Impact of milk protein genotypes on milk coagulation properties

Effekt av genetiske melkeproteinvarianter på melkens koaguleringsegenskaper

Philosophiae Doctor (PhD) Thesis

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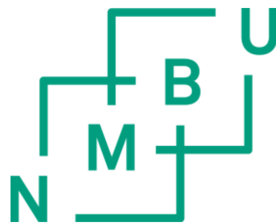

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Isaya Appelesy Ketto
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Summary

Evaluation of the milk coagulation properties is very important for the dairy industry, because it gives information on the processability of milk for both cheese and yoghurt/cultured milk. Milk that takes a shorter time to coagulate is more appropriate for the production of cheese with improved texture compared to the non-coagulating and poor coagulating samples (that take longer time to coagulate). Several parameters are used for studying milk coagulation properties, for example, time taken for the milk to coagulate, speed of gel formation and final gel firmness. Low amplitude oscillation rheometry (LAOR) and Formagraph (Lattodinamografo) are the most popular methods used to monitor milk coagulation properties. LAOR has been widely used in studying both the rennet and acid coagulation properties of milk, while Formagraph was designed for studying the rennet coagulation process. LAOR is limited by the fact that it measures only one sample at a time while Formagraph takes more than one sample (parallels) at the same time. An alternative method to LAOR is needed because a large throughput analysis on the acid coagulation properties of milk is needed. Differences in rennet coagulation properties of milk have been associated with the milk protein genotypes in most of the commercial dairy cattle breeds. However, limited studies are available on the effects of milk protein genotypes, salts (Ca, Mg and P) distribution, casein

micelle size and milk protein composition on the acid coagulation properties of milk. Hence, the main objective of this project was to study the effects of milk protein genotypes on the rennet and acid coagulation properties in the Norwegian Red cattle / Norsk Rødt Fe (NRF).

Paper I describes a comparison of LAOR and Formagraph for milk acid coagulation properties. Formagraph and LAOR obtained similar patterns for gelation time and gel firming rate. However, in some samples, the gel firmness at 60 minutes did not show similar patterns for the two methods, especially for those with weaker gels. In general, Formagraph could be used in studying acid coagulation properties of milk, especially on many samples.

Paper II modeled the acid coagulation process using data retrieved from the Formagraph. Acid coagulation parameters were estimated from model equation and compared with the traditional parameters derived from the Formagraph output. MATLAB was used to fit the acid coagulation curves in four milk samples analyzed 10 times (except for one sample, which was tested 9 times). Thirty-nine model equations were fitted. Results showed good correlation between the model parameters and the traditional parameters. Less variation within parallels (replicates) was obtained for the model parameters (gel firming rate and final gel strength) than for traditional parameters. The results showed that milk acid coagulation parameters could be estimated from the

model with good repeatability especially for the gel-firming rate and the final gel strength.

Paper III describes the effects of milk protein polymorphism, salts distribution and casein micelle size on the rennet, and acid coagulation properties of the milk. More favorable rennet coagulation properties were obtained by α_{s1}-CN BC, β-CN A^1A^2 and κ-CN BB compared to the BB, A^2A^2 and BE genotypes of the respective caseins, while composite genotype BC-A^2A^2-BB was associated with improved rennet coagulation properties compared to the rest of the composite genotypes. Surprisingly, improved acid coagulation properties were favored by κ-CN AA and composite genotype BB-A^2A^2-AA, which have been associated with poor rennet coagulation properties; moreover, acid coagulation properties were not significantly influenced by α_{s1}- and β-CN genotypes. Calcium (Ca) distribution in milk was associated with variations in the rennet coagulation properties only, while phosphorus (P) content was associated with both rennet and acid coagulation properties. In brief, higher levels of total and micellar Ca were associated with improved rennet coagulation properties (shorter rennet clotting time; RCT) and shorter rennet curd firming time (k_{20}), while soluble calcium was associated with higher rennet curd firmness at 30 minutes. Higher total phosphorus lowered the time taken for the gel formation (both rennet and acid gels). Higher soluble P favored acid coagulation properties (shorter gelation time and higher gel

firmness). A higher amount of phosphorylation in α_{s1}-CN (i.e., α_{s1}-CN-9P) impaired rennet and acid coagulation properties of milk. Conclusively, some milk protein variants associated with improved rennet coagulation properties impaired acid coagulation properties. Whereas milk protein genotypes that improved acid coagulation properties impaired rennet coagulation properties.

Paper IV investigated the effects of milk protein genotypes (α_{s1}-CN, κ-CN and β-LG) on the physical and chemical properties of cultured skim milk on the fresh (one-day storage; D1) and stored cultured milk (fourteen days storage; D14). The particle size distribution and elastic properties of the gel (G′) were not significantly influenced by the milk protein genotypes. Significant effects of κ-CN/β-LG composite genotype were observed on the yield stress and degree of syneresis in the D14 samples of cultured milk (i.e., the samples with AA/AB and BB/AB composite genotypes of κ-CN/β-LG had higher yield stress and lower degree of syneresis compared to AA/BB and BB/BB). However, the inclusion of protein content in the models reduced the effects of κ-CN/β-LG composite genotypes on the yield stress. This indicates that protein content could be the main cause of the differences in the yield stress between the samples. On the other hand, the effect of κ-CN/β-LG composite genotype combinations on the degree of syneresis were not influenced by the protein content in the model. The concentrations of lactic acid and orotic acid in the D1 cultured milks were influenced by the

α_{s1}-CN genotypes and κ-CN/β-LG composite genotypes, respectively. These effects were not observed after the inclusion of the protein content of the fresh milk in the model. Therefore, differences in the concentration of lactic acid and orotic acid are explained by the protein content in the milk rather than by the κ-CN/β-LG composite genotypes. The concentration of acetoin was influenced by the α_{s1}/κ-CN composite genotypes both before and after the inclusion of protein content in the model as covariate. Since the protein content explained variations in the rheological properties of the samples analyzed, future research should evaluate effects of milk protein genotypes at equal protein concentration. Results could provide possibilities for improving water-holding capacity in low fat acid gels by using milk protein genomics.

Sammendrag

Evaluering av melkens koaguleringsegenskaper er svært viktig for meieriindustrien, fordi disse gir informasjon om melkens egnethet for produksjon av både ost og fermentert melk. Melk med kortere koaguleringtid er mer hensiktsmessig for produksjon av ost sammenlignet med melk som ikke koagulerer eller har dårlige koaguleringsegenskaper (melk som tar lengre tid å koagulere). Flere parametere brukes til å studere melkens koaguleringsegenskaper, dvs. tiden frem til melken starter å koagulere, geldannelsens hastighet og endelig fasthet på gelet. Low amplitude oscillation rheologi (LAOR) og Formagraph (Lattodinamografo) er de mest populære metodene som brukes til å overvåke melkens koaguleringsegenskaper. LAOR har blitt mye brukt til å studere både løpe og syre koagulering av melk, mens Formagraph opprinnelig ble designet for å studere løpekoagulering. LAOR er begrenset av det faktum at det bare måler én prøve om gangen i forhold til Formagraph, som kan måle mer enn én prøve (paralleller) samtidig. En alternativ metode til LAOR er nødvendig for å måle syrekoagulering fordi det er nødvendig å kunne analysere flere prøver samtidig. Forskjeller ved løpekoagulering av melk har vært assosiert med de ulike genotypene av melkeprotein i de fleste kommersielle raser av melkeku. Imidlertid er det begrensede studier tilgjengelig på effekter av de ulike genotypene av melkeprotein, salter (Ca, Mg

og P), kaseinmicellestørrelse og melkeproteinets sammensetning på melkens syrekoaguleringsegenskaper. Hovedmålet med dette prosjektet var derfor å undersøke effektene av melkens genotyper av protein på løpe- og syrekoaguleringsegenskapene til Norsk Rødt Fe (NRF).

Artikkel I beskriver en sammenligning av metodene LAOR og Formagraf for å måle melkes syrekoaguleringsegenskaper. Både Formagraf og LAOR oppnådde lignende mønstre for geleringstid og hastighet på geldannelsen. For noen av prøvene fikk man imidlertid ikke likt mønster for gelfasthet etter 60 minutter for de to metodene, spesielt for de prøvene med svakere geler, men det ble konkludert med at generelt kan Formagraf brukes til å studere syre koagulasjonsegenskaper av melk, spesielt når man har mange prøver.

I artikkel II ble syrekoaguleringsprosessen modellert ved å bruke data hentet fra Formagrafen. Syrekoaguleringsparametere ble estimert fra en ligning som beskriver modellen og sammenlignet med de tradisjonelle parameterne avledet fra resultater på Formagrafen. MATLAB ble brukt til å tilpasse modellene for koaguleringskurvene til de fire melkeprøvene som ble analysert 10 ganger (bortsett fra en prøve, som ble testet 9 ganger, dette gav 39 modell ligninger). Resultatene viste god korrelasjon mellom modellparameterne og de tradisjonelle

parameterne. Mindre variasjon innenfor paralleller (replikater) ble oppnådd for modellparameterne (koagueringshastighet og endelig gelstyrke) enn for de tradisjonelle parametere. Resultatene viste at syrekoaguleringsparametere kunne estimeres fra modellen, med god repeterbarhet, spesielt for koaguleringshastigheten og den endelige gelstyrken.

Artikkel III beskriver effekter av genetiske kaseinvarianter (α_{s1}-, β- og κ-CN), av de sammensatte kaseinvariantene (α_{s1}-β-κ-CN), av genotyper av myseproteinet β-LG, og av fordelingen av salter, størrelsen på kaseinmiceller, på melkens sammensetning og på melkenes løpe og syre koaguleringsegenskaper. En mer gunstig løpekoagulering ble funnet ved α_{s1}-CN BC, β-CN A^1A^2 og κ-CN BB sammenlignet med BB, A^2A^2 og BE-genotypene av de respektive kaseinene, mens de sammensatte kaseinvariantene BC-A^2A^2-BB var assosiert med forbedrete løpekoaguleringsegenskaper sammenlignet med resten av de sammensatte genotypene. Overraskende ble forbedrede syrekoaguleringsegenskaper favorisert av κ-CN AA og den sammensatte kaseinvarianten BB-A^2A^2-AA, som har vært assosiert med dårlige løpekoaguleringsegenskaper, og dessuten var syrekoaguleringsegenskapene ikke signifikant påvirket av α_{s1}- og β-CN-genotypene. Kalsiumfordelingen i melk var bare knyttet til variasjoner i løpekoaguleringsegenskapene, mens fosforinnholdet

var forbundet med både løpe og syre koaguleringsegenskapene. Kort fortalt ble høyere nivåer av total og micellært Ca assosiert med forbedrede løpekoagulasjonsegenskaper; kortere løpekoaguleringstid (RCT) og raskere koaguleringshastighet (k_{20}), mens løselig kalsium (Ca) var assosiert med økt koagelfasthet etter 30 minutters løpekoagulering. Høyere totalt fosfor (P) senket koagulasjonstiden (både for løpe og syre geler). Høyere mengde oppløselig P favoriserte syrekoaguleringsegenskapene (kortere geleringstid og høyere gelfasthet). Høyere grad av fosforylering av α_{s1}-CN (dvs. α_{s1}-CN-9P) svekket løpe og syre koaguleringsegenskapene til melk. Arbeidet konkluderes med at noen genetiske varianter av melkeprotein som er assosiert med forbedrede løpekoaguleringsegenskaper faktisk fører til nedsatte syrekoaguleringsegenskaper, mens genetiske varianter av melkeprotein som forbedret syrekoaguleringsegenskapene svekket løpekoaguleringsegenskapene.

Artikkel IV undersøkte effekten av de ulike genotypene av melkeprotein (α_{s1}-CN, K-CN, β-LG) på de fysiske og kjemiske egenskapene til skummet kulturmelk. Partikkelstørrelsesfordelingen og de elastiske egenskapene til gelen (*G′*) ble ikke signifikant påvirket av de ulike melkproteingenotypene. Signifikante effekter av κ-CN/β-LG-genotypekombinasjonene ble observert på flytgrense og

synereseg rad (dvs. prøvene med AA/AB og BB/AB genotyper av κ-CN/β-LG hadde høyere flytgrense og lavere grad av synerese sammenlignet med AA/BB og BB/BB genotypene). Imidlertid ved å inkludere protein i modellene reduserte en effekten av genotype-kombinasjon (κ-CN/β-LG) på flytgrense. Dette indikerer at proteininnholdet kan være hovedårsaken til forskjellene i flytgrense mellom prøvene. På den annen side ble effekten av κ-CN/β-LG genotype kombinasjonene på graden av synerese ikke påvirket av proteininnhold i modellen. Konsentrasjonene av melkesyre og orotinsyre i fersk kulturmelk ble påvirket av henholdsvis α_{s1}-CN og κ-CN/β-LG kombinasjonene av genotypene, disse effektene ble ikke observert etter at proteininnhold ble inkludert i modellen. Forskjeller i konsentrasjonen av melkesyre og orotinsyre kan forklares av proteininnholdet i melken i stedet for av K-CN / β-LG-genotypene. Konsentrasjonen av acetoin ble påvirket av de sammensatte genotypene av α_{s1}-/κ-CN både før og etter inklusjon av proteininnhold i modellen som kovariater. Siden proteininnholdet kunne forklarte variasjonene i de reologiske egenskapene til de analyserte prøvene, bør fremtidig forskning evaluere effekter av melkeproteingenotyper ved lik proteinkonsentrasjon. Resultatene kunne da gi muligheter for å forbedre vannbindingskapasiteten i syregeler med lavt fettinnhold ved å ta i bruk kunnskap om de genetiske variantene av melkeproteiner.

List of papers

Paper I

Ketto, I.A., Schüller, R.B., Rukke, E., Johansen A-G., Skeie, S.B. (2015). Comparison between Formagraph and Low-amplitude oscillation rheometry in monitoring acid induced gels in bovine milk. *Annual Transactions of the Nordic Rheology Society, Volume 23, 181-187.*

Paper II

Ketto, I. A., Skeie, B.S., Schüller, R.B. (2016). Modelling of acid coagulation data analyzed by Formagraph and estimation of milk coagulation parameters. *Annual Transactions of the Nordic Rheology Society, Volume 24, 87-92.*

Paper III

Ketto, I. A., Knutsen, T. M., Øyaas, J., Heringstad, B., Ådnøy, T., Devold, T. G., & Skeie, S. B. (2017). Effects of milk protein polymorphism and composition, casein micelle size and salt distribution on the milk coagulation properties in Norwegian Red cattle. *International Dairy Journal*, 70, 55-64.

Paper IV

Ketto, I.A., Øyaas, J., Tormod Ådnøy., Johansen A-G., Schüller, R.B., Narvhus, J., Skeie, S.B. (2017). The influences of milk protein genotypes on the physical properties of the cultured milk. (Submitted Manuscript).

General abbreviations

a_{30}	Maximum width of curves at 30 min
BSA	Bovine Serum Albumin
CCP	Colloidal calcium phosphate
CSLM	Confocal laser microscopy
CMP	Caseinomacropeptide
CN	Casein
G′	Storage modulus/Elastic properties
G″	Loss modulus/Viscous properties
G30	Gel firmness at 30 minutes
G60	Gel firmness at 60 minutes
GFR	Gel firming rate
GT	Gelation time
k_{20}	Time taken for the width of the curves to increase to 20 mm
LA	Lactalbumin
LAOR	Low amplitude oscillation rheometry
LG	Lactoglobulin
NRF	Norsk Rødt Fe (Norwegian Red cattle)
RCT	Rennet clotting time
SNP	Single nucleotide polymorphism
SRB	Swedish Red breed
P	Phosphorus
Mg	Magnesium
Ca	Calcium
Pa	Pascal

Amino acid abbreviations

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
Cys	Cysteine
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Table of contents

Acknowledgements i
Summary iv
Sammendrag ix
List of papers xiv
General abbreviations xv
Amino acid abbreviations xvi
Table of contents xvii
1. Introduction 1
1.1. Bovine milk gross composition 1
1.2. Milk protein composition 2
1.3. Molecular aspects of the milk proteins 5
1.4. Milk coagulation properties 21
1.5. Analysis of milk coagulation properties 23
1.5.1. Low amplitude oscillation rheometry 24
1.5.2. Formagraph (Lattodinamografo) 27
1.6. Research justification 29
2. Objectives 33
3. Materials and methods 35
3.1. Blood samples and genotyping 35
3.2. Milk analyses 35
3.3. Analyses on the cultured milk 36
4. Results and discussion 38
4.1. Method development (Paper I and II) 38
4.2. Milk coagulation properties (Paper III) 40
4.3. Properties of cultured skim milk (Paper IV) 44
4.3.1. Physical properties 45
4.3.2. Fermentation metabolites 47
5. Conclusions and research outlook for the future 50
6. References 52
7. Papers (I to IV) 63

1. Introduction

1.1. Bovine milk gross composition

Milk serves as an important diet for the growth and development of, since it contains protein (3.4 %), fat (3.7 %), lactose (4.8 %), ash (0.7 %), water (87.3 %) and the minor nutrients (e.g., vitamins (Fox et al. 2015). Milk components occurs in the three phases: the true solution (of lactose, organic and inorganic salts and vitamins in water), dispersed proteins (whey proteins and large colloidal aggregates/casein micelles), and finally, is the milk lipids. Milk lipids are expressed in a fat globule with a diameter of 0.1 to 15 µm, depending on the breed and stage of lactation (Fox et al. 2015). The diameter of the fat globules can be reduced to about 1 µm by the mechanical treatment of the milk, i.e., homogenization (Michalski et al. 2001). Milk proteins (mainly caseins) replace disrupted membranes of the fat globules during homogenization (Walstra et al. 2006). This was found improve the technological properties of the fermented milks (Lee & Lucey 2010). Salts of the milk exist in the dynamic equilibrium between soluble phase of the milk and colloidal phase of the milk (Figure 1). Both pH and temperature were found to affect this distribution (Gaucheron 2005). Calcium, magnesium, phosphorus and citrate are partly associated with casein micelles, while sodium, potassium and chloride are associated with the diffusible (soluble) phase of the milk (Gaucheron 2005). The details on the milk protein chemistry will be discussed in the next sections.

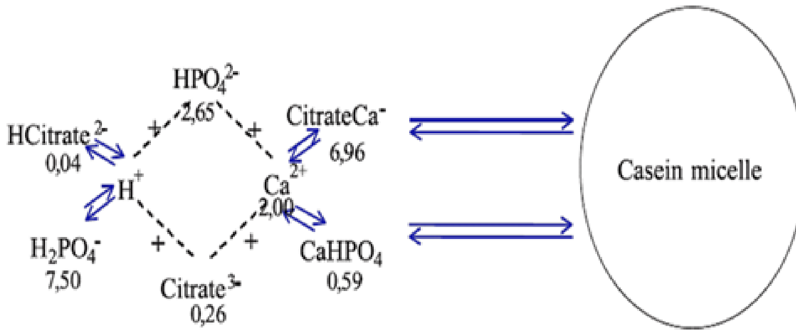

Figure 1: Salt equilibrium between the soluble and the colloidal phase of the milk.
Source: Gaucheron (2005)

1.2. Milk protein composition

Milk proteins play an important role for the nutritional and technological properties of milk and milk products. Two major milk protein classes have been identified: caseins and whey proteins (about 80% and 20%, respectively, in the bovine milk). Caseins (α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN) in their native state aggregate with calcium phosphate to form colloidal aggregates known as casein micelles, with a mean diameter of about 200 nm (Dalgleish 2011), while the whey proteins (e.g., β-LG, α-LA and BSA) occur as soluble monomers or oligomers in the serum phase of the milk. Caseins and whey proteins differ in terms of their amino acid composition, i.e., higher contents of proline (especially in β-CN) and lower levels of cysteine in caseins compared to the lower levels of proline and higher levels of cysteine in whey proteins (Table 1). So caseins have very low contents of α-helix or β-sheets compared to whey proteins, which, in turn, makes caseins more sensitive to proteolytic enzymes than to heat denaturation compared to whey proteins (Fox et al. 2015).

Another difference between caseins and whey proteins is the existence of the various forms of phosphorylation in caseins compared to the whey proteins, which are not phosphorylated. A high number of apolar amino acid residues (Val, Leu, Ile, Phe, Tyr and Pro) and uneven distributions of amino acids cause caseins to have more open structures compared to whey proteins. This give caseins a unique feature of adsorbing air-water and oil-water interfaces (Dickinson 2006; Fox et al. 2015).

Table 1: The overall compositional differences between caseins and whey proteins; **Source:** Fox et al. (2015).

Property	Caseins				Whey proteins	
	α_{s1}-CN B-8P	α_{s2}-CN A-11P	β-CN A²-5P	κ-CN A 1P	α-LA B	β-LG B
Molecular weight	23.614	25.230	23.983	19.023	14.176	18,363
Residue/molecule						
Amino acids	199	207	209	169	123	162
Proline	17	10	35	20	2	8
Cysteine	0	2	0	2	8	5
Disulphides[a]	0	0	0	0	4	2
Phosphate	8	11	5	1	0	0
Carbohydrate	0	0	0	[b]	0	[c]
Hydrophobicity (kJ/residue)	4.9	4.7	5.6	5.1	4.7	5.1
Charged residue/molecule	34	36	23	21	28	30

[a]Intramolecular disulphide bonds, [b]Variable (0 to 6 glycans per molecules), [c]Only in Dr variant

Most of the physicochemical properties of milk (i.e., thermal stability and rheological properties) depend on the properties of caseins and how they are assembled into micelles in milk. Hence, a better understanding of the chemistry of caseins and their structural organisation (casein micelles) is considered to be essential in the understanding of the various dairy processes (de Kruif et al. 2012). This has led to intensive scientific debates on the structure and the

physicochemical properties of casein micelles (Holt & Horne 1996; Horne 1998; Walstra 1999). However, a common opinion about the casein micelle structures has been elaborated in most of the models established (Dalgleish 2011; de Kruif et al. 2012; Horne 1998; Horne 2002). These models show that κ-CN is found on the surface of the casein micelles with the N-terminal being attached to the casein supramolecular structure hydrophobically, while the C-terminal/CMP (residue 106 to 169) protrudes on the surface of casein micelles. CMP provides the steric stability to casein micelles and the high negative charges, which makes the casein micelles stable and prevent them from aggregating (Dalgleish 2011; Dalgleish & Corredig 2012). The internal structure of the casein micelle is explained by the crosslinking between calcium phosphate nanoclusters and the highly phosphorylated caseins (α_s- and β-CN) (Dalgleish 2011). Furthermore, Dalgleish and Corredig (2012) provided extra details on the internal structure of the casein micelles, i.e., presence and role played by the water channels which are unevenly distributed through the casein micelle structure (Figure 2).

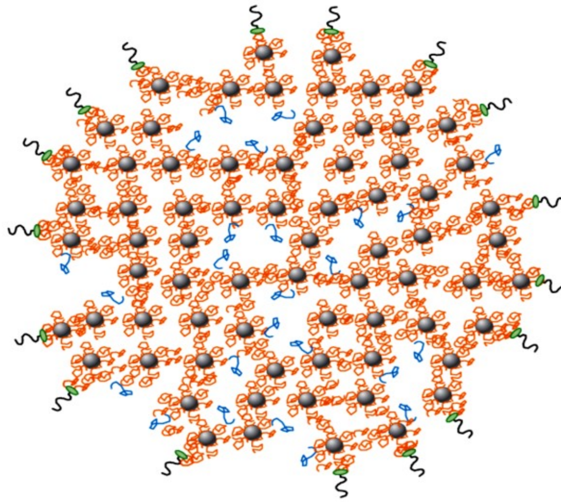

Figure 2 The structure of the casein micelles with protruding electrolyte brushes of κ-CN/CMP (black) and para-κ-CN (green), calcium phosphate nanoclusters (grey circles), α_s- and β-CN (orange) and hydrophobically bound β-CN (blue) which can be drained out of the micelles by cooling (Dalgleish & Corredig 2012).

1.3. Molecular aspects of the milk proteins

Casein genes, i.e., *CSN1S1, CSN2, CSN1S2* and *CSN3* in the bovine genome, which code for α_{s1}-CN, β-CN, α_{s2}-CN and κ-CN, respectively, are closely linked along 250-kilobase-pairs (kb) in chromosome 6 (Threadgill & Womack 1990). Their effects on milk coagulation properties have been estimated together as aggregate/composite genotypes of α_{s1}-β-α_{s2}-κ-CN (Threadgill & Womack 1990). While the genes which code for the whey proteins (α-LA and β-LG), i.e., *LAA* and *LGB*, are located on chromosome 5 and 11 on around 2- and 4-kb of the bovine genome, respectively (Caroli et al. 2009) (Figure 3). Milk proteins are polymorphic due to post-translational modifications (i.e., phosphorylation (only α_S-, β- and κ-CN), glycosylation (only κ-CN)) and genetic polymorphism

caused by single nucleotide polymorphism (SNP) and/or nucleotide deletions or insertions (Caroli et al. 2009). Genetic polymorphisms and post-translation modifications change the physicochemical properties of the proteins due to the change of the net charge, isoelectric point and the hydrophobicity of the proteins (Martin et al. 2013). Different methods used to detect milk protein polymorphism have been described in recent reports (Le et al. 2016; Martin et al. 2013). The following paragraphs will provide an overview of the chemistry of the milk proteins and the nature of genetic polymorphism in bovine milk.

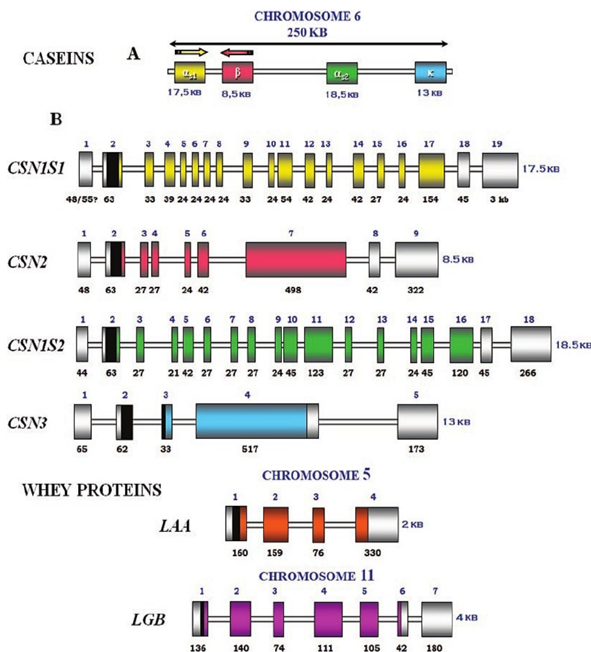

Figure 3: The structural organization of the genes coding for caseins (i.e., *CSN1S1, CSN2, CSN1S2* and *CSN3* for α_{s1}-, β-, α_{s2} - and κ-CN, respectively) and whey proteins (*LAA* and *LGB* which code for α-LA and β-LG, respectively)
Source: Caroli et al. (2009)

The α_{s1}-Casein (α_{s1}-CN) accounts for approximately 40% of the casein fraction of the bovine milk and contains 199 amino acid residues on its primary structure with 16 serine residues (Figure 4). The reference (wild type) protein for α_{s1}-CN is phosphorylated on eight Ser residues (α_{s1}-CN B-8P, where B and 8P stand for the reference genetic variant/genotype and the number of phosphorylations). It differs from the minor component (α_{s1}-CN B-9P) that has an extra phosphorylation on Ser41 (Farrell Jr et al. 2004). Huppertz (2013) reported two phosphorylation centers on α_{s1}-CN (on residue 41-51 and 61-70). Phosphorylated centers are the important sites for the calcium phosphate nanocluster formation (McMahon & Oommen 2013).

