International Dairy Journal 119 (2021) 105064

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Variation in casein distribution and mineralisation in the milk from Holstein-Friesian cows

Thom Huppertz [a, b, *], Jeroen Heck [a], Etske Bijl [b], Nina Aagaard Poulsen [c], Lotte Bach Larsen [c]

[a] FrieslandCampina, Amersfoort, the Netherlands
[b] Wageningen University and Research, Wageningen, the Netherlands
[c] Aarhus University, Aarhus, Denmark

ARTICLE INFO

Article history:
Received 29 January 2021
Received in revised form
15 March 2021
Accepted 15 March 2021
Available online 20 March 2021

ABSTRACT

In this study, protein and salt distributions in the milk of 48 Holstein-Friesian cows were studied. Gross composition of milk samples and variation between individual cows was in line with expectation. However, the mineralisation of the casein fraction, expressed in mmol protein-associated calcium per 10 g casein, differed >2-fold between the milk samples. Reasons for this variation are unknown and could not be correlated to casein composition and/or genetic variants of caseins. Furthermore, notable differences (>5-fold) in non-sedimentable (100,000×*g* for 60 min) casein were observed. Percentages of non-sedimentable caseins differed for κ-casein genetic variants (BB > AB > AA) and increased with increasing degree of glycosylation. Higher levels of non-sedimentable caseins with increasing degree of glycosylated κ-casein could be attributable to increased repulsion between the micelles, leading to the formation of a less cohesive sediment on centrifugation.

1. Introduction

The casein micelles in milk play a pivotal role in the dairy sector. Their controlled coagulation is crucial for the conversion of milk into popular dairy products such as cheese and yoghurt, as well for the phased release of proteinaceous material from the stomach into the intestine during digestion (Huppertz & Chia, 2020). The (uncontrolled) coagulation of casein micelles can also have negative effects, e.g., in the heat-induced coagulation of milk during sterilisation (Dumpler, Huppertz, & Kulozik, 2020) or the instability observed in the form of either sediment formation or age gelation of UHT milk (Anema, 2019). In addition, the ability of casein micelles to transfer large quantities of sparingly soluble calcium and phosphate in bioavailable form plays a key role in the development and maintenance of various physiological and biological functions of mammalian neonates, but also for humans in stages of life post-infancy (Holt, Carver, Ecroyd, & Thorn, 2013). Given this pivotal role of casein micelles, it is hardly surprising that a large body of scientific research has been dedicated to the structure, stability and properties of casein micelles over the past century.

Continuous advances in our understanding of caseins, casein interactions and casein-mineral interactions, combined with (bio) physical insights have shaped our understanding on the casein micelle to the present day. Key elements in the casein micelle structure are the calcium phosphate nanoclusters (De Kruif & Holt, 2003; Holt et al., 2013; Huppertz et al., 2017). A typical casein micelle contains several hundreds of these nanoclusters (De Kruif & Holt, 2003; Holt, De Kruif, Tuinier, & Timmins, 2003; Huppertz et al., 2017), which represent most of the so-called micellar calcium phosphate (MCP); however, some micellar calcium is also associated with SerP, Glu and Asp residues that are not the specific parts of the peptide chain that cover the calcium phosphate nanoclusters (Bijl, Huppertz, van Valenberg, & Holt, 2019). For bulk milk, MCP content is typically reported as 5–7% of the dry mass of casein micelles. However, Bijl et al. (2019) presented detailed mineral partitioning calculations on the milk from 16 Holstein-Friesians cows and indicated notable variation in the ratio of MCP present in the milk and the amount of MCP that could theoretically be stabilised by the casein fraction present in the milk. This degree of casein mineralisation varied from <55 to >90% between milk samples (Bijl et al., 2019).

* Corresponding author. Tel.: +31 6 11187512.
E-mail address: thom.huppertz@frieslandcampina.com (T. Huppertz).

https://doi.org/10.1016/j.idairyj.2021.105064

This variation in casein mineralisation was also previously noted by Malacarne et al. (2014), who also reported a correlation to rennet coagulation properties; milk samples with a higher level of mineralisation were found to have better rennet coagulation properties. Furthermore, adjustment of MCP content of bulk milk has been shown to affect various properties, including rennet coagulation (Shalabi & Fox, 1982), acid coagulation (Anema, 2009), heat stability (Fox & Hoynes, 1975) and gastric coagulation and digestion (Huppertz & Lambers, 2020), but also bacterial inactivation during high pressure treatment (Black, Huppertz, Fitzgerald, & Kelly, 2007). To build further upon the aforementioned findings of differences in casein mineralisation and their effect on casein micelle properties, we studied the variation in mineral and casein partitioning between 48 milk samples from Holstein-Friesian cows in the Netherlands and establish whether differences found correlate to genetic variation in milk protein composition.

