Surviving ratio of milk microflora during storage at freezing temperature

- A pilot study

Mikrobiologisk överlevnad i mjölk under förvaring i frystemperaturer – en pilotstudie

Louise Gustafsson

Degree project/Independent project • 30 credits
Swedish University of Agricultural Sciences, SLU
SLU Department of Molecular Science
Agricultural programme – Food Science
Molecular Sciences, 2022:56
Uppsala, 2022

Surviving ratio of milk microflora during storage at freezing temperature – a pilot study

Mikrobiologisk överlevnad i mjölk under förvaring i frystemperaturer – en pilotstudie

Louise Gustafsson

Supervisor:	**Monika Johansson, SLU, Department of Molecular Sciences**
Examiner:	Åse Lundh, SLU, Department of Molecular Sciences

Credits:	30 credits
Level:	A2E
Course title:	Master thesis in Food Science
Course code:	EX0877
Programme/education:	Agricultural Programme – Food Science
Course coordinating dept:	Department of Molecular Sciences
Place of publication:	Uppsala
Year of publication:	2022
Cover picture:	Pernilla Ivarsson
Copyright:	All featured images are used with permission from the owner.
Title of series:	Molecular Sciences
Part number:	2022:56

Keywords: *Raw cow´s milk, raw milk quality, milk microflora, culturing methods, MALDI-TOF MS, cold storage of milk*

Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Molecular Sciences

Abstract

Milk contains beneficial and non-beneficial bacteria which contribute with different outcomes to milk and dairy products. Beneficial bacteria are important during manufacturing while non-beneficial bacteria cause spoilage, food related outbreaks but also economical losses for the dairy industry. It is therefore important to control the survival of wanted and unwanted bacteria through microbial analyses. For this purpose, it would be beneficial if milk samples could be stored frozen for some time until analyses will be performed. The aim of this study was to investigate changes in ratio of milk microbiota during storage at two freezing temperatures, -20°C and -80°C, with respect to time. The hypothesis was that no change of milk microbiota would occur. The microbial community in the fresh milk was investigated directly after collection followed by once-a-week cultivation during eight weeks. Analysis was performed through pure culture streaking method both aerobically on Plate Count agar (PCA) and anaerobically on De Man Rogosa and Sharpe (MRS) agar. Bacterial identification was performed using MALDI-TOF MS. Results revealed an effect of storage temperature, where the number of CFU/ml cultivated from milk samples stored at -80°C was higher than after storage at -20°C, irrespectively if bacteria were cultured on PCA or MRS. However, the effect was only significant for bacteria cultured on MRS. There was a significant effect of storage time on both aerobes and anaerobes when cultivating milk samples stored at -80°C and -20°C, respectively. However, for aerobes the difference between these two temperatures was not significant. The colony characterization by MALDI-TOF MS identified 14 different species from PCA and 9 species from MRS media. In conclusion, to maintain the original microbial community in milk during storage at freezing temperatures, storage of milk samples at -80°C is recommended.

Keywords: raw cow´s milk, raw milk quality, milk microflora, culturing methods, MALDI-TOF MS, frozen storage of milk

Sammanfattning

Mjölk innehåller bakterier som kan ha såväl en positiv respektive negativ inverkan på mjölk och mejeriprodukter. Fördelaktiga bakterier har en betydelsefull roll inom förädlingsprocesser medan ofördelaktiga bakterier kan orsaka livsmedelsrelaterade utbrott och förstörelse av mejeriprodukter och slutligen leda till ekonomiska förluster för mejeriindustrin. Följaktligen är det av stor vikt att utföra kontroller av både fördelaktiga och ofördelaktiga bakterier via mikrobiella analyser. I detta sammanhang vore det fördelaktigt om mjölkprover kunde förvaras frysta under en kortare tid tills analyserna kan genomföras. Syftet med denna studie var att undersöka förändringar i mjölkens mikroflora över tid under förvaring i två frystemperaturer, -20°C och -80°C. Hypotesen var att ingen förändring i sammansättningen av mjölkens mikroflora skulle uppstå. Mikrofloran i den färska mjölken undersöktes direkt efter provtagningen med hjälp av odlingsbaserade metoder, och analyserna följde upp veckovis under åtta veckor. Mjölkprover odlades aerobt på Plate Count Agar (PCA) samt anaerobt på De Man, Rogosa and Sharpe (MRS) agar. MALDI-TOF MS användes för identifiering av bakteriekolonier. Resultaten visade att antalet CFU/ml odlade från mjölkprover förvarade i -80°C var högre för båda bakterietyperna men att det högre antalet enbart var signifikant för anaeroba bakterier. Odlingarna visade en signifikant effekt av lagringstid för aeroba bakterier förvarade i -80°C respektive för anaeroba bakterier förvarade i -20°C. Däremot var skillnaden mellan de två temperaturerna inte signifikant för de aeroba bakterierna. MALDI-TOF MS kunde identifiera 14 olika arter från PCA- och 9 från MRS agar. Sammanfattningsvis rekommenderar studien att mjölkprover förvaras i -80°C för att bibehålla mjölkens mikroflora vid förvaring innan mikrobiella analyser genomförs.

Nyckelord: obehandlad komjölk, kvalité i obehandlad mjölk, mikrobiellt innehåll i mjölk, odlingsbaserade metoder, MALDI-TOF MS, frysförvaring av mjölk

Table of contents

List of tables

Abbreviations

CFU	Colony-Forming Units
DT	Direct transfer
EMP	Emden-Meyerhof-Parnas
HCCA	α-cyano-4-hydroxycinnamic acid
LAB	Lactic Acid Bacteria
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MRS	De Man, Rogosa and Sharpe
MTP	Maldi Target Plate
NGS	Next Generation Sequencing
PCA	Plate Count Agar
RCM	Raw Cow´s Milk
SBSEC	*Streptococcus bovis*/*Streptococcus equinus* complex

1. Introduction

Milk is consumed worldwide and can be connected to human consumption, due to rock drawings, back to 4000 BC. Thousands of years later, in 1600s US, the first dairy herd was formed and in connection to the industrial revolution in 1830s, the first milking machine was used. Since then, the dairy industry has grown and advanced to produce high quantities and variations of milk and dairy products (Miller et al. 2000).

Microorganisms in milk include yeasts and moulds but mainly bacteria. Milk consists of a complex microbiota which makes a simplified overview complicated to achieve. Bacteria in milk influence its environment while the environment selects for certain bacteria. In this way, each milk and dairy product creates an individual ecosystem. The environment in this case consists of the nutritious components of milk and the surrounding conditions where ambient temperature is of main importance. RCM (raw cow´s milk) left without any package is an open ecosystem, enabling growth of roughly any bacteria present. However, the majority of milk products are in a closed ecosystem. In other words, the alteration in milk is therefore a result of the particular bacteria causing the contamination. Consequently, composition of milk microbiota can vary widely (Walstra et al. 2005).

Due to high concentration of nutrients, in relation to its caloric energy, milk is convenient for human consumption and hence connected to well-being and health-benefits (Miller et al. 2000). In contrast, milk and dairy products have also caused food-related outbreaks because of microbial contaminations. Food-related outbreaks, disease within the herd and spoilage of milk and dairy products are all consequences of microbial contaminations somewhere thorough the dairy value chain. As a result, the microbial contaminations also cause economical losses within the dairy industry (Adams 2016). To prevent these negative effects, and for scientific purposes, microbial analyses are performed on milk and dairy products. To manage handling of high quantities of milk samples, storage at freezing temperatures is often used (Hubáčková & Ryšánek 2007).

1.1 Purpose and objective

This project was performed as an explorative pilot study. Microbial analysis of raw milk is a common approach to prevent food related outbreaks, sickness within the

herd and hence economical losses within the dairy industry. Freezing of milk samples is a common storage method before analyses of different milk quality attributes, however, microbial analysis based on culturing methods is usually performed using fresh milk samples. The purpose of this study was to monitor the surviving ratio of different types of bacteria in the raw milk after storage at freezing temperatures, to determine the risk of misdiagnosis during microbial analyses on frozen and thawed raw milk. The study included three batches of bulk milk collected during three different occasions at the Swedish Livestock Research Centre, Lövsta. Bacteria in the milk were analysed with cultivation techniques followed by MALDI-TOF MS as an identification method. The hypothesis of the project was that bacterial surviving ratio would maintain the same or that bacteria numbers would possibly decrease with time.

1.2 Delimitations

During this pilot study, bulk milk was collected on three occasions within two weeks. More collections of bulk milk during a longer period would have been preferable as it would result in broader overview of the initial milk microflora. Also, duplicates of each concentration during plate spreading would enable an average of colony forming units on each agar plate. Finally, colonies analysed by MALDI-TOF MS were only made on one storage temperature and spontaneously chosen from cultivation resulting in an uneven selection. Consequently, results from this pilot study cannot be generalized but they can give an indication of the course, where further research is needed.

2. Literature review

2.1 Microbiota in raw cow´s milk

Milk is a nutritious food product which contributes with a high amount of macro- and micronutrients in relation to its energy value measured in calories. The macronutrients in milk are proteins, mainly casein- and whey proteins (3.5%), carbohydrates in the form of lactose (4.8%), fat mainly as triglycerides (3.9%) and water (88%). The micronutrients consist of vitamins and minerals where calcium, phosphorus and various vitamins e.g. vitamins A and B, among others are of importance from a nutritional point of view (Miller et al. 2000; Jay et al. 2005). High nutritional value in combination with high water activity and pH around 6.7 make milk a beneficial medium for microbial growth (Adams 2016).

Bacteria which are present in raw cow´s milk (RCM) can influence dairy products in various ways, resulting in both wanted and unwanted outcomes. Unwanted outcomes caused by bacteria include spoilage of the product or pathogenic effects for humans. Bacteria therefore serve as hygiene and quality indicators for dairy plants, industries and food quality inspectors. Lactic acid bacteria originally isolated from milk are often part of commercial starter cultures used in the production of fermented dairy products. Certain strains with probiotic features could also generate additional benefical outcomes.

The composition of the bacteria in RCM is dependent on various factors such as the surrounding environment, health condition of the cow and hygiene of the milking equipment (Nero & Carvalho 2019). It was also shown that the feed could affect the composition of bacteria in RCM (Zhang et al. 2015).

In this litterature rewiev the bacteria will be divided in two groups; (1) beneficial bacteria and (2) spoilage and pathogenic bacteria of cow´s milk. The most common bacteria in raw milk are summarized in Tables 1 and 2. As seen in the tables, some genera are present in both table 1 and 2. This is due to different species within one genus could contribute to different effects to raw milk or milk products.

