Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

DNA based analyses of microbiota in fat fraction of raw milk

DNA- baserad analys av mikrobiota i råmjölkens fettfraktion

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Department of Molecular Sciences
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Abstract

The microbiota of raw milk is complex and the analysis methods have during the years developed from culturing to molecular based methods, allowing more precise descriptions of the composition. This project included both cultivation and molecular methods to compile the distribution of microorganisms in the whole milk, since the most common way to day to investigate milk microbiota is based on defatted milk. The goal was to explore how big part of the microbiota that is connected to the milk fat fraction and if the already existing method for DNA based analyses of milk microbiota is suitable also for detection of microbiota in whole milk. In this project, raw milk samples were spiked with L*actobacillus reuteri* DSM, *Lactobacillus reuteri* PTA, *Escherichia coli* and *Staphylococcus aureus*. The samples were centrifuged and the fractions (pellet, supernatant and fat) were cultured to identify the bacterial distribution. The molecular method consisted of DNA extractions, Terminal Restriction Fragment Length Polymorphism (T-RFLP) and quantitative PCR (qPCR) also based on whole milk, spiked with the same bacteria as during the cultivation part. PowerFood Microbial DNA Isolation kit (MoBio Laboratories Inc.) was used for four different extraction methods. For comparing the DNA extraction methods and bacterial community, the T-RFLP was used. Additionally, qPCR was performed for total and specific targeting of bacteria and quantification. The results from the cultivation part showed that the highest amounts of colony forming units (CFU) originated from the fat fraction. The molecular methods confirmed the pattern as the T-RFLP analyses from the whole milk samples showed more traces of bacteria. The qPCR showed that the whole milk samples contained more DNA than the skim milk samples. In conclusion, the PowerFood kit without additions of solvents can be used for DNA preparation followed by PCR and/or T-RFLP/sequencing when analyzing the microbiota in whole milk.

Keywords: Raw milk, microbiota, whole milk, DNA T-RFLP, PCR

Sammanfattning

Mjölkråvarans mikrobiota är komplex och genom åren har analysmetoderna utvecklats från odling på agarplattor till molekylära metoder, vilket resulterat i en tydligare bild av de förekommande bakterierna. Detta projekt har inkluderat både odling och molekylära metoder för att jämföra distributionen av bakterier i helmjölk, eftersom dagens analyser är baserade på avfettad mjölk. Syftet med detta projekt är att undersöka hur stor del av mikrobiotan som binder till fettet och om befintliga metoder för DNA-analyser av mjölk även går att applicera för analyser av mjölkfettets mikrobiota. Första delen av projektet innebar att undersöka hur bakterierna fördelades i pellet-, supernatant- eller fettfraktionerna efter centrifugering. Detta gjordes genom att inokulera mjölkprover separat med *Lactobacillus reuteri* DSM, *Lactobacillus reuteri* PTA, *Escherichia coli* and *Stafylococcus aureus* och därefter gjordes spädningar som odlades på selektiva agarplattor. Nästa del bestod av DNA-extrahering med hjälp av PowerFood Microbial DNA Isolation kit (MoBio Laboratories Inc.) med några avvikelser from tillverkarens protokoll, vilket resulterade i fyra metoder. Tre av dessa användes för vidare analys med metoden Terminal Restriction Fragment Length Polymorphism (T-RFLP) för att jämföra mikrobiotan i proverna och om det förekom fler typer av bakterier i helmjölkproverna. För undersökning av de specifika bakterierna och kvantifiering av dem utfördes även kvantitativ PCR (qPCR). Odlingen på agarplattorna resulterade i att flest kolonibildande enheter (CFU) bildades från fettfraktionerna, vilket betyder att mjölkfettet innehåller en stor del bakterier som annars kan förbises vid analyser då avfettad mjölk används. För T-RFLP analysen visade det sig att det inte var någon större skillnad i extraheringsmetoderna, men vid analys med metoder baserade på helmjölk tenderade proverna att innehålla högre antal bakterier. Som slutsats kan det konstateras att PowerFood kittet går att använda utan att ursprungsprotokollet modifieras för DNA-isolering, PCR och T-RFLP/sekvensering för att analysera bakterier även i helmjölk.

Nyckelord: Mjölkråvara, mikrobiota, helmjölk, DNA, T-RFLP, PCR

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Abbreviations

BHI	Brain heart infusion
BP	Base pair
CFU	Colony forming units
LAB	Lactic acid bacteria
MRS	de Man Ragosa Sharpe
MS	Mannitol salt
NTC	No template control (negative control)
PCR	Polymerase chain reaction
qPCR	Quantity polymerase chain reaction
SC	Somatic cells
SLU	Swedish University of Agricultural Sciences
SSU	Small subunit
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
VRB	Violet red bile

1 Introduction

Milk is tested on daily bases worldwide to verify good quality and safe milk for consumption and further processing (Rodrigues *et al*., 2017). Since the 1990s, bulk tank milk has been used to diagnose problems in dairy herds related to quality and mastitis (Jayarao *et al*., 2004). There are many factors that influence the milk and the composition of microbiota is in turn directly influencing the subsequent development of dairy products (Quingley, *et al*., 2013b). Through the years, many methods have been established for different milk analyses and in recent years, DNA based methods have become an important tool for microbial food constituents. However, since milk is a complex media, it makes it difficult to analyze in order to get a high quantity and quality of DNA. PCR inhibitors like fat, protein and calcium reduce the ability to generate a successful amplification of the DNA. A usual step in these molecular based methods is discarding the fat fraction during DNA extraction. This can result in underestimation of bacteria, partitioned in the fat fraction, or that they will not be detected at all (Quingley, *et al*., 2012; Poms, Glössl & Foissy, 2001). Since these existing methods used for analysing microbiota in milk are based on defatted sample, there is still room for improvement for methods analysing the microbiota in whole milk samples.

This thesis is a part of a big on-going survey where the overall objective is to increase the long-term profitability of the Swedish dairy sector by improving quality management in the dairy value chain.

1.1 Aim

The aim of this project is to explore the proportion of the microbiota associated to the milk fat fraction and if the already existing method for DNA based analyses of milk microbiota is suitable also for detection of microbiota in whole milk.

2 Literature review

2.1 Composition of milk

Milk is a highly nutritious food and includes all the nutrients in a good balance that newborns need; proteins, fats, carbohydrates, vitamins and minerals. The pH of milk is near neutral and at a high water activity and in combination with all the nutrients it makes an ideal environment for microorganisms (Quigley *et al*., 2013; Smoczynski, Staniewski & Kielczewska, 2012). Bovine milk consists of water (87%), lactose (4.6%), protein (3.4%), fat (4.2%), minerals (0.8%) and vitamins (0.1%). The composition is influenced by factors like breed, feeding strategies, management of the cow, lactation stage and seasonal changes. Since these factors can vary between farmers and the facts that there is always on going development for e.g. feed, milk composition continuously undergoes changes (Lindmark Månsson, 2008). According to FAO (2013), milk can for example be classified according to its fat content: whole milk, skimmed milk, semi-skimmed milk, low-fat milk and standardized milk etc. Processing of the milk is another kind of classification, like pasteurized and extended shelf-life milk. FAOSTAT definition (FAO, 2013) for skim milk of cows is "milk from which most of the fat has been removed" and the definition for whole milk is:

> "Cow milk, whole, fresh: Production data refer to raw milk containing all its constituents. Trade data normally cover milk from any animal, and refer to milk that is not concentrated, pasteurized, sterilized or other-wise preserved, homogenized or peptonized."

