



Characterisation of non-coagulating milk and effects of milk composition and physical properties on rennet-induced coagulation in Swedish Red Dairy Cattle

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ABSTRACT

Non-coagulating milk is a serious problem in the cheese industry, since it decreases cheese yield, resulting in decreased economic output. This study evaluated rennet-induced coagulation properties and composition of milk from individual Swedish Red Dairy Cattle. Milk samples from 679 individual cows were rheologically evaluated, of which 18.1% of the cows produced non-coagulating milk and 18.9% produced poor-coagulating milk. This resulted in 37% of the milk samples being non-optimal in cheese production, which is an alarmingly high figure. A comparison between non-coagulating and coagulating milk showed a significantly lower calcium content and less free Ca^{2+} in non-coagulating milk. The results provide more information about non- and poor-coagulating milk and will be further used to understand the genetic background of non-coagulating milk and breed against this undesired milk property.

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1. Introduction

In 2016, 24% of all bovine milk in Sweden was used for cheese production (LRF Dairy Sweden, 2017). During cheese processing, rennet-induced coagulation of casein micelles is the first step and the coagulation is divided into two phases; the enzymatic and the aggregation phase, respectively. In the enzymatic phase, the κ -casein on the surface of the casein micelles is hydrolysed by the added rennet, which destabilises the casein micelles due to reduction of both static and steric repulsion. This leads to the aggregation phase, which starts when sufficient κ -casein is hydrolysed, where the destabilised casein micelles aggregate to a three-dimensional gel network (Fox & McSweeney, 2017; Walstra, Wouters & Geurts, 2006). From an industrial perspective, a short coagulation process is desired, mainly for economic reasons. The

industrial cheese process is preferably optimised, e.g., by time, and therefore it is not desirable to prolong the processing time of the coagulation step. However, also other factors affect the coagulation rate, for example temperature, pH and rennet concentration (Walstra et al., 2006). According to Troch, Lefébure, Baeten, and Colinet (2017) milk that has not coagulated within 30 min after rennet addition could be defined as non-coagulating (NC) milk.

One dairy breed previously linked to a high proportion of NC milk is the Swedish Red (SR) breed (Gustavsson et al., 2014a), which is the second most common dairy cow breed in Sweden (Växa Sverige, 2017) and is also popular internationally for its animal health benefits (Växa Sverige, 2017). For many years, there has been a migration between SR and other red breeds in breeding programs based on the breeding values of the individual animals. This combined breeding has resulted in the Swedish Red Dairy Cattle (RDC) breed that had the following breed distribution in bulls born between 2008 and 2010: 37% original SR, 32% Finnish Ayrshire, 12% Norwegian Red, 6% Danish Red and 6% Canadian Ayrshire. Since 2010, the Swedish RDC is also a part of the VikingRed breeding

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program, which is a cooperation between Sweden, Finland and Denmark (VikingGenetics, 2017). This exchange means that the red breeds, especially in the Nordic countries, are becoming increasingly genetically linked to each other. Therefore, by researching NC milk in the Swedish RDC, this could be used not only to improve rennet coagulation properties of milk from the Swedish RDC, but might also be useful for milk from other international red breeds.

The presence of NC milk in bulk milk is known to impair rennet coagulation properties (Frederiksen et al., 2011a). They showed that an increasing percentage of NC milk in cheese milk resulted in higher rennet coagulation time (RCT) and slower curd firming rate (CFR). They showed that the CFR decreased with about 10% after adding 5% NC milk and further decreased 30% after adding 25% NC milk, while RCT increased by 20% after addition of 25% NC milk. The frequency of NC milk differs also between different breeds. Gustavsson et al. (2014a) reported that 18% of sampled SR cows produced NC milk but other breeds have also been identified with NC milk: 13% Finnish Ayrshire (Ikonen, Morri, Tyrisevä, Ruottinen, & Ojala, 2004), 4% Danish red and white, 3% Danish Jersey and 2% Danish Holstein-Friesian (Frederiksen et al., 2011b). However, the frequencies of NC milk in different breeds are hard to compare since these studies differed in, e.g., milk fat content (skim or whole milk), type of rennet composition (ratio chymosin/pepsin), rennet concentration, pH adjustment and coagulation temperature, as well as the definition of NC milk.

The ability of milk to coagulate can be determined using rheological parameters. One of these is RCT, which can be defined in different ways. One way to define RCT is the time between rennet addition and the onset of viscosity increase (Amenu & Deeth, 2007). Another way to define RCT is to measure the elastic modulus (G'), and the viscous modulus (G''). G' describes how the milk behaves as an elastic solid and G'' as a viscous liquid, and the relationship between these is described by the phase angle (δ) (Horne & Lucey, 2017). When $\delta > 45^\circ$, the liquid behaviour dominates and, when $\delta < 45^\circ$, the solid behaviour dominates. RCT can therefore be defined as $\delta = 45^\circ$, i.e., when the liquid changes to solid behaviour (Horne & Lucey, 2017). However, there are few studies that compare both approaches, which could be useful in providing detailed information to further strengthen the evidence for the NC property.