2. Materials and methods

2.1. Milk sample collection

Milk samples were collected from 48 different Holstein-Friesian cows (days in milk 23–309, parity 1–8) from a single farm in the Netherlands in the space of one week in February 2018. Immediately after collection, sodium azide (0.02%, w/w) and cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (1 tablet per L milk; Sigma–Aldrich, St. Louis, MO, USA) were added to the milk and milk samples were cooled to 0–5 °C and transported to the laboratory.

2.2. Sample fractionation

After overnight storage at 5 °C, samples were first defatted by centrifugation at 2000×*g* for 20 min at 5 °C, followed by filtration of the subnatant through glass wool. The skimmed milk was subsequently warmed to 20 °C and centrifuged at 100,000×*g* for 60 min at 20 °C, followed by separation of the pellet and supernatant by decanting. Part of the ultracentrifugal supernatant was subsequently filtered over a 10 kDa Amicon Ultra-15 centrifugal filter (Merck KAaG, Darmstadt, Germany) and the filtrate was collected for analysis.

2.3. Analysis

Total nitrogen (TN), soluble nitrogen (SN) and non-protein nitrogen (NPN) in milk were determined using the Kjeldahl method as described by ISO/IDF (2014), ISO/IDF (2004) and ISO/IDF (2016), respectively. Ash content was determined as described by AOAC (2012), whereas fat content was determined as described by ISO/IDF (2010). Lactose content was determined as described by ISO/IDF (2002). The levels of calcium, magnesium, potassium, sodium and phosphorus in the milk samples and the 10 kDa-permeate of the milk were determined by ICP-AES as described by Cruijsen, Poitevin, and Brunelle (2019).

Protein composition of the skim milk samples and the ultracentrifugal supernatants were determined by LC/ESI-MS, which included identification of glycosylated and non-glycosylated forms of κ-casein in the LC analysis and genetic variants of κ-casein, β-casein and β-lactoglobulin by MS analysis, as described by Jensen et al. (2012). Quantification for the different caseins was done by summing the respective peak areas from the of the caseins from the LC chromatogram based on the UV detection at 214 nm. For total casein, the sum of the peak area for all caseins was used. Non-sedimentable casein was defined as the proportion of each casein, or total casein, that was recovered in the ultracentrifugal supernatant.

Z-average mean particle diameter of the skimmed milk was determined using a Malvern NanoSizer (Malvern Instruments, Malvern, UK) at a scattering angle of 173° after 50-fold dilution of the samples with milk permeate using refractive index values of 1.570 for casein micelles and 1.341 for milk permeate (Huppertz, Smiddy, & de Kruif, 2007).

2.4. Statistical analysis

Statistical significance of the influence of genetic variants of κ-casein, β-casein and β-lactoglobulin on particle size, casein mineralisation and non-sedimentable casein were analysed by one-way ANOVA followed by Tukey-HSD post-hoc test using XLSTAT (Addinsoft, Paris, France), with differences considered significant when $P < 0.05$.

3. Results and discussion

3.1. Milk composition

Gross composition of the 48 milk samples from Holstein-Friesians cows that were studied is shown in Table 1. Average crude protein (calculated as TN × 6.38), fat and lactose content were ~3.6, 4.6 and 4.5%, respectively, which are in line with expected composition for milk from Holstein-Friesian cows in the Netherlands (https://www.cooperatie-crv.nl/downloads/stamboek/bedrijven-en-koeien-in-cijfers/). As expected, notable variation could be observed in the protein and fat content, whereas lactose content showed far less variation. This is in line with the fact that lactose, together with the soluble salts, is the main determinant of the osmotic pressure of the milk, which should equal that of the blood of the cow. Accordingly, the ash content of milk, which averaged ~0.8% (w/w) also showed little variation (Table 1). pH of the milk samples averaged 6.7, with limited variation observed (Table 1).

The different nitrogen fractions for the 48 milk samples are also shown in Table 1. The variation in protein content (TN × 6.38) was reflected in both SN × 6.38, which represents the whey protein fraction, and (TN-SN) × 6.38, which represents the casein fraction. In contrast, NPN × 6.38 showed only limited variation (Table 1). SN/TN and (TN-SN)/TN did not vary greatly between samples (data not shown), indicating that casein:whey protein ratio was rather constant.