2.2 Milk fat

Milk is produced in the mammary gland of mammals and the fat consists of lipids present in fat globules. These are formed at the endoplasmatic reticulum located in

the epithelial cells in the alveoli. During the secretion, they are enveloped by the plasma membrane of the cell, so that each fat globule contains residues from it. The composition and structure of the membrane is mainly composed of polar lipids and membrane-bound and associated proteins. The lipid fraction of membrane material in the dry matter constitutes of 30% where 25% consist of phospholipids, 3% of cerebrosides and 2% of cholesterol (Lindmark Månsson, 2008; Smoczynski, Staniewski & Kielczewska, 2012).

For processes and analyses, fat is often separated from milk through centrifugation, based on differences in density between the fat particles and the dispersing phase or plasma. Since fat has lower density than plasma it will form a layer on the top during centrifugation that can be removed. Other particles like dirt particles, somatic cells (SC) and microorganisms, will also be removed during the centrifugation. Moreover, the fat globules readily agglutinates at lower temperatures and even more so in raw milk than in homogenized milk, since the fat globules exist in more diverse sizes in raw milk. In cold agglutination, also SC and several bacteria readily participate. At 37°C, no agglutination occurs but with temperatures falling below 35°C, an increasing number of cells will be lost within the cream fraction with declining temperature (Walstra, Wouters & Geurts, 2006; Griffiths, 2010).

2.3 Microbiota in milk

Milk is usually thought to be sterile in a healthy udder but becomes colonised by microorganisms from the environment after milking (Quigley *et al*., 2013b). The microbial composition of the milk is diverse and influenced by different factors. Microbes can be present in the teat canal, on the surface of the teat, in the air, in feed, in water and on different types of equipment (Quigley, *et al*., 2013a). Michal, Hauwuy & Chamba (2001) investigated diversity and influence of production conditions on 27 farms. The difference between milk supplier practises in terms of cleaning the milk equipment, by pre-milking and post-milking udder preparation, resulted in differences in the microbial load and composition in the milk. Indoor and outdoor feeding also have an impact on the milk, especially the increase of *Staphylococcus* spp. during outdoor feeding (Hagi, Kombayashi & Nomura, 2010). Most of the bacteria found in milk belong to lactic acid bacteria (LAB), like *Lactococcus*, *Streptococcus* and *Leuconostoc*. Other commonly found bacteria include Gram-positive bacteria like *Bacillus*, *Microbacterium*, *Micrococcus* and *Staphylococcus*. Gram-negative bacteria that can be found are *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Sternotrophomonas* and *Chryseobacte-*

rium as well as some *Enterobacteriaceae* such as *Enterobacter*, *Hafnia* and *Klebsiella* (von Neubeck *et al*., 2015). However, the research on raw milk microbiota has mainly been focusing on pathogens and therefore is the knowledge about the natural occurring microbiota in raw milk still limited (Fricker *et al*. 2011).

In addition to all the nutrients in milk, SC are also natural presented components. They mainly consist of milk-secreting epithelial cells and white blood cells, and are used as a measure of the milk quality and the state of udder health, since it is involved in the innate immune system. High number of SC is associated with udder inflammation and leads to bacteriological problems. Therefore, alteration of milk composition leads to major variation of dairy products characteristics compared to normal values. In healthy quarters of dairy cows the somatic cell count in 1 ml is 2×10^4 to 2×10^5 and in an infected quarter it ranges from 3×10^5 to 9×10^7 cells/ml. Levels above 4×10^5 is considered to be unfit for human consumption (Pokorska, 2016; Usman *et al*., 2014; Li *et al*., 2014).

A heating process, i.e. pasteurization is used for elimination of pathogens in the raw milk and for eliminating a large proportion of potential spoilage organisms. However, thermoduric organisms like spore formers and some gram-positive vegetative species in the genera *Enterococcus*, *Microbacterium* and *Arthrobacter* can survive pasteurization (Adams & Moss, 2008) and can therefore influence a product in a negative manner.

2.4 Microbiota and its impact on cheeses

The microbiota in raw milk is of big importance for sensory richness and variety of traditional cheeses. A French term that is used to refer to the conditions in which food is grown or produced is *terroir*. It is the *terroir* that gives the food its unique sensory characteristics and the meaning of the word is homological with local area and productions systems, i.e. the milk varies between farms. Turbes *et al*. (2001) who investigated the evidence of *terroir* in milk and its influence on Cheddar cheese flavour suggested that the geographical location of the milk source has an effect on the flavour. However, the commingling of milk and the heat treatment likely reduce the effect of geographical location and even more during the time the cheese ages. Even though the microbiota contributes to flavour and characteristics in cheeses, they can also be the ones that are responsible for flavour defects and can even be of health risk (Fricker *et al*., 2010). A famous example of spoilage bacteria is representatives of *Clostridia*, which cause late cheese blowing and can result in massive economical losses for the industry

(Quigley *et al*., 2011). Coliforms and yeasts can also cause gas formation and *Bacillus* spores and *Pseudomonas* species can produce proteolytic and lipolytic enzymes that mainly affect hard and semi-hard cheeses. *Staphylococcus aureus*, a pathogenic bacterium, can also contaminate milk. The variety of microorganisms is an important factor, which the cheese-maker has to contend (Griffiths, 2010).

2.5 Methods and problems for detecting microbes in milk

Testing of the microbiological status of both raw and pasteurized milk occurs on a daily basis with different methods in order to maintain quality and safe products. Many of the techniques that have been used for identifying and quantification involve culturing on agar plates, which makes bacteria not easily cultured hard to detect or not detected at all. More recently, DNA based methods have been used for the purpose, since it is a way to examine the bacteria without introducing cultured-based biases (Quigley *et al.*, 2012). Despite the positive aspects of DNA methods, there are also negative ones. They can over- or underestimate specific bacterial groups due to certain biases caused by different levels of DNA extraction efficiencies, preferential PCR amplification of certain species or interspecies 16S rRNA operon copy number heterogeneity (Fricker *et al*. 2011). DNA based methods also detect dead cells and extracellular DNA, e.g. DNA from SC (Weber *et al*., 2014). According to Fricker *et al.* (2011), a combination of the two types of methods are preferred for more accurate results on the microflora of the raw milk due to the benefits and disadvantages of both of them.

