Coagulation temperature affects the microstructure and composition of full fat Cheddar cheese

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Dairy Sci. & Technol. (2011) 91:739–758
DOI 10.1007/s13594-011-0033-6

ORIGINAL PAPER

Coagulation temperature affects the microstructure and composition of full fat Cheddar cheese

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Received: 28 December 2010 / Revised: 15 May 2011 / Accepted: 16 May 2011 /
Published online: 20 July 2011

Abstract An understanding of coagulation and factors that affect cheese microstructure is important, as this microstructure influences cheese texture and flavour. Of particular importance to many producers is the loss of milk fat during the cheese-making process, which reduces the inherent value of the product. The aim of this study was to investigate the effect of coagulation temperature on the microstructure of gel, curd and cheese samples during the manufacture of full fat Cheddar cheese. The microstructure of the gel formed at 27 °C consisted of a fine interconnected protein network as compared to a coarse, irregular and more discontinuous protein network in gel formed at 36 °C. At a higher coagulation temperature (36 °C), the size of the casein micelle aggregates in the protein strands increased when observed using confocal laser scanning microscopy possibly due to increased hydrophobic and ionic interactions and the rearrangement of casein micelles. This characteristic microstructure observed in the gel was retained in the curd collected prior to whey draining and may be responsible for the increased loss of fat in the whey. The concentration of fat in dry matter in cheese prepared from cheese-milk coagulated at 27 °C and 30 °C was significantly ($P<0.05$) higher than in cheese made from milk coagulated at 33 °C and 36 °C possibly due to the observed differences in microstructure and the direct effect of coagulation temperature on physical properties of the fat and the casein micelles. Our results suggest the need to control milk

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coagulation temperature, as this parameter may affect product microstructure and fat retention.

凝乳温度对全脂切达干酪微观结构和组成的影响

摘要 干酪的微观结构能够影响干酪的质地和风味，因此了解影响干酪微观结构的因素是非常重要的。在干酪加工中脂肪的损失是许多种类干酪加工中存在的重要问题，脂肪的损失会引起干酪内在性质的改变。本文目的是调查在全脂切达干酪生产中凝乳温度对凝胶、凝块和干酪微观结构的影响。研究表明，在 27 ℃ 形成的凝胶是由微小的蛋白质相互连接成的网络结构，而在 36 ℃ 形成的凝胶结构则是由粗糙的、不规则的蛋白质形成的不连续的蛋白网络结构。共聚焦激光扫描显微镜的观察发现在较高的凝乳温度（36 ℃）下，在蛋白链中酪蛋白胶束聚合体的尺寸增大，原因是酪蛋白胶束表面疏水性和离子相互作用的增加以及酪蛋白胶束的重新组合。所形成的这种凝胶结构一直保持到排乳清之前，这有可能是脂肪向乳清中流失量增加的原因。在 27 ℃ 和 30 ℃ 下凝乳后制备的干酪总固形物中脂肪的含量显著（$P<0.05$）地高于在 33 ℃ 和 36 ℃ 下凝乳制备的干酪，原因是前后两者形成的微观结构的不同，以及凝乳温度对脂肪和酪蛋白胶束物理性质产生了影响。本研究结果说明，在工业生产中很有必要控制凝乳温度，因为其直接影响最终干酪产品的微观结构。

Keywords Cheddar cheese · Confocal · Fat retention · Microstructure

关键词 切达干酪 · 共聚焦激光扫描显微镜 · 脂肪的截留 · 微观结构

Abbreviations

CLSM	Confocal laser scanning microscopy
CM	Casein micelle
Cryo-SEM	Cryo scanning electron microscopy
FDM	Fat in dry matter
FL	Fat lost
FRec	Fat recovery
FRet	Fat retention
PL	Protein lost
PRec	Protein recovery
PRet	Protein retention
Ya	Total cheese yield
YDM	Yield in dry matter

缩写

CLSM	共聚焦激光扫描显微镜
CM	酪蛋白胶束
Cryo SEM	低温扫描电镜
FDM	干物质中的脂肪
FL	脂肪损失
FRec	脂肪回收率
FRet	脂肪截留
PL	蛋白损失
PRec	蛋白回收率
PRet	蛋白截留率
Ya	干酪总产量
YDM	干物质产量

1 Introduction

The coagulation of milk is one of the first steps in the manufacture of many dairy products, including cheese. This process is known to affect the microstructure of cheese, which consists of a complex arrangement of fat, protein and minerals (Madadlou et al. 2006; Everett 2007). An understanding of the coagulation process and factors that affect product microstructure is important as this microstructure influences the texture and flavour of cheese (Green 1987; Euston et al. 2002). In rennet-induced coagulation two processes occur; a hydrolysis reaction due the enzymatic activity of rennet and the physical aggregation of the coagulant-altered casein micelles (CM). The CM in milk are a composite structure composed of casein molecules held together by salt bridges, hydrophobic and electrostatic interactions and hydrogen bonding (Euston et al. 2002). A 'hairy' layer of κ-casein molecules at the surface stabilises the casein micelles, providing a combination of electrostatic and steric stabilisation (Horne and Banks 2004). In rennet-induced coagulation, the micelle is destabilised by the enzymatic hydrolysis of κ-casein. The loss of κ-casein reduces the range of steric stabilisation and charge repulsion and the micelles aggregate (Horne and Banks 2004).

A number of factors greatly influence the rennet-induced coagulation of milk during cheese formation. These include the coagulation temperature (Esteves et al. 2003; Wium et al. 2003; Madadlou et al. 2006), duration of coagulation (Wium et al. 2003), the type of rennet (plant-, microbial- and coagulant; Esteves et al. 2003) and concentration of rennet (Wium et al. 2003; Madadlou et al. 2005). The coagulation temperature typically varies from 20 °C to 40 °C, depending on the type of cheese being produced. Rennet can act on casein at temperatures as low as 0 °C but milk does not clot at temperatures below 18 °C (Dalgleish 1983). Typical coagulation temperatures used for Cheddar cheese production range from 31 °C to 33 °C (Fox and Cogan 2004). Increasing the coagulation temperature is known to increase the rate of the enzymatic reactions such as the cleavage of κ-casein by rennet but the influence of coagulation temperature on the rate of micelle aggregation is considered more significant (Esteves et al. 2003). The increase in enzymatic activity and the rate of aggregation at higher temperatures reduces the coagulation time. However, if the temperature of the milk is too high (>36 °C), the coagulation time increases due to the heat-induced inactivation of rennet (Dalgleish 1983). Several investigations suggest that factors relating to the rate of aggregation of the CM may affect the way in which the micelles aggregate and rearrange themselves in the gel and this will also affect the microstructure of the cheese produced (Euston et al. 2002).

