thick with an ultramicrotome (Ultracut UCT; Leica Biosystems, Buffalo Grove, IL) and a diamond knife (DiATOME Ultra 45; EMJapan Co., Tokyo) and mounted on copper grids. Thin sections were dyed in the EM Stainer for 15 min and then dyed with acetate uranium (1% final concentration) to enable observation. The examination was performed with a transmission electron microscope (JEM-2010; JEOL) at an acceleration voltage of 140 kV. We defined the white circle observed in the image as the fat globule and measured its diameter at three locations; the average value was calculated.

Statistical analysis

A one-way repeated measures analysis of variance (ANOVA) and Tukey-Kramer adjustment were used to investigate the effects of differences in test conditions on the yogurts' physical properties and the sensory evaluation. The data were analyzed using Bellcurve for Excel (ver. 3.10, Social Survey Research Information, Tokyo) to find significant differences among the samples. Differences were considered significant when $P < 0.05$.

Results

Physical characterization of the yogurt

The particle sizes of the fat globules depended on the homogenization pressure (Table 1). Homogenization at 10 + 5 MPa reduced the fat globule size to 1.0 µm, and homogenization at 35 + 5 MPa reduced the fat globule size to 0.5 µm. Table 1 lists the physical characteristics of the yogurt. The curd firmness was highest in the experimental yogurts processed at 95°C-35 + 5 MPa and lowest in the experimental yogurts processed at 130°C-10 + 5 MPa. Regarding the penetration angle, the yogurt processed at 95°C 35 + 5 MPa had the highest penetration angle of the two. The control yogurt made with the 95°C-10 + 5 MPa processing conditions showed the greatest syneresis, and the 130°C-35 + 5 MPa condition produced the lowest syneresis. These results were consistent with those obtained in our earlier study (Ichimura *et al.*, 2022).

The microstructure of the yogurt

Figures 1 and 2 are SEM images of the network structure of yogurt. A lower magnification image is provided at online Supplementary Fig. S3. Figure 1 shows a detailed yogurt network structure. The network structure obtained with the 130°C-10 + 5 MPa condition tended to be thin, and the network from the 95°C-35 + 5 MPa condition tended to be thick. Figure 2 depicts the appearance of casein micelles in the yogurt network. The casein micelles in the yogurt network made at 130°C had a pleated surface and many gaps inside the casein micelle had aggregate. In contrast, the yogurt made with the 95°C treatment had fine ~10-nm particles bonded to the surface of casein micelles instead of a pleated structure. In addition, there were few gaps in the casein micelle assembly, and the structure was dense.

Regarding the size of casein micelles in the yogurt network, there was no significant difference between the 95 and 130°C heat treatments, as the size of the micelles for both heat treatment values were approx. 100–200 nm. Figure 3 is TEM images of the internal structure of the network of yogurt made by 130°C heat treatment; small fat globules ≤0.4 mm tended to be included in the yogurt network. The yogurt made by homogenization at 10 + 5 MPa tended to have fat globules outside the network, whereas in the yogurt made by homogenization at 35 + 5 MPa, fat globules tended to exist inside the network.

Discussion

To the best of our knowledge, this study is the first to examine in detail the microstructure of set yogurt made by 130°C heat treatment. There is a report by Savello and Dargan (1995) regarding the physical properties of set yogurt made by heating at 130°C, but it does not mention the microstructure. The effects of the 130°C heat treatment and homogenization pressure on the microstructure of yogurt were clarified by our present findings. The

Table 1. The conditions used to make the yogurt mixes and the resulting particle-size distributions

Conditions	Homogenization[1] pressure, MPa	Heat treatment			Particle size of fat globules $d_{4,3}$, µm	Curd firmness[2] g·s	Syneresis[2], %	Penetration angle[2], °
		Temp., °C	Time	System				
95°C, 10 + 5 MPa	10 + 5	95	5 min	Vat[3]	0.982 ± 0.227	1600 ± 277^{b}	22.0 ± 3.7^{a}	61.3 ± 2.5^{b}
130°C, 10 + 5 MPa	10 + 5	130	2 s	Plate[4]	0.974 ± 0.219	517 ± 137^{c}	19.0 ± 2.8^{ab}	20.0 ± 4.1^{d}
95°C, 35 + 5 MPa	35 + 5	95	5 min	Vat[3]	0.506 ± 0.129	2310 ± 386^{a}	14.3 ± 1.4^{bc}	72.5 ± 6.5^{a}
130°C, 35 + 5 MPa	35 + 5	130	2 s	Plate[4]	0.509 ± 0.127	1508 ± 257^{b}	10.1 ± 1.4^{c}	32.5 ± 4.8^{c}