Table 1. Features of common spoilage and pathogenic bacteria in raw cow´s milk

Genus	Growth temperature (optimum) °C	Temperature group	Spoilage/ pathogenic	Origin	G^+/G^-	Aerobe/ anaerobe	Spore-former
Acinetobacter	<5-35 (25-30)[1]	Psychotropic[1]	Spoilage[1]	Soil, water[2]	-[2]	Aerobe[2]	No[1]
Bacillus	-5-35 (25-30)[1]	Psychotropic[5]	Spoilage/ Pathogenic[4]	Soil, water[1]	+[1]	Aerobe [4]	Yes[1]
Campylobacter	5-47 (30-40)[1]	Mesophilic[1]	Pathogenic[1]	Alimentary tract[1]	-[1]	Aerobe[2]	No[1]
Clostridium	-5-35 (25-30)[1]	Psychotropic[5]	Spoilage[5]	Soil, silage, water, dung[1]	+[1]	Anaerobe[1]	Yes[1]
Enterococcus	40-90 (55-75)[1]	Thermophilic/ Mesophilic[1,5]	Pathogenic/spoilage[3,5]	Endogenous[6], intestinal tract, soil, water[3]	+[3]	Facultative anaerobe[5]	No[3]
Escherichia	5-47 (30-40)[1]	Mesophilic[1]	Pathogenic/spoilage[1]	Intestinal tract[1]	-[1]	Facultative anaerobe[1]	No[1]
Lactobacillus	5-47 (30-40)[1]	Mesophilic[3]	Spoilage[3]	Endogenous[6], milking utensils[4]	+[2]	Anaerobe[2]	No[3]
Lactococcus	5-47 (30-40)[1]	Mesophilic[3]	Spoilage[4]	Endogenous[7], milking utensils[4]	+[2]	Facultative anaerobe[3]	No[4]
Listeria	0-42 (30-35)[1]	Psychotropic[1]	Pathogenic[1]	Water, soil, silage[1]	+[1]	Facultative anaerobe[1]	No[1]
Pseudomonas	-5-35 (25-30)[1]	Psychotropic[4]	Spoilage[4]	Milking utensils, cold storage of milk[4]	-[1]	Aerobe[4]	No[4]
Salmonella	5-47 (30-40)[1]	Mesophilic[1]	Pathogenic[1]	Intestinal tract, dung[1]	-[1]	Facultative anaerobe[1]	No[1]
Staphylococcus	5-47 (30-40)[1]	Mesophilic[1]	Pathogenic/spoilage[1]	Skin, teat canal, interior udder[4]	+[1]	Aerobe[4]	No[4]
Streptococcus	40-90 (55-75)[1]	Thermophilic[4]	Spoilage[4]	Endogenous[6], interior udder, milking parlour[4]	+[3]	Facultative anaerobe[3]	No[4]
Yersinia	-5-35 (25-30)[1]	Psychotropic[1]	Pathogenic[1]	Soil, water, intestinal tract[1]	-[1]	Facultative anaerobe[1]	No[1]

Table 2. Features of common beneficial bacteria in raw cow´s milk

Genus	Growth temperature (optimum) °C	Temperature group	Fermentation	Origin	G^+/G^-	Aerobe/ anaerobe	Spore-former
Lactococcus	5-47 (30-40)[1]	Mesophilic[3]	Homo[1]	Endogenous[7], dairy environment[4]	+[2]	Facultative anaerobe[3]	No[4]
Lactobacillus	5-47 (30-40)[1]	Mesophilic[3]	Homo/ Hetero[1]	Endogenous[6], Milking utensils[4]	+[2]	Anaerobe[2]	No[3]
Streptococcus	40-90 (55-75)[1]	Thermophilic[4]	Homo[1]	Endogenous[6], Interior udder[4]	+[3]	Facultative anaerobe[3]	No[4]
Leuconostoc	5-47 (30-40)[1]	Mesophilic[3]	Hetero[1]	Endogenous[7], plant material[3]	+[3]	Facultative anaerobe[3]	No[4]
Pediococcus	5-47 (30-40)[1]	Mesophilic[3]	Homo[1]	Intestinal tract[3]	+[3]	Facultative aerobe[3]	No[4]
Enterococcus	40-90 (55-75)[1]	Thermophilic[1]	Homo[3]	Endogenous[6], Intestinal tract[3]	+[3]	Facultative aerobe[5]	No[3]

[1] Adams 2016
[2] Jay et al. 2005
[3] Holzapfel & Wood 2014
[4] Walstra et al. 2005
[5] Nero & Carvalho 2019
[6] Albesharat et al. 2011
[7] Cabrera-Rubio et al. 2012

The knowledge of milk microbiota is of great importance at both farm and industry level. To reduce the risk of food related outbreaks or disease within the herd, continual microbial control measurements are required. By controlling and reducing the microbial activity of unwanted microorganisms the above-mentioned events can be prevented and economical losses within the dairy industry can be minimised. To make analyses of large numbers of milk samples practically possible, storage of samples usually occurs in freezing temperatures (Biddle et al. 2004; Hussein & Sakuma 2005; Hubáčková & Ryšánek 2007). The present study aimed to investigate how milk microbiota changes over time during storage of RCM at freezing temperatures.

2.1.1 Pathogens and spoilage bacteria in milk

Psychotropic bacteria

Psychotropic bacteria can grow and multiply during storage at cooling temperatures, 4-10°C. They are common in nature and can easily contaminate milk throughout the dairy value chain. Psychotropic bacteria with pathogenic properties common in milk include species of *Listeria* and *Bacillus* while psychotropic spoilage bacteria include species of *Pseudomonas, Clostridium and Acinetobacter*. Common feature of most psychotropic bacteria is their sensitivity to heat treatment, which makes pasteurisation an important tool to inhibit growth. However, *Bacillus and Clostridium* are spore formers and can thereby survive heat treatments (Nero & Carvalho 2019).

The abovementioned pathogenic genera, *Bacillus* and *Listeria,* include species which can be associated to human illness. *Bacillus cereus* (*B. cereus)* is a spore-forming bacterium and can thereby survive pasteurization. Contaminated milk will give rise to bad flavour and sweet curdling leading to milk deterioration and higher food waste. However, large numbers are needed for food poisoning which is usually not occurring in milk (Walstra et al. 2005). The genus of *Listeria* includes six known species whereas *Listeria monocytogenes* (*L. monocytogenes)* is the most important due to its association to human illness. Listeria outbreaks have mainly been connected to raw milk and soft cheeses (Adams 2016). *L. monocytogenes* originates from the environment nature and could cause sickness within the herd. Since the bacteria is heat sensitive, it should not cause any hazard when proper pasteurization is applied (Walstra et al. 2005).

Some genera among the psychotropic spoilage bacteria, such as *Pseudomonas,* can produce heat-resistant lipolytic and proteolytic enzymes. The lipolytic enzymes produced by *Pseudomonas* hydrolyse triglycerides present in milk, leading to a bitter taste, while the proteolytic enzymes produced by *Pseudomonas* act mainly on k-casein, leading to an unwanted consistency such as gel formation. Since the

enzymes are heat-resistant they can contribute to lowering of milk quality after heat treatment (Scatamburlo et al. 2015, Capodifoglio et al. 2016). Studies have shown higher activity of *Pseudomonas*-produced lipases during dry seasons and higher activity of *Pseudomonas*-produced proteases during rainy seasons. Consequently, the type of spoilage in milk by *Pseudomonas* could be seasonal and vary between these (Capodifoglio et al. 2016).

Clostridium is an important bacterium among the spore-forming genera, mainly the species *Clostridium botulinum* (*C. botulinum*), *Clostridium perfringens* (*C. perfringens*) and *Clostridium tyrobutyricum* (*C. tyrobutyricum*). The first two mentioned are considered as pathogenic. The genus *Clostridium* includes species of psychotropic, mesophilic and thermophilic bacteria (Jay et al. 2005). This is one example of the complexity of bacterial categorization as mentioned in the introduction. In this study, *Clostridium* species are referred as psychotropic (table 1). *C. tyrobutyricum* grow under anaerobic conditions and the spores survive pasteurization. During cheese maturation, the spores develop into bacteria which ferments lactic acid into butyric acid, carbon dioxide and hydrogen gas. After a long period of time, *C. tyrobutyricum* cause an unpleasant flavour, taste and texture associated to a phenomena called "late blowing" (Walstra et al. 2005). The third spoilage genera within psychotropic bacteria is *Acinetobacter*. Species within *Acinetobacter* can according to Adams (2016) spoil milk during storage at refrigerated temperatures but is also commonly spoiling the milk after pasteurization. The contamination causes off-flavours, off-odours and texture defects.

Mesophilic bacteria

Common characteristics of mesophilic bacteria is their ability to grow at temperatures ranging from 5-47°C with optimal growth at 37°C (Adams 2016). Mesophilic spoilage bacteria are often used as hygiene indicators as they origin from the surrounding environment such as the animal itself, equipment, water and air. The pathogenic bacteria with mesophilic features include species from the genera *Staphylococcus, Escherichia, Mycobacterium, Campylobacter* and *Salmonella* while the mesophilic spoilage bacteria common in milk are species from *Lactobacillus, Lactococcus* and some *Enterococcus* (Nero & Carvalho 2019). The spoilage mesophilic bacteria are also important during manufacturing, which will be described further (section 2.1.2).

Mesophilic bacteria prefer growth at room temperatures and their growth is therefore inhibited at refrigerated temperatures, below 4°C. Inhibited growth does not always mean death, instead refrigerated temperatures may induce a phenomenon called cold shock (Adams 2016). This was shown in a study published in 2019 where mesophilic bacteria were present in RCM stored at cold temperatures, the results indicating their survival during storage in refrigerator

(Hahne et al. 2019). The phenomena of cold shock will be further presented in section 2.2.1 below.

The abovementioned mesophilic spoilage bacteria produce acid, hence destroying the milk. However, they are also considered to have beneficial properties and will be presented further in section 2.1.2 below (Adams 2016; Nero & Carvalho 2019).

Some species of the pathogenic mesophilic genera are toxin producing, including *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). Even though these species are destroyed by heat treatment, their toxins may remain in the milk and cause food hazards for humans. In milk also two species of *Mycobacterium* occur, associated to hazards for human: *Mycobacterium tuberculosis* and *Mycobacterium bovis.* These, along with species from the genera *Campylobacter* and *Salmonella* do not produce any toxin but are pathogenic for both animals and human. During inadequate heat treatments, the bacteria survive and consequently cause illness (Adams 2016).