Many components in milk have been associated with improved rennet coagulation properties, such as high concentrations of casein, and Ca^{2+} (Lucey, 2009), high total calcium content (Joudu, Henno, Kaart, Püssa, & Kärt, 2008; Malacarne et al., 2014; Nájera, De Renobales, & Barron, 2003), and small casein micelle size (Amenu & Deeth, 2007; Glantz et al., 2010). Of these, a high concentration of Ca^{2+} is the main parameter to improve RCT (Walstra et al., 2006). The cause of NC milk is not yet fully understood, but both genetic and phenotypic factors, including both concentration and genetic variants of milk protein fractions, have been studied earlier and linked with NC milk (Bittante, Penasa, & Cecchinato, 2012; Hallén, Lundén, Tyrisevä, Westerlind, & Andrén, 2010; Poulsen et al., 2016). NC milk has been found to be significantly positively genetically correlated with protein content (Gustavsson et al., 2014c), suggesting that breeding for a high protein content could increase the frequency of NC milk.

However, other studies on coagulating milk have found both positive and negative genetic correlations between protein content and RCT when studying different breeds, e.g. Italian Holstein-Friesian (−0.11), Italian Brown Swiss (−0.02), Finnish Ayrshire and Finnish Friesian (0.49), Danish Jersey (0.55), and Danish Holstein (−0.74) (Cecchinato et al., 2011; Ikonen, Ahlfors, Kempe, Ojala, & Ruottinen, 1999; Poulsen, Buitenhuis, & Larsen, 2015). Phenotypic factors that could be linked with NC milk include lower fat (Jensen et al., 2012) and protein contents (Jensen et al., 2012;

Malacarne et al., 2014), lower content of total calcium (Gustavsson et al., 2014b; Jensen et al., 2012), lower amount of Ca^{2+} (Gustavsson et al., 2014b) as well as lower casein and lactose contents (Gustavsson et al., 2014c). However, Malacarne et al. (2014) did not find significant differences between NC and coagulating milk regarding fat content and total amount of calcium, and nor did Gustavsson et al. (2014b) find a strong phenotypic correlation between NC milk and fat and protein contents. Additionally, most of these studies are based on a low number of animals with NC milk.

Therefore, this study evaluated a larger set of NC milk samples from 679 individual cows from the Swedish RDC, to determine the frequency of NC milk and to distinguish differences in phenotypic traits between milk with different coagulation ability. This revealed effects of compositional and physical properties of milk that influence rennet-induced coagulation and provide data that can be used indirectly in breeding programs to reduce the frequency of NC milk and thereby improve milk quality and cheese processing in the long run.

2. Material and methods

2.1. Milk sampling

A representative aliquot of morning milk samples from 724 individual Swedish RDC were collected during the stable period between December 2015 to April 2016 and September 2016 to April 2017 from 31 conventional farms (one farm was sampled per occasion) in the same geographical region in the south of Sweden. Each farm was sampled once, except for one large herd that was sampled on three separate occasions, and no cow was sampled more than once. The sampled cows were in parity 1–8, with the majority (87%) in parity 1–3, and were between 10 and 436 days in milk (DIM), with 78% in mid-lactation (70–280 DIM). All cows were milked twice a day and fed according to standard practices. The sampled cows were selected based on a number of criteria, such as ancestry, SCC, calving dates, and registration in the Swedish national cow database, thus representing the Swedish RDC population at the time of the study. Parity, DIM, and milk yield data were collected from the Swedish national cow database.

Milk was collected and cooled for transport to Lund University the same day as the milking and kept at 7 °C, without any preservatives, until analyses or freezing at −18 °C for later analyses. Skim milk was obtained by centrifugation (Allerga X-15R Centrifuge, Beckman Coulter, Brea, CA, USA) at 2000 × g, 4 °C for 30 min.

2.2. Milk composition

On the day of milking, milk samples were also sent to a certified dairy analysis laboratory (Eurofins Steins Laboratorium AB, Jönköping, Sweden) for determination of fat, protein, lactose and total calcium contents, as well as somatic cell count (SCC). Fat, protein and lactose contents were measured on fresh milk using Fourier Transform Infrared (FTIR) analysis in a CombiFoss 6000 (FOSS, Hillerød, Denmark) and SCC was measured using flow cytometry fluorescent in a Fossomatic (FOSS). The total amount of calcium was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES), according to the certified method NMKL NO 161 1998 MOD (Nordic Committee on Food Analysis, Oslo, Norway). Milk samples that had a SCC >300,000 cells mL^{−1}, a total of 45 samples, were discarded from further analyses, resulting in 679 individual milk samples used in the study.

Frozen milk samples were thawed overnight at 4 °C and thereafter heated in a water bath at 40 °C before measuring casein, protein and citric acid contents using FTIR in a MilkoScan FT1 (FOSS). The casein and protein contents of frozen milk samples

were used to calculate the casein:protein ratio. The samples were measured in technical duplicates in the MilkoScan.

2.3. pH and titratable acidity

pH was measured on fresh skim milk samples at ambient temperature using a pH meter (744, Metrohm Nordic AB, Bromma, Sweden). Titratable acidity (TA) was measured on a randomly selected subset of 54 fresh milk samples by titration of 50 mL whole milk with batch-wise additions of 0.5 mL NaOH (0.5 M) until the samples reached pH 12. These 54 milk samples were collected from cows belonging to 11 of the 31 farms. TA is an estimation of buffering capacity between the natural pH of the milk and pH 8.3 and is expressed as mmol added NaOH L⁻¹ of milk (°N) (Walstra et al., 2006). A linear regression was used to calculate the added amount of 0.5 M NaOH to reach pH 8.3 and was thereafter used to calculate °N.