[1]Two-stage homogenization (first stage and second stage) at 75°C.
[2]Values with different letters in the same column are significantly different ($P < 0.05$).
[3]Vat heat treatment.
[4]Indirect plate exchange system (Powerpoint International, Tokyo).

https://doi.org/10.1017/S0022029923000523 Published online by Cambridge University Press

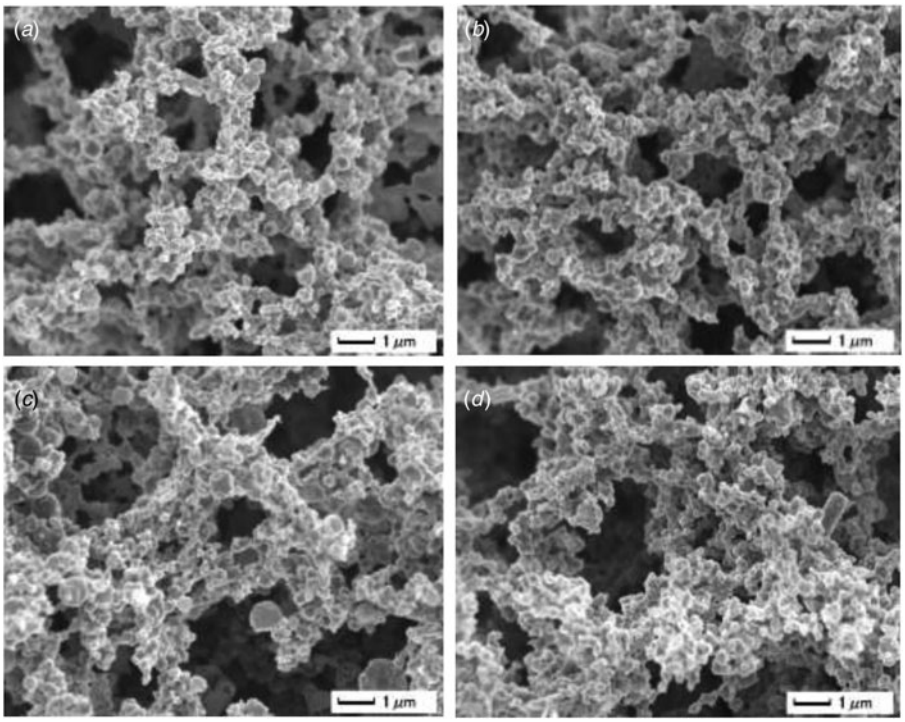

Figure 1. Scanning electron micrographs of yogurts with different conditions of homogenization and heat-treatment temperature. A: Heat treatment at 95°C for 5 min and homogenization at 10 + 5 MPa. B: Heat treatment at 130°C for 2 s and homogenization at 10 + 5 MPa. C: Heat treatment at 95°C for 5 min and homogenization at 35 + 5 MPa. D: Heat treatment at 130°C for 2 s and homogenization at 35 + 5 MPa.

highest curd firmness (obtained in yogurt produced at 95°C-35 + 5 MPa) tended to have a thick network, and the lowest curd firmness (the yogurt produced at 130°C-10 + 5 MPa) tended to have a thin network (Fig. 1). That is, the network thickness affected the curd firmness of the yogurt. A thin and dense structure is less likely to produce agglomerates when the network collapses, and we therefore speculated that this is why the penetration angle, which indicates the roughness of the texture, became low in both yogurt types (Table 1).