Unfortunately, due to the milk fat, milk is a difficult source for extracting high quality and quantity DNA. The fat and protein play roles as inhibitors and interfere with most of the DNA extraction methods (Usman *et al*., 2014; Mertens *et al*., 2014). Phenol-chloroform is a classical extraction method, which has been used for DNA extraction from different tissues, but today there are also several kits used; Nucleospin Blood, modified TianGen, PowerFood, MasterPure Gram Positive and UltraClean e.g. (Usman *et al*. 2014; Rodrigues et al. 2017; Thomas *et al*., 2013). The problem to obtain high quantity and quality genomic DNA from milk still exists, but through the years, the analyses have improved and with extraction kits the extracted DNA amounts have increased (Usman *et al*. 2014). The kits that are used for DNA extraction though are often designed for extractions from pure cultures with highly concentrated cells and are not necessarily optimized for scenarios requiring high sensitivity (Thomas *et al*., 2013). Even though milk analyses are performed as routines in the dairy industry, fat and proteins is still a

problem for high quantity and quality DNA and there is still not a kit that is optimized for DNA extractions from milk (Usman *et al*. 2014).

The extraction methods normally start with cell disruption by chemical or physical methods and thereafter separation of membrane lipids and cell debris using detergents and centrifugation (Mertens *et al*., 2014). After centrifugation, the supernatants are discarded. In the protocol followed with the PowerFood kit, used in this project, it is also specially mentioned to remove as much fat as possible. By discarding the supernatant and fat, however, there is loss of bacteria. In fact, it has been known for long time that high proportions of bacteria exist in the milk fat fraction (Anderson, 1909). Nevertheless, there seems to be researchers that are unaware of this and therefore also the effect of removing the fat fraction on assay sensitivity. According to Poms, Glössl & Foissy (2001), free DNA is in fact also in the highest concentration in the cream layer. By discarding the fat layer during extraction procedures, there can be a great loss of DNA and this could be of significant importance if there are small amounts of target DNA present in the sample. Brewster & Moushumi (2016) investigated the recovery of bacteria in pellet after centrifugation, which in raw milk with spiked bacteria was only 2.2-6.1%. In homogenized and pasteurized samples however, there were much higher recoveries, with average values between 52.2-100%. The partitioning of bacteria was also very rapid; 80% of added inoculated bacteria partitioned into the cream layer within 5 minutes and over 95% partitioned after 45 minutes. Furthermore, the cream layer also exhibited a high capacity for bacteria, reaching half-saturation at a bacterial load of ca 5×10^8 CFU/ml. It would therefore require very high levels of non-target bacteria to saturate the cream layer and fully release the target bacterium for centrifugal recovery. The binding mechanism of bacteria to milk fat is mainly due to antibody-mediated binding, but there are also other mechanisms suggested, like hydrophobic, lectin-sugar and lymphocyte mediated mechanisms (Brewster & Paul 2016).

2.6 Polymerase chain reaction (PCR) methods

Polymerase chain reaction (PCR) is a rapid, sensitive and specific molecular method. The 16s rRNA gene (16srDNA) has frequently been used as a target due to its structural characteristics. Through evolution, some parts of the sequences of 16sr DNA have been highly conserved, which therefore can be used for all bacterial species. Regions that have species-specific variable sequences can in turn be used for identification of unique bacterial species (Maeda *et al*., 2003).

Another version of PCR is quantitative PCR (qPCR), also called Real-time PCR. It is also a fast method, has a high sensitivity and specificity, low contamination risk and is easy to perform (Wang et al., 2014). As the name reveals, qPCR is a quantitative method and makes it possible to determine the amount of bacteria, both of specific and unspecific bacteria depending on what the target is. To be able to quantify them, there must be a standard curve for every run, with known amounts of DNA in a number of dilutions. As the PCR products (also called amplicons) increase exponentially, a signal of fluorescence is obtained after every cycle, caused by a fluorescent-labelled probe that binds to DNA. The increase of fluorescence is plotted against the cycle number and generates an amplification curve, from which a quantification cycle (Cq) value can be determined. This value can in turn be linked to the initial concentration of the target nucleic acid. qPCR has become a method of choice when quantifying genes (Postollec *et al*., 2011). PCR inhibitors like fat, enzymes and minerals have been identified in milk. According to Bickley *et al*. (1996) however, fat have only minor influence on PCR and the inhibition is mainly due to the concentration of calcium. Plasmin was suggested by Powell *et al*. (1994) as a naturally occurring inhibitor in milk that degrades Taq polymerase. Due to the importance of removing PCR inhibitors, there have been a number of techniques developed to overcome this problem, e.g. by the use of silica columns (Kemp *et al*., 2006).

2.7 Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a methodology that makes it possible to assess the diversity of a complex mix of bacteria. The distribution of microorganisms also includes uncultivable organisms and can be divided into clusters or groups (Andoh *et al*., 2008). The method is an inexpensive, robust, reproducible and rapid (Parkash *et al*. 2014). When the technique was firstly introduced, it was by using the small subunit (SSU) rRNA gene for identification, specifically the 16s rRNA gene. The methodology has primarily been used in analysis of the genetic diversity, change and composition of complex microbiota communities within environmental samples, even though it is also utilized for marine denitrifiers, ammonia-oxidisers and methanogens among others (Elliot *et al*., 2012). The T-RFLP method starts with extraction of DNA. The 16s rRNAs present in the community are PCR amplified using primers, which can be designed to be non-discriminating, amplifying almost all 16s rRNAs, or selective, targeting specific domains or groups. The 5’ primer is fluorescently labelled to tag the products. The products are thereafter digested with restriction enzymes, normally 4-base cutters, and the digested products obtained are visualised with gel electropho-

resis. The output for a given sample will be detected as series of peaks based on the size and amount of the fragments (Marsh *et al*., 2000, Andoh *et al*., 2008).

3 Materials and methods

The method was divided in two procedures and the first part consisted of cultivation on selective agar plates. The distribution of four chosen bacteria species where examined in the three milk fractions after centrifugation; fat, supernatant or pellet. The second part consisted of the molecular based methods, starting with DNA extraction. Also for this part, the three milk fractions and the same bacteria species were used in order to investigate the distribution of microbiota. By using a commercial kit with some deviations from the instructions of manufacturer's, different methods were tested to see which one of them resulted in highest yield of extracted DNA. The steps following DNA extraction were PCR amplification for T-RFLP analysis and qPCR for identification and quantification of bacteria in the milk.

3.1 Milk samples

The milk used in this project was whole milk obtained from Lövsta, the Swedish livestock research centre on the 5th of December 2016. It was stored in -20°C until use.

3.2 Bacteria and strains

Bacteria used for spiking of milk samples: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Lactobacillus reuteri* PTA 4659 (hydrophobic) and *L. reuteri* DSM 17938 (hydrophilic).