2.4. Determination of free amount of Ca²⁺

The level of free Ca²⁺ was measured on fresh milk samples according to the method described by Glantz, Lindmark Månsson, Stålhammar, and Paulsson (2011). A calcium electrode (9720BNWP, Thermo Fisher Scientific, Waltham, MA, USA) was connected to a standard pH meter (PHM210, Radiometer analytical, Loveland, CO, USA) and set to measure the electric potential. The electrode was filled with a fill solution (Optimum Results A, Thermo Fisher Scientific, Chelmsford, UK), and a calibration curve for 10⁻², 10⁻³ and 10⁻⁴ M Ca²⁺ using a calcium standard (Calcium Standard 0.1 M Ca²⁺, Thermo Fisher Scientific) was recorded prior to measurement, according to the manufacturer's manual. Calcium ISA (Thermo Fisher Scientific; 0.45 mL) was added to 22.5 mL skim milk and thereafter heated to 32 °C for 30 min in a water bath before measurement. Level of Ca²⁺ was measured in technical duplicates.

2.5. Casein micelle size distribution

The casein micelle size distribution was measured on fresh milk samples according to Gustavsson et al. (2014b) using laser light scattering (Mastersizer 2000, Malvern Instruments, Worcester-shire, UK). Skim milk was added to the dispersing unit, containing approximately 100 mL of deionised water at ambient temperature, until the obscuration was around 8%. The refractive index for casein micelles was 1.57 (Griffin & Griffin, 1985) and for water 1.33, and the stirrer speed was set to 1500 rpm prior to addition of skim milk. The size distribution was measured in duplicate. The average volume-weighted diameter (D[4,3]) was determined using the supplied software (Malvern application, v 5.60, Malvern Instruments) where only particles between 0.02 and 0.83 µm were included, to remove interference from any remaining fat globules.

2.6. Rheological measurements

Fresh skim milk samples were adjusted to pH 6.50 ± 0.02 using 5% lactic acid (Lactic acid 90%, VWR Chemicals, Leuven, Belgium) at ambient temperature before being pre-heated to 32 °C for 30 min in a water bath. The pH adjustment was made to mimic industrial cheese production and also to minimise the effect of pH variation on the coagulation properties. The rheological properties of rennet-induced coagulation on individual milk samples were thereafter measured using two different instruments.

All samples were measured in duplicate in a free oscillation rheometer (ReoRox 4, MediRox AB, Nyköping, Sweden) at 10 Hz as described by Poulsen et al. (2013). Chymosin (Chy-Max Plus, 200 IMCU mL⁻¹, Christian Hansen A/S, Hoersholm, Denmark) was

added to 10 mL of pH adjusted skim milk pre-heated to 32 °C for 30 min, to a final concentration of 0.09 IMCU mL⁻¹ milk. The recording started immediately and 1 mL of the milk was transferred to the measuring cup after 30 s of stirring. Over 45 min, the system recorded the coagulation time (RCT_{rox}), maximum gel strength (G'_{max_rox}) and the curd-firming rate (CFR_{rox}). RCT_{rox} is defined as the time when δ = 45°, G'_{max_rox} is the highest gel strength during the recording and CFR_{rox} is the rate of gel formation between RCT_{rox} and the end of the measurement.

Furthermore, 324 skim milk samples were randomly selected for measurement in a low-amplitude oscillation rheometer (StressTech, ReoLogica Instruments AB, Lund, Sweden) with controlled strain at 0.01 and 1 Hz using the method described by Glantz et al. (2009). 30 mL of pH-adjusted skim milk pre-heated to 32 °C for 30 min was induced with chymosin (Chy-Max Plus, 200 IMCU mL⁻¹) to a final concentration of 0.09 IMCU mL⁻¹ milk. The sample was transferred to the measuring system comprising a vane-cup system. The vane was lowered to a gap of 2 mm and the recording of the coagulation was started and ran for 40 min. Parameters obtained were coagulation time (RCT_{stress}) and gel strength at 40 min (G'_{40_stress}), where RCT is defined as the time when the gel strength increases continuously. After 40 min, a stress sweep followed with increased shear stress from 0.1 to 300 Pa, where the yield stress (σ_{y_stress}) was obtained. σ_{y_stress} is defined as the shear stress when 90% of the maximum recorded viscosity is reached.

Milk samples were defined as NC if RCT_{rox} > 40 min and as poor coagulating (PC) if RCT_{rox} was between 16 and 39 min, based on the mean RCT_{rox} of the two duplicates. The rest of the samples were defined as coagulating based on RCT_{rox} (Table 1). For the randomly selected samples measured with low-amplitude oscillation rheometry (StressTech), the same grouping as from the free oscillation rheometry (ReoRox 4) was used to define the different coagulation groups. The choice for defining NC as RCT_{rox} > 40 min was made to allow comparison of the results to earlier studies on Swedish RDC (Gustavsson et al., 2014a; Poulsen et al., 2013), and to avoid over-estimating the frequency of cows producing NC milk.

2.7. Statistical analyses

For each measurement, one value per milk sample was calculated as the average of the technical duplicates, and was further used to calculate means, standard deviations and coefficients of variance for the measured traits. To evaluate the influence of coagulation group, herd, parity and DIM on the different traits and, to estimate least square means for each coagulation group, the following general linear model was used:

$$Y_{ijk} = \mu + coag_i + herd_j + parity_k + DIM + e_{ijk}$$

where Y_{ijk} is the phenotypic traits, coagulation group is a fixed effect ($i = 1, 2, 3$) herd is a fixed effect ($j = 1, 2, \dots, 31$), parity is a fixed effect ($k = 1, 2, 3$) for which parities 3–8 were grouped together, DIM is a covariate, and e_{ij} is the random residual effect. The effect of lactation stage (DIM) was tested by means of a Wilmink function (Wilmink, 1987). Only the linear regression on DIM was found to be significant for milk yield, and fat and protein content ($P < 0.05$), and a linear DIM covariate was therefore assumed for all traits. To obtain normally distributed residuals from the general linear model, some of the traits were transformed and outliers were removed prior to analysis with the general linear model. SCC was transformed using a log-transformation, whereas CFR_{rox} and G'_{max_rox} were transformed using a box-cox transformation. Results from the analyses were back-transformed, and all reported estimates are on the original scale of measurement. Outliers were

removed for ratio casein:protein and total calcium, which was 11 and 6 values, respectively.

