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Abstract

Frozen storage is an efficient and convenient way to preserve and transport milk, but the effect from frozen storage needs to be further researched. The objective of this study was to investigate how storage at freezing temperatures (-80°C and -20°C) of frozen and freeze-dried bovine milk samples, respectively, affects pH, casein micelle size, ethanol stability, rheological properties and curd yield. The short-term changes after cold storage at 4°C were also examined. The results were evaluated using one-way ANOVA and Tukey's pairwise comparison test. A significant decrease in pH was observed during the short-term storage at 4°C. A significant difference was shown in ethanol stability after storage of the freeze-dried milk samples in week 4 while comparing the freezing temperatures. The pH measurements showed a significant difference between frozen and freeze-dried samples stored at -20°C and in week 1 and 2. The result from the coagulation time measurements showed significant difference in all treatments, except during the first week. The gel firmness was significantly different when comparing frozen milk samples stored at -80°C and -20°C, respectively for 2, 3, 5 and 6 weeks. The study concluded that milk is affected by frozen storage, resulting in softer cheese curd, prolonged coagulation time and decreased gel firmness. The study indicated that the optimal freezing temperature and time for frozen bovine milk samples are one week at -80°C. Further research with higher number of replicates and longer storage time could result in stronger conclusions about the effects of frozen storage of milk. Inclusion of caprine and ovine milk samples would make the results more useful for small-scale cheese manufacturers.

Keywords: Casein micelle size, milk coagulation time, gel strength, freezing, drying, curd yield

Sammanfattning

Frysning är ett effektivt och enkelt sätt att konservera och transportera mjölk, men mer forskning om infrysningens effekter på mjölk behövs. Studiens syfte var att undersöka hur frysta och frystorkade komjölkprover påverkas av lagring i -80°C och -20°C genom att mäta pH, kaseinmicellstorlek, etanolstabilitet, reologiska egenskaper och ostutbyte före och under som längst sex veckors fryslagring. Resultatet utvärderades statistiskt med envägs-ANOVA och Tukey's post-hoc test. Förändringarna i samtliga parametrar mättes även under kylförvaring i +4°C under tre dagar. Resultatet påvisade en signifikant minskning i pH under kylförvaringen. Under frysningen påvisades en statistisk skillnad i etanolstabilitet för frystorkade prover lagrade i -80°C och inom vecka 4 vid jämförelse av provmedelvärde. Även för pH påvisades statistiska skillnader under lagring av frysta och frystorkade prover i -20°C och inom vecka 1 och 2. Statistiska skillnader mellan ?? påvisades även för resultatet för de reologiska egenskaperna. Statistisk skillnad påvisades inom alla jämförelser i koaguleringstid, förutom från vecka 1. Skillnader i gelstyrka påvisade statistisk signifikans från de frysta mjölkproverna lagrade i -80°C, -20°C och inom vecka 2, 3, 5 och 6. Studien påvisade att mjölk påverkas av infrysning med ökat ostutbyte, förlängd koaguleringstid och försvagad gelstyrka. Resultatet indikerade att den optimala infrysningstiden och -temperaturen för komjölksprover som ska användas för ostproduktion är en vecka i -80°C. Vidare forskning med högre antal replikat och förlängd infrysningstid skulle kunna resultera i tydligare utvärdering av effekter av frysförvaring av mjölk som ska användas för ystning. Inkludering av get- och fårmjölk i studien skulle göra resultatet användbart även för småskaliga get- och fåroststillverkare.

Nyckelord: micellstorlek, koaguleringstid, gelstyrka, ostutbyte, frysning, frystorkning

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Abbreviations

ANOVA	Analysis of Variance
AMS	Automatic milking systems
CCP	Colloidal calcium phosphate
CT	Coagulation time
FD	Freeze-dried
G20	Gel firmness 20 minutes after rennet addition
G’	Elastic modulus
GF	Gel firmness
IMCU	International milk clotting units
NTA	Nanoparticle tracking analysis
SCC	Somatic cell count
SH	Swedish Holstein
SRB	Swedish Red Cattle

1. Introduction

Frozen storage of milk has been used as a method of preservation since the 1930's (Gaber et al. 2020). Despite its high energy consumption (Baldwin 2012), freezing is considered a sustainable preservation method due to convenience, greater storability and decrease in waste (Pollack 2001). Frozen foods can in some cases have a lower carbon footprint impact compared to fresh, refrigerated products due to the decreased waste in food services and households (Martindale 2014). Freezing food also makes it possible to use more time-consuming and sustainable ways of transportation, e.g., maritime instead of airborne, which also reduces the transportation costs (Alinovi et al. 2020).

The control of effects from frozen storage, i.e., ice crystal formation and nucleation, on ice cream and frozen desserts have been extensively researched as it is essential to its quality and sensory properties. The effects from frozen storage on milk and cheese has been less studied (Alinovi et al. 2020). It is a research field of growing interest, as freezing is an easy way of transporting high-quality foods to markets with limited access of fresh dairy products (Bertola et al. 1996; Tejada et al. 2000). It also allows transportation of dairy ingredients for further processing into fresh products on other locations (Picon et al. 2013; Vélez et al. 2015).

For cheese manufacturers, freezing is a preservation method mostly used for those who face seasonality (Bertola et al. 1996; Tejada et al. 2000), e.g., sheep and goat herders, to be able to maintain cheese production in periods with few or no lactations (Zhang et al. 2006; de Garnica et al. 2011; Fava et al. 2013; Kljajevic et al. 2016). The effects of frozen storage on milk are a subject of interest for the small-scale dairies, which often freeze their small milk batches while awaiting sufficient volumes for cheese production. It is also important to know whether there is an optimal frozen storage time for their milk.

1.1 Aim and objective

The objective of this study was to investigate how pH, casein micelle size, ethanol stability, rheological properties and curd yield are affected by freezing and freeze-drying of bovine milk. The milk quality parameters of fresh milk were compared with the same parameters after storage at -80°C and -20°C for various periods of

time, also comparing differences between freezing and freeze drying as method of preservation.

2. Background

2.1 Milk composition

Milk is an aqueous solution composed of proteins, lactose, vitamins, minerals, water and lipids. The raw milk composition is affected by the animal species and breed, e.g., the cow breed Swedish Red Cattle (SRB) produces milk with higher fat and protein content than Swedish Holstein (SH) (Larsen et al. 2010).

Milking systems and the milking frequency also affect the composition. In automatic milking systems (AMS) where the cows are kept in loose housing systems, the milking frequency is higher than in tie stall systems. Johansson et. al (2017) found that fat and protein content in bulk milk from AMS was lower compared to bulk milk from milking parlours, where cows were milked twice daily. Other factors affecting the composition are e.g., individual variations, composition of feed, stage of lactation, age and health of the animal (Walstra 2006; Hallén 2008; Coultate 2016; Nilsson 2017; Priyashantha 2021).

The average composition of cow's milk is 4 % fat, 3.3 % protein and 4.6 % lactose with a pH around 6.7. Approximately 80 % of the protein consist of the casein fraction. Together with a complex called the colloidal calcium phosphate (CCP), the caseins form the casein micelle. The CCP consist of phosphate, calcium and small amounts of citrate. The remaining 20 % consists of the water-soluble whey, or serum, proteins (Walstra 2006; Coultate 2016). Traditionally, the proteins are defined by their solubility when adjusting pH of the milk to 4.6. At this pH the caseins precipitate in the milk, while the whey proteins remain in solution (Walstra 2006; Hallén 2008).

The caseins have an amphiphilic structure due to polar and non-polar regions on their peptide chain and have a negative net charge. With their high proline content, the caseins show little secondary and tertiary structure, making them heat resistant for up till 100°C and stable during storage (Walstra 2006; Hallén 2008). There are four main caseins with different primary structures. They can vary slightly in their amino acid sequence, e.g., the casein α_{s1}C differ from α_{s1}B in having a glycine residue at position 192 in the polypeptide chain instead of a glutamic acid residue (Walstra 2006; Coultate 2016). α_{s1}-casein has a high number of phosphate groups esterified to serine residues. It has a high negative net charge, partly due to the

phosphate groups being ionized at the pH 6.7. Usually, this casein has eight phosphate groups but it also occurs in a variant with nine groups. α_{s2}-casein contains two cysteine residues which form an intramolecular disulphide bridge. Like α_{s1}-casein, it also occurs in different variants with different amounts of phosphate groups, i.e., 10 -14 per molecule. β-casein is the most hydrophobic casein with many proline residues. Its charge is unevenly distributed resulting in a molecule with a polar end and a long-chained non-polar end. In the presence of plasmin, the C-terminal, i.e. the hydrophobic end of β-casein is split off, resulting in a degradation product prior thought to be another separate casein, γ-casein. κ-casein is different from the other caseins. Two thirds of these molecules are glycosylated, with a carbohydrate group esterified to threonine. The carbohydrate groups are negatively charged, resulting in a hydrophilic C-terminal. κ-casein has two cysteine residues able to form intermolecular disulphide bonds and is usually present in polymers of 2-9 molecules (Walstra 2006).

