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FOOD TECHNOLOGY

Association of casein micelle size and enzymatic curd strength and dry matter curd yield

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ABSTRACT: *The aim of the present study was to explore the association between milk protein content and casein micelle size and to examine the effects of casein micelle size on enzymatic curd strength and dry matter curd yield using reduced laboratory-scale cheese production. In this research, 140 bulk tank milk samples were collected at dairy farms. The traits were analyzed using two linear models, including only fixed effects. Smaller micelles were associated with higher κ-casein and lower α_s-casein contents. The casein micellar size (in the absence of the α_s-casein and κ-casein effects) did not affect the enzymatic curd strength; however, smaller casein micelles combined with higher fat, lactose, casein and κ-casein contents exhibited a favorable effect on the dry matter curd yield. Overall, results of the present study provide new insights into the importance of casein micelle size for optimizing cheese production.*
Key words: *caseins, cheese, whey proteins.*

Associação do tamanho das micelas de caseína com a força e produção de massa da coalhada

RESUMO: *Este trabalho foi desenvolvido com o objetivo de investigar a associação da composição proteica do leite com o tamanho das micelas de caseína, e o efeito do TMCN sobre a firmeza do coágulo enzimático e da produção de massa seca do coágulo produzido em escala reduzida. Foram coletadas 140 amostras de leite cru de diferentes fazendas. Os dados foram analisados usando dois modelos lineares, incluindo somente efeitos fixos. Menores micelas de caseína foram associadas com maior conteúdo de k-caseína e menor conteúdo de α_s-caseína. O tamanho das micelas de caseína sem o efeito da α_s-caseína e k-caseína não apresentou efeito sobre a firmeza do coágulo, porém apresentou efeito significatico sobre a produção de massa seca do coágulo. Esses resultados demonstram a importância do tamanho das micelas de caseína para otimizar a produção de queijo.*
Palavras-chave: *caseínas, proteínas do soro, queijo.*

INTRODUCTION

Milk caseins (CN), i.e., α_S-CN, β-CN, and κ-CN, aggregate into spherical micelles with average diameters ranging from 150 to 200 nm (DE KRUIF, 1998). Thus far, the detailed micellar structure is not completely known. κ-CN predominates on the outer surface, influencing the physico-chemical stability of micelles in milk, while other CNs are located inside the sphere (FOX & MCSWEENEY, 2003). Variations in the CN and whey protein contents, particularly higher κ-CN contents or higher degrees of κ-CN glycosylation in milk, have been associated with smaller micelles (DEVOLD et al., 2000). Variations in casein micelle size (CMS) appear to influence the effect of κ-CN content and glycosylation degree on milk gelation properties and cheese production (DZIUBA & MINKIEWICZ, 1996). Consequently, CMS represents a potential indicator trait for exploration in animal breeding to enhance the technological quality of milk, particularly in cheese production (GLANTZ et al., 2010).

Aside from the κ-CN genotype, content and degree of glycosylation, associations between CMS and milk protein composition have received little attention (BIJL et al., 2014). In addition, as smaller micelles are associated with increased κ-CN and CN contents, it is not yet clear whether the effect of CMS on milk gelation reflects differences in milk protein content/composition or whether the effect is

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directly due to variations in the CMS. Studies on the effect of CMS associated with other factors affecting dry matter cheese yield, such as fat and protein contents, are scarce (GLANTZ, et al., 2010). Moreover, previous studies have demonstrated that gel strength is not always associated with cheese yield (BONFATTI et al., 2014), and the production of model cheeses through laboratory cheese-making processes can more appropriately indicate cheese yields in comparison to milk coagulation properties (BONFATTI et al., 2014; CIPOLAT-GOTET et al., 2014).

The aims of the present study were i) to investigate the associations between CMS and milk composition and ii) to evaluate the association between CMS and enzymatic curd and dry matter curd yield, as measured in laboratory-scale cheese production.

