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# Rennet gelation properties of milk: Influence of natural variation in milk fat globule size and casein micelle size

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ABSTRACT

Seasonal and farming practises can influence milk composition and functionality. An understanding of changes in milk fat globule (MFG) and casein micelle (CM) size may help to guide the selection of milk on the basis of MFG or CM size for manufacturing of different products and product quality. Milk was obtained from cows known to produce predominantly large or small MFG and CM. The rennet gelation properties of this milk were investigated by measuring the rheological properties during gel development. The structure of the CM and MFG network within the rennet gel were characterised by a series of microscopy techniques. Milk with small CM produced firmer curds, and the combination of large MFG (4.49–5.38 μm) with small CM (164–168 nm) produced the firmest curd of any of the combinations tested. MFG size can influence rennet gel firmness, an effect that is dependent upon the pore structure of the CM network.



## 1. Introduction

Milk fat globules (MFG) and casein micelles (CM) are integral structural components of milk responsible for the texture and flavour development of dairy products (Goudédranche, Fauquant, & Maubois, 2000; Lucey, Johnson, & Horne, 2003; Michalski et al., 2006; O'Mahony, Auty, & McSweeney, 2005). Individual caseins form in the endoplasmic reticulum (ER) of the bovine mammary gland and assemble into micellar form, incorporating calcium phosphate nano-particles in the Golgi complex prior to secretion by exocytosis, and pass into the alveolar lumen along with lactose and some minerals (Mather, 2011). The MFG are secreted via a different pathway, where globules encased in a single layer of ER membranous proteins and polar lipids are progressively enveloped by the bi-layer of the apical plasma membrane, creating the tri-layer MFG membrane structure (Heid & Keenan, 2005). Initially, small lipid droplets are formed within the ER and released into the cytosol. Droplets may fuse together and coalesce to form larger entities during transportation toward the apical plasma membrane (Heid & Keenan, 2005). MFG range in size between 0.5 and 4 μm prior to secretion (Deeney, Valivullah, Dapper, Dylewski, & Keenan, 1985). Inherent differences in both CM and MFG size exist within the milk of individual cows (Bijl, de Vries, van Valenberg, Huppertz, & Van Hooijdonk, 2014; Glantz et al., 2010; Logan, Auldist, Greenwood, & Day, 2014a; Williams, Auldist, Greenwood, & Puvanenrhiran, 2014), and it has been shown that MFG size can be influenced further by diet (Couvreur, Hurtaud, Marnet, Faverdin, & Peyraud, 2007), stage of lactation (Mesilati-Stahy & Argov-Argaman, 2014) and other environmental factors, such as milking frequency (Wiking, Nielsen, Båvius, Edvardsson, & Svennersten-Sjaunja, 2006) or coalescence of MFG into larger globules post-secretion (Evers, 2004).

Mechanical techniques to separate milk on the basis of MFG or CM size have been investigated (Lopez et al., 2011, Logan et al., 2014b; Niki et al., 1994; O'Mahony et al., 2005; Timmen & Patton, 1988; Walsh et al., 1998); however, processing functionality of raw milk with naturally occurring differences in MFG and/or CM size has not been investigated. This may provide opportunities for milk to be selected on-farm to improve processing efficiency, product quality and development of novel dairy products.

Differences in milk composition and structure, including MFG and CM size, can influence functionality. Improved texture, flavour and sensory characteristics have been shown in Cheddar (O'Mahony et al., 2005) and Emmental (Michalski et al., 2007) cheeses made from milk with small MFG. In addition, cheese made from milk with small CM size resulted in higher yield and better

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curd-forming properties (Walsh et al., 1998). Our previous work has shown that milk with small CM size form firmer curds with a faster curd firming rate than milk with large CM (Logan et al., 2014b). Moreover, the curds produced from milk with larger MFG, obtained via centrifugation of milk into differently sized fractions, were firmer when in combination with small CM skim compared with smaller MFG in combination with small CM skim (Logan et al., 2014b). The question is whether such properties displayed by milk fractionated through centrifugation and recombined with skim to create milk of a particular MFG size could also be achieved through natural selection of milk with varied MFG size on-farm. In this work, we aimed to investigate the rennet gelation properties of milk naturally containing either small or large MFG, in combination with small and large CM, eliminating the need for further fractionation steps.

The rheological examination of rennet gelation properties, coupled with structural characterisation, provides further understanding of the relative contribution of MFG and CM selected naturally through cow selection on the processing functionality of milk. This knowledge provides new insights for on-farm milk selection for cheese production.

## 2. Methods and materials

### 2.1. Milk preparation

Three separate rennet experiments (E-1, E-2 and E-3) were performed on pooled milk collected at three different occasions from cows known to produce CM and MFG of a particular size. Milk was sourced from the herd of dairy cattle maintained at the Department of Environment and Primary Industry (DEPI) research farm in Ellinbank, Australia. Cows were selected on the basis of previous analysis for CM (Williams et al., 2014) and MFG (Logan et al., 2014a) size and known to produce either small MFG and small CM (SMFG-SCM), small MFG and large CM (SMFG-LCM), large MFG and small CM (LMFG-SCM), or large MFG and large CM (LMFG-LCM) in combination.