2.2 The casein micelle

The major function of milk is to transport nutrients to the new-born mammal (Walstra 2006). The organization of the casein together with calcium and phosphate in the casein micelle solves two problems; without the aggregation, the caseins would form a viscous solution due to its open structure physically hard to secret. Calcium and phosphate would also form insoluble crystals of calcium phosphate without the micelle, inhibiting the secretion and digestion of the milk (Coultate 2016).

The structure of the casein micelle has been researched and reviewed over time. In 1814, Berzelius was the first to describe a method to separate caseins from milk (Fox & Brodkorb 2008; Hallén 2008). The first model of the casein micelle was described by Waugh in 1958, who also introduced the general usage of the word ‘micelle’ for the structure (Waugh 1958; Fox & Brodkorb 2008). In the sub-micelle model described by Slattery and Evard in 1973 and subsequently worked on by Schmidt, the casein micelle was composed of ‘submicelles’, spherical aggregates composed of 25-30 casein molecules, held together by hydrophobic bonds (Müller-Buschbaum et al. 2007; Coultate 2016). The dual-binding model by Horne (1998) described how caseins were crosslinked through their hydrophobic regions while the hydrophilic regions interacted through calcium phosphate bridges (Müller-Buschbaum et al. 2007). Since then, as research has shown an unanimous result for the detailed structure of the casein micelle, the following properties of the micelle are widely accepted (Fox & Brodkorb 2008). According to the electron micrographs, the casein micelle is roughly spherical while the prevalence of ‘sub-micelles’ needs further research. Its core is hydrophobic, despite containing 4 g H_2O per gram casein. The core consists of roughly equal amounts of α_s- and β-

casein with small amounts of κ-casein. The outer parts of the micelle consist of equal amounts of κ- and α_s-casein with small amounts of β-casein. At pH 6.7, the glycosylated κ-casein on the surface give the micelle hydrophilic properties which is essential in providing the structure stability. Forces further stabilizing the micelle are ionic and hydrophobic interactions, as well as cross-linking through the CCP between the casein proteins (Walstra 2006). Their size differ within a portion of milk, from between 50 to 600 nm, with mean around 150 nm (Fox & Brodkorb 2008). 50 % of the micelles have a diameter between 130 to 250 nm. The casein micelle contains 10^4 to 10^5 casein molecules and one litre of milk consist of approximately 10^{15} micelles (Walstra 2006; Coultate 2016).

Several factors affect the size of the casein micelle. In a study by Priyashantha et al. (2019), large casein micelles had a mean size of 161.6±6.63 nm and small casein micelles had a mean of 149.8±4.15. One factor affecting the casein micelle size is the lactation phase, e.g., the number of large micelles is lower in the early stage of lactation (Walstra 2006). The size differs between breeds, e.g., the SH have larger average casein micelle size than the SRB. Casein micelle size also vary with seasons, with smaller micelles in the summer than in the winter (Glantz et al. 2010). Individuals within the same breed, independent of age, milk production and lactation stage, have different sizes of casein micelles (de Kruif & Huppertz 2012). The average size is also influenced by feeding regime, genotype of αs1- and κ-casein, pH, casein- and whey content (Devold et al. 2000), e.g., the amount of κ-casein is proportional to the surface area of the casein micelle (Walstra 2006). A study by Bijl et al (2014) showed that low average size was correlated with the κ-casein B variant with a higher concentration of glycosylated κ-casein.

The casein micelle constantly exchanges components with its surroundings. The casein proteins diffuse in and out of micelles, presumably in form of submicelles, together with components from the CCP. The equilibria depend on factors e.g., pH, temperature and Ca^{2+}-activity. By lowering the pH, the colloidal calcium phosphate will go into solution. The free Ca^{2+} and hydrogen ions will neutralize the negative charges in the micelle and its hydrophilic κ-casein. In unstirred milk kept at 30°C, this causes aggregation of the micelles in so-called acid coagulation. Due to weaker bonds after the dissolving of phosphate, the micelle will swell and increase in size. Higher pH in combination with increased temperature will dissolve the casein molecules. At temperatures above 100°C and pH below 6.2 no dissolution will occur but at pH 7.2, the dissolution is almost complete. With no change in pH, high temperature will reduce the size of the micelles and increase the amount of colloidal phosphate. At lower temperatures, e.g., 5°C, the colloidal stability of the micelle increases together with the voluminosity. This is partly due to β-casein protruding from the micelle surface (Walstra 2006). Priyashantha et al (2019) found that addition of calcium in milk decreases the casein micelle size while addition of

citrate slighlty increased the voluminosity. Citrate reduced the levels of free Ca^{2+} in milk by acting as a chelating agent (Odagiri & Nickerson 1965).

2.3 Cheese making, milk coagulation and gelling

Cheese making, involving several production steps and biochemical transformations, is a complicated process in which each step affects composition, quality and yield of the cheese (Walstra 2006).

The milk is immediately cooled to 4°C after milking, to inhibit microbial growth, enzyme activity and chemical changes, e.g. oxidation (Walstra 2006). Depending on the duration of refrigerated storage of the raw milk, growth of psychrotrophs may impair its quality. Given good hygienic practices at the farm, the bacterial count is initially almost constant during the first 2-3 days storage of the bulk tank milk. With food cold chain management, the bacterial count increases after 4-5 days. A bacterial number greater than $5*10^5$ cfu/ml may indicate that psychrotrophs have produced heat-stable enzymes, e.g., lipases and proteinases. Pre-treating the milk before processing with a heat treatment, e.g., thermization at 65°C for 15 sec, usually reduces the number of psychrotrophs. This moderate heat-treatment extends the storage for another 3 or 4 days at 6-7°C without substantial bacterial increase (Walstra 2006). Other changes during cold storage are dissolution of β -casein into the serum, due to weakening of hydrophobic bonds, and reduced rennetability. The leakage of β -casein causes swelling of the micelle (Gaber et al. 2020). Also lipase activity increases during cold storage, especially with temperature fluctuations (Walstra 2006).

An essential part in cheese making is the milk coagulation, which mechanism in general is well understood. By addition of proteolytic enzymes in milk, e.g., chymosin, the hydrophilic part of κ-casein is split at a specific peptide bond, Phe105-Met106. The casein micelle is destabilized by the release of the glucomacropeptide since the electrostatic repulsion between micelles is diminished. Ca^{2+} also contributes to the aggregation by forming salt bridges between the para-κ-caseins, which is the κ-casein without the C-terminal, on the micelle surfaces. At pH 6.7, aggregation between the micelles starts when about 70 % of the κ-casein have been split, transforming the milk into a gel. The aggregation will be faster with declining pH and at pH 6.2, aggregation starts at 60 % splitting. Temperature also affects the aggregation rate. At low temperatures, the splitting of κ-casein will occur, but the micelles will not aggregate and above 35°C, the heat will inactivate the chymosin activity. In cheese making, the traditional coagulation temperature is around 30°C (Walstra 2006) Addition of calcium is another way of shortening the coagulation time (CT) and achieve firmer gels. Calcium chloride is regularly added to milk to speed up the clotting and reduce the amount rennet needed. Addition of citrate on the other hand give opposite results; an increased CT and weaker gels, as

it chelates Ca^{2+} (Hallén 2008; Glantz et al. 2010; Priyashantha et al. 2019). Higher rennet concentration will also decrease the CT (Walstra 2006).

Syneresis, the expulsion of the water phase and the whey proteins from rennet gels, does usually not occur spontaneously but is enhanced by cutting. Depending on the desired level of moisture in the cheese, the syneresis should be stopped or slowed down at a certain time. Factors that affect the syneresis are e.g., the firmness of the cut gel. A softer gel can tend to have lower syneresis than a firmer gel. This can also decrease the curd yield as a large amount of curd fines can be released from the softer gel. The surface area of the curd also affects the amount of syneresis. Cutting into smaller cubes, resulting in a larger total surface area, results in faster syneresis. Other factors affecting syneresis are pressure, acidity, temperature and composition of the milk (Walstra 2006).

To save time and expenses in production, clotting is preferred to be fast, and concentrations of rennet used preferred to be low (Ikonen et al. 2004). When rennet is added to milk it takes time for coagulation to start and the micelles to aggregate. The measured time from addition of rennet in the milk until coagulation with a certain firmness, i.e., the coagulation time (CT), is an important parameter in cheese making and is inversely proportional to the enzyme concentration. CT is a useful parameter to evaluate the amount of rennet needed to produce a certain type of cheese. Generally, a shorter CT results in a firmer gel (Walstra 2006).

Another coagulation measurement is the ethanol stability test (Horne & Muir 1990). The test indicates milk heat stability and milk quality by evaluating which ethanol concentration is needed to cause protein precipitation. It predicts processability for heat treatments, e.g., ultra-high temperature (UHT) milk and milk powder. For sour milk, with a lower pH, the coagulation will occur at lower ethanol concentrations (Walstra 2006). Visual coagulation at 72 % (v/v) or less is considered low ethanol stability while coagulation at 78 % (v/v) or more is considered high ethanol stability (Chavez et al. 2004).