MATERIALS AND METHODS

Bulk tank milk samples were obtained from 140 crossbreed (Holstein x Zebu) dairy herds located in the state of Minas Gerais, Brazil. For each sample, two aliquots of milk were collected: one aliquot was collected in a 150-mL sterilized flask for laboratory-scale cheese production, and the second aliquot was collected in a 50-mL tube, mixed with a preservative (Bronopol, 2-bromo-2-nitropropane-1,3-diol, 0.6:100 v/v) and analyzed for gross composition, protein composition, and somatic cell counting. Samples were stored at 4°C during transport to the laboratory.

The fat, total protein, CN, lactose, total solids, non-fat solids, and milk urea nitrogen contents (IDF, 2000) and somatic cell (IDF, 1995) count were analyzed using a CombiScope FTIR 400® analysis system (Delta Instruments; Drachten, Denmark) equipped with Fourier transform infrared (FTIR) and flow cytometry technology. Milk pH was measured with a digital pH meter (DM22, Digimed; São Paulo, Brazil). Milk samples for total bacteria count (TBC) were collected in a vial containing azidiol, and determined using a Bactocount ICB 150$^{\dot{O}}$ unit (Bentley Instruments, Chaska, USA). The casein number – CN number (%) was calculated as the percentage ratio of total casein to total protein of milk.

Milk protein composition (α_S-CN, β-CN, κ-CN, α-lactoalbumin, β-lactoglobulin) was measured based on electrophoretic mobility, following the methods of VERDI et al. (1987), with some modifications. The SDS-PAGE was performed with a 20×20cm vertical cube (Prolab, São Paulo, Brazil) using a 5% stacking gel in 0.5M Tris-HCl buffer, pH 6.8, and 12-20% separating gels in 1.5M Tris-HCl buffer, pH 8.8 with 10% SDS. Samples (2mg) were dissolved in 200µL of Tris-HCl buffer, pH 6.8 with 10% SDS, 5% β-mercaptoethanol, 5% glycerol, and bromophenol blue and heated at 100°C for 3 min. Electrophoresis of 4-µL aliquots was conducted for 4h at 120V. Protein identification was conducted by comparing the peaks with those obtained using five protein standards (Sigma Aldrich, St. Louis, MO, USA): κ-CN (cat. no. C-0406), α-CN (cat. no. C-6780), β-CN (cat. no. C-6905), α-lactoalbumin (α-LA; cat. no. L-5385 type I), and β-lactoglobulin (β-LG; cat. no. L-4756).

Gel images were captured and processed using Image J 1.48 software (NIH; Bethesda, MD) for the quantification of proteins. Image J deconvolution was used to improve the α_S-CN, β-CN and κ-CN baseline curves for band quantification. The X and Y coordinates were analyzed using Origin Pro8.6 software (OriginLab; Northampton, MA). The relative proportion of each protein fraction was obtained as the percentage of each peak with respect to the sum of the α_S-CN, β-CN, κ-CN, α-LA, and β-LG peaks. Relative proportions of CN fractions were transformed to concentrations, based on the FTIR measurement of total CN.

The average CMS was estimated within a few hours of sample collection through photon correlation spectroscopy (DEVOLD et al., 2000) using a Zeta sizer 3000HS (Malvern Instruments Ltd., Malvern, UK) with a He-Ne laser set to 632.8nm.

