**Impact of processing conditions on microstructure, texture and chemical properties of
model cheese from sheep milk**

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Abstract

Cutting and cooking settings have a strong effect on curd particle features, whey syneresis and cheese properties. In the present study, the impact of curd grain size and cooking temperature on the microstructure, texture and composition of cheese, whey losses and cheese yield was studied with specific focus on sheep milk. Cooking temperature especially affected cheese microstructure, texture and composition, while cutting process was largely responsible for fat losses in the whey. Additionally, cheese yield increased with a bigger curd grain size and lower cooking temperatures. Higher cooking temperatures reduced the moisture content of the curd grains and cheese and lead to cheeses with reduced porosity and more free fat in their structure, resulting in harder and chewier cheeses. Interactions between the microstructural arrangement of fat and textural parameters were also observed. These results contribute with new data on the relationships between curd grain size and cooking conditions on the microstructure and physico-chemical properties of cheese. In addition, reducing the compound losses in whey would have a direct effect on the improvement of processing, cheese quality and yield, and the ulterior byproduct management.

Keywords: Confocal microscopy, cryo-scanning electron microscopy, fat loss, curd grain size, cooking temperature, cheesemaking settings.

Highlights

- Cutting and cooking settings affect cheese microstructure and texture properties
- A higher cooking temperature raised free fat formation and reduced cheese porosity
- The hardness and chewiness of cheese increased with higher cooking temperature
- A bigger curd grain size increased the globular fat volume in cheese microstructure
- A smaller curd grain size enhanced the fat loss and decreased cheese yield

1. Introduction

The technological conditions used during cheese processing, particularly curd cutting and cooking process, have a direct effect on cheese composition, yield and compound losses in the whey. During cutting and cooking, syneresis of curd occurs and this is affected by several factors well documented in the scientific literature such as: milk composition, firmness of the gel at cutting, gel acidity, temperature, time of cutting, speed of rotation of the cutting and stirring tools and the surface area of the curd (Banks 2007; Dejmek and Walstra 2004; Everard et al. 2008; Johnston et al. 1991; Johnston et al. 1998; Walstra et al. 2006).

While some authors (Johnston et al. 1991; Whitehead and Harkness 1954) have suggested that the size of the curd grain plays an important role in the moisture of the final cheese product, others have found that different curd cutting intensities have little effect on the extent of syneresis, although enhanced losses of fat and casein fines were reported (Everard et al. 2008). The relationship between fat and protein losses from the curd and a reduction in cheese yield with curd grain size has been shown to be influenced not only by the cutting revolutions but by a combination of the total revolutions applied during cutting and cooking processes (Everard et al. 2008; Johnston et al. 1991; Johnston et al. 1998). Even though cheesemaking processing conditions affect chemical properties of cheese, the studies about the interactions of cutting and cooking settings with microstructure and texture are scarce. Additionally, the comparison of cutting and stirring conditions in this prior studies is made difficult due to technical differences in the cheesemaking processes applied. In this study, the curd grain size was assessed by image analysis to provide information about the curd grain size obtained at the end of the cutting or cooking processes (Aldalur et al. 2019). This method may reduce some of the comparative issues described by other authors, such as the use of different vat designs and sizes, cutting knives, stirrers and cutting and stirring conditions (Everard et al. 2008; Johnston et al. 1998).

Temperature is another factor that enhances moisture expulsion from curd grains, as the permeability of the curd matrix increases at higher temperatures (Fagan et al. 2007). A rapid

increase in temperature, however, may lead to a shrunken outer layer of the curd grain, impairing permeability and slowing syneresis (Dejmek and Walstra 2004). Moreover, temperature greatly affects the properties of fat globules and the spatial arrangement of other components in cheese, which ultimately determine the structure of the cheese and its physico-chemical and sensory properties (Lamichhane et al. 2018; Lopez et al. 2006). As a result, the microstructural observation of cheese and samples taken during the cheesemaking process can be very useful to predict and control the properties of the final cheese product (El-Bakry and Sheehan 2014). Confocal laser scanning microscopy (CLSM) is a powerful tool, which allows two-dimensional (2D) thin optical sections of a sample to be digitally captured and reassembled to obtain three-dimensional (3D) information (Gunasekaran and Ding 1998). This imaging allows both fat and protein to be visualised and quantified, providing numerical information about the size, shape and distribution of key components of the curd and cheese matrix. In addition, cryo-scanning electron microscopy (cryo-SEM) provides a detailed image of the surface features of hydrated samples without chemical staining and conventional sample drying (Ong et al. 2011).

Cheese produced from raw sheep milk is particularly important in European countries such as France, Italy, Greece, Spain and Romania (EUROSTAT 2016). This type of milk is suitable for cheesemaking due to the higher concentration of milk components and improved coagulation properties compared to cow milk (Kammerlehner 2009; Park et al. 2007). While considerable differences have been observed, the effects of the conditions used during the cheesemaking process using sheep milk in the cheese product, compound losses and yield have been scarcely reported.

The objective of the current study was to investigate the effect of the processing parameters curd grain size, assessed by image analysis, and cooking temperature on the microstructure, texture, composition and yield of cheese from sheep milk.

2. Materials and methods

2.1. Experimental design and sampling

A two-level full factorial experimental design was employed, which looked at the effects of curd grain size (*CGS*) and cooking temperature (*CT*) on the final cheese product (Table 1). Two levels of *CGS* were selected: big and small, and two levels of *CT*: 36 °C and 45 °C, all other cheesemaking conditions remained fixed. The four experimental cheeses were made in two consecutive days using the same bulk raw ewe milk collected from a local farm (Meredith Dairy, Truganina, Victoria, Australia) one day prior to the first day of cheesemaking. Simultaneous treatments were carried out in two identical vats. On the first day the big *CGS* factor level was selected and the *CT* factor was varied in the two identical vats. The following day, the same procedure was followed except the *CGS* factor level selected was small. The experiments were then replicated the following week using a second collection of bulk milk. [Online Resource 1](#) summarises the most relevant physico-chemical and rheological parameters of the milk used for the experimental cheesemaking.