Transmission electron microscopy (TEM) was used by Wium et al. (2003) who studied the effects of varying rennet concentration and coagulation temperature on the microstructure of feta cheese. This study found that increasing the chymosin dosage and increasing the coagulation temperature from 25 °C to 35 °C enhanced the rearrangement of casein networks leading to compact CM aggregates and a coarse protein network structure in the final cheese. Another similar study using TEM for feta-, camembert-, danbo- and gouda-type cheeses also found that increasing the rennet concentration and the coagulation temperature from 25 °C to 35 °C led to larger and more densely packed aggregates in the network structure

(Euston et al. 2002). In both studies (Euston et al. 2002; Wium et al. 2003), the differences in product microstructure between 25 °C and 35 °C treatment were enhanced when a higher concentration of coagulant was added.

Other studies with low-fat Iranian white cheese found the microstructure of cheese coagulated at 41.5 °C to be clearly different from that observed in cheese from milk coagulated at lower temperatures (34 °C and 37 °C; Madadlou et al. 2006). Notably, the casein matrix observed by scanning electron microscopy (SEM) was more compact and the size of the pores was smaller in the cheese formed from milk coagulated at 41.5 °C. Esteves et al. (2003) found that the characteristics of skim milk gel produced using plant coagulants and examined using confocal laser scanning microscopy (CLSM) were influenced less by the changes in coagulation temperature from 25 °C to 35 °C as compared to the equivalent gel produced using microbial rennet. This may be an important consideration in using plant-origin coagulants in the production of cheeses with a wider range of coagulation temperatures.

Of particular importance to many full fat cheese producers, is the loss of milk fat that can occur during the cheese-making process. In industrial Cheddar cheese manufacture, a total of 85 $g.kg^{-1}$ of milk fat can be lost in the whey of which approximately 76% of this is lost from the cheese vat (Fox et al. 2000). This loss of fat reduces the inherent value of the final product. There is not a clear understanding in the literature as to the cause of this fat loss, how it relates to the microstructure of the gel and curd, or how it is influenced by coagulation temperature. Indeed, the effect of coagulation temperature on the microstructure of samples collected from the intermediate stages of full fat renneted cheese manufacture appears limited to the SEM studies of Green (1987). An increase in the coarseness of gel networks was observed when the coagulation temperature of concentrated milk was increased from 22 °C to 38 °C. Prior treatment of the milk with chilled rennet gave a much finer protein network which retained fat better than curd formed normally possibly due to slower rennet action.

The microstructure of a full fat cooked curd collected prior to the draining of whey is therefore worthy of investigation. The objective of the present study was to examine the influence of coagulation temperature on the microstructure of the gel, curd and cheese samples collected during the manufacture of full fat Cheddar cheese. The influence of microstructure on the composition of the final cheese product was also examined.

2 Materials and methods

2.1 Manufacture of Cheddar cheese

Cheddar cheese was made using four different temperature treatments for coagulation (27 °C, 30 °C, 33 °C and 36 °C). Each cheese was made with 4 L of pasteurised cheese-milk (Murray Goulburn Co-Operative Co. Ltd., Cobram, VIC, Australia), where cheese-milk is defined as milk prepared for Cheddar cheese manufacture. The milk was obtained from a variety of farms across regional Victoria and was pooled during collection. The milk was then standardised by blending whole raw milk with raw ultrafiltered milk (UF) retentate before pasteurisation at 72 °C for 15 s. The cheese making was performed within 2 days of milk delivery.

The milk was tempered to 33 °C before inoculation with 0.05 g.kg^{-1} of freeze-dried mixed strain direct vat set mesophilic starter culture, *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (Chr. Hansen, Bayswater, VIC, Australia). Rennet (Hannilase, 690 IMCU.mL^{-1}; Chr. Hansen) was added at a concentration of 0.1 mL.kg^{-1} of milk. The milk was then allowed to coagulate at 27 °C, 30 °C, 33 °C or 36 °C until it reached a set gel strength (see determination of cutting time in Section 2.2). Gel cutting to 1 cm^3 was performed with cheese wire knives by inserting a horizontal and vertical wire knife of 1 cm spacing into the gel simultaneously and slicing the sample from left to right with one knife and right to left with another knife. The cutting process was completed within 30 s. The process continued as per the method reported in a previous study (Ong et al. 2010b). A total of 12 cheeses (three batches of cheese for each temperature treatment) were made in random order using cheese-milk with protein composition of 37.4±0.8 g.kg^{-1} and fat composition of 47.2±0.7 g.kg^{-1}.

2.2 Determination of cutting time

Previous studies have shown that the firmness of the gel at cutting may affect the final product yield (Fenelon and Guinee 1999). If the gel is quite weak, the knives tend to shatter the curd during cutting, leading to curd fines, which are partly lost in the whey. If the gel is too firm, more energy is needed to cut the curd, which will also lead to the production of fines. It is therefore important to cut the gel when it reaches a predetermined level of firmness for each of the temperature treatments. Previously, we have found that a cutting time of 45 min for milk coagulated at 33 °C resulted in cheese with good texture, yield and a composition standard for Cheddar cheese (data not shown).

The cutting time for the other temperature treatments was determined as the time needed for the gel to reach a similar firmness as that of the gel incubated at 33 °C for 45 min. Prior to cheese manufacture, the firmness of the gel (indicated by the gel strength) was measured at different coagulation temperatures. Acidified and renneted milk was prepared as described in Section 2.1. The milk was then poured into 13 glass containers (52 mm in diameter and 54 mm in height) each containing 50 mL of milk and incubated in a water bath (Thermoline, Wetherill Park, NSW, Australia) at the chosen temperature (27 °C, 30 °C, 33 °C or 36 °C) for 60 min. The gel strength of each sample was then determined as the milk set at an interval of 5 min using a TA-XT2 texture analyser (Stable Micro Systems, Godalming, England, UK). The TA-XT2 was equipped with a 2-kg load cell and a cylindrical acrylic probe (2 cm in diameter and 35 mm in height). A test speed of 1 mm.s^{-1} was used and the sample was compressed to 50% of the original sample height (15 mm). The maximum force measured in these tests was used as a measure of the gel strength. Each gel strength test was repeated on three independent samples and the data presented is the mean of the three readings (n=3).

2.3 Setting time of the cheese-milk

The setting time of the cheese-milk was determined during the cheese-making process. After the addition of rennet, 15 ml of cheese-milk was transferred to 15 microfuge tubes (Eppendorf, North Ryde, NSW, Australia) so that each tube

contained 1 mL of sample and the samples incubated in a water bath at 27 °C, 30 °C, 33 °C or 36 °C. Single samples were then taken out from the water bath and inverted at 2-min intervals until the milk coagulated within the tube. The setting time of the milk was defined as the time needed for the milk to set within the tube so that the sample was self-supporting and would not collapse when the tube was inverted.