3.3 Growth mediums and solutions

3.3.1 Broths

Broths for overnight cultures of LAB were de Man Rogosa Sharpe (MRS; VWR Chemicals) and for *S. aureus* and *E. coli* Brain heart infusion (BHI; OXOID), prepared according to the manufacturer´s instructions. The solutions were divided between four bottles containing 50 ml each. The broth was autoclaved (121°C for 30 min) and stored at 2°C.

3.3.2 Agar plates

Agar plates used for culturing LAB were de Man Rogosa Sharpe MRS (from National veterinary institute), *E. coli* were on Violet red bile (VRB) (OXOID) and *S. aureus* on Mannitol salt (MS) (US Biological), prepared according to the manufacturer´s instructions.

3.3.3 Saline solution

NaCl solution with the concentration 9 mg/ml was made at a total volume of 500 ml, autoclaved and stored at 2°C.

3.4 Culturing of bacteria

3.4.1 Overnight cultures and bacteria calculations

An overnight culture for each bacterium species was made with 9 ml of broth inoculated from a stock that had been stored at -80°C. The culture was incubated for 24 h at 37°C, shaking. Thereafter, a new overnight culture for each bacterium species was made by taking 500 µl from the first overnight culture to new tubes containing 5 ml broth, incubated for 24 h at 37°C, shaking. The number of bacteria in the second overnight culture was calculated using a haemocytometer (Petroff-Hausser Counting Chamber). Dilutions of the overnight culture were made to count the bacteria with the equation:

$$Cells/ml = number\ of\ cells\ counted \times 0.00005 \times (1000 \times dilution\ factor)$$

where 0.0005 is the volume of the smallest square of the chamber and 1000 is to get the amount in ml.

3.4.2 Culturing on agar plates

Frozen whole milk was heated in a water bath at 37°C for 20 minutes. A milk sample of 5 ml was spiked with approximately 10^7 bacteria cells. The spiked milk

was incubated for one hour at 4°C during shaking. After the cold incubation, 1 ml of the spiked milk was pipetted to a 1.5 ml eppendorf tube and centrifuged for 4 minutes, 13000 x *g* at 4°C (Eppendorf™ 5424R Microcentrifuge). The fat fraction was removed to a new eppendorf tube using a spatula. The fat was weighed and the tube was filled with saline solution to a total volume of 1 ml (equal to 1 g). Thereafter, the tube was incubated in a water bath at 37°C for 15 minutes to resolve the fat. Dilutions were made and the ones with concentration 10^{-2}, 10^{-4} and 10^{-6} were spread on agar plates. The serum fraction was removed with a pipette to a new eppendorf tube. After vortexing, 100 µl was pipetted to a tube with 900 µl saline solution. Dilutions were made and the ones with concentration 10^{-2}, 10^{-4} and 10^{-6} were spread on agar plates. The remaining micelle fraction located in the pellet was suspended with 50 µl saline solution. The volume of pellet was measured by pipetting the total volume and subtracted by 50 (saline solution). The tube was adjusted to 1 ml with saline solution. Dilutions were made and the ones with concentration 10^{-2}, 10^{-4} and 10^{-6} were spread on agar plates. All plates with LAB were put in anaerobic jars with Microbiology Anaerocult® A (Merck) and Mikrobiologie Anaerotest® (Merck) for generation and detection of an anaerobic atmosphere.

Controls were made by spreading 100 µl of unspiked whole milk (10^{0}) and dilutions with concentration 10^{-1} and 10^{-2} were spread on each type of agar plate used for the four bacteria. Controls of the dilutions 10^{1} and 10^{3} from the overnight cultures (100 µl) were also spread on the four different agar plates.

3.5 PCR amplification and T-RFLP

3.5.1 Sample preparation

Sample preparation was made for each bacterium species by spiking 1.8 ml whole milk with 200 µl saline solution containing 10^{7} cells of bacteria. Petroff-Hausser Counting Chamber was used in order to calculate cells. Controls of 2 ml milk was prepared and treated in the same way as the spiked samples. Each sample was made in replicates of two. The skim milk controls were defatted through centrifugation (Eppendorf™ 5424R Microcentrifuge) in 13 000 x *g* in 4 minutes. The samples were stored at -20°C until further analysis.

3.5.2 DNA extraction

Before beginning, the hood and all equipment were cleaned with 70% ethanol. Tips, solutions and tubes provided with the kit were subjected to UV light for 15-30 minutes before use. Aliquots of PF1 and PF3 solutions provided by the DNA

kit were pipetted into separate tubes for each milk sample. After the UV treatment, tubes with solutions of PF1 and PF3 were placed in a heating block at 55°C. Gloves were used during the whole procedure, including preparations.

DNA was isolated from the milk samples using PowerFood DNA isolation kit (MoBio Laboratories, Immuno diagnostics OY #21000-50) according to the manufacturer´s instructions but with some deviations in order to try to optimize the bacteria DNA yield from the fat fraction, resulting in four methods (see below).

The milk samples prepared during the DNA preparation step were thawed and vortexed for homogenization. An aliquot of 1 ml of every sample was pipetted into a new 2 ml tube and centrifuged at 13000 x *g* for 5 minutes. The following steps were different for each of the tested methods:

i. *A method*: (whole milk) after centrifugation, the supernatant was discarded. Cell pellet and the fat fraction were re-suspended by adding 450 µl warm PF1 solution (lysing reagent that includes a detergent to break cell walls).

ii. *B method*: (skim milk) fat and the supernatant were discarded and cell pellet was re-suspended by adding 450 µl warm PF1 solution (method according to PowerFoods protocol).

iii. *n-A method*: (whole milk) the supernatant was discarded. Cell pellet and the fat fraction were treated with 30 µl of n-hexane, vortexed and heated in a heating block at 65°C for 10 minutes. Thereafter, the sample were re-suspended by adding 450 µl warm PF1 solution.

iv. *m-A method*: (whole milk) the supernatant was discarded. Cell pellet and the fat fraction were treated with 30 µl of methanol, vortexed and heated in a heating block at 65°C for 10 minutes. Thereafter, the sample were re-suspended by adding 450 µl warm PF1 solution.