Cheese production was carried out in a small cheesemaking facility located at The Bio21 Molecular Science and Biotechnology Institute (The University of Melbourne, Melbourne, Australia). The cheesemaking process carried out was a typical manufacture of semi-hard sheep cheeses which are usually ripened for a minimum of 2 months. For that purpose, 20 L cubic-shaped stainless-steel double jacketed open vats were used. Each experimental cheese was made using 10 kg of raw sheep milk, which was stored at 5 °C for 1 or 2 d before cheese production. First, the cheese milk was tempered to 25 °C in the vat with constant stirring at 30 rpm for ~35 min before adding 0.02 g/kg of freeze-dried mesophilic starter culture, *Lactococcus lactis*, subsp. *lactis* and *Lactococcus lactis* subsp. *Cremoris* (Danisco, Copenhagen, Denmark). The temperature was increased from 25 °C to 30 °C over 20 min and 0.03 g/kg of rennet (IMCU ~1400, Hansen Naturem, Chr. Hansen, Hørsholm, Denmark) added. Stirring was stopped 2 min after rennet addition to ensure the correct blending of the mixture. The cutting time was set to 40 min after rennet addition and the gel strength of each batch was determined at this time point. Cutting was performed using two manual cheese cutting frames with nylon wires (Grunt, Victoria, Australia) stretching in the horizontal (29 wires) or vertical direction (23 wires). The spacing

between the wires in both directions was 1 cm. First, both cutting frames were inserted in the left side of the vat and moved simultaneously from left to right once and removed. The two frames were then inserted again at the back of the vat and were pushed through the curds to the front of the vat. This whole movement was repeated either twice to obtain big curd grains or six times to obtain small curd grains. The assessment of the curd grain size (big and small) was performed after cutting and stirring (Table 1) by image analysis, as previously reported (Aldalur et al. 2019) to ensure that two well defined and significantly different curd grain sizes were produced during cheesemaking. After cutting, the curd grain and whey mixture was stirred at 40 rpm for 50 min while the temperature increased from 30 °C to either 36 °C or 45 °C, depending on the *CT* selected. The cooking temperature rate was different for both *CT*, with mean values of 0.12 and 0.30 °C/min when the temperature was raised to 36 °C and 45 °C, respectively. Whey was then drained from the vat and weighed. The curd was placed manually into a metallic cheese mould with a wet cloth and pressed at 4.2 kg/cm² in a horizontal hydraulic press for ~4-5.5 h at room temperature (~ 20 °C) until the cheese pH dropped to 5.50 ± 0.07 . The pressed cheese was removed from the mould and weighed (± 0.5 g). The final cheese had a cylindrical shape 15 cm in diameter and ~12 cm in height, with an approximate weight of 2 kg. Within this study, the brining and ripening typically following cheesemaking were not carried out. The pH values at rennet addition (i.e. coagulation pH), cutting, stirring, whey draining and after pressing are shown in Table 1. The cheese was subsequently cut into four quarters or subsamples, vacuum packed and stored at 5 °C until physico-chemical and microstructure analyses.

During the cheesemaking process, intermediate samples of curd, curd grains after cutting (fresh curd grains, FCG), curd grains after cooking (stirred curd grains, SCG) and whey were collected. For image analysis of the curd grains, FCG and SCG samples were collected using a round steel mesh sieve 13 cm in diameter with 0.7 mm mesh openings. The sieve was submerged to a depth approximately halfway between the top and bottom of the vat right after the end of either cutting, to collect FCG or after stirring or to collect SCG, the grains were extracted by draining the excess whey. For CLSM analysis, intermediate samples were stored at 5 °C for no more than 3 h. For

compositional analysis, curd grain samples were frozen at -30 °C until analysis. CLSM, analysis to determine fat globule size and rheological analysis of milk samples was carried out on the day of milk collection. Samples were then kept frozen (-30 °C) for compositional analysis.

2.2. Physico-chemical analysis

The fat content was determined by the Babcock method (AOAC 2005). Protein content was obtained by analysing the nitrogen content using the combustion method (ISO/IDF 2002) with a nitrogen determinator (TruMac CNS, LECO Corporation, Saint Joseph, MI, USA); this value was then multiplied by a factor of 6.38 (ISO/IDF 2014). Dry matter (DM) was measured by drying the samples overnight (ISO/IDF 2004; 2010). The pH during the cheesemaking process and in the final product was assessed using a pH meter (S220 SevenCompact, Mettler Toledo, Port Melbourne, Australia).

The total calcium, magnesium and phosphorous content of samples was analysed using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (720ES; Varian Inc., Palo Alto, CA, USA) (Chen et al. 2016). Liquid samples were prepared for the ICP-OES analysis by diluting the sample 100 times in high purity water (resistivity of 18.2 mΩ·cm; Millipore, Billerica, MA, USA) followed by filtering through a 0.22 µm pore size filter (Millipore). Solid samples were dried overnight at 100 °C before ashing at 600 °C for 4 h using a chamber furnace equipped with a temperature controller (FP93, Shimaden, Tokyo, Japan) to remove the organic matter. The ashes were then dissolved in nitric acid (68%) and hydrochloric acid (32%) (1:1; vol/vol; both from Univar, Ingleburn, NSW, Australia) before diluting 1000 times in high purity water and filtering through a 0.22 µm pore size filter for ICP-OES analysis. All physico-chemical analyses were performed in duplicate for each sample.

The particle size distribution of the milk fat globules was measured by Light Scattering (LS) using a particle size analyser (Mastersizer 3000; Malvern Instruments, Malvern, UK), as described previously by Ong et al. (2010). Briefly, sheep milk samples were diluted (1:1; vol/vol) in ethylenediaminetetraacetic acid (EDTA, 50 mM, pH 7) 2 hours before analysis. Samples were

then added to a circulating cell containing a mixture of water and sodium dodecyl sulphate (SDS, 0.05%). The refractive indices of the milk fat and water were set at 1.460 and 1.334, respectively. The volume weighted mean diameter $D[4,3]$ was calculated by the particle analyser software.