2.4 CLSM of gel, curd and cheese samples

The gel samples were prepared for CLSM observation during the cheese-making process using the method reported in a previous study (Ong et al. 2010a). Briefly, protein was labelled with Fast Green FCF and fat with Nile Red and dual-channel images were obtained using 488 and 633 nm laser excitation to visualise the protein and fat, respectively. An aliquot of 12 μL of the renneted and stained milk was transferred to a cavity slide (ProSciTech, Thuringowa, Queensland, Australia) and covered with a 0.17 mm thick glass coverslip (ProSciTech) so that the sample was flush with the coverslip. The slide was then incubated at 27 °C, 30 °C, 33 °C or 36 °C for a period equal to the cutting time (determined in Section 2.2) before observation using an inverted CLSM (Leica Microsystems, Heidelberg, Germany). For image analysis, single optical sections (1,024×1,024 pixels) were acquired for each cheese using ×63 magnification objective.

The cooked curd samples were collected from the cheese vat just prior to the whey-draining process. The cheese samples were collected after 16 h of pressing. The samples were prepared for CLSM observation using the method described in a previous study (Ong et al. 2010a). All samples were then inverted for analysis by CLSM as described above for gel samples.

2.5 Cryo scanning electron microscopy

The gel, curd and cheese samples were prepared for cryo-SEM using the method reported in a previous study (Ong et al. 2010b). Briefly, samples 5×2×2 mm were rapidly immersed into a liquid nitrogen slush (−210 °C). Following freezing, the frozen specimens were immediately transferred using a vacuum transfer device into an attached cryo-preparation chamber. With the aid of an externally fitted binocular microscope, the sample was fractured using a chilled scalpel blade in the chamber which was maintained at −140 °C under a high vacuum ($<10^{-4}$ Pa). The specimen was then etched (facilitating the removal of ice from the surface of the fractured sample by vacuum sublimation) at −95 °C for 30 min and coated using a cold magnetron sputter coater using 300 V, 10 mA of sputtered gold/palladium alloy (60/40) for 120 s (~6 nm). It was then transferred under vacuum onto a nitrogen gas cooled module, maintained at −140 °C and observed using a field emission gun SEM (Quanta; Fei Company, Hillsboro, Oregon, USA). The detector used for the SEM observation was a solid state backscattered electron detector.

2.6 Image analysis

Image analysis of CLSM micrographs was performed using Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA, USA) equipped with Fovea Pro 4

image analysis plug-in (Reindeer Graphics, Asheville, NC, USA). The images from the two channels used to capture the fat and the protein were collected separately.

A stereological approach was used to analyse the CLSM micrographs. This allows 3-D structural information to be estimated based upon observations made on 2-D sections (Russ 2004). The star volume of the fat was calculated using a line intercept length measurement (Langton and Hermansson 1996). Briefly, segmented binary images of the fat (1,024×1,024 pixels) collected using ×63 magnification objectives (numerical aperture of 1.4) and a zoom factor of 2 were overlaid with a regular array of 200–400 points. The horizontal line intercepts of the selected features were measured using the line intercept measurement plug-in function of Fovea Pro 4 image analysis software. The mean equivalent diameter of the fat was calculated from the star volume (Langton and Hermansson 1996). The volume fraction of the fat was calculated from the global measurement of the area fraction of the fat according to stereological procedures (Russ 2004). For the analysis of the pores, two images were collected separately at an excitation wavelength of 488 nm for the fat and at an excitation wavelength of 633 nm for the protein. These images were overlaid, binarized and inverted so that the pore features appeared black and the protein and the fat globules appeared white. The pore volume fraction was then calculated as described for the fat volume fraction.

Two micrographs were analysed for each batch of cheese. Three batches of cheese were made for each temperature treatment, giving a total of six analyses for each data point presented from image analysis.

2.7 Compositional analysis

The fat and protein content of milk and whey were analysed using a Milko Scan FT120 (FOSS, North Ryde, NSW, Australia). Grated cheese samples were analysed for fat using the Rose–Gottlieb method (IDF 1996), protein using the Kjeldahl method (IDF 1993) and total solids using an oven-drying method (IDF 1982). Finally, salt content was determined using potentiometric titration with silver nitrate (IDF 1988). The moisture content was calculated based on the total solids content of the cheese. The analysis was performed in duplicate for each batch of cheese. Three batches of cheese were made for each temperature treatment, giving a total of six measurements at each experimental condition.

The pH of the milk, whey and gel was measured using a pH metre (Orion 720A, Orion Pacific Pty Ltd., Frankston, VIC, Australia) on site at Bio21 after calibrating with freshly prepared pH 4.0 and 7.0 standard buffers. The pH of the curd and cheese was measured in a curd or cheese slurry made by blending 20 g of grated curd or cheese with 12 mL of deionised H_2O (Millipore, Billerica, MA, USA; purified to a resistivity of 18.2 mΩ). This later method follows Australian Standard 2300.1.6 (1989).

2.8 Fat and protein recovery and cheese yield

The amount of milk fat and protein lost in the whey collected during Cheddar cheese making (fat loss, $g.kg^{-1}$ and protein lost, $g.kg^{-1}$, respectively) or retained in the cheese (fat retention, $g.kg^{-1}$ and protein retention, $g.kg^{-1}$, respectively) was

calculated on the basis of fat or protein levels in the cheese-milk using the formula described by Guinee et al. (2006). The total fat recovery ($g.kg^{-1}$) is the sum of the fat lost in the whey and the fat retained in the cheese product. Similarly, the total protein recovery ($g.kg^{-1}$) is the sum of the protein lost in the whey and the protein retained in the cheese product.

The cheese yield was calculated as the yield of cheese per kg of cheese-milk (Ya, $g.kg^{-1}$). Dry matter cheese yield ($g.kg^{-1}$) was calculated as dry matter yield ($Ya \times 1{,}000 - \text{moisture content of the cheese}$) per kg of cheese-milk. The moisture adjusted cheese yield ($g.kg^{-1}$) was calculated to eliminate the direct effect of differences in cheese moisture to yield where $Yma = Ya \times [(100 - \textit{moisture in cheese})/(100 - \textit{reference moisture in cheese})]$ using a reference moisture in cheese of 350 $g.kg^{-1}$. The yield adjusted by the compositional difference in protein and fat content was calculated as $Yafp = Ya \times [(\textit{reference fat in milk}/\textit{reference proteinin milk})/(\textit{fat in milk}/\textit{protein in milk})]$ using a reference protein in milk of 37 $g.kg^{-1}$ and reference fat in milk of 47 $g.kg^{-1}$.