Thermophilic bacteria

Thermophilic bacteria grow at temperatures ranging from 40-90°C with an optimum at 55-75°C (Adams 2016). Some studies categorize bacteria which grow at 90°C as hyperthermophiles. This group has 80-100°C as an optimal growth temperature. However, hyperthermophiles are unable to grow at 60°C and hence, they differ from thermophilic bacteria (Stetter 1996). Unlike mesophilic bacteria, thermophilic bacteria can survive pasteurization even though they are not spore-formers and include the genera *Streptococcus* and *Enterococcus* (Mendonca et al. 2020). *Enterococcus* and *Streptococcus* include acid producing species and they can therefore have spoilage potential in milk. Some species are also toxin producers and thereby considered as pathogenic. The pathogenic species of *Streptococcus* and *Enterococcus* could lead to human illness, and species of *Streptococcus* could additionally cause bovine mastitis (Holzapfel & Wood 2014). Some species within these genera are however also common as beneficial bacteria described in section 2.1.2.

According to Watterson et al., (2014) and Martinez et al., (2017) thermophilic bacteria have been commonly found in highly processed dairy products such as condensed milk and milk powder. This indicates the importance of understanding and controlling the contamination risk of thermophilic bacteria on dairy industry level.

2.1.2 Beneficial bacteria in milk

Lactic acid bacteria

Lactic acid bacteria (LAB) are considered to be beneficial bacteria in milk due to their ability to ferment lactose which makes them important from a technological point of view in the dairy industry. Once LAB have been isolated, identified and characterized it can be added to milk as a starter culture. Further, the starter culture generates desirable properties such as flavour, appearance and texture of the milk hence important for fermented dairy products. However, the biological role of LAB in milk is not as well defined as its technological role during processing (Nero & Carvalho 2019).

Most of the literature refers to RCM as a sterile medium. However, the theory of milk being a sterile has been challenged. Studies using DNA-based methods have shown that how human breast milk contains bacteria which did not enter the milk through contaminations. Studies also showed changes in milk microbiota composition throughout different lactation stages. The natural microbiota which have been found in milk include among others *Leuconostoc, Lactococcus,* and *Streptococcus* (Albesharat et al. 2011; Cabrera-Rubio et al. 2012).

Common features for LAB are that they are gram-positive, non-spore-forming and aerotolerant anaerobes. LAB get their energy from fermentation of lactose to lactate. The production of lactate can be derived from two different pathways, the homofermentative pathway and heterofermentative pathway (table 2) (Adams 2016). Homofermentative LAB produce basically only lactic acid as an end product. The energy is provided from glucose, and through a glycolytic pathway called Emden-Meyerhof-Parnas (EMP) the bacteria produce lactic acid. Genera that go under the classification of homofermentative bacteria include *Lactococcus, Streptococcus, Pediococcus* and some species from *Lactobacillus.* They are also known as the most acid producing bacteria which makes them suitable for food fermentation (Adams 2016; Nero & Carvalho 2019).

Heterofermentative LAB include the genus *Leuconostoc* and some species from *Lactobacillus*. Due to the lack of the enzyme aldolase, which is present in homofermentative LAB, the heterofermentative LAB are dependent on another pathway called 6P-Gluconate and phosphoketolase pathway. Consequently, ethanol/acetate, CO_2 and lactic acid is produced as end products (Adams 2016).

Apart from the primary property of LAB (fermentation), some of the bacteria can additionally produce bacteriocins. Bacteriocins are peptides produced in the ribosome. These peptides have antimicrobial effect against both spoilage and pathogenic bacteria. Studies have shown that bacteriocins due to their microbial effects could be used as preservatives instead of chemicals added to foods (Kruger et al. 2013). Examples of bacteriocins are niacin, lacticin and leuconocin. These are

produced by *Lactobacillus* spp., *Lactococcus* spp., *Enterococcus* spp. among others (Nero & Carvalho 2019).

2.2 Sources of contamination

Since milk is a good medium for microbial growth it the demand on hygiene procedures is high in every handling step, starting at the farm, through transportation, processing and finally in the home of the consumers. Through history, contamination of milk or milk products have caused food related outbreaks. In 1982 there was one of the most extensive outbreaks caused by milk contaminated by *Yersinia enterocolitica* (*Y. enterocolitica*). Even though *Y. enterocolitica* is not common in milk, the contamination occurred while jugs with waste milk were transported between a pig's farm and dairy plant. Another outbreak connected to RCM was caused by *E. coli* in undercooked milk. The cattle seemed healthy, yet *E. coli* was found and isolated from 0.9-8.2% of the cows in UK, seemingly a good reservoir for the bacteria. Even processed milk products have been associated to outbreaks. In the last 40 years there has been 17 outbreaks related to dried milk powder where *S. aureus* and *Salmonella* have played a major role in the contamination (Adams 2016).

The outbreaks presented above are examples of recontaminations through bad handling during heat treatment (*E. coli*), and poor hygiene associated to equipment (*Y. enterocolitica*) and processing (*S. aureus* and *Salmonella*). Another source of contamination could be the cow herself during sickness or poor udder health, i.e., in case of mastitis. Bacteria typically involved in mastitis are *S. aureus, E. coli* and *Streptococcus agalactiae* (*S. agalactiae*). Clinical mastitis could be indicated by visible changes in the milk but also by measuring total bacteria counts (CFU/ml) which tend to rise extensively during mastitis, up to 10^8 CFU/ml (Adams 2016). Another mastitis indicator is elevated somatic cell count (SCC) which is the current standard method for detection of subclinical mastitis in the herd (Nero & Carvalho 2019). However, subclinical mastitis could only be detected by previously mentioned analyses and can consequently be present in the herd without farmer´s knowledge. Contamination could also occur by bacteria originating from the surrounding environment especially wet bedding material and soil, further transferred to the milking equipment and finally to the RCM. However, during grazing periods there is a lower risk of infection as well as during dry periods (Adams 2016).

Because of the reasons mentioned above, good hygiene in connection with milking and during milk handling procedures, and keeping the cow´s healthy is essential for good quality of RCM.

2.2.1 How to prevent contamination and growth of bacteria in the milk

Good hygiene handling

An important application to decrease the contamination risk of RCM is to keep good hygiene practises during milk handling on farm and dairy plant. Storage tanks, teat cups, milk holders and other wet equipment constitute a good growth medium which increase the contamination risk in RCM (Adams 2016). The temperature used during cleaning along with bad sanitizing and disinfection could select for different bacteria. When high temperature is used during cleaning, *Streptococcus* and spore-formers will be responsible for the main microbial growth on the equipment. While using low temperature, *Lactococcus* and *Pseudomonas* will be the main bacteria growing (Walstra et al. 2005). Even though bad hygiene during handling of the milk could contribute to high numbers of bacteria, the majority will not survive pasteurization (Adams 2016). However, heat-resistant enzymes of microbial origin can survive pasteurization hence maintaining the risk of spoilage of milk and dairy products (Walstra et al. 2005) as described in section 2.1.1.

In a study from 2004, milk was collected from the farm and microbial analyses were performed. The results showed that the milk contained pathogenic organisms such as *E. coli* and *S. aureus*. The authors concluded that bad hygiene practices could be the reason for food-related public health diseases, specially while consuming non-pasteurized milk or dairy products (Ekici et al. 2004).

Cold storage

Immediately after milking, the RCM is chilled to a temperature below 4°C. The temperature is held in the bulk tank, in the milk truck and finally at the dairy where it will be stored until processing. Since psychotropic bacteria have the capacity of growing at refrigerated temperatures, a selection occurs during cold storage as the growth of mesophilic bacteria will be inhibited. Mesophilic bacteria undergo a phenomenon called “cold shock” which destroys the membrane whereby cytoplasm will leach out. Since cold shock phenomena destroy the membrane, gram-negative bacteria will be affected to a greater extent than gram-positive bacteria since gram-positive bacteria have a thicker membrane (Adams 2016).

It has been shown that refrigeration may not be the optimal procedure to prevent microbial growth. In a study by Vithanage et al., (2016) the microbiota of RCM was analysed with focus on psychotropic bacteria considering storage conditions and seasonal variability. The samples were collected for one year and stored at different temperatures (2, 4, 6, 8 and 10°C) for 10 days, and further analysed using pure culture streak plate method, MALDI-TOF MS and 16S rRNA sequencing. The results showed that heat stable enzymes produced by psychotropic bacteria (proteases and lipases) were the main reason of spoilage and were dependent on

both storage temperature, season and microbial composition. The authors meant that it requires more thorough standard practices to keep track on the spoilage by psychotropic enzymes, than only storage temperature (Vithanage et al. 2016).

Regarding storage of RCM or dairy products at freezing temperatures, Alrabadi (2015) stated that freezing temperature is a good storage method, freezing temperatures delaying microbial growth, hence maintaining good quality of RCM or dairy product for a longer period (up to eight weeks) compared to cold storage. However, a study from 2007 showed varying effects of freezing temperatures on different types of bacteria in the milk, resulting in decreased counts in *E. coli* while the counts of *S. aureus* increased. According to the authors, keeping RCM and dairy products at freezing temperatures is therefore not a suitable storage method in cases where milk is collected for further microbial analyses. This, since freezing temperatures could result in misdiagnosis of the bacterial composition when culturing methods are used (Hubáčková & Ryšánek 2007).

Consequently, even though bacteria get injured during cold storage, there is no guarantees of elimination in the same way as for heat treatment presented below.

Pasteurization

Pasteurization of milk is a commercial application used by the dairy industry since 1890's with the main purpose to kill all pathogenic bacteria in the raw milk and prevent food related disease (Adams 2016). Today, pasteurization still eliminates pathogenic bacteria such as *Salmonella*, *E. coli*, *L. monocytogenes* and *Campylobacter jejuni* (*C. jejuni*). The treatment is also used for prolonged shelf life by eliminating spoilage bacteria, such as mesophilic LAB and psychotropic bacteria, and inactivation of lipolytic enzyme which destroy the milk fat (Walstra et al. 2005). It is therefore of great importance to avoid recontamination during post-pasteurization milk handling (Richardson & American Public Health Association 1985). Heat resistant *Streptococcus* and bacterial spores produced by *C. tyrobutyricum*, *C. sporogenes* and *B. cereus* could survive pasteurization but most of them do not grow rapidly (Jay et al. 2005; Walstra et al. 2005). In RCM from healthy cows, the microbial concentration is usually 10 000 CFU/ml, after pasteurization the bacterial count is generally 500-1000 CFU/ml. When spoilage bacteria grow to 5-10 x 10^6 CFU/ml in the RCM, the effects become observable, and the milk is not suitable for consumption (Walstra et al. 2005).