Cheese production was simulated in a small-scale method devised by MELILLI et al. (2002), with slight modifications. Raw milk samples (25g) were poured into 50-mm beakers, and 300µL of diluted acetic acid (1.1:10 v/v) was added for acidification, followed by agitation for 20 s and incubation in a water bath at 35°C for 10min. Subsequently, the acidified milk was mixed with 230µL of diluted rennet (HA-LA®, Chr. Hansen) (1:10 v/v), agitated for 20s, and incubated in a water bath at 35°C for 30min. Curd strength was measured using a TA-XT2 Texture Analyzer (Stable Micro Systems, Reading, UK) equipped with a TA-10 1/2" diameter AOAC cylinder probe moving downward at 1mm/s, and the strength (g) was measured at a depth of 4mm. Subsequently, the curd sample was cut into 4 uniform pieces through the y-axis, transferred into a 50-mL tube, and centrifuged (1,100 x g, 30min, 10ºC). The supernatant containing whey was carefully poured into a tube, whereas the precipitated gel was poured onto metal plates, oven dried (100°C ± 2°C, 4 h), and weighed. The dry matter curd yield was calculated as

the percentage ratio of the dry matter weight over the raw milk weight.

Statistical analysis

Associations between the average CMS and milk composition were investigated through estimates of Pearson's product-moment correlation between the traits and the effects of the milk protein composition on the average CMS estimated using a linear model, with α_s-CN, β-CN and κ-CN contents as independent variables. The CN content was expressed in g/L, and the CN levels were grouped according to three ranges: class 1 (concentration $<\bar{x}-$ 0.5 SD), class 2 ($\bar{x}-$ 0.5 SD$\leq$concentration$\leq\bar{x}+$ 0.5 SD), and class 3 (concentration$>\bar{x}+$0.5 SD). Although, a cause-effect relationship between average milk CMS, milk urea nitrogen, and milk pH has been reported, these variables were not included in the model to avoid multicollinearity, as these parameters presented a high correlation with κ-CN content.

The effect of CMS on the gel strength and on the production of cheese dry matter was estimated using linear regression. The fat, lactose, total CN, % α_s-CN, % β-CN, and % κ-CN contents and average CMS, without the effect of α_s-CN or κ-CN, were used as independent variables. However, the original values of the micelle size were not used, rather the estimated residue was obtained using a model of linear regression, with the average CMS as a dependent variable and % α_s-CN and % κ-CN as independent variables. The estimated residue was not correlated (P>0.05) with the other variables of the model. All variables were included in the model as continuous variables. The total CN content was included in the model with respective fractions to investigate the potential effects of individual CNs, in the absence of quantitative effects, as this parameter has been reported to exert a significant influence on cheese dry matter production. The somatic cell and total bacteria counts were not included in the model as these values were not significant. Statistical analyses were performed using the Statistical Analysis System, SAS 9.2 (SAS Institute Inc.; Cary, NC).

RESULTS

Pearson's correlation was used to estimate the association of CMS and κ-CN with composition and other milk quality parameters. The CMS variations were associated with the protein (0.23; P<0.05), casein (0.17; P<0.05), urea (-0.40; P<0.01), α_s-CN (0,46; P<0.001), κ-CN (-0.52; P<0.001), β-LG (-0.37; P<0.001) and α-LA (-0,15; P<0.05) contents, CN number (-0.22; P<0.001) and milk pH (0.21; P<0.05). κ-CN variations were associated with milk pH (-0.31; P<0.001).

The association of milk protein composition with CN micelles, based on the least square means for each class, is presented in Table 1. The β-CN content did not affect the CMS, but smaller micelles were detected in milk samples with lower α_s-CN and higher κ-CN contents. The average micelle size for milk samples with contents below 2.5 g/L (class 1) was 178.79.85 ± 1.47nm (average ± SD), while for samples with contents higher than 3.43 g/L (class 3), the average size was 177.35 ± 1.50nm.

Effect of CMS on gel strength and cheese dry matter production was evaluated using a linear regression model with CMS (without the effect of α_s-CN or κ-CN) and fat, lactose, and total CN contents as independent variables.Results are reported in Table 2. CMS did not affect the gel strength when the effect of the residue CMS was examined using the statistical model.

Smaller micelles of casein (without the effect of α_s-CN and κ-CN) exhibited favorable effect on dry matter cheese yield. Conversely content

Table 1 - Least squares means (LSMEANS) and standard error (SE) of the effect of casein micelle size grouped according to concentration levels of its fractions.