The re-suspended cells were transferred to Bead Beating tubes and placed in a bead beater machine (FastPrep-24, Bio medicals) at speed 5 for 1 minute, paused for 1 minute and run for 1 minute again. Thereafter, the samples were centrifuged at 13000 x *g* for 1 minute. All of the supernatant (approximately 400 µl) and fat from the whole milk samples were carefully transferred to a new 2 ml tube using a 200 µl pipette. 100 µl of PF2 (inhibitor removal reagent) was added and vortexed

briefly and the tubes were incubated at 4°C for 5 minutes. After the cold incubation, the tubes were centrifuged at 13000 x *g* for 1 minute. Supernatant, and fat fraction from the whole milk samples, were transferred to a new 2 ml collection tube. The pellet was discarded. Furthermore, 900 µl of warm PF3 solution (a highly-concentrated salt solution) was added and the tubes were vortexed. The supernatant was transferred through taking 660 µl at each time onto a Spin filter and centrifuged at 13000 x *g* for 1 minute. The flow through was discarded and the procedure was repeated until all of the supernatant were centrifuged. The filter baskets were placed into new clean 2 ml tubes. The PF4 solution (alcohol based wash solution) was shaken and 650 µl was added to the tubes, which thereafter were centrifuged at 13000 x *g* for 1 minute. The flow through was discarded and 650 µl of PF5 (solution used for complete removal of PF4) were added and the tubes were centrifuged again at 13000 x *g* for 1 minute. The flow through was discarded and the tubes were centrifuged dry at 13000 x *g* for 2 minutes. The Spin filter baskets were placed into clean 1.5 ml tubes and 50 µl of PF6 (sterile elution buffer) was added to the centre of the Spin filters and incubated in room temperature for 5 minutes. The tubes were centrifuged at 13000 x *g f*or 1 minute. The spin filter baskets were discarded. The DNA was stored in -20°C for further analysis.

Quantification of DNA was examined after the DNA extraction using Qubit 3.0 Flourometer (Thermo Fisher Scientific) according to the manufacturer´s instructions. Amounts of 2 µl of each extracted DNA sample was used for quantification.

3.5.3 PCR

The PCR product was based on the 16S rRNA genes from each DNA extract using the general bacteria primers Bact-8FAM (forward) end-labelled with 6-carboxyfluorescein and BAct-926r (reverse), see table 1. Samples were analysed in two technical replicates from each DNA replicate. The total reaction volume was 25 µl containing 12.5 µl master mix, 9.5 µl sterile water, 1 µl forward primer, 1 µl reverse primer and 1 µl DNA template.

Table 1. *General bacterial primers used for PCR amplification*

Target gene	Primer	Sequence (5'→3')	Reference
16S rRNA	Bact-8FAM F	AGAGTTTGATCCTGGCTCAG	Dicksved *et al*., 2008
	BAct-926r R	CCGTCAATTCCTTTRAGTTT	

The PCR amplification (using CFX96 Real-Time System, Bio Rad) started with an initialization step at 95°C for 3 minutes followed by 30 cycles consisting of 20

seconds at 95°C (denaturation), 20 seconds at 49°C (annealing), 30 seconds at 72°C (elongation) and a final elongation at 72°C for 5 minutes.

After PCR amplification, the DNA products were confirmed by agarose gel (1%) electrophoresis using GeneRuler 1 kb Plus DNA ladder, ready-to-use (Thermo Fisher Scientific) as a size marker. The gel was made by mixing 3 g agarose and 300 ml TBE (0.5x). Electrophoresis was carried out at 110 V for approximately one hour. 5 µl of each PCR product was mixed with 2 µl DNA loading dye (6X) (Thermo Fisher Scientific).

3.5.4 PCR purification

The technical replicates obtained from the PCR amplification were pooled together by taking 18 µl from each one into a 2 ml tube. To each tube, 29 µl of Agencourt AMPure beads was added ($0.8 \times V_{PCR}$) followed by vortexing for 10 seconds and incubation in room temperature for 5 minutes. Thereafter, the tubes were placed onto Agencourt SPRIPlate for 5 minutes to separate beads. The pure solution was discarded and 200 µl of 70% ethanol was added and incubated for 30 seconds in room temperature. The ethanol was aspirated and the wash was repeated once again. The tubes were placed on a rack without magnetism and air dried for 10 minutes. 38 µl of nuclease free H_2O was added to each tube, vortexed and placed onto the SPRIPrack again for 2 minutes. The eluted PCR product was transferred to new tubes and stored in a freezer. After purification, DNA concentrations were examined with Qubit according to the manufacturer´s instructions by using 5 µl from each purified PCR sample. Gel electrophoresis was made to verify the purification, in the same way as previous gel electrophoresis.

3.5.5 Digestion of PCR product

After purification of DNA, the PCR products were digested with the restriction enzyme HaelII 10U/µl (Thermo Fisher Scientific) (Recognition sequence CC/GG). The total reaction volume was 30 µl containing 27 µl PCR reaction mixture, 0.5 µl HaeIII and 3 µl 10x Buffer R. The mixture was incubated at 37°C for 2 hours followed by a thermal inactivation by incubation at 80°C for 20 minutes. The PCR reaction mixture was based on the results from DNA concentration (C_{PCR}) after purification. The needed volume of PCR product (V_{PCR}) for every sample was calculated by the equation:

$$V_{PCR}\frac{30\ (\mu l)\ \times\ 7.14\ (ng/\mu l)}{C_{PCR}\ (ng/\mu l)}$$

and the needed volume of nuclease free water by the equation:

$$V_{H2O} = 30\ (\mu l)\ \ - V_{PCR}\ (\mu l)$$

Together, these two volumes made up PCR reaction mixture, containing 200 ng of DNA in each sample.

3.5.6 T-RFLP

T-RFLP analysis was prepared by making dilutions of 1:20 and 1:100 of digested PCR products with ultra-pure water. The samples were submitted in a 96-well plate covered by small lids. The plate was put into a freezer and thereafter sent to Uppsala Genome Center for T-RFLP analysis. The resulting T-RFLP profiles were thereafter processed by Peak Scanner software (Applied Biosystems). The relative abundance of the individual T-RFs was calculated by dividing the peak area by the total area of all peaks, using Excel. Peaks shorter than 30 base pairs (BP) were excluded as noise.

3.6 qPCR

For assessment of the bacterial load by quantification of the samples, a standard curve was made using TOPO TA kit for sequencing (Thermo Fisher Scientific) according to the manufacturers protocol and TOP10 cells were used. The plasmids were purified with plasmid purification kit (Quiagen). Triplicate measurements of the DNA concentration was made with Qubit. $V_{plasmid}$ were calculated and 10 dilutions from 10^8 down to 10^0 were made in small PCR tubes. The standard curve was included in each qPCR assay in duplicates and with 3 negative controls (no template controls, NTC). The program started with an initialization step at 95°C for 5 minutes followed by 50 cycles consisting of 45 seconds at 95°C (denaturation), 20 seconds at 60°C (annealing) and 30 seconds at 72°C (elongation). After the PCR, a melting curve was constructed in the range of 55°C to 95°C. The average values of the cycle thresholds were used for calculation of the bacterial load.

Five procedures of qPCR with different targets were run using forward and reverse primers (table 2). Before each analysis, a 96-well plate, empty Eppendorf tubes, tubes with ultrapure water and filter tips were treated by UV light for 15 minutes. A reaction mixture was prepared with 730 µl SYBR Green qPCR master mix No ROX (Thermo Fisher Scientific), 36.5 µl of each forward and reverse primer (Thermo Fisher Scientific, see table 2) and 438 µl ultrapure water. The total reaction volume in each well was 20 µl, consisting of 17 µl of reaction mixture

and 3 µl of template. Two replicates for standard curves were run, ranging from 10^8-10^0 and additionally three NTC were included in each run. qPCR amplification program was run in the same way as the standard curves. At each cycle and end of the run, accumulation of PCR products was detected by fluorescence of the reporter dye of SYBR Green.