2.9 Texture analysis

Texture profile analysis (TPA) was performed on cheese samples at room temperature (~20 °C) using the texture analyser TA-XT2 as described above. The probe used was a 5 cm cylindrical flat probe. Each sample ($1.5 \times 1.5 \times 1.5$ cm in size) was cut from the central part of the cheese using a sharp knife and held at room temperature (~20 °C) for 1 h in a closed container prior to analysis to prevent moisture loss. TPA simulates the human chewing action by subjecting a sample to a compressive deformation (first bite), followed by a relaxation and a second deformation (second bite) (Halmos et al. 2003). In this experiment, a test using 50% sample compression was applied to the cheese using two compression cycles at a constant crosshead speed of 2 $mm.s^{-1}$. The texture analyses were performed twice for two independent samples from each batch of cheese.

2.10 Statistical analysis

Data analysis was carried out using a statistical package from Minitab (Minitab Inc, State College, PA, USA). One-way analysis of variance and Tukey's paired comparison were used to study differences between means with a significance level of $\alpha = 0.05$.

3 Results and discussion

3.1 Adjustments to the cheese making process

The influence of coagulation temperature on the gel strength is shown in Fig. 1. The gel strength gives a good indication of the firmness of the sample (Kalab et al. 1970). Gel firmness increased more rapidly in samples coagulated at higher temperatures (Fig. 1). This was expected as an increase in temperature is known to enhance rennet activity and CM aggregation (Dalgleish 1983). The gel strength of

the milk coagulated at 33 °C was 0.54±0.02 N after 45 min of incubation. The cutting time for the gels produced at different temperatures was defined here as the time needed for the gel to reach a similar firmness, which was 60, 50 and 35 min for cheese-milk coagulated at 27 °C, 30 °C and 36 °C, respectively, as indicated by the line in Fig. 1. This adjustment was incorporated to the cheese-making process as shown in Table 1. The time required for the cheese-milk to set prior to cutting was significantly affected by the coagulation temperature ($P<0.05$), consistent with the results presented in Fig. 1. A difference of 19 min was observed between the setting time for the cheese-milk at the highest (36 °C) and lowest (27 °C) temperatures, further confirming that an increase in coagulation temperature enhances the rate of CM aggregation.

A second adjustment to the cheese-making process was the cooking time. The length of cooking time was altered to ensure the curds had a pH of 6.1 at draining. The actual pH of the curds measured at draining and the cooking time are recorded in Table 1. Draining of the whey at pH 6.1 is critical as the pH of the curds at draining dictates the amount of lactose remaining in the curd, the amount of lactic acid in the final product and the pH of the cheese. More rennet and less plasmin will be retained in the curd at a lower pH and this will have implications for the extent of proteolysis during the maturation of the cheese. A low pH at draining also results in a lower mineral content within the final cheese. Minerals such as calcium participate in the protein networks that form the structural matrix of the cheese and hence any change to the pH at draining will indirectly affect the cheese texture (Lucey and Fox 1993).

The cooking time required for the curd generated from milk coagulated at 36 °C was reduced significantly compared to that required for curd generated from milk coagulated at a lower temperature ($P<0.05$). This was probably due to the increased growth of the starter culture at the higher coagulation temperature. Overall, the processing time from the addition of the starter culture to the draining and the milling was also reduced significantly for samples coagulated at 36 °C, as compared to samples coagulated at lower temperatures ($P<0.05$; Table 1).

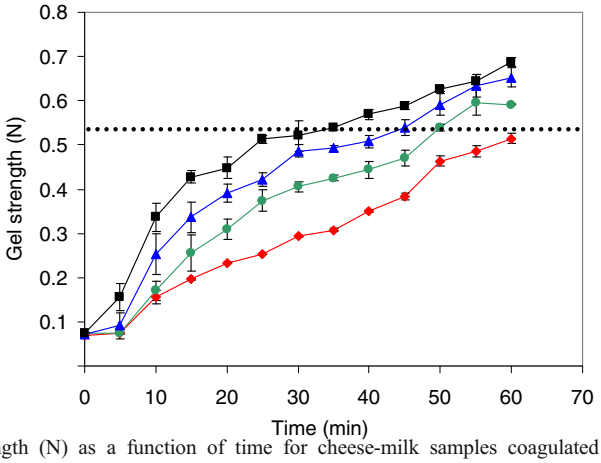

Fig. 1 Gel strength (N) as a function of time for cheese-milk samples coagulated at 27 °C (*filled diamond*), 30 °C (*filled circle*), 33 °C (*filled triangle*) and 36 °C (*filled square*). Time zero corresponds to the addition of rennet to the milk. The *dotted line* indicates firmness at cutting time. Results are the mean± the standard deviation of mean ($n=3$ individual gel samples)

Table 1 Cheddar cheese-making process parameters and adjustments

Coagulation temperature (°C)	pH at renneting	Setting time[a] (min)	Cutting time[b] (min)	pH at cutting	pH at draining	Cooking time[c] (min)	Time between starter addition to drain (min)	pH at milling	Cheddaring time[c] (min)	Time between starter addition to milling (min)
27	6.54±0.02[a]	32±0[a]	60	6.52±0.03[a]	6.15±0.04[a]	105±3[a]	238±3[a]	5.39±0.04[a]	90±0[a]	328±3[a]
30	6.54±0.03[a]	22±1[b]	50	6.50±0.01[a]	6.10±0.01[a]	110±0[b]	230±1[b]	5.33±0.02[a]	95±2[b, c]	327±2[a]
33	6.53±0.03[a]	20±1[b]	45	6.51±0.03[a]	6.11±0.03[a]	112±4[a, b]	217±4[c]	5.44±0.05[a]	90±3[a, b]	307±4[b]
36	6.52±0[a]	13±0.7[c]	35	6.47±0.02[a]	6.12±0.05[a]	82.0±4[d]	191±3[d]	5.38±0.07[a]	97±2[c]	285±5[c]

Means in a single column with different letters are significantly different ($P<0.05$)

The results are expressed as the mean±the standard deviation of mean ($n=3$ for three cheese-making experiments)

[a] The setting time is the time needed for the cheese-milk to set and form a self-supporting gel within a microfuge tube when the tube was inverted

[b] The cutting time is the time when the gel is firm enough to start cutting the gel, based on predetermined gel strength (Fig. 1)

[c] The cooking and Cheddaring time were adjusted based on the pH of the curds during Cheddar cheese manufacture

3.2 Influence of the coagulation temperature on the microstructure of the gel

The microstructure of the gels formed at 27 °C, 30 °C, 33 °C and 36 °C observed by CLSM is shown in Fig. 2A–D. Spherical fat globules were evenly dispersed within the porous structure of the protein matrix. Qualitatively, there was no clear distinction between the microstructure of the gels coagulated at 27 °C, 30 °C or 33 °C. However, the clusters of aggregated CM appeared bigger and more compact in the gel formed at 36 °C, possibly due to increased hydrophobic interactions and rearrangement of CM particles during coagulation. Hydrophobic interactions, which influence the aggregation of casein particles, are favoured at higher temperatures (Madadlou et al. 2006). An increase in temperature (<40 °C) also promotes calcium binding, resulting in a decrease in protein charge and decrease in electrostatic repulsion thus promoting aggregation (Dalgleish 1983). In addition, after a network has been formed, the outer surface of the casein strands and nodes is made from casein that is still reactive. Further collisions between CM aggregates and the network strands leads to further aggregation and rearrangement of the protein network (van Vliet et al. 1991).