Pearson correlation coefficients were calculated among the phenotypic traits and among residuals from the general linear model. NC milk was added as a binary trait to the correlation study, where 1 = non-coagulating and 0 = poor and coagulating samples. All statistical analyses were calculated in IBM SPSS Software (IBM Analytics, Armonk, NY, USA).

3. Results and discussion

3.1. Coagulation properties

Descriptive statistics of the raw data used in the study can be seen in Table 2. The rheological measurements in the present study resulted in 18.1% of the milk samples being defined as NC (Table 1). In the study of Gustavsson et al. (2014a), 18% of milk from SR breed was defined as NC milk. This suggests that the Swedish RDC still has a high proportion of NC milk, which negatively affects cheese yield in Sweden and could also affect cheese yield in countries where Swedish RDC have been used in breeding programs. In the present study, pH was adjusted to 6.5 to mimic industrial cheese production. Although this pH adjustment was not performed by Gustavsson et al. (2014a), a high proportion of NC milk still remains in the Swedish RDC even after adjustment of pH to industrial conditions. Furthermore, in the present study, an additional 18.9% of the milk samples were defined as PC (Table 1), resulting in a high figure of 37% of the milk from the sampled Swedish RDC having impaired coagulation properties.

Fig. 1 shows the distribution of cows producing NC and PC milk across the 31 sampled farms to various extents. Notably, all farms have cows producing PC milk and 26 of the farms also have cows producing NC milk. RCT has been shown to be influenced by herd and feeding regimen, but this influence is much lower than that of other factors (Bittante et al., 2012). However, Fig. 1 shows variations between the farms due to unidentified reasons, even if the farms were selected to have similar genetic background. The variation could be because the presence of NC milk requires a specific genotype and environmental factor that are unknown, or it could be that NC milk is caused by combinations of specific alleles that occurs by chance.

The findings in this study show that the Swedish RDC still has a high amount of cows producing NC milk (Table 1), compared with 4% of Danish Red, 3% of Danish Jersey and 2% of Danish Holstein-Friesian (Frederiksen et al., 2011b) to name a few. However, other red breeds have also been linked with a high proportion of NC milk. Tyrisevä, Ikonen, and Ojala (2003) re-sampled cows of the Finnish Ayrshire breed multiple times over two years and they found that around 25% of the sampled cows produced NC milk at least once during lactation and about 10% on almost every sampling occasion. In their study, Tyrisevä et al. (2003) stated that most of the cows producing NC milk had ancestors commonly used in breeding, both sires and dams, from not only Finnish Ayrshire but also Norwegian Red breed and Canadian Ayrshire, which further supports that NC milk is an issue in many red breeds. It has also been shown in earlier studies that cross-breeding Holstein cows with SR sires increased the frequency of NC milk compared with the purebred Holstein cows, from 11.0% to 15.7% (Malchiodi, Cecchinato, Penasa, Cipolat-Gotet, & Bittante, 2014).

Table 1

Definition and frequency for the three coagulation groups and mean \pm standard error of rennet coagulation time from the screening rheometer (RCT_{rox}).^a

Parameter	Non-coagulating milk (n = 123)	Poor coagulating milk (n = 128)	Coagulating milk (n = 428)
Definition based on RCT_{rox}	≥ 40 min	16–39 min	≤ 15 min
Frequency (%)	18.11	18.85	63.03
RCT_{rox} (min)	$\geq 40^a$	21.64 ± 0.58	10.68 ± 0.10

^a RCT_{rox} for non-coagulating samples is right-censored data.

Table 2

Descriptive statistics of the milk samples with mean value, standard deviation (SD), coefficient of variance (CV), minimum and maximum value.^a

Trait	n	Mean	SD	CV (%)	Min	Max
CFR_{rox} (Pa min ⁻¹)	679	3.70	4.57	123.51	0.00	42.10
RCT_{rox} (min)	679	18.92	13.29	70.24	5.35	45.00
$G'_{\text{max,rox}}$ (Pa)	679	188.33	145.46	77.24	0.00	839.77
$G'_{40\text{,stress}}$ (Pa)	324	129.16	74.41	57.61	0.57	362.10
RCT_{stress} (min)	324	9.53	9.10	95.49	1.25	40.00
$\sigma_{y\text{,stress}}$ (Pa)	324	17.13	8.15	47.58	0.00	39.62
Milk yield (kg day ⁻¹)	676	31.84	7.59	23.84	8.10	55.20
Days in milk	679	166.01	82.66	49.79	10.00	463.00
Parity	679	2.12	1.21	57.08	1.00	8.00
SCC (1000 cells mL ⁻¹)	678	52.53	53.52	101.88	2.00	300.00
Fat (%)	678	4.50	1.58	35.11	1.00	10.24
Lactose (%)	678	4.63	0.23	4.97	3.59	5.17
Protein (%)	678	3.65	0.36	9.86	2.49	5.04
Casein (%)	652	2.86	0.31	10.84	1.97	3.96
Casein:Protein	652	0.77	0.02	2.60	0.61	0.97
Calcium (mg kg ⁻¹)	678	1238.66	199.21	16.08	180.00	2500.00
Ca ²⁺ (mmol L ⁻¹)	414	2.65	0.71	26.79	1.59	6.21
Citric acid (%)	652	0.19	0.03	15.79	0.11	0.33
pH	679	6.69	0.08	1.20	6.31	7.04
Casein micelle size, D[4,3] (μm)	607	0.14	0.02	14.29	0.11	0.23
Titrate acidity ($^{\circ}\text{N}$)	54	20.14	2.46	12.21	14.61	26.86