----------Protein fraction class*----------						
	----------Class 1----------		----------Class 2----------		----------Class 3----------	
Effect	LSMEANS	SE	LSMEANS	SE	LSMEANS	SE
α_s-casein	178.79[b]	1.47	185.58[a]	1.37	186.08[a]	1.60
β-casein	182.93	1.41	184.46	1.37	183.07	1.53
κ-casein	189.85[a]	1.50	183.26[b]	1.40	177.35[c]	1.50

[a-c]Different superscripted letters[a,] within a row indicate significant differences (P≤0.05) among the values. *Casein fraction was classified as follows: class 1 (concentration< $\bar{x}$ – 0.5 SD), class 2 ($\bar{x}$ – 0.5 SD ≤concentration≤ $\bar{x}$ + 0.5 SD), class 3 (concentration> $\bar{x}$ + 0.5 SD).

Table 2 - Regression coefficients and standard error (SE) of the effects of milk composition and average casein micelle size on gel strength and cheese dry matter production (the magnitude of these effects is expressed in SD units of traits).

Trait	----------Cheese gel strength----------			----------Dry matter cheese yield---------		
	Mean	SE	P value	Mean	SE	P value
Fat (g 100 g^{-1})	-0.15	0.44	P>0.05	0.53	0.13	P<0.001
Lactose (g 100 g^{-1})	-0.05	1.26	P>0.05	0.25	0.37	P<0.001
Casein (g 100 g^{-1})	0.25	1.29	P<0.05	0.17	0.38	P<0.01
α_s-casein, %	-0.07	0.04	P>0.05	-0.07	0.01	P>0.05
β-casein, %	-0.04	0.05	P>0.05	-0.05	0.01	P>0.05
κ-casein, %	0.24	0.08	P>0.05	0.61	0.02	P<0.001
Casein micelle size,[a]	0.12	0.02	P>0.05	-0.14	0.00	P<0.01

[a] Residue of the statistical model: Casein micelle size = α_s-casein + k-CN: k-casein + error.

of fat, lactose, casein and κ-CN was positively associated with dry matter cheese yield. Increases in dry matter cheese yield were 0.53, 0.25, 0.17 and 0.61 percentage points for 1-SD unit of fat, lactose, casein and κ-CN, respectively.

DISCUSSION

The positive correlation of pH with CMS reflects the influence of milk acidity on the CMS. Indeed, as found here, MCDERMOTT et al. (2016) also reported a negative correlation between pH and protein fractions, such as κ-CN, which in turn affect CMS. GLANTZ et al. (2010) reported similar results, demonstrating that pH reduction results in colloidal calcium phosphate migration to the whey phase and affects the micellar surface and/or alter the stability of κ-CN layer. Thus, micellar aggregation or dissociation into sub-micellar particles are resulted from environmental alterations, such as pH, which in turn disturb micelle stability as a consequence of the lack of rigid three-dimensional tertiary conformation in casein micelles (WALSTRA, 1990). In addition, VASBINDER & DE KRUIF (2003) showed that small alterations in pH had a great influence on whey protein denaturation and gelation properties in milk.

Results of the relationship between α_s-CN and CN micelle size have not been described in previous studies (DALGLEISH et al., 1989; BIJL et al.. 2014). CN micelles have dynamic structures that can be disrupted or reorganized into smaller micelles, with CN loss or solubilization to the whey phase (LIU & GUO, 2008). Our research showed that micellar dissociation may be associated with pH variation and urea content; therefore, micellar reorganization or CN loss might affect the content of α_s-CN in CN micelles.