The size of the micelle appears to be critical in coagulation but further research on the subject is needed, as studies show contradictive results on whether the smaller or larger micelles have the most beneficial properties. Dalgleish et al (1981) found the coagulation properties to be unaffected by micelle size. Glantz et al (2010) and Logan et al (2014) found smaller micelles associated with shorter CT and firmer gels, while research by Ekstrand et al. (1980) showed smaller casein micelles resulting in longer CT. Priyashantha et al. (2019) found that micelles with larger size resulted in shorter CT and stronger GF. (Glantz et al. 2010; Logan et al. 2014).

2.4 Freezing and freeze drying of milk

Both freezing and freeze drying are preservation methods based on the reduction of water activity without heating the food (Fellows 2016). While freezing is an easy and commonly used preservation method both in households and industry since the 1930's (Gaber et al. 2020), freeze drying is an more expensive and energy and time-consuming method. It is suitable for processing of small quantities and mainly used in e.g., production of lactic starters (Walstra 2006).

During freezing, the milk goes through a phase transformation. It goes from a liquid suspension to a solid substance with a mixed phase of ice crystals and a supersaturated solution (Alinovi et al. 2020). The water freezes into ice and the remaining milk becomes more concentrated (Walstra 2006). The process makes the water mobility and mass transfer kinetics decrease, resulting in a glassy, or amorphous, state. It also minimizes chemical and enzymatic reactions, e.g., proteolysis and oxidation, and physical changes in the milk, e.g. recrystallizations or phase separations (Alinovi et al. 2020). Generally, the kinetics of deteriorative reactions will slow down as the temperature goes down (Verdini & Rubiolo 2002). By increasing the temperature of a glassy material, it will shift to a viscous state at a point called the glass transition temperature. This point is dependent on sample composition and temperature history. Above that point, the viscosity decreases with temperature. The stability of the glassy material is provided by the temperature below the glass transition temperature. Lactose, which is an important component in milk during freezing, affecting water content and water activity, has a glass transition temperature at -28°C. It is important to control the conditions during the freezing process as they can influence the milk quality, e.g. the growth of ice crystals affected the matrix and dehydration by punctuation of the cells (Alinovi et al. 2020). Above the glass transition temperature, crystallisation can occur, and diffusion rate increases considerably (Walstra 2006).

When lactose has crystallized and frozen milk is thawed, aggregation of micelles is observed. The coalescence is presumably a result of the calcium phosphate in the micelle dispositioning and salting out during the freezing. The protein molecules tend to associate and attain a compact conformation due to the changes in ionic strength, pH and salt equilibria. The casein micelle coalesce which causes them to increase in size (Walstra 2006). According to Walstra (2006), the aggregation of the casein micelles remains in the thawed milk. The aggregation can be undone by stirring at low temperatures, but can also be irreversible, e.g., if the milk has been stored for a longer period at -18 degrees. One way of avoiding aggregation of the casein micelles is to prevent lactose crystallization during the freezing, e.g., by rapid freezing. At -20°C, i.e., slow freezing rate, the ice formation is slow and lactose crystals develop due to the higher amount of available unfrozen water. At faster freezing rates, e.g., at -23°C and below, the lactose turns into an amorphous state and no or few crystals are formed due to lack of free water (Gaber et al. 2020).

The milk fat is also affected by the freezing process. During freezing and thawing of whole milk, partial coalescence, or clumping, of the fat globules generally occur. The fat globules are pressed together due to the ice crystals, evolved by the freezing. This can be prevented by rapid freezing and prior homogenization (Walstra 2006).

Frozen storage and thawing conditions can cause structural and rheological changes to food, thus it is also important to control the thawing conditions. Gaber et al. (2020) compared casein micelle size and free calcium ion levels in non-frozen samples and samples frozen at -40°C to compare the effect of three thawing methods and found no significant changes between them. The three methods included thawing at room temperature, overnight at 4°C and warming of samples in water bath to 40°C (Gaber et al. 2020).

During freeze drying, the water is first frozen and then converted to vapour by sublimation under vacuum. The process causes minimal damage to nutritional and sensory qualities while producing a porous structure that quickly rehydrates to more than 90 % of its original moisture (Fellows 2016). During frozen storage, the stability of freeze dried (FD) milk will decrease due to lowering pH, increased viscosity, loss of soluble calcium and phosphate, casein precipitation and formation of aggregates (Saito et al. 1963; Wells & Leeder 1963). According to Koschak et al (1981), FD milk at slow freezing at -20°C obtains higher protein stability than FD milk in rapid freezing at -80°C, due to increased calcium concentration in solution and preventing precipitation of phosphate at -80°C.

3. Materials and methods

3.1 Milk sampling

The milk samples were collected from a bulk tank from the experimental dairy herd at the Swedish Livestock Research Centre, Lövsta, Uppsala, Sweden.

Milk samples were collected on three different occasions (Oct 7th, 8th and 11th 2021), referred to as sampling 1, 2 and 3, resulting in three replicates for the different measurements. The herd is kept in an indoor loose housing system, fed conventional silage ad libitum and concentrate according to individual milk production. At sampling, the herd consisted of approximately 55% SRB and 45 % SH. The herd was divided into four large stalls, in which different research projects were running. Milking was conducted by AMS with robots from DeLaval.

For the first and third sampling, the bulk milk consisted of milk from all four stalls as the bulk tank had been emptied the previous morning. For the second sampling, the bulk tank had been emptied earlier the same day. All three samples were collected in the afternoon.

Milk sample preparation

After sampling, half of the milk containers were pre-warmed in a water bath at 32°C for the milk fat to be homogenously distributed. The whole milk was aliquoted in 50 ml Falcon tubes and stored in freezers at -20°C and -80°C. The remaining milk was defatted by centrifugation (Sorvall, Super T21, centrifugal rotor ST-H750, Sorvall Products L.P., Newton, Connecticut, USA), 10 min for 3000 RPM at 4°C. The fat layer on the surface of the milk was removed using a cotton swab. The defatted sample was poured into a new Falcon tube and stored in freezers at -20°C and -80°C. Before analysis, the samples were thawed overnight at 4°C according to method by Gaber et al (2020).

From sampling 2 and 3, both whole and defatted milk was also poured into freeze drying vessels. The vessels were kept at -20°C for 3 days before freeze-drying in Epsilon 2-6D LSCplus (Martin Christ, Osterode am Harz, Germany). The freeze drying proceeded in four steps: 1) freezing: -20°C for 10 min; 2) warmup: 0°C for 10 min; 3) main drying: 0°C and vacuum 0.200 mbar for 20 hours; 4) final drying:

15°C and vacuum 0.0100 mbar for 15 hours. After freeze-drying, the vessels were stored at -20°C and -80°C. Before analysis, the samples were resolved in distilled water, shaken, and kept at 4°C overnight. Whole milk samples, freeze-dried in 10 ml vessels, were resolved with 9 ml distilled water while the defatted milk samples, freeze-dried in 3 ml vessels, were resolved in 2.7 ml distilled water.

The frozen samples were analysed once a week while the FD samples were analysed every other week during a six-week period. Milk from all three sampling occasions was also analysed as fresh milk, before freezing, during day 0-3. Day 0 was the sampling day, considered as the reference for the frozen and FD samples. The milk for days 1, 2 and 3 was stored at 4°C and used for evaluation of short-term changes of the measured parameters during refrigerated conditions.

The whole milk samples were used for the making of mini cheeses, analysis of SCC, gross composition, and pH while the defatted milk samples were used for the ethanol stability test, and rheology and casein micelle size measurements. pH for the FD was measured in defatted milk. Each analysis was performed with two technical replicates except for the making of mini-cheese and pH measurement, where in total four and one technical replicate, respectively, was used.

3.2 Production of mini cheeses

Rennet preparation

For each cheese making occasion, rennet diluted in tap water with a final concentration of 18 international milk clotting units (IMCU) was prepared from bovine rennet consisting of 75 % chymosin and 25 % pepsin with a concentration of 180 IMCU (Scandirenn Kemikalia AB, Skurup, Sweden).

Milk sample preparation

The production of mini cheeses was performed according to Othmane et al. (2002) with few modifications (see Figure 1). The whole milk sample was pre-warmed in a water bath at 32°C for 30 min for the milk fat to melt. Milk (10 grams) was weighed in a pre-weighed 15 ml Falcon tubes in four technical replicates and returned to the water batch at 32°C.