3.2. Salt composition and distribution

Concentrations of Ca, Mg, P, K and Na in the milk samples are shown in Table 1. Values are in line with expected values for Dutch milk (Bijl, Van Valenberg, Huppertz, & Van Hooijdonk, 2013). Some variation between milk samples was, as expected, observed. The proportion of Ca, Mg and P in the 10 kDa-permeable fraction was also determined, as shown in Table 1. For Ca, ~30% of total Ca was found to be 10 kDa-permeable, which is in line with expected values based on the work of Bijl et al. (2013) and White and Davies (1958). Likewise, ~65% of total Mg and ~45% of total P were found in the 10 kDa-permeable fraction (Table 1) is also conform expectation (Bijl et al., 2013; White & Davies, 1958). For K and Na, virtually all was found to be 10 kDa-permeable (Table 1).

The difference between total Ca, Mg and P and 10 kDa-permeable Ca, Mg and P, i.e., the 10 kDa non-permeable fraction, represents the fraction that is associated with the proteins. Particularly with the casein fraction of milk, a lot of Ca, Mg and P is associated, both in the form of calcium phosphate nanoclusters, but also via ionic bonds with amino acid residues (Bijl et al., 2019). The so-called mineralisation of the casein fraction is shown in Fig. 1,

Table 1
Compositional properties of the milk samples and their 10 kDa permeates from 48 Holstein-Friesian cows.[a]

Component		Mean ± SD	Minimum	Maximum
Fat (%, w/w)		4.57 ± 0.71	3.05	6.06
Lactose (%, w/w)		4.53 ± 0.18	4.18	5.01
TN × 6.38 (%, w/w)		3.58 ± 0.43	2.57	4.46
SN × 6.38 (%, w/w)		0.87 ± 0.17	0.57	1.34
NPN × 6.38 (%, w/w)		0.14 ± 0.01	0.12	0.16
(TN-SN) × 6.38 (%, w/w)		2.71 ± 0.35	1.91	3.42
Ash (%, w/w)		0.76 ± 0.04	0.67	0.83
Ca (mmol L^{-1})	Milk	31.1 ± 2.4	26.1	37.3
	Permeate	9.6 ± 1.5	6.3	12.0
Mg (mmol L^{-1})	Milk	4.6 ± 0.5	3.5	5.4
	Permeate	3.1 ± 0.3	2.4	4.0
P (mmol L^{-1})	Milk	31.6 ± 2.4	27.8	36.3
	Permeate	14.2 ± 1.6	11.3	18.1
Na (mmol L^{-1})	Milk	13.8 ± 2.3	10.1	21.0
	Permeate	13.7 ± 2.2	10.0	21.0
K (mmol L^{-1})	Milk	40.8 ± 2.4	34.1	45.0
	Permeate	40.2 ± 1.6	31.2	45.4
pH		6.75 ± 0.05	6.63	6.86
Casein micelle size (nm)		182.2 ± 22.8	154.6	295.0

[a] Abbreviations are: SD, standard deviation; TN, total nitrogen; SN, soluble nitrogen; NPN, non-protein nitrogen.

where levels of 10 kDa non-permeable Ca, Mg and P are expressed as mmol of Ca, Mg or P per 10 g of casein (which was calculated as (TN-SN) × 6.38). For Ca, the average mineralisation level was ~8.4 mmol Ca per 10 g casein, whereas for Mg this was ~0.6 and for P this was ~6.9 mmol per 10 g casein (Fig. 1). In all cases, notable variation between samples was observed, indicating that casein mineralisation levels are not constant, but vary notably between the milk from individual cows. As outlined in Fig. 2, however, variation in levels of mineralisation of casein did not affect the Ca/P ratio of the micellar fraction, which was very constant. Furthermore, a weak correlation was observed between total Ca and total P, whereas no correlation was observed between 10 kDa-permeable Ca and P (Fig. 2). However, a strong correlation was observed between micellar Ca and micellar P (Fig. 2).

The expected trend with majority of calcium and approximately half of phosphorus being protein-associated, but far less magnesium, sodium and potassium being protein-associated was found. This is, e.g., in line with previous work by Bijl et al. (2013, 2019), Bijl, de Vries, van Valenberg, Huppertz, and Van Hooijdonk (2014) and White and Davies (1958). When considering levels of protein-associated calcium as a function of total casein, large variations are observed between the milk from different cows, with the level of casein mineralisation on the basis of calcium ranging from 6.7 to 12.9 mmol of Ca per 10 g casein (Fig 1). Mineralisation expressed on a phosphorus basis varied equally (Fig. 1) and levels of protein-associated Ca and P in milk samples were strongly correlated (Fig. 2).