2.3. Coagulation properties and texture

The coagulation properties of the milk at renneting were analysed using a Twin Drive Rheometer (MCR702, Anton Paar, North Ryde, Australia), equipped with a CC27 cup and bob accessory. A sample of milk containing rennet was loaded into the cup while the temperature was maintained at 30 °C. The analysis was carried out at an angular frequency of 0.8 Hz and 0.1% shear strain. Changes in storage modulus (G'), loss modulus (G'') and loss factor $\tan(\delta)$ were measured every 10 s for 60 min. The onset of gelation (s) was defined as the time at which the G' first reached 0.05 Pa, the curd firming rate (Pa/s) was obtained from the maximum slope determined over 12 consecutive points and the final curd firmness defined as the G' value at 60 min after rennet addition (Logan et al. 2014). All samples were analysed in duplicate.

The gel strength at the cutting point was analysed using a texture analyser (TA.HD plus; Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell and a cylindrical probe (10 mm diameter). Immediately after rennet addition and blending, two 50 mL samples of milk were taken directly from the cheese vat, poured into a plastic container 42 mm in diameter and 55 mm in height and incubated for 40 min at 30 °C. A test speed of 1 mm/s was used and the penetration was set to 20 mm with a trigger force of 1g. The maximum force obtained during the penetration was used as the measure of gel strength. The measurement was performed in duplicate on the two samples collected for each cheese vat and the mean value of gel strength was used for each vat.

Texture profile analysis was carried out using the texture analyser following a published method with some modifications (Ong et al. 2012). Briefly, cheese samples were taken from the centre of the cheese subsample using a knife and a cylindrical cheese corer to obtain three samples 2.5 cm in diameter and 2.5 cm in height. These were equilibrated at room temperature (~20 °C) for 1 h in a closed container prior to analysis to prevent moisture loss. A 35 mm cylindrical flat probe

was used and a 50% compression test was applied to the cheese using two compression cycles at a constant crosshead speed of 2 mm/s. Three analyses were performed for each cheese treatment.

2.4. Determination of cheese yield

The actual cheese yield (kg of cheese/100 kg of milk) per vat was estimated as the ratio of the total weight of fresh cheese after pressing to the weight of milk used in the vat. Moisture-adjusted cheese yield (Y_{MA}) was calculated using the equation (1) described by Guinee et al. (2006).

$$Y_{MA} = Y_A \times ((100-M_A)/(100-M_R)) \quad (1)$$

where Y_A was the actual cheese yield and M_A and M_R were the actual and reference (45.0%) moisture contents of the cheese, respectively. The theoretical cheese yield (Y_T) was calculated using equation (2) described by Van Slyke (Emmons and Modler 2010):

$$Y_T = (0.93 \times F + (C - 0.1)) \times 1.09 \times 100/(100 - (M)) \quad (2)$$

where F and C were the fat and casein content of the milk respectively and M was the desired cheese moisture content, which was 45% in this instance. Casein content of milk was estimated as the 80 % of the total protein (Park et al. 2007). The yield efficiency (Y_E) was calculated as the percentage of the ratio between Y_{MA} and Y_T (Barbano and Sherbon 1984). The amount of fat, protein and minerals lost in the whey were calculated on the basis of milk weight following methods described by Guinee et al. (2006). Fat loss percentage (FL) was calculated as described in equation 3:

$$\% FL = (W_W \times W_F)/(M_W \times M_F) \times 100 \quad (3)$$

where W_W and M_W were the weight (kg) of the drained whey and milk, respectively and W_F and M_F were the fat content (wt/wt) of whey and milk, respectively. The protein and mineral losses were calculated using similar formulas described in the same reference.

2.5. Microstructure analysis

Milk, gel, curd grain and cheese samples were prepared for microstructural examination using CLSM by applying a method reported previously (Ong et al. 2011). Briefly, solid samples were cut into approximately 5 x 5 x 2 mm in size using a surgical blade and stained at ~ 4 °C using Nile Red (0.1 mg/mL) (Sigma-Aldrich, Castle Hill, Australia) and fast green FCF (0.1 mg/mL) (Sigma-Aldrich). For milk samples, Nile Red (0.02 mg/mL), fast green FCF (0.02 mg/mL), agarose (0.5%) (Sigma-Aldrich) and milk were mixed and the stained sample was then placed on a cavity slide and covered with a coverslip (0.17 mm thick) secured with nail polish (Maybelline, LLC, NY, USA). Microstructural analysis was performed using an inverted confocal microscopy (SP8; Leica Microsystems, Heidelberg, Germany) with excitation wavelengths of 488 nm for fat and 638 nm for protein. Leica confocal software was used to acquire two-dimensional images 512 x 512 pixels in size.

Cheese samples were also analysed by cryo-SEM, as described by Ong et al. (2011). Briefly, cheese samples 5 x 5 x 2 mm were placed on the sample holder and immersed into a liquid nitrogen slush (-210 °C) for 10 s. The frozen samples were then transferred into the cryo preparation chamber, which was maintained at -140 °C under high vacuum conditions ($< 10^{-4}$ Pa). The sample was fractured horizontally using a cooled scalpel blade and etched at -95 °C for 30 min. The temperature was reduced to -140 °C and the cheese sample was coated with 10 mA of sputtered gold/palladium alloy (60/40) using a cold magnetron sputter coater (300V) for 120 s generating a layer ~ 6 nm thick. The sample was then placed onto a nitrogen gas cooled module under vacuum, maintained at -140 °C and observed using a field emission SEM (Quanta; Fei Company, Hillsboro, OR, USA) with a spot size of 2 and acceleration voltage of 15 kV. The detector used for observation was a solid-state backscattered electron detector.