After the second incubation in the water bath (32°C for 30 min), 100 µl rennet was added, resulting in final concentration of 0.18 IMCU/ml in the milk samples. The tubes were turned upside down three times to ensure even distribution of rennet in the milk. The tubes were kept in the water bath at 32°C for 30 min for coagulation to occur. The curd was vertically cut using a special cross-shaped tool, which was pre-warmed in the water bath at 32°C for 30 min before usage. The cross-shaped tool was wiped down with a tissue between samples to avoid whey to move between tubes. The cut curd was incubated again for another 30 min in the water bath at

32°C to allow syneresis. To separate the whey and curd, the tubes were then centrifuged at 1650 RPM (Sorvall, Super T21, Sorvall Products L.P., Newton, Connecticut, USA) at 22°C for 20 min. The expelled whey was poured into a new Falcon tube and weighed. The curd remaining in the tubes was weighed and the weight from the Falcon tubes subtracted to calculate the curd yield.

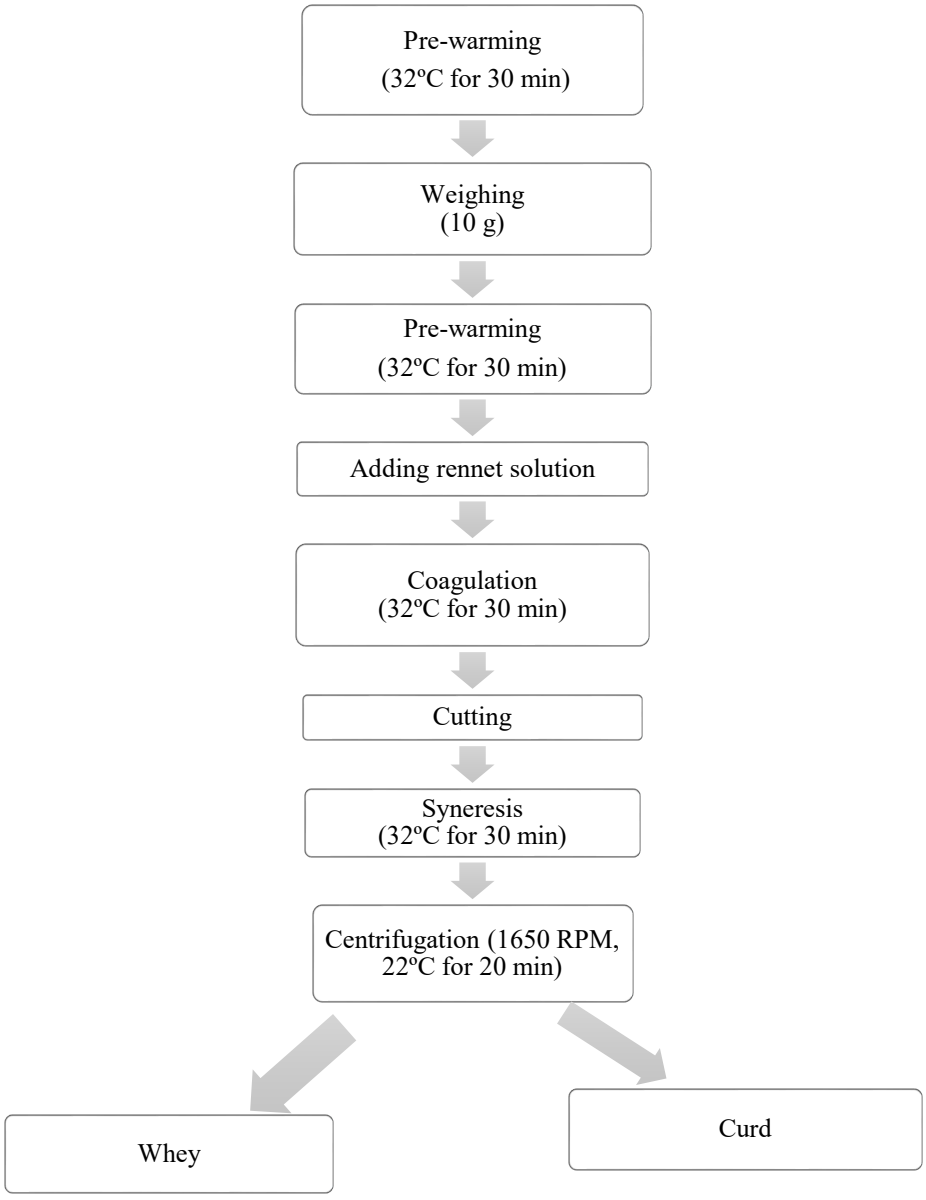

Figure 1. Flow chart over mini-cheese production steps

3.3 Casein micelle size measurements

The casein micelle size was analysed using NanoSight NS300 (Malvern Panalytical, Malvern, United Kingdom) with software 3.3 and a 405 nm laser setting. Method used was according to Priyashantha et al. (2019).

The technology, Nanoparticle Tracking Analysis (NTA) uses the properties of light scattering and Brownian motion to measure size distribution and particle concentration in liquid suspensions. The particles were captured in video files in 20x magnification and the software used the Stokes-Einstein equation to calculate the hydrodynamic diameter (*Malvern Panalytical* 2021). The defatted milk samples were pre-heated in a water bath at 32°C for 30 min. Prior to analysis the milk samples were diluted 2000 times using Falcon tubes with distilled water. Settings used during the recording of the sequences were Screen Gain 1 and Camera Level 16. The Auto Setup was used to find best focus, then focus was manually adjusted. Settings used during the calculation of the sequences were Screen Gain 17 and Detection Threshold 14.

3.4 Ethanol stability test

Falcon tubes with different dilutions of 99.5% ethanol and distilled water, ranging between 50-100 % in 2 % increments, were prepared. 0.5 ml defatted milk sample, pre-warmed in a water bath at 32°C for 15 min, and 1 ml of each ethanol dilution was mixed in Eppendorf tubes. The tubes were vortexed and incubated at room temperature for 30 min. Coagulation was ocularly observed, to determine for concentrations of ethanol resulting in low, medium, and high precipitation (Horne & Muir 1990; Chavez et al. 2004).

3.5 pH measurements

For the frozen milk samples, pH was measured on whole milk after thawing, when the milk reached room temperature using a Mettler Toledo, SevenCompact pH meter S210. For FD milk samples, pH was measured in the defatted milk at room temperature.

3.6 Rheological measurements

The rheological properties were measured using a hybrid rheometer (Discovery HR-3, TA Instruments, New Castle, USA) with a Peltier plate (Hard Anodized

Aluminium with Solvent Trap, 40 mm), used with settings temperature 35°C, strain 1 % and frequency 1 Hz.

Rennet solution (15 µl; see 3.2 Material preparation) was added to 1.5 ml of milk sample, pre-warmed in a water bath at 35°C for 15 minutes. CT was measured in seconds from the time rennet solution was added to the milk until the elastic modulus (G') had reached 1 Pa. The gel firmness (G20, Pa) was determined twenty minutes after the addition of rennet solution.

3.7 Milk gross composition

Milk samples from all three sampling occasions were analysed in one biological replicate for gross composition at the Department of Animal Nutrition and Management, SLU. The analysis was performed on Nov 3rd, 2021, on whole milk samples after storage at -20°C for 27, 26 and 23 days in agreement with the routine procedure of the laboratory.

Method used for total fat, total protein, total solids and lactose concentration was Fourier Transform Infrared Spectroscopy (FTIR, Hillerød, Denmark). The somatic cell count was analysed by electronic fluorescence-based cell counting (Fossomatic, Hillerød, Denmark).

3.8 Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's pairwise comparison test was used to evaluate results generated after refrigerated storage for 0 – 3 days, and results obtained at different storage temperatures after storage for different periods of time. Results for the analysed parameters were compared with results generated on day 0, to evaluate if changes due to the different treatments were statistically significant at the levels 0.05, 0.01 and 0.001. Statistical analysis was performed using Minitab® Version 19.2020.1.0 (Minitab, LLC., United States).

4. Results

4.1 Milk gross composition

Milk gross composition for the three sampling occasions is shown in Table 1. The results show that the milk collected on the three occasions was very similar in composition. However, the SCC was somewhat lower in milk collected on the second occasion. There was also a slightly higher level of total solids (TS) and fat in the second and third samplings, respectively, compared to milk from the other two samplings. However, the differences were only 0.4% for TS and 1.3% for fat.

Table 1. Milk gross composition for the three sampling occasions

Sampling occasion	Fat (%)	Protein (%)	Lactose (%)	TS (%)	Density (g/ml)	SCC (*10^3/ml)
1	4.36	3.65	4.76	13.37	1.030	130
2	4.36	3.64	4.77	13.42	1.030	107
3	4.42	3.65	4.77	13.37	1.030	137

Abbreviations: TS=total solids. SCC=somatic cell count.

4.2 Short-term changes in fresh milk

All parameters were analysed on the same day as the milk was collected, day 0, and on the three following days (day 1-3) to evaluate short term changes in the milk during storage at 4°C. Comparison between means for results obtained during day 0 to 3, as well as p-values for the observed differences, is shown in Table 2. There were no significant differences in the investigated parameters except for pH. Here, the value was 3.8 % lower at day 3 compared to day 0 ($p = 0.007$).