Described above are consequences when milk is low pasteurized, i.e., heated to 72°C for 15 seconds. Other properties of RCM such as flavour could be slightly changed, but nutritious value does not get affected by low pasteurization. Other heat treatments could also be applied, such as high pasteurization and ultra-high temperature (UHT) treatment. In high pasteurization treatment, milk is usually heated to 85°C for 15 seconds, which will kill all bacteria but not spores. In UHT treatment milk is heated to 130°C for 30 seconds or 145°C for 15 seconds. This will

eliminate all bacteria including spores which makes it possible for UHT treated milk to be stored at ambient temperatures in adequate packaging. However, high pasteurization and UHT treatment could develop Maillard reaction causing altered colour and off-flavours compared to RCM (Walstra et al. 2005).

2.3 Quantitative techniques for investigation of bacteria in milk

2.3.1 Cultural technique

Cultural techniques roughly refer to growth of microorganisms and allowing them to multiply in a specific agar gel. Agar consists of a polysaccharide produced by different species of red algae. The agar is mixed with various nutrients to promote growth or inhibition of wanted bacteria. Beneficial properties of agar which makes it convenient for microbial growth is its stability towards hydrolysis performed by microorganisms, even though it is composed by a polysaccharide. Another beneficial property is its ability to form a gel even at low concentrations which allow the agar to maintain good water activity (Adams 2016). Traditionally, cultural techniques have been used to identify microbes. Advantages with theses methods include simplicity and the possibility of recultivation for further analysis. However, disadvantages with cultural methods is the high labour intensity and the fact that some bacteria will not grow under the conditions applied during cultivation, leading to an underestimation of the microbial diversity in the sample (Dicksved 2008).

2.3.2 Pure culture streak plate method

Pure culture streak plate method allows the microorganism of request under certain incubation conditions grow and multiply and as a result form a colony. The colony can be detected by the naked eye, making it possible to count colonirs resulting in the unit “colony forming units per millilitre” (CFU/ml). This unit differs from the numbers of microorganisms per millilitre since there could be more than one cell forming a colony and thereby give a false result considering the number of bacteria (Lightfoot & Maier 1998). After incubation, plates with 30-300 colonies are considered as accurate for further analysis. Counts under 30 colonies is considered not trustworthy due to a wide variation (+/-) in how many CFU/ml which could be present in the sample. Plates with more than 300 colonies are difficult to manually count and could also lead to competition between microorganisms and thus limit some microorganisms from being detected (Adams 2016).

MRS agar

One frequently used agar for growth of *Lactobacillus* is De Man, Rogosa and Sharpe (MRS) agar. The name refers to the developers who aimed for producing an agar containing standard ingredients and still promote good growth of *Lactobacillus*. Using standard ingredients the authors meant that the agar could be used for commercial practices (De Man et al. 1960). According to today’s manufacturer of MRS agar, the agar is mainly selecting for *Lactobacillus*, but some growth of *Leuconostocs* and *Pediococcus* may also occur (Oxoid - Product Detail

n.d.). MRS agar is a selective medium meaning it contains ingredients which inhibit other microorganisms from growing. It will not affect the microorganisms wanted for isolation and identification (Adams 2016; Oxoid - Product Detail n.d.).

Plate count agar

Plate count agar (casein-peptone dextrose yeast agar) is a general agar used for growth of microorganisms in milk and dairy products. Possible bacteria which could grow include *E. coli, B. subtilis, L. lactis, L. monocytogenes, S. aureus, S. agalactiae,* and *L. acidophilus* and is according to the manufacturer recommended by the American Public Health Association (APHA) (Sigma Aldrich n.d.). Plate count agar is a non-selective agar meaning it does not include inhibitory agents and hence does not intentionally inhibit any microorganisms exposed to this agar. However, some selection still occur since there is a limited variation of nutrients, which means that nutrients required by some microorganisms for growth is not available (Adams 2016).

2.4 Other identification methods

2.4.1 MALDI-TOF MS

MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) is an identification method used for bacteria, viruses and fungi. MALDI-TOF MS is a relatively rapid, easy to use and cheap method which makes it useful in various commercial applications. The principle of MALDI-TOF MS is detection of mass and charge ratio of the ions and the time it takes for the ions to reach a detector (Giebel et al. 2010). The identified spectrum of an unknown organism is later compared to a reference library. Further, a logarithmic score value is calculated and interpreted (Lista et al. 2011).

In a study from 1996, MALDI-TOF MS was investigated as an identification method using bacteria cultivated by cultural techniques. The result showed that MALDI-TOF MS easily identified and distinguished many species. With the possibility of fast and accurate identification and differentiation between bacteria, the authors suggested this method to be suitable for commercial and regulatory use in food-related applications among others (Holland et al. 1996).

2.4.2 Sequencing

Sanger-sequencing is the first generation of sequencing technology named after its developer Edward Sanger and it was for a long time considered as the gold standard of sequencing. In recent years there have been demands on further development of the method which resulted in the second generation of sequencing technology

called Next Generation Sequencing (NGS). NGS is considered to be faster and more cost-effective than the first generation of sequencing techniques (Grada & Weinbrecht 2013).

Regardless of the generation there are some fundamental steps for implementing this technique. DNA sequencing initially requires extraction. isolation and purification of DNA from the organism of interest. Together with DNA-primers, DNA-polymerase and DNA-bases with and without fluorescent tags, the technique produces small sequences duplicated from the original DNA-sequence. Though separation by length and size, a laser is used to induce the fluorescent tagged DNA-bases which are eventually detected and assembled by a computer. As a result, the full DNA-sequence from the organism of interest is shown (Sigma Aldrich 2022).

Even though the technique has developed, is cheaper and faster and more used today, sequencing is still considered to be expensive, time consuming and requires special knowledge which limits its use in many commercial laboratories (Grada & Weinbrecht 2013).

3. Material and method

3.1 Literature review

The databases PUBMED, Google Scholar, ScienceDirect and *Web of science* were used to search for relevant information in books and previously published scientific studies on milk microbiota and its behaviour during cold storage. Additionally, scientific books available in the SLU library were used for further information. Following words was used to find sources; *raw cow´s milk, raw milk quality, milk microbiota, culturing methods, MALDI-TOF MS, cold storage of milk microbiota.*

3.2 Collection of milk samples

One litre of RCM was collected directly from the bulk tank at the Swedish Livestock Research Centre at Lövsta, Uppsala, during four different occasions with at least two days in between collections. The milk was transported to SLU, Ultuna, and prepared for immediate analysis. Initially, it was planned to collect the milk from Lövsta on three occasions. Since there were multiple errors during the analyses on the first collection, another sample had to be collected to re-do the analyses. Consequently, the results throughout this study are based on milk collected during three different occasions. These will further be referred as batch 1, batch 2 and batch 3.

3.3 Sample storage and preparation

Bacteria in the milk were cultivated on two different agars, De Man, Rogosa and Sharpe (MRS) and Total Plate Count agar (PCA). Cultivation was performed on the fresh bulk milk directly after collection. Sub-samples were then frozen, and stored at -20°C and -80°C followed by once-a-week cultivation during eight weeks. The volume of milk applied on the MRS plates ranged from 100 µl to 500 µl. The MRS agar plates were placed in an anaerobic jar along with Anaerocult® A and Anaerotest® (Merck KGaA, Darmstadt, Germany) and incubated in 37°C for 48h.

The samples for cultivation on PCA agar were diluted in peptone water to obtain a concentration of 1:10, 1:100 and 1:1000 before 100 µl was plated. The PCA agar plates were incubated at 37°C for 24h. In total it resulted in eight agar plates from each batch of RCM, five on MRS agar and three from PCA agar.

The remaining milk was divided in 28 falcon tubes, 10 ml each. Each falcon tube was marked with batch number and temperature and stored at two different freezing temperatures, -20°C and -80°C.

During the following 8 weeks, once a week one sample from each batch and temperature were thawed at a 30°C water bath to avoid a cream layer in the samples. The sample from each batch and temperature were plated on both agars in the concentrations and incubation conditions described above. All chemicals and equipment, if not indicated differently, were obtained from VWR Chemicals (VWR International, Leuven, Belgium).

3.4 Re-cultivation by streaking method

After incubation, the colonies were counted and noted in Microsoft® Excel version 16.59. For the samples which were used for MALDI-TOF MS analysis, colonies were isolated using a sterile plastic loop and spread on new agar, MRS or PCA. The agar plates were incubated aerobically (PCA) for 24 hours or anaerobically (MRS) for 48 hours at 37°C for re-cultivation. After incubation, the isolated colonies on PCA agar were stored at 4°C until further analysis. The isolated colonies on MRS agar were transferred to a glycerol stock. The stock was prepared using 99.5% glycerol and distilled water to obtain a concentration of 50% glycerol. The glycerol stock was autoclaved at 125°C for 15 minutes. The isolated colonies were transferred to a screw top micro tube containing 1 ml glycerol, vortexed, and stored in a freezer at -80°C. Before further analysis with MALDI-TOF MS, the colonies from the stock were incubated anaerobically on MRS agar as described above.

3.5 MALDI-TOF MS

Bacterial identification was performed using MALDI-TOF MS (MALDI Biotyper, Microflex, Bruker Daltonik GmbH). Colonies were analysed week 0 (reference), and after 1, 4 and 8 weeks storage at -20°C. Ten colonies from each batch and agar type were analysed, resulting in 60 colonies from each week (three batches and two agar types). Each colony was run in duplicates and in total 240 colonies were analysed and 480 runs were performed.