2.6. Image analysis

Three-dimensional (3D) image analysis was carried out for the CLSM micrographs of milk, curd, curd grains and cheese samples. For each sample, a minimum of 30 adjacent planes were acquired with a separation of 0.5 μm and reconstructed for an observation depth of 15 μm . This 3D

reconstruction was carried out using ImageJ software (National Institutes of Health, Bethesda, MD, USA). First, the 2D image layers were stacked together to reconstruct the 3D image. Stacks were equalized using Stack Normalizer plug-in and the channels were split to separate the data for fat and protein. For both stacks, the background was subtracted to enhance image quality. For the fat micrograph stack, a 3D Median filter was also used to smooth the image and improve analysis. The stacks were then thresholded using the IsoData thresholding method (Ridler and Calvard 1978). A Watershed function was applied to separate fat globules that appeared coalesced in the samples. To quantify image features, a 3D Object Counter was used to obtain volume and area surface measurements. This procedure allowed the fat globule diameter and sphericity to be determined together with the network porosity. In addition, two distinct populations of fat globules were identified in samples that were quantified by image analysis. These were the population of globular fat droplets and the population of non-globular or coalesced fat droplets. Globular fat droplets were defined as those droplets with a diameter smaller than the mean diameter of the fat globule in milk. The diameter of these fat droplets ranged from 0.57 to 5.93 μm , which were clearly not disrupted by the handling encountered during the cheesemaking process. Non-globular fat droplets were defined as those droplets with a diameter higher than 10 μm , as these larger areas of fat are most likely pools of free fat (Everett and Auty 2008). Fat droplets with a diameter between 6-10 μm did not fit these descriptions and were excluded from this analysis. In each case, the percentage of fat volume corresponding to globular or non-globular fat was quantified.

2.7. Statistical analysis

The IBM-SPSS Statistics software version 24.0 (New York, NY, USA) was used for statistical analysis. The general linear model of Analysis of Variance (ANOVA) was used to study the effects of the two factors, *CGS* ($l = 2$) and *CT* ($l = 2$) on the composition, microstructure, texture and yield variables measured in this study. The fixed effects model $Y_{ijk} = \mu + GS_i + CGS_j + CT_k + day_l + (CGS*CT)_{jk} + \varepsilon_{ijkl}$ was applied to study simultaneously the effect of both factors and the gel strength (*GS*) was used as a covariable. The relationship between texture and microstructure

parameters of cheese samples were studied using Pearson bivariate correlations. Statistical significance was defined as $P \leq 0.05$.

3. Results and discussion

3.1. Curd grain size and cooking temperature

Cheesemaking experiments were performed where the curd grain size (CGS) and cooking temperature (CT) were varied to assess the impact of these parameters. The area of the large curd grains assessed by image analysis was significantly ($P \leq 0.05$) greater than the area of the small curd grains (Table 1). As expected, this difference was significant for grains assessed both after cutting (FCG) and after cooking (SCG), indicating the effectiveness of the manual wire mesh technique and number of cuts used to alter curd grain size. Direct comparisons between the curd grain size produced here and in previous studies is made difficult by the use of different measures of curd sizes, including previous indirect measures of knife spacing and curd weight after sieving. Nevertheless the method used here provides a good span of sizes (Aldalur et al. 2019; Johnston et al. 1991; Whitehead and Harkness 1954).

Table 1. Technical parameters controlled during the experimental cheesemaking design assessing the effect of curd grain size (CGS) and cooking temperature (CT). Two replicates ($n = 2$) were carried out for each of the four treatments and the data presented is the mean $\pm$ standard deviation.

Factors		FCG area (mm ²)	SCG area (mm ²)	Milk pH	Coagulation pH	Cutting pH	Cooking pH	Whey draining pH	Cheese pH after pressing
CT	CGS								
36°C	Big	52.53 ^a $\pm$ 5.30	7.48 ^a $\pm$ 0.66	6.54 $\pm$ 0.01	6.53 $\pm$ 0.01	6.51 $\pm$ 0.01	6.49 $\pm$ 0.01	6.41 $\pm$ 0.04	5.46 $\pm$ 0.01
45°C	Big	45.06 ^a $\pm$ 8.35	6.46 ^a $\pm$ 1.03	6.53 $\pm$ 0.04	6.52 $\pm$ 0.01	6.50 $\pm$ 0.01	6.48 $\pm$ 0.18	6.35 $\pm$ 0.04	5.56 $\pm$ 0.11
36°C	Small	14.42 ^b $\pm$ 3.21	4.54 ^b $\pm$ 1.41	6.56 $\pm$ 0.04	6.52 $\pm$ 0.00	6.51 $\pm$ 0.04	6.38 $\pm$ 0.04	6.41 $\pm$ 0.04	5.41 $\pm$ 0.01
45°C	Small	12.69 ^b $\pm$ 4.44	3.63 ^b $\pm$ 1.04	6.54 $\pm$ 0.04	6.52 $\pm$ 0.01	6.50 $\pm$ 0.03	6.40 $\pm$ 0.02	6.33 $\pm$ 0.03	5.48 $\pm$ 0.01

FCG, curd grain after cutting; SCG, curd grain after cooking.

^{a,b} Different superscripts in the same column indicate statistically significant differences ($P \leq 0.05$).

3.2. Physico-chemical composition and cheese yield

Table 2 shows the physico-chemical parameters measured for the curd grains during cooking (SCG) and cheese from the trials described in Table 1. The significant ($P \leq 0.05$) differences observed among treatments are mainly due to an increase in dry matter content of the curd grains

and cheese samples when subjected to higher cooking temperatures, as temperature greatly increases the rate of curd syneresis (Dejmek and Walstra 2004). Regarding the effect of curd grain size, measured with varied indices, previous authors have reported a higher moisture content in cheese made from bigger curd grains (Johnston et al. 1991; Whitehead and Harkness 1954). In other studies, however, the effect of curd grain size was reported have no impact on the dry matter content of the cheese (Everard et al. 2008). In this study, no significant difference ($P > 0.05$) was observed for the dry matter of the cheese samples using both big and small curd grain sizes, consistent with the results obtained by Everard et al. (2008).