In addition, the 130°C heat treatment tended to reduce the aggregation of casein micelles and increase the surface exposed to voids in the binding of casein particles, which resulted in small gaps in the network and high density (Fig. 1) as well as least syneresis (Table 1). We thus inferred that the mechanism by which syneresis was reduced by 130°C heat treatment originated from an increase in the hydration area of the casein micelles. We also infer that the mechanism by which syneresis was decreased by a reduction in the fat globule size originated

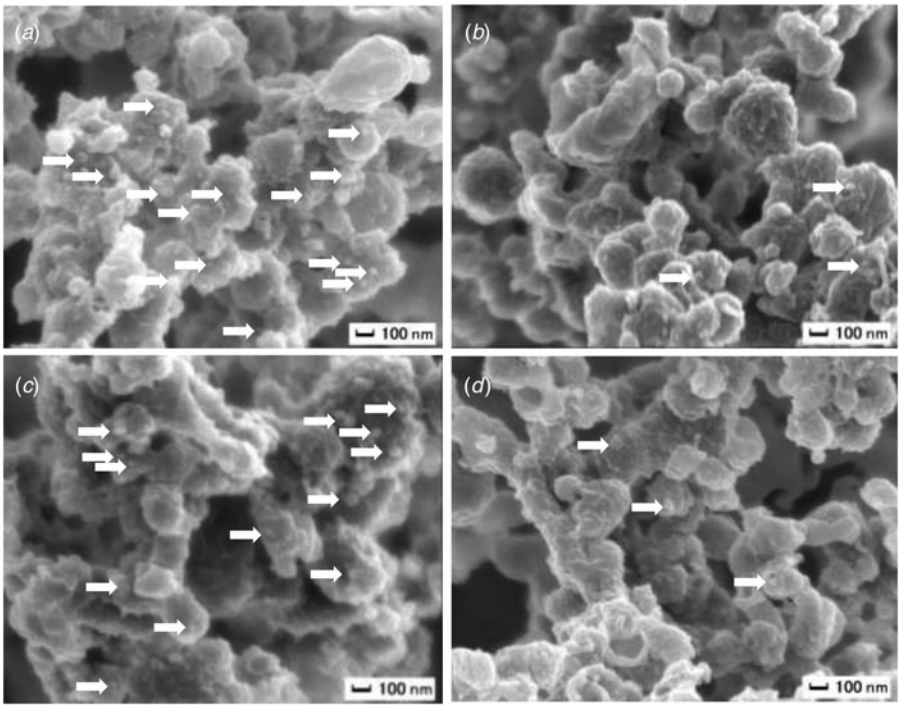

Figure 2. Scanning electron micrographs of yogurts with different conditions of homogenization and heat-treatment temperature. A: Heat treatment at 95°C for 5 min and homogenization at 10 + 5 MPa. B: Heat treatment at 130°C for 2 s and homogenization at 10 + 5 MPa. C: Heat treatment at 95°C for 5 min and homogenization at 35 + 5 MPa. D: Heat treatment at 130°C for 2 s and homogenization at 35 + 5 MPa. *White arrows:* fine particles on the surface of the casein micelles.

https://doi.org/10.1017/S0022029923000523 Published online by Cambridge University Press

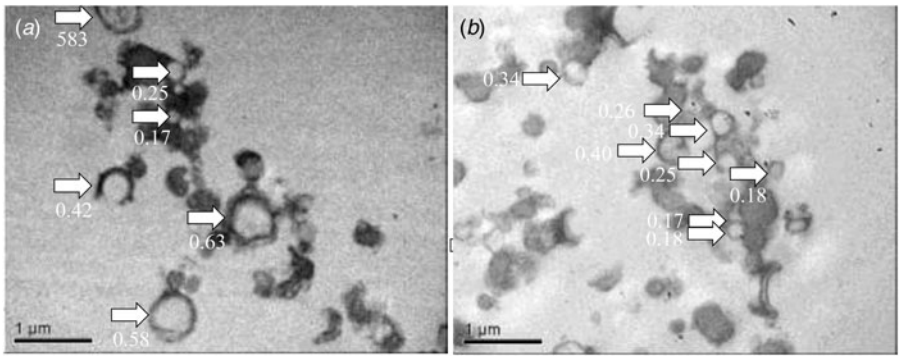

Figure 3. Transmission electron micrographs of yogurts made by heat treatment at 130°C with different conditions of homogenization pressure. A: Heat treatment at 130°C for 2 s and homogenization at 10 + 5 MPa. B: Heat treatment at 130°C for 2 s and homogenization at 35 + 5 MPa. Casein micelles are *black* and fat globules are *white*. *White arrows:* fat globules. *Numbers*: The sizes of fat the globules (μm).