At the time of each experiment, fresh milk was sourced from 8 individual cows. Milk was pooled from a consecutive evening and morning, and stored at 4 °C immediately after milking and maintained at 4 °C throughout the experiment prior to analysis. Milk from paired cows of similar CM and MFG size was pooled together into bulk samples to minimise variables that may have been introduced from individual cow-to-cow variation. The control was produced by mixing equal portions of these samples, and all samples were standardised on the basis of fat concentration using skim milk acquired via centrifugation (2500 × *g* for 30 min at 4 °C). All analyses were completed within 2 days of milk collection. E-1 was conducted in October 2013, near the beginning of lactation. E-2 and E-3 were conducted in January and February 2014, during mid-lactation. Each milk was standardised once in bulk, and replicate analysis was performed from the standardised milk. The replication in sampling, i.e., the size range of MFG and CM in combination, was obtained by performing the experiment in triplicate (E-1, E-2 and E-3).

Routine somatic cell count analysis of the 8 individual cows indicates the bulk milk cell count (BMCC) of milk at the time of each experiment was low (i.e., $1.6 \times 10^5$ cells mL$^{-1}$ or less, average of $8 \times 10^4$ cells mL$^{-1}$), with the exception of one cow at E-1 that contributed milk to the LMFG-LCM pooling (BMCC ~$1 \times 10^6$ cells mL$^{-1}$) and a second cow that contributed milk to the SMFG-SCM pooling (BMCC ~$3 \times 10^5$ cells mL$^{-1}$) at E-2. All milk was normal in appearance, and the elevated BMCC for the two milk samples collected at E-1 and E-2 did not seem to influence the trends across the three experiments.

### 2.2. Milk characterisation

Fat and protein concentration (%, w/v), the volume weighted mean MFG diameter ($d_{4,3}$ μm) and the average CM diameter (nm) were measured as described earlier by Logan et al. (2014b). In brief, fat and protein concentrations were measured using a Lactoscope FTIR 20 (Delta Instruments, Melbourne, Australia), and the MFG and CM size were determined using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) and a Zetasizer Nano ZS (Malvern Instruments), respectively. The milk samples were brought to room temperature (~20 °C) prior to the size measurements. The MFG size distribution and proximate analysis was carried out in duplicate, while CM size distribution was measured in triplicate.

### 2.3. Determination of renneting behaviour

Fermentation-derived chymosin rennet (Chymax plus, Chr. Hansen, Bayswater, Victoria, Australia) was used to prepare a rennet stock solution (200 IMCU mL$^{-1}$), stored at 4 °C. Fresh rennet solution (4 IMCU mL$^{-1}$) was prepared daily by diluting the stock (0.2 mL) with deionised water (9.8 mL) and stored on ice. The milk sample (25 mL) was first warmed to room temperature (~20 °C) and the pH was adjusted to 6.55. The milk was then heated to 32 °C in a water bath and held for 15 min before the rennet solution (0.25 mL) was added with stirring using a magnetic stirrer (300 rpm for 15 s). The rennet gelation properties were measured using an Anton Paar-Physica rheometer (MCR 300, Anton Paar Physica, Physica Meßtechnik GmbH, Stuttgart, Germany) fitted with a CC27 cup and bob geometry, according to the method of Auldist, Mullins, O'Brien, and Guinee (2001). An aliquot (~17 mL) of the treated milk was carefully poured into the preheated cup and maintained at 32 °C. A constant frequency of 1 Hz and a low amplitude shear strain of 0.025 was applied to the sample, so that the measurement was within the linear viscoelastic region (Guinee et al., 1997). The rennet gelation properties of milk were followed by monitoring the storage ($G'$, Pa) and the loss modulus ($G''$, Pa). Data were collected at 15 s intervals over 60 min and the final value of $G'$ (corresponding to the final curd firmness), $G''$ and tan $\delta$ (the loss tangent) were recorded after 60 min from rennet addition. The onset of gelation (s) was defined as the time at which the $G'$ first reached 0.05 Pa and the curd firming rate (mPa s$^{-1}$) was obtained as the maximum slope value over 12 consecutive points. All samples were analysed in duplicate.

### 2.4. Visualisation of rennet gels using confocal laser scanning microscopy

Rennet gels were prepared by adding fluorescein isothiocyanate solution (0.1% in dimethylsulphoxide, 50 μL), Nile Red solution (0.05% in dimethylsulphoxide, 20 μL) and fresh rennet solution (50 μL) to cold milk (5 mL). For visualisation using confocal laser scanning microscopy (CLSM), the milk was then injected into two purpose-built sample holders and placed with the built-in cover-slip facing sideways in a 32 °C water bath for 60 min to form rennet gels that would have the final curd firmness equivalent to gels measured in the rheometer. The sideway positioning of the sample in the water bath during curd formation enabled visualisation of the top, middle and bottom sections of the established curd. In this way, occurrences of MFG creaming and variation in rennet gel network density within a sample could be identified. When removed from the water bath, the sample holder was carefully rotated 90° for the cover-slip to face upwards. Images were captured using a Leica TCS SP5 upright confocal microscope (Leica Microsystems, Mannheim, Germany) at room temperature using a