The data from quantitative image analysis of the microstructure of the gel samples formed at 27 °C, 30 °C, 33 °C and 36 °C by sterology are shown in Fig. 2E–G. The

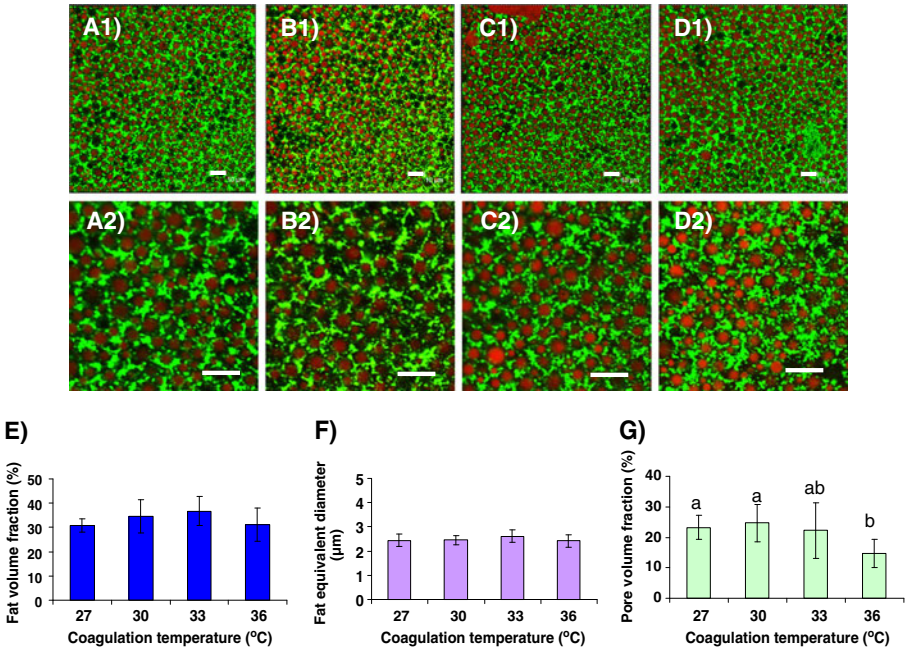

Fig. 2 The microstructure and image analysis of gel samples coagulated at different temperatures. **A–D** The microstructure of gel samples prepared using cheese-milk coagulated at 27 °C (**A**), 30 °C (**B**), 33 °C (**C**) and 36 °C (**D**) observed by CLSM. *A1–D1*, ×2 digital zoom; *A2–D2*, ×5 digital zoom with ×63 objective lenses. The Nile Red stained fat appears *red* and the fast green FCF stained protein appears *green* in these images. *Scale bars* 10 μm in length. **E–F** Stereological image analysis of the CLSM micrographs showing: the fat volume fraction (**E**), the fat equivalent diameter (**F**) and the pore volume fraction (**G**). Results are the mean±the standard deviation of mean (n=6 where three cheeses were each sampled twice). Results with different superscripts are significantly different (P<0.05). Results without superscripts are not significantly different (P>0.05)

compactness of the CM aggregates observed qualitatively was reflected quantitatively in the significantly ($P<0.05$) lower pore volume fraction measured for gels formed at 36 °C, compared to gels formed at 27 °C or 30 °C. The volume fraction of the fat and the size of the fat globules in the gel samples were not significantly affected by the temperature treatments ($P<0.05$).

Previous studies using skim, reconstituted and UF milk provide some indication that the casein networks of rennet induced gels formed at high temperatures have denser clusters of aggregated CM (Green 1987; Lagoueyte et al. 1994; Wium et al. 2003). Lagoueyte et al. (1994) observed that the casein aggregates fused together more tightly as the coagulation process continued at a higher coagulation temperature, resulting in a more open structure in the gel network prepared using reconstituted milk. This was not observed in the gel prepared in our study, possibly due to the different type of milk used and the difference in the coagulation period which was 35–60 min in our study compared to 162–300 min for gels coagulated at 26–40 °C in the previously published study. This comparison highlights the need to adjust the coagulation period of gels formed at higher coagulation temperatures to prevent the opening of the pores, which may increase the amount of fat lost in the whey (Lagoueyte et al. 1994).

The microstructure of the gel samples formed at the temperature extremes of 27 °C and 36 °C and observed by cryo-SEM are shown in Figs. 3A1 and A2, respectively. In agreement with Green (1987), the gel structure of samples formed at 36 °C was coarse and irregular. In contrast, the microstructure of the gel formed at 27 °C consists of thin protein strands which formed a very fine and continuous gel

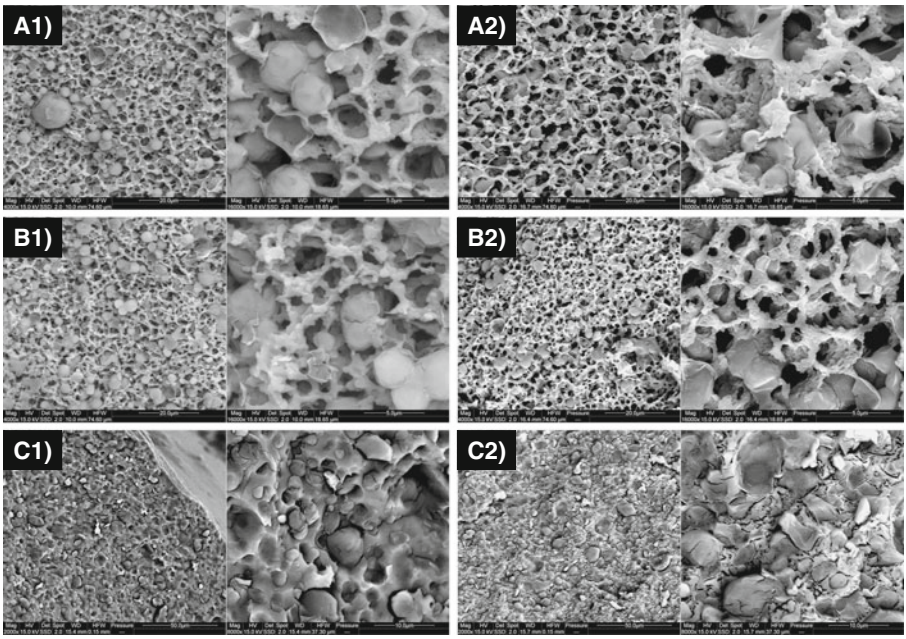

Fig. 3 Cryo SEM micrographs of the gel (**A**), cooked curd (**B**) and Cheddar cheese (**C**) samples prepared using cheese-milk coagulated at 27 °C (*A1*, *B1*, *C1*) and 36 °C (*A2*, *B2*, *C2*). *Scale bars* within the images obtained using ×4,000 and ×16,000 magnification are 20 and 5 μm in length, respectively

network. These differences in the initial gel structure may in turn affect the microstructure and composition of the curd at the later stages of manufacturing.