1 10 20
Arg-Pro -Lys- His - Pro- Ile - Lys - His - Gln- Gly-Leu- Pro- Gln -Glu - Val- Leu-Asn-Glu-Ans-Leu-
21 30 40
Leu-Arg -Phe- Phe - Val- Ala - Pro - Phe- Pro -Glu-Val- Phe- Gly - Lys- Glu- Lys-Val-Asn- Glu-Leu-
41 50 60
Ser – lys – Asp –Ile - Gly- SerP- Glu - SerP-Thr-Glu-Asp- Gln- Ala- Met- Glu- Asp-Ile - Lys- Gln- Met-
61 70 80
Glu-Ala – Glu -SerP- Ile – SerP- SerP- SerP- Glu-Glu-Ile - Val- Pro- Asn- SerP-Val-Glu-Gln- Lys-His-
81 90 100
Ile – Gln -Lys- Glu - Asp- Val - Pro - Ser- Glu- Arg-Try- Leu-Gly- Tyr- Leu - Glu-Gln-Leu-Leu-Arg-
101 110 120
Leu-Lys- Lys- Try - Lys- Val - Pro - Gln- Leu -Glu-Ile – Val - Pro-Asn-SerP- Ala-Glu-Glu- Arg-Leu-
121 130 140
His- Ser- Met- Lys – Glu- Gly - Ile - Hist- Ala - Gln-Gln -Lys -Glu- Pro-Met - Ile- Gly- Val-Asn- Gln-
141 151 160
Glu- Leu- Ala- Tyr - Phe- Try - Pro - Glu - Leu-Phe-Arg- Gln - Phe- Tyr-Gln - Leu-Asp-Ala-Tyr-Pro-
161 170 180
Ser- Gly - Ala- Trp - Tyr- Tyr – Val - Pro - Leu-Gly- Thr-Gln - Tyr- Thr-Asp -Ala - Pro-Ser-Phe-Ser-
181 190 200
Asp- Ile - Pro- Asn- Pro - Ile - Gly - Ser -Glu -Asn- Ser- Glu – Lys -Thr-Thr- Met-Pro-Leu- Trp

Figure 4: Amino acid sequence in the α_{s1}-CN B-8P, with 8 phosphorylated Ser residues (red underlined), **Source**: Farrell et al. (2004)

Several mutations on the *CSN1S1* gene (coding for α_{s1}-CN) have been identified, ranging from those caused by deletion or exon skipping (i.e., variants A and H) and those caused by amino acid

substitutions (C, D, E, F and G). Exon skipping resulting in deletion of the 13 amino acid residue 14 to 26 in variant A involves the deletion of the hydrophobic N-terminal including the residues cleaved by residual chymosin during cheese ripening (Phe23-Phe24-Val25) (Farrell Jr et al. 2004). Variant C differs from variant B on residue 192 where Gly substitutes Glu. Other variants (D, E, F, G and H) are shown in Table 2.

Table 2: Genetic variants in αs_1-CN compared to the reference variant **α_{s1}-CN B-8P** (Allmere et al. 1997; Caroli et al. 2009; Huppertz 2013; Ketto et al. 2017)

Variant	Amino acid position						Examples
	14-26	51-58	53	59	66	192	
A	Deletion						Red Friesian and German Red
B			**Ala**	**Gln**	**SerP**	**Glu**	**Common in NRF, SRB**
C						Gly	Danish Jersey
D			ThrP				Jersey cattle
E				Lys		Gly	*Bos grunnies* (Yak)
F					Leu		German black and white cattle
G						Glu	Italian brown cattle
H		Deletion					Kuri cattle

The reference/major fraction of α_{s2}-CN (α_{s2}-CN A-11P) in dairy cows has 207 amino acid residues on its structure with 11 phosphorylated residues (Figure 5) (Farrell Jr et al. 2004; Huppertz 2013). This protein constitutes about 10% of the total casein fraction in bovine milk. It consists of two cysteine residues (Cys36 and Cys40) that make α_{s2}-CN form intramolecular and intermolecular di-sulphide bindings (Huppertz 2013). However, more than 85% of this protein exist as a monomer while the rest exists as dimers in either parallel (i.e., amino-to-carboxyl-terminus direction) or antiparallel

configuration (i.e., opposing amino-to-carboxyl-terminus direction) or both (Huppertz 2013; Rasmussen et al. 1992; Rasmussen et al. 1994). Three phosphorylation centers have been found in α_{s2}-CN (residue 8-16, 56-63 and 126-133), therefore, it has more charged regions and hence it is considered the most hydrophilic casein (Farrell Jr et al. 2004; Huppertz 2013). Apart from the reference protein (α_{s2}-CN A-11P), three other forms exist due to the different levels of phosphorylation (i.e., α_{s2}-CN A-10P, α_{s2}-CN A-12P and α_{s2}-CN A-13P) and four other forms due to genetic polymorphism (Farrell Jr et al. 2004; Martin et al. 2013). Interestingly, recent results by Fang et al. (2016) on French Montbélliarde cattle found three extra phosphorylation sites on αs_2-CN, i.e., αs_2-CN-9P, αs_2-CN-14P and αs_2-CN-15P.

1 10 20
Lys-Asn-Thr-Met-Glu –His -Val-SerP-SerP-SerP- Glu- Glu- Ser- Ile - Ile- SerP –Gln - Glu – Thr -Tyr-
21 30 40
Lys- Gln- Glu- Lys- Asn-Met -Ala- Ile- Asn - Pro – SerP- Lys-Glu- Asn- Leu- Cys - Ser –Thr - Phe- Cys-
41 50 60
Lys- Glu- Val- Val- Arg- Asn-Ala- Asn-Glu - Glu – Glu - Tyr- Ser- Ile - Gy- SerP -SerP-SerP-Glu- Glu-
61 70 80
SerP-Ala-Glu- Val- Ala- Thr- Glu- Glu-Val - Lys – Ile - Thr- Val –Asp-Asp- Lys- His- Tyr - Gln- Lys-
81 90 100
Ala- Leu- Asn -Glu- Ile - Asn- Gln- Phe-Tyr- Gln – Lys - Phe- Pro- Gln- Tyr- Leu- Gln –Tyr - Leu- Tyr-
101 110 120
Gln- Gly –Pro - Ile- Val- Leu - Asn-Pro-Trp- Asn - Gln - Val- Lys - Arg-Asn -Ala- Val - Pro- Ile - Thr-
121 130 140
Pro- Thr- Leu- Asn-Arg-Glu - Gln -Leu-SerP-Thr - SerP-Glu-Glu - Asn- Ser- Lys -Lys-Thr –Val - Asp-
141 150 160
Met- Glu- SerP-Thr-Glu-Val -Phe- Thr -Lys- Lys - Thr - Lys-Leu - Thr- Glu- Glu- Glu - Lys- Asn -Arg-
161 170 180
Leu- Asn- Phe - Leu -Lys-Lys-Ile – Ser -Gln- Arg - Tyr - Gln -Lys – Phe -Ala- Leu -Pro -Gln- Tyr -Leu-
181 190 200
Lys- Thr- Val – Tyr - Gln-His-Gln- Lys-Ala –Met - Lys – Pro - Trp – Ile – Gln -Pro - Lys- Thr- Lys -Val-
201 210
Ile- Pro- Tyr - Val - Arg- Tyr

Figure 5: Amino acid sequence in α_{s2}-CN A-11P, with 11 phosphorylated Ser residues (red underlined), **Source**: Farrell et al. (2004).

Genetic polymorphisms identified on the αs_2-CN locus of the bovine species are αs_2-CN A, B, C and D (Table 3). Variant B was

found in Zebu cattle of South Africa (Caroli et al. 2009); but its complete site specific mutation has not yet been identified (Huppertz 2013). Variant C has Gly at position 37, Thr at 47 and Ile at position 130, instead of Glu, Ala and Thr, respectively. Variant D is characterized by the loss of the potential phosphorylation sites due to exon deletion of nine amino acid residues (51 to 59) (Martin et al. 2013).

Table 3: Genetic variants in α_{s2}-CN compared to the reference variant **α_{s2}-CN A 11P** (Caroli et al. 2009; Huppertz 2013)

	Amino acid position				
Variant	33	47	51-59	130	Examples
A	**Glu**	**Ala**		**Thr**	**Most breeds**
B					Zebu cattle (South Africa)
C	Gly	Thr		Ile	Yaks in Nepalese valley and in Mongolia
D			Deleted		Simmental, Ayrshire and some Spanish breeds

The β-Casein (β-CN) contribute approximately 35% of the total casein content in the bovine milk, with β-CN A^2-5P as a reference protein. It has 209 amino acid residues and one phosphate center (residue 14-21, Figure 6), where the five phosphorylated serine residues are found (De Kruif & Holt 2003). This protein is more hydrophobic compared to other caseins. It has a less hydrophobic N-terminal (residue 1-40) with a high net charge and a higher hydrophobic C-terminal end (residue 136-209) with little charge, and a moderate hydrophobic on its intermediate residues (residue 41-135). β-CN is sensitive to the native protease in the milk (i.e., plasmin), which leads to the formation of different peptides/fragments of β-CN, i.e., γ_1, γ_2 , and γ_3-CN which correspond to several peptides on the β-

CN (29-209, 106-209 and 108-209, respectively) (Farrell Jr et al. 2004).

```
1                                        10                                              20
Arg-Glu-Leu-Glu- Glu- Leu-Asn-Val - Pro-Gly- Glu- Ile - Val - Glu- SerP- Leu-SerP-SerP-SerP-Glu-
21                                       30                                              40
Glu –Ser -Ile –Thr- Arg- Ile- Asn- Lys-↓Lys-Ile - Glu- Lys –Phe -Gln- SerP-Glu-Glu- Gln- Gln -Gln-
41                                       50                                              60
Thr-Glu- Asp-Glu - Leu- Gln-Asp- Lys- Ile- His - Pro- Phe-Ala - Gln- Thr- Gln -Ser- Leu- Var -Try-
61                                       70                                              80
Pro-Phe- Pro – Gly- Pro- Ile - Pro- Asn–Ser–Leu– Pro -Gln-Asn - Ile- Pro- Pro–Leu –Thr -Gln -Thr-
81                                       90                                              100
Pro-Val- Val – Val - Pro- Pro- Phe- Leu-Gln-Pro- Glu -Val- Met– Gly –Var- Ser- Lys – Val - Lys - Glu-
101                                      110                                             120
Ala -Met- Ala - Pro- Lys-↓His- Lys-↓Glu- Met-Pro- Phe- Pro- Lys –Try –Pro –Val -Glu- Pro- Phe -Thr-
121                                      130                                             140
Glu- Ser- Gln – Ser -Leu- Thr- Leu-Thr- Asp-Val- Glu -Asn- Leu - His- Leu- Pro-Leu- Pro -Leu - Leu-
141                                      150                                             160
Gln- Ser- Trp - Met- His- Gln- Pro- His - Gln- Pro -Leu- Pro- Pro -Thr- Val- Met –Phe -Pro- Pro -Gln-
161                                      170                                             180
Ser- Val – Leu -Ser- Leu- Ser- Gln- Ser - Lys-Val- Leu - Pro-Val - Pro- Gln- Lys- Ala - Val - Pro- Try-
181                                      190                                             200
Pro- Gln- Arg –Asp-Met - Pro- Ile- Gln- Ala –Phe–Leu -Leu-Tyr- Gln- Glu - Pro –Val - Leu- Gly- Pro-
201
Val- Arg –Gly -Pro-Phe - Pro- Ile- Ile - Val
```

Figure 6: Amino acid sequence in β-CN A^2-5P, with 5 phosphorylated Ser residues (red underlined) and the amino positions for plasmin cleavage (blue arrows), **Source**: Farrell et al. (2004).

Different polymorphisms, i.e., genetic polymorphisms (A^1, A^2, A^3, B C, D, E, F, G, H^1, H^2 and I; Table 4) and phosphorylation sites (5P and 4P) have been reported for β-CN. The genetic variants A^1, A^2, A^3 and B are common to most of the *Bos taurus* breeds. The milk protein genotypes identified for β-CN so far are all due to amino acid substitution. For example, the β-CN A^1 differs from β-CN A^2 at position 67, because of amino acid substitution of His for Pro, while β-CN A^3 has Gln instead of His at position 106. Variant B of β-CN has Arg instead of Ser at position 122. Table 3 shows the rare variants (C to H) which have been discovered so far according to the review by Caroli et al. (2009).

Table 4: Genetic variants in β-CN compared to the reference variant **β-CN A^2-5P** (Caroli et al. 2009; Ketto et al. 2017; Martin et al. 2013; Poulsen et al. 2017)

	Amino acid position														
Variant	18	25	35	36	37	67	72	88	93	106	122	137/8	152	?	Examples
A^1						His									Most breeds
A^2	**SerP**	**Arg**	**SerP**	**Glu**	**Glu**	**Pro**	**Gln**	**Leu**	**Met**	**His**	**Ser**	**Leu/Pro**	**Pro**	**Gln**	**All breeds /Most frequent in NRF**
A^3										Gln					Jersey and Holstein Friesian
B						His					Arg				Most Taurus breeds
C			Ser		Lys	His									Guernsey and Piemontese
D	Lys														East African Boran
E				Lys											Piemontese
F						His							Leu		Mouse-Rhine-Yssel, Danish Red and Jutland Cattle
G						His						Leu			Holstein Friesian
H^1		Cys						Ile							Korean Cattle
H^2							Glu		Leu					Glu	Normande
I									Leu						Italian red, Jersey and German Holstein

The κ-Casein (κ-CN) represents about 15% of the total casein in bovine milk. κ-CN A-1P is the reference protein for κ-CN. It has 169 amino acid residues with no phosphate center (De Kruif & Holt 2003; Holland 2008). The existence of intermolecular disulphide linkages, glycosylation and missense mutations makes κ-CN exist in many forms with different physicochemical properties in bovine milk. The two cysteine residues on the κ-CN structure (Cys11 and Cys88) form inter-molecular disulphide bonds, corresponding to oligomers, in a large proportion of κ-CN (~ 90%), while the remaining 10% occur as monomers (Huppertz 2013) via intra-molecular disulphide bonds (Holland 2008). The mono-phosphorylated form is phosphorylated at Ser149 (Figure 13), whereas di-phosphorylated and tri-phosphorylated forms have additional phosphate groups at Ser121 and Thr145, respectively (Huppertz, 2013).

1 10 20
Gln-Glu-Gln-Asn-Gln- Glu -Gln- Pro -Ile- Arg- Cys- Glu - Lys- Asp-Glu –Arg -Phe-Phe-Ser–Asp-
21 30 40
Lys -Ile- Ala- Lys-Tyr- Ile - Pro –Ile –Gln - Tyr- Val- Leu- Ser- Arg- Tyr - Pro- Ser-Tyr-Gly-Leu-
41 50 60
Asn-Tyr-Tyr-Gln-Gln- Lys- Pro- Val- Ala - Leu- Ile- Asn- Asn- Gln- Phe- Leu- Pro-Tyr-Pro-Tyr-
61 70 80
Tyr-Ala- Lys- Pro-Ala-Ala- Val- Arg – Ser - Pro- Ala- Gln -Ile- Leu -Gln- Trp- Gln-Val- Leu- Ser-
81 90 100
Asn-Thr-Val- Pro-Ala- Lys- Ser- Cys- Gln - Ala- Gln- Pro- Thr-Thr- Met- Ala- Arg- His- Pro-His-
101 110 120
Pro- His- Leu- Ser-Phe-Met- Ala- Ile- Pro - Pro- Lys- Lys- Asn- Gln- Asp- Lys- Thr- Glu- Ile -Pro-
121 130 140
Thr -Ile- Asn - Thr-Ile- Ala- Ser- Gly- Glu - Pro- Thr- Ser- Thr -Pro- Thr- Thr –Glu -Ala-Val-Glu-
141 150 160
Ser- Thr- Val- Ala-Thr-Leu- Glu- Asp- SerP- Pro- Glu- Val- Ile- Glu- Ser- Pro - Pro-Glu- Ile-Asn-
161
Thr-Val -Gln -Val-Thr-Ser - Thr- Ala - Val

Figure 7: Amino acid sequence in κ-CN A-1P, with 1 phosphorylated Ser residues (red underlined) and the amino positions for chymosin cleavage (blue arrows), **Source**: Farrell et al. (2004).

About 40% of the κ-CN in bovine milk occur in the non-glycosylated form, the rest is glycosylated with up to six glycans on their C-terminal fragment (residue 106 to 169) (Huppertz 2013). The glycoforms include galactose (Gal), *N*-acetylgalactosamine (GalNAc) and *N*-acetyl neuraminic acid (NANA) (Fox et al. 2015; Huppertz 2013). The mono-glycosylated κ-CN has glycan on residue Thr131, while the di-glycosylated κ-CN has extra glycan on Thr142. In tri-glycosylated κ-CN there is an additional glycan on residue Thr133, while tetra-glycosylated κ-CN (κ-CN B) has an extra glycan on residue Thr145. The extra two glycans on residue Thr121 and Thr165 are not confirmed to date (Huppertz 2013). The four glycans on κ-CN B increase the surface charge on the casein micelles. This has stabilizing and size-controlling effects

on the casein micelles (Bijl et al. 2014a; Holland 2008) and probably gives different physicochemical properties compared to the less glycosylated κ-CN. The differences between κ-CN B and A is at amino acid positions 136 and 148. At position 136 of κ-CN B, Thr substitutes Ile, while at amino acid position 148, Ala substitutes Asp. κ-CN E differs from the A variant at position 155 where Gly substitutes Ser (Martin et al. 2013). Table 5 shows the other genotypes discovered in κ-CN (E to J) in different breeds as shown in Martin et al. (2013).

Table 5: Genetic variants in κ-CN compared with the reference variant **κ-CN A-1P** (Caroli et al. 2009; Hallén et al. 2007; Ketto et al. 2017; Martin et al. 2013)

	Position							
Variant	10	97	104	135	136	148	155	Examples
A	**Arg**	**Arg**	**Ser**	**Thr**	**Thr**	**Asp**	**Ser**	**Common in *Bos taurus cattle***
B					Ile	Ala		Most of breeds
C		His						Grey Alpine, German Simmental etc.
E							Gly	Holstein Friesian, Ayrshire etc
F[1]						Val		Yakuti
F[2]	His				Ile	Ala		Finish Ayrshire
G[1]		Cys				Ala		Pinzgauer
G[2]						Ala		*Bos grunniens* (Yak)
H					Ile			Madagascar Zebu and White Fulani cattle
I			Ala					Ivory coast cattle etc.
J						Ala	Arg	Some *Bos taurus* cattle

The β-lactoglobulin (β-LG) and alpha-lactalbumin (α-LA) are the major whey proteins in bovine milk. β-LG accounts for approximately 50% of the total whey proteins (Farrell Jr et al. 2004; Fox et al. 2015). The reference protein for this protein is β-LG B with 161 amino acids (Figure 14) (Farrell Jr et al. 2004). Compared to caseins, whey proteins in their native form are dispersed in a solution of lactose and minerals and has higher amounts of sulphur containing amino acids and lower levels of proline. This lets β-LG maintain its globular structure compared to caseins (Fox et al. 2015). Unlike caseins, β-LG is prone to heat denaturation. Upon β-LG denaturation, it interacts with κ-CN via disulphide bonds to form β-LG/casein micellar complex. This has been found to improve the rheological properties of acid milk gels (Lucey 2004).

1 10 20
Leu- Ile- Val - Thr- Gln-Thr -Met-Lys- Gly - Leu-Asp-Ile -Gln- Lys- Val- Ala -Gly- Thr -Trp -Tyr-
21 30 40
Ser- Leu- Ala - Met-Ala-Ala - Ser- Asp- Ile - Ser-Leu - Leu-Asp-Ala- Gln-Ser- Ala – Pro -Leu Arg-
41 50 60
Val- Tyr- Val - Glu- Glu-Leu- Lys- Pro- Thr-Pro-Glu- Gly-Asp-Leu- Glu-Ile- Leu –Leu- Gln- Lys-
61 70 80
Trp- Glu- Asn - Gly- Glu- Cys-Ala- Gln- Lys-Lys- Ile – Ile - Ala- Glu- Lys-Thr-Lys – Ile - Pro- Ala-
81 90 100
Val - Phe- Lys – Ile - Asp- Ala- Leu- Asn-Glu-Asn- Lys-Val- Leu-Val- Leu-Asp-Thr- Asp -Tyr-Lys-
101 110 120
Lys - Tyr- Leu- Leu- Phe- Cys- Met- Glu-Asn-Ser- Ala- Glu -Pro- Glu-Gln-Ser-Leu- Ala - Cys-Gln-
121 130 140
Cys – Leu -Val - Arg-Thr- Pro- Glu- Val- Asp-Asp- Glu-Ala- Leu-Glu-Lys-Phe-Asp –Lys - Ala-Leu-
141 150 160
Lys - Ala- Leu- Pro - Met- His- Ile - Arg- Leu- Ser- Phe- Asn-Pro-Thr- Gln-Leu-Glu- Glu - Gln-Cys-
161
His - Ile

Figure 8: Amino acid sequence in β-LG, **Source**: Farrell et al. (2004).

Different genotypes of β-LG have been associated with different denaturation temperatures and/or pressure treatments.

For example, the β-LG B variant is more prone to denaturation compared to the A variant at low pressure treatments, while at higher temperatures, β-LG A denatures more rapidly compared to B (Anema et al. 2005). Furthermore, Li (1997) reported a higher proportion of denatured β-LG at 85 °C for 15 min in the milk samples with κ-CN AA compared to BB genotypes (91% vs. 78.5%). β-LG is neither phosphorylated nor glycosylated, except for the very rare genetic variant Dr discovered in the Droughtmaster breed of Australia, which was found to be glycosylated at Asn28 (Bell et al. 1970; Bell et al. 1981). This variant (Dr) has the same sequence as the A variant, except Dr contains the carbohydrate moiety at Asn28 (Bell et al. 1981). Several β-LG genotypes have been discovered. A, B and C are the most common variants in *Bos taurus*. A differs from B with the amino acid substitution at two positions, i.e., 64 and 118, where Asp and Val substitute Gly and Ala, respectively. Variant C differs from B at position 59, where His substitutes Gln. The remaining genotypes for β-LG are presented in Table 6.

Table 6: Genetic variants in β-LG compared to the reference variant **β-LG B** (Caroli et al. 2009; Hallén et al. 2007; Ketto et al. 2017; Martin et al. 2013)

	Position													
Variants	28	45	50	56	59	64	70	78	108	118	126	129	158	Examples
A						Asp				Val				Common to all breeds
B	**Asp**	**Glu**	**Pro**	**Ile**	**Gln**	**Gly**	**Lys**	**Ile**	**Glu**	**Ala**	**Pro**	**Asp**	**Glu**	**All breeds, including NRF and SRB**
C					His									Jersey
D		Gln												German Holstein and German Simmental
Dr	Asn													Droughtmaster
E													Gly	Nepal grunniens and Australian javanicus
F			Ser										Gly	Rare
G								Met					Gly	Rare
H						Asp	Asn			Val				Italian Friesian
I									Gly					Polish red
J											Leu			Hungary grey
W				Leu										Jersey and Red Holstein×Simmental

The α-Lactalbumin (α-LA) is the second major whey protein in milk and it contributes 20% of the whey proteins in bovine milk (Fox et al. 2015). Cysteine and methionine are present in α-LA as a chief source of sulphur. Cysteine facilitates the formation of intramolecular disulphide (S-S) bonds (Fox et al. 2015; Martin et al. 2013). The reference protein for α-LA is α-LA B. It has 123 amino acid residues on its primary structure (Figure 9).

1 10 20
-Glu-Gln-Leu-Thr- Lys- Cys-Glu-Val- Phe- Arg- Glu-Leu-Lys-Asp-Leu- Lys-Gly-Tyr-Gly-Gly-
21 30 40
Val- Ser -Leu- Pro-Glu-Trp-Val- Cys - Thr-Thr- Phe-His-Thr- Ser -Gly-Tyr-Asp-Thr-Gln-Ala-
41 50 60
Ile - Val- Gln- Asn- Asn-Asp-Ser- Thr - Glu-Tyr-Gly- Leu-Phe-Gln -Ile- Asn-Asn-Lys -Ile- Trp-
61 70 80
Cys - Lys-Asp-Asp- Gln- Asn-Pro –His - Ser-Ser- Asn- Ile- Cys- Asn-Ile- Ser - Cys-Asp-Lys-Phe-
81 90 100
Leu- Asp-Asp-Asp- Leu- Thr-Asp-Asp-Ile - Met- Cys-Val- Lys-Lys - Ile- Leu- Asp-Lys-Val-Gly-
101 110 120
Ile - Asn-Tyr-Trp - Leu- Ala-His-Lys- Ala - Leu- Cys-Ser- Glu-Lys-Leu-Asp-Gln- Trp-Leu-Cys-
121
Glu- Lys-Leu-

Figure 9: Amino acid sequence in α-LA B, **Source**: Farrell et al. (2004).

Nutritionally, *α*-LA is a good source of essential amino acids in the human diet (e.g., cysteine and methionine). In presence of β-LG, α-LA interacts with other molecules via disulphide (S-S) bonding during thermal denaturation (Wijayanti et al. 2014) since α-LA is more stable to thermal denaturation than β-LG. To date, four genotypes have been discovered for α-LA, i.e., A, B, C and D (Table 7). Variant A differs from B at position 10 where, Gln substitutes Arg, while variant D has His at position 65 instead of Gln. The C variant differs from B by Asn to Asp or Gln to Glu substitutions (Farrell Jr et al. 2004).

Table 7: Genetic variants in α-LA compared to the reference variant **α-LA B** (Caroli et al. 2009; Martin et al. 2013)

Variant	Position			Examples
	10	?	65	
A	Gln			All *indicus* and some *taurus* breeds
B	**Arg**	**Asp/Glu**	**Gln**	**Common to all breeds**
C		Asn/Gln		Bali cattle (*Bos Javanicus*) in Australia
D			His	Some *Bos taurus* breeds

1.4. Milk coagulation properties

Casein micelle destabilization is the key step in manufacturing cheese and fermented milks (such as yoghurt). Methods used to destabilize the micellar structure are, for example, enzymatic coagulation by using chymosin (EC.3.4.23.4) in rennet coagulation and glucono-δ-lactone (GDL) in the acid coagulation of milk. During rennet coagulation, casein micelles are destabilized enzymatically by specific enzymes (i.e., Chymosin), which cleave the CMP of κ-CN and, hence, reduce the steric stability on the casein micelles by the removal of the hairy layer on the C-terminal of the κ-CN and the reduction of the negative charges (zeta-potential) on the surface of the casein micelles. Rennet coagulation of milk involves two main phases: the primary phase and the secondary/aggregation phase. During the primary phase of rennet coagulation, the specific enzyme (Chymosin) cleaves at Phe105-Met106 of κ-CN residue to form two fragments, i.e., Para-κ-CN and CMP (Corredig & Salvatore 2016). Para-κ-CN is incorporated into the cheese, while the soluble CMP is drained with the whey (Fox et al. 2017). The second phase of rennet coagulation (aggregation phase) involves the self-aggregation of casein micelles under the influence of the free calcium

ion. These processes result in the formation of a three-dimensional gel network. Figure 10 shows the micellar aggregation after the cleavage of the CMP residue of κ-CN.

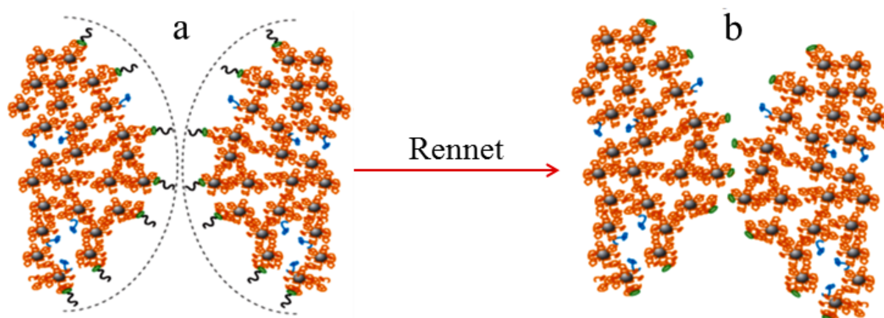

Figure 10: Rennet coagulation process: (a) The native casein micelles with the CMP stabilizing the micelles sterically; (b) Micellar aggregation after collapse of CMP
Source: Dalgleish and Corredig (2012)

During acid coagulation, the casein micelles are destabilized by the reduction surface negative charge on the CMP and by solubilisation of the colloidal calcium phosphate (Lucey 2016). Figure 11 shows what happens during acid coagulation, i.e., the collapse of the hairy layer of casein micelles (Dalgleish & Corredig 2012), which reduces the steric stabilization of the casein micelles, hence micellar aggregation and the solubilisation of colloidal calcium phosphate. Since whey proteins are more heat labile than caseins. At temperatures above 70 °C, most of whey proteins, especially β-LG, are denatured and incorporated into the surface of casein micelles through the -SH groups on κ-CN to form the intermolecular di-sulphide linkages. This was found to improve the

rheological properties of acid-induced gels (Anema et al. 2004; Lucey & Singh 1997).