Analyses were performed using the Direct Transfer (DT) method. The isolated colonies were transferred with a toothpick to a spot on a Maldi target plate (MTP). For colonies of bacteria which were assumed to be gram-positive, 1µl of freshly

prepared 70% formic acid was added and left at room temperature (20-25°C) to evaporate. The formic acid was added to break the cell wall allowing proteins to be released and detected, and this step was not needed for bacteria that were assumed to be gram-negative as proteins were released without adding formic acid. Once evaporated, 1µl of matrix containing α-cyano-4-hydroxycinnamic acid (HCCA) mixed with 250µl 50% acetonitrile and 2.5% trifluoroacetic acid was added to each colony. For gram-negative bacteria, matrix was directly added on the colony before analysing. Once again, MTP was left at room temperature allowing the matrix to evaporate. Before identification, each colony was marked with a sample-ID in the software program used (MBT Compass version 4.1). The sample-ID was based on batch, week and agar type. Finally, MTP was inserted to the MALDI-TOF MS and identification of bacterial colonies was performed and compared to a reference library.

The comparison generated a logarithmic score value raging from 0-3. Logarithmic score values raging from 0.000-1.699 indicated "no organism identification possible" due to incomplete match between bacteria spectra and reference library. Logarithmic score value between 1.700-1.999 resulted in "low-confidence identification", indicating identification at genus-level but strain identification was not reliable. Finally, logarithmic score value between 2.000-3.000 resulted in "high-confidence identification". In this case, a reliable result with both genus and strain was obtained.

According to the manufacturer, the logarithmic score value should only be interpreted as a *probability* of identified microorganism. For a more confident identification, further analyses should be performed on the microorganism such as gram staining, colony morphology identification and growth characteristics.

3.6 Statistical analysis

Statistical analyses were performed using Minitab® Version 19.2020.2.0 as software (Minitab, LCC., United states). To evaluate the effects of different batches, temperatures and storage time, univariate analysis was performed using ANOVA with 95% confidence interval. Additionally, Tukey´s pairwise comparison test was used for evaluation of significant differences between time of storage and temperatures, respectively.

4. Results

4.1 Cultivation

Significant and non-significant parameters based on CFU/ml in samples cultivated aerobically on PCA and anaerobically on MRS agar are presented in table 3. The p-value indicates the effect of storage at the different temperatures (Temperature) and the effect of storage time (Time at -20°C and Time at -80°C) on CFU/ml for the respective agar type. Significant differences in number of CFU/ml between the temperatures were observed for anaerobic (p=0.000) cultivations. The numerical values for CFU/ml in milk samples stored at -80°C were higher on both PCA and MRS compared to CFU/ml in milk samples stored at -20°C, however the difference was significant only for the anaerobes cultured on MRS (table 4 and 5).

Storage time had a significant effect (p = 0.010) on CFU of aerobic bacteria cultivated on PCA and stored at -80°C, and for anaerobic bacteria (p = 0.000) cultivated on MRS agar and stored at -20°C (table 3).

The results are based on bulk milk collected on three different occasions to include natural variation in microflora of bulk tank milk between days. Data for the individual bulk milk samples are available in appendix 1.

Table 3. Effects of storage temperature (Temperature) and time (Time at -20°C and Time at -80°C, respectively) on number of colony forming units per millilitre (CFU/ml) for milk samples thawed and cultivated on Plate Count Agar (PCA) and De Man, Rogosa and Sharpe agar (MRS), respectively, on a weekly bases during eight weeks

Agar	Significant parameters	P-value	Not significant parameters	P-value
PCA	Time at -80°C	0.010	Time at -20°C	0.137
			Temperature	0.457
MRS	Time at -20°C	0.000	Time at -80°C	0.938
	Temperature	0.000		

In total, n=396, where n is the number of milk samples that were analysed. Of these, milk stored at -20°C (n=67) and at -80°C (n= 64), thawed and cultured on PCA, as well as milk stored at -20°C (n=133) and at -80°C (n= 132), thawed and cultured on MRS. P <0.05 was considered significant.

Table 4. Average number of colony forming units per millilitre milk (CFU/ml) after storage of milk samples at -20°C and -80°C, respectively, and cultivated on Plate count agar (PCA) where n = number of samples from all weeks

Agar	Temperature	n	CFU/ml
PCA	-20	67	15225 ± 20238^{a}
PCA	-80	64	18897 ± 3455^{a}

Values that do not share superscripts are significant different ($p < 0.05$). Standard deviation is indicated.

Table 5. Average number of colony forming units per millilitre milk (CFU/ml) after storage of milk samples at -20°C and -80°C, respectively and cultivated on De Man, Rogosa and Sharpe agar (MRS) where n = number of samples from all weeks

Agar	Temperature	n	CFU/ml
MRS	-20	132	82 ± 70^{a}
MRS	-80	134	179 ± 124^{b}

Values that do not share superscripts are significant different ($p < 0.05$). Standard deviation is indicated.

4.1.1 Total plate count agar

The means of CFU/ml in the milk samples cultivated on PCA agar after storage at -20°C and -80°C for 8 weeks in comparison with the reference sample from day 0 are shown in table 6.

For samples stored at -20°C, no significant differences were observed in relation to period of storage at -20°C. In samples stored at -80°C, the number of CFU/ml in milk stored for 1 week at -80°C was significantly higher compared to milk stored for 0, 2, 5, 7 and 8 weeks. No significant difference was observed for CFUs in milk stored for 3, 4 and 6 weeks.

Table 6. Average number of colony forming units per millilitre milk (CFU/ml) cultivated aerobically on Plate count agar (PCA) after storage of milk samples at -20°C and -80°C, respectively. The statistical significance of differences between numbers at day 0 and after storage for 1-8 weeks are indicated column wise

Week	n	CFU/ml -20°C	n	CFU/ml -80°C
0	9	9689 ± 4854^{a}	9	9689 ± 4854^{b}
1	8	30275 ± 14807^{a}	9	62822 ± 745^{a}
2	8	10988 ± 5610^{a}	7	7843 ± 7820^{b}
3	8	23913 ± 47082^{a}	6	23383 ± 35631^{ab}
4	8	24350 ± 21407^{a}	8	18713 ± 12383^{ab}
5	7	11543 ± 11057^{a}	6	7567 ± 6939^{b}
6	7	6414 ± 3452^{a}	6	12783 ± 787^{ab}
7	7	5014 ± 3478^{a}	7	6900 ± 2857^{b}
8	5	11180 ± 11569^{a}	6	6917 ± 707^{b}

Values that do not share superscripts are significant different ($p < 0.05$). Standard deviation is indicated.

4.1.2 De Man, Rogosa and Sharpe agar

The mean values of CFU/ml cultivated from the frozen milk samples on MRS agar after storage at -20°C and -80°C for 8 weeks compared with the reference sample from day 0 are shown in table 7.

For samples stored at -20°C, the number of CFU/ml from week 0 was significantly higher than the numbers for samples that had been stored 1-8 weeks. For samples stored at -80°C, no significant differences between the weeks in cold storage were observed.

Table 7. Average number of colony forming units per millilitre milk (CFU/ml) cultivated anaerobically on De Man, Rogosa and Sharpe (MRS) agar after storage of frozen milk samples at -20°C and -80°C, respectively. The statistical significance of differences between means at day 0 and after storage for 1-8 weeks are indicated column wise

Week	n	Mean CFU/ml -20°C	n	Mean CFU/ml -80°C
0	14	196 ± 102^{a}	14	196 ± 102^{a}
1	15	92 ± 47^{b}	15	168 ± 121^{a}
2	15	69 ± 45^{b}	15	152 ± 114^{a}
3	15	87 ± 73^{b}	14	201 ± 149^{a}
4	15	77 ± 51^{b}	15	196 ± 150^{a}
5	15	80 ± 54^{b}	15	203 ± 147^{a}
6	15	48 ± 40^{b}	15	161 ± 104^{a}
7	15	53 ± 36^{b}	15	163 ± 121^{a}
8	14	46 ± 35^{b}	14	168 ± 115^{a}

Values that do not share superscripts are significantly different ($p < 0.05$). Standard deviation is indicated

4.2 MALDI-TOF MS

4.2.1 Total plate count agar

Table 8 shows bacterial composition of collected milk samples from all batches after storage at -20°C for 1, 4 and 8 weeks in comparison with the reference sample (week 0) where n = number of colonies detected. Samples were cultivated and colonies re-cultivated on PCA agar and further identified using MALDI-TOF MS instrument. For week 0, 1 and 4, each week 30 colonies were characterized and for week 8 only 22, making a total of 112 observations. In total, 14 species were identified whereof the most abundant were *Staphylococcus aureus, Kluyvera intermedia*, *Acinetobacter pittii, Acinobacter lactucae* and *Stenotrophomonas maltophilia*. Values are based on numbers of observations per week and calculated in percentage. The background data is available in appendix 2.

Table 8. Identified bacteria in milk samples stored in -20°C for 0 (reference), 1, 4 and 8 weeks, cultivated aerobically on Plate count agar and identified by MALDI-TOF MS , In total n=112; n=30 within week 0, 1, 4 and n=22 for week 8 where n = number of colonies detected

Bacteria	**Week 0 (%)**	**Week 1 (%)**	**Week 4 (%)**	**Week 8 (%)**
Acinetobacter junii		3		
Acinetobacter lactucae	7	10	3	18
Acinetobacter pittii	10	13	23	9
Aeromonas veronii	7		3	
Buttiauxella gaviniae	10			
Bacillus pumilus				5
Chryseobacterium taichungenese	3	3	3	5
Kluyvera intermedia	13	20	27	
Pantoea agglomerans			3	
Pseudomonas fragi	3			
Staphylococcus aureus	30	47	33	46
Staphylococcus chromogenes		3		
Staphylococcus epidermidis	3			
Stenotrophomonas maltophilia	13		3	18

4.2.2 De Man, Rogosa and Sharpe agar

Table 9 shows bacterial composition of collected milk samples from all batches after storage at -20°C for 1, 4 and 8 weeks in comparison with the reference sample (week 0) where n = number of colonies detected. Samples were cultivated and colonies re-cultivated on MRS agar and further identified using MALDI-TOF MS instrument. For week 0, 1 and 4, each week 30 colonies were characterized and for week 8 29, making a total of 119 observations. In total, 9 species were detected whereof the most abundant were *Streptococcus lutetiensis*, *Streptococcus equinus*, *Weissella paramedenteroides*, *Streptococcus infantarius* and *Lactobacillus paracasei*. Values are based on numbers of observations per week and calculated in percentage. The background data is available in appendix 3.