3.3 Influence of coagulation temperature on the microstructure of cooked curd

The microstructure of the cooked curd samples collected just prior to whey draining and observed by CLSM is shown in Fig. 4A–D. The shape of the pores is more regular in the cooked curd formed using cheese-milk coagulated at 27 °C, 30 °C and 33 °C compared to the pores in the cooked curd that had been coagulated at 36 °C. The irregularity of the cooked curd formed from milk coagulated at 36 °C was particularly evident at lower magnifications (Fig. 4D1). The heat treatment applied during cooking, which is 38 °C for each sample regardless of coagulation temperature resulted in further fusion of CM particles. As a result, the protein strands have drawn closer together. The tightening of the protein network is known to cause a stress in the strands, increasing the endogenous pressure on the whey and potentially inducing syneresis (van Vliet et al. 1991). The data from quantitative image analysis of the microstructure of the cooked curd samples are shown in Fig. 4E–G. The volume fraction and the size of the fat globules in the cooked curd

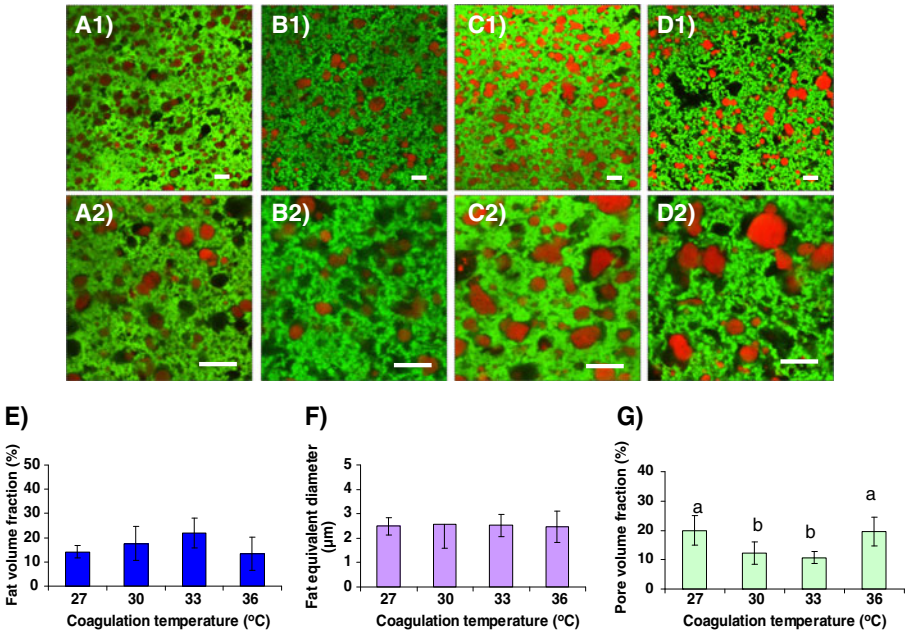

Fig. 4 The microstructure and image analysis of cooked curd samples prepared using cheese-milk coagulated at different temperatures. **A–D** The microstructure of cooked curd samples prepared using cheese-milk coagulated at 27 °C (**A**), 30 °C (**B**), 33 °C (**C**) and 36 °C (**D**) observed by CLSM. *A1–D1*, ×2 digital zoom; *A2–D2*, ×5 digital zoom with ×63 objective lenses. The Nile Red stained fat appears *red* and the fast green FCF stained protein appears *green* in these images. *Scale bars* 10 μm in length. **E–F** Stereological image analysis of the CLSM micrographs showing: the fat volume fraction (**E**), the fat equivalent diameter (**F**) and the pore volume fraction (**G**). Results are the mean±the standard deviation of mean (n=6 where three cheeses were each sampled twice). Results with different superscripts are significantly different (P<0.05). Results without superscripts are not significantly different (P>0.05)

samples were not significantly affected by the temperature treatment ($P<0.05$; Fig. 4E–F). There was no clear trend in the volume fraction of the pores in the curd prepared from milk coagulated at different temperatures (Fig. 4G). The pore volume fraction at 36 °C was significantly higher ($P<0.05$) than that in the curd formed from milk coagulated at 33 °C or 30 °C (Fig. 4G) possibly due to the irregular gel structure, which can be a limiting factor for protein fusion during cooking. The higher pore volume fraction at 27 °C may be a result of the significantly longer cutting time for these samples (Table 1), leading to an increase in the serum within these samples that is only visible after cutting and cooking.

The microstructure of the cooked curd samples was also observed using cryo-SEM, as shown in Fig. 3B. The network contracted consistent with CLSM and qualitatively the cryo-SEM micrographs show that the pore size and the size and number of the fat globules in the cooked curd samples formed from milk coagulated at high (36 °C) and low (27 °C) temperatures were similar. However, magnification of the structure at ×16,000 revealed that the protein strands in the cooked curd prepared using cheese-milk coagulated at 27 °C were fine and regularly structured (Fig. 3B1), whereas the protein strands in the cooked curd prepared using cheese-milk coagulated at 36 °C contained an irregular, coarse and less continuous protein network (Fig. 3B2) consistent with the coagulated gel (Fig 3A).

Lucey et al. (1997) suggested that breakage of protein network may occur as the size of CM aggregates increases. Such breakage may reduce the ability of the protein network to retain fat. Breakage of the protein strands was not observed in the gel or cooked curd samples in our study. This is probably due to the shorter processing time used in our study as compared to the long processing time used in other studies. For example, local breaking of protein strands was observed in casein gels 17 h after the addition of glucono delta-lactone for acid-induced aggregation (Lucey et al. 1997).

3.4 Influence of coagulation temperature on the microstructure of Cheddar cheese

The microstructure observed by CLSM within Cheddar cheese samples prepared using cheese-milk coagulated at different temperatures is shown in Fig. 5A–D and the data from quantitative image analysis are shown in Fig. 5E–G. The micrographs show a continuous protein network permeated by fat with different shapes; fat globules, heat-induced coalesced fat globules and pools of free fat or non-globular fat. The non-globular fat and the elongation of the fat observed in all cheese samples is evidence of the disruption of the milk fat globules during the earlier stages of cooking, Cheddaring and pressing during the cheese making process. Differences in the microstructure of Cheddar cheese samples prepared using cheese-milk coagulated at 27 °C, 30 °C and 33 °C were not apparent. However, in cheese prepared from cheese-milk coagulated at a higher temperature (36 °C), the protein strands were thicker and less occupied by fat (Fig. 5D).