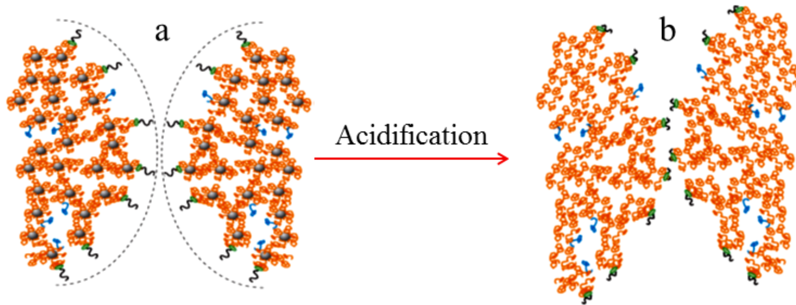

Figure 11: Acid coagulation process of milk. (a) The native casein micelles with the CMP stabilizing the micelles by sterically with the colloidal calcium phosphate (grey circles). (b) Micellar aggregation after the collapse of the hairy layer and the solubilisation of the colloidal calcium phosphate. **Source**: Dalgleish and Corredig (2012).

1.5. Analysis of milk coagulation properties

Milk coagulation involves the transformation of casein micelles, which are stable colloidal aggregates, into a coagulum (gel) by either acid or rennet. During rennet coagulation, the formed gel network entrap the fat globules (Fox et al. 2017). While in acid gels from heated and homogenized milks, the fat globules are part of the formed gel of casein-whey protein network. This process can be monitored by different methods, i.e., Low Amplitude Oscillation Rheometry (LAOR) and use of Formagraph (Lattodinamografo) (Fox et al. 2017). The former involves a non-destructive measurement of the milk gelation process while the latter is destructive. The rennet coagulation processes has been frequently studied by both methods (Auldist et al. 2001; Ipsen et al. 1997), while

acid coagulation properties have been studied by LAOR (Hallén et al. 2009; Lucey et al. 1996; Lucey et al. 1998b).

1.5.1. Low amplitude oscillation rheometry

The most used rheometers in studying milk coagulation properties are Physica MCR (Anton Paar GmbH, Graz, Austria. Figure 12a) and Bohlin VOR Rheometer (Malvern Instruments, Nordic AB, Lund, Sweden). Milk coagulation in both instruments is monitored in a bob-cup measurement system (Figure 12b), where the milk sample (about 14 mL) to be enzymatically modified or acidified is added into the cup and inserted into the temperature-controlled measurement cell of the rheometer. After insertion of the bob into the sample, the bob is set to oscillates at a very low amplitude (strain value defined below the upper-limit of linear viscoelastic region range (LVR)) to ensure that the formed gel/structure is not destroyed; hence, LAOR is a non-destructive measurement (Foegeding et al. 2011).

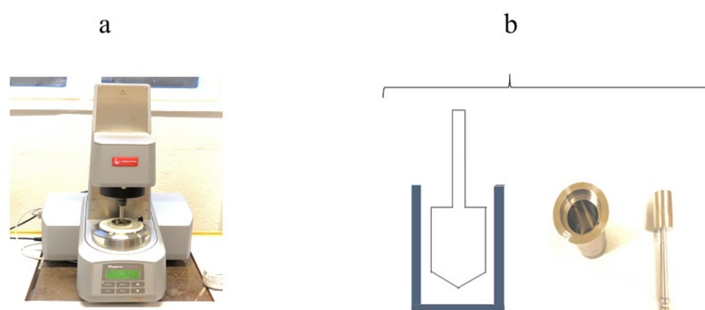

Figure 12: **a**: Physica MCR 301 rheometer (Anton Paar. GmbH, Graz, Austria). **b:** bob and cup measurement system (CC27/Ti with diameter 26.657 mm and 40.03 mm length for the bob specifications and C-CC27/T200/Ti with 28.926 mm diameter for the cup specifications).

The important milk coagulation parameters, i.e., gelation time (GT, min), gel-firming rate (GFR, Pa/min) and gel strength/firmness (in Pa) at a time t ($G't$) can be obtained from the LAOR. These parameters are depicted in the curve of storage modulus (G′) and loss modulus (G″) *vs.* time (Figure 13). G' indicates the magnitude of energy stored per cycle of deformation, while G'' indicates the energy loss per the cycle of deformation (Rao 2014). Before the gelation point of the milk (e.g., at acidification/rennet addition), the process is fully dominated by the viscous behavior ($G'' > G'$), while at the gelation point and the later stage the process is dominated by the elastic behavior ($G' >> G''$) (Foegeding et al. 2011; Rao 2014). The increase in G' is related to the strength and the number of bonds in the gel network (Foegeding et al. 2011). Hence, it is a measure of the stiffness/firmness of the gel (Lucey 2004).

Different authors have expressed GT differently; Fox et al. (2017) defined GT as the time when G' reached a threshold value of 0.2 Pa. Others defined GT as the time when the phase angle (δ) was equal to 45° or the crossover point between G' and G'' (when $G' = G''$) on the coagulation curve (Ipsen et al. 1997; Kristo et al. 2003; Poulsen et al. 2013a). Finally, other authors have defined GT as the point when G' was ≥ 1Pa (Bikker et al. 2000; Lucey et al. 1998b; Srinivasan & Lucey 2002; Waungana et al. 1998). The use of the cross over point between G' and G'' is limited by the fact that some samples do not show any crossing over point throughout the coagulation process (Ketto et al. 2015). Gel firming rate (GFR) is calculated as the maximum slope of G' *vs.* time curve (in

Pa/min), while *G′*60 is the storage modulus of the gel 60 min after the addition of rennet or acidification (in Pa). Furthermore, Fox et al. (2017) defined an extra, important, parameter for the rennet coagulation in rheometers: the set-to-cut time (SCT), as the time between rennet addition and the gel cutting at a proper firmness, i.e., 40Pa (SCT40Pa). A milk sample with good coagulation ability will take a short time to coagulate and have a higher gel strength. Low-amplitude oscillation rheometry is precise for the monitoring of milk coagulation properties and milk gel characterization. However, with the instruments available, only one sample at a time is analyzed. This limits numbers of samples that may be analyzed.

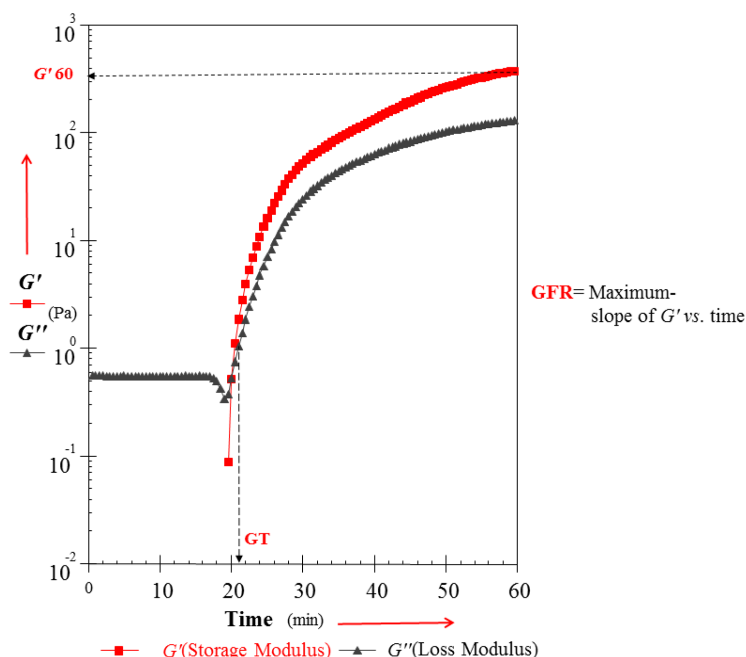

Figure 13: Acid coagulation pattern of milk samples by using glucono-δ-lactone (GDL) as analyzed by Physica MCR 301 rheometer at 32 °C

1.5.2. Formagraph (Lattodinamografo)

The Formagraph is an instrument designed to monitor the rennet coagulation properties of the milk. The working principle of the older version of the Formagraph was described by McMahon and Brown (1982), while Fox et al. (2017) reviewed the working principle of the modern Formagraph (Lattodinamografo). Both versions contain a metal block with ten cuvettes where milk samples are oscillated. In each cuvettes, a pendulum loop registers the viscosity of the milk samples. In the beginning, when the milk is less viscous, the pendulum remains at its original vertical position (zero position) and describes a straight line. After the gel formation, the samples become more viscous and the pendulum loop is dragged by the moving samples from its vertical position resulting in the bifurcation of the line (Figure 14). In the modern Formagraph (i.e., Lattodinamografo), the milk coagulation curves are captured electronically and displayed in a computer output as shown in Fox et al. (2017). While in the older version, the milk coagulation curves are captured on photographic chart paper (McMahon & Brown 1982).

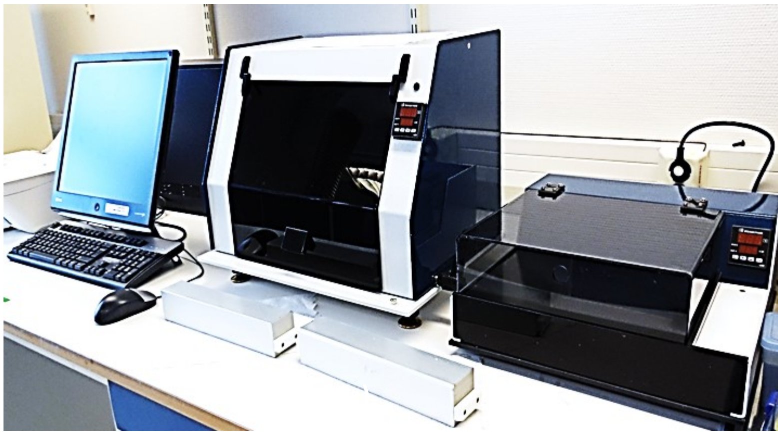

Figure 14: Lattodinamografo unit (LAT; Foss-Italia SpA, Padova, Italy)

Apart from the graphical screen output (or printout) from the computer, Lattodinamografo software also saves files with data that may be used to calculate milk coagulation parameters and to plot the milk coagulation curves manually. These files provide useful information for studying the rennet coagulation properties of milk. One parameter used to study rennet coagulation properties of milk is rennet-clotting time (RCT): the time taken from rennet addition until the point of bifurcation (gelation point). The time taken (in minutes) from the bifurcation point (where the curve splits) until the width of the bifurcate reaches 20 mm (k_{20}). This parameter is equivalent to the time for cutting of the cheese curd. Curd-firming rate (CFR) is the measure $1/k_{20}$ in the Formagraph data. The width (in mm) of the curve (bifurcate) at 30 minutes, which represents the curd strength at 30 minutes, is denoted a_{30} (Figure 15).

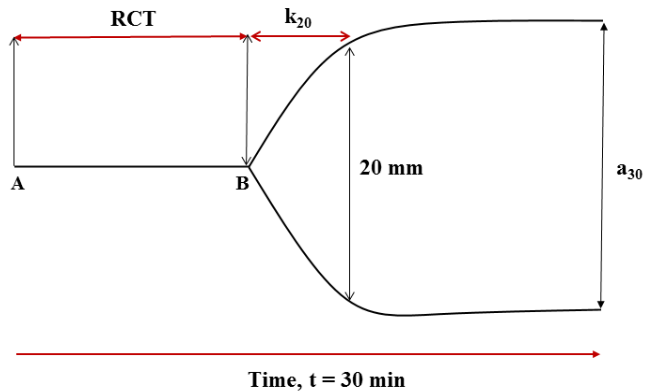

Figure 15: A typical rennet coagulation curve made by the Formagraph monitored for 30 min at 32 °C. RCT = rennet clotting time (min), k_{20} = curd firming time (min) and a_{30}= width of the curves at 30 minutes (mm). A= rennet addition point, B=gelation point

The major differences between low amplitude oscillation rheometry (LAOR) and Formagraph measurements is that LAOR uses a very low amplitude of oscillation; hence, it involves non-destructive measurements. This makes LAOR more sensitive to minor changes during milk coagulation. However, LAOR measures one sample at a time. On the other hand, Formagraph involves destructive measurements, but can measure ten samples at a time. A comparison between rennet coagulation process measured by Formagraph and LAOR has been established, with good correlation between the two methods (Auldist et al. 2001; Ipsen et al. 1997).

1.6. Research justification

Milk protein genomics has been researched intensively within the last four decades. The most frequent alleles discovered so far in modern dairy cattle breeds are α_{s1}-CN (B>C), β-CN (A^2>A^1>B>A^3), κ-CN

(A>B>E) and β-LG (B>A) (Gustavsson et al. 2014a; Hallén et al. 2007; Heck et al. 2009; Lundén et al. 1997). The Danish Jersey breed is an exception as it has been reported to have a higher frequency of κ-CN B compared to A and a slightly higher frequency of α_{s1}-CN C compared to the other modern Scandinavian dairy breeds (Poulsen et al. 2013a). α_{s2}-CN and α-LA are monomorphic in most of the dairy cattle breeds (Farrell Jr et al. 2004).

Genotype BB of α_{s1}-CN was associated with higher milk and protein yield and lower protein concentration in milk, while allele α_{s1}-CN G was associated with lower α_{s1}-CN relative to the other proteins (α_{s2}-, β- and κ-Casein) (Aleandri et al. 1990; Ng-Kwai-Hang et al. 1984). Several publications on milk protein genomics have found significant effects of milk protein genotypes on the rennet coagulation properties in most of the *Bos taurus* cattle. For example, in Italian Holstein, Swedish Red, Finnish Ayrshire, Estonian Native cattle and in Danish breeds rennet coagulation properties were favored by κ-CN B>A>E, β-CN A^1>A^2, β-LG B>A and α_{s1}-CN C>B (Comin et al. 2008; Gustavsson et al. 2014a; Jõudu et al. 2007; Poulsen et al. 2013b). The effects of milk protein variants on the casein micelle size, casein content and casein number (Glantz et al. 2010; Hallén et al. 2009; Heck et al. 2009) could explain these effects. The study by Jensen et al. (2012b) showed that the α_{s1}-β-κ-CN composite genotype (BB-A^1A^2-AB) is associated with good renneting properties, while the composite genotype BB-A^2A^2-AA was associated with poor rennet coagulation properties in both Danish

Holstein and Danish Jersey. Similarly, composite genotypes of β-κ-CN A^1A^2-AE and A^2A^2-AA were associated with poor renneting properties in Italian Holstein and Swedish Red (Comin et al. 2008; Gustavsson et al. 2014a). Studies on the effects of casein post-translational modifications (PTM's) on the milk coagulation properties have also focused on rennet coagulation properties (Bijl et al. 2014a; Jensen et al. 2012a). Improved rennet coagulation properties in Danish Holstein cows were associated with lower fractions of phosphorylated caseins (Frederiksen et al. 2011). On the other hand, higher levels of glycosylation on κ-CN B were associated with improved rennet coagulation (Bijl et al. 2014b; Jensen et al. 2012a).

A limited number of studies have been made on the effects of milk protein polymorphisms on the milk acid coagulation properties. A few studies on the effects of milk protein genetic polymorphism on the acid coagulation properties have been established, for example, in the Swedish Red Breed (SRB) (Allmere et al. 1998a; Allmere et al. 1998b; Hallén et al. 2009). A study on SRB by Allmere et al. (1998a), reported a higher elastic modulus with β-LG B allele compared to A. Another study by Hallén et al. (2009) reported shorter gelation time in the milk samples with β-LG AA compared to AB and a higher elastic modulus with AA and AB variants compared to BB in the milk from Swedish Red. These findings were linked to the effect to the influence of allele A of β-LG on the concentration of β-LG. The same study by Hallén et al. (2009) reported an opposite trend at equal concentration of β-LG, i.e., the higher

elastic modulus in samples with β-LG BB compared to samples with β-LG AA. This agreed with Allmere et al. (1998a). More studies are needed to investigate the effects of milk protein polymorphism, salt distribution and casein micelle size on the milk coagulation properties, especially acid coagulation properties. A rapid method that can generate large data set compared to the conventional method, i.e., LAOR is needed.

2. Objectives

The main objective of this work was to study the effects of milk protein genotypes on the rennet and acid coagulation properties of the milk in Norwegian Red cattle.

The following were the specific objectives of this work

i. **To establish a rapid method to study the acid coagulation properties of milk by comparing the conventional method (Low Amplitude Oscillation Rheometry) with the Formagraph (Lattodinamografo) [paper I]**

   This was intended to establish an alternative method for analysis of acid coagulation properties in many samples during shorter time compared to the conventional method (LAOR) which takes only one sample at a time.

ii. **To model the acid coagulation processes and estimate the acid coagulation parameters measured by Formagraph [paper II]**

   Aimed at the estimation of the acid coagulation parameters from the model used previously in rennet gels and comparison of the parameters estimated from the model with conventional parameters.

iii. **To study the effects of milk protein polymorphism, composition, casein micelle size and salts distribution on the rennet and acid coagulation properties of milk in Norwegian Red cattle [paper III]**

This study was aimed to provide information to the breeders' association and the dairy industry on important aspects of the association between milk protein genes and milk coagulation properties in Norwegian Red cattle.

iv. **To study effects of α_{s1}-CN, κ-CN and β-LG genotypes on the physical properties of cultured skim milk [paper IV]**

Excessive whey separation and poor consistency may limit the quality of cultured milk. This study was aimed at the investigation of the effects of milk protein genotypes on the physical properties of acid gels made by commercial starter cultures.

3. Materials and methods

3.1. Blood samples and genotyping

Blood samples from 118 NRF cows were collected in 9 mL Vacutainer® plastic whole blood tubes spray-coated K3EDTA. Samples were prepared for the paired-end sequencing (2 ×125 bp) using a TruSeq DNA PCR-free library preparation kit and sequenced with the manufacturer's V4 kit (Illumina, San Diego, CA, USA). Nine non-anonymous missense SNPs were identified and the 118 cows were genotyped for the SNPs using the MassArray genotyping platform (Agena Biosciences, San Diego, CA, USA). All reads were aligned against the bovine reference genome UMD 3.1 using BWA-mem version 0.7.10 while the variant calling was established using Freebayes version 1.0.2 (Paper III).

3.2. Milk analyses

The analyses made on the fresh milk samples were the pH (PHM61; Radiometer, Copenhagen, Denmark), gross chemical composition (total protein, fat, casein and lactose content) by MilkoScan FT1 (Foss Electric A/S, Hillerød, Denmark) and milk coagulation properties (i.e., rennet and acid coagulation properties) by Formagraph (LAT; Foss-Italia SpA, Padova, Italy; Papers I, II and III). In addition, casein micelle size was determined by ZetaSizer 3000HS (Malvern Instruments Ltd., Malvern, UK) on the fresh milk samples. Separation of the soluble and colloidal phases of the milk was established by ultracentrifugation of skim milk by a Sorvall discovery 100SE (Kendro

Laboratory Aseville, North Carolina, USA; Paper III) on fresh samples. Fat globule size was measured by MasterSizer 3000HS (Malvern Instrument Ltd., Malvern, UK), while the total salts (calcium, magnesium and phosphorus) in skim milk and supernatant (colloidal) after ultracentrifugation were determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) on cold stored samples (4 °C; Paper III). Milk protein composition and the different phosphorylation states of caseins (α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, β-LG and α-LA) were determined on frozen samples by capillary electrophoresis (G1600AX) coupled with ChemStation software (Agilent technologies, Germany; Paper III).

3.3. Analyses on the cultured milk

Cultured milks were made from fresh milk samples with known milk protein genotypes (α_{s1}-CN, β-CN, κ-CN and β-LG; Paper IV). The physical and chemical properties of the cultured milk were analyzed on the fresh (1 day old; D1) and stored samples of the cultured milk (14 days old; D14). With the exception of the gel microstructure, which was analyzed on the D1 cultured milk samples by Confocal Laser Scanning Microscopy (Leica, Microsystems, CSM, GmbH, Mann Heim, Germany). Rheological properties of the D1 and D14 samples were analyzed by Rheometer (Physica MCR 301, Anton Paar. GmbH, Graz, Austria), while particle size distribution was analyzed by MasterSizer 3000HS (Malvern Instrument Ltd., Malvern, UK) on the D1 and D14 cultured milk. Aromatic compounds were analyzed on the D1 and D14 samples of the cultured milk by Head Space Gas Chromatography

(Agilent, Santa Clara, CA, USA), and organic acids and carbohydrates by High Pressure Liquid Chromatography (Perkin-Elmer, Norwalk, CT, USA).

4. Results and discussion

4.1. Method development (Paper I and II)

The aim of Paper I was to compare acid coagulation measurements between Formagraph and Low amplitude oscillation rheometry (LAOR). The results showed good agreements between the acid gelation time and gel-firming rate for the two methods, similar to previous reports (Auldist et al. 2001; Ipsen et al. 1997), which compared rennet coagulation data between controlled stress rheometer and the Formagraph. In the Fomagraph, some samples (curve Q) showed a typical coagulation pattern (Figure 16). These samples had whey expulsion from the gel during the analysis. This could be explained by the fact that in a weaker gel, the Formagraph pendulum loop loses its contact with the sample over time due to whey expulsion from the gel resulting in a continuous decrease in the width of the curve (gel firmness) towards the end of the measurement (McMahon et al. 1984). This is different from the LAOR that has non-destructive measurements. This could be the reason for slight differences in the maximum gel firmness between the two methods. Other parameters, i.e., gelation time and speed of gel formation, showed similar coagulation patterns between the two methods. The results showed that the Formagraph could be used as an alternative method for monitoring acid coagulation properties in many milk samples, fulfilling the need for higher throughput data for the acid coagulation analysis.

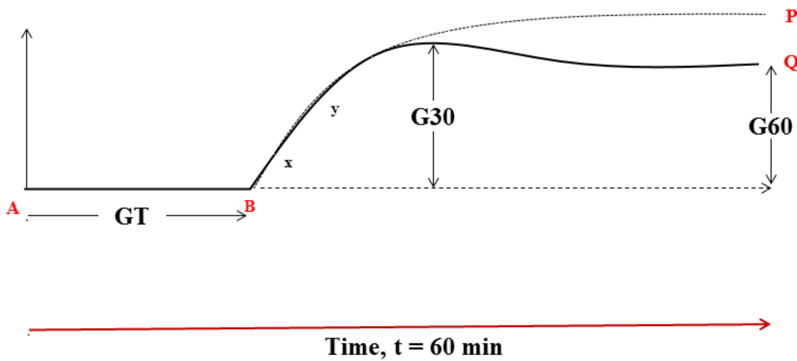

Figure 16: Acid coagulation pattern by Formagraph in a sample with high extent of gel syneresis (GT=acid gelation time, G60=Gel firmness at 60 minutes and GFR=Slope of between point line XY=DG/DT. A = acid addition point, B = gelation point)

The aim of Paper II was to model the acid coagulation curves in order to estimate the acid coagulation parameters from the model and compare these with the traditional parameters derived from the Formagraph output. All samples showed normal coagulation curves without the gel shrinkage previously reported in some samples which experienced whey expulsion from the gel (Ketto et al. 2015). The traditional gelation time (GT) showed lower variation within the sample (between parallels) similar to the modelled gelation time (CC). Other traditional parameters (i.e., gel-firming rate (GFR)) and final gel strength (G60) showed slightly higher variations between the parallels, compared to the modelled gel firming rate/time constant (CB) and modelled gel firmness at 60 minutes (CA). A strong correlation between parameter estimates from the model and traditional parameters was found (CC *vs.* GT; $R^2 = 0.93$ and CA *vs.* G60; $R^2 = 0.97$) similar to the study by Bittante (2011), that modelled the rennet coagulation curves. Only the time

constant (CB) and gel firming rate (GFR), showed a weak correlation coefficient (R^2 = 0.40). These results showed that acid coagulation parameters could be estimated from the model equations.

4.2. Milk coagulation properties (Paper III)

In paper III, the effect of milk protein polymorphism, composition, micelle size and salt distribution on the rennet and acid coagulation properties were investigated in milk from Norwegian Red cattle (NRF). The most frequent alleles in the 118 NRF cattle genotyped were α_{s1}-CN B, β-CN A^2, κ-CN B, β-LG B (Table 8), similar to most of the Scandinavian and Baltic cattle (Gustavsson et al. 2014b; Poulsen et al. 2013a; Pärna et al. 2012). However, the present study reported a higher frequency of β-CN A^2A^2 genotype compared to A^1A^2, similar to previous reports on Estonian Native cattle and Danish Holstein (Jõudu et al. 2007; Poulsen et al. 2013b). The higher frequency of β-CN A^2A^2 was not reported before in NRF (Devold et al. 2000) or in the Swedish Red cattle (Gustavsson et al. 2014a; Hallén et al. 2007). The composite casein genotypes BB-A^2A^2-BB and BB-A^2A^2-AA were the most dominant composite genotypes in the 118 cows genotyped compared to BB-A^1A^2-AA, BC-A^2A^2-BB and BB-A^1A^2-BE which occurred at lower frequencies (10%). Other composite genotypes occurred at still lower frequencies (< 7%). Similar to the findings in the current research, a high frequency of BB-A^2A^2-AA was found in Estonian Native and Danish-Holstein cattle (Gustavsson et al. 2014b; Jõudu et al. 2007) and less frequently in Swedish Red and Danish Jersey (Poulsen et al. 2013a).

Table 8: Allele frequencies for the four milk protein loci in 118 NRF cows genotyped

Locus	Allele	Frequency, %
α_{s1}-CN	B	91.1
	C	8.9
β-CN	A^1	19.1
	A^2	79.7
	A^3	0.0
	B	1.2
κ-CN	A	48.3
	B	45.7
	E	6
β-LG	A	34.3
	B	65.7

Casein genotypes, i.e., αs_1-CN BC and β-CN A^1A^2, κ-CN BB and BC-A^2A^2-BB gave favorable rennet coagulation properties in NRF milk. These findings agrees with the previous reports in most dairy cattle breeds studied (Comin et al. 2008; Hallén et al. 2007; Jensen et al. 2012b; Jõudu et al. 2009). These effects of casein genotypes on the rennet coagulation properties could be explained by their negative correlation with casein micelle size (Devold et al. 2000; Glantz et al. 2010; Jõudu et al. 2009). In the current study, milk protein genotypes which impaired rennet coagulation properties were linked to larger casein micelles. This may be because the small casein micelle size may improve rennet coagulation properties, since they provide a larger surface area for the gel network formation compared to the larger casein micelles.

Rennet coagulation properties of NRF milk were impaired by the BB-A^2A^2-AA and BB-A^1A^2-BE composite genotypes. Negative effects of BB-A^2A^2-AA on rennet coagulation properties were also previously reported in other modern dairy breeds (Comin et al. 2008; Jensen et al. 2012b). This could be due to the negative effect of this composite

genotype on the relative concentration of κ-CN and their positive correlation with larger casein micelle size.

Favored acid coagulation properties (shorter GT, high GFR, G30 and G60) were associated with the κ-CN AA and α_{s1}-β-κ-CN composite genotype BB-A^2A^2-AA, while κ-CN BB and BC-A^2A^2-BB were associated with poor acid coagulation properties (Figure 17). β-CN and α_{s1}-CN did not affect the acid coagulation properties of milk. These effects of κ-CN and composite genotypes (α_{s1}-β-κ-CN) on acid coagulation properties were not reported in previous research on Swedish Red cattle (Hallén et al. 2009). In the study by (Hallén et al. 2009), acid coagulation was favored by β-LG AA compared with BB. This was linked with the effect of AA genotype on the concentration of β-LG (Hallén et al. 2009).

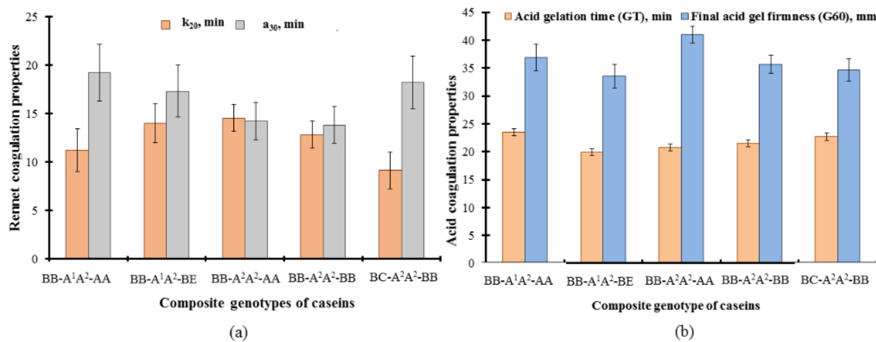

Figure 17: Effects of composite genotypes on a) rennet and b) acid coagulation properties of milk

Our findings showed that higher casein, total protein and lactose contents were associated with improved rennet and acid coagulation properties of milk. High fat content was associated with the shorter time needed for rennet curd formation and reduced acid gel firmness (G60). Fat globule size distribution did not influence the rennet coagulation properties of milk. However, larger fat globule size influenced acid coagulation properties (slow gel formation and a weaker gel at 60 min). This could be explained by the fact that larger fat globules (unhomogenized milks) have lower surface area and are weakly attached to the milk acid gel network (Ji et al. 2011), hence they create weaker connection points in the gel network which makes the gel more prone to syneresis.