The fat in dry matter content in the cheese samples was significantly ($P \leq 0.05$) affected by the curd grain size, indicating that the bigger particles retained more fat (Table 2). Similarly, the fat loss was also higher when the curd grain was smaller (Table 3). Reducing the curd grain size increases the surface area to volume ratio and the loss of fat globules to the whey is consequently enhanced (Banks 2007). This process could be visualised using confocal microscopy, as shown in [Online Resource 2](#), where the fat globules near the edge of the curd grain are observed readily entering the surrounding solution after the protein matrix of the curd is cut and this process is likely responsible for the differences in fat loss observed with different sized curd. No visual differences were observed, however, between the edges of the curd with difference grain sizes.

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Table 2. General composition (mean $\pm$ standard deviation) of curd grains after cooking and cheese samples from the experimental cheesemaking design combining the factors curd grain size (*CGS*) and cooking temperature (*CT*).

	Big <i>CGS</i>		Small <i>CGS</i>		Significance		
	36 °C	45 °C	36 °C	45 °C	<i>CGS</i>	<i>CT</i>	<i>CGS*CT</i>
Curd grains after cooking							
Fat, g/100 g	17.1 $\pm$ 1.6	24.0 $\pm$ 0.7	17.5 $\pm$ 1.1	22.5 $\pm$ 0.4	ns	**	ns
Fat in dry matter, g/100 g	42.0 $\pm$ 1.7	48.0 $\pm$ 2.7	44.9 $\pm$ 1.5	47.4 $\pm$ 1.3	ns	ns	ns
Protein, g/100 g	16.10 $\pm$ 1.97	20.50 $\pm$ 1.85	14.24 $\pm$ 0.24	20.41 $\pm$ 0.18	ns	**	ns
Protein in dry matter, g/100 g	39.45 $\pm$ 2.72	40.92 $\pm$ 2.61	36.55 $\pm$ 1.61	43.02 $\pm$ 0.89	ns	ns	ns
Dry Matter, g/100 g	40.74 $\pm$ 2.17	50.07 $\pm$ 1.31	38.97 $\pm$ 1.07	47.44 $\pm$ 0.57	*	***	ns
Calcium, mg/100 g	661 $\pm$ 50	842 $\pm$ 11	623 $\pm$ 55	874 $\pm$ 35	ns	**	ns
Phosphorous, mg/100 g	426 $\pm$ 27	527 $\pm$ 53	399 $\pm$ 32	545 $\pm$ 23	ns	**	ns
Magnesium, mg/100 g	42 $\pm$ 3	52 $\pm$ 1	40 $\pm$ 4	53 $\pm$ 2	ns	**	ns
Cheese							
Fat, g/100 g	28.5 $\pm$ 0.0	30.0 $\pm$ 0.7	27.9 $\pm$ 0.2	29.0 $\pm$ 0.0	ns	*	ns
Fat in dry matter, g/100 g	52.8 $\pm$ 0.4	52.6 $\pm$ 0.8	51.4 $\pm$ 0.6	50.1 $\pm$ 0.3	*	ns	ns
Protein, g/100 g	21.90 $\pm$ 0.07	24.00 $\pm$ 0.26	23.12 $\pm$ 0.33	24.27 $\pm$ 0.16	*	**	*
Protein in dry matter, g/100 g	40.52 $\pm$ 0.44	42.10 $\pm$ 0.82	42.66 $\pm$ 1.36	41.91 $\pm$ 0.54	ns	ns	ns
Dry Matter, g/100 g	54.04 $\pm$ 0.40	57.02 $\pm$ 0.49	54.22 $\pm$ 0.96	57.91 $\pm$ 0.36	ns	**	ns
Calcium, mg/100 g	986 $\pm$ 28	1062 $\pm$ 34	990 $\pm$ 1	1104 $\pm$ 21	ns	**	ns
Phosphorous, mg/100 g	596 $\pm$ 4	648 $\pm$ 21	610 $\pm$ 2	675 $\pm$ 8	ns	**	ns
Magnesium, mg/100 g	56 $\pm$ 1	61 $\pm$ 2	59 $\pm$ 1	65 $\pm$ 6	*	**	ns

*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant where $P > 0.05$.

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The fat content in the whey obtained in this study was also higher than in previous studies (0.79 ± 0.13 vs $0.30\text{--}0.48$ g/100 g in previous studies). The difference in fat content could be due to several factors such as a higher milk fat concentration (6.14 ± 0.17 vs $2.9\text{--}5.1$ g/100g in previous studies), the use of ovine milk rather than bovine milk, a higher stirring speed (40 rpm in a 20 L capacity vat vs 10 to 22 rpm in 11 L vats or 4 to 7 rpm in over 20,000 L vats), differences in the cheesemaking processing conditions, or the method of manual cutting and use of nylon wires for cutting the curd (Everard et al. 2008; Johnston et al. 1991; Johnston et al. 1998). Such wires can break the gel, producing curd grains with coarse surfaces causing a high number of mini curd particles, increasing fat losses in the whey (Kammerlehner 2009). There was some evidence of such small particles in all treatments here observed by CLSM, where the curd junction could be seen to surround the curd particle, where particles were typically $\sim 200\text{--}400$ μm in diameter (Online Resource 3). Similar consistent observations were made by cryo-SEM.

The total number of revolutions has also been shown previously to influence fat losses. Several authors have shown using cow milk that when the total revolutions of the cutting process are insufficient, the subsequent stirring leads to the shattering of curd particles and increases the amount of fat in the whey, whereas fat losses can be contained when the number of revolutions during cutting is increased (Everard et al. 2008; Johnston et al. 1991; Johnston et al. 1998). In a recent study using raw sheep milk, a higher number of stirring revolutions also caused higher fat losses in the whey (Aldalur et al. 2019). In the present study, the number of total revolutions (or equivalent number of cuts) appears higher than the above cited studies, suggesting that greater curd cutting and stirring has the potential to also be damaging.