The volume fraction of fat globules observed in the cheese made using cheese-milk coagulated at 36 °C was significantly lower ($P<0.05$; Fig. 5E) than in cheese made using cheese-milk coagulated at 27 °C, 30 °C and 33 °C. The size of the fat globules estimated by sterology was not significantly different ($P>0.05$; Fig. 5E–G) for the cheese samples produced from milk coagulated at different temperatures. The pressing process also closed the pores of the network, resulting

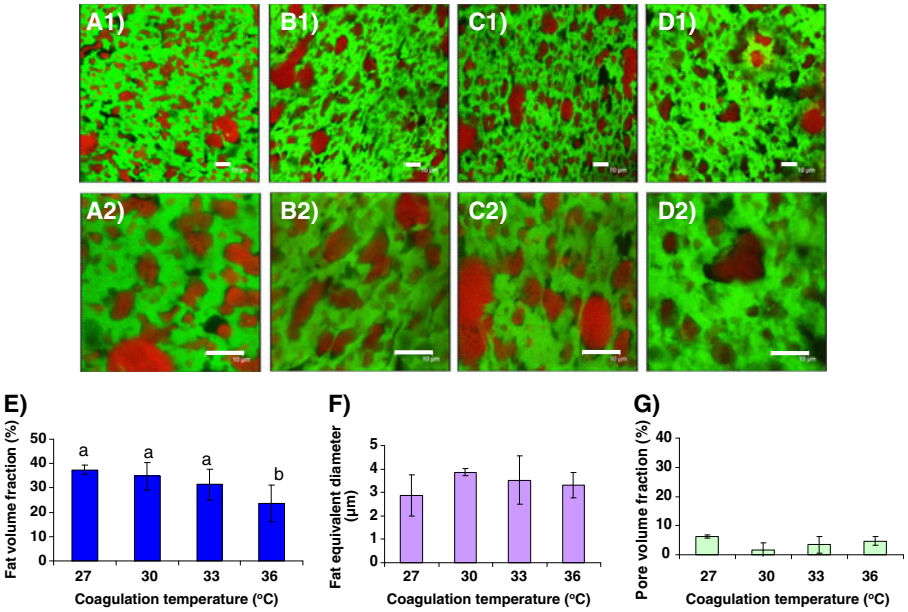

Fig. 5 The microstructure and image analysis of cheese samples prepared using cheese-milk coagulated at different temperatures. **A–D** the microstructure of cheese samples prepared using cheese-milk coagulated at 27 °C (**A**), 30 °C (**B**), 33 °C (**C**) and 36 °C (**D**) observed by CLSM. *A1–D1*, ×2 digital zoom; *A2–D2*, ×5 digital zoom, with ×63 objective lenses. The Nile Red stained fat appears *red* and the fast green FCF stained protein appears *green* in these images. *Scale bars* 10 μm in length. **E–F** Stereological image analysis of the CLSM micrographs showing: the fat volume fraction (**E**), the fat equivalent diameter (**F**) and the pore volume fraction (**G**). Results are the mean±the standard deviation of mean (n=6 where three cheeses were each sampled twice). Results with different superscripts are significantly different (P<0.05). Results without superscripts are not significantly different (P>0.05)

in a significantly lower pore volume fraction for all cheese samples compared to the cooked curd and gel samples, regardless of the initial coagulation temperature (P<0.05; Fig. 5E).

The microstructure of the cheese samples formed from milk coagulated at 27 °C and 36 °C was also observed using cryo-SEM (Fig. 3C). The protein matrix in the cheese prepared using cheese-milk coagulated at 27 °C appeared smoother than the matrix observed in the cheese prepared using cheese-milk coagulated at 36 °C consistent with observations made for the gel and cooked curd (Fig. 3A1–A2 and B1–B2).

3.5 Influence of coagulation temperatures on composition, yield and texture of cheese

The influence of the coagulation temperature on the fat and protein lost in the whey or retained in the cheese is shown in Table 2. The percentage of fat lost within the whey of samples coagulated at 33 °C and 36 °C was significantly higher than for samples coagulated at 27 °C and 30 °C (P<0.05; Table 2). Only about 884 g.kg^{-1} of the total fat in cheese-milk was retained in the cheese prepared using cheese-milk coagulated at 36 °C. This was possibly due to the irregular and less continuous

Table 2 Fat and protein lost in the whey or retained in the cheese and the final Cheddar cheese yield of samples prepared using cheese-milk coagulated at 27 °C, 30 °C, 33 °C or 36 °C

Coagulation temperature (°C)	Concentration of fat and protein in milk		Concentration of fat and protein in whey		Fat and protein lost in whey		Fat and protein retained in cheese		Recovery		Cheddar cheese yield		
	Fat ($g.kg^{-1}$)	Protein ($g.kg^{-1}$)	Fat ($g.kg^{-1}$)	Protein ($g.kg^{-1}$)	FL ($g.kg^{-1}$)	PL ($g.kg^{-1}$)	FRet ($g.kg^{-1}$)	PRet ($g.kg^{-1}$)	FRec ($g.kg^{-1}$)	PRec ($g.kg^{-1}$)	Ya ($g.kg^{-1}$)	YDM ($g.kg^{-1}$)	Yma ($g.kg^{-1}$)
27	48.2 ± 0.4^{b}	38.0 ± 0.1^{a}	2.8 ± 0.2^{a}	10.7 ± 0.2^{a}	48.5 ± 2.6^{a}	237 ± 3.4^{a}	915 ± 7.8^{a}	767±5.9 a	964 ± 9.4^{a}	1000 ± 5.7^{a}	120.3 ± 0.8^{a}	80.0 ± 0.7^{a}	122.0 ± 1.2^{a}
30	46.6 ± 0.3^{a}	36.7 ± 0.5^{b}	2.5 ± 0.3^{a}	11.3 ± 0.7^{a}	45.0 ± 5.1^{a}	259 ± 15^{b}	$907\pm1.9^{a,\ b}$	772±2.7 a	952 ± 3.5^{b}	1030 ± 17^{b}	114.3 ± 0.3^{b}	75.5 ± 0.1^{b}	115.9 ± 0.3^{b}
33	47.3 ± 0.1^{a}	38.2 ± 0.2^{a}	3.2 ± 0.1^{b}	10.8 ± 0.1^{a}	56.4 ± 1.2^{b}	237 ± 4.9^{a}	$899\pm5.6^{b,\ c}$	768±5.9 a	$956\pm4.8^{a,\ b}$	1000 ± 8.1^{a}	119.3 ± 0.8^{a}	78.0 ± 0.7^{c}	120.1 ± 0.1^{a}
36	46.6 ± 0.3^{a}	36.7 ± 0.5^{b}	3.8 ± 0.5^{b}	11.2 ± 0.4^{a}	67.7 ± 9.6^{c}	257 ± 10^{b}	884 ± 8.0^{c}	771±7.1 a	$952\pm13^{a,\ b}$	1030 ± 19^{b}	118.8 ± 1^{a}	75.5 ± 0.6^{b}	116.5 ± 1.1^{b}