Calcium distribution in the milk (between the soluble and micellar phase) did not influence acid coagulation properties. However, higher concentrations of the micellar Ca and P were associated with improved rennet coagulation properties similar to previous reports

(Gustavsson et al. 2014c; Malacarne et al. 2014). Poor rennet coagulation properties in the samples with less micellar Ca and P could be explained by less micellar aggregation (Malacarne et al. 2014). The higher amount of the highly phosphorylated caseins (α_{s1}-CN 9P and α_{s2}-CN 12P) were associated with poorer milk coagulation properties similar to findings in Danish Holstein cows (Frederiksen et al. 2011). In the current study, α_{s1}-CN 9P was associated with lower casein and protein content compared to α_{s1}-CN 9P.

The present study reported poor rennet and acid coagulation properties of the milk at high pH of the raw milk similar to previous reports (Cassandro et al. 2008; Jõudu et al. 2008) which reported a significant effect on RCT and k_{20} for different pH. This was previously explained by a higher rennet activity at reduced pH (Foltmann 1959; Tsioulpas et al. 2007). Poor acid coagulation properties (longer gelation time, low gel firming rate and low gel firmness) were observed in the samples with high pH (> 6.8). This could be explained by the longer time needed for the colloidal calcium phosphate to dissolve from the casein micelles.

4.3. Properties of cultured skim milk (Paper IV)

The aim of Paper IV was to study the effects of milk protein genotypes on the rheological properties, degree of syneresis, particle size distribution and concentration of the fermentation metabolites of cultured skim milk. Twenty-eight milk samples from individual NRF cattle were used to produce the cultured skim milk. The samples were analyzed on the first day (fresh; D1) and fourteenth day (stored; D14) after the

production day. Microstructure images on the cultured milk samples were taken on D1 samples of the cultured milk using confocal laser scanning microscopy (CSLM).

4.3.1. Physical properties

We did not find an influence of the α_{s1}/κ-CN composite genotypes on the physical properties of the cultured milk. The effects of milk protein genotypes (β-LG and κ-CN/β-LG composite genotypes) on the rheological properties, degree of syneresis and particle size distribution were revealed more on D14 samples than on D1 samples, as expected. This could be due to the improved structural changes in the stored samples compared to the fresh samples, which could be linked to the higher values of storage modulus (G') on the stored cultured milk compared to the fresh cultured milk (91.56 ± 8.04 *vs*. 72.80 ± 8.04 Pa). Since G' is related to the strength and the number of bonds (Lucey et al. 1998a), the higher value of G' could be related to an increase in the protein-to-protein network and improved microstructure in the casein gels.

In the D14 samples, yield stress and degree of syneresis were influenced ($P < 0.05$) by the β-LG genotypes and the κ-CN/β-LG composite genotypes. Higher values of yield stress and lower degree of syneresis were observed in the samples with AB genotype of β-LG compared to the samples with BB genotypes. Furthermore, samples with κ-CN/β-LG AA/AB and BB/AB composite genotypes showed a lower degree of syneresis with higher yield stress compared to AA/BB and

BB/BB. In the present study, the samples with lower degrees of syneresis had higher yield stress and vice-versa (Figure 18). The inclusion of the protein content in the statistical model rendered the effects of the κ-CN/β-LG composite genotypes on the yield stress insignificant, while the effect of the κ-CN/β-LG composite genotype on the degree of syneresis was not affected by the protein content. Previous research reported improved rheological properties with A allele of β-LG as also reported by Hallén et al. (2009) who found higher gel strength with AA and AB genotypes of β-LG in the milk from Swedish Red breed (SRB).

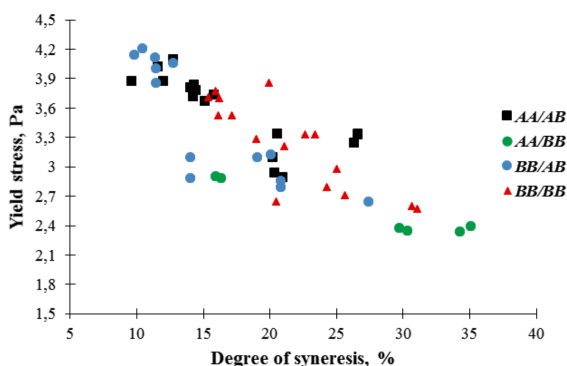

Figure 18: The relationship between yield stress and the degree of syneresis based on the κ-CN/β-LG genotypes

The effects of β-LG genotypes (AA and AB) on the rheological properties was linked with its effect on the β-LG concentration, where AA and AB genotypes were associated with higher concentration of β-LG (Hallén et al. 2009). This shows that the concentration of whey proteins in the pre-heat treated low fat acid gels is very important for the structure of the finished gels. A higher concentration of β-LG was associated with an increase in the strength of the bonds formed in the

whey-casein micelle complex. This would lead to the formation of denser networks, larger aggregates and improved microstructure hence improved water holding capacity and elastic properties (Chua et al. 2017; Jørgensen et al. 2015; Krzeminski et al. 2011; Laiho et al. 2017). These findings agree with the CSLM images where samples with AB genotypes of β-LG had more compact (denser) gels with fewer pores compared to the samples with BB in all combinations of κ-CN (AA or BB).

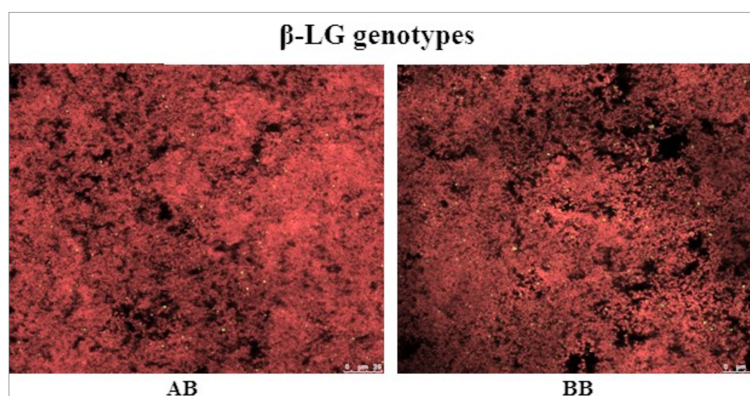

Figure 19: Confocal laser scanning microscopy images to show the microstructure of the cultured milk gels with the different genotypes of β-LG

4.3.2. Fermentation metabolites

Milk protein genotypes such as α_{s1}-CN and κ-CN/β-LG genotypes influenced the concentration of lactic acid and orotic acid in the fresh samples. In the stored samples, concentrations of lactic acid and acetoin were significantly influenced by the κ-CN/β-LG and the α_{s1}/κ-CN composite genotypes, respectively. Other fermentation metabolites analyzed were not significantly influenced by the milk protein genotypes. There was higher concentration of lactic acid in the D1 samples in the

BC genotype of α_{s1}-CN compared to the BB genotype ($P < 0.05$), while the higher concentration of orotic acid in the D1 samples was more associated with κ-CN/β-LG AA/AB and BB/AB composite genotypes, compared to AA/BB. In the D14 samples, a higher concentration of lactic acid was more associated with the κ-CN/β-LG BB/AB, AA/BB and AA/AB compared to the BB/BB composite genotypes ($P < 0.05$). However, the effects of α_{s1}-CN and κ-CN/β-LG composite genotypes on the concentrations of lactic acid and orotic acid were not observed when protein content was included in the statistical model as covariate. This implies that the differences in the lactic acid and orotic acid may be due to differences in total protein content.

Furthermore, the concentration of acetoin was higher ($P < 0.05$) in α_{s1}/κ-CN composite genotypes BB/AA and BC/BB compared to BB/BB. Since the concentration of citric acid and diacetyl were similar for the α_{s1}/κ-CN composite genotypes, the differences in acetoin could reflect the transformation of the acetoin to 2,3-butandiol. This means that in α_{s1}/κ-CN composite genotypes BB/AA and BC/AA could have a reduced transformation of acetoin to 2,3-butandiol is limited compared to in BB/BB, which had a very low concentration of acetoin. The significant effect of the α_{s1}/κ-CN genotypes on the concentration of acetoin was still apparent even when including protein covariate. The reason for a reduced transformation of acetoin to 2,3-butandiol in the samples with α_{s1}/κ-CN composite genotypes BB/AA and BC/AA is not known.

The current research showed favored rheological properties and water-holding capacity with AB genotypes of β-LG compared to BB. However, the differences in yield stress of the product was explained by differences in the protein content. Future research should substantiate the effects of κ-CN/β-LG composite genotypes at a controlled protein concentration. These results might provide new possibilities for improving the rheological properties of low fat cultured milk through milk protein genomics in the future.

5. Conclusions and research outlook for the future

Current findings showed that κ-CN B, α_{s1}-CN C, β-CN A^1 and the BC-A^2A^2-BB of the α_{s1}-β-κ-CN composite genotype were associated with improved rennet coagulation properties, while poorer rennet coagulation properties were associated with α_{s1}-CN B, κ-CN A and E, β-CN A^2, the BB-A^2A^2-BB and BB-A^2A^2-AA composite genotypes. On the other hand, acid coagulation properties when using GDL were favored by κ-CN A and the composite genotype BB-A^2A^2-AA. κ-CN BB and composite genotypes including BB-A^2A^2-BB and BC-A^2A^2-BB were associated with poor acid coagulation properties. In addition, the β-LG AB was associated with higher water-holding capacity in the low fat cultured milk compared to the BB. This might impose a challenge to breeders when selecting the best genotypes for both cheese and cultured/fermented milks, since A for both κ-CN and β-LG loci was associated with poor cheese making properties.

The findings from the current research reveal that the research to be considered in the future within milk genomics should focus on:

- The effects of milk protein genotypes on the cheese yield and quality. This will provide evidence for the best alleles in NRF for efficient cheese processing in Norway.

- The effects of the milk protein genotypes that favor both cheese and fermented milk products for cow health traits (i.e., somatic cell count) and reproductive traits (i.e., fertility).
- The effects of milk protein genotypes on the rheological properties of the cultured milk at a standardized protein concentration.

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7. Papers (I to IV)

Paper I

Comparison between Formagraph and low-amplitude oscillation rheometry in monitoring coagulation properties of acid induced gels in bovine milk

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ABSTRACT

Acid coagulation properties of milk (gelation time, gel-firming rate and maximum gel firmness) were analysed simultaneously by Formagraph and LAOR. Good agreement between acid coagulation data by the two methods was perceived. Results from this investigation shows that Formagraph can be used in acid coagulation studies when analysing a larger set of milk samples.

INTRODUCTION

Preparation of acid milk protein gels involve the structural destabilization of casein micelles through decrease in pH. In milk, the steric repulsive forces caused by the outer hairy structure of κ-CN and the hydrophobic binding via colloidal calcium phosphate from the interior of the micelles accounts for the stability of casein micelles[1]. During acidification, the steric repulsive force is reduced by an increase in the positive charges while the colloidal calcium phosphate become more soluble and it is released from the interior of the micelles[2].

Several factors are known to affect coagulation properties of milk. For example, temperature history of the milk, pH, enzyme concentration[3], genetic polymorphism[4,5] and feeding regime[6]. Heat treatment of milk at higher temperature leads to whey protein denaturation[7]. The denatured whey proteins interact with caseins through their hydrophobic sites[8], which improve the texture and strength of yoghurt gels.

Milk gelation properties are determined by gelation time, gel strength and gel-firming rate. These parameters plays an important role in order to optimize the production of fermented milk products at large scale. Several methods are used to evaluate gelation or coagulation properties of milk. For example, Formagraph is used to measure rennet coagulation[9,10,11]. The Formagraph can analyse many samples at the time. Low-amplitude oscillation rheometry (LAOR)[4,12] is the conventional method used to measure acid coagulation properties of milk, but only one sample can be analysed at the time. Optical methods, as for example, Fourier transform infrared spectroscopy has also been used[13]. Several studies have been used to compare rennet coagulation properties between Formagraph and LAOR[14,15]. To our knowledge, there is no study, which has evaluated the acid coagulation properties between Formagraph and LAOR. As LAOR is time consuming and is limited to one sample at the time, a more effective method is needed for a larger number of samples. Hence, the current study was anticipated to evaluate if the Formagraph could be used to give reliable results on acid gelation properties.

MATERIALS AND METHODS

Milk samples

The methods of gel characterisation were tested in two steps. In the first step, (four-sample test) individual milk samples from four cows were analysed in quintuplet by Formagraph and Small amplitude oscillation rheometry to test the repeatability within each sample. In the second step, (ten-sample test) individual milk samples were analysed in duplet by Formagraph and once by LAOR to test if the two methods showed a similar variation between different samples.

Four-sample test: Individual morning milk samples from four (4) lactating cows were collected in two consecutive weeks, and samples from two cows were analysed in each week.

Ten-sample test: Ten (10) samples from ten cows were collected and analysed the same week.

All experimental animals were kept in the same housing and feeding management at the Centre for Animal Research (SHF) of Norwegian University of Life Sciences in Ås, Norway. The milk samples from 10-sample test were analysed once in triplicate for total protein and casein by using MilkoScan FT1 (Foss Electric A/S, Hillerød, Denmark) as described by Inglingstad et al [6]. Before acidification, milk samples were transferred into 15mL Falcon tubes and heat treated at 95^{0}C for 5 minutes in a temperature controlled water bath and then cooled to 32^{0}C in ice water.

Formagraph analysis

Acid coagulation properties were monitored by Formagraph (LAT; Foss-Italia, Padova, Italy). The working principle of the apparatus has previously been described [11,14,16]. 3% Glucono-δ-lactone (GDL) was added into the wells of the Formagraph blocks followed by addition of 10 mL of milk samples maintained at 32^{0}C. The mixture was mixed simultaneously by using the Formagraph multiple spoon for approximately 30 seconds and transferred to the Formagraph recording system. Parameters recorded in the Formagraph were Formagraph acid Gelation Time (FGT), defined as the time interval in minutes from start acidification to the time at which the width of bifurcate was increased to 1.2 mm; Formagraph Maximum Gel Firmness, (FMGF) defined as the point with the maximum width in mm of the bifurcate. Formagraph Gel-firming rate, mm/min (FGFR) defined as the slope of width of the curve *vs.* time curve (Figure 1). The width of the bifurcate as a measure of gel development was measured at 15 seconds interval for 60 minutes.

Rheometry analyses

Rheological analyses on the acidified milk gels was monitored simultaneously by using Paar Physica universal dynamic spectrometer (MCR 301, Anton Paar) equipped with a bob-cup measurement system (CC27/*Ti* with diameter 26.657 mm and 40.03 mm length: bob specifications and C-CC27/T200/*Ti* with 28.926 mm diameter: cup specifications). 14mL of milk was acidified with 3% GDL and shaken vigorously for 30 seconds. The mixture was transferred into the measuring system maintained at 32^{0}C with angular frequency of 10 rad/sec at a constant strain of 0.1% within the linear visco-elastic range (LVR).

The following parameters were recorded on the LAOR, gelation time (GT), which was defined as the time in minutes when G'≥1Pa[17]. Maximum gel firmness (MGF), defined as the maximum gel strength attained measured in Pa and gel-firming rate (GFR'), defined as the slope of G' *vs*. time curve (Pa/min)[15]. Storage modulus (G') as a measure of gel strength was recorded at 45 seconds interval for 60 minutes.

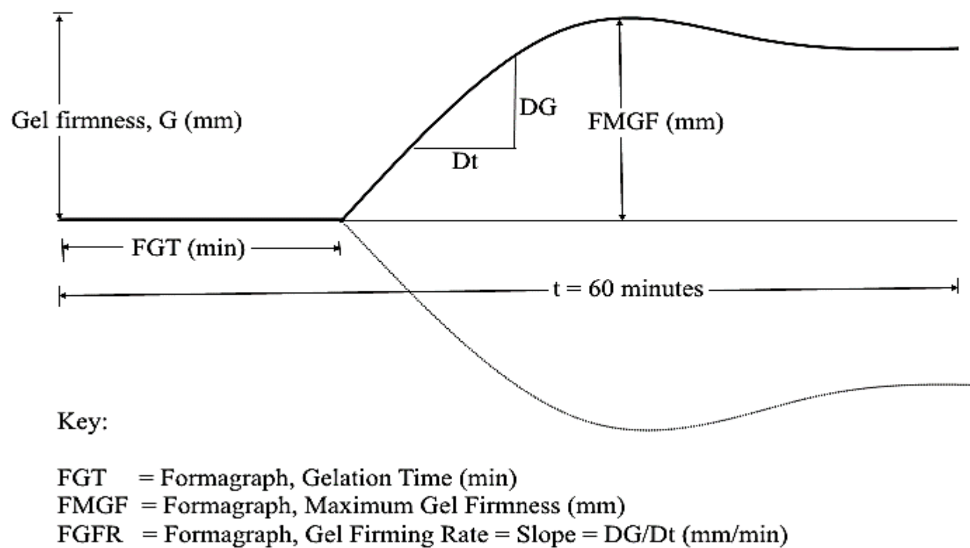

Figure 1: Acid coagulation parameters as analysed by Formagraph

Statistical analysis

The regression procedure of Statistical Analytical Software (SAS) was carried out to test the relationship between acid coagulation properties (GT *vs.* FGT, GFR *vs.* FGFR and MGF *vs.* FMGF) on the four and ten samples test between Formagraph and LAOR.

RESULTS

The four-sample test aimed at assessing the repeatability of the acid coagulation results between the two methods.
The ten-sample test intended to assess the differences between the samples analyzed by the two methods.

Four-sample test

The standard deviation (SD) within each sample in the four-sample test showed that the repeatability was similar between the two methods (Fig. 2, 3 & 4).

Regression analysis on the four sample test shows weaker correlation coefficients in all variables tested (Table 1), compared to the ten sample tests (Table 2).

Table 1: Regression analysis of the acid coagulation data as analysed by Formagraph and LAOR

Variable	n	R^2	CV	p
GT vs. FGT	20	20.99%	7.93	0.0422
GFR vs. FGFR	20	48.00%	13.78	0.0007
MGF vs. FMGF	20	43.00%	9.96	0.0017

Despite of the weak correlation coefficients (Table 1) in the four-sample test, there is a sound agreement in gelation time between the two methods, (Figure 2). Rheometry analyses showed shorter gelation time in average compared to the Formagraph. These results show that the LAOR as a non-destructive type of measurement has a higher sensitivity compared to the Formagraph. Hence, it takes shorter time for the instrument to detect gel development.

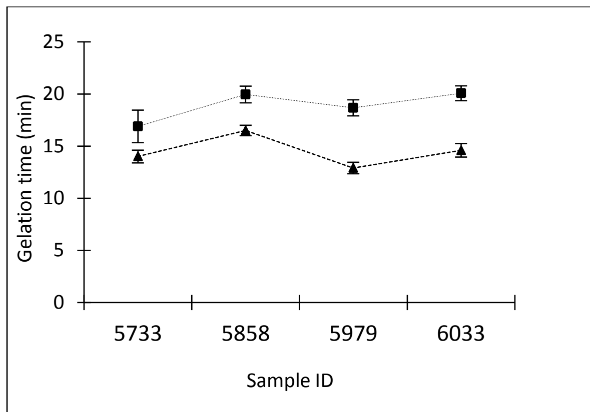

Figure 2: Means with standard deviation (SD) for gelation time between Formagraph (■) and LAOR (▲)

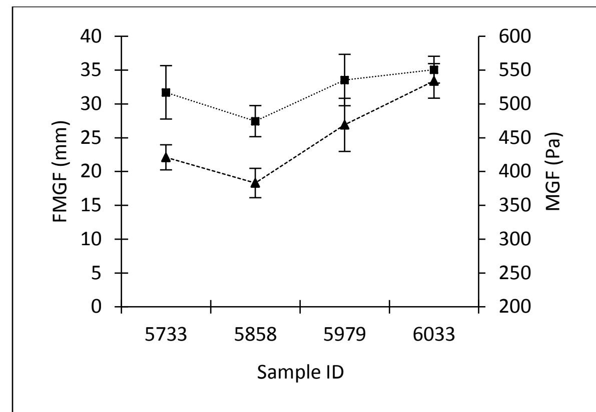

Figure 4: Means and SD for the maximum gel firmness between Formagraph (■) and LAOR (▲)

Figure 3 discloses the pattern for the gel-firming rate by the two methods investigated. A similar pattern of gel-firming rate was achieved by both methods, where higher gel-firming rate was perceived in sample 6033 compared to 5858 in both the Formagraph and the LAOR.

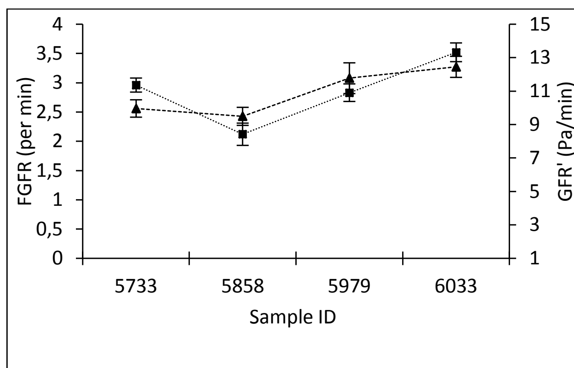

Figure 3: Means and SD for the gel-firming rate between Formagraph (■) and LAOR (▲)

The results for the maximum gel firmness for the two instruments tallies to each other, as shown in Figure 4. Both the Formagraph and LAOR showed a similar trend for maximum gel firmness.

Ten-sample test.

The ten-sample test aimed to verify the similarity of the methods by studying the steadiness of the results between samples analysed by the two methods. Sample 6033 showed higher total protein content (4.10%) and casein (3.05%) compared with sample 5616 with mean 3.25% and 2.48% for total protein and casein respectively.

Table 2 shows the regression analysis when comparing the formagraph and LAOR data was achieved between the methods on Gelation time and Gel-firming rate respectively.

Table 2: Regression analysis of the acid coagulation data as analysed by Formagraph and LAOR

Variables	n	R^2	C.V	p
GT *vs.* FGT	10	81.2%	10.21	0.0004
GFR *vs.* FGFR	10	83.57%	11.37	0.0002
MGF *vs.* FMGF	10	42.03%	21.52	0.043

Figure 5 shows the pattern for the gelation time in ten samples between the two methods. Reliable agreement on acid gelation time between the two methods was noticed as in the four-sample test.

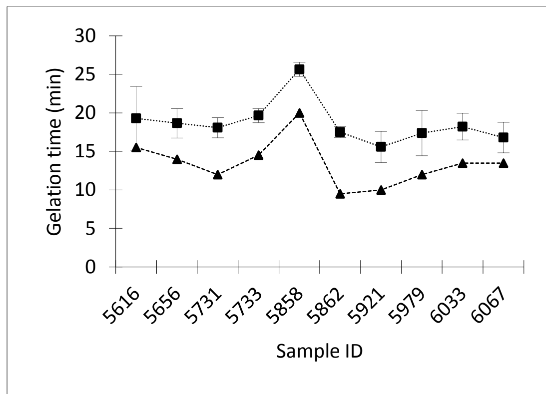

Figure 5: Gelation time pattern between Formagraph (■) and LAOR (▲).

As in the four-sample test, good agreement on the gel-firming rate between the two methods was observed in the 10-sample test, as illustrated in Figure 6. Sample 5858 showed a slower rate of gel formation compared to other samples by both methods.

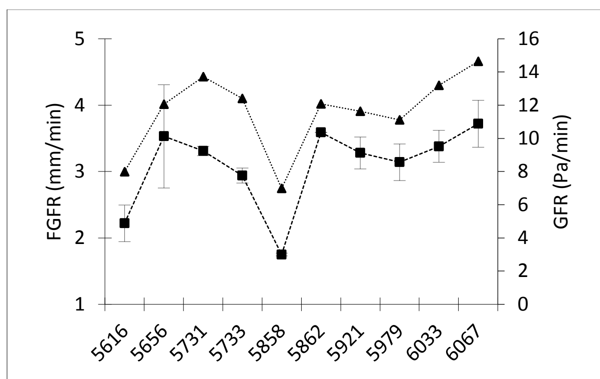

Figure 6: Gel-firming rate pattern between Formagraph (■) and LAOR (▲).

A weaker correlation was found between the methods for maximum gel firmness (R^2=42.03 %) compared to the other variables in the 10 sample trial (Table 2). However, the correlation was comparable to that obtained in the four-sample test. However, in the four-sample test, the SD between the samples were quite large for the maximum gel firmness compared to the SD of the gelation time and gel-firming rate. Therefore, the lower correlation between the methods on maximum gel firmness could be expected.

Maximum gel firmness analysed in the 10 sample-test (Figure 7) showed a similar agreement between the two methods as in the 4-sample test. For example, sample 5858 showed low gel strength in each method as expressed in the four and ten-sample test. However, some samples also showed an opposite trend with the two methods, i.e. sample 5731 and 5733.

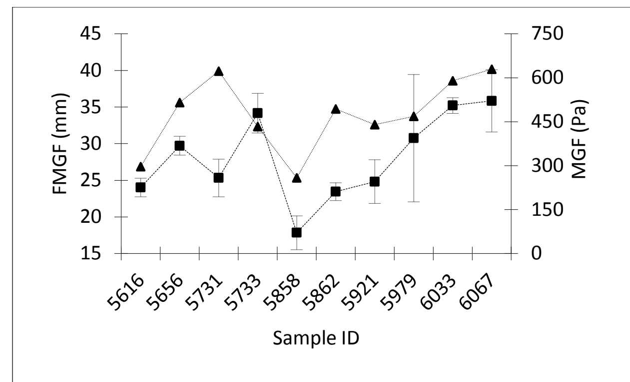

Figure 7: Maximum gel firmness between Formagraph (■) and LAOR (▲)

CONCLUSION

Comparable results for the acid coagulation parameters were obtained for gelation time and gel-firming rate between Formagraph and Small amplitude oscillation rheometry, and our results shows that Formagraph can be used as an alternative method for analyzing the acid coagulation properties of milk on large sample sizes.

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Paper II

Modelling of acid coagulation data analysed by Formagraph and estimation of milk coagulation parameters

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ABSTRACT

Milk acid coagulation data from a Formagraph have been modelled in order to determine the main parameters of the dynamic coagulation process i.e. Gel firmness at 60 min (CA), firming rate (CB) and delay time (CC). Traditional parameters (single point estimates) were gelation time (GT), gel firming rate (GFR) and final gel firmness (G60). Strong correlation was achieved between A *vs*. G60 and CC *vs*. GT (i.e. 0.97 and 0.93 respectively, while CB *vs*. GFR showed moderate correlation (0.40). CA and CC could be used in studying acid coagulation process of the milk, however the use CB needs further investigation.

INTRODUCTION

Acid coagulation properties of milk have gained significant concern for many years, this is because of their association with texture and consistency of milk protein gels of cultured milk products i.e. yoghurt gels. Caseins and whey proteins are the major proteins found in milk. In fresh milk, caseins (α_{s1}-, α_{S2}-, β- and κ-Casein) are organized in the form of colloidal aggregates known as casein micelles, while, whey proteins (β-lactoglobulin, α-lactalbumin) are globular in nature and are presented in the soluble phase of the milk. Casein micelles are covered with the hairy layer of κ-CN which provide steric stabilization against aggregation while the interior of micelle contains highly phosphorylated caseins (α_s-and β-CN), which participates in the formation of calcium phosphate nanocluster this provide colloidal stability to the casein micelles due to non-covalent crosslinkings[1].

Production of acid milk gels involve structural destabilization of the structure casein micelles through acidification by using acidulants (e.g. glucono-δ-lactone) or by lactic acid produced by starter cultures. Acidification of milk decreases the hydrophobicity of micelles through dissolution of colloidal calcium phosphate and neutralization of surface negative charges. This leads to the reduction in the colloidal stability and steric de-stabilization on the casein micelles, these events induce the aggregation of casein micelles[1].

For many years acid coagulation properties of milk have been analysed by low-amplitude oscillation rheometry, which is based on a non-destructive measurement[2, 3]. Recently, a new method for acid gel characterization, especially for a large number of samples have been established[4]. Traditional parameters obtained from the Formagraph print-out and coagulation pattern between two different samples are presented in Figure 1. There is a possibility of modelling the acid coagulation data retrieved from the Formagraph and estimate important acid coagulation parameters from the model by using all observations obtained from the computer storage, since the modelling of rennet coagulation data from Formagraph have already established[5-8], to our knowledge there is no information in the literature on the modelling of the acid coagulation properties measured by Formagraph. Hence, the current study was intended to model the acid coagulation data derived from Formagraph to estimate the main parameters derived from the dynamic coagulation process and compare them with traditional parameters.