^a Abbreviations are: CFR, curd firming rate; RCT, rennet coagulation time; G'_{max} , maximum gel strength during recording; G'_{40} , gel strength after 40 min of recording; $\sigma_{y\text{,stress}}$, yield stress; SCC, somatic cell count. Rox or stress refers to the rheometric system used, free oscillation or low amplitude oscillation, respectively. RCT values are right-censored data, i.e., the measurement was stopped after 40 min (RCT_{stress}) or 45 min (RCT_{rox}).

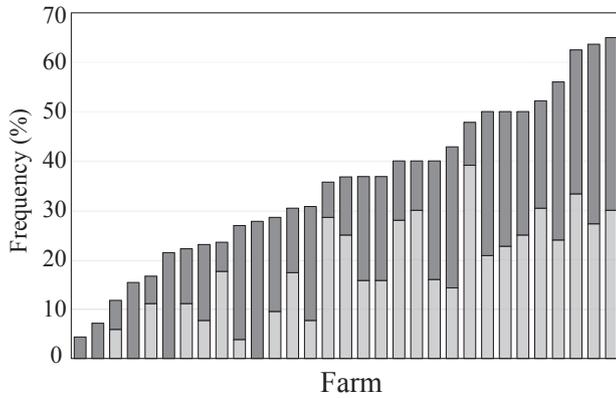


Fig. 1. The frequency of cows producing non- (■) and poor- (■) coagulating milk on the 31 farms studied.

Since the Swedish RDC is exported worldwide and used in breeding programs of other red breeds, there is an urgent need to reduce the occurrence of NC milk from this breed. However, RCT is known to be a heritable trait, ranging from 0.21 to 0.28 (Cecchinato et al., 2011; Chessa et al., 2014; Vallas et al., 2010), which suggests that NC milk can be reduced by selective breeding. Swedish RDC are known to have many superior animal health benefits, such as low frequency of stillborn calves and low frequency of udder diseases (Växa Sverige, 2017). Therefore, it is important to increase the versatility of the milk from the Swedish RDC, and there is a need for the genetic potential to produce milk suited for various dairy products. As previously mentioned, researching NC milk from Swedish RDC is also believed to yield useful information about NC milk from other breeds, especially other red breeds, due to their common ancestry.

All the rheological traits from both instruments were significantly correlated to each other (Table 3). The Pearson correlation coefficient between the coagulation times RCT_{rox} and RCT_{stress} was 0.82 and between the gel strengths from the two instruments G'_{40_stress} and $G'_{\text{max_rox}}$, the Pearson correlation coefficient was 0.94. This shows that the two rheological systems used have a high correlation for the same parameters (coagulation time and gel strength) even if they are measured using different oscillation techniques. From this it can be confirmed that the free oscillation instrument can be used as a screening method and low-amplitude oscillation as a more sensitive measurement. The duplicates in the free oscillation instrument were consistent, and the majority of the samples varied by $\pm 6 \text{ Pa min}^{-1}$, $\pm 4 \text{ min}$ and $\pm 65 \text{ Pa}$ between the duplicates for the CFR_{rox} , RCT_{rox} and $G'_{\text{max_rox}}$, respectively (data not shown). The rheological traits measured are all significantly influenced by coagulation group, as expected due to the model used, whereas parity has no significant influence on the rheological traits (Table 4). Herd influenced all rheological traits except, for G'_{40_stress} and RCT_{stress} , and DIM has a strong influence on all rheological traits except on RCT_{stress} (Table 4).

Milk composition is known to vary within lactation and due to different feeds (Panthi, Jordan, Kelly, & Sheehan, 2017), which explains the influence of DIM and herd on the different coagulation properties. Comparisons with results of Gustavsson et al. (2014a), where the same low-amplitude oscillation rheometer (StressTech) and the SR breed were used, indicates improved coagulation properties in the present study on all three parameters (G'_{40_stress} , RCT_{stress} and σ_{y_stress}). The present samples had even better coagulation properties than when Gustavsson et al. (2014a) excluded the NC samples from the means (Table 2). This could be explained by the pH adjustment prior to the measurement that was not

Table 3
Calculated Pearson correlation coefficients for rheological and phenotypical traits. Above the diagonal are the correlations among the residuals from the general linear model and below the diagonal are the correlations among the raw traits.^a