Table 2. Calculated ratio of volume of casein micelles and fat globules in the fat-containing yogurt mix

Conditions	Particle size of fat globules[a] $d_{4,3}$, μm	<0.4 μm fat volume[a], μm^3	Ratio of volume of <0.4 μm fat globules to volume of casein micelles[b], %
95°C, 10 + 5 MPa	0.982 ± 0.227	1.723×10^{10}	0.794
130°C, 10 + 5 MPa	0.974 ± 0.219	1.460×10^{10}	0.672
95°C, 35 + 5 MPa	0.506 ± 0.129	6.847×10^{11}	31.530
130°C, 35 + 5 MPa	0.509 ± 0.127	6.505×10^{11}	29.959

aCalculated from the particle-size distribution and the amount of fat.
bCalculated from <0.4 μm fat volume and the amount of protein.

from an increase in the surface area of the fat globules emulsified by milk proteins, resulting in an increase in the hydration area.

In the 130°C heat treatment, unlike the 95°C heat treatment, little binding of fine particles was detected on the surface of the casein micelles (Fig. 2). We also observed that the yogurt made with 130°C heat treatment had many gaps in the structure of networks and that there were a few aggregates due to the coalescence of the casein micelles (Fig. 1). With heat treatment, β-Lg binds to κ-casein *via* disulfide bonds (Edwin *et al.*, 2004), which increases the hydrophobicity of the casein micelles and enhances the curd strength of yogurt (Mottar *et al.*, 1989). On the other hand, heat treatment above 120°C reduces the curd strength of yogurt regardless of the whey denaturation rate (Krasaekoopt *et al.*, 2003). Phoebe *et al.* (2015) confirmed that UHT sterilization (135°C for 2 s) partially disrupts the secondary and tertiary structures of whey protein (β-Lg and α-La) which results in the formation of an amorphous string-like structure around the casein micelles (Li *et al.*, 2021) We speculated that these changes inhibited the interaction between casein micelles after heat treatment at 130°C, that is, yogurt network formation.

Figure 2 shows that the yogurt made with 130°C heat treatment had a thinner network structure, and the yogurt made with 35 + 5 MPa homogenization had a thicker network structure. Not only casein micelles but also fat globules were taken into the network, and they reinforced the structure (Fig. 3). We speculated that fat globules ≤0.4 mm in size would be taken into the yogurt networks. When we calculated the ratio of the volume of fat globules <0.4 μm ($F_{0.4}$) to the volume of casein from the specific density of casein micelles (1.333) and fat globules (0.916), the results confirmed that in the 130°C-35 + 5 MPa condition, the ratios were particularly high (Table 2). The density of fat globules was described by Cano-Ruiz and Richter (1997), and we estimated the density of the casein micelles from the report by Ono (2005). Our present findings thus indicate that the reason that the curd strength increases when the homogenization pressure is increased is because the fine fat globules became network constituents, like pseudo-casein micelles.

In conclusion, we have investigated the differences in the physical properties of yogurt made with the use of different heat-treatment temperatures and homogenization conditions, focusing on the network structure. Electron microscopy revealed that fat globules smaller than 0.4 μm were present in the network, increasing the network density. Whey proteins on the surface of casein micelles were clearly visible with the 95°C heat treatment, whereas no clear structure was observed in the 130°C heat treatment. We speculate that the 130°C heat treatment changed the structure of whey proteins on the surface of casein micelles, preventing network formation, whereas smaller fat globules reinforced the network as pseudo-proteins. Our present evaluation of the network structure clarified the causes underlying the effects of the manufacturing conditions on the physical properties of yogurt.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029923000523

Acknowledgements. We thank Hiroshi Horiuchi, Takashi Osada, and Megumi Takai for the many useful comments on this research. We also thank Tomoko Ichiba and Nao Takagi for their assistance with the yogurt samples' preparation and evaluation.

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https://doi.org/10.1017/S0022029923000523 Published online by Cambridge University Press