The curd yield and casein micelle size increased 5.2 % and 16.4 % respectively after three days storage compared to the reference value from day 0. The ethanol stability test showed no changes over time in ethanol concentration needed for protein precipitation. The CT was 7.2 % longer and the GF 13.2 % softer in day 3 compared to day 0.

Table 2. Short-term changes in the investigated parameters measured after refrigerated storage of the milk at +4°C for up to 3 days. The mean values of triplicates within rows differ significantly if they do not share a superscript (n=3 replicates, n=2 replicates).*

Day	0	1	2	3	p-value
Curd yield (g/100g)	50.68±1.22	51.95±2.11	50.08±1.84	53.32±0.25	0.120
Casein micelle size (nm)	158.83±16.46	165.50±35.80	192.83±12.10	184.83±10.21	0.245
EtOH stability (%)	86±0.58	86±0.00	85±1.73	86±0.00	0.528
pH	6.63±0.06[a]	6.47±0.05	6.45±0.07[b]	6.38±0.08[b]	**0.007**
Coagulation time (sec)	584±28.90	638±17.70*	626±38.20*	626±35.80	0.303
Gel firmness (Pa)	47.73±6.84	39.97±2.04*	42.08±6.20*	41.45±5.56	0.474

4.3 Curd yield

The mean curd yield in g/100g milk from frozen and FD milk samples after storage at -20°C and -80°C compared with the reference curd yield from day 0 is shown in Table 3 and 4. There were no statistical differences between the periods of freezing (see Table 3) and no significant differences between the freezing temperatures except for week 1 (p = 0.05). For week 1, the curd yield was 11.7 % higher for the frozen milk samples stored at -80° compared to the reference value (see Table 4).

Table 3. Average curd yield (g/100g milk) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of differences between means at day 0 and after storage for 1-6 weeks are indicated row wise by their p-value. P<0.05 is considered significant. Standard deviation indicated. n=3 for frozen milk samples, n=2 for freeze-dried milk samples

Reference	Weeks of storage							
value/day 0	Temp	1	2	3	4	5	6	p-value
50.68±1.22	-80°C	56.60±3.48	55.26±2.58	55.15±2.58	52.67±2.66	52.44±1.64	55.28±2.68	0.115
50.68±1.22	-20°C	54.68±1.52	54.20±2.08	55.31±2.45	51.68±1.36	50.59±3.33	52.31±1.61	0.060
50.68±1.22	FD -80°C	NA	49.58±3.89	NA	52.83±0.67	NA	51.24±2.42	0.563
50.68±1.22	FD -20°C	NA	49.95±3.15	NA	53.33±3.46	NA	53.65±2.62	0.411

Abbreviations: FD=freeze-dried. NA=not analysed.

The curd yield of frozen milk samples stored at -80°C increased with 9 % after two weeks and maintained a higher yield also after 6 weeks. For the freeze-dried samples, the curd yield was still numerically higher after six weeks at both storage temperatures. For all treatments, the increases in curd yield were only numerical, and not significant.

Table 4. Average curd yield (g/100g milk) measured after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of differences between means at day 0 and after storage at different temperatures is indicated column wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. Values are statistically different if they do not share a superscript.

Day 0	50.68±1.22[b]	50.68±1.22	50.68±1.22	50.68±1.22	50.68±1.22	50.68±1.22
Weeks of storage	**1**	2	3	4	5	6
-80°C	56.60±3.48[a]	55.26±2.58	55.15±2.58	52.67±2.66	52.44±1.64	55.28±2.68
-20°C	54.68±1.52	54.20±2.08	55.31±2.45	51.68±1.36	50.59±3.33	52.31±1.61
FD -80°C	NA	49.58±3.89	NA	52.83±0.67	NA	51.24±2.42
FD -20°C	NA	49.95±3.15	NA	53.33±3.46	NA	53.65±2.62
p-value	**0.050**	0.098	0.066	0.612	0.560	0.158

Abbreviations: FD=freeze-dried. NA=not analysed.

4.4 Casein micelle measurements

The comparison of casein micelle size in nanometres between the reference milk at day 0 and frozen and FD milk stored at different temperatures for up to 5 weeks is shown in Table 5 and 6. There were no statistical differences in the casein micelle size when comparing between different periods of freezing (Table 5) nor between freezing temperatures (Table 6).

Table 5. Casein micelle size (nanometre) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and week 1-6 are indicated rowwise by their p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Reference	Weeks of storage						
value/day 0	Temp	1	2	3	4	5	p-value
158.83±16.46	-80°C	176.33±15.33	164.00±36.30	162.80±24.10	151.20±28.30	123.25±10.96*	0.346
158.83±16.46	-20°C	175.83±10.77	146.80±36.70	148.50±17.40	139.70±47.80	127.75±2.47*	0.509
158.83±16.46	FD -80°C	NA	172.80±19.40	NA	141.30±59.80	NA	0.669
158.83±16.46	FD -20°C	NA	156.00±12.02	NA	128.50±76.40	NA	0.708

Abbreviations: FD=freeze-dried. NA=not analysed.

Table 6. Casein micelle size (nanometre) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and the different storage temperatures is indicated column wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2.*

Day 0	158.83±16.46	158.83±16.46	158.83±16.46	158.83±16.46	158.83±16.46
Weeks of storage	1	2	3	4	5
-80°C	176.33±15.33	164.00±36.30	162.80±24.10	151.20±28.30	123.25±10.96*
-20°C	175.83±10.77	146.80±36.70	148.50±17.40	139.70±47.80	127.75±2.47*
FD -80°C	NA	172.80±19.40	NA	141.30±59.80	NA
FD -20°C	NA	156.00±12.02	NA	128.50±76.40	NA
p-value	0.309	0.879	0.671	0.949	0.066

Abbreviations: FD=freeze-dried. NA=not analysed.

4.5 Ethanol stability test

The results for the ethanol stability test, comparing the values for the reference milk with values from the frozen and FD milk samples stored at -20°C and -80°C are shown in Table 7 and 8. There was a significant difference for the FD samples stored at -80°C when comparing freezing time with a p-value of 0.017 (Table 7). The pH value from week 4 was significantly 3.4 % higher than the reference value and the pH in week 6.

Table 7. Ethanol stability (percent ethanol at visual precipitation) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and week 1-6 is indicated row wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Reference	Weeks of storage							
value/day 0	Temp	1	2	3	4	5	6	p-value
86±0.58	-80°C	87±2.31	87±10	86±0	86±0.58	86±0.58	87±1.16	0.229
86±0.58	-20°C	87±2.31	87±1.16	86±0	86±0.56	85±2.31	87±1.16	0.514
86±0.58[b]	**FD -80°C**	NA	87±1.41	NA	89±0[a]	NA	86±0[b]	**0.017**
86±0.58	FD -20°C	NA	86±0	NA	86±0	NA	86±2.83	0.742

Abbreviations: FD=freeze-dried. NA=not analysed.

Comparing the result in respect to storage at the different freezing temperatures (Table 8) showed a significant difference between results at week 4 (p = 0.001), where the FD samples stored at -80°C was 3.4 % significantly higher than the reference values of day 0.

Table 8. Ethanol stability (percent ethanol at visual precipitation) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and storage at the different temperatures is indicated column wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Day 0	86±0.58	86±0.58	86±0.58	86±0.58[b]	86±0.58	86±0.58
Weeks of storage	1	2	3	**4**	5	6
-80°C	87±2.31	87±10.00	86±0.00	86±0.58[b]	86±0.58	87±1.16
-20°C	87±2.31	87±1.16	86±0.00	86±0.56[b]	85±2.31	87±1.16
FD -80°C	NA	87±1.41	NA	89±0.00[a]	NA	86±0.00
FD -20°C	NA	86±0.00	NA	86±0.00[b]	NA	86±2.83
p-value	0.509	0.434	0.422	**0.001**	0.946	0.447

Abbreviations: FD=freeze-dried. NA=not analysed.

4.6 pH measurements

The comparison of results for pH measured at room temperature between the reference value at day 0 and frozen and FD milk stored in -20°C and -80°C is shown in Table 9 and 10. Significant differences in pH for frozen and FD milk samples stored at -20°C were observed after storage at various periods of time. The result after one week of storage of the frozen milk at -20°C was significantly lower (p=0.013) compared to the pH of the reference sample, but also lower than the pH after storage for 4 and 5 weeks. For the FD samples, pH was significantly higher after 4 and 5 weeks of storage at -20°C, than pH at day 0 (Table 9).

Table 9. pH measured at room temperature after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and week 1-6 is indicated row wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Reference	Weeks of storage							
value/day 0	Temp	1	2	3	4	5	6	p-value
6.63±0.06	-80°C	6.57±0.02	6.65±0.04	6.68±0.05*	6.68±0.05	6.6±0.08	6.62±0.04	0.191
6.63±0.06[a]	**-20°C**	6.48±0.04[b]	6.60±0.05	6.61±0.05*	6.69±0.03[a]	6.65±0.01[a]	6.60±0.11	**0.013**
6.63±0.06	FD -80°C	NA	6.46^	NA	6.69±0.09	NA	6.68±0.01	0.106
6.63±0.06[b]	**FD -20°C**	NA	NA	NA	6.78±0.01[a]	NA	6.67±0.01	**0.038**

Abbreviations: FD=freeze-dried. NA=not analysed.