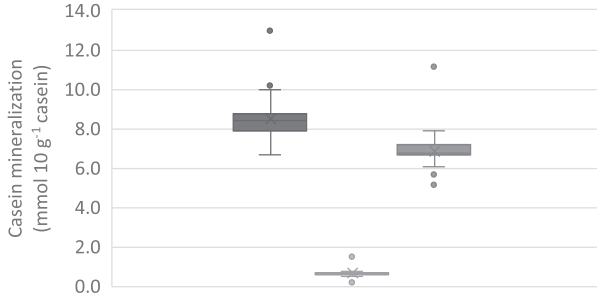

Fig. 1. Casein mineralisation with respect to calcium (■), magnesium (■) and phosphorus (■) for milk samples from 48 Holstein-Friesian cows. Values are expressed as a mmol of protein-associated Ca, Mg and P per 10 g casein.

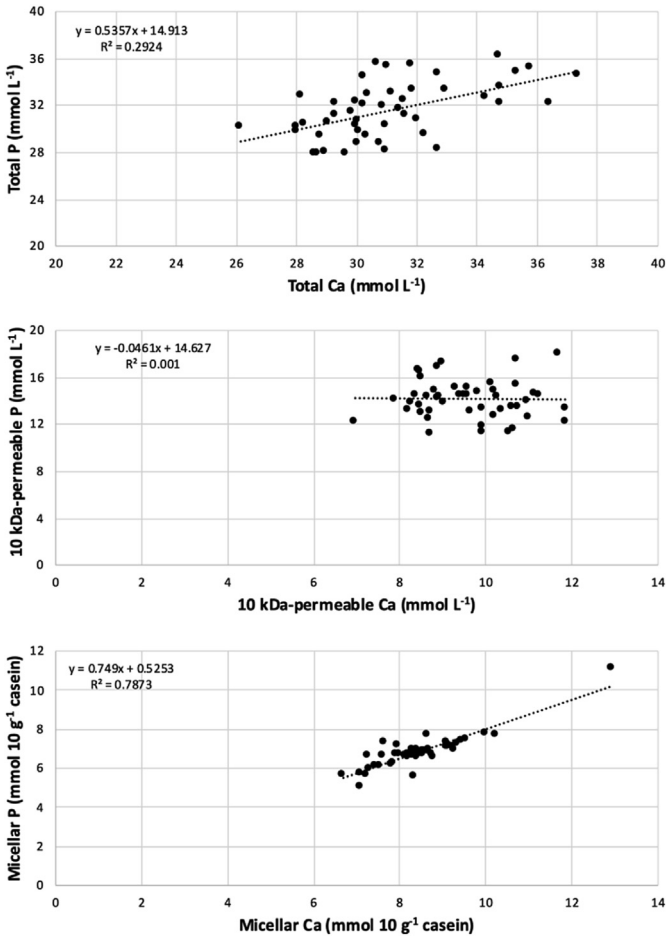

Fig. 2. Correlation between total (top), 10 kDa-permeable (middle) and micellar (bottom) Ca and P in milk samples from 48 Holstein-Friesian cows.

Differences in the degree of mineralisation of the casein fraction between milk from individual cows are also apparent from the data of Bijl et al. (2019) and Malacarne et al. (2014), as well as the data of White and Davies (1958). However, these differences have gone largely unreported to date when it comes to natural variation in milk properties, with the exception of Malacarne et al. (2014) who showed that milk with a higher level of casein mineralisation showed better rennet coagulation properties. Variation in casein mineralisation can also be induced by processing, and these induced variations have been shown to affect, e.g., rennet coagulation (Shalabi & Fox, 1982), acid coagulation (Anema, 2009), heat stability (Fox & Hoynes, 1975) and in vitro gastric digestion (Huppertz & Lambers, 2020) of milk products.