Table 2. *Primers used for the qPCR amplification.*

Target	Primer	Sequence (5'→3')	Reference
E. coli	395 F	CATGCCGCGTGTATGAAGAA	Huijsdens *et al*., 2002
	490 R	CGGGTAACGTCAATGAGCAAA	
L. reuteri DSM	1694 F	TTAAGGATGCAAACCCGAAC	Protocol made by Department of Microbiology, SLU
	1694 R	CCTTGTCACCTGGAACCACT	
L. reuteri PTA	0733 F	GACAGTGGCTAAACGCCTTC	Protocol made by Department of Microbiology, SLU
	0733 R	AATTCCACTTGCCATCTTCG	
S. aureus	472 F	AGTGATGAAGGTCTTCGGATCGTAAA	Wang *et al*., 2014
	575 R	CGTGGCTTTCTGATTAGGTACCGTC	
Total bacteria	U1048 F	GTGSTGCAYGGYTGTCGTCA	Maeda *et al*., 2003
	U1371 R	ACGTCRTCCMCACCTTCCTC	

SLU = Swedish University of Agricultural Sciences

4 Results

4.1 Culturing of bacteria

The four cultured species of bacteria resulted in numbers of colony forming units (CFU) as shown in table 3. The highest amounts of CFU were presented in the samples containing 10^{-2} bacteria cells; the other dilutions seemed to be too low for culturing. Furthermore, the highest number of bacteria originated from the fat fraction compared to the supernatant and pellet. Looking at figure 1, both of the LABs were present in the highest amounts in the fat fraction, even though *L. reuteri* DSM is hydrophilic. There are even more bacteria from the samples spiked with *S. aureus* and *E. coli* in the fat fractions, which clearly shows where the bacteria partitioned when centrifuged.

Table 3. *Colony distribution in the milk fractions (fat, supernatant, pellet) of the four bacteria species detected on selective agar plates*

Fraction	Dilution	*L. reuteri* PTA (CFU)	*L. reuteri* DSM (CFU)	*S. aureus* (CFU)	*E. coli* (CFU)
Fat	10^{-6}	0	0	0	0
	10^{-4}	2	3	3	1
	10^{-2}	666	190	750	210
Supernatant	10^{-6}	0	0	0	0
	10^{-4}	0	0	0	1
	10^{-2}	268	68	188	72
Pellet	10^{-6}	0	0	0	0
	10^{-4}	1	3	0	1
	10^{-2}	613	143	166	93

CFU = Colony forming units

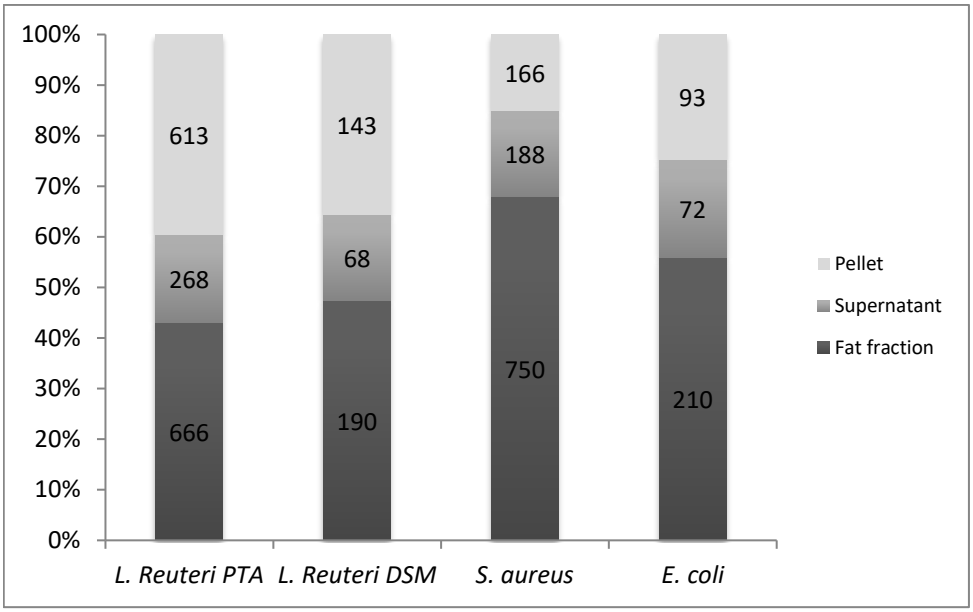

Figure 1. The percentage of bacteria colonies originating from different fractions after culturing on agar plats. The results are based on milk samples spiked with 10^5 bacteria cells. Number of colony forming units (CFU) of each fraction is indicated in the bars.

The amounts of fat removed from the centrifuged tubes and the number of bacteria calculated back to the original spiked 5 ml whole milk are shown in table 4. The highest amount of fat was in the sample spiked with *E. coli*, probably due to better technique when removing the fat. Controls are shown in table 5 and 6 (Appendix 1). The spiked number of cells (table 4) were lower than 10^7 as it was supposed to be.

Table 4. *Fat fraction (mg) and amounts of spiked bacteria in 5 ml milk*

Bacterium	Fat (mg)	Total bacteria in 5 ml milk*
L. reuteri PTA	71.6	7×10^6
L. reuteri DSM	67.4	2×10^6
E. coli	62.5	5×10^6
S. aureus	85.5	2×10^6

*Based on results obtained from culturing the fractions with dilutions 10^{-2}.

4.2 DNA extraction and concentration

DNA was extracted with the MoBio PowerFood kit with some deviations from the manufacturer´s instructions. The method that included methanol was excluded since it made the fat difficult to transfer between tubes; it got stuck to the walls of the tubes and inside the pipettes. Concentration of extracted DNA was measured

by using Qubit directly after the extraction. The results show diverse amounts of extracted DNA for each bacterium and the there is no method in general that seems to be better than the others (figure 2). However, the whole milk (analysed with methods A and n-A) gives more DNA than skim milk (analysed with method B). The A-method generated higher amounts of DNA than the B-method for all bacteria except for *S. aureus*. It is worth to mention that the results do not only show DNA from the spiked bacteria; it also contains DNA from other naturally occurring bacteria in the milk and SC. The replicates differed from each other in some samples, like *L. reuteri* PTA (both A and n-A method), leading to a high standard deviation.