The protein content, unlike the fat, appeared less affected by the curd grain size and cooking temperature. While differences were observed for selected conditions (a smaller *CGS* and higher *CT* and the interaction term *CGS*CT* indicated higher protein) these were not significant once corrected for moisture and compared on the basis of protein in dry matter.

The cooking temperature was the main factor affecting the mineral composition of the cheese and the curd grain size had no significant effect (Table 2). The calcium, phosphorous and magnesium contents were significantly ($P \leq 0.05$) higher in cheeses cooked at the higher temperature of 45 °C. Consequently, the calcium in the whey was also significantly ($P \leq 0.05$) higher when the cooking temperature was lower at 36 °C (Table 3). Interestingly, these differences occurred despite the similar pH at whey draining (Table 1); another factor known to influence calcium loss during cheesemaking (Johnson and Lucey 2006).

Table 3. Actual yield, moisture-adjusted yield, yield efficiency and component losses (mean $\pm$ standard deviation) obtained from the experiments assessing the effect of curd grain size (*CGS*) and cooking temperature (*CT*).

	Big <i>CGS</i>		Small <i>CGS</i>		Significance		
	36 °C	45 °C	36 °C	45 °C	<i>CGS</i>	<i>CT</i>	<i>CGS*CT</i>
Fat loss (%)	8.60 $\pm$ 0.78	9.11 $\pm$ 1.46	10.81 $\pm$ 0.23	11.35 $\pm$ 0.06	*	ns	ns
Protein loss (%)	22.39 $\pm$ 0.66	23.82 $\pm$ 4.44	22.91 $\pm$ 1.01	22.76 $\pm$ 0.50	ns	ns	ns
Calcium loss (%)	23.67 $\pm$ 4.87	19.83 $\pm$ 1.63	22.17 $\pm$ 3.37	20.05 $\pm$ 0.86	ns	*	ns
Phosphorous loss (%)	36.98 $\pm$ 1.71	36.71 $\pm$ 2.07	37.23 $\pm$ 2.02	36.87 $\pm$ 3.38	ns	ns	ns
Magnesium loss (%)	60.20 $\pm$ 0.48	60.90 $\pm$ 1.17	61.14 $\pm$ 1.52	62.50 $\pm$ 0.59	ns	ns	ns
Actual yield (kg/100 kg)	19.21 $\pm$ 0.74	18.43 $\pm$ 0.37	19.16 $\pm$ 0.24	17.96 $\pm$ 0.48	**	***	**
Moisture-adjusted yield (kg/100 kg)	18.87 $\pm$ 0.58	19.10 $\pm$ 0.22	18.89 $\pm$ 0.09	18.91 $\pm$ 0.38	ns	ns	ns
Yield efficiency (%)	97.09 $\pm$ 0.63	98.29 $\pm$ 2.57	97.23 $\pm$ 4.13	97.28 $\pm$ 1.67	ns	ns	ns

*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant where $P > 0.05$.

The actual yield (Y_A) was statistically affected by both, the curd grain size and cooking temperature ($P \leq 0.05$; Table 3). The yield increased when the curd grain size was bigger and the cooking temperature was lower. The interaction term *CGS*CT* was also statistically significant ($P \leq 0.05$), indicating that both factors had an impact on the cheese yield. These differences were mainly associated with the dry matter content of the cheese samples, however, as once corrected for moisture the moisture adjusted yield (Y_{MA}) was not significantly different for these treatments ($P > 0.05$; Table 3). The relationship between cheese yield and dry matter has been well documented by other authors (Lucey and Kelly 1994; Whitehead and Harkness 1954). The moisture-adjusted yield was expected to be different due to an increased amount of fat loss in the whey observed for small curd grains (Table 3), however, this loss appears to be insignificant compared to the weight of the cheese samples. Consequently, we consider the amount of fat loss

a better indicator of cheese yield when observing small differences between temperatures and curd grain sizes in cheesemaking. Other studies have similarly observed fat loss to be a more sensitive measure when operating a small pilot scale (Ong et al. 2015). The high fat losses in this study were also reflected in the low cheese yield efficiency (Table 3), as the average values for Y_{MA} were below the calculated theoretical cheese yield (19.44 ± 0.55 kg/100 kg). This again may be due to several factors including the composition of milk, the method of cutting, number of total revolutions and pilot scale of the study.

3.3. *Microstructure of curd and cheese*

Changes in the microstructure of dairy matrices were followed by confocal microscopy during the cheesemaking process to visualise the effects of *CGS* and *CT*. The rennet-induced gel had a compact structure for all cheesemaking treatments, with a mean porosity of 0.36 ± 0.08 . This low porosity was expected, due to the high fat and protein content of raw sheep milk ([Online Resource 4](#); [Online Resource 1](#)). In comparison, Ong et al. (2010) reported 0.49 ± 0.02 porosity for gels formed from raw cow's milk where the fat and protein content were lower with a concentration of 4.45 g/100 g and 3.53 g/100 g, respectively.

Fat droplets were numerous in rennet-induced gel samples. The mean diameter determined by image analysis was 7.25 ± 0.74 μ m, which was not significantly ($P > 0.05$) different to the mean diameter of milk samples determined using the same method (5.93 ± 0.48 μ m). In agreement with other studies, the fat droplets appear to act as inner fillers, as the casein network was formed around the fat globules with no visible interaction between the protein and fat components (Lopez et al. 2007; Michalski et al. 2002). Some fat globules formed aggregates or coalesced droplets but generally the globular structure of fat was retained throughout this stage of the cheesemaking process ([Online Resource 4](#)).