Means in a single column with different letters are significantly different ($P<0.05$)

The results are expressed as the mean±standard error of mean ($n=6$ for three cheese making experiments sampled twice)

FL fat lost in whey, *PL* protein lost in whey, *FRet* fat retained in cheese, *PRet* protein retained in cheese, *FRec* fat recovery (FL+FRet), *PRec* protein recovery (PL+PRet). FL, PL, FRet, PRet, FRec and PRec were calculated on the basis of protein or fat levels in the cheese-milk. *Ya* total cheese yield per kg of cheese-milk, *YDM* yield in dry matter per kilogram of cheese-milk, *Yma* moisture-adjusted yield per kilogram of cheese-milk

Table 3 Composition of Cheddar cheese prepared using cheese-milk coagulated at 27 °C, 30 °C, 33 °C or 36 °C

Coagulation temperature (°C)	Fat ($g.kg^{-1}$)	Protein ($g.kg^{-1}$)	Salt ($g.kg^{-1}$)	pH	Moisture ($g.kg^{-1}$)	S/M ($g.kg^{-1}$)	FDM ($g.kg^{-1}$)
27	365.2 ± 4.3^{a}	242.9 ± 1.2^{a}	$13.7\pm0.8^{a, b}$	5.40 ± 0.03^{a}	345.7 ± 5.7^{a}	40.1 ± 1.1^{a}	559.2 ± 2.4^{a}
30	366.4 ± 1.5^{a}	243.3 ± 1.0^{a}	14.0 ± 0.5^{b}	5.33 ± 0.03^{a}	341.3 ± 1.7^{a}	41.0 ± 1.1^{a}	556.2 ± 1.5^{a}
33	356.8 ± 1.6^{b}	246.1 ± 1.2^{b}	$13.0\pm0.4^{a, c}$	5.36 ± 0.03^{a}	345.6 ± 3.2^{a}	$37.6\pm1.0^{a, b}$	545.3 ± 3.9^{b}
36	343.7 ± 4.5^{c}	242.5 ± 1.9^{a}	12.5 ± 0.4^{c}	5.38 ± 0.04^{a}	367.0 ± 5.1^{b}	34.5 ± 1.0^{b}	544.2 ± 3.2^{b}

Means in a single column with different superscripts are significantly different ($P<0.05$)

The results are expressed as the mean±the standard error of the mean ($n=6$ where three cheeses were each sampled twice)

S/M salt in moisture, *FDM* fat in dry matter

network structure observed in the gel and cooked curd formed from milk coagulated at this temperature. A strengthening of the gel network at higher coagulation temperature could also lead to a greater loss of fat in the whey.

The higher percentage of fat lost to the whey may also be due to the direct effect of the coagulation temperature on the physical properties of the fat globule. The softening point of milk fat ranges from 31 °C to 36 °C and the average melting point of milk fat is 37 °C (Jensen and Clark 1999; Lopez et al. 2006). At 36 °C, the milk fat globule is less viscous, which could increase fat mobility in the protein network during coagulation. Heat also induces the aggregation of fat globules (Lopez et al. 2007). The increased mobility, aggregation and coalescence of fat globules at higher temperatures may allow the fat to form pools that may subsequently leak out from the protein matrix during cheese manufacture (Richoux et al. 2008), although there was little evidence of the aggregation or coalescence of fat in the gel formed from milk coagulated at 36 °C. The increased temperature may also affect interactions between the fat globule membrane components and the casein matrix but further study is clearly required to fully understand the mechanism by which coagulation temperature affects fat retention.

Despite the significant influence of coagulation temperature on fat lost in the whey ($P<0.05$), there was no observable trend for the protein lost, the total cheese yield, the yield in dry matter and the moisture adjusted cheese yield for the different samples (Table 2). The yield adjusted by the compositional difference in the cheese-milk was 118.9±0.8, 116.9±1.4, 118.0±0.9 and 117.5±0.9 $g.kg^{-1}$ for cheese prepared using cheese-milk coagulated at 27 °C, 30 °C, 33 °C and 36 °C, respectively. The higher percentage of fat lost in the whey at higher coagulation temperatures (33 °C and 36 °C) was reflected in the significantly lower final fat content and the lower fat in dry matter for these cheese samples, as shown in Table 3 ($P<0.05$). There was no observable trend for the effect of temperature on the other compositional parameters of the cheese. The textural profile of the cheese measured within a week of cheese manufacture was also unchanged ($P<0.05$) by the microstructure of the curd (data not shown). However, the development of texture within Cheddar cheese continues during ripening. Further studies on the influence of coagulation temperature on the texture and microstructure of Cheddar cheese will therefore be required to assess the possible long term effects of coagulation temperature on Cheddar cheese properties. The translation of these results to a pilot scale setting will also reveal whether these findings can be adopted at an industrial scale.

4 Conclusions

We have clearly demonstrated that the coagulation temperature affects the microstructure observed within the gel, curd and cheese during Cheddar cheese manufacture. This in turn appears to affect the ability of the curd to retain fat. The fat content and fat in dry matter were higher in the cheese samples prepared using cheese-milk coagulated at lower temperatures (27 °C and 30 °C) compared to those prepared using cheese-milk coagulated at higher temperatures (33 °C and 36 °C). This was possibly related to the differences in the casein micelle building blocks

within the gel network. The microstructure in the gel coagulated at 27 °C consisted of fine protein strands that interconnected to form a regularly structured protein network. In contrast, the microstructure for the gel formed from milk coagulated at 36 °C consisted of irregular, coarse and less continuous protein networks. The characteristic structure observed in samples generated from milk coagulated at higher temperatures may have reduced the capability of the cooked curd to retain fat during whey draining. The different coagulation temperatures will also have affected the physical properties of the fat and the casein micelles during coagulation.

Acknowledgements This work was funded by Dairy Innovation Australia Limited and the Australia Research Council (LP0883300). The authors would like to thank the Bio21 Molecular Science & Biotechnology Institute and the Particulate Fluids Processing Centre, which is a Special Research Centre of the Australian Research Council at The University of Melbourne, for access to equipment. We would also like to thank Murray Goulburn, Warrnambool Cheese and Butter Factory, National Foods and Burra Foods for their involvement. We appreciate the technical input from Mr. Roger Curtain (Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne) and his help operating the scanning electron microscope in cryo-mode. We also thank the reviewers for their constructive input to the manuscript.

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