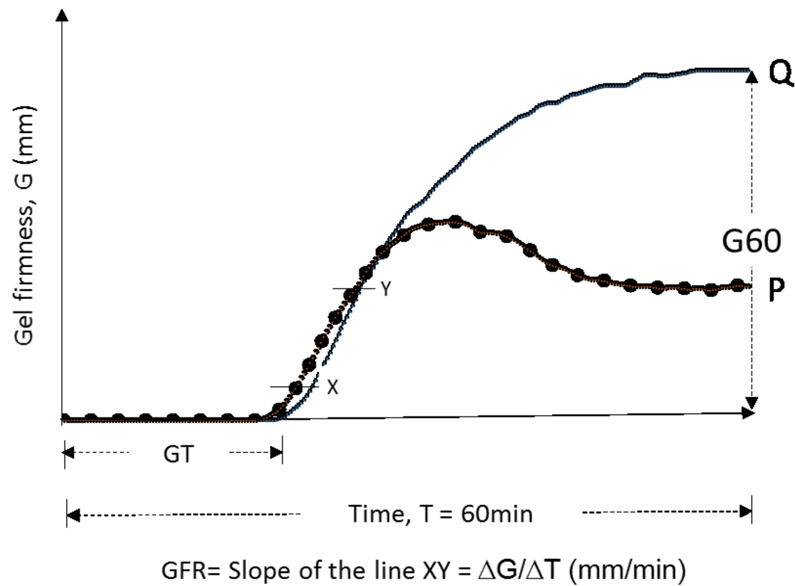

Figure 1: Parameters obtained from Formagraph output/single point estimates (GT=gelation time, GFR=gel firming rate and G60=final gel firmness at 60 minutes) between two different samples. Sample P showed gel shrinkage (Syneresis) at 60 minutes while sample Q showed continuous increase in gel firmness over time.

MATERIALS AND METHODS

Milk samples

Fresh milk samples from four (4) lactating cows were collected during the day from the Centre of Animal Research of Norwegian University of Life Sciences (SHF). Milk samples were cooled to 4°C immediately after sampling before transported to the Dairy technology laboratory and stored overnight at 4°C until the next day when the tests were done. At the dairy technology laboratory milk samples were analysed for fat, lactose, total protein and casein by MilkoScan FT1 (Foss Electric A/S, Hillerød, Denmark) and pH by pH meter (PHM61; Radiometer, Copenhagen, Denmark), before acidification.

Acid coagulation was monitored by Formagraph (LAT; Foss-Italia, Padova, Italy) for 60 minutes as described[4]. Acid coagulation parameters obtained were gelation time (GT, min; time taken from acid addition until the width of bifurcates were increased to 1.2 mm), gel firming rate (GFR, mm/min; the steepness of the curve) and final gel firmness (G60, mm; gel firmness at 60 minutes after acid addition). The model was fitted on the 4 samples (1×10 =10 equations/sample) except for one sample where only 9 parallels were made (= 39 model equations). All samples showed a continuous increase in the gel firmness over time as shown by sample Q in Figure 1.

Model description

A simple growth model was tested over 60 minutes after acid addition, the model was adopted from the model established by Bittante[5] and McMahon et al[8] on the rennet gels.

$$\hat{y} = CA \times (1 - e^{-CB*(x-CC)}) \qquad \text{(Eq. 1)}$$

Where ŷ is the gel firmness (mm) modelled against time (x, min); CA is the asymptotical potential value at infinite time

(mm); CB is the time constant (1/minutes) and CC is the delay time (minutes).

By using the model described above it was possible to estimate the acid coagulation parameters i.e. acid gelation time (CC), gel firming rate (CB) and gel firmness at 60 minutes (CA).

Statistical analysis

Acid coagulation data were modelled by using MATLAB[9]. Standard deviation and coefficient of variation were estimated from each model parameter for all samples tested and compared with the traditional parameter estimates derived from the Formagraph output. Simple linear regression was used to determine the linear relationship between the parameters.

RESULTS AND DISCUSSION

Milk composition and pH

Table 1 presents the chemical composition and pH on the samples analysed. The content of fat and total protein had the largest variation between samples whereas the content of lactose and casein were more stable while the pH had little variation between the milk samples. Sample 5704 showed higher G60 compared to 6169, 5616 and 6114 (Figure 2). The high gel firmness in 5704 compared to 6114 could be explained by the differences in casein, total protein and fat content between the two samples.

Table 1: Chemical composition of the milk samples

Sample	pH	Lactose	Fat	Protein	Casein
5616	6.8	4.78	4.23	3.18	2.47
6169	6.72	4.63	4.05	3.6	2.73
6114	6.73	4.38	2.86	3.07	2.38
5704	6.74	4.54	4.34	3.62	2.71

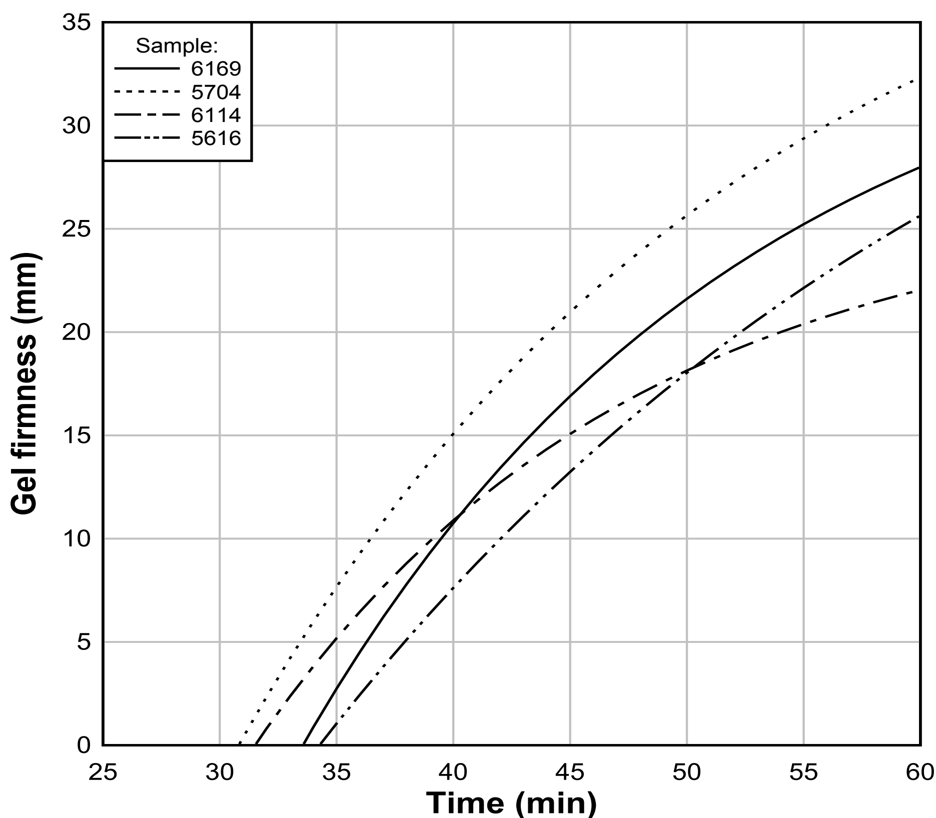

Figure 2: Modelled curves between the samples (average of the parallels)

Descriptive statistics

Table 2 shows the descriptive statistics for the traditional and the model parameters. Good repeatability was achieved model parameters compared to single point estimates, especially in CB showed low standard deviation within the parallels compared CA. In traditional estimates, GT showed good repeatability compared to GFR and G60. Samples expressed weaker gel showed poor repeatability on the single point estimate (G60) compared to the samples with strong gel. This could be explained by the fact that a stronger gel gives a constant movement of the Formagraph pendulum loop with less gel destruction compared with a weaker gel which most probably gives an irregular movement of the pendulum loop. The weaker gel most probably results in the loss of intimate contact between the loop and the gel[8]. Perhaps this effect would be less pronounced in conventional rheometry analysis because the analysis are made within the linear visco-elastic range (LVR).

Table 2: Descriptive statistics for the parameters within the samples between model parameters ad traditional parameters

Sample	Traditional parameters	n	Mean	SD	CV (%)	Model parameters	Mean	SD	CV (%)
5616	GT	10	35.29	1.5	4.25	CC	34.27	1.51	4.41
	G60	10	22.99	1.56	6.79	CA	23.79	1.22	5.14
	GFR	10	1.16	0.09	7.76	CB	1.58	0.08	5.34
6114	GT	9	32.37	1.12	3.46	CC	31.51	0.99	3.14
	G60	9	20.05	2.07	10.32	CA	20.67	1.65	7.99
	GFR	9	1.2	0.17	14.17	CB	0.73	0.06	7.73
6169	GT	10	34.06	0.56	1.64	CC	33.56	0.56	1.67
	G60	10	26.37	1.93	7.32	CA	27.43	1.84	7.99
	GFR	10	1.62	0.14	8.64	CB	1.04	0.06	5.36
5704	GT	10	31.17	1.04	3.34	CC	30.81	1.17	3.80
	G60	10	29.72	2.03	6.81	CA	30.86	1.72	5.54
	GFR	10	1.58	0.08	5.06	CB	1.06	0.03	3.12

Relationship between model parameters *vs.* single point estimates.

The current results showed stronger linear relationship (R^2=0.93, Figure 3) between gelation time (GT) as a single estimate parameter and delay time (CC) of the model estimate , similar to Bittante[5] who reported similar values between model estimates and single point estimates.

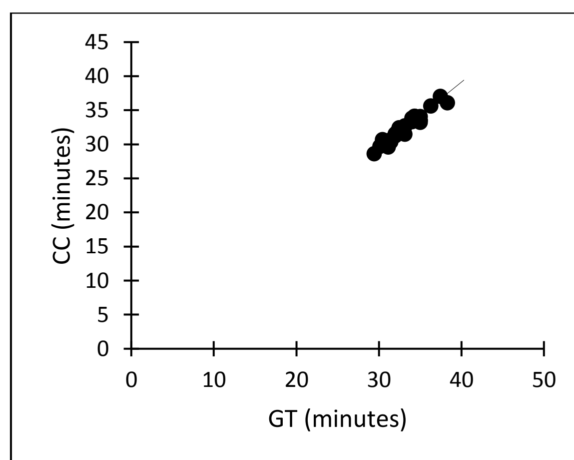

Figure 3: Correlation between delay time (C) and traditional gelation time (GT) (R^2=0.93, CC=0.987*GT)

The relationship between the estimated gel firming rate (CB) and the traditional gel firming rate (GFR) is presented in Figure 4. The two parameters showed moderate correlation (R^2 =0.40).

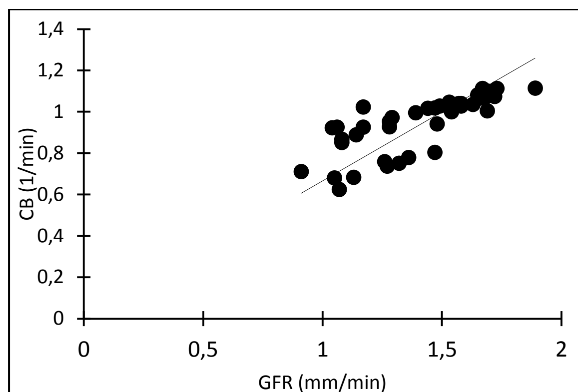

Figure 4: Correlation between model time constant (CB) and traditional gel firming rate (R^2 =0.40, CB=0.667* GFR)

Gel firmness at 60 minutes estimated from the model (CA) and observed final gel firmness (G60) showed stronger linear relationship (R^2 = 0.97, Figure 5), similar to Bittante[5].

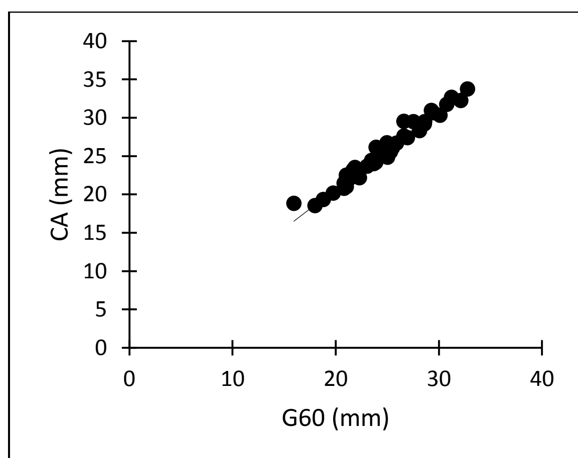

Figure 5: Correlation final gel firmness and traditional final gel firmness (R^2=0.97, CA= 1.035*G60)

CONCLUSION

Good repeatability was achieved the model parameters compared to single point estimates. CC *vs.* GT and CA vs G60 showed stronger linear relationship. This implies that gelation time and final gel firmness at 60 minutes can be estimated from the model and used in studying acid coagulation properties of milk by Formagraph, since they showed good repeatability in all samples tested. The use of estimated gel firming rate needs further investigation.

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Paper III

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Effects of milk protein polymorphism and composition, casein micelle size and salt distribution on the milk coagulation properties in Norwegian Red cattle

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A B S T R A C T

Effects of milk protein polymorphism and composition, casein micelle size and salts distribution on the coagulation properties of milk from 99 Norwegian Red cattle (NRF) were studied. Genetic variants of α_{S1}-casein (CN), β-CN, κ-CN and β-lactoglobulin (LG) affected rennet coagulation properties of milk. Significant effects of κ-CN and the composite genotype α_{S1}-β-κ-CN were observed on acid coagulation properties. Relative concentrations of milk proteins were significantly affected by individual casein genotypes and the composite genotype of α_{S1}-β-κ-CN while, the relative concentration of β-LG was only affected by β-LG genotypes. The salts distribution in milk and the concentration of milk proteins affected both rennet and acid coagulation properties. Milk protein genotypes associated with better rennet coagulation, impaired the acid coagulation properties. However, α_{S1}-β-κ-CN BB-A^1A^2-BE and BB-A^2A^2-BB were associated with poor rennet and acid coagulation properties. Breeding programs should focus on decreasing these genotypes in NRF cattle.

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1. Introduction

The major milk protein genes of dairy cattle [α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, β-LG and α-lactalbumin (LA)] are polymorphic due to genetic polymorphism, which is caused by single nucleotide polymorphisms (SNP) and/or nucleotide deletion or insertion or post-translational modifications, i.e., phosphorylation (only α_{S1}-, α_{S2}-, β- and κ-CN) and glycosylation (only κ-CN) (Caroli, Chessa, & Erhardt, 2009). Milk protein genetic polymorphism, milk protein composition and concentration, concentration of κ-CN relative to total caseins, total milk salts and casein micelle size have been reported to affect rennet coagulation properties of milk (Glantz et al., 2010; Gustavsson et al., 2014c; Jõudu, Henno, Kaart, Püssa, & Kärt, 2008; McMahon, Brown, Richardson, & Ernstrom, 1984).

Milk salts exist in a dynamic equilibrium between the soluble phase (serum phase) and colloidal phase (micellar phase). Factors affecting the distribution of salts between the two phases of milk have been described (Gaucheron, 2005); both pH and temperature influences its distribution. Since the micellar salts are associated with the stability of the casein micelles (Dalgleish & Corredig, 2012), research towards salt distribution in milk and their effects on milk processability is important.

Limited studies have been made related the distribution of milk salts (Ca, Mg and P) between micellar and serum phases with rennet coagulation properties (Jensen et al., 2012; Udabage, McKinnon, & Augustin, 2001). It is still unclear whether the distribution of salts between the two phases of milk affects milk coagulation properties. Recent studies on the effects of individual casein genotypes and composite genotypes of caseins (α_{S1}-β-κ-CN) on the rennet-induced gels have been published (Bijl et al., 2014b; Gustavsson et al., 2014a; Perna, Intaglietta, Gambacorta, & Simonetti, 2016); however there are limited studies on their

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influence on acid-induced gels (Allmere, Åkerlind, & Andrén, 1999; Hallén, Allmere, Lundén, & Andrén, 2009).

In Danish Jersey and Danish Holstein cattle (Frederiksen et al., 2011; Jensen et al., 2012) an association between the effects of degree of phosphorylation of the caseins and the milk coagulation properties have been reported. None of these studies has been made on milk from Norwegian Red cattle (NRF), hence, the current study was intended to analyse the effects of their genetic polymorphism on individual caseins and β-LG, in addition to the effect of the composite genotype (α_{S1}-β-κ-CN) on rennet and acid coagulation properties of milk. In addition, the effects of the gross composition of milk, the salt distribution between whey and micellar phases of milk, casein micelle size on the rennet and acid coagulation properties of milk were investigated.

2. Materials and methods

2.1. Blood samples and genotyping

Blood was collected from 118 cows in 9 mL Vacutainer® plastic whole blood tubes with spray-coated K3EDTA. To assess the frequency of genetic variants of milk proteins and to identify putative novel variants, 31 female Norwegian Red cattle from the high protein yield (HPY) and low clinical mastitis (LCM) selection lines were sequenced. Sequencing was performed by the Norwegian Sequencing Centre, Oslo, Norway using a HiSeq 2500 platform according to the manufacturer's protocols. Samples were prepared for paired-end sequencing (2 × 125 bp) using TruSeq DNA PCR-free library preparation kits and sequenced with the manufacturers V4 kit (Illumina, San Diego, CA, USA) to generate an average of 9 × coverage. Sequence data from 21 Norwegian Red bulls used for artificial insemination were also available from another project (Olsen et al., unpublished). All reads were aligned against the bovine reference genome UMD 3.1, using BWA-mem version 0.7.10 (Li, 2013). Variant calling was done with FreeBayes version 1.0.2 (Garrison & Marth, 2012). Nine non-synonymous missense SNPs where identified and the 118 sampled cows were genotyped for the SNPs using the MassArray genotyping platform (Agena Biosciences, San Diego, CA). Marker IDs as well as primer IDs and sequences are shown in Table 1.

2.2. Milk samples

Individual morning milk samples from 99 NRF with known genetic milk protein variants were collected. These cows belonged to two different selection lines, i.e., high protein yield line (HPY, n = 40) and low clinical mastitis line (LCM, n = 59). The experimental animals were kept indoors at the centre for animal research (SHF) of Norwegian University of Life Sciences, Ås, Norway. As the cows are kept in an automatic milking system, cows were milked in a separate milking parlour to take specific milk samples. Some cows (49) were sampled twice in their second and fourth month of lactation, while the rest (50) were sampled once in their second month of lactation. Individual fresh milk samples were analysed for protein, fat, casein and lactose by using MilkoScan FT1 (Foss Electric A/S, Hillerød, Denmark) (Inglingstad et al., 2014). Milk pH was analysed at 20 °C using a pH meter (PHM61; Radiometer, Copenhagen, Denmark).

Milk samples for casein micelle size and ultracentrifugation (described later) were centrifuged at 2000 × g for 20 min at 25 °C as described by Inglingstad et al. (2014), followed by crystallization of milk fat at −20 °C for 10 min before fat removal. Skim milk samples for micelle sizing and ultracentrifugation were kept at room temperature for >3 h before micelle size measurements and ultracentrifugation. Whole milk samples for capillary electrophoresis (CE) were stored at −20 °C.

2.3. Quantification of milk proteins by capillary electrophoresis

Capillary electrophoresis (CE) analysis was made using an Agilent (G1600AX), with Agilent ChemStation software (Agilent technologies, Germany), as described by Jørgensen et al. (2016). Sample and run buffers were prepared according to Heck et al. (2008). Identification of peaks representing milk proteins and their isoforms (α-LA, β-LG , α_{s1}-CN-8P and α_{s1}-CN-9P, α_{s2}-CN-10P, α_{s2}-CN -11P, α_{s2}-CN-12P, κ-CN-1P and β-CN) was made by comparing our results with electropherograms reported by others (Heck et al., 2008; Otte, Zakora, Kristiansen, & Qvist, 1997). Relative concentration of milk proteins (α-LA, β-LG, total α_{S1}-CN, α_{S1}-CN-8P and -9P, total α_{S2}-CN, α_{S2}-CN-10P, -11P and -12P, κ-CN-1P and β-CN) were estimated according to Gustavsson et al. (2014b) and Heck et al. (2008).

2.4. Casein micelle size

The mean diameter of the casein micelles was analysed on the individual fresh skim milk samples by Photon Correlation Spectroscopy (PCS) by Zetasizer 3000HS particle size analyser (Malvern Instruments Ltd., Malvern, UK) as described by Devold, Brovold, Langsrud, and Vegarud (2000). In brief, before analysis skim milk samples were diluted (1:1000) using simulated milk ultrafiltrate (SMUF) prepared as described by Jenness and Koops (1962). Prior to dilution, SMUF was filtered through 0.22 μm filters (Millex®GP, Millipore Ltd, Cork, Ireland) to remove foreign particles that may interfere with the results. Diluted samples were filtered through 0.8 μm filters (Millex®GP, Millipore Corp, Cork, Ireland) and then transferred to the polystyrene cuvettes (DTS0012, Malvern, Germany), then incubated at 26 °C for 5–10 min before measurements. Measurements were made in triplicate for all samples at a scattering angle of 90° at 25 °C.

2.5. Milk fat globule size

The mean size of milk fat globules was determined through the best-fit light scattering mode (Mie) theory and measured by light

Table 1
Single nucleotide (SNIP ID) polymorphism and primer sequences for the genotyped markers.

SNP ID	Chromosome	Position (bp)	Forward primer sequence	Reverse primer sequence	Extended primer sequence
CSN1S1_192	6	87157262	ACGTTGGATGCACACAATACACTGATGCCC	ACGTTGGATGTTACCACCACAGTGGCATAG	CAGTGGCATAGTAGTCTTT
CSN2_122	6	87181453	ACGTTGGATGCCAAAGTGAAGGAGGCTATG	ACGTTGGATGTCAACATCAGTGAGAGTCAG	ATCAGTGAGAGTCAGGCTCTG
CSN2_106	6	87181501	ACGTTGGATGTCAACATCAGTGAGAGTCAG	ACGTTGGATGCCAAAGTGAAGGAGGCTATG	GCTATGGCTCCTAAGCA
CSN2_67	6	87181619	ACGTTGGATGTAAAATCCACCCCTTTGCCC	ACGTTGGATGAGAGGAGGGATGTTTTGTGG	TTTGTGGGAGGCTGTTA
CSN3_136	6	87390576	ACGTTGGATGACTTGGACTGTGTTGATCTC	ACGTTGGATGCCTACCATCAATACCATTGC	CTACAAGTACACCTACCA
CSN3_148	6	87390612	ACGTTGGATGACTTGGACTGTGTTGATCTC	ACGTTGGATGCCTACCATCAATACCATTGC	GCACTGTAGCTACTCTAGAAG
CSN3_155	6	87390632	ACGTTGGATGCCTACCATCAATACCATTGC	ACGTTGGATGACTTGGACTGTGTTGATCTC	GTGTTGATCTCAGGTGGGC
LGB_64	11	103303475	ACGTTGGATGGCAATGATCTTCTTCTGAGC	ACGTTGGATGATGAAAATGGTCCATGCCCG	GTCTTTCAGGGAGAACG
LGB_118	11	103304757	ACGTTGGATGTGCTCTTCTGCATGGAGAAC	ACGTTGGATGAGGACCACACAGCTGGTCTC	ACCCACCCAGGCACTGGCAG

scattering pattern using Mastersizer 3000HS (Malvern Instrument Ltd., Malvern, UK) as described by Logan et al. (2014). Measurements were made after adding 8 to 12 drops of milk samples in a working cell filled with distilled water until the obscuration rate was between 3 and 10% at 0.001 absorbance. The refractive index for water and milk fat globules were 1.33 and 1.46, respectively. The mean particle size was computed as the volume weighted mean diameter $d_{4,3}$ (De Brouckere mean diameter) by the following equation:

$$d_{4,3} = \sum n_i di^4 \Big/ \sum n_i di^3$$

where d_i = the square root of the upper × lower diameter or geometric mean, and n_i = the discrete number of particles.

2.6. Milk coagulation properties

2.6.1. Rennet coagulation

Rennet coagulation properties was monitored using a Formagraph (LAT; Foss-Italia SpA, Padova, Italy) as described by Inglingstad et al. (2014). In brief, milk samples were tempered at 63 °C for 30 min before mixed with 200 μL of rennet (CHY-MAX; Chr. Hansen A/S, Hørsholm, Denmark), which was prepared by diluting (1:50) with acetate buffer (pH 5.6). The following parameters were recorded, time from rennet addition to the time of curd formation (RCT, rennet clotting time in minutes), time taken for the width of the curves to increase to 20 mm (k_{20}, in minutes) and the maximum width of the curves at 30 min (a_{30}, in mm). Rennet coagulation was examined at 32 °C for 60 min and all samples were tested twice.

2.6.2. Acid coagulation

Acid coagulation properties were monitored by Formagraph (LAT; Foss-Italia, Padova, Italy). The protocol was adopted from the method described in Ketto, Schüller, Rukke, Johansen, and Skeie (2015). Before acidification milk samples were heat treated at 95 °C for 5 min and cooled to 32 °C in ice water before acidification. Milk samples (10 mL) were acidified with 0.30 g of glucono-δ-lactone (GDL), then mixed simultaneously by using the Formagraph multiple spoon for approximately 15 s and transferred to the Formagraph recording system. Acid coagulation was monitored at 32 °C for 60 min. Parameters recorded in the Formagraph were, acid gelation time (GT), defined as the time interval in minutes from start of acidification to the time at which the width of the bifurcate increased to 1.2 mm; Gel firmness (width of the curve) in mm at 30 and 60 min (G30 and G60, respectively) and the gel firming rate, mm min^{-1} (GFR) defined as the slope of the points after gelation point, assuming linear increase in gel firmness with time. All measurements were made in duplicate.

2.7. Salts distribution (Ca, P and Mg) in milk

The micellar and soluble phase of milk was separated by ultracentrifugation of skim milk by using a Sorvall discovery 100SE (Kendro Laboratory Aseville, North Carolina, USA) equipped with a T-641 rotor at 100,000 × *g* for 1 h at 40 °C (Adams, Hurt, & Barbano, 2015). Clear supernatant representing the soluble/whey fraction was carefully removed from the centrifugation tubes (Thermo Scientific, Asheville, North Carolina, USA) and transferred into a 5 mL Eppendorf and kept at 4 °C before analysis. Total salts in the skim milk samples and supernatant were analysed for calcium, magnesium and phosphorus by the method described by Jørgensen et al. (2015). Salts in the micellar phase of the milk was calculated by subtracting the contents in the supernatant from the total contents measured in the skim milk before ultracentrifugation according to Frederiksen et al. (2011). Percentage of salts in the micelles was calculated as the ratio of the micellar salts to the total salts.

2.8. Statistical analyses

The effects of milk protein genotypes on the milk coagulation properties and protein composition of milk were analysed by using the MIXED procedure of SAS (SAS, 2013), where the effect of cow was treated as a random effect. Effects of parity, selection line and stage of lactation were not found to be significant and therefore excluded from the further statistical analysis.

The less frequent genotypes (<4%) of β-CN (A^1B, A^2B and A^1A^1) and κ-CN AE were excluded from the statistical analysis. The fixed effects of the individual casein genotypes and β-LG on the milk coagulation properties and relative concentration of milk proteins were tested in model 1:

$$Y_{ijklmn} = \text{Mean} + \text{Cow}_i + \alpha_{S1}\text{CNgen}_j + \beta\text{CNgen}_k + \kappa\text{CNgen}_l + \beta\text{LGgen}_m + \varepsilon_{ijklmn} \quad \text{(M1)}$$

where Y_{ijklmn} = milk coagulation properties or protein composition; Cow_i = random cow (i = 1, 2, 3 ..., 99), α_{S1}-CNgen_j (j = BB or BC), β-CNgen_k (k = A^1A^2 or A^2A^2), κ-CNgen_l (l = AA, AB, BB or BE), β-LGgen_m (m = AA, AB, BB) and ε_{ijklmn} = Error term.

Effects of α_{S1}-β-κ-CN composite genotypes (with frequency >7%) were used to evaluate the effect of the composite genotypes of α_{S1}-β-κ-CN on the milk coagulation properties and milk protein composition by using model 2:

$$Y_{ijk} = \text{Mean} + \text{Cow}_i + \alpha_{S1}\cdot\beta\cdot\kappa\text{CNcompgen}_j + \beta\text{LGgen}_k + \varepsilon_{ijk} \quad \text{(M2)}$$

where Y_{ijkl} = Milk coagulation properties or protein composition, Cow = random cow (i = 1, 2, 3 ..., 99), α_{S1}-β-κ-CN_j (j = BB-A^1A^2-AA, BB-A^1A^2-BE, BB-A^2A^2-AA, BB-A^2A^2-BB, or BC-A^2A^2-BB), β-LGgen_k (k = AA, AB, BB) and ε_{ijkl} = Error term.