Traits	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1. NC																					
2. CFR_{rox} (Pa min^{-1})	-0.38*																				
3. RCT_{rox} (min)	0.92*	-0.55*																			
4. $G'_{\text{max_rox}}$ (Pa)	-0.61*	0.88*	-0.77*																		
5. G'_{40_stress} (Pa)	-0.70*	0.87*	-0.83*	-0.63*																	
6. RCT_{stress} (min)	0.81*	-0.42*	0.82*	-0.69*	-0.69*																
7. σ_{y_stress} (Pa)	-0.67*	0.63*	-0.69*	0.81*	0.81*	-0.68*															
8. Milk yield (kg day^{-1})	0.02	-0.29*	0.05	-0.20*	-0.25*	0.11*	-0.23*														
9. SCC ($1000 \text{ cells mL}^{-1}$)	-0.07	0.14*	-0.09*	0.09*	0.09*	-0.09*	0.05	-0.20*													
10. Fat (%)	-0.09*	0.21*	-0.10*	0.20*	0.22*	0.07	0.15*	-0.48*	0.18*												
11. Lactose (%)	0.09*	-0.24*	0.13*	-0.19*	0.20*	0.22*	0.11*	-0.23*	0.33*	-0.39*											
12. Protein (%)	-0.02	0.50*	-0.06	0.38*	0.42*	-0.07	0.44*	-0.07	0.44*	-0.23*	-0.46*										
13. CN (%)	-0.03	0.49*	-0.08	0.39*	0.38*	-0.05	0.41*	-0.53*	0.16*	0.37*	0.32*	0.93*									
14. CN:Protein	-0.09*	0.08*	-0.11*	0.13*	0.05	-0.05	0.08*	-0.09*	0.05	0.24*	0.03	0.20*	0.20*								
15. Calcium (mg kg^{-1})	-0.20*	0.41*	-0.25*	0.35*	0.41*	-0.20*	0.38*	-0.24*	-0.03	0.14*	0.06	0.39*	0.38*	0.01							
16. Ca^{2+} (mmol L^{-1})	-0.22*	0.22*	-0.25*	0.27*	0.21*	-0.23*	0.22*	-0.12*	0.05	0.24*	-0.38*	0.05	-0.01	0.19*	-0.01						
17. Citric acid (%)	-0.01	-0.02*	-0.01	0.00	0.05	-0.05	0.00	-0.01	-0.11*	0.05	-0.16*	-0.12*	0.18*	0.18*	0.18*	0.20*					
18. pH	0.00	-0.09*	0.00	-0.10*	0.06	-0.02	-0.13*	0.02	0.12*	-0.07	-0.02	-0.14*	-0.15*	0.06	-0.02	-0.26*	0.07	0.04	0.06	0.03	
19. D[4,3] (nm)	0.00	-0.06	-0.02	-0.04	-0.07	-0.03	0.03	-0.09*	0.06	0.11*	-0.24*	0.04	0.06	0.17*	-0.03	0.23*	0.00	-0.10*	-0.08	-0.39*	0.09
20. TA (°N)	0.33*	-0.12	0.35*	-0.16	-0.10	-0.06	0.06	-0.27	0.09	0.25	-0.08	0.40*	0.40*	-0.16	-0.09	-0.31*	0.21	-0.42*	-0.42*	0.12	

^a Abbreviations are: NC, binary trait for non-coagulating milk samples; CFR, curd firming rate; RCT, rennet coagulation time; G'_{40} , maximum gel strength during recording; G'_{40} , gel strength after 40 min of recording; σ_{y_yield} stress; SCC, somatic cell count; CN, casein; D[4,3], CN micelle size; TA, titratable acidity. Rox or stress refers to the rheometric system used, free oscillation or low amplitude oscillation, respectively. An asterisk indicates $P \leq 0.05$. No correlations between NC or RCT_{rox} and residuals from general linear model could be calculated, as RCT_{rox} was a fixed effect in the model, and NC is based on RCT_{rox} .

Table 4
Influence of coagulation group, herd, parity and days in milking (DIM) on the measured traits.^a

Trait	n	Coagulation group	Herd	Parity	DIM
CFR _{rox} (Pa min ⁻¹)	679	***	*	NS	***
G' _{max_rox} (Pa)	679	***	***	NS	***
G' _{40_stress} (Pa)	324	***	NS	NS	***
RCT _{stress} (min)	324	***	NS	NS	NS
σ _{y_stress} (Pa)	324	***	***	NS	***
Milk yield (kg day ⁻¹)	676	*	***	***	***
Somatic cell count (log ₁₀ 1000 cells mL ⁻¹)	678	NS	***	***	***
Fat (%)	678	NS	***	***	***
Lactose (%)	678	NS	***	***	***
Protein (%)	678	NS	***	NS	***
Casein (%)	652	NS	***	NS	***
Casein:Protein	641	NS	***	**	**
Calcium (mg kg ⁻¹)	672	***	***	*	***
Ca ²⁺ (mmol L ⁻¹)	414	***	***	NS	NS
Citric Acid (%)	652	NS	***	NS	NS
pH	679	NS	***	***	NS
Casein micelle size, D[4,3] (μm)	607	NS	***	NS	***
Titrate acidity (°N)	54	NS	NS	NS	NS

^a Abbreviations are: CFR, curd firming rate; RCT, rennet coagulation time; G'_{max}, maximum gel strength during recording; G'₄₀, gel strength after 40 min of recording; σ_{y_stress}, yield stress. Rox or stress refers to the rheometric system used, free oscillation or low amplitude oscillation, respectively. Asterisks indicate P-values: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, NS, P > 0.05; n differs from table with descriptive statistics (Table 2) due to removal of outliers.

performed in Gustavsson et al. (2014a) but was performed in the present study, since a reduction of milk pH is known to improve rennet coagulation. Okigbo, Richardson, Brown, and Ernststrom (1985) showed that the coagulation time was shortened, and the gel strength was increased, when reducing pH of good and poor coagulating milk.