3.3. Casein composition and casein distribution

Casein composition of the milk samples and their ultracentrifugal supernatants was determined by LC-ESI/MS. A notable proportion of casein was observed to be non-sedimentable for all caseins in milk samples, with variation from <10% to >60% of caseins observed (Fig. 3). Strong linear correlations were observed between the non-sedimentable individual caseins (Fig. 3). This suggests that the differences in levels of non-sedimentable casein between the milk samples relate to the entire casein fraction, rather

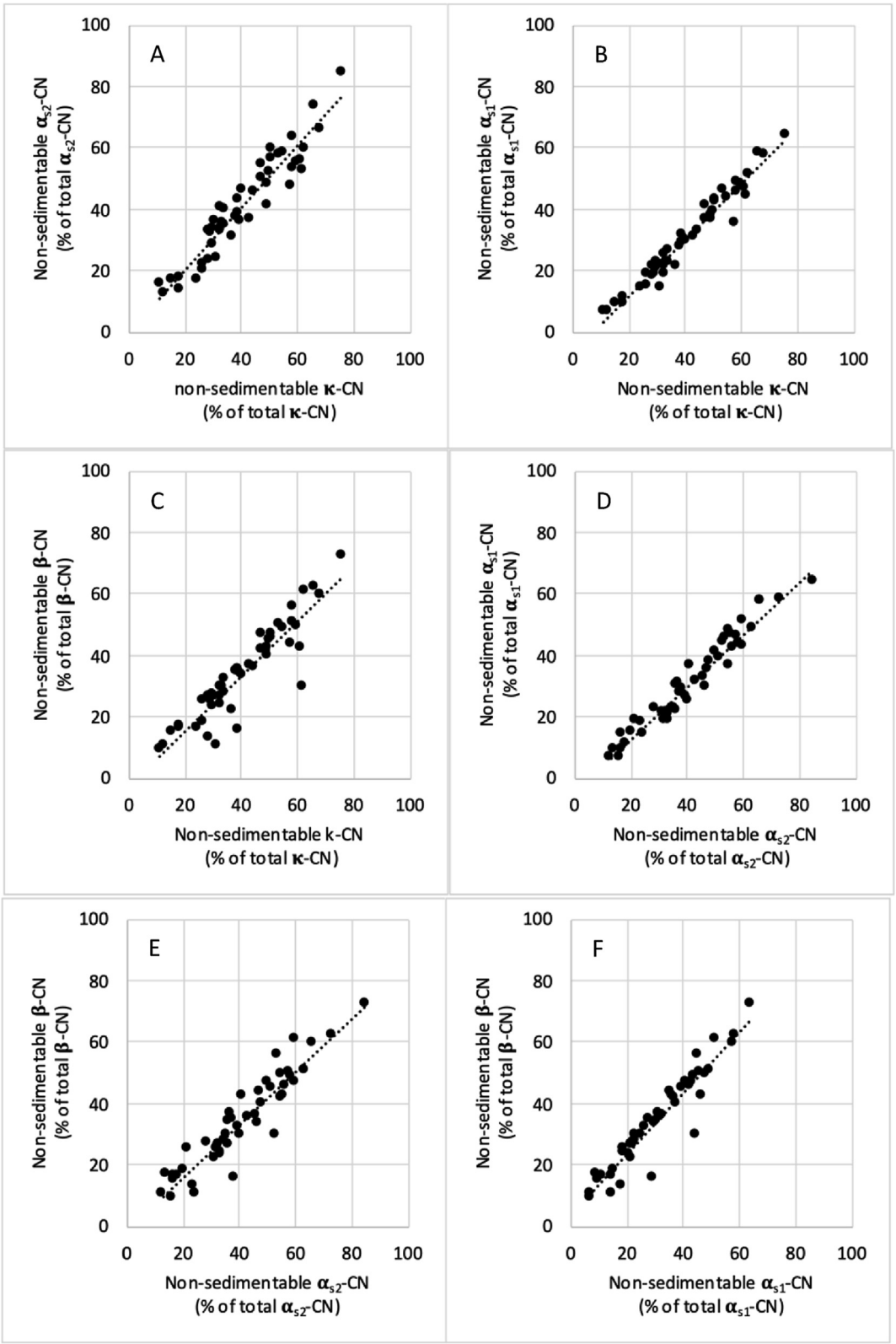

Fig. 3. Correlation between levels of non-sedimentable α_{S1}-casein (α_{S1}-CN), α_{S2}-casein (α_{S2}-CN), β-casein (β-CN) and κ-casein (κ-CN) in the milk from 48 Holstein-Friesian cows: A, B and C, non-sedimentable α_{S2}-CN, α_{S1}-CN and β-CN versus non-sedimentable κ-CN, respectively; D, non-sedimentable α_{S1}-CN, versus non-sedimentable α_{S2}-CN; E and F, non-sedimentable β-CN versus non-sedimentable α_{S2}-CN and α_{S1}-CN, respectively.

than individual casein. It is interesting to note, however, that correlations involving β-casein, which is known to dissociate from the micelles on cooling (Atamer et al., 2017; Schäfer, Schubert, & Atamer, 2019), were somewhat weaker than those involving the other three caseins (Fig. 3).