The relationships between the protein concentration of milk, salts distribution, casein micelle size, fat globule size, gross chemical composition (fat, total protein, total casein and lactose) and pH with milk coagulation properties were analysed by Pearson's correlation procedure of SAS (SAS, 2013).

3. Results

3.1. Allele and genotype frequencies

Distribution of the allele frequencies of α_{S1}-CN, β-CN and β-LG between the two breeding lines were generally similar, except for the frequency κ-CN B allele, which was the most frequent allele in the low-clinical mastitis selection line (LCM) (50%) compared with the high protein yield line (HPY) (40%). In general, the most common alleles for each of the four loci were α_{S1}-CN B, β-CN A^2, κ-CN A and β-LG B (Table 2). Genotype frequencies found for the milk protein genes and the composite genotypes of the caseins (α_{S1}-β-κ-CN) are shown in Table 3. The BB genotype of α_{S1}-CN was the most frequent (83%) compared with BC (16%) and CC (1%). The A^2A^2 genotypes constituted 64% of the genotypes of β-CN with the A^1A^2 being the second most frequent (30%), while <3% of the genotyped cows had A^1A^1, A^1B and A^2B. The AA and BB variants of κ-CN were most frequent (43 and 36%, respectively), whereas BE, AB and AE were present in <10% of the cows genotyped. The BB (45%)

Table 2
Allele frequencies for the four milk protein loci in 118 NRF cows genotyped.

Locus	Allele	Frequency (%)
α_{S1}-Casein	B	91.1
	C	8.9
β-Casein	A^1	19.1
	A^2	79.7
	A^3	0.0
	B	1.2
κ-Casein	A	48.3
	B	45.7
	E	6
β-Lactoglobulin	A	34.3
	B	65.7

Table 3
Genotype frequencies for individual caseins, β-lactoglobulin and composite genotypes for α_{S1}-β-κ-casein.

Locus	Genotype	n (of 118)	Frequency (%)
α_{S1}-CN	BB	98	83
	BC	19	16
	CC	1	1
β-CN	A^1A^1	4	3
	A^1A^2	35	30
	A^1B	2	2
	A^2A^2	76	64
	A^2B	1	1
κ-CN	AA	51	43
	AB	10	9
	AE	2	2
	BB	43	36
	BE	12	10
β-LG	AA	16	14
	AB	49	41
	BB	53	45
α_{S1}-β-κ-CN	BB-A^1A^2-AA	14	11.86
	BB-A^1A^2-BE	9	7.63
	BB-A^2A^2-AA	27	22.88
	BB-A^2A^2-BB	28	23.73
	BC-A^2A^2-BB	10	8.47
	Others (<7%)	30	25.43

genotype of β-LG was the most common genotype compared with the AB (41%) and AA (14%) variants. The composite genotypes (α_{S1}-β-κ-CN) BB-A^2A^2-BB and BB-A^2A^2-AA occurred at higher frequencies (about 23%) compared with BB-A^1A^2-AA, BC-A^2A^2-BB and BB-A^1A^2-BE, which occurred at frequencies around 10%. Other composite genotypes were rare (<7%).

3.2. Summary statistics for the random and fixed effects

Means and variance component estimates for the milk gross composition, salts distribution, casein micelle size, fat globule size, distribution and significant effects of Model 1 and 2 are presented in Table 4. Estimates of residual variance within the cow were higher than the variation between cows (within fixed effects of the model) in most of the dependent variables, except for casein micelle size, total α_{S2}-CN, α_{S2}-CN-11P, α_{S2}-CN-12P and κ-CN-1P. No cow variance component estimate was found for lactose or fat content and fat globule size. Fat percentage was significantly affected by κ-CN genotypes (Model 1), a higher fat percentage was associated with the BB (4.7 ± 0.3%) variant of κ-CN compared with BE (3.9 ± 0.3%) and AA (3.7 ± 0.3%). About 57% of the calcium was found within the micelle, while 45% of the phosphorus and 25% of the magnesium were found in the micellar phase. The salt distribution in milk was not found to be influenced by milk protein genetic polymorphism. Casein micelle size was affected by α_{S1}-CN, κ-CN, and the composite genotypes (α_{S1}-β-κ-CN). Milk fat globule size was not significantly affected by the milk protein genetic polymorphism. The relative concentration of milk proteins and their phosphorylation states (for α_{S1}- and α_{S2}-CN) were affected by milk protein genetic polymorphism.

3.3. Milk coagulation properties

3.3.1. Individual casein genes

Table 5 presents the effects of the individual caseins and β-LG genotypes on the rennet and acid coagulation properties of milk. The genotypes of α_{S1}- and β-CN and β-LG affected the rennet coagulation properties. Rennet coagulation properties was favoured by the BC variant of α_{S1}-CN ($p < 0.05$) (shorter curd formation time, $k_{20} = 8.8$ min, and higher curd firmness at 30 min, $a_{30} = 24.5$ mm, compared with the BB variant, $k_{20} = 13.5$ min and $a_{30} = 17.7$ mm). For β-CN the A^1A^2 variant showed better coagulation properties i.e., shorter RCT (16.8 min), lower k_{20} (9.3 min) and higher a_{30} (24.4 mm) compared with the β-CN A^2A^2 variant (RCT = 19.5 min, $k_{20} = 13$ min and $a_{30} = 17.8$ mm). The rennet clotting time RCT and a_{30} were significantly affected by the β-LG genotypes ($p < 0.05$), where genotype AB showed shorter RCT and higher a_{30} compared with BB and AA genotypes (Table 5). Genotypes of the κ-CN gene showed significant effects ($p < 0.05$) on k_{20}. A higher value of k_{20} was observed in the BE variant (14.5 min) compared with the rest of the κ-CN genotypes, i.e., AA (11.2 min), AB (9.0 min) and BB (9.9 min).

Acid gel firming rate (GFR) and firmness at 60 min (G60) were affected by the κ-CN genotypes ($p < 0.05$). Milk with the κ-CN AA genotype had a higher GFR (3.1 mm min^{-1}) and a slightly higher G60 (40.7 mm) compared with the AB and BB genotypes (GFR<3 min and G60 < 38 mm). Genetic polymorphism in β-CN, β-LG and α_{S1}-CN did not affect the acid coagulation properties of the milk from the investigated NRF cows.

3.3.2. Composite genotype of caseins (α_{S1}-β-κ-CN)

The composite genotype of the caseins (α_{S1}-β-κ-CN) affected both k_{20} and a_{30} ($p < 0.05$), while RCT was not significantly affected (Table 6). The composite genotypes BC-A^2A^2-BB and BB-A^1A^2-AA showed improved ($p < 0.05$) rennet coagulation properties ($k_{20} < 11.2$ min and $a_{30} > 18.2$) compared with BB-A^2A^2-BB, BB-A^1A^2-BE and BB-A^2A^2-AA ($k_{20} > 12.8$ min and $a_{30} < 17.3$).

Acid coagulation properties (GT, GFR, G30 and G60) were significantly affected ($p < 0.05$) by the α_{S1}-β-κ-CN composite genotypes. The BB-A^2A^2-AA genotype was associated with better acid coagulation properties, i.e., higher values for GFR (3.2 mm min^{-1}) and higher gel strength both at 30 and 60 min (41.0 mm) compared with the rest of the composite genotypes (GFR<2.9 mm min^{-1}, G60 < 36.9 mm).

3.4. Casein micelle size

3.4.1. Effect of casein genes and β-LG

The casein micelle size was affected by the genetic polymorphism of α_{S1}-CN ($p < 0.001$) and κ-CN ($p < 0.05$) (Fig. 1), while the other milk protein genes investigated (β-CN and β-LG) did not affect the micelle size. Smaller micelle sizes were found with the BC variant of α_{S1}-CN (156.9 ± 3.3 nm) compared with the BB variant (170.3 ± 1.8 nm) (Fig. 1). Milk with the κ-CN BE variants had a significantly ($p < 0.05$) larger casein micelle size (178.6 ± 3.7 nm) compared with the AA (159.4 ± 2.5 nm), BB (164.8.1 ± 2.5 nm) and AB (152.0.1 ± 5.3 nm) variants (Fig. 1).

3.4.2. Composite genotypes of the caseins (α_{S1}-β-κ-CN)

A significant effect of the different composite genotypes was found on the casein micelle size ($p < 0.001$, Fig. 1), larger micelles were found in the α_{S1}-β-κ-CN composite genotype BB-A^1A^2-BE

Table 4
Means and variance components (σ^2 estimates) for the overall composition and pH in milk from Norwegian Red cattle (NRF).[a]

Variable	N	Mean	σ^2 estimates		Model 1	Model 2
			Residual	Cow	Caseins+β-LG	α_{S1}-β-κ-CN
pH and milk composition						
pH	168	6.70	0.006	0.003	NS	NS
Fat (%)	168	4.14	2.24	0	κ-CN*	NS
Lactose (%)	168	4.65	0.09	0	NS	NS
Protein (%)	168	3.36	0.06	0.001	NS	NS
Casein (%)	168	2.53	0.03	0.0028	NS	NS
Salt distribution in milk						
Total Ca (g kg^{-1})	160	1.20	0.007	0.0003	NS	NS
Soluble Ca (g kg^{-1})	160	0.51	0.004	0.0005	NS	NS
Micellar Ca (g kg^{-1})	160	0.69	0.009	0.004	NS	NS
Ca in the micelles (%)	160	57	27.28	8.69	NS	NS
Total Mg (g kg^{-1})	160	0.12	0.0001	0.00001	NS	NS
Soluble Mg (g kg^{-1})	160	0.09	0.0001	0.00003	NS	NS
Micellar Mg (g kg^{-1})	160	0.03	0.0005	1.7×10^{-7}	NS	NS
Mg in the micelles (%)	160	25.01	21.99	3.55	NS	NS
Total P (g kg^{-1})	160	1.01	0.0068	0.0016	NS	NS
Soluble P (g kg^{-1})	160	0.56	0.004	0.002	NS	NS
Micellar P (g kg^{-1})	160	0.46	0.004	0.001	NS	NS
P in the micelles (%)	160	44.89	24.83	10.86	NS	NS
Particle size distribution						
Fat globule size, μM	168	4.28	0.88	0	NS	NS
Casein micelle size, nm	168	168.13	64.58	92.34	α_{S1} and κ-CN**	**
Milk protein composition						
Total α_{S1}-CN	156	33.74	1.01	0.5	α_{S1}-CN***	α_{S1}-β-κ-CN*
α_{S1}-CN-8P	156	23.40	0.54	0.46	α_{S1}** and β-CN*	α_{S1}-β-κ-CN*
α_{S1}-CN-9P	156	7.38	0.60	0.39	NS	α_{S1}-β-κ-CN*
Total α_{S2}-CN	156	7.81	0.75	1.05	NS	NS
α_{S2}-CN-10P	156	1.02	0.14	0.04	NS	NS
α_{S2}-CN-11P	156	4.1	0.16	0.31	NS	NS
α_{S2}-CN-12P	156	2.70	0.27	0.14	NS	NS
β-CN	156	33.5	3.05	1.61	α_{S1}* and β-CN**	α_{S1}-β-κ-CN**
κ-CN-1P	156	4.55	0.11	0.13	α_{S1}*-, β*- and κ-CN*	NS
α-LA	156	3.34	0.18	0.03	NS	NS
β-LG	156	8.28	0.42	0.30	NS	NS

[a] Statistical influence (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.0001$) of the genetic variants on milk composition (Model 1 and 2 respectively), σ^2 estimates were derived from model 1.

Table 5
Effects of individual casein (CN) and β-lactoglobulin (β-LG) genes on the milk coagulation properties.[a]

Genotypes		Rennet coagulation properties			Acid coagulation properties			
		RCT (min)	k_{20} (min)	a_{30} (mm)	GT (min)	GFR (mm min^{-1})	G30 (mm)	G60 (mm)
α_{S1}-CN	BB	19.2 ± 0.8	13.5 ± 0.7[a]	17.7 ± 1.1[a]	21.7 ± 0.4	2.9 ± 0.1	20.9 ± 1.1	35.7 ± 1.0
	BC	16.4 ± 1.5	8.8 ± 1.4[b]	24.5 ± 1.9[b]	21.2 ± 1.8	2.9 ± 0.1	20.5 ± 1.8	37.9 ± 1.6
p-value		NS	$p < 0.01$	$p < 0.01$	NS	NS	NS	NS
β-CN	A^1A^2	16.8 ± 1.1	9.3 ± 1.2	24.4 ± 1.8[a]	22.2 ± 0.7	2.9 ± 0.1	19.6 ± 1.7	36.7 ± 1.5
	A^2A^2	19.5 ± 1.3	13.0 ± 1.1	17.8 ± 1.5[c]	21.6 ± 0.6	2.9 ± 0.1	21.7 ± 1.4	37.0 ± 1.3
p-value		$p < 0.05$	$p < 0.01$	$p < 0.01$	NS	NS	NS	NS
κ-CN	AA	17.4 ± 1.1	11.2 ± 1.0[a]	21.5 ± 1.5	21.7 ± 0.6	3.1 ± 0.1[b]	21.9 ± 1.4	40.7 ± 1.3[b]
	AB	16.8 ± 2.4	9.0 ± 2.3[a]	24.6 ± 3.1	23.4 ± 1.3	2.8 ± 0.2[a]	17.3 ± 3.1	36.2 ± 2.8[a]
	BB	18.8 ± 1.2	9.9 ± 1.1[a]	20.2 ± 1.5	22.5 ± 0.6	2.8 ± 0.1[a]	19.3 ± 1.5	34.9 ± 1.3[a]
	BE	18.2 ± 1.7	14.5 ± 1.6[b]	18.1 ± 2.2	20.2 ± 0.9	2.9 ± 0.1[a]	24.1 ± 2.2	35.4 ± 2.0[a]
p-value		NS	$p < 0.05$	NS	NS	$p < 0.05$	NS	$p < 0.01$
β-LG	AA	19.3 ± 1.7[a]	11.4 ± 1.6	18.7 ± 2.2[a]	21.5 ± 0.9	2.9 ± 0.1	20.9 ± 2.1	35.6 ± 2.0
	AB	15.4 ± 1.2[b]	11.1 ± 1.1	23.7 ± 1.6[b]	22.1 ± 0.6	2.9 ± 0.1	20.8 ± 1.5	35.8 ± 1.4
	BB	18.6 ± 1.0[a]	11.1 ± 1.0	20.9 ± 1.4[ab]	22.2 ± 0.5	2.9 ± 0.1	20.3 ± 1.3	39.1 ± 1.1
p-value		$p < 0.05$	NS	$p < 0.05$	NS	NS	NS	NS

[a] Values are least square means ± standard error. For explanation of the rennet coagulation properties and acid coagulation properties see text. Statistical influence of the genetic variants on coagulation (Model 1) is shown in a separate row under the results of each protein, different superscript letters within each protein and column shows significant differences ($p < 0.05$).

(187.2 ± 4.1 nm) followed by BB-A^2A^2-BB (174.6 ± 2.7 nm), compared with BB-A^1A^2-AA (162.5 ± 3.7 nm), BB-A^2A^2-AA (168.7 ± 2.7 nm) and BC-A^2A^2-BB (157.9 ± 4.2 nm). Apart from milk protein genetic polymorphisms, the relative concentration of κ-CN-1P, α-LA and soluble phosphorus were negatively correlated to the casein micelle size (Supplementary Table S1).

3.5. Relative concentration of milk proteins

3.5.1. Individual caseins genes and β-LG

The genetic variants of α_{S1}-CN influenced the relative concentration of total α_{S1}-CN, α_{S1}-CN-8P, β-CN and κ-CN-1P (Table 7). A higher concentration of total α_{S1}-CN and κ-CN-1P was observed in

Table 6
Effect of composite casein genotypes (α_{S1}-β-κ-CN) on the milk coagulation properties.[a]

Genotype		Rennet coagulation properties			Acid coagulation properties			
		RCT (min)	k_{20} (min)	a_{30} (mm)	GT (min)	GFR (mm min^{-1})	G30 (mm)	G60 (mm)
α_{s1}-β-κ-CN	BB-A^{1}A^2-AA	18.5 ± 2.3	11.2 ± 2.2^b	19.2 ± 2.9^b	23.5 ± 1.1^a	2.8 ± 0.2^a	16.8 ± 2.6^a	36.9 ± 2.4^a
	BB-A^{1}A^2-BE	18.3 ± 2.1	14.0 ± 2.0^a	17.3 ± 2.7^a	19.9 ± 1.0^b	2.8 ± 0.1^a	23.6 ± 2.4^b	33.6 ± 2.1^c
	BB-A^{2}A^2-AA	21.1 ± 1.5	14.5 ± 1.4^a	14.2 ± 1.9^a	20.8 ± 0.7^{cb}	3.2 ± 0.1^b	24.1 ± 1.9^b	41.0 ± 1.5^b
	BB-A^{2}A^2-BB	22.5 ± 1.5	12.8 ± 1.4^a	13.8 ± 1.9^a	21.5 ± 0.7^a	2.9 ± 0.1^a	22.3 ± 1.7^b	35.7 ± 1.6^a
	BC-A^{2}A^2-BB	20.6 ± 2.1	9.1 ± 1.9^b	18.2 ± 2.7^b	22.7 ± 1.0^a	2.6 ± 0.4^a	18.2 ± 2.4^a	34.7 ± 2.0^c
p-value		NS	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.05$

[a] Values are least square means ± standard error. For explanation of the rennet coagulation properties and acid coagulation properties see text. Statistical influence of the composite genetic variants on coagulation (Model 2) is shown in the last row, different superscript letters within each protein and column shows significant differences ($p < 0.05$).

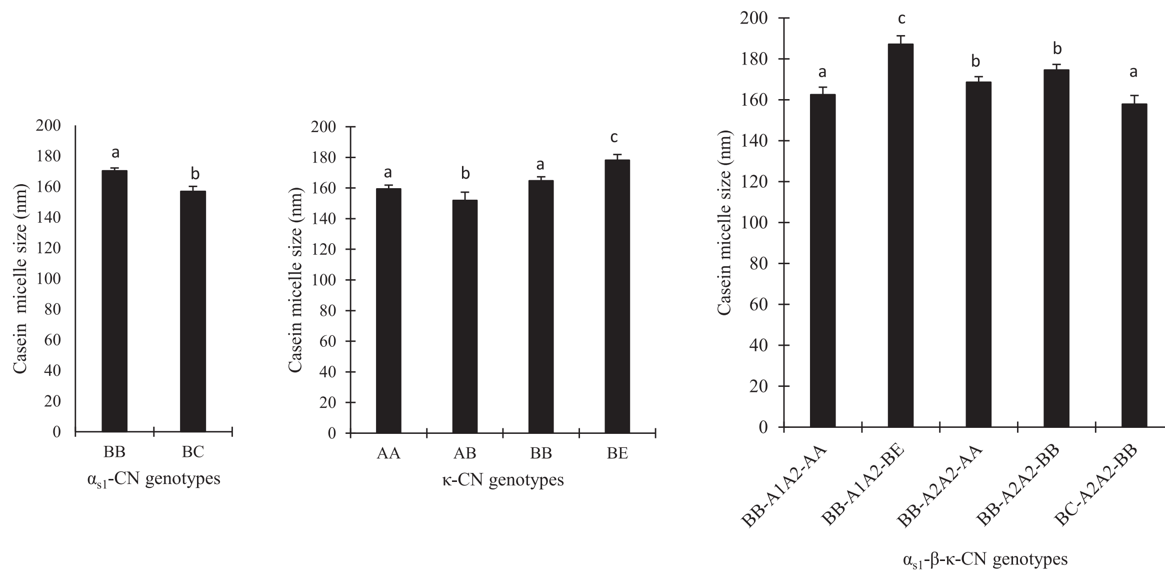

Fig. 1. Effects of α_{S1}-, κ-CN and α_{S1}-β-κ-CN composite genotypes on the casein micelle size different letters shows significant differences in micelle sizes ($p < 0.05$).

Table 7
Effects of individual casein (CN) and β-lactoglobulin (β-LG) polymorphism on the relative concentration of milk proteins.[a]

Genotypes		Relative concentration of milk proteins (%)										
		α_{S1}-CN	α_{S1}-CN-8P	α_{S1}-CN-9P	α_{S2}-CN	α_{S2}-CN-10P	α_{S2}-CN-11P	α_{s2}-CN-12P	β-CN	κ-CN	α-LA	β-LG
α_{S1}-CN	BB	32.9 ± 0.2	22.8 ± 0.2	7.1 ± 0.1	7.8 ± 0.2	1.0 ± 0.1	4.1 ± 0.1	2.7 ± 0.1	33.9 ± 0.3	4.5 ± 0.1	3.4 ± 0.1	8.9 ± 0.3
	BC	34.5 ± 0.3	23.8 ± 0.3	7.6 ± 0.3	7.4 ± 0.4	0.9 ± 0.1	3.7 ± 0.2	2.7 ± 0.1	32.7 ± 0.6	4.9 ± 0.1	3.4 ± 0.1	8.7 ± 0.4
p-value		$p < 0.0001$	$p < 0.01$	NS	NS	NS	NS	NS	$p < 0.05$	$p < 0.01$	NS	NS
β-CN	A^{1}A^2	33.3 ± 0.3^a	22.8 ± 0.2^a	7.3 ± 0.2	7.5 ± 0.3^a	1.0 ± 0.1^b	3.9 ± 0.1	2.7 ± 0.1	34.4 ± 0.5^b	4.9 ± 0.1^b	3.3 ± 0.1	8.7 ± 0.2
	A^{2}A^2	34.2 ± 0.2^b	23.8 ± 0.3^b	7.5 ± 0.1	7.7 ± 0.3^b	0.9 ± 0.1^a	3.9 ± 0.3	2.7 ± 0.1	32.1 ± 0.4^a	4.4 ± 0.1^a	3.5 ± 0.1	8.5 ± 0.2
p-value		$p < 0.01$	$p < 0.05$	NS	NS	$p < 0.01$	NS	NS	$p < 0.01$	$p < 0.05$	NS	NS
κ-CN	AA	33.8 ± 0.3	23.4 ± 0.3	7.3 ± 0.2	7.5 ± 0.3	1.0 ± 0.1	4.0 ± 0.1	2.5 ± 0.1	33.6 ± 0.4	4.7 ± 0.1^a	3.3 ± 0.1	8.8 ± 0.2
	AB	33.1 ± 0.5	23.1 ± 0.4	7.0 ± 0.4	7.6 ± 0.6	0.9 ± 0.2	4.0 ± 0.3	2.8 ± 0.3	33.1 ± 1.0	4.8 ± 0.2^a	3.5 ± 0.2	8.8 ± 0.4
	BB	33.6 ± 0.3	23.2 ± 0.2	7.5 ± 0.2	7.8 ± 0.3	1.0 ± 0.1	3.9 ± 0.2	2.8 ± 0.1	32.9 ± 0.4	4.9 ± 0.1^a	3.5 ± 0.1	8.9 ± 0.2
	BE	34.4 ± 0.4	23.7 ± 0.3	7.5 ± 0.3	7.8 ± 0.4	0.9 ± 0.1	3.9 ± 0.2	2.7 ± 0.2	33.5 ± 0.7	4.3 ± 0.2^{ab}	3.3 ± 0.1	8.6 ± 0.3
p-value		NS	NS	NS	NS	NS	NS	NS	NS	$p < 0.05$	NS	NS
β-LG	AA	33.0 ± 0.4^a	22.8 ± 0.3^a	7.4 ± 0.3	7.2 ± 0.4	1.0 ± 0.1	3.7 ± 0.2	2.5 ± 0.3	33.0 ± 0.7	4.7 ± 0.2	3.4 ± 0.1	10.0 ± 0.3^a
	AB	33.6 ± 0.3^a	23.5 ± 0.2^b	7.2 ± 0.2	8.0 ± 0.3	1.1 ± 0.1	4.0 ± 0.2	2.7 ± 0.3	32.9 ± 0.5	4.6 ± 0.1	3.4 ± 0.1	9.2 ± 0.2^a
	BB	34.5 ± 0.2^b	23.8 ± 0.2^b	7.6 ± 0.2	7.6 ± 0.3	1.0 ± 0.1	4.0 ± 0.1	2.6 ± 0.2	33.9 ± 0.4	4.7 ± 0.1	3.5 ± 0.1	7.1 ± 0.2^b
p-value		$p < 0.05$	$p < 0.05$	NS	NS	NS	NS	NS	NS	NS	NS	$p < 0.0001$

[a] Values are least square means ± standard error. Statistical influence of the genetic variants (Model 1) is shown in a separate row under the results of each protein, different superscript letters within each protein and column shows significant differences ($p < 0.05$).

the BC compared with the BB variant of α_{S1}-CN, while a lower relative concentration of β-CN was observed in the BC compared with the BB variant of α_{S1}-CN ($p < 0.05$). The genetic variants of β-CN affected the relative concentration of total α_{S1}-CN, α_{S1}-CN-8P, α_{S2}-CN-10P, β-CN and κ-CN-1P. The genetic variant A^{1}A^2 of β-CN showed a 2% higher relative concentration of β-CN compared with the A^{2}A^2 variant. The β-CN variant A^{1}A^2 showed higher concentration of κ-CN-1P compared with the A^{2}A^2 variant (4.9 versus 4.4%). The genetic polymorphism of κ-CN affected the relative concentration of κ-CN-1P, where, the variants BB, AA and AB

showed a 0.5% higher concentration of κ-CN-1P than the κ-CN variant BE. The genetic polymorphism of the β-LG gene affected ($p < 0.05$) the relative concentration of β-LG, where, the AA and AB variant of β-LG showed a 2% higher relative concentration of β-LG than the BB variant. The relative concentration of α_{S1}-CN-9P, total α_{S2}-CN, α_{S2}-CN-11P, α_{S2}-CN-12P and α-LA were not affected by the genetic polymorphism of the individual caseins or β-LG.

3.5.2. Composite genotype of the caseins (α_{S1}-β-κ-CN)

Effects of the composite genotypes of α_{S1}-β-κ-CN on the relative concentration of milk proteins is presented in Table 8. The composite genotype of α_{S1}-β-κ-CN affected the relative concentration of total α_{S1}-CN and its phosphorylation states (8 and 9 P) ($p < 0.05$) and β-CN. A higher concentration of total α_{S1}-CN, α_{S1}-CN-8P, and α_{S1}-CN-9P and a lower concentration of β-CN was observed in the composite genotype BC-A^2A^2-BB compared with the rest of the α_{S1}-β-κ-CN composite genotypes.

3.6. Effects of milk composition, salts distribution and protein composition and particle size distribution on milk coagulation properties

The relative concentration of total α_{S1}-CN, total α_{S2}-CN, α_{S2}-CN-10P, α_{S2}-CN-11P were not correlated with acid or rennet coagulation properties (Table 9). With a higher relative concentration of α_{S1}-CN-8P, the rennet coagulation properties and acid coagulation were improved, compared with the α_{S1}-CN-9P, which was negatively correlated to rennet and acid coagulation properties (Table 9). The increase in the relative concentration of α_{S2}-CN-12P impaired rennet and acid-induced gelation. The concentration of κ-CN-1P was positively correlated with a_{30} ($p < 0.0001$) and negatively correlated with k_{20} ($p < 0.0001$), while the β-CN relative concentrations correlated with improved rennet coagulation properties (a_{30}) and acid coagulation properties (i.e., high GFR, G30 and G60). The relative concentration of α-LA was positively correlated with RCT and GT ($p < 0.05$ and $p < 0.01$, respectively) and negatively correlated with GFR and G30 ($p < 0.0001$); this implies that at higher relative concentration of α-LA, both rennet and acid coagulation properties were impaired. The β-LG relative concentration showed a significant negative correlation ($p < 0.0001$) with acid gel strength at 60 min (G60). Increase in the fat globule size was associated with poor acid coagulation properties (GFR and G60). Milk samples with larger casein micelles produced weaker rennet and acid gels ($p < 0.0001$).

Table 10 shows the relationship between salts distribution in milk with rennet and acid coagulation properties of milk. Higher concentration of total Ca and micellar Ca improved rennet coagulation properties (higher a_{30} and low k_{20}), while the acid coagulation properties were not correlated with total and micellar Ca. Total P and soluble P were associated with improved rennet (high a30 and low k20) and acid (high gel strength, gel firming rate and shorter acid gelation time) coagulation properties, while a higher micellar P was associated with shorter curd firming time (k_{20}). Total Mg was positively correlated with GFR and G30 ($p < 0.01$), while soluble and micellar Mg were not correlated with acid and rennet coagulation properties.