Table 9. Identified bacteria in milk samples stored at -20°C for 0 (reference), 1, 4 and 8 weeks. Colonies were cultivated anaerobically on De Man, Rogosa and Sharpe agar and identified by MALDI-TOF MS. In total n=119; n= 30 within week 0, 1, 4 and n=29 for week 8 n = number of colonies detected

Bacteria	**Week 0 (%)**	**Week 1 (%)**	**Week 4 (%)**	**Week 8 (%)**
Lactobacillus dextrinicus				3
Lactobacillus mucosae		3		
Lactobacillus paracasei	33		3	
Pediococcus pentosaceus			3	7
Streptococcus equinus	23	23	27	3
Streptococcus gallolyticus		3		
Streptococcus infantarius		7		47
Streptococcus lutetiensis	37	40	43	17
Weissella paramesenteroides	7	23	23	20

5. Discussion

5.1 Total plate count agar

5.1.1 Cultivation

Based on present results, for cultivation of the aerobic bacteria on PCA agar, milk samples should preferably to be stored at -20°C since storage time had no significant effect on CFU/ml at this temperature (table 3). This is also shown in table 6 where mean CFU/ml share superscripts independent on duration of storage at -20°C. According to Hubáčková & Ryšánek (2007), similar results were observed in milk samples stored at -20°C and further cultivated on PCA agar. However, obtained results are in contrast to the study performed by Alrabadi (2015), where storage of RCM
at -20°C for eight weeks showed significant differences in CFU/ml and the counts were continuously decreasing with storage time. The differences between the Alrabadi (2015) and present study could depend on dissimilar incubation conditions during cultivation but are most likely depending on divergent handling of milk samples during analyses.

Significant effects of storage time on CFU/ml were observed for bacteria cultivated on PCA agar stored at -80°C (table 3). However, at this storage temperature there was fluctuation in the number of CFU/ml milk between the weeks. The largest deviation was noticed between week zero and week two. Here, the number of CFU/ml milk increased by 85% (table 6). This increase is most likely depending on an extended thawing time of the milk samples before culturing, which allows the bacteria to grow right before culturing. The thawing time was unfortunately not standardized in this study, and this is a bias which could permeate throughout the entire study.

The difference related to the effect of storage time between the two temperatures for bacteria cultured on PCA could depend on the taxonomy of the bacteria. Since bacteria cultivated on PCA agar are gram-negative, they may undergo the phenomena of cold-shock during storage at -80°C (Adams 2016). Possibly, similar outcome would occur for bacteria stored at -20°C during longer storage time.

However, there is a need to standardise the thawing procedure in order to exclude the deviations between the weeks during cold storage.

As shown in table 3, no significant differences in numbers of CFU/ ml were observed in milk samples between the two temperatures for the aerobically cultivated bacteria on PCA. This is also illustrated in table 4, the samples stored at the two temperatures share superscripts although the number of CFU/ml cultivated from milk samples stored at -80°C was numerically higher compared to -20°C. Obtained results therefore indicate that both temperatures are suitable for storage of aerobic bacteria before the performance of microbial analyses.

5.1.2 MALDI-TOF MS

Bacteria cultivated on PCA included aerobic, gram-negative and gram-positive bacteria. In total, 14 species were identified by MALDI-TOF MS whereof the most abundant were *Staphylococcus aureus, Kluyvera intermedia*, *Acinetobacter pittii, Acinobacter lactucae* and *Stenotrophomonas maltophilia* (table 6). Suggestions by the manufacturer regarding the bacterial selection of PCA agar (see section 2.3.2) does not entirely correspond to the results from MALDI-TOF MS presented in table 6, except for *S. aureus.* However, the suggestions by the manufacturer will not include all possible bacteria growing on the culture media. Since the agar is a non-selective medium, it allows growth of any bacterium with nutrient requirements similar of those provided by the agar. The suggestions by the manufacturer of possible bacterial growth can therefore not be used as a reference.

As seen in table 8, *S. aureus* was the dominating bacteria during all weeks. As previously mentioned, (section 2.2), *S. aureus* is one of the most common bacteria causing mastitis in Sweden. Possibly, this bacterium could have multiplied during thawing before culturing, thus explaining the increase in CFU/ml between week 0 and week 1 for samples stored at -80°C. It could also be the reason for the high number of CFU/ml in general, compared to the expected 10 000 CFU/ml of healthy cows (table 4) (Walstra et al. 2005). Furthermore, other studies have also reported an increase in *S. aureus* during storage at freezing temperatures (Hubáčková & Ryšánek 2007; Alrabadi 2015). Considering that *S. aureus* is a gram-positive bacteria , the dominance of this bacteria might become more apparent during the storage at -80°C, as other gram-negative bacteria may more easily undergo cold shock (Adams 2016). Even though results from MALDI-TOF MS were performed on samples stored at -20°C, a similar microbial composition can be assumed in samples stored at -80°C considering that the RCM was collected from the same bulk on the same occasion. However, since the study lacks results from MALDI-TOF MS on milk stored at -80°C, this assumption cannot be established.

The other most abundant genus detected by MALDI-TOF MS were, *Stenotrophomonas, Kluyvera* and *Acinetobacter spp.*. All these species are commonly found in soil, water and milk (Jay et al. 2005; Cooney et al. 2014; Adams

2016). *Acinetobacter spp.* are as previously mentioned psychotropic bacteria and are capable to spoil the bulk milk during storage at cooling temperatures but also after pasteurization, consequently causing recontamination of the milk (Adams 2016). Species from *Kluyvera* and *Stenotrophomonas* could cause human diseases such as urinary tract infections and infections of respiratory tract, respectively (Cooney et al. 2014; Hauben et al. n.d.) However, to our knowledge, there are no scientific studies that have investigated their impact on raw milk nor their survival in cold storage of the milk.

5.2 De Man, Rogosa and Sharpe agar

5.2.1 Cultivation

As shown in table 3, there was a significant effect of storage temperature on CFU/ml milk, as well as of duration of storage of milk samples at -20°C. This was also shown in Alrabadi (2015), where the number of bacteria cultivated on MRS agar was significantly lower after storage at -20°C for eight weeks. Furthermore, there was a decrease in the number of CFU/ml milk by 76% after 8 weeks storage in comparison with the reference (table 6) Here, in contrast to cultivation on PCA plates, the number of CFU/ml did not show any larger fluctuations between weeks, which is in agreement with the results by Alrabadi (2015). Since MRS is a more selective agar compared to PCR, it yields in a narrower diversity in bacterial species growing on the plate. This may explain the larger fluctuation on PCA agar. It can also depend on the taxonomy of the bacteria as gram-positive bacteria are more resistant to cold shock (Adams 2016). These assumptions can however not be established since a more thoroughly explanation is not available.

No-significant effects of time on CFU/ml were observed for bacteria cultivated on MRS agar from -80°C (table 3). This was also shown in table 7 where mean CFU/ml share superscripts during all weeks. Furthermore, significant differences were observed in milk samples between the two temperatures (table 3). This was further observed in table 5 as the samples do not share superscripts and the number of CFU/ml cultivated from milk samples stored at -80°C was higher compared to -20°C. It could therefore be assumed that storage at -80°C is more preferable for anaerobic bacteria. Unfortunately, both for MRS and PCA agar, no studies have been found on cultivation and storage of milk samples at -80°C. This fact makes it difficult to discuss if the obtained results from the present study are in line or in contrast to others. However, according to obtained results, it might be more preferrable to store RCM at -80°C as it does not alter the milk microbiota in a significant way.

5.2.2 MALDI-TOF MS

Bacteria cultivated on MRS agar include anaerobic, gram-positive microorganisms. In total, 9 species were identified by MALDI-TOF MS, whereof the most abundant were *Streptococcus lutetiensis* (*S. lutetiensis*), *Streptococcus equinus* (*S. equinus*), *Wiessella paramesenteroides* (*W. paramesenteroides*), *Streptococcus infantarius* (*S. infantarius)* and *Lactobacillus paracasei* (*Lb. paracasei*) (table 7). The results obtained by MALDI-TOF MS analysis was in agreement with bacteria suggested by the manufacturer (see section 2.3.2) to a larger extent than for bacteria identified from the PCA agar. However, the results for bacteria isolated on MRS agar were limited to genus level, whereas on PCA agar the suggested bacteria were limited to species level. Once again, the results from MALDI-TOF MS analysis did not entirely reflect the expected genus. Nevertheless, the identified bacteria were all sharing characteristics of the expected genus and have all been reported to be present in raw milk (Holzapfel & Wood 2014).

As shown in table 7, the majority of the bacteria identified after 8 weeks of storage consisted of bacteria from the genus *Streptococcus*. As previously mentioned (section 2.1.1), bacteria included in the genera of *Streptococcus* could cause bovine mastitis (Holzapfel & Wood 2014). According to Chen et al. (2021), *Streptococcus lutetiensis* is a common mastitis pathogen. The remaining species in the genera of *Streptococcus* detected during MALDI-TOF MS analysis were *Streptococcus equinus*, *Streptococcus gallolyticus* (*S. gallolyticus)* and *Streptococcus infantarius* are all included in the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC). SBSEC have been isolated from both human and animal infections (Schlegel et al. 2003) whereas *S. gallolycticus* has been shown to be involved in mastitis (Herrera et al. 2009). The occurrence of mastitis in the herd producing the RCM used in this study is thereby likely, also considering the occurrence of *S. aureus* discussed in section 5.1.2.

W. paramesenteroides and *Lb. paracasei* were among the five most abundant bacteria detected by MALDI-TOF MS. The genus *Weissella* has been isolated from fish, meat, vegetable and soil. The species *W. paramesenteroides*, has been isolated from soil and has an important role during fermentation of vegetables. *Lb. paracasei* has been isolated from dairy products and may also contribute with health-promoting effects as probiotic bacteria (Holzapfel & Wood 2014).

5.3 Further research

Further research of changes in the milk microflora during storage at freezing temperatures should include more batches of bulk tank milk, collected during a longer period. Also, cultivation on additional agar types, longer storage times and additional methods for analysis, such as sequencing of DNA. This would enable a broader overview of initial milk microbiota but also more consequent and reliable results. More research is especially needed on milk samples stored at different freezing temperatures to obtain a comparation of changes in milk microbiota between these.

6. Conclusion

The purpose of this pilot study was to monitor the surviving ratio of different bacteria in milk after eight weeks of storage at -20°C and -80°C, respectively. The study was performed by collecting milk from the Swedish Livestock Research Centre at Lövsta, followed by storage of the milk samples at the two temperatures. Once a week, the number of colony forming units per millilitre of milk was measured using culturing methods. Two different agars were used to include a wide range of bacterial species. Further, the identity of selected colonies growing on the different agars was determined using MALDI-TOF MS analysis.