Comparing the effect of the different freezing temperatures on pH, pH measured after storage at -20°C for one week was significantly lower than at day 0 (Table 10).

Table 10. pH measured at room temperature after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and storage at the different temperatures is indicated column wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Day 0	6.63±0.06[a]	6.63±0.06	6.63±0.06	6.63±0.06	6.63±0.06	6.63±0.06
Weeks in storage	**1**	2	3	4	5	6
-80°C	6.57±0.02	6.65±0.04	6.68±0.05*	6.68±0.05	6.60±0.08	6.62±0.04
-20°C	6.48±0.04[b]	6.60±0.05	6.61±0.05*	6.69±0.03	6.65±0.01	6.60±0.11
FD -80°C	NA	6.46^	NA	6.69±0.09	NA	6.68±0.01
FD -20°C	NA	NA	NA	6.78±0.01	NA	6.67±0.01
p-value	**0.009**	0.061	0.472	0.115	0.610	0.631

Abbreviations: FD=freeze-dried. NA=not analysed.

4.7 Rheological measurements

Coagulation time

Storage of the milk for different periods of time at both -20°C and -80°C resulted in significant differences in CT compared to the reference value (see Table 11). The shortest significant CT for the frozen samples occurred for the samples stored at -80°C, with increased with 7.2 % prolonged CT after one week of storage (p=0.001).

Table 11. Coagulation time (seconds) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and week 1-6 is indicated row wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Reference	Weeks in storage							
value/day 0	Temp	1	2	3	4	5	6	p-value
584±29[c]	**-80°C**	629±65[bc]	692±19[ab]	719±32[ab]	730±14[a]	703±15[ab]	725±37[a]	**0.001**
584±29[b]	**-20°C**	670±25	661±59	717±50[a]	728±40[a]	700±21[a]	705±38[a]	**0.009**
584±29[b]	**FD -80°C**	NA	743±59	NA	708±59	NA	751±54[a]	**0.032**
584±29[b]	**FD -20°C**	NA	646±16	NA	693±74	NA	750±21[a]	**0.025**

Abbreviations: FD=freeze-dried. NA=not analysed.

The comparison between the effects of different freezing temperatures on the CT results showed significant differences for all weeks except week 1 (see Table 12). The smallest significant change in CT compared with the reference value occurred in week 5, with 19.8 % longer CT for the frozen sample stored in -20°C.

Table 12. Coagulation time (seconds) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and storage at the different temperatures is indicated column wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Day 0	584±29	584±29[b]	584±29[b]	584±29[b]	584±29[b]	584±29[b]
Weeks in storage	1	**2**	**3**	**4**	**5**	**6**
-80°C	629±65	692±19	719±32[a]	730±14[a]	703±15[a]	725±37[a]
-20°C	670±25	661±59	717±50[a]	728±40[a]	700±21[a]	705±38[a]
FD -80°C	NA	743±59[a]	NA	708±59	NA	751±54[a]
FD -20°C	NA	646±16	NA	693±74	NA	750±21[a]
p-value	0.126	**0.022**	**0.007**	**0.015**	**0.001**	**0.003**

Abbreviations: FD=freeze-dried. NA=not analysed.

Gel firmness

Comparing the effects of freezing time on GF showed significant differences for the frozen samples stored at -80°C and -20°C (see Table 13). For the frozen samples stored at -80°C, the reference value was significantly higher compared to weeks 4, 5 and 6 (p=0.009). The frozen samples stored at -20°C had significantly lower GF compared to the reference value at week 1, 4, 5 and 6 (p=0.022). Week 5 at -80°C had the least affected samples, with 39.3 % softer gel than the reference value while week 1 in -20°C was least affected. Here, the gel was 32.4 % softer than the reference value.

Table 11. Gel firmness (Pa, 20 min after rennet addition) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time The statistical significance of the differences between means from day 0 and week 1-6 is indicated row wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Reference	Weeks in storage							
value/day 0	Temp	1	2	3	4	5	6	p-value
47.73±6.84[a]	**-80°C**	44.31±13.29	32.34±2.90	29.96±3.54	28.87±0.10[b]	28.93±4.89[b]	27.40±6.53[b]	**0.009**
47.73±6.84[a]	**-20°C**	31.30±4.63[b]	32.30±7.77	31.54±6.67	29.14±4.40[b]	30.35±4.79[b]	30.74±5.10[b]	**0.022**
47.73±6.84	FD -80°C	NA	34.06±2.60	NA	39.25±10.94	NA	30.25±9.50	0.193
47.73±6.84	FD -20°C	NA	46.24±5.49	NA	40.20±14.90	NA	31.63±0.75	0.278

Abbreviations: FD=freeze-dried. NA=not analysed.

Comparing the effects of different freezing temperatures on GF showed significant differences for all weeks except week 1 and 4 (see Table 14). For all weeks with

significant differences, except week 2, the frozen sample stored at -80°C was significantly lower than the reference value. For week 3 and 5, the GF of frozen samples stored at -20°C were also significantly lower than the reference value. The result with the least affected GF is during week 3 for the frozen sample stored in -20°C, which is 33.9 % softer than the reference value.

Table 12. Gel firmness (Pa, 20 min after rennet addition) after storage of frozen and freeze-dried milk samples at -20°C and -80°C, respectively, for various periods of time. The statistical significance of the differences between means from day 0 and storage at the different temperatures is indicated column wise by its p-value. P<0.05 is considered significant. Standard deviation is indicated. n=3 for frozen milk samples. n=2 for freeze-dried samples. n=2. Values are statistically different if they do not share a superscript.*

Day 0	47.73±6.84	47.73±6.84	47.73±6.84[a]	47.73±6.84	47.73±6.84[a]	47.73±6.84[a]
Weeks in storage	1	**2**	**3**	4	**5**	**6**
-80°C	44.31±13.29	32.34±2.90	29.96±3.54[b]	28.87±0.10	28.93±4.89[b]	27.40±6.53[b]
-20°C	31.30±4.63	32.30±7.77	31.54±6.67[b]	29.14±4.40	30.35±4.79[b]	30.74±5.10
FD -80°C	NA	34.06±2.60	NA	39.25±10.94	NA	30.25±9.50
FD -20°C	NA	46.24±5.49	NA	40.20±14.90	NA	31.63±0.75
p-value	0.141	**0.029**	**0.018**	0.076	**0.011**	**0.029**

Abbreviations: FD=freeze-dried. NA=not analysed.

5. Discussion

5.1 Milk gross composition

The second sampling at the milk collection (see Table 1) had slightly different composition than at the other two samplings. This was expected as the bulk tank had been emptied earlier during the day of the collection, in contrast to the previous two occasions, where the milk tank had been emptied the day before sampling. The level of milk was lower in the tank on the second day of sampling compared to the other two sampling occasions and presumably, all cows had not been milked at the time of sampling.

With a herd consisting of both SRB and SH and individual variations within the breeds, the composition of the bulk milk will be affected depending on whether the whole herd has been milked at the time of sampling or not. It would also matter if a larger proportion of one of the breeds was milked and it will be affected by the individuals that were milked before sampling. According to Larsen et al. (2010), SRB produce milk with higher levels of fat and protein than SH. This could be a possible explanation for the above average concentrations in the milk composition as there was higher proportion of SRB than SH in the herd.

The first and third sampling also differed slightly from each other in composition. The third sampling had slightly higher fat- and lactose concentration, but the differences was so small, that it can be considered as unimportant. The higher fat concentration could possibly be explained by a higher amount of milkings from SRB cows before the third sampling. Another explanation could be that a higher proportion of the milked cows were in late lactation, which is associated to higher levels of fat in the milk (Walstra 2006). The hypothesis is supported by the correlation between increasing somatic cell count (SCC) at later lactation stage for healthy cows as the third sampling also had the highest SCC. Shorter time between milkings can also increase the fat content. According to Walstra (2006), feed composition may have an impact on the fat concentration. Since the milk was collected at the Livestock Research Centre with continuing research projects concerning feed composition, it is possible that cows fed with e.g., a higher level of concentrate was milked before collection.

The composition also includes the levels of SCC. In bulk milk, SCC is used to evaluate general udder health in the herd and it is used as a marker for milk quality (Walstra 2006). Processing milk with high SCC levels into cheese will cause reduced GF (Barbano et al. 2006) and decreased curd yield due to increased proteolytic activity (Senyk et al. 1985). Swedish bulk tank milk originating from a healthy herd in most cases has a SCC below 150 000 cells/ml. The dairies also request that the milk delivered should have SCC below certain levels to process the milk. The EU-legislated upper limit in bulk tank milk is 400 000 cells/ml while Swedish dairies accept levels up to 200 000 cell/ml (*Juverportalen* 2020). The SCC was low at all three samplings and the minor differences in the composition had presumably no effect on the results obtained within this project. Other factors increasing the levels of SCC, apart from disease and late lactation stage, include higher number of lactations (Walstra 2006). The usage of AMS could also increase the SCC, possibly due to the robotic teat cleaning being less efficient (Svennersten-Sjaunja & Pettersson 2008). The reason for the lower SCC in the second sampling compared to the other two is presumably uneven milkings of the herd affecting factors above.