Total protein content was positively correlated ($p < 0.0001$) with a_{30}, GFR and G30, and negatively correlated with k_{20} (Supplementary Table S2). Casein content was negatively correlated with k_{20} and GT ($p < 0.05$) and positively correlated ($p < 0.0001$) with a_{30}, GFR and G30. Samples with high fat content used shorter time to form rennet curd ($p < 0.001$) and produced weaker acid gels (G60). Higher lactose content was associated with improved rennet and acid coagulation properties, since it was positively correlated with a_{30}, GFR, G30 and G60 and negatively correlated with k_{20}. A high pH (>6.8) of the raw milk impaired milk coagulation properties (low a_{30}, GFR, G30 and G60).

4. Discussion

The mean chemical composition of milk reported from this work on the NRF breed were in agreement with previous values reported for other dairy cattle breeds (Gustavsson et al., 2014a; Schopen et al., 2009; Vallas et al., 2010). Association of the κ-CN B allele with a high fat percentage was also reported in milk from the Finish Ayrshire cattle (Ikonen, Ojala, & Ruottinen, 1999). In the current study, the proportion of salts in the micelles (i.e., micellar Ca, P and Mg) were slightly lower compared with that reported for Danish dairy breeds (Jensen et al., 2012) and in Dutch Holstein-Friesian cattle (Bijl, van Valenberg, Huppertz, & van Hooijdonk, 2013). This could be due to the differences in stage of lactation, as in our case sampling was between lactation week 8 and 16, while in Jensen et al. (2012), the sampling was between week 19 and 32. A slight difference in the average micelle size for the same breed between the current study and the study by Devold et al. (2000) could be due to different feeding and stage of lactation.

The most common genotype frequency for β-CN was A^2A^2, similar to what was found for Estonian Cattle (Jõudu et al., 2007) and Danish Jersey cows (Gustavsson et al., 2014b; Poulsen et al., 2013). However, this trend has not been found previously in Norwegian Red cattle (Devold et al., 2000) and Swedish Red cattle (Gustavsson et al., 2014a; Poulsen et al., 2013). Selection towards protein yield could probably be the reason for the increase in the β-CN A^2 allele in the NRF breed, since β-CN A^2 was associated with increased protein yield (Heck et al., 2009). The composite genotype of α_{S1}-β-κ-CN BB-A^2A^2-BB and BB-A^2A^2-AA were more common in the current study, similar to the results of Jõudu et al. (2007), who found that BB-A^2A^2-AA was one of the most common composite casein genotypes in Estonian cattle. This composite genotype (BB-A^2A^2-AA) was also common in Danish Holstein, but less common in Swedish Red and Danish Jersey cows (Gustavsson et al., 2014b; Poulsen et al., 2013).

Table 8
Effects of composite genotype on the relative concentration of milk proteins.[a]

α_{S1}-β-κ-CN	Relative concentration of milk proteins (%)										
	α_{S1}-CN	α_{S1}-CN-8P	α_{S1}-CN-9P	α_{S2}-CN	α_{S2}-CN-10P	α_{S2}-CN-11P	α_{S2}-CN-12P	β-CN	κ-CN-1P	α-LA	β-LG
BB-A1A2-AA	32.6 ± 0.5^a	22.6 ± 0.4^a	7.1 ± 0.4^a	7.4 ± 0.5	1.0 ± 0.2	3.9 ± 0.2	2.5 ± 0.2	35.4 ± 0.6^a	4.6 ± 0.2	3.2 ± 0.2	9.0 ± 0.3
BB-A1A2-BE	33.4 ± 0.4^a	23.0 ± 0.3^{ab}	7.5 ± 0.3^a	7.3 ± 0.5	0.9 ± 0.2	3.8 ± 0.2	2.5 ± 0.2	35.3 ± 0.6^a	4.3 ± 0.2	3.4 ± 0.1	8.9 ± 0.3
BB-A2A2-AA	33.4 ± 0.3^a	23.1 ± 0.2^{ab}	7.4 ± 0.4^a	7.6 ± 0.3	1.0 ± 0.1	4.0 ± 0.2	2.5 ± 0.1	33.2 ± 0.4^b	4.2 ± 0.1	3.2 ± 0.1	8.9 ± 0.2
BB-A2A2-BB	33.5 ± 0.3^a	23.4 ± 0.2^{ab}	7.5 ± 0.2^a	7.7 ± 0.3	0.9 ± 0.1	4.1 ± 0.2	2.7 ± 0.1	33.0 ± 0.4^b	4.3 ± 0.1	3.6 ± 0.1	8.9 ± 0.2
BC-A2A2-BB	35.1 ± 0.4^b	23.7 ± 0.3^b	8.4 ± 0.3^b	7.6 ± 0.5	0.8 ± 0.1	3.7 ± 0.2	3.00 ± 0.2	30.5 ± 0.6^b	4.6 ± 0.2	3.6 ± 0.1	8.8 ± 0.3
p-value	$p < 0.05$	$p < 0.05$	$p < 0.05$	NS	NS	NS	NS	$p < 0.0001$	NS	NS	NS

[a] Values are least square means ± standard error. Statistical influence of the composite genetic variants (Model 2) is shown in the last row, different superscript letters within each protein and column shows significant differences ($p < 0.05$).

Table 9
Correlation matrix between the relative concentration of milk proteins, fat globule size, micelle size and milk coagulation properties.[a]

Milk proteins and variables	Milk coagulation properties						
	Rennet coagulation properties			Acid coagulation properties			
	RCT	k_{20}	a_{30}	GT	GFR	G30	G60
Total α_{S1}-CN	NS	NS	NS	NS	NS	NS	NS
α_{S1}-CN-8P	−0.22**	NS	0.25**	−0.16*	0.23**	0.23**	0.29**
α_{s1}-CN-9P	0.18*	NS	−0.20*	NS	−0.22**	NS	−0.20*
Total α_{S2}-CN	NS	NS	NS	NS	NS	NS	NS
α_{S2}-CN-10P	NS	NS	NS	NS	NS	NS	NS
α_{S2}-CN-11P	NS	NS	NS	NS	NS	NS	NS
α_{S2}-CN-12P	0.25*	NS	−0.23**	0.26**	−0.33***	−0.29***	−0.33***
κ-CN-1P	NS	−0.40***	0.36***	NS	NS	NS	NS
Total β-CN	−0.34***	NS	0.30***	NS	0.35***	0.19*	0.33***
α-LA	0.18*	NS	NS	0.24**	−0.33***	−0.31***	NS
β-LG	NS	NS	NS	NS	NS	NS	−0.35***
Other variables							
Fat globule size (μm)	NS	NS	NS	NS	−0.22**	NS	−0.37***
Micelle size (nm)	NS	0.34***	−0.28***	NS	NS	NS	−0.54***

[a] Numbers in the table indicates the coefficients of correlation: NS, non significant; *** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$.

Table 10
Correlation matrix between the salt distribution in milk and milk coagulation properties.[a]

Salt distribution	Milk coagulation properties						
	Rennet coagulation properties			Acid coagulation properties			
	RCT	k_{20}	a_{30}	GT	GFR	G30	G60
Calcium, Ca							
Total Ca	−0.21**	−0.23**	0.27***	NS	NS	NS	NS
Soluble Ca	NS	NS	0.19*	NS	NS	NS	NS
Micellar Ca	NS	−0.16*	NS	NS	NS	NS	NS
Phosphorus, P							
Total P	NS	−0.22**	0.22**	−0.16*	0.22**	0.26**	NS
Soluble P	NS	NS	0.20**	−0.21**	0.26***	0.27***	0.25**
Micellar P	NS	−0.16*	NS	NS	NS	NS	NS
Magnesium, Mg							
Total Mg	NS	NS	NS	NS	0.18**	0.22**	NS
Soluble Mg	NS	NS	NS	NS	NS	NS	NS
Micellar Mg	NS	NS	NS	NS	NS	NS	NS

[a] Numbers in the table indicates the coefficients of correlation: NS, non-significant; *** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$.

The current results showed that genotype BC of α_{S1}-CN improved the rennet coagulation properties (i.e., a low k_{20} and a high a_{30}), this is in agreement with previous studies (Jensen et al., 2012; Jõudu et al., 2009; Poulsen et al., 2013), this could be associated with the effect of α_{S1}-CN BC on the casein micelle size (Devold et al., 2000). Improved rennet coagulation properties (low k_{20} and high a_{30}) was expressed by β CN A^1 compared with A^2, which was in accordance with previous studies, which found an association of the β-CN A^1 allele with good rennet coagulation properties compared with the A^2 alleles (Comin et al., 2008; Jensen et al., 2012). A low acid gel firming rate represented with a large value of k_{20} expressed by the BE variant of κ-CN could be associated with the negative impact of the E allele on the casein micelle size and rennet coagulation properties (Glantz et al., 2010; Jõudu et al., 2009). Shorter RCT and higher a_{30} observed with the β-LG AB genotype compared with the other genotypes (BB and AA), these results are in accordance with Bonfatti, Di Martino, Cecchinato, Degano, and Carnier (2010), while a study on Swedish Red cattle by Hallén, Allmere, Näslund, Andrén, and Lundén (2007) found a non-significant effect of the β-LG variants on RCT.

The inclusion of the C variant of α_{S1}-CN in the composite genotype α_{S1}-β-κ-CN (BC-A^2A^2-BB) resulted in lower values of k_{20} compared with the other composite genotypes, most probably since α_{S1}-CN BC was linked to smaller casein micelle size and better rennet coagulation properties compared with the BB genotype, similar to the findings of Jensen et al. (2012) and Devold et al. (2000). In the current study BB-A^2A^2-AA was linked to poor rennet coagulation properties, which was also shown in previous studies (Comin et al., 2008; Gustavsson et al., 2014b; Jensen et al., 2012).

The κ-CN genotypes imposed a large variation on the acid coagulation properties, while surprisingly the β-CN and β-LG genotypes did not influence the acid coagulation properties. This disagrees with the results of Hallén et al. (2009), who reported a significant effect of the β-LG genotypes and a non-significant effect of casein genotypes on the acid coagulation properties of milk in Swedish Red cattle; these differences could be explained by the different stages of lactation. The composite α_{S1}-β-κ-CN genotypes significantly affected the acid coagulation properties, while the previous study by Hallén et al. (2009) found no effect of the β-κ-CN composite genotype on acid coagulation properties.

Genotype BE of the κ-CN was associated with large casein micelles compared with the AB and BB genotypes, this agrees with studies on other breeds (Bijl, de Vries, van Valenberg, Huppertz, & van Hooijdonk, 2014a; Hristov et al., 2014).

The results from the current study showed a significant effect of the casein composite genotypes on the casein micelle size, with smaller sized micelles in the α_{S1}-β-κ-CN BC-A^2A^2-BB genotype and large micelles associated with the BB-A^1A^2-BE genotype. Others reported smaller casein micelle size in the composite genotype of

β-κ-CN A^1A^2-AB compared with the A^2A^2-AA and A^1A^1-EE composite genotypes (Gustavsson et al., 2014c), this shows that the presence of κ-CN E in the composite genotype of caseins favours a micelle of larger size compared with the A and B alleles of κ-CN.

A higher concentration of κ-CN-1P and total α_{S1}-CN were associated with the C allele of α_{S1}-CN compared with the B allele. The A^2A^2 variant of β-CN showed a higher concentration of α_{S1}-CN-8P and total α_{S1}-CN, while the A^1A^2 variant of β-CN showed a higher concentration of α_{S2}-CN-10P, β-CN and κ-CN-1P. The current results showed the effects of the κ-CN genotypes on the relative concentration of κ-CN-1P, a slightly higher concentration of κ-CN-1P was associated with the BB and BA variants compared with the AA variant, which was also observed by Heck et al. (2009). The association of β-LG BB with a lower concentration of β-LG compared with AB and AA was observed, this is similar to the results reported by others (Allmere, Andrén, Lindersson, & Björck, 1998; Hallén et al., 2009; Ng-Kwai-Hang, Hayes, Moxley, & Monardes, 1987). A slightly higher concentration of β-CN was found in the α_{S1}-β-κ-CN composite genotype BB-A^1A^2-AA and BB-A^1A^2-BE compared with other composite genotypes investigated, including BB-A^2A^2-AA, which is similar to the observation found in Danish Holstein cattle (Gustavsson et al., 2014b).

Association between casein micelle size distribution with rennet coagulation properties was similar to the findings by Glantz et al. (2010) who reported improved rennet coagulation properties in the samples with smaller casein micelle size. This could be explained by the fact that, smaller micelles provide large surface area for the gel-network formed during milk coagulation compared with that provided by the larger casein micelles. The present study reported low gel strength (acid and rennet) with the increase in the relative concentration of α_{S1}-CN-9P compared with α_{S1}-CN-8P in agreement with Frederiksen et al. (2011), who found poor coagulation properties with higher fractions of highly phosphorylated α_{S1}- and α_{S2}-CN. This could be due to the negative effect of the α_{S1}-CN-9P with casein content and protein percentage. Association of a higher concentration of κ-CN-1P with better milk coagulation properties was in agreement with previous reports (Hallén, Lundén, Tyrisevä, Westerlind, & Andrén, 2010; Wedholm, Larsen, Lindmark-Mansson, Karlsson, & Andren, 2006), this is because κ-CN-1P is associated with smaller casein micelle size, higher casein content and protein percentage, which were associated with improved rennet coagulation properties. Furthermore, the association of β-CN concentration with good rennet coagulation properties was in agreement to previous observations made by Wedholm et al. (2006) who found a positive correlation between an increase in the concentration of β-CN and cheese yield. Similar to previous studies (Abeykoon et al., 2016; Jensen et al., 2012), α-LA and β-LG concentrations were associated with poor acid and rennet coagulation properties, respectively. Negative effect of α-La and β-LG on the milk coagulation properties could be explained by their negative effect on the casein content, protein percentage and casein micelle size. On the other hand, in the study by Hallén et al. (2009), β-LG concentration was associated with high acid gel strength at 4, 8 and 10 h; these differences could be explained by the different lactation stages or methodological approaches (in the present gel was monitored for 1 h). A higher proportion of larger sized fat globules resulted into weaker acid gels, this agrees with findings made by Ji, Lee, and Anema (2011), who observed less interaction of the native fat globules (unhomogenised milk) with the casein matrix, hence weaker acid gel.

The salts distribution between the micellar and soluble phase explained a large part of the variation in the rennet coagulation properties, where higher total salts and micellar Ca and P were associated with better rennet coagulation properties, this is in line with previous studies (Gustavsson et al., 2014a; Jensen et al., 2012; Malacarne et al., 2013). Poor rennet coagulation properties in milk samples with the low content of micellar bound salts (Ca and P) could be due to the low amount of phosphate groups available for aggregation of casein micelles during the non-enzymatic phase of rennet coagulation (Malacarne et al., 2013). Effects of total P, soluble P and total Mg on the acid coagulation properties is still unclear; however, in the current study a negative correlation between soluble P and casein micelle size was found.

Milk coagulation properties were improved in samples with a high dry matter content (casein, protein fat and lactose), which was in accordance with the results of Malacarne et al. (2013). However, weaker acid gels (G60) were obtained in the samples with higher fat content. Differences in pH contributed to the variation in RCT and a_{30}, in accordance to previous reports (Cassandro et al., 2008; Jõudu et al., 2008). This could be explained by the fact that rennet activity increases with reduced pH (Foltmann, 1959). Likewise, GFR, G30 and G60 negatively correlated with high the pH of the raw milk. The explanation for the longer GT and poor acid coagulation properties (GFR, G30 and G30) in the samples with high pH (>6.8) could be due to the longer time needed to dissolve the colloidal calcium phosphate and for micelle disintegration.

5. Conclusions

Improved rennet coagulation properties were associated with α_{S1}-CN C, β-CN A^1 and BC-A^2A^2-BB composite genotype of α_{S1}-β-κ-CN, while κ-CN A and BB-A^2A^2-AA were associated with good acid coagulation properties. Milk protein genotypes that favoured better rennet coagulation properties (i.e., BC-A^2A^2-BB and κ-CN BB) were associated with poor acid coagulation properties, while those that favoured good acid coagulation properties (i.e., κ-CN AA and BB-A^2A^2-AA) were associated with poor rennet coagulation properties. It is challenging for the dairy industry to choose the best genotypes for both cheese and cultured milk production. However, the two composite genotype of α_{S1}-β-κ-CN BB-A^2A^2-BB and BB-A^1A^2-BE were associated with both poor rennet and acid coagulation properties. Therefore, the breeding program for NRF cattle should focus on decreasing the BB-A^2A^2-BB and BB-A^1A^2-BE genotypes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.idairyj.2016.10.010.

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Paper IV

1 **The influences of milk protein genotypes on the physical properties of the cultured milk**

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27 **Abstract**

28 The objective of the current research was to study the effect of milk protein genotypes on the
29 physical properties of cultured milk focusing on the rheological properties, the degree of
30 syneresis, particle size distribution. Analyses were made at the first (D1) and fourteenth day after
31 production (D14). Significant effects of genotypes of β-LG (lactoglobulin) and the κ-CN/β-LG
32 composite genotypes were found on the degree of syneresis and yield stress in D14 samples.
33 However, effect of κ-CN/β-LG composite genotypes on yield stress were not observed after
34 including protein content in the statistical model. The degree of syneresis and acetoin
35 concentration in the D14 samples were affected by the respective κ-CN/β-LG and α_{s1}-/κ-CN
36 composite genotypes, even if the protein contents was introduced in the statistical model. These
37 results provide the possibilities of using milk protein genomics for improvement of the water-
38 holding capacity in the cultured milk. However, further studies at equal protein concentration are
39 needed.

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45 # 1 Introduction

46 The major problems affecting the quality of the fermented milks are poor consistency, poor
47 texture, and excessive whey separation, especially in low fat products. Heat treatment, incubation
48 temperature and the dry matter content of the milk have been found to be important
49 technological factors for properties of low fat acid gels (Jørgensen, et al., 2015; Laiho, Williams,
50 Poelman, Appelqvist, & Logan, 2017; Lucey, Tamehana, Singh, & Munro, 1998b). Milk used
51 for the production of fermented milk is normally heat-treated at 90-95 °C for 5-10 minutes and
52 cooled to 42 °C or 22 °C (for yoghurts or cultured milk, respectively) before starter addition
53 (Zhao, Wang, Tian, & Mao, 2016). During the heat treatment of milk, whey proteins, especially
54 β-lactoglobulin (β-LG), are denatured and attached to the surface of the casein micelle to form
55 whey protein-casein micelle complexes via hydrophobic interaction and intermolecular
56 disulphide bonds (Lucey, Tamehana, Singh, & Munro, 1998c). This has been associated with
57 shorter gelation time and improved structural properties of the milk acid gels (Lucey, Tamehana,
58 et al., 1998b; Lucey, 2004).

59 Single nucleotide polymorphisms and nucleotide deletion/insertion on the genes (*CSN1S1*,
60 *CSN2*, *CSN1S2* and *CSN3*, *LAA* and *LGB*), which codes for milk proteins (α_{s1}-CN, β-CN, α_{s2}-
61 CN, κ-CN, α-LA, and β-LG, respectively) alters the properties of proteins. This is due to the
62 modifications in the amino acid sequence of the proteins (by either, amino acid substitution or
63 deletion/insertion), which leads to the change in the isoelectric point, net charge and
64 hydrophobicity of the proteins (Martin, Bianchi, Cebo, & Miranda, 2013). These modifications
65 will lead to the change in the milk properties and product characteristics, for example, milk
66 composition, heat denaturation of proteins, casein micelle size, fat globule size, salt/mineral
67 distribution in milk and cheese making properties (Allmere, Andrén, & Björck, 1997; Bijl, de

68 Vries, van Valenberg, Huppertz, & van Hooijdonk, 2014; Ketto, et al., 2017; Poulsen, et al.,
69 2013). Understanding how these variations at the gene level (genotypes) influence milk
70 properties is an important tool for the dairy industry in marking the best possible gene
71 combinations linked to the product quality.
72 Elastic modulus (*G′*), yield stress, viscosity and particle size in yoghurt gels were found to
73 increase with the increase in the concentration of β-LG (Chua, Deeth, Oh, & Bansal, 2017;
74 Jørgensen, et al., 2015; Laiho, et al., 2017). Previous reports on milk from individual cows
75 showed variations in concentration of β-LG with the different milk protein genotypes (Hallén,
76 Wedholm, Andrén, & Lundén, 2008; Hallén, Allmere, Lundén, & Andrén, 2009; Heck, et al.,
77 2009; Ketto, et al., 2017). Studies relating milk protein genotypes with acid coagulation
78 properties of milk using glucono-δ-lactone (GDL) have been published, mainly in milk from the
79 Swedish Red (SRB) and Norwegian Red (NRF) breeds (Allmere, Andrén, Lindersson, & Björck,
80 1998; Hallén, et al., 2009; Ketto, et al., 2017). A study on the SRB by Allmere, Andrén,
81 Lindersson, et al. (1998) reported a higher *G′* with the B allele of β-LG compared to A, which
82 was explained by higher aggregation of β-LG B to casein micelles compared to A (Allmere,
83 Andrén, Lindersson, et al., 1998; Allmere, Andrén, Lundén, & Björck, 1998). A study on the
84 same breed by Hallén, et al. (2009), reported a shorter gelation time with β-LG AA compared to
85 AB and higher *G′* with β-LG AA and AB compared to BB when the statistical model was not
86 adjusted for the concentration of β-LG. On the same study, an opposite trend was found at equal
87 β-LG concentration, when β-LG BB showed a higher *G′* compared to AB. Furthermore, a study
88 by Ketto, et al. (2017) which investigated acid coagulation properties in NRF and found no
89 effects of β-LG genotypes, but a shorter gelation time and higher gel firmness with κ-CN AA
90 compared to those with κ-CN AB and BB.

91 Studies relating the effects of milk protein genotypes on the acid gelation using commercial
92 starter cultures are needed to confirm these findings. Hence, the aim of the current research was
93 to study the effects of α_{s1}-CN, κ-CN and β-LG genotypes on the properties of the cultured milk
94 produced by mesophilic starter culture focusing on the rheological properties, degree of
95 syneresis, particle size distribution and fermentation metabolites such as organic acids and
96 volatile compounds.

97

2 Materials and Methods

2.1 Blood samples and genotyping

Genotyping of the cow was established previously by Ketto, et al. (2017). In brief, blood samples were collected in 9 mL Vacutainer® plastic tubes coated K3EDTA (Greiner bio-one GmbH, Austria). Samples were prepared for the paired-end sequencing (2 ×125 bp) using a TruSeq DNA PCR-free library preparation kit and sequenced with the manufacturer's V4 kit (Illumina, San Diego, CA, USA). Sequencing was performed by the Norwegian Sequencing Centre, Oslo, Norway, using a Hiseq 2500 platform according to the manufacturers' protocol. All reads were aligned against the bovine reference genome UMD 3.1 using BWA-mem version 0.7.10 and variant calling was established using Freebayes version 1.0.2. Nine non-anonymous missense SNPs were identified and the cows were genotyped for the SNPs using the MassArray genotyping platform (Agena Biosciences, San Diego, CA, USA).

2.2 Milk samples

Morning milk samples were collected from twenty-eight individual cows of NRF cattle that had calved between September 2016 and February 2017 (within 30 to 150 days after calving). These cows belong to the Centre for Animal Research (SHF) of the Norwegian University of Life Sciences (NMBU). Cows were milked individually in a separate milking parlor as described by Ketto, et al. (2017). Immediately after sampling, the milk samples were transported to the Dairy pilot plant at the Faculty of Chemistry, Biotechnology and Food Science (KBM) for production of cultured skim milk. Table 1 shows the grouping of the cows by the different milk protein genotypes, all cows had the same genotype for β-CN (A^2A^2), since it was the most frequent genotype in NRF cattle (Ketto, et al., 2017).

2.3 Production of cultured milk

Milk samples were preheated to 55 °C before cream separation using a 10 L batch electrical cream separator (Janschitz GmbH., Althofen, Austria). After separation, the skim milk samples were analyzed for gross chemical composition i.e. protein, fat, casein and lactose using a MilkoScan FT1 (Foss Electric A/S, Hillerød, Denmark), before homogenization at 180 bar at 55 °C (Rennie Works Ltd., Albertslund, Denmark). About 5 liters of the homogenized milk sample was heat treated at 95 °C for 5 minutes in a special pasteurization unit (5 L process tank) linked to steam and cold water. After heat treatment, samples were cooled to 22 °C, transferred to a 5 L sterile steel container with a lid before the addition of starter culture (0.1%). The starter culture was prepared by adding 2 mL of a frozen Direct Vat Set (DVS) mesophilic DL culture (XT-303; Chr.Hansen A/S, Hørsholm, Denmark) into a 200 mL ultra-heat treated milk (TINE SA, Oslo, Norway) and incubated at 22 °C for 24 ± 0.5 h until the production day. After inoculation, samples were transferred into four sterile glass jars before transferring to a temperature controlled water bath and incubated at 22 °C until pH of 4.5±0.01. The samples were then immediately transferred into a container with ice water before storage at 4 °C. One glass jar was used for pH measurements using pH meter (PHM61; Radiometer, Copenhagen, Denmark), while the other jars were used for rheological measurements, analysis of particle size distribution, degree of syneresis and fermentation metabolites on the first day (D1) and fourteenth day (D14) after production.

2.4 Physical properties of cultured milk

2.4.1 Particle size distribution

Particle size distribution in the D1 and D14 samples of cultured milk was analyzed by laser diffraction technique using Mastersizer 3000HS (Malvern Instruments Ltd., Malvern, UK) by the

143 method described by Jørgensen, et al. (2015), with some modifications. In brief, the refractive
144 indexes of the dispersant solution and the sample were set at 1.33 and 1.461, respectively. 4
145 drops of the sample was added into a large volume wet sample dispersion unit (Hydro LV;
146 Malvern Instruments Ltd., Malvern, UK) with distilled water, within the obscuration range of 3
147 to 10%. The samples were stirred at 3500 rpm for 1 min, to ensure uniform dispersion of the
148 particles. Measurements were made at an absorption index of 0.001 and at 1500 rpm stirring
149 speed at room temperature (20±2 °C). Ten (10) measurement sequences were made on each
150 sample and two parallels were made for each sample, making 20 observations per sample. Before
151 proceeding to the next sample, data quality and reproducibility between measurement sequences
152 were checked. Volume weighted mean diameter of the particles (d[4, 3]) and the diameter below
153 which 90% of particles by volume were found (Dv 0.9) was reported as a measure of the
154 presence of larger particles (Ciron, Gee, Kelly, & Auty, 2010; Laiho, et al., 2017).

155 **2.4.2 Rheological properties**

156 Rheological measurements were made by using Physica MCR 301 rheometer (Anton Paar.,
157 GmbH, Graz, Austria) using a bob-cup measurement system (CC27/Ti with diameter 26.657 mm
158 and 40.03 mm length for bob specifications and C-CC27/T200/Ti with 28.926 mm diameter for
159 the cup specifications). Measurements were made at 4 °C by using three techniques (i.e., strain
160 sweep, frequency sweep and rotational viscometry) according to the method established by
161 Allmere, Andrén, Lindersson, et al. (1998), with a few modifications. Strain sweep was made to
162 define the linear viscoelastic range (LVR) by determining the strain applied before the gel
163 ruptured at frequency of 0.5 Hz and a strain level of 0.0002 to 0.206. The strain below the upper
164 limit of the LVR obtained from strain sweep was used in the frequency sweep at 0.10 to 0.5 Hz
165 to determine the elastic modulus (G') and viscous modulus (G'') within the LVR. These values

166 (G' and G'') were measured at 0.5 Hz. Finally, the viscosity measurements were made on the
167 samples to determine the flow properties of the samples at a shear rate range of 0.02 to 1.46 s^{-1} at
168 357 s interval and 9 measurement points. Two parallels were made for each sample.

169 After fitting the viscometry or flow data (Shear Stress vs. Shear Rate data) to several rheological
170 models (data not shown), only modified Cross model (E1) with yield stress established by
171 Rayment, Ross-Murphy, and Ellis (1995), gave good fit in all data ($R^2 = 0.99$). Important
172 rheological parameters were estimated from this model, i.e., yield stress (τ_0), zero-shear viscosity
173 (η_0) and the model fitting constants K and n.