For aerobe bacteria cultivated on PCA agar, the preferrable storage temperature was -20°C as no significant differences were observed during eight weeks of storage. For samples stored at -80°C, significant differences were observed over time indicating a less suitable temperature for bacteria cultivated on PCA agar. However, no significant differences in bacteria growth were observed between -20°C and -80°C, which could indicate that both storage temperatures are suitable for aerobes. As for the anaerobe bacteria cultivated on MRS agar, it seemed to be more suitable to store milk samples at -80°C as no significant differences were observed during eight weeks of storage. For milk samples stored at -20°C, a significant decrease in CFU was observed over time making this temperature not preferrable for cultivated on MRS agar. Also, a significant difference was observed between -20°C and -80°C which strengthens the assumption that -80°C is more suitable for anaerobes. Finally, the number of CFU/ml cultivated from milk samples stored at -80°C was higher for both types of bacteria. The fluctuation in CFU per ml could depend on non-consequent milk sample handling during thawing. The bacteria numbers could therefore be affected by the variation in time before they were plated which further generates inconclusive results. The results from MALDI-TOF MS indicated presence of bacteria causing bovine mastitis. This could contribute to the high numbers of CFU from culture analyses. However, the MALDI-TOF MS analysis was not performed on samples stored at -80°C which hinders the general connection between microbial quantity and quality throughout this study.

In conclusion, to reduce the risk of alteration in the ratio of different bacteria in the raw milk during storage before microbial analyses, milk samples should be stored at -80°C.

During this pilot study, bulk milk was collected on three occasions. More collections of bulk milk during a longer period and a wider variation in agar medium would be preferable as it would result in broader overview of the initial milk microflora. Also, a more advanced sample analysis and more consequent sample handling would be needed to obtain more conclusive results. Consequently, results from this pilot study cannot be generalized but conclude that further research is needed regarding comparisons between different freezing temperatures and effects on milk microbiota.

References

Adams, M.R. (2016). *Food microbiology*. 4th edition. Cambridge: Royal Society of Chemistry.

Albesharat, R., Ehrmann, M.A., Korakli, M., Yazaji, S. & Vogel, R.F. (2011). Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. *Systematic and Applied Microbiology*, 34 (2), 148–155. https://doi.org/10.1016/j.syapm.2010.12.001

Alrabadi, N.I. (2015). The Effect of Freezing on Different Bacterial Counts in Raw Milk. *International Journal of Biology*, 7 (4), p9. https://doi.org/10.5539/ijb.v7n4p9

Biddle, M.K., Fox, L.K., Hancock, D.D., Gaskins, C.T. & Evans, M.A. (2004). Effects of Storage Time and Thawing Methods on the Recovery of Mycoplasma Species in Milk Samples from Cows with Intramammary Infections. *Journal of Dairy Science*, 87 (4), 933–936. https://doi.org/10.3168/jds.S0022-0302(04)73237-3

Cabrera-Rubio, R., Collado, M.C., Laitinen, K., Salminen, S., Isolauri, E. & Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *The American Journal of Clinical Nutrition*, 96 (3), 544–551. https://doi.org/10.3945/ajcn.112.037382

Capodifoglio, E., Vidal, A.M.C., Lima, J.A.S., Bortoletto, F., D'Abreu, L.F., Gonçalves, A.C.S., Vaz, A.C.N., Balieiro, J.C. de C. & Netto, A.S. (2016). Lipolytic and proteolytic activity of Pseudomonas spp. isolated during milking and storage of refrigerated raw milk. *Journal of Dairy Science*, 99 (7), 5214–5223. https://doi.org/10.3168/jds.2015-10453

Chen, P., Qiu, Y., Liu, G., Li, X., Cheng, J., Liu, K., Qu, W., Zhu, C., Kastelic, J.P., Han, B. & Gao, J. (2021). Characterization of Streptococcus lutetiensis isolated from clinical mastitis of dairy cows. *Journal of Dairy Science*, 104 (1), 702–714. https://doi.org/10.3168/jds.2020-18347

Cooney, S., O'Brien, S., Iversen, C. & Fanning, S. (2014). Bacteria: Other Pathogenic Enterobacteriaceae – Enterobacter and Other Genera. I: Motarjemi, Y. (red.) *Encyclopedia of Food Safety*. Waltham: Academic Press, 433–441. https://doi.org/10.1016/B978-0-12-378612-8.00104-9

De MAN, J.C., Rogosa, M. & Sharpe, M.E. (1960). A Medium for the Cultivation of Lactobacilli. *Journal of Applied Bacteriology*, 23 (1), 130–135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

Dicksved, J. (2008). Exploring the Human Intestinal Microbiome in Health and Disease. 75

Ekici, K., Bozkurt, H. & Isleyici, O. (2004). Isolation of Some Pathogens from Raw Milk of Different Milch Animals

Giebel, R., Worden, C., Rust, S.M., Kleinheinz, G.T., Robbins, M. & Sandrin, T.R. (2010). Chapter 6 - Microbial Fingerprinting using Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS): Applications and Challenges. *Advances in Applied Microbiology*.

Academic Press, 149–184. https://doi.org/10.1016/S0065-2164(10)71006-6
Grada, A. & Weinbrecht, K. (2013). Next-Generation Sequencing: Methodology and Application. *Journal of Investigative Dermatology*, 133 (8), 1–4. https://doi.org/10.1038/jid.2013.248
Hahne, J., Isele, D., Berning, J. & Lipski, A. (2019). The contribution of fast growing, psychrotrophic microorganisms on biodiversity of refrigerated raw cow's milk with high bacterial counts and their food spoilage potential. *Food Microbiology*, 79, 11–19. https://doi.org/10.1016/j.fm.2018.10.019
Hauben, L., Vauterin, L., Moore, E.R.B., Hoste, B. & Swings, J.Y. 1999 (u.å.). Genomic diversity of the genus Stenotrophomonas. *International Journal of Systematic and Evolutionary Microbiology*, 49 (4), 1749–1760. https://doi.org/10.1099/00207713-49-4-1749
Herrera, P., Kwon, Y.M. & Ricke, S.C. (2009). Ecology and pathogenicity of gastrointestinal Streptococcus bovis. *Anaerobe*, 15 (1), 44–54. https://doi.org/10.1016/j.anaerobe.2008.11.003
Holland, R.D., Wilkes, J.G., Rafii, F., Sutherland, J.B., Persons, C.C., Voorhees, K.J. & Lay Jr., J.O. (1996). Rapid Identification of Intact Whole Bacteria Based on Spectral Patterns using Matrix-assisted Laser Desorption/Ionization with Time-of-flight Mass Spectrometry. *Rapid Communications in Mass Spectrometry*, 10 (10), 1227–1232. https://doi.org/10.1002/(SICI)1097-0231(19960731)10:10<1227::AID-RCM659>3.0.CO;2-6
Holzapfel, W.H. & Wood, B.J.B. (2014). *Lactic Acid Bacteria: Biodiversity and Taxonomy*. Hoboken, UNITED KINGDOM: John Wiley & Sons, Incorporated. http://ebookcentral.proquest.com/lib/slub-ebooks/detail.action?docID=1684368 [2022-03-16]
Hubáčková, M. & Ryšánek, D. (2007). Effects of Freezing Milk Samples on the Recovery of Alimentary Pathogens and Indicator Microorganisms. *Acta Veterinaria Brno - ACTA VET BRNO*, 76, 301–307. https://doi.org/10.2754/avb200776020301
Hussein, H.S. & Sakuma, T. (2005). Shiga Toxin–Producing Escherichia coli: Pre- and Postharvest Control Measures To Ensure Safety of Dairy Cattle Products. *Journal of Food Protection*, 68 (1), 199–207. https://doi.org/10.4315/0362-028X-68.1.199
Jay, J.M., Loessner, M.J. & Golden, D.A. (2005). *Modern food microbiology*. 7th ed. New York: Springer. (Food science text series)
Kruger, M.F., Barbosa, M. de S., Miranda, A., Landgraf, M., Destro, M.T., Todorov, S.D. & Gombossy de Melo Franco, B.D. (2013). Isolation of bacteriocinogenic strain of Lactococcus lactis subsp. lactis from rocket salad (Eruca sativa Mill.) and evidences of production of a variant of nisin with modification in the leader-peptide. *Food Control*, 33 (2), 467–476. https://doi.org/10.1016/j.foodcont.2013.03.043
Lightfoot, N.F. & Maier, E.A. (red.) (1998). Chapter 7 - Quantitative method and procedure assessment. *Microbiological Analysis of Food and Water*. Amsterdam: Elsevier Science B.V., 109–148. https://doi.org/10.1016/B978-044482911-5/50024-2
Lista, F., Reubsaet, F.A., De Santis, R., Parchen, R.R., de Jong, A.L., Kieboom, J., van der Laaken, A.L., Voskamp-Visser, I.A., Fillo, S., Jansen, H.-J., Van der Plas, J. & Paauw, A. (2011). Reliable identification at the species level of Brucella isolates with MALDI-TOF-MS. *BMC Microbiology*, 11 (1), 267. https://doi.org/10.1186/1471-2180-11-267
Martinez, B.A., Stratton, J. & Bianchini, A. (2017). Isolation and genetic identification of spore-forming bacteria associated with concentrated-milk