The lactose level in milk affects primarily the pH of milk during storage as it is the primary energy source of lactic acid bacteria (Priyashantha 2021). The negligible differences in lactose, the short storage time and a cooled temperature, was probably not the reason to slightly changed pH observed in this study. Also, the tolerable SCC levels in all three samplings and small differences in the composition have presumably no effect on the results obtained within this project.

5.2 Short-term changes in fresh milk in cold storage

At collection, the temperature in the bulk tank was 3-5°C according to its display. The milk samples, in 500 ml containers, were kept at ambient temperature for approximately 30-40 min during transportation to the lab for analysis. Half of the milk was cooled shortly after arrival after being defatted in the centrifuge, the other half was directly placed in a water bath at 32°C for the milk fat to melt. There was no heat treatment of the samples before analysis or preservation to prevent microbial growth. In this study, analysis of total bacteria count was not included. But it is possible to assume that the microbial growth in the whole milk stored in 4°C samples should be higher than in the defatted milk. According to Walstra (2006), raw milk without heat treatment kept at 4°C will have a bacterial count above $5*10^5$ cells/ml after 4-5 days after the first milking. This is the considered as the desirable upper limit for processing. During the collection of first and third sampling, the tank had been emptied the morning before. The samplings were collected in the afternoon, therefore 1½ day had already gone from the first milking to collection time. It is possible to assume that the microbial growth, specifically of

psychotropic bacteria, had increased by the second analysis day, especially as the cold chain was broken during transportation to the lab. A higher bacterial count results in reduced curd yield due to proteolytic enzymes (Skeie 2007). However, in this study the curd yield increased during the short-term storage at 4°C which is an interesting result (see Table 2). According to earlier reasoning, the microbial count should be at its peak at day 3 with highest levels of enzymes and protein denaturation, especially as the curd yield is based on the whole milk. According to Walstra (2006), the β-casein dissolute during cold storage. According to this reasoning, it is contradictory with the highest curd yield at day 3. But the increase in day 3 is not significant according to the statistical analysis either.

Values for CT and GF were inversely related, i.e. the highest CT corresponded to the softest gel and the shortest CT to firmest gel, which was expected. The result with a longer CT and softer gel at day 3 is in agreement with Walstra (2006) and explained by decreased rennetability in milk after cold storage.

The results in the rheological properties do not correlate with the increased curd yield in day 3 where the yield is supposed to be decreasing. Possibly, the result could be affected by handling errors during the cheese making process. One handling error could be additional addition of rennet, which was added in very small volumes, to the samples.

Another interesting observation related to the short-term changes during storage at +4°C, is the change in micelle size and curd yield, respectively. The micelle size seemed to increase during storage, even if the value fluctuated over the days. The curd yield was lowest in day 0 and 3, with 50.68 g/100g milk and 53.32 g/100g milk, while the smallest micelle size was observed in day 0, 158.83 nm, and the largest in day 2, 192.83 nm. If there was a statistical difference found, it could be possible to conclude that the curd yield is unaffected by the micelle size by comparing their values.

Ethanol stability of milk is negatively affected by a lower pH level. The ethanol concentration needed for milk to coagulate, as the ethanol stability is almost constant over the days. However, the changes in pH in this study were significant and could be explained by the lactic acid bacteria in the milk converting lactose into lactic acid. But the rate of conversion was probably affected by the cold storage and not big enough to impact on the ethanol stability.

According to the result from this study, there was no strong correlation between casein micelle size and the rheological properties. The smallest micelle size was found in day 0, the time point which also showed the most favourable rheological properties with shortest CT and highest GF (in terms of numerical values). This would agree with the results from Glantz et al (2010) and Logan et al (2014). Otherwise, it is not possible to make any clear conclusion by comparing the other days during the short-term changes. In this study the micelle size increased throughout the days 0 - 3, with irregular variations, whereas the CT is almost stable

after one day of storage. One factor affecting the result of micelle measurement day 0 could be an error in the method. Due to equipment service, the machine was not available for testing until shortly before collection of the 0 samples. This mainly affected the reading of the samples, with accidental addition of bubbles with the syringe. This may have caused mistakes in the calculation of micelle size during the short-term changes and the first weeks of measurements. Despite this, considering that the methods showed correct results, it can be concluded that for short-term changes, there were no significant differences between the parameters investigated in this project, except for pH (see Table 2). This means that milk stored at 4°C can be used for this type of analysis within at least three days.

5.3 Curd yield and rheological properties in frozen storage

According to the result, the curd yield increased with 11.7 % and 7.9 % in the frozen milk samples after one week of storage in -80°C and -20°C respectively (see Table 3). This increase was significant only for -80°C (see Table 4). According to Walstra (2006), the casein micelles aggregate during freezing and this can be avoided by rapid freezing, which occurs during freezing at -80°C. Not specified by Walstra, is the effect of casein micelle aggregation on cheese production and curd yield. It is possible to assume that the aggregation may inhibit the enzymatic coagulation, with the splitting of the κ-casein, maybe resulting in a softer gel. According to the result from the GF measurements (see Table 13), the frozen milk samples stored at -80°C had 41.6 % firmer gel than the frozen milk samples stored at -20°C after one week of storage. Neither the increase in curd yield at -20°C nor the higher gel firmness at -80°C were significant. If there would have been a significant difference, it could have confirmed the hypothesis that micelle aggregation interferes with coagulation. Even though, the curd yield from both storage temperatures is quite similar, with 3.4 % lower yield from frozen milk stored in -20°C than from storage in -80°C. Both storage temperatures resulted in numerically higher yields compared to prior freezing in day 0, in all weeks, except for week 5 in -20°C, indicating that the micelle aggregation during slow freezing may have no effect on curd yield.

The FD samples showed a small decrease in curd yield after two weeks storage, with 2.2 % and 1.4 % in -80°C and -20°C respectively. After six weeks the yield increased with 1.1 % at -80°C compared to the reference value in day 0. This increase was numerical lowest compared to the other milk samples at the different temperatures. According to Koschak et al. (1981) storage at -80°C provides higher protein stability for FD samples. There was no difference in GF for FD samples stored at -80°C compared to the other freezing temperatures. There is hence

difficult to conclude that the freezing temperature is favourable for FD samples considering the result.

The GF for the frozen samples significantly decreased slightly over the storage weeks; more at -80°C than at -20°C. After six weeks, the CT was 24.1 % and 20.7 % longer after storage in -80°C and -20°C respectively. This correlated with the softest gel formation week 6 in both temperatures.

The GF of the FD samples decreased at both temperatures after storage for two weeks, unfortunately not significant compared to the reference value. An interesting result is the small decrease, just 3.1 %, in GF from the FD samples stored in -20°C compared to the 28.6 % decrease from the FD samples stored in -80°C. The difference was equalized after four weeks of storage with a 17.8 % and 15.7 % at -20°C and -80°C respectively, compared to the reference values in day 0. After six weeks of storage, the GF was stabilized with 36.6 % and 33.7 % in respective temperatures. In agreement with expectations, the GF for the FD samples seemed inversely correlated with the CT, with the shortest CT after short freezing corresponding to the firmest gel. The result indicated that the FD sample stored at -20°C at first was relatively unaffected by the frozen storage while starting to react to its storage environment by week 4. According to Gaber (2020), the casein micelle stability is most affected in the early days of frozen storage due to gradual ice formation and concentration of solids still occurring (Gaber et al. 2020). As the FD samples are dehydrated, it is presumable that the effect of crystallisation would be very small. The curd yield was similar despite the differences in GF. This observation is in agreement with Koschak et al. (1981), showing higher protein stability for FD samples at -20°C.

One interesting observation during the cheese making process was the floating aggregates in the serum phase after the centrifugation of the curd made with the reconstituted FD samples stored at both -80°C and -20°C. Since the floating aggregates would separate from the curd with the serum phase, this was probably the reason for the slightly lower yield from the FD samples . According to Gaber et al. (2020), this is due to destabilisation of the casein micelle during freezing which was observed in several studies. It would have been interesting to examine the aggregates closer to identify the particle type.

To conclude, the numerical curd yield values increased over time for all types of samples at all temperatures but only the frozen sample stored at -80°C showed a significant increase in yield. The higher yield can be connected to higher whey content because of lower syneresis rate. Also, in all cases the gel became softer with storage time for all types of samples at all temperatures. The higher yield and softer gel could, however, be suitable for specific type of cheeses based on softer curds. Storage of frozen milk at -80°C is most beneficial regarding curd yield but the

differences are small. The rheological properties were best maintained after storage at -20°C for frozen milk regarding coagulation time though GF show small difference between freezing temperatures.