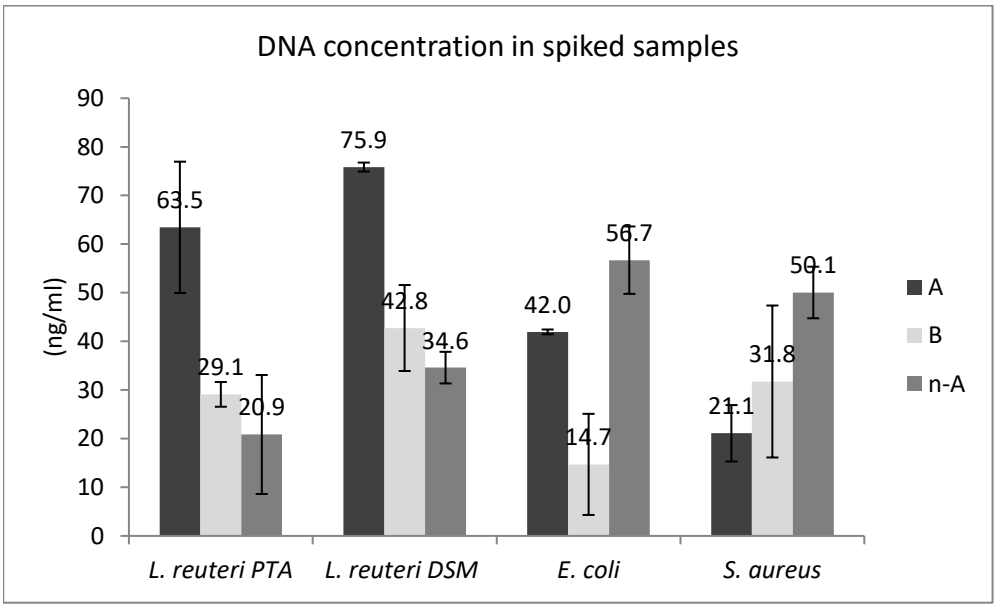

Figure 2. DNA concentrations based on three different methods (A, B and n-A) in the spiked milk samples.

Controls, in figure 3, showed high yield of DNA. There is also a higher DNA yield from the control samples analysed with method A and n-A than from method B.

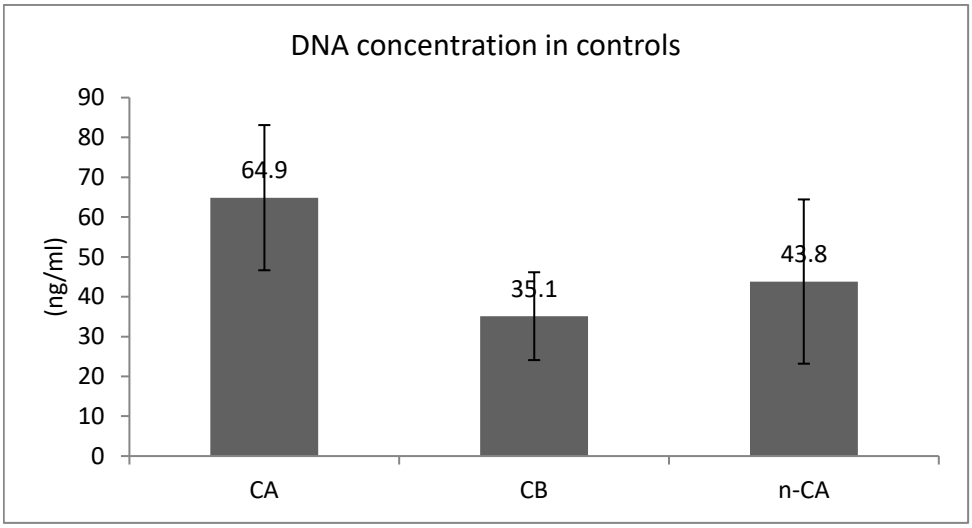

Figure 3. The DNA concentration in the milk controls. CA = control for method A, CB = control for method B and n-CA = control for method n-A.

4.2.1 Gel electrophoresis

Gel electrophoresis was done after PCR amplification, purification of PCR products (figure 4) and digestion of PCR product (figure 5) to confirm the DNA products obtained from the extraction methods (A, B and n-A). The bands in figure 4 were single and clear and showing the same size, between 1000 and 700 base pairs (BP). The results indicate that the described methods can be used to extract DNA. Well 2-7 in every gel in figure 4 contains DNA from the spiked samples (well 2-3: A-method, well 4-5: B-method and well 6-7: n-A-method) and shows brighter bands than the controls that are in the other wells (well 8-13 in 4A and 4C and well 8-9 in 4B and 4D). The DNA product in the controls assumes to be DNA originated from natural occurring milk bacteria. In figure 4A and 4B, well 6-7 in both gels contains DNA from the n-A method and shows the brightest bands. It is specially seen in figure 4B, with DNA from samples spiked with *S. aureus*.

In figure 5A and 5C, the results are difficult to see, which is probably due to contamination in the gel, but it is possible to see some very diffuse bands in 5C. In figure 5B though, the wells 8-9 show brighter bands, containing DNA from samples spiked with *L. reuteri* PTA analysed with the n-A method. That indicates that higher amount of DNA was extracted with n-A method. In figure 5B, well 2-3 and 10-11 (products from controls for n-A-method) shows that the DNA products have been cut different than the spiked samples, between bands 700 and 500 BP.

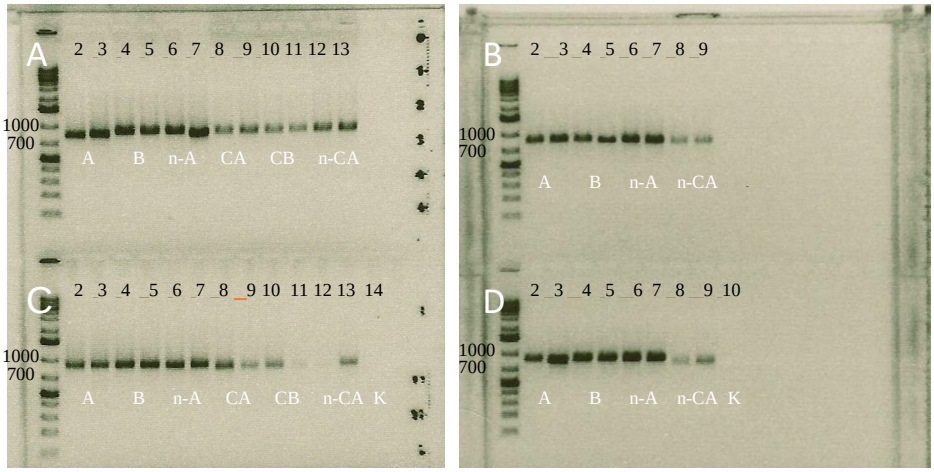

Figure 4. Gel electrophoresis showing products after DNA purification. 5 µl from purified PCR sample is loaded and GeneRuler 1 kb Plus DNA ladder is used in every of the 1th well. The products are between 1000 and 700 BP. (A): well 2-7: samples with *E. coli* and wells 8-13: controls. (C): well 2-7: samples with *L. reuteri* DSM, well 8-13: controls and a negative control, K (well 14). (B): wells 2-7: samples with *S. aureus*, wells 8 and 9: controls only for n-A method. (D): wells 2-7: samples with *L. reuteri* PTA, wells 8 and 9: controls only for n-A method, well 10: negative control.