processing in Nebraska. *Journal of Dairy Science*, 100 (2), 919–932. https://doi.org/10.3168/jds.2016-11660
Mendonca, A., Thomas-Popo, E. & Gordon, A. (2020). Chapter 5 - Microbiological considerations in food safety and quality systems implementation. I: Gordon, A. (red.) *Food Safety and Quality Systems in Developing Countries*. Academic Press, 185–260. https://doi.org/10.1016/B978-0-12-814272-1.00005-X
Miller, G.D., Jarvis, J.K. & McBean, L.D. (2000). *Handbook of dairy foods and nutrition*. 2nd ed. Boca Raton, FL: CRC Press. (CRC series in modern nutrition)
Nero, L.A. & Carvalho, A.F. de (red.) (2019). *Raw milk: balance between hazards and benefits*. London, United Kingdom ; San Diego, CA, United States: Academic Press is an imprint of Elsevier.
Oxoid - Product Detail (u.å.). http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0361&org=82 [2022-02-21]
Richardson, G.H. & American Public Health Association (red.) (1985). *Standard Methods for the examination of dairy products*. 15. ed. Washington, D.C.
Scatamburlo, T.M., Yamazi, A.K., Cavicchioli, V.Q., Pieri, F.A. & Nero, L.A. (2015). Spoilage potential of Pseudomonas species isolated from goat milk. *Journal of Dairy Science*, 98 (2), 759–764. https://doi.org/10.3168/jds.2014-8747
Schlegel, L., Grimont, F., Ageron, E., Grimont, P.A.D. & Bouvet, A. 2003 (2003). Reappraisal of the taxonomy of the Streptococcus bovis/Streptococcus equinus complex and related species: description of Streptococcus gallolyticus subsp. gallolyticus subsp. nov., S. gallolyticus subsp. macedonicus subsp. nov. and S. gallolyticus subsp. pasteurianus subsp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 53 (3), 631–645. https://doi.org/10.1099/ijs.0.02361-0
Sigma Aldrich (2022). *Sequencing*. https://www.sigmaaldrich.com/SE/en/applications/genomics/sequencing [2022-03-02]
Sigma Aldrich (n.d.). Sigma-aldrich.
Stetter, K.O. (1996). Hyperthermophilic procaryotes. *FEMS Microbiology Reviews*, 18 (2–3), 149–158. https://doi.org/10.1111/j.1574-6976.1996.tb00233.x
Vithanage, N.R., Dissanayake, M., Bolge, G., Palombo, E.A., Yeager, T.R. & Datta, N. (2016). Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential. *International Dairy Journal*, 57, 80–90. https://doi.org/10.1016/j.idairyj.2016.02.042
Walstra, P., Wouters, J.T.M., Geurts, T.J. & Geurts, T.J. (2005). *Dairy Science and Technology*. Baton Rouge, UNITED STATES: CRC Press LLC. http://ebookcentral.proquest.com/lib/slub-ebooks/detail.action?docID=263085 [2020-03-27]
Watterson, M.J., Kent, D.J., Boor, K.J., Wiedmann, M. & Martin, N.H. (2014). Evaluation of dairy powder products implicates thermophilic sporeformers as the primary organisms of interest. *Journal of Dairy Science*, 97 (4), 2487–2497. https://doi.org/10.3168/jds.2013-7363
Zhang, R., Huo, W., Zhu, W. & Mao, S. (2015). Characterization of bacterial community of raw milk from dairy cows during subacute ruminal acidosis challenge by high-throughput sequencing. *Journal of the Science of Food and Agriculture*, 95 (5), 1072–1079. https://doi.org/10.1002/jsfa.6800

Popular science summary

Did you know that milk directly collected from the cow, often referred as “raw cow´s milk”, contains a huge variety of microorganisms? Do you know what they do and the importance of controlling them?

These microorganisms, especially bacteria, form an ecosystem within the milk and provide the milk with both beneficial and non-beneficial properties. Beneficial properties can include probiotic features, flavour and fermentation enhancement but can also serve as hygiene indicators during quality controls. Non-beneficial properties provided by bacteria include off-flavours, shortened shelf-life and disease within the herd but also human outbreaks after consumption. The non-beneficial outcomes result in economic losses within the dairy industry. The unwanted bacteria are commonly removed after application of different treatments, such as heat-treatments. However, some bacteria are highly viable and can survive such treatments and the problems provided by the unwanted bacteria remains.

To avoid negative consequences, microbial analyses of milk is performed on dairy level but also for scientific purposes. However, since bacteria are living organisms, they could multiply even during short times such as when the milk is waiting to be analysed. In some cases, the milk must be stored for a longer period which may change the microbial composition and, in the end, cause non-trustworthy results. It its therefore important to find a storage method which would not alter the microbial composition of the raw milk, i.e., not increasing the bacterial diversity and not decreasing it (in other words, not killing the bacteria).

In this pilot study, the aim was to investigate the bacterial numbers in raw cow’s milk after it had been stored at freezing temperatures of -20°C and -80°C for eight weeks by performing microbial analyses once a week. The hypothesis was that nothing would happen to the microbial flora, meaning that the number and types of bacteria would be the same when comparing results from analyses directly after the milk was collected (week 0) and from week 8. To establish which kind of bacteria the milk contained, MALDI-TOF MS analysis were performed.

The bacteria in this study could roughly be divided into two groups; Aerobic bacteria (grow in the presence of oxygen) and anaerobic bacteria (do not require oxygen, or even die in the presence of oxygen). The results suggested that the aerobic bacteria should be stored at -20°C as no microbial changes occurred within eight weeks. As for the anaerobic bacteria, the preferrable temperature was -80°C,

where no changes in the milk microflora could be observed during the eight weeks of storage. In the cases where changes were observed (-80°C for aerobes and -20°C for anaerobes), it was probably a consequence of letting the milk samples thaw for a longer time, resulting in wrong results. However, the results indicated no differences in aerobic bacteria when -20°C and -80°C was compared. It can therefore be assumed that aerobic bacteria can be stored at both temperatures. The results from MALDI-TOF MS analysis of the milk samples indicated the presence of mastitis bacteria, but also a diversity of different species.

The conclusion of this study is therefore that milk samples should be stored at -80°C although for the purpose of aerobic bacteria, milk could be stored at both temperatures.

Acknowledgements

First, I would like to thank the dairy science group at the Department of Molecular Sciences (SLU, Uppsala) for inputs and guidance during the practical work of this thesis. I would also like to thank my course mate, Frida Willdén, for valuable brainstorming sessions, discussions, and support throughout the entire process. Lastly, thank you Monika Johansson for being the best supervisor one could possibly wish for. Thank you for always sparing time for me whenever needed. Your professionalism combined with your thoughtfulness have given me the best final term at SLU I could possibly get.

Appendix 1 – Significance in CFU/ml of the parameters temperature and bulk.

Table 10. Effect of milk collection occasion (n=3) on colony forming units per mL in milk stored for up to 8 weeks at -20°C and -80°C, respectively, and cultivated on a weekly basis. Results for cultivation on Plate count agar (PCA) and De Man, Rogosa and Sharpe agar (MRS),s respectively.

Significant parameters	P-value	Not significant parameters	P-value
PCA agar			
Bulk -80°C	0.031	Bulk -20°C	0.225
MRS agar			
Bulk -20°C°C	0.000		
Bulk -80°C	0.000		

Appendix 2 – Microbial composition in bulk milk from the three different milk collection occasions, cultivated on total plate count agar.

Bulk 1

Table 11. Microbial composition of milk samples from collection occasion 1 stored in -20°C for 0 (reference), 1,4 and 8 weeks. Colonies were cultivated aerobically on total plate count agar and identified using MALDI-TOF MS

Bacteria	Week 0 (%)	Week 1 (%)	Week 4 (%)	Week 8 (%)
K. intermedia	40	60	80	
A. lactucae	20			
A. pittii	20			
B. gaviniae	20			
S. aureus		30		50
S. chromogenes		10		
A. veronii			10	
P. agglomerans			10	
B. pumilus				50

In total n= 32. For week 0, 1 and 4 n=10 per week and n=2 for week 8.

Bulk 2

Table 12. Microbial composition of milk samples from collection occasion 2 stored in -20°C for 0 (reference), 1,4 and 8 weeks. Colonies were cultivated aerobically on total plate count agar and identified using MALDI-TOF MS

Bacteria	Week 0 (%)	Week 1 (%)	Week 4 (%)	Week 8 (%)
S. maltophilia	40		10	40
A. veronii	20			
B. gaviniae	10			
C. taichungenese	10	10	10	10
P. fragi	10			
A. pittii	10	40	70	10
A. lactucae		30	10	40
A. junii		10		
S. aureus		10		

In total n= 40 (n=10 per week).

Bulk 3

Table 13. Microbial composition of milk samples collection occasion 3 stored in -20°C for 0 (reference), 1,4 and 8 weeks. Colonies were cultivated aerobically on total plate count agar and identified using MALDI-TOF MS

Bacteria	Week 0 (%)	Week 1 (%)	Week 4 (%)	Week 8 (%)
S. aureus	90	100	100	90
S. epidermidis	10			
A. pittii				10

In total n= 40 (n=10 per week).

Appendix 3 – Microbial composition in bulk milk from the three different milk collection occasions, De Man, Rogosa and Sharpe agar.

Bulk 1

Table 14. Microbial composition of milk samples collection occasion 1 stored in at 20°C for 0 (reference), 1, 4 and 8 weeks. Colonies were cultivated anerobically on De Man, Rogosa and Sharpe agar and identified using MALDI-TOF MS

Bacteria	Week 0 (%)	Week 1 (%)	Week 4 (%)	Week 8 (%)
S. equinus	60	40	30	
S. lutetiensis	40	50	70	40
S. infantarius		10		60

In total n= 40 (n=10 per week).

Bulk 2

Table 15. Microbial composition of milk samples collection occasion 2 stored at -20°C for 0 (reference), 1, 4 and 8 weeks. Colonies were cultivated anerobically on De Man, Rogosa and Sharpe agar and identified using MALDI-TOF MS

Bacteria	Week 0 (%)	Week 1 (%)	Week 4 (%)	Week 8 (%)
S. lutetiensis	70	60	50	
W. paramedenteroides	20			
S. equinus	10	30	50	10
S. infantarius		10		90

In total n= 40 (n=10 per week).

Bulk 3

Table 16. Microbial composition of milk samples collection occasion 3 stored at -20°C for 0 (reference), 1, 4 and 8 weeks. Colonies were cultivated anerobically on De Man, Rogosa and Sharpe agar and identified using MALDI-TOF MS

Bacteria	Week 0 (%)	Week 1 (%)	Week 4 (%)	Week 8 (%)
L. paracasei	100		10	
W. paramedenteroides		70	70	60
S. lutetiensis		10	10	
S. gallolyticus		10		
L. mucosae		10		
P. pentosaceus			10	
L. dextrinicus				10
P. pentosaceus				20

In total n= 32 samples. For week 0, 1 and 4 n=10 per week and n=9 for week 8.

Publishing and archiving

Approved students' theses at SLU are published electronically. As a student, you have the copyright to your own work and need to approve the electronic publishing. If you check the box for **YES**, the full text (pdf file) and metadata will be visible and searchable online. If you check the box for **NO**, only the metadata and the abstract will be visible and searchable online. Nevertheless, when the document is uploaded it will still be archived as a digital file. If you are more than one author, the checked box will be applied to all authors. Read about SLU's publishing agreement here:

☒ YES, I/we hereby give permission to publish the present thesis in accordance with the SLU agreement regarding the transfer of the right to publish a work.

☐ NO, I/we do not give permission to publish the present work. The work will still be archived and its metadata and abstract will be visible and searchable.