From LC-ESI/MS data, the genetic variants of the proteins in the milk could also be identified. For κ-casein, β-casein and β-lactoglobulin, a number of phenotypes were identified and casein micelle size, casein mineralisation and the percentage of summed non-sedimentable casein were classified per phenotype (Table 2). For κ-casein, milk samples with phenotype AA have notably larger casein micelles and the lowest percentage of non-sedimentable casein. In contrast, milk with phenotype BB has the smallest casein micelles and the highest proportion of non-sedimentable casein. Differences in casein micelle size and non-sedimentable casein between phenotypic groups of κ-casein were found to be significant ($P < 0.05$) and are in line with previous results (Bijl et al., 2014; Day, Williams, Otter, & Augustin, 2015). A similar inverse ranking of casein micelles size and the percentage of non-sedimentable casein was observed for the β-lactoglobulin phenotypes (Table 2), though differences were not significant. For the β-casein phenotypes, this was not observed. However, in this case, it should be noted that a large number of β-casein phenotypes were observed, many of which were only observed once. Differences in casein mineralisation between the phenotypic groups for the different milk proteins were not found to be significant (Table 2).

When the degree of casein mineralisation, often expressed as the concentration of colloidal or micellar calcium phosphate (CCP or MCP, respectively) is decreased or increased, respectively, trough processing, increases or decreases in non-sedimentable casein are typically observed (Huppertz & Lambers, 2020). In the current study, studying natural variation in casein mineralisation, this trend was not observed, despite the fact that notable variation in levels of non-sedimentable casein was observed between milk samples.

In fact, in the present study, very high levels of non-sedimentable casein were observed in some samples, with values exceeding 60% of total casein for some samples. Such findings have not been reported previously; it should be noted, however, that in most cases, casein balances after ultracentrifugation have been studied in bulk milk rather than in milk from individual cows. For sedimentation of caseins by centrifugation, particle size would be expected to be the main factor governing the rate of sedimentation based on Stokes' law. Indeed, a significant ($P < 0.05$) negative correlation was observed between particle size and non-sedimentable casein (data not shown). However, the typical variation observed in particle size (~160–220 nm) can, according to Stokes' law, only account for a 2-fold difference in rate of sedimentation, which seems insufficient to explain the large variation in non-sedimentable casein observed.

A further consideration to explain the large variation in non-sedimentable casein may be found in the consistency of the pellet formed; i.e., a weaker pellet may be partially dislodged on decanting. As pellet consistency would be based on inter-micellar coherence, the significant effect of κ-casein phenotype on non-sedimentable casein (Table 2) is of particular interest. It is well known that κ-casein B is more highly glycosylated than κ-casein A (Bijl et al., 2014; Day et al., 2015; Poulsen et al., 2016). As these glycans, which can carry a negative charge as a result of the presence of sialic acid, are found on the micelle surface, they can interfere with formation of a cohesive pellet on ultracentrifugation. This is in line with milk samples with κ-casein phenotype AB and BB having significantly higher levels of non-sedimentable casein than milk samples with κ-casein phenotype AA (Table 2).

To explore this further, correlation analysis was performed between the proportion of glycosylated κ-casein (on a total κ-casein basis) and the percentage of non-sedimentable casein. For this, a significant ($R = 0.49$, $P < 0.01$) positive correlation was observed (data not shown); i.e., milk samples with a higher proportion of glycosylated κ-casein showed more non-sedimentable casein. This thus supports the hypothesis that the high levels of non-sedimentable casein observed in some samples are related to the composition of the casein micelle surface, and in particular the κ-casein glycosylation. At high levels of glycosylation, an insufficiently cohesive sediment may form, which is partly dislodged on sedimentation. This aspect requires further study in future and may have applications in limiting aggregation of casein micelles in dense casein systems.