DALGLEISH et al. (1989) and DALGLEISH (2011) reported a similar correlation between κ-CN levels and CMS. The κ-CN outer layer, particularly the glycosylated molecules, is primarily responsible for the steric and electrostatic repulsive forces between micelles and is a major factor for CMS variations. Animals that are homozygous for κ-CN variant B produce milk with a higher ratio of glycosylated κ-CN compared with animals homozygous for variant A (DALGLEISH, 2011; BIJL et al., 2014).

WEDHOLM et al., (2006) and BONFATTI et al. (2010) reported positive associations between the κ-CN content and gel strength. In the present study, Pearson's correlation coefficient between gel strength and κ-CN content was 0.35 (P<0.001), indicating that the association between smaller CN micelles and higher gel strength may partially reflect the higher content of κ-CN. DZIUBA & MINKIEWICZ (1996) reported that a higher level of κ-CN glycosylation, associated with smaller and more hydrophobic micelles, favors firmer rennet gels, reflecting increased κ-CN hydrolysis through chymosin and a closer packing arrangement (and aggregation between) of para-CN micelles forming the basic building blocks (para-CN aggregates) of the gel matrix. Thus, animals carrying the CNS3 B allele produce milk with a higher degree of κ-CN glycosylation, smaller micelles and enhanced cheese gel strength in comparison to animals carrying the A allele (WEDHOLM et al., 2006; BIJL et al., 2014; BONFATTI et al., 2014).

Smaller CN micelles (without the effect of α_s-CN or κ-CN) exhibited favorable effects on dry

matter cheese yield. Moreover, the fat, lactose, CN and κ-CN contents were positively associated with the dry matter cheese yield. Consistent with the study of VERDIER-METZ et al. (2001), the relationship between fat and CN contents and cheese yield was positive and linear. Hence, the effect of fat was considerably greater than the effect of CN. Generally, fat and CN represent approximately 94% of the dry matter of cheese (LUCEY & KELLY, 1994).

The CMS was considered in the model as a residue; hence, it was difficult to interpret the regression coefficient generated based on these results. In other words, it was not possible to quantify the effect of CMS on the dry matter cheese yield based on each unit of size decrease. However, a key finding of the present study was that milk samples with smaller CN micelles and higher proportions of fat, CN and κ-CN might lead to an optimized production of dry matter cheese yield. This result suggested that the highest κ-CN content would be associated with the smallest micelles, independent of the cause-effect relationship between these variables, and might be beneficial to the gel structure. WALSH et al. (1998) showed that milk samples from animals homozygous for the κ-CN B gene were associated with smaller CN micelles and produced cheese with smaller pores, implying that compact micelles form more interactions between molecules during gel formation. ZHAO et al. (2014) reduced the CMS using ultrasonification and observed that the gel structure presented smaller and more uniform pores, likely contributing to the retention of more milk components in cheeses with better yield (HALLÉN et al., 2010).

Results of the present study provided novel insights into the positive effects of small CN micelles and higher fat, CN and κ-CN contents on dry matter cheese yield, indicating that the effect of CMS on dry matter cheese yield does not result from a different milk protein content/composition, but is rather an effect directly resulting from variations in the CMS. It is likely that small micelles exert two favorable effects during the initial cheese processing. First, small CN micelles have more surface area than large CN micelles, likely increasing the number of junctions between micelles during the initial cheese processing and increasing the incorporation of micelles into the gel network. Consequently, this effect facilitates a more compact and uniform arrangement of the gel network, likely reducing losses in whey through improved entrapping. Second, small CN micelles may reduce the coefficient of diffusion between the enzyme molecules and the CN micelles, potentially further decreasing the rennettime and consequently enhancing cheese curd firmness and overall cheese yield.

CONCLUSION

Smaller micelles increase cheese dry matter production, without affecting the cheese gel strength. Although, influence of CMS on cheese yield should be further investigated, these findings provide new insights into the combined effects of small micelles and higher fat, lactose, CN and κ-CN contents on cheese production, suggesting that the selection of smaller CN micelles would aid in optimizing cheese production.

DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest.

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AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript**.**

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