5.4 Ethanol stability and pH in frozen storage

The results for ethanol stability (see Table 7 and 8) and pH (see Table 9 and 10) showed small changes throughout the weeks of storage for both frozen and FD samples at both temperatures. An ethanol stability above 78 % is considered as a high ethanol stability (Chavez et al. 2004). The correlation between unchanged values for ethanol stability and pH are expected, as the levels of ethanol concentration needed for precipitation is dependent on the pH in the milk (Walstra 2006). The high ethanol stability confirmed that frozen storage preserved the milk without affecting the heat stability.
There was a statistically significant effect on pH from storage at -20°C for various periods (Table??) , for both the FD and frozen samples, however, differences were small. The effect of storage at -80°C on ethanol stability for FD samples, also with small variation, was also significant. Unfortunately, a correlation with significant difference for both ethanol stability and pH cannot be found comparing the statistical analysis.

The result indicates that both frozen and FD milk is suitable for heat treatment processing after storage at freezing temperatures. Both freezing for various time periods times and at the different temperatures resulted in almost unaltered pH and ethanol stabilities.

5.5 Casein micelle size measurements in frozen storage

The casein micelle will typically increase in size during frozen storage (Walstra 2006) which is contradictory with the result in this study, both for both frozen and FD samples (Table 5 and 6). According to the results, the decrease in numerical values for micelle size was gradual and linear with storage time and not significant. It would have been interesting to do the measurements at 6 weeks also for the FD samples to evaluate if the decrease was in agreement for both sample types. This was unfortunately not possible due to machine service.

The frozen milk samples stored in -80°C had the most unchanged casein micelles size except for week 5, indicating that the rapid freezing is more favourable in keeping the micelles in original size. The result from FD milk stored in -80°C also has the most similar casein micelle sizes compared to the reference value in day 0. This result is contradictory from Koschak et al. (1981) who showed that slow

freezing in -20°C results in higher protein stability than rapid freezing. Without significant differences, it is difficult to draw any conclusions. It is also difficult to decide the wanted outcome from the measured casein micelle sizes as studies show contradictive result on what size is most beneficial in coagulation (Ekstrand et al. 1980; Dalgleish et al. 1981; Glantz et al. 2010).

Comparing the effects of freezing on both the rheological properties and casein micelle size, it appears as -80°C would be most beneficial in maintaining the rheological characteristics. It is also the temperature which maintain the casein micelle size closest to its original size, suggesting that if the micelle size is not impacted, milk will coagulate better.

According to Walstra (2006), the casein micelle size will increase in size as pH in milk decreases. Comparing the lowest points of pH with the casein micelles size from same sample showed that the two lowest pH values were associated to two of the largest values for casein micelle sizes. Milk with pH 6.48 had the average micelle size of 175.83 nm (see Table 9 and 10) and milk with pH 6.46 had an average micelle size of 172.8 nm. It is also disproven by the fact that milk stored in -80°C for one week with a higher pH, 6.57, has a larger micelle size. The conclusion is also disproven as the size of the micelle is affected by temperature, and pH and casein micelle size are measured at different temperatures. It would have been interesting to measure the pH at 30°C to be able to learn more about a possible correlation.

To conclude, there was no significant difference in casein micelle size. The storage in -80°C for both sample types appear to be most beneficial to maintain the rheological properties.

5.6 General discussion

As freeze-drying is an expensive process which requires special equipment (Walstra 2006), the small-scale cheese manufactures are likely prone to use freezers. The result of this study showed that the optimal freezing time and temperature for frozen milk with respect to curd yield and coagulation time was one week at -80°C. Since freezing of milk to come up to the volumes required for a cheese batch, it would be interesting to do further studies on caprine and ovine milk. With a different milk composition based on the animal species, it is possible that the result after frozen storage will be different from those of bovine milk.

The small number of replicates makes it difficult to make conclusions about the results, especially for the FD samples. Increasing the number of replicates would mean a great improvement in further studies.

It would also be interesting to analyse the protein profile of the samples to understand denaturation, especially during cold storage and freeze-drying. According to Gaber et al (2020), the whey proteins are likely to denature and unfold

during freezing, affecting the formation of aggregates and gel characteristics. The whey and casein profile would hence be an interesting addition to better understand the properties of milk after freezing.

6. Conclusion

In this study, composition and properties, i.e., casein micelle size and pH, and the processability, i.e., curd yield, ethanol stability and rheological properties, of frozen and freeze-dried bovine milk samples were studied, both prior and after frozen storage at -80°C and -20°C for up to 6 weeks. The short-term changes on fresh milk during cold storage at 4°C were investigated. The result from the gross composition showed similar levels of all factors from the three sampling occasions, with slightly lower levels of somatic cells in the second sampling. During refrigerated storage, the only significant change observed was a small decrease in pH, from x to y.

The result from the frozen storage showed no significant difference in curd yield except for the first week, where the yield increases 11.7 % in the frozen samples stored in -80°C. The ethanol stability test showed significant difference for the freeze-dried samples stored in -80°C comparing freezing temperatures, with 3.2 % increased stability after four weeks. There was also a significant difference in week 4 when comparing freezing time. The pH measurements showed significant difference for the frozen and freeze-dried samples stored at -20°C and in week 1 and 2 and showed small variation, at most 2.3 % increase, during the storage for six weeks. For the rheological properties, many factors showed significant difference in the result. For the coagulation time, all comparisons between means showed significant differences, except for week 1. The gel firmness measurements showed significant difference for the frozen milk samples stored at both temperatures as for week 2, 3, 5 and 6. The result showed increased coagulation time and decreased gel firmness for all samples during six weeks of storage. The samples with shortest coagulation time were the frozen samples stored in -80°C for one week, with a 7.7 % prolonged coagulation time. The firmest gel was measured in the frozen sample stored in -20°C, where the gel firmness decreased with 33.9 % after three weeks. There was no significant difference shown in casein micelle size. The result indicates that the most beneficial storage for cheese production is one week at -80°C for frozen milk samples. In further research, it would be interesting to execute the same study on ovine and caprine milk to make the result more useful for small-scale producers of sheep and goat cheese. With different milk composition, it is possible that the result will be differ from the bovine milk result.

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Popular science summary

Did you know that we know very little about how milk is affected by frozen storage?

We all have used a freezer. We use them at home and at the grocery stores. Sometimes in our workplaces as well. Which dairy product is normally kept in the freezer? I know we think about the same product – that's right, ice cream! But the other dairy products, why are they never kept in the freezer? Why are they always just stored cold, in the fridge?

One reason is that the cows in this part of the world produces milk for us every day. We always have access to fresh milk; we don't need to store it for longer periods of time in the freezer. Also, the process of transforming milk into cheese, crème fraiche or yoghurt, helps to preserve the milk by heat treatment, lower water content and low pH. After that, cold storage is enough to keep products in good condition until they're bough and eaten.

But what about parts in the world that don't have milk produced for them all year around? Or the places that have limited access to dairy? Understanding the effect of frozen storage on milk can also open up for more sustainable ways of transporting milk – suddenly the transport doesn't need to be time-depended. The frozen milk can be transported during longer time for lower transportations costs and lower environmental impact, for example by boat instead of flights.

Another group that would need to know more about the effects of frozen storage is the small-scale sheep- and goat cheese manufacturers; with animals that produces milk seasonally, or for those who have small herds and need to store the milk while awaiting to get enough milk for production of one cheese batch. The freezer is an easy and convenient way for them to preserve their milk. But is the frozen storage impacting their cheese production? How? Could there be an optimal freezing time that can benefit their production?

During this study, fresh milk was kept in a freezer at -20°C and -80°C, respectively, and analysed once a week for six weeks to understand more about the effects of frozen storage over time. The fresh milk was also freeze-dried and stored at the same freezing temperatures.

The result showed that gel firmness, i.e., how firm the cheese curd is, was greatly affected after frozen storage of the milk. The least impact could be found in the

sample stored at -20°C after three weeks, where the gel was 33.9 % softer than before freezing.

The frozen and freeze-dried milk was also analysed for how long time it took for the milk to coagulate after adding rennet. All samples showed significant increased coagulation time, or it took all samples longer time to coagulate after frozen storage. The storage time that was most beneficial, with the smallest negative effect on coagulation time, was one week of storage in -80°C from the frozen milk which increased with just 7.7 %.

A surprising result is the cheese curd yield analysis. It showed that the curd yield increased significantly (!) after one week of storage at -80°C with 11.7 %, which means that the cheese manufacturer would get more cheese per 100 gram of milk after frozen storage for one week in comparison with using fresh milk.

After the study, it was possible to conclude that frozen storage affects the milk and its proteins, but all changes are not for the worst. It can even improve the production by resulting in higher curd yield. But for the cheese manufacturers to be able to cherry-pick, the result indicates that they should invest in -80°C freezers, as most beneficial results are after one week of storage in that temperature. The result also indicate that the frozen milk is most suitable for the making of softer cheese types, with the large decreases in gel firmness.

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