Table 2
Influence of κ-casein, β-casein or β-lactoglobulin phenotypes on particle size, casein mineralisation and non-sedimentable total casein in milk from 48 Holstein-Friesian cows.[a]

Phenotype		n	Particle size (nm)		Mineralisation (mmol Ca 10 g^{-1} casein)		Non-sedimentable casein (% of total casein)	
			Mean	SD	Mean	SD	Mean	SD
κ-CN	AA	10	209.1[a]	32.8	8.6	0.7	20.7[a]	5.7
	AB	25	178.4[b]	12.5	8.5	1.3	35.7[b]	15.6
	AE	1	191.2	–	7.9	–	23.8	–
	BB	12	166.8[b]	7.1	8.2	0.6	42.9[b]	11.1
β-CN	A1A1	5	182.6	11.2	8.5	0.5	35.0	7.1
	A1A2	15	182.3	6.8	8.8	1.1	31.4	16.6
	A1B	1	166.3	–	8.5	–	47.8	–
	A1F	1	200.1	–	8.1	–	40.3	–
	A1I	1	158.4	–	7.6	–	57.5	–
	A2A2	15	176.3	21.2	8.3	0.6	37.6	16.1
	A2A3	1	193.0	–	8.2	–	31.0	–
	A2B	5	198.5	55.0	8.5	0.3	25.8	9.5
	A2F	1	215.6	–	7.6	–	18.1	–
	A2I	3	175.3	15.7	7.6	0.7	34.3	21.9
β-LG	AA	19	188.0	29.5	8.6	0.7	30.0	15.3
	AB	27	180.1	15.8	8.3	1.1	36.5	14.6
	BB	2	154.8	0.4	9.0	1.7	40.1	6.9

[a] Abbreviations are: κ-CN, κ-casein; β-CN, β-casein; β-LG, β-lactoglobulin; SD, standard deviation. Values within a column for a single protein with different superscript lowercase letters differ significantly ($P < 0.05$) based on one-way ANOVA followed by Tukey-HSD post-hoc test.

4. Conclusions

In this study, the milk from 48 Holstein-Friesian cows was examined for salt and protein distribution. Milk samples showed notable variation in casein mineralisation, when expressed on a Ca-basis, with an approximate 2-fold difference observed between samples with the highest and lowest level of mineralisation. Based on previous work, natural variation in this previously rarely acknowledged factor is likely to impact the stability of milk against heat-, acid- and enzyme-induced coagulation. Notable variations in non-sedimentable casein observed between milk samples were likely related to differences in micelle size as well as the coherence of the sediment formed on ultracentrifugation; higher levels of glycosylation appeared to result in less coherent pellets.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Mr. Siem Nijskens and Ms. Anne van de Pijpekamp (FrieslandCampina) and FrieslandCampina Laboratory & Quality Services for skilful technical assistance as well as Ms. Gitte Hald Kristiansen (Department of Food Science, Aarhus University) for technical assistance in the LC-ESI/MS analyses.