The protein matrix contracted during the cheesemaking process (Fig. 1), as the dry matter content significantly ($P \leq 0.05$) increased due to the syneresis (Table 2). The size of the fat globules was similar to within the gel after cutting (7.58 ± 0.86 μ m) but these droplets began to change in shape

Fig. 1: Confocal laser scanning microscopy images of (a to d) curd grains after cutting, (e to h) curd grains after cooking and (i to l) cheese, of the combination of factors curd grain size (*CGS*) and cooking temperature (*CT*) during the cheesemaking process. Cheesemaking treatments were coded as: big *CGS* and 36 °C *CT* (a, e, i); big *CGS* and 45 °C *CT* (b, f, j); small *CGS* and 36 °C *CT* (c, g, k); small *CGS* and 45 °C *CT* (d, h, l). Images were obtained using a 63x objective lens and digitally magnified 2x. Fat appears red and protein appears green in these images. The scale bars are 25 μm in length. AFG = aggregates of fat globule, CFG = coalesced fat globule, NGF = non-globular fat.

following cooking and pressing (Fig. 1, e to l). Some fat globules fused together during cooking generating coalesced fat globules, whilst some non-globular or free fat could also be observed (Fig. 1, f and j). After pressing, the fat volume significantly increased ($P \leq 0.05$) and the structure of nearly all fat globules was disrupted (Fig. 1, i to l).

The cooking temperature was found to significantly affect the physical properties of the cheese measured by 3D image analysis of the CLSM micrographs. When the cooking temperature increased to 45 °C, the volume of non-globular fat increased significantly ($P \leq 0.05$) in cheese samples (Table 4). This was observed by both CLSM (Fig. 1; j and l) and cryo-SEM imaging

(Fig. 2; b, d, f and h). Most of the fat droplets in these micrographs appeared to have lost their globular structure and the fat appeared as coalesced non-globular fat. Conversely, the volume contributed by globular fat significantly decreased ($P \leq 0.05$) when the stirring temperature was 45 °C; few globular fat droplets were also observed when the curd grain size was smaller (Table 4), indicating that these smaller fat globules fused to form larger coalesced fat globules and free fat (Lopez et al. 2006). Additionally, a smaller curd grain size can lead to a higher compaction of the curd during pressing (Walstra et al. 2006); this could cause further disruption of the fat droplets, resulting in more non-globular fat as detected by image analysis here.

Fig. 2: Cryo-scanning electron microscopy images of cheese of the combination of factors curd grain size (CGS) and cooking temperature (CT) during the cheesemaking. Cheesemaking treatments were coded as: big CGS and 36 °C CT (a, e); big CGS and 45 °C CT (b, f); small CGS and 36 °C CT (c, g); small CGS and 45 °C CT (d, h). Representative images were presented at 4000x magnification (a to d) and 8000x magnification (e to h). The scale bars are 20 µm (A) and 10 µm (B) in length. AFG = aggregates of fat globule, CFG = coalesced fat globule, NGF = non-globular fat.

The effect of temperature on the cheese matrix structure could be also observed by analysing the porosity (Table 4), as the curd grains heated at 36 °C or 45 °C had porosities of 0.17 ± 0.03 and 0.10 ± 0.03 , respectively, with the higher temperature reducing network porosity ($P \leq 0.05$). This effect of temperature could also be observed visually by cryo SEM, as gaps could be seen at the interface between the fat and protein network of the cheese cooked at 36 °C with big curd granules (Fig. 2e).

The surface of the curd grains contained little fat content once the curd was pressed and the curd junctions were evident as thick protein strands within the cheese microstructure ([Online Resource 5](#)). Several calcium phosphate crystalline inclusions were also observed (white arrows in [Online Resource 5](#)) and these generally occurred along the curd junction in all four treatments, as calcium in dry matter basis was not significantly different ($P > 0.05$) in these cheese samples ([Online Resource 6](#)). This structural arrangement has also been reported in other cheeses (Brooker et al. 1975; D’Incecco et al. 2016; Ong et al. 2015), with the number of inclusions increasing after calcium addition. D’Incecco et al. (2016) attributed the increased number of crystalline inclusions within the curd junction to be a result of incomplete aggregation of the curd granules and the higher volume of whey initially present within the curd junctions. The resulting dense spherulite crystals likely arise from non-crystalline branching from a nuclei core. These nuclei could form from the supersaturated concentration of solutes originating in the whey; a condition that is known to lead to spherulite formation in gels (Shtukenberg et al. 2011), such as the protein network observed here.

3.4. Cheese texture

Cooking temperature was the main factor found to affect the textural parameters of the cheese (Table 4). A higher cooking temperature (45 °C) produced cheeses that were significantly ($P \leq 0.05$) harder and chewier than those produced at lower temperatures (36 °C). Cheese texture is known to be affected by many factors such as the composition, microstructure, fat droplet size and distribution, casein matrix bond strength and interactions between fat globules and the protein matrix (Everett and Auty 2008; Hickey et al. 2015). Here, the differences observed with higher temperature were likely due to the higher fat, protein, mineral and dry matter content in the cheeses cooked at 45 °C (Table 2). Conversely, the curd granule size was not found to significantly impact ($P > 0.05$) on the cheese textural parameters.

Table 4. Physical properties obtained by 3D image analysis and texture parameters (mean $\pm$ standard deviation) of cheese samples from experiments assessing the effect of curd grain size (*CGS*) and cooking temperature (*CT*).