174 $$\eta = \eta_0 + [\eta_0 - \eta_\infty]/[1 + (\mathrm{K}\dot{\gamma})^{\mathrm{n}}] + (\tau_0/\dot{\gamma}) \quad [E1]$$

175 Where:

176 η = apparent viscosity ($\tau/\dot{\gamma}$), η_0 = zero shear viscosity, K = is the structural relaxation time
177 associated with the rupture of the linkages in the gel network (Cross, 1965), n= exponent related
178 to shear thinning behavior and η_∞ = viscosity at infinite shear rate. Since η_∞ is very low, close to
179 zero ($\eta_\infty \sim 0$) it is difficult to estimate (Rao, 2014). Therefore the cross equation (E1) was
180 reduced to:

181 $$\tau = \tau_0 + (\eta_0/[1 + (\mathrm{K}\dot{\gamma})^{\mathrm{n}}])\dot{\gamma} \quad [E2]$$

182 **2.4.3 Susceptibility to syneresis**

183 The degree of syneresis on the cultured milk gels was determined according to the method by
184 Zhang, Folkenberg, Amigo, and Ipsen (2016). Briefly, 30 g of the sample was weighed into 50
185 mL Falcon tubes and each tube was placed in a 50 mL conical tube bucket connected to a TX-
186 750 swing-out rotor (Thermo Fisher Scientific LED., GmbH, Osterode Germany). Centrifugation
187 was carried out using a Heraus Multifuge X3R centrifuge (Thermo Fisher Scientific LED,

188 GmbH, Osterode Germany) at 500 $\times g$ for 20 min at 4 °C. The amount of whey was calculated to

189 express the degree of syneresis according to the following formula:

190 $$Syneresis, \% = {W1}/{W2} * 100 \qquad \text{E3}$$

191 Where, W1 is the weight of the supernatant and W2 is the weight of the sample (W2 = 30g).

192 **2.4.4 Gel microstructure**

193 The microstructure of the D1 samples of the cultured milk was analyzed by confocal laser

194 scanning microscopy (CLSM) using an inverted microscope Leica TCS SPS fitted with

195 Ar/DPPS laser (Leica, Microsystems, CSM, GmbH, Mann Heim, Germany) as described by

196 Jørgensen, et al. (2015), with some modifications. The top layer of the sample was discarded

197 before sampling, the mid-layer was carefully sampled and stained by using Fast green (CFC dye;

198 Sigma-Aldrich, Saint Louis, MO, USA) for proteins and Nile red (Nile red, Oxazone, Sigma-

199 Aldrich, Saint Louis, MO, USA) for fat. The excitation emissions used were 633 nm and 488

200 nm, at emission wavelength of 643 to 695 nm and 498 to 570 nm for protein and fat,

201 respectively. After staining, samples were allowed to rest at 4 °C for 2 h before analyses. An

202 objective lens with 63 × magnification was used to take five images (resolution: 1024×1024) at

203 random positions on each sample. One representative image was chosen within each sample with

204 respect to each κ-CN/β-LG genotype.

205 **2.4.5 Fermentation metabolites**

206 Organic acids (citric acid, acetic acid, pyruvic acid, orotic acid, succinic acid, α-keto glutaric

207 acid, lactic acid and uric acid) and carbohydrates (glucose, galactose and lactose) were

208 determined by using High Pressure Liquid Chromatography, HPLC (Perkin-Elmer, Norwark,

209 CT, USA). Aromatic compounds (acetaldehyde, acetoin, acetone/3-hydroxybutanone, diacetyl,

210 3-methyl butanal and 3-methyl butanol) were determined by using Headspace sampler unit HP
211 7694 coupled with a 6890 GC system (Agilent, Santa Clara, CA, USA). Both methods were
212 previously described by Narvhus, Østeraas, Mutukumira, and Abrahamsen (1998), with further
213 modifications by Grønnevik, Falstad, and Narvhus (2011).

214 2.5 Statistical analysis

215 The Mixed prodecure of SAS (SAS, 2015) was used to analyse the efects of the milk protein
216 genotypes on the rheological properties, degree of syneresis, particle size distribution and
217 fermentation metabolites in the D1 and D14 samples by using the following mixed model:

218 $\mathrm{Y} = \mathrm{X}\beta + \mathrm{Z}u + \mathrm{residual}$ E4

219 Where Y is the vector for the response variable (the rheological properties, syneresis, particle
220 size distribution or fermentation metabolites), β is an unknown vector for the fixed effects (α_{s1}-
221 CN, κ-CN, β-LG and κ-CN/β-LG or α_{s1}/κ-CN composite genotypes), and *u* is a vector random
222 variables (Cow: 1, 2, 3,….,28), in addition to the residual. X and Z are known design matrices
223 for the fixed and random effects.

224 The fixed effects were evaluated by using Type 3 tests, using two steps. In the first step, fixed
225 effects of the milk protein genotypes, such as α_{s1}-CN (BB and BC), κ-CN (AA and BB), β-LG
226 (AB and BB) and κ-CN/β-LG composite genotypes (AA/AB, AA/BB, BB/AB and BB/BB) were
227 tested, while in the second step, the fixed effect of the α_{s1}/κ-CN composite genotypes (BB/AA,
228 BB/BB and BC/BB) were analysed. Restricted Maximum Likelihood (REML) was used to
229 estimate the residual variance and the cow variance components. The statistical analyses were
230 repeated with protein contents as covariate (in Xβ) in order to test if the physical properties and

231 the concentration of fermentation metabolites were due to the milk protein genotypes or protein
232 content.

233 Correlation coefficients between the quality parameters were computed by correlation procedure
234 of SAS (SAS, 2015).

235 3 Results

236 3.1 Milk composition and fermentation metabolites

237 The average composition of skim milk samples with their covariance estimates is presented in
238 Table 2. In the data set analyzed, the milk protein genotypes (α_{s1}-CN, κ-CN, and β-LG and α_{s1}-
239 /κ-CN genotypes) had no significant effect on the protein and lactose contents. However, the
240 residual variances (within sample variation) were small compared to the between cow variation,
241 for all milk components within the fixed effects of the milk protein genotypes (Table 2).
242 However, a slightly higher protein content was associated with κ-CN/β-LG composite genotypes
243 AA/AB and BB/AB compared to κ-CN/β-LG composite genotypes BB/BB and AA/BB (Figure
244 1).

245 The concentrations of other fermentation metabolites (such as, pyruvic acid, acetoin,
246 acetaldehyde, diacetyl, and ethanol) were not significantly influneced by the α_{s1}-CN, κ-CN, β-
247 LG and κ-CN/β-LG composite genotypes, neighter in D1 nor D14 samples of cultured milk.
248 Surprisingly, a higher concentration of lactic acid at D1 was observed in cultured milk with the
249 BC genotype of α_{s1}-CN compared to the BB genotype ($P<0.05$), while a higher concentration of
250 orotic acid was observed in the AA/AB, BB/BB and BB/AB composite genotypes of κ-CN/β-LG
251 compared to the AA/BB genotype (Table 5). In D14 cultured milk samples the concentration of
252 lactic acid increased with BB/AB, AA/BB and AA/AB composite genotypes of κ-CN/β-LG

253 compared to the BB/BB composite genotype. To confirm the effects of α_{s1}-CN genotypes and κ-
254 CN/β-LG composite genotypes on the concentration of fermentation metabolites observed, the
255 protein content of the fresh milk was included as a covariate in the statistical model. However,
256 after including the protein content in the model, the effects of α_{s1}-CN and κ-CN/β-LG composite
257 genotypes on the lactic acid and orotic acid concentrations on the D1 cultured milk samples were
258 not observed, nor the effect of κ-CN/β-LG composite genotypes on the lactic acid concentration
259 in the D14 samples.

260 A higher (P<0.05) concentration of acetoin was observed in cultured milk at D14 with the α_{s1}/κ-
261 CN composite genotypes BB/AA and BC/BB (259.56±44.56 and 138.56±49.88 ppm,
262 respectively) compared to the BB/BB genotype (138.56±49.88 ppm), and this was not altered by
263 inclusion of protein content in the statistical model.

264 3.2 Physical properties

265 Milk protein genotypes did not have significant effect on the elastic modulus (G') or particle size
266 distribution of the cultured milk samples in neither D1 nor D14 samples. Table 3 summarize the
267 effect of milk protein genotypes on the yield stress and degree of syneresis in the D14 samples of
268 the cultured milk, before and after adjustment of the protein content. In the D14 samples of
269 cultured milk, the β-LG and the κ-CN/β-LG composite genotypes significantly influenced the
270 degree of syneresis and the yield stress (P<0.01). Higher values of yield stress and a lower
271 degree of syneresis were observed in cultured milk with the AB genotypes of β-LG compared to
272 the BB, which was more susceptible to syneresis (Figure 2). However, the milk protein
273 genotypes (both β-LG and κ-CN/β-LG composite genotype) did not influence yield stress when
274 protein contents was included in the model, while the degree of syneresis was still influenced by
275 the β-LG and κ-CN/β-LG composite genotypes even when protein content was included in the

276 model. Figure 2b shows that the AA/AB and BB/AB composite genotypes of κ-CN/β-LG were
277 associated with a lower degree of syneresis, compared to AA/BB and BB/BB, which were more
278 susceptible to syneresis.

279 The images from CSLM analysis (Figure 3), shows the differences of the gel microstructure
280 between samples with different combinations of κ-CN/β-LG composite genotypes,
281 corresponding to the susceptibility to syneresis. Samples of cultured milk with AA/AB and
282 BB/AB κ-CN/β-LG composite genotypes showed a less porous structure and a lower degree of
283 syneresis compared to samples with AA/BB and BB/BB κ-CN/β-LG composite genotypes. In the
284 present study, elastic properties (G') and yield stress of the gels were positively correlated to the
285 protein content and particle size distribution, while the degree of syneresis was negatively
286 correlated with the G', yield stress and the particle size distribution (Table 4). Furthermore, an
287 effect of the α_{s1}-CN genotypes was observed on the syneresis of the cultured milk, with a lower
288 degree of syneresis in the samples with the BC genotype of α_{s1}-CN compared to the BB
289 genotype.

290 4 Discussion

291 Yield stress, elastic properties, gel microstructure and water-holding capacity are the important
292 parameters used in evaluation of the physical properties of milk acid gels (Kalab, Allan-Wojtas,
293 & Phipps-Todd, 1983; Lucey, Teo, Munro, & Singh, 1997). A study by Lucey, Munro, and
294 Singh (1998a) associated the higher yield stress with higher elasticity and a resistance of the gel
295 networks to break after applying the shear. The samples with higher yield stress would have a
296 more compact microstructure/denser networks and hence the lower degree of syneresis compared
297 to the samples with lower yield stress. This could explain why a more compact microstructure,
298 higher yield stress and a lower degree of syneresis was obtained in the samples with κ-CN/β-LG

AA/AB compared to samples with the BB/BB composite genotype, which had a more open microstructure and loose network which were easy to break and loosed whey.

The results from the current study showed that the effect of the κ-CN/β-LG composite genotypes on the yield stress was confounded with the protein content, while the degree of syneresis was not influenced by the differences in protein content. Previous reports showed a higher concentration of β-LG with the A allele for κ-CN and β-LG (Hallén, et al., 2009; Heck, et al., 2009; Ketto, et al., 2017). A higher concentration of β-LG could contribute to a denser network between the aggregated particles, improved microstructure and hence improved elastic properties and water-holding capacity of acid milk gels. Studies on low fat yoghurt prepared from heated milk samples reported higher elastic properties and yield stress at a higher concentration of β-LG (Chua, et al., 2017; Jørgensen, et al., 2015; Laiho, et al., 2017). A higher concentration of β-LG could provide a higher degree of β-LG aggregates to each casein micelle; this would lead to the formation of larger particles and improved elastic properties and higher yield stress (Chua, et al., 2017; Laiho, et al., 2017; Mahomud, Katsuno, & Nishizu, 2017; Zhao, et al., 2016). However, the presence of coarser particles (Dv 0.9 > 150 μm) in the yoghurt gels was associated with the increase in the graininess and roughness perception in yoghurts (Cayot, Schenker, Houzé, Sulmont-Rossé, & Colas, 2008; Krzeminski, Großhable, & Hinrichs, 2011; Laiho, et al., 2017). However, in the current study only small sized particles were measured (Dv 0.9 < 50 μm), this was expected in cultured milk since the above-mentioned yoghurts were fortified with skim milk powder and whey protein isolate (Chua, et al., 2017; Laiho, et al., 2017). This was found to increases the protein content and hence the formation of larger soluble protein complexes, because of the larger amount of denatured whey protein associated to the casein micelle (Mahomud, et al., 2017)

Since the proportion of denatured whey protein associated to the surface of casein micelles was found to influence the physical properties of the fermented milk gels, studies on the effect of β-LG denaturation were established, for example, Li (1997) showed a decrease in the heat stability of β-LG in milk with κ-CN AA compared to the BB genotype. The AA genotype of κ-CN was associated with a higher proportion of denatured β-LG when heat-treated at 80 °C for 15 min compared to the BB genotype (91% *vs.* 78.5%). The same report showed that the milk samples with κ-CN/β-LG composite genotypes AA/BB and AB/AA were easily denatured (31% at 70 °C and 91% at 80°C) compared to the BB/AA and BB/BB (25% at 70 °C and 77.5% at 80 °C). This was similar to previous report (Imafidon, Ng-Kwai-Hang, Harwalkar, & Ma, 1991). During heat treatment, the denatured β-LG and casein micelles aggregate together to form a β-LG/casein micelle complex (Kalab, et al., 1983; Lucey, 2004).

Aforementioned, only few studies have been performed on the effects of milk protein genetic polymorphism on the acid coagulation properties of milk. For example, a study by Hallén, et al. (2009) on the Swedish Red cattle (SRB) reported the shorter acid gelation time with the AA genotype of β-LG compared to AB and a higher gel firmness at 60 min with AA and AB genotypes of β-LG compared to BB. However, after adjusting the concentration of β-LG, Hallén, et al. (2009) found a higher gel firmness with BB compared to AB and AA genotypes of β-LG, this was similar to observations in their previous study on the SRB by Allmere, Andrén, Lindersson, et al. (1998) who reported an increase in *G′* with β-LG BB, compared to AB and AA. Ketto, et al. (2017) reported (on milk from Norwegian Red cattle (NRF)) favored acid coagulation properties of milk (i.e. gelation time, gel firming rate and gel firmness at 60 min) with κ-CN AA and composite genotype (α_{s1}-β-κ-CN) BB-$A^{2}A^{2-}$AA compared to BB and BC-A2A2-BB respectively.

The values for the total protein, casein and lactose obtained from the cows analyzed correspond with values reported previously in Norwegian Red cattle (Devold, Brovold, Langsrud, & Vegarud, 2000; Ketto, et al., 2017). Similar to Ketto, et al. (2017), the present study reported a non-significant effect of the α_{s1}-CN, κ-CN, β-LG and casein composite genotypes on the protein, casein and lactose contents. However, the total protein content influenced the concentrations of lactic acid and orotic acid. The effect of milk protein content on the concentration of lactic acid and orotic acid could be explained by the buffering capacity of the milk, which was found to be determined by the protein content, inorganic phosphate and citrate concentration (Salaün, Mietton, & Gaucheron, 2005). Acetoin together with other aromatic/carbonyl compounds in fermented milk (e.g., acetate, diacetyl and 2,3-butanediol) are the key products of citrate metabolism in the milk by starter bacteria (Cheng, 2010; Hugenholtz, 1993; Tamime, Skriver, & Nilsson, 2007). As there was no significant difference in the content of citrate and diacetyl between the samples, a possible explanation could be a reduced transformation of acetoin to 2,3-butanediol in the cultured milk with α_{s1}/κ-CN composite genotypes BB/AA compared to BB/BB. The reason for the reduced transformation of acetoin to 2,3-butanediol in BB/AA needs further investigation.

5 Conclusions

The effect of the milk protein genotypes of β-LG (lactoglobulin) and the κ-CN/β-LG composite genotypes on the yield stress and the concentrations of organic acids (lactic and orotic acid) was confounded with the protein content. The ffects of milk protein κ-CN/β-LG and α_{s1}-/κ-CN composite genotypes on the degree of syneresis and the concentration of acetoin, respectively were not masked by protein content. These findings could provide the possibility of improving the water-holding capacity of the fermented milk gels in the future through genomic selection.

However, the future experiments are needed to study the effects of milk protein genotypes on the rheological properties of the cultured skim milk at a standardized protein concentration.

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493

Figure 1

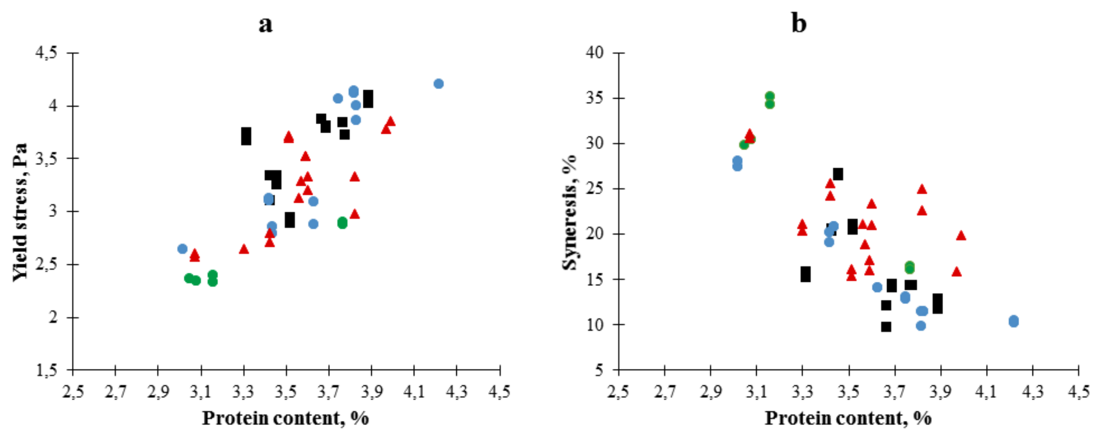

Figure 2

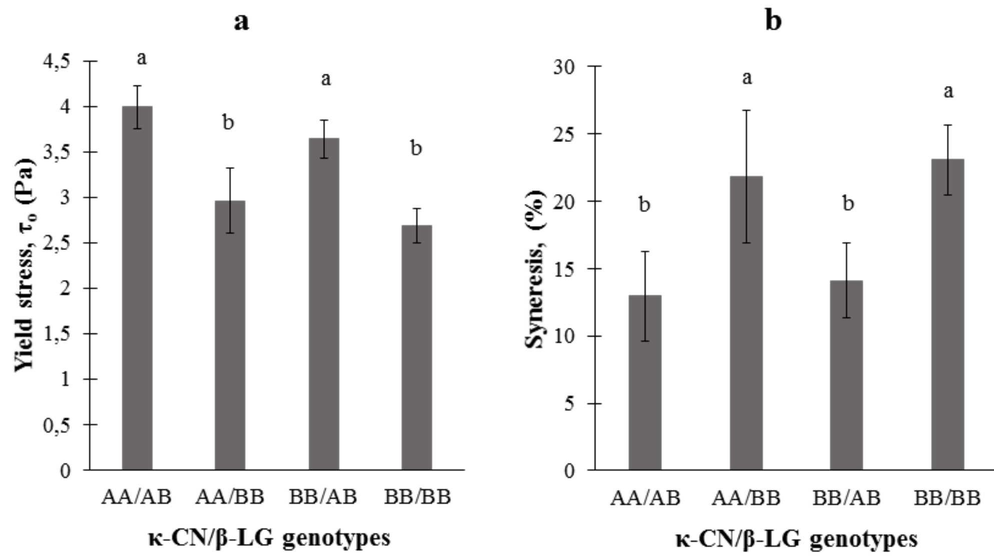

Figure 3

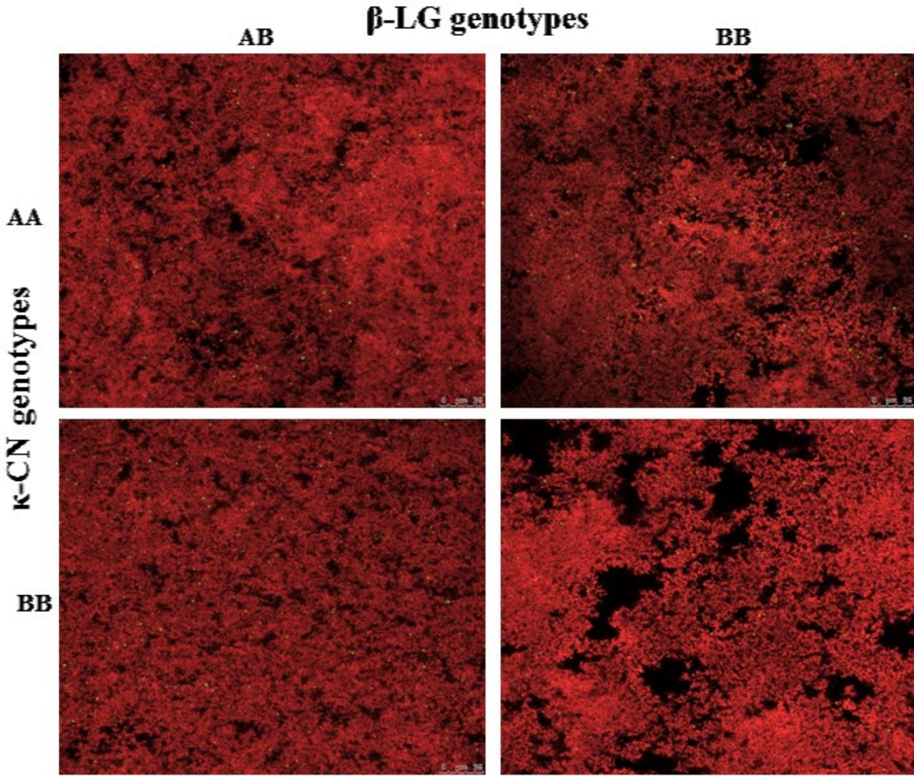

Figure legends

Figure 1: Effects of κ-CN/β-LG genotypes (● AA/AB, ● AA/BB, ● BB/AB and ▲ BB/BB) on (a) yield stress and (b) degree of syneresis in relation to the protein content.

Figure 2: Effects of κ-CN/β-LG genotypes on (a) yield stress and (b) the degree of syneresis in the stored cultured milk: Different letters indicate statistical differences (P<0.05) between κ-CN/β-LG genotypes: without including protein and lactose content in the statistical analysis.

Figure 3: Confocal laser scanning microscopy images of selected samples to display the microstructure of cultured skim milk gels with different κ-CN/β-LG genotypes.

TABLES

Table 1: The number (n) of cows investigated by each milk protein genotype.

	α_{s1}-CN		κ-CN		β-LG		κ-CN/β-LG genotypes				α_{s1}/κ-CN genotypes		
Genotypes	BB	BC	AA	BB	AB	BB	AA/AB	AA/BB	BB/AB	BB/BB	BB/AA	BB/BB	BC/BB
n	23	5	11	7	16	13	8	3	7	10	12	11	5

Table 2: Variance component estimations for the skim milk components.

Milk composition, %	Mean	σ^2 estimates		Type III of the fixed effects			
		Cow	Residual	(α_{s1}-CN	κ-CN	β-LG	κ-CN/ β-LG)
Total protein	3.6	0.09	0.003			NS	
Casein	2.6	0.08	0.002			NS	
Lactose	4.7	0.03	0.001			NS	
Fat	0.1	0.01	0.0001			NS	

NS=No significant effect

Table 3: The effects of the milk protein genotypes on yield stress and degree of syneresis in the stored (D14) samples

Yield stress and degree of syneresis in stored (D14) samples				
	Before adjusting for total protein		After adjusting for total protein	
Genotypes	Yield stress (τ_o), Pa	Syneresis, %	Yield stress (τ_o), Pa	Syneresis, %
α_{s1}-CN				
BB	3.18±0.10	22.33±1.67	3.25±0.11	20.12±0.92
BC	3.42±0.27	13.82±3.34	3.56±0.26	15.64±0.92
p-value	NS	*	NS	NS
κ-CN				
AA	3.18±0.24	17.68±3.30	3.53±0.21	17.34±1.83
BB	3.42±0.13	18.47±2.02	3.29±0.13	18.40±1.11
p-value	NS	NS	NS	NS
β-LG				
AB	$3.64±0.17^a$	$12.67±2.65^b$	3.54±0.17	15.41±1.46
BB	$2.96±0.17^b$	$23.48±2.74^a$	3.28±0.19	20.35±1.51
p-value	**	**	NS	*
κ-CN /β-LG				
AA/AB	$3.71±0.22^a$	$12.60±3.45^b$	3.75±0.23	$14.75±1.92^b$
AA/BB	$2.65±0.30^b$	$22.76±4.78^a$	3.31±0.31	$19.96±2.64^a$
BB/AB	$3.57±0.20^a$	$12.74±3.08^b$	3.32±0.20	$16.08±1.69^b$
BB/BB	$3.27±0.15^b$	$24.20±2.49^a$	3.25±0.16	$20.73±1.36^a$
p-value	**	*	NS	*

NS=Non-significant ($P>0.05$), *$P<0.05$, **$P<0.01$

τ_o= Yield stress (Pa): Determine the resistance to breakage of the junctions between aggregating particles in the gel.
D14= Analysis made on the 14th day after production (stored cultured milk)

Table 4: Correlations of the estimated rheological properties with milk composition, particle size distribution and the degree of syneresis in cultured milk.

	Milk composition			Particle size distribution		Susceptibility to syneresis
Viscoelastic properties	Casein, %	Protein, %	Lactose, %	$D_{[4,3]}$, µm	Dv 0.9, µm	Syneresis, %
Storage modulus , G' (Pa)	**0.49**	**0.71**	-0.20	**0.69**	**0.69**	**-0.50**
Loss modulus, G'' (Pa)	**0.31**	**0.74**	**-0.20**	**0.71**	**0.71**	**-0.50**
Flow properties						
Yield Stress, τ_o (Pa)	**0.42**	**0.71**	-0.20	**0.60**	**0.60**	**-0.80**
Zero shear viscosity, η_0 (Pas)	**0.30**	**0.61**	-0.14	**0.82**	**0.82**	**-0.40**
Structural relaxation time, K	-0.06	**-0.37**	0.09	**-0.51**	**-0.57**	**0.18**

Bolded numbers indicate correlation coefficients significantly different from zero at P<0.05

↑G' = Shows the increase in elastic properties (firmness) of the gel and is related to the strength and the number of bonds between protein particles (Lucey, Munro, & Singh, 1998).

↑G'' = Shows the increase viscous properties of the gel (Foegeding, Vardhanabhuti, & Yang, 2011) .

↑τ_o = Determine the resistance to breakage of the junctions between aggregating particles in the gel (Lucey, et al., 1998).

↑η_0 = Resistance to deformation (Foegeding, et al., 2011)

↑K = Increase in the rate of structural break down (Cross, 1965).

↑$D_{[4,3]}$, = Increase in the mean diameter of the particles (Ciron, Gee, Kelly, & Auty, 2010; Laiho, Williams, Poelman, Appelqvist, & Logan, 2017) .

↑Dv 0.9 = Increase in the proportion of the coarser particles (Ciron, et al., 2010; Laiho, et al., 2017)

Table 5: Effects of milk protein genotypes on concentration of lactic acid and orotic acid in cultured milk before including the protein content in the statistical analysis.

	D1 samples		D14	
Genotypes	Lactic acid	Orotic acid	Lactic acid	Orotic acid
α_{s1}-CN				
BB	8219.08±103.13	41.04±3.55	8566.68±128.13	40.06±3.88
BC	8781.18±243.83	38.41±8.40	8633.63±278.13	22,23±8.39
p-value	*	NS	NS	NS
κ-CN				
AA	8416.35±205.90	39.43±7.10	8636.96±254.94	26.21±7.70
BB	8583.90±126.05	40.03±4.34	8563.34±139.52	36.07±4.21
p-value	NS	NS	NS	NS
β-LG				
AB	8586.94±164.37	44.89±5.65	8690.45±188.13	32.97±5.68
BB	8413.31±172.10	34.57±5.94	8509.85±216.18	29.32±6.53
p-value	NS	NS	NS	NS
κ-CN /β-LG				
AA/AB	8427.48±217.04	52.04 ± 7.46^{a}	8485.21 ± 254.57^{ab}	34.23±7.70
AA/BB	8405.22±299.01	26.81 ± 10.33^{b}	8788.72 ± 390.77^{ab}	18.19±11.80
BB/AB	8746.41±190.15	37.73 ± 6.54^{ab}	8895.70 ± 211.72^{a}	31.70±6.39
BB/BB	8421.40±149.61	42.32 ± 5.10^{ab}	8230.98 ± 171.52^{b}	40.45±5.18
p-value	NS	*	*	NS

NS=Non significant ($P>0.05$), *$P<0.05$, **$P<0.01$

D1= Analysis on the first day after production (fresh cultured milk) and

D14= Analysis made on the 14th day after production (stored cultured milk)