References

Anema, S. G. (2009). Role of colloidal calcium phosphate in the acid gelation properties of heated skim milk. *Food Chemistry, 114*, 161–167.
Anema, S. G. (2019). Age gelation, sedimentation, and creaming in UHT milk: A review. *Comprehensive Reviews in Food Science and Food Safety, 18*, 140–166.
AOAC. (2012). *AOAC official method 930.30: Ash of dried milk. Official methods of analysis of AOAC* (19th edn.). Gaithersburg, MA, USA: AOAC International.
Atamer, Z., Post, A. E., Schubert, T., Holder, A., Boom, R. M., & Hinrichs, J. (2017). Bovine β-casein: Isolation, properties and functionality. A review. *International Dairy Journal, 66*, 115–125.
Bijl, E., de Vries, R., van Valenberg, H., Huppertz, T., & Van Hooijdonk, T. (2014). Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of κ-casein. *International Dairy Journal, 34*, 135–141.
Bijl, E., Huppertz, T., van Valenberg, H., & Holt, C. (2019). A quantitative model of the bovine casein micelle: Ion equilibria and calcium phosphate sequestration by individual caseins in bovine milk. *European Biophysics Journal, 48*, 45–59.
Bijl, E., Van Valenberg, H. J. F., Huppertz, T., & Van Hooijdonk, A. C. M. (2013). Protein, casein, and micellar salts in milk: Current content and historical perspectives. *Journal of Dairy Science, 96*, 5455–5464.
Black, E. P., Huppertz, T., Fitzgerald, G. F., & Kelly, A. L. (2007). Baroprotection of vegetative bacteria by milk constituents: A study of *Listeria innocua*. *International Dairy Journal, 17*, 104–110.
Cruijsen, H., Poitevin, E., & Brunelle, S. L. (2019). Determination of minerals and trace elements in milk, milk products, infant formula, and adult nutrition: Collaborative study 2011.14 method modification. *Journal of AOAC International, 102*, 1845–1863.
Day, L., Williams, R. P. W., Otter, D., & Augustin, M. A. (2015). Casein polymorphism heterogeneity influences casein micelle size in milk of individual cows. *Journal of Dairy Science, 98*, 3633–3644.
De Kruif, C. G., & Holt, C. (2003). Casein micelle structure, functions and interactions. In P. F. Fox, & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry. Vol. 1. Proteins* (3rd edn., pp. 233–276). Boston, MA, USA: Springer.
Dumpler, J., Huppertz, T., & Kulozik, U. (2020). Invited review: Heat stability of milk and concentrated milk: Past, present, and future research objectives. *Journal of Dairy Science, 103*, 10986–11007.
Fox, P. F., & Hoynes, M. C. T. (1975). Heat stability of milk: Influence of colloidal calcium phosphate and β-lactoglobulin. *Journal of Dairy Research, 42*, 427–435.
Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. *Journal of Dairy Science, 96*, 6127–6146.
Holt, C., De Kruif, C. G., Tuinier, R., & Timmins, P. A. (2003). Substructure of bovine casein micelles by small-angle X-ray and neutron scattering. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 213*, 275–284.
Huppertz, T., & Chia, L. W. (2020). Milk protein coagulation under gastric conditions: A review. *International Dairy Journal, 113*, Article 104882.
Huppertz, T., Gazi, I., Luyten, H., Nieuwenhuijse, H., Alting, A., & Schokker, E. (2017). Hydration of casein micelles and caseinates: Implications for casein micelle structure. *International Dairy Journal, 74*, 1–11.
Huppertz, T., & Lambers, T. T. (2020). Influence of micellar calcium phosphate on in vitro gastric coagulation and digestion of milk proteins in infant formula model systems. *International Dairy Journal, 107*, Article 104717.
Huppertz, T., Smiddy, M. A., & de Kruif, C. G. (2007). Biocompatible micro-gel particles from cross-linked casein micelles. *Biomacromolecules, 8*, 1300–1305.
ISO/IDF. (2002). *International Standard ISO 5765-2:2002 [IDF 79-2:2002]. Dried milk, dried ice-mixes and processed cheese — determination of lactose content — Part 2: Enzymatic method utilizing the galactose moiety of the lactose*. Geneva, Switzerland: International Standardisation Organisation/Brussels, Belgium: International Dairy Federation.
ISO/IDF. (2004). *International standard ISO 17997-1:2004 | IDF 29-1:2004. Milk — determination of casein-nitrogen content — Part 1: Indirect method (reference method)*. Geneva, Switzerland: International Standardisation Organisation/Brussels, Belgium: International Dairy Federation.
ISO/IDF. (2010). International standard ISO 1211:2010 |IDF 1:2010. Milk — Determination of fat content — Gravimetric method (Reference method). *I geneva, Switzerland: International standardisation organisation/brussels, Belgium*. International Dairy Federation.
ISO/IDF. (2014). *International standard ISO8968-1|IDF20-1. Milk and milk products — determination of nitrogen content — Part 1: Kjeldahl principle and crude protein calculation*. Geneva, Switzerland: International Standardisation Organisation/Brussels, Belgium: International Dairy Federation.
ISO/IDF. (2016). *International Standard ISO8968-4|IDF20-4. Milk and milk products – Determination of nitrogen content — Part 4: Determination of protein and non-protein nitrogen content and true protein content calculation (Reference method)*. Geneva, Switzerland: International Standardisation Organisation/Brussels, Belgium: International Dairy Federation.
Jensen, H. B., Poulsen, N. A., Andersen, K. K., Hammershøj, M., Poulsen, H. D., & Larsen, L. B. (2012). Distinct composition of bovine milk from Jersey and Holstein-Friesian cows with good, poor, or noncoagulation properties as reflected in protein genetic variants and isoforms. *Journal of Dairy Science, 95*, 6905–6917.
Malacarne, M., Franceschi, P., Formaggioni, P., Sandri, S., Mariani, P., & Summer, A. (2014). Influence of micellar calcium and phosphorus on rennet coagulation properties of cows milk. *Journal of Dairy Research, 81*, 129–136.
Schäfer, J., Schubert, T., & Atamer, Z. (2019). Pilot-scale β-casein depletion from micellar casein via cold microfiltration in the diafiltration mode. *International Dairy Journal, 97*, 222–229.
Shalabi, S. I., & Fox, P. F. (1982). Influence of pH on the rennet coagulation of milk. *Journal of Dairy Research, 49*, 153–157.
White, J. C. D., & Davies, D. T. (1958). 712. The relation between the chemical composition of milk and the stability of the caseinate complex: I. General introduction, description of samples, methods and chemical composition of samples. *Journal of Dairy Research, 25*, 236–255.