Physical properties	Big <i>CGS</i>		Small <i>CGS</i>		Significance		
	36°C	45°C	36°C	45°C	<i>CGS</i>	<i>CT</i>	<i>CGS*CT</i>
Fat sphericity	0.60 $\pm$ 0.00	0.58 $\pm$ 0.02	0.61 $\pm$ 0.04	0.56 $\pm$ 0.05	ns	ns	ns
Porosity	0.18 $\pm$ 0.05	0.11 $\pm$ 0.00	0.17 $\pm$ 0.01	0.09 $\pm$ 0.05	ns	*	ns
Volume of globular fat (%)	12 $\pm$ 1	4 $\pm$ 2	7 $\pm$ 2	3 $\pm$ 2	*	**	ns
Volume of non-globular fat (%)	69 $\pm$ 6	92 $\pm$ 0	78 $\pm$ 11	96 $\pm$ 2	ns	*	ns
Cheese texture							
Hardness (g)	5389 $\pm$ 518	7796 $\pm$ 649	6653 $\pm$ 1307	8414 $\pm$ 296	ns	*	ns
Chewiness (g)	2989 $\pm$ 560	4834 $\pm$ 308	3845 $\pm$ 1086	5284 $\pm$ 403	ns	*	ns
Springiness (s/s)	0.846 $\pm$ 0.002	0.858 $\pm$ 0.004	0.858 $\pm$ 0.007	0.865 $\pm$ 0.011	ns	ns	ns
Cohesiveness (g·s/g·s)	0.654 $\pm$ 0.056	0.716 $\pm$ 0.004	0.669 $\pm$ 0.065	0.726 $\pm$ 0.022	ns	ns	ns
Resilience (g·s/g·s)	0.338 $\pm$ 0.029	0.373 $\pm$ 0.000	0.360 $\pm$ 0.026	0.391 $\pm$ 0.020	ns	ns	ns

*, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant where $P > 0.05$.

In addition, the textural parameters measured were correlated with the microstructural properties of cheese quantified by 3D image analysis. The cheeses in which a higher volume of globular fat was retained (i.e. cheese cooked at 36 °C and or using a larger curd grain size) had lower hardness, chewiness, cohesiveness and resilience ($r > |0.7|$, $P \leq 0.05$), while those cheeses with a higher volume of non-globular fat (i.e. cheese cooked at 45 °C) had higher values in textural parameters ($r > 0.7$, $P \leq 0.05$; i.e. were harder, chewier and had greater cohesiveness and resilience). Fat is known to act as an inert filler when the globular structure is maintained, retaining a softer cheese texture (Everett and Auty 2008), as was observed here. Cheese springiness also showed an interesting negative correlation with the porosity of the protein network ($r = |0.7|$; $P \leq 0.05$), with more porous cheeses demonstrating a reduced ability to spring back on compression; this may be due to the higher number of cavities and network imperfections observed in the most porous cheese structures (e.g. Fig. 2e).

4. Conclusions

This study indicates that curd grain size and cooking temperature affect the structural and textural characteristics of cheese made using raw sheep milk, in addition to affecting the yield and composition of the whey generated during the cheesemaking process. The cooking temperature

had a major effect on cheese composition, increasing the dry matter content of the cheese due to the enhanced syneresis during cooking. A higher cooking temperature significantly altered the texture of the cheese, increasing hardness and chewiness. Additionally, changes in the microstructure were observed with different cooking temperatures, with high temperatures increasing non-globular fat and reducing the porosity of the cheese protein matrix. These changes of the structural arrangement of fat were correlated with the textural properties of cheese. A small curd grain size was the main factor responsible for increased fat losses in the whey. Consequently, the actual cheese yield was impaired when the cutting process was more intense and cooking temperatures higher. These results contribute with new data on the interactions between curd grain size and cooking conditions on the microstructural, textural and chemical properties of cheese. These insights are useful for the dairy sector in order to improve and control the cheesemaking process and the composition of the whey generated for ulterior processing.

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Online Resource 1. Chemical composition, rheological properties and fat globule size (mean $\pm$ standard deviation) of the raw ewe's milk used for experimental cheesemaking. Bulk milk was collected from a commercial farm on two consecutive weeks (n = 2).

Fat, g/100 g	6.14 $\pm$ 0.17
Protein, g/100 g	5.25 $\pm$ 0.14
Dry matter, g/100 g	17.91 $\pm$ 0.38
Calcium, mg/100 mL	158 $\pm$ 8
Magnesium, mg/100 mL	16 $\pm$ 1
Phosphorous, mg/100 mL	134 $\pm$ 14
Fat globule size D[4,3], μm^1	4.29 $\pm$ 0.12
Onset gelation, s	882 $\pm$ 55
Curd firming rate, Pa/s	0.16 $\pm$ 0.03
Final curd firmness, Pa	320 $\pm$ 42

¹Measured by light scattering.

Online Resource 2: Confocal laser scanning microscopy micrograph in 2-D (left) and 3-D reconstruction (right) showing the release of fat globules from the curd grain (small size) after cooking (45 °C). Images were acquired using a 63x objective lens. Fat appears red and protein appears green in this image. The scale bar is 50 μm in length.

Online Resource 3: Confocal laser scanning microscopy images showing mini curd particles and curd junctions in cheese samples. Cheesemaking treatments were coded as: big CGS and 36 °C CT (a); big CGS and 45 °C CT (b); small CGS and 36 °C CT (c); small CGS and 45 °C CT (d). Fat appears red and protein appears green. The scale are 250 μm in length. The white outline indicates a mini curd particle completely surrounded by curd junctions.

Online Resource 4: Confocal laser scanning microscopy image of rennet-induced gel obtained using a 63x objective lens. Fat appears red and protein appears green in this image. The scale bar is 50 μm in length. AFG = aggregates of fat globule, CFG = coalesced fat globule.

Online Resource 5: Confocal laser scanning microscopy (left) and cryo-scanning electron microscopy (right) images showing curd grain junctions in cheese samples. For CLSM image, fat appears red and protein appears green. Scale bars of both images are 50 μm in length. White arrows indicate the presence of crystalline inclusions of calcium phosphate. CJ = curd junction.

Online Resource 6: Cryo-scanning electron microscopy images showing curd grain junctions and crystalline inclusions of calcium phosphate in cheese samples. Cheesemaking treatments were coded as: big CGS and 36 °C CT (a); big CGS and 45 °C CT (b); small CGS and 36 °C CT (c); small CGS and 45 °C CT (d). Scale bars of images 1, 2 and 4 are 300 μm and of image 3 is 200 μm in length.