All measured rheological traits significantly differed between the three coagulation groups (Table 5). From Tables 1 and 5, however, it is also clear that the definition of coagulation time affects the definition of NC milk, as RCT_{rox} and RCT_{stress} are based on different definitions and thereby give different mean coagulation times. This indicates difficulties in comparing absolute values between different oscillating techniques; however, relative comparisons may be valid based on the strong correlation between RCT_{rox} and RCT_{stress} (0.82; Table 3). When coagulation time is based on a continuous increase of viscosity, as RCT_{stress}, it will give an earlier indication of the ability of the milk to coagulate. If, instead, it is based on δ = 45°, as in RCT_{rox}, it will more closely resemble the actual cutting time within cheese processing, since it is not optimal to cut the coagulum if δ < 45°. In this study, NC milk has been defined in relation to RCT_{rox}, as it was more important to link the coagulation of the milk to cheese production and its effect in the industry. It was, however, important in this study to also include the low-amplitude oscillation rheometry, as it is a more sensitive method that can ensure accurate results from the screening method (free oscillation rheometry).

3.2. Milk composition and physical properties

Coagulation groups differed significantly in milk yield, amount of calcium and level of free Ca²⁺ (Table 4). However, coagulation group was not significantly associated with SCC, fat, lactose, protein or casein contents, nor with casein:protein ratio, citric acid content, pH, casein micelle size or TA (Table 4). Table 5 shows that NC and PC milk were associated with a higher milk yield than coagulating milk. The total amount of calcium was significantly different between all three coagulation groups and decreased with increasing coagulation time (Table 5), which is in agreement with previous studies (Gustavsson et al., 2014b; Jensen et al., 2012; Ketto et al., 2017). This has also been observed in abnormal milk with a coagulation time >75 min (van Hooydonk, Hagedoorn, & Boerrigter, 1986).

The level of free Ca²⁺ was significantly lower for NC and PC milk, compared with coagulating milk, in agreement with findings of Gustavsson et al. (2014b) and van Hooydonk et al. (1986). CaCl₂ is sometimes added to milk prior to rennet addition to improve the coagulation process (Fox & McSweeney, 2017), which could be argued to solve the issue of NC and PC milk. Okigbo et al. (1985) showed that addition of 0.02% of CaCl₂ reduced the coagulation time of milk samples with PC properties and increased curd firmness; however, the curd firmness was still lower than untreated milk samples with good coagulation properties. This suggests that the coagulation properties of PC milk can be improved to a certain

Table 5
Marginal means ± standard error of rheological parameters, milk composition traits and physical properties for different coagulation groups in milk from Swedish Red Dairy Cattle.^a

Trait	Non-coagulating milk	Poor coagulating milk	Coagulating milk
CFR _{rox} (Pa min ⁻¹)	0.01 ^a (n = 123)	0.11 ^b (n = 128)	4.41 ^c (n = 428)
G' _{max_rox} (Pa)	0.07 ^a (n = 123)	86.98 ^b (n = 128)	256.08 ^c (n = 428)
G' _{40_stress} (Pa)	11.63 ± 5.87 ^a (n = 52)	90.61 ± 5.08 ^b (n = 67)	171.55 ± 2.92 ^c (n = 205)
RCT _{stress} (min)	25.90 ± 0.78 ^a (n = 52)	9.02 ± 0.68 ^b (n = 67)	5.28 ± 0.39 ^c (n = 205)
σ _{y_stress} (Pa)	5.17 ± 0.76 ^a (n = 52)	16.49 ± 0.66 ^b (n = 67)	20.50 ± 0.38 ^c (n = 205)
Milk yield (kg day ⁻¹)	32.69 ± 0.53 ^a (n = 123)	32.87 ± 0.51 ^a (n = 128)	31.42 ± 0.27 ^b (n = 425)
Calcium (mg kg ⁻¹)	1146.59 ± 12.00 ^a (n = 122)	1194.41 ± 11.44 ^b (n = 128)	1281.99 ± 6.25 ^c (n = 422)
Ca ²⁺ (mmol L ⁻¹)	2.65 ± 0.06 ^a (n = 80)	2.73 ± 0.05 ^a (n = 80)	2.87 ± 0.03 ^b (n = 254)

^a The samples measured using low-amplitude oscillation (StressTech) were randomly chosen. Marginal means are adjusted based on general linear model. Only traits where coagulation group had significant impact are presented. Abbreviations are: CFR, curd firming rate; RCT, rennet coagulation time; G'_{max}, maximum gel strength during recording; G'₄₀, gel strength after 40 min of recording; σ_y, yield stress. Rox or stress refers to the rheometric system used, i.e. free oscillation or low amplitude oscillation, respectively. Different superscript letters indicate significant differences (P ≤ 0.05) within each row. To present means for CFR_{rox} and G'_{max_rox} on the original scale, a back-transformation was applied to the marginal means. Standard errors cannot be back-transformed this way and were therefore not reported.

extent by addition of CaCl_2 . Hallén et al. (2010) showed that, by adding 0.05% CaCl_2 to NC milk samples, some of the samples showed improved coagulation properties, but not all of them, which means that this does not fully solve the issue of NC milk. According to van Hooydonk et al. (1986), the reason that addition of CaCl_2 improves rennet coagulation is due to a combined effect of increasing calcium concentration but also a reduction in pH. Since Hallén et al. (2010) did not reduce the milk pH before rheological measurements, it could be argued that the improvement of NC milk they obtained by addition of CaCl_2 , could be the effect of a pH reduction rather than an increase of calcium. In the present study, the milk pH was adjusted before rheological measurements and therefore it cannot be concluded that addition of CaCl_2 would have the same effect as in Hallén et al. (2010). Additionally, 0.05% is also a very high addition of CaCl_2 and addition of even less than 0.04% of CaCl_2 can result in a cheese with bitter taste (Schwarz & Mumm, 1948), which is undesirable. Another approach to minimising addition of CaCl_2 and still obtaining good coagulation properties of the milk could be to breed for milk with a higher calcium content, as calcium level has been found to have a high heritability (0.57; van Hulzen, Sprong, van der Meer, & van Arendonk, 2009).

3.3. Phenotypic correlations

Comparison between raw traits and residual correlations show that many of the correlations between phenotypical and rheological traits are no longer significant for the residuals (Table 3). The most evident example was lactose content, which was significantly correlated with all rheological traits when looking at the raw traits but, when the correlations of the residuals were calculated instead, was no longer significantly correlated with any of the rheological traits. Level of free Ca^{2+} is another trait that has a much less significant correlation to rheological traits when the correlations of the residuals are evaluated. However, the opposite can be observed for casein micelle size, where no significant correlation was observed for raw traits but, when investigating the correlations of the residuals, there were significant correlations to curd firming rate and gel strengths (Table 3). This suggests that further investigation of phenotypes affecting coagulation traits should be focused on casein micelle size rather than level of lactose or free Ca^{2+} , as the residual correlations of the latter traits are reduced in significance when correcting for coagulation group, herd, parity and DIM in the statistical model. Earlier studies have shown that casein micelle size is a very interesting and important trait for rennet coagulation (Amenu & Deeth, 2007; Troch et al., 2017; Walsh et al., 1998) that should be further investigated for NC milk, even if the results in this study does not link NC milk with casein micelle size (Table 3).

Residual correlations for the NC trait, could not be calculated, as RCT_{rox} was a fixed effect in the model, and NC is based on RCT_{rox} . Instead, raw Pearson correlation coefficients showed that the binary NC trait was significantly correlated with all the measured rheological traits (Table 3). Furthermore, NC properties of milk could also be negatively correlated with fat content, casein:protein ratio, as well as both total and free calcium content. Moreover, NC milk was positively correlated with lactose content as well as TA. Even though milk coagulation was measured on skim milk, the lower fat content is in agreement with the results of Jensen et al. (2012), where significant differences regarding fat content were found between NC and coagulating skim milk.

NC milk was significant correlated with a high level of lactose (Table 3). A higher lactose content for NC milk has not been found previously in other studies. In contrast, in studies with fewer milk samples, coagulating milk has been observed to have a higher lactose content than NC milk (Gustavsson et al., 2014c; Hallén et al., 2010; Malacarne et al., 2014). Considering the casein:protein ratio,

NC milk showed a significant negative correlation (Table 3). This is interesting since there were no significant correlations for casein or protein contents with NC milk. This could indicate the importance of analysing individual milk protein variants and correlating these to coagulation properties, which could reveal more information about the protein composition of NC milk. The correlations between NC milk and both total and free calcium content are in agreement with the results from the general linear model (Table 5).

The buffering capacity of milk is an important characteristic that affects many of the physicochemical properties of milk during processing, such as rennet-induced coagulation (McCarthy & Singh, 2009). To evaluate the buffering capacity of the milk samples, a subgroup of samples were titrated with NaOH and TA was estimated. The results show that NC milk can be correlated with a high TA (Table 3). This is the opposite of what has been found in a previous study, in which TA was highest for milk with good coagulation properties and lowest for NC milk (Malacarne et al., 2014). TA is expected to increase with increased contents of casein, serum proteins, phosphate and other compounds (Walstra et al., 2006) and therefore, it could be interesting to further investigate buffering capacity or ion composition of NC milk to see if this would strengthen or weaken the correlation.

All the significant correlations between NC milk and the phenotypical traits were, however, weak (<0.4 ; Table 3). This can indicate that the traits measured in this study do not fully explain non-coagulating milk, but only to a certain degree. This means that none of the correlated parameters could be measured in a milk sample and then used to estimate NC milk behaviour, the rheological measurements would still be needed.

4. Conclusions

The rheological evaluation of milk from a large data set of 679 individual cows from the Swedish RDC resulted in 18.1% NC and 18.9% PC samples according to the definitions used. This indicates that milk from the Swedish RDC has an alarming high prevalence of milk with impaired coagulation properties, which may influence industrial cheese production and breeding of Swedish RDC in a negative way. All the farms sampled in this study had cows producing PC milk and most of them also had cows producing NC milk. However, the frequency of NC and PC milk differed among the farms, due to reasons that remain unclear.

The general linear model showed that NC milk can be linked to lower amounts of total calcium and Ca^{2+} compared to coagulating milk. Furthermore, this study showed that NC milk can be correlated with higher lactose and less fat content, less casein in relation to total protein, and lower concentrations of both total calcium and Ca^{2+} , as well as a higher titratable acidity. However, the results from evaluating the correlations show that more research is needed regarding phenotypes that can be linked to NC milk, since all the correlations were weak (<0.4).

This study has provided more information about the composition and physical properties of NC milk from the Swedish RDC. However, studies are needed to also evaluate the genetic influence on NC milk in a larger data set and to investigate the possibility of breeding against the undesirable property of NC milk.

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