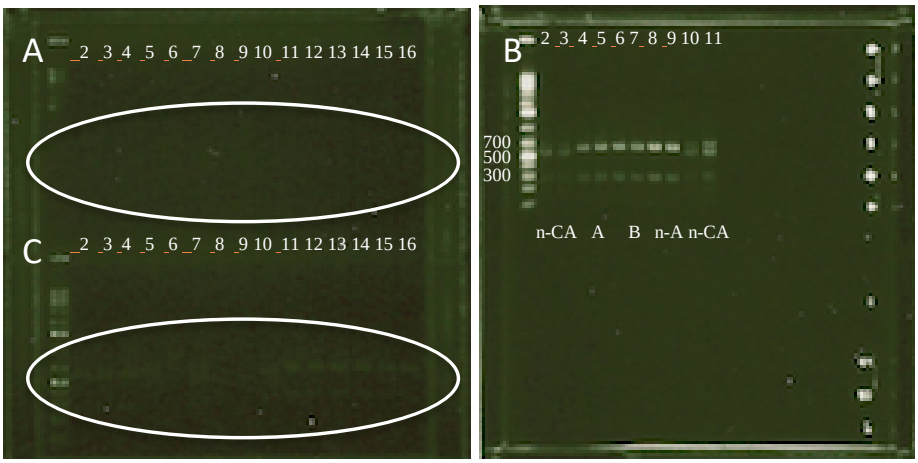

Figure 5. Gel electrophoresis showing the results after digestion with restriction enzymes. The gels were loaded with GeneRuler1 kb Plus DNA ladder in every of the first well and loaded with 5 µl from each purified PCR sample. Bands from samples were between lanes of 700 and 300 BP. (A) and (C): gel loaded with samples from, *E. coli*, *L. reuteri* DSM, *S. aureus* and controls but the gel is probably contaminated. (B): well 2-3: controls for n-A method, well 4-9 samples with *L. reuteri* PTA, well 10-11: controls only for n-A method.

4.3 T-RFLP

The results obtained from the T-RFLP analyses were diverse and the most dominant T-RF in all samples consisted of 24 BP, but was considered as "noise" and was therefore excluded. The results are only showed for dilution 1:20 since concentration 1:100 was too diluted.

The result for the samples spiked with *L. reuteri* DSM (figure 6) showed that the methods where the fat was kept (methods A and n-A) showed more traces of bacteria than the B-method. The most abundant T-RF was 63 BP, which is the T-RF for LAB when using the restriction enzyme HaeIII. Other T-RFs that are shown, like T-RF 32, 199, 275, 307 and 317 BP can also be seen in figure 10, showing the microbiota in the unspiked controls for each method. One T-RF that cannot be seen in the controls is 335 BP, which is high in comparison to the other in the spiked samples (figure 6 and 8).

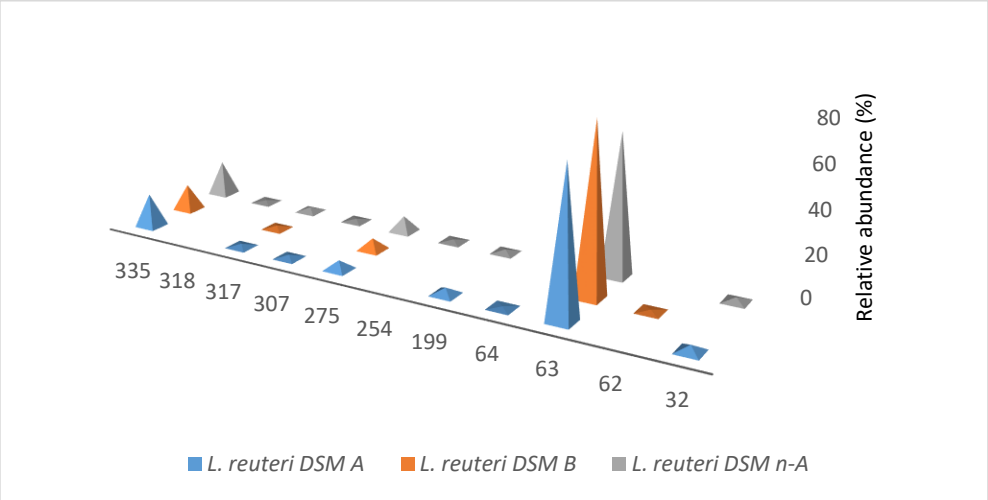

Figure 6. T-RFLP results based on three different methods (A, B, n-A) from the samples spiked with *L. reuteri* DSM.

Results obtained in the samples spiked with *S. aureus* are shown in figure 7. The most abundant T-RF is 309 BP; the one showing the presence of *S. aureus*. There are also small amounts of T-RF 32 and 308 BP, which are present in the controls (figure 10).

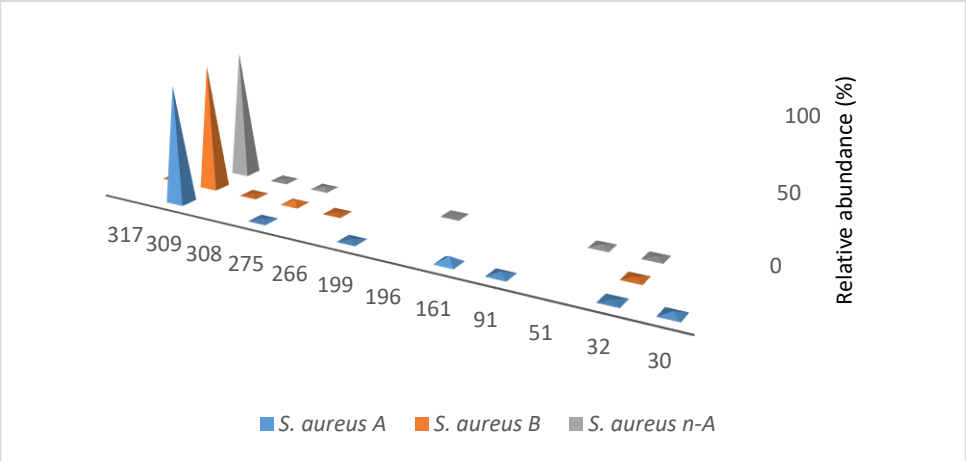

Figure 7. T-RFLP results based on three different methods (A, B, n-A) from the samples spiked with *S. aureus*.

The results obtained for *L. reuteri* PTA (figure 8) resulted in most T-RFs 63 BP for methods A and n-A but only T-RF 30 BP in the samples analysed with B-method, which might be due to some error. The T-RFs 62 and 65 BP can be considered as T-RF 63 BP, since ±5 BP can differ from the site that HaeIII cuts. Like the results in figure 6, T-RF 335 Bp can be seen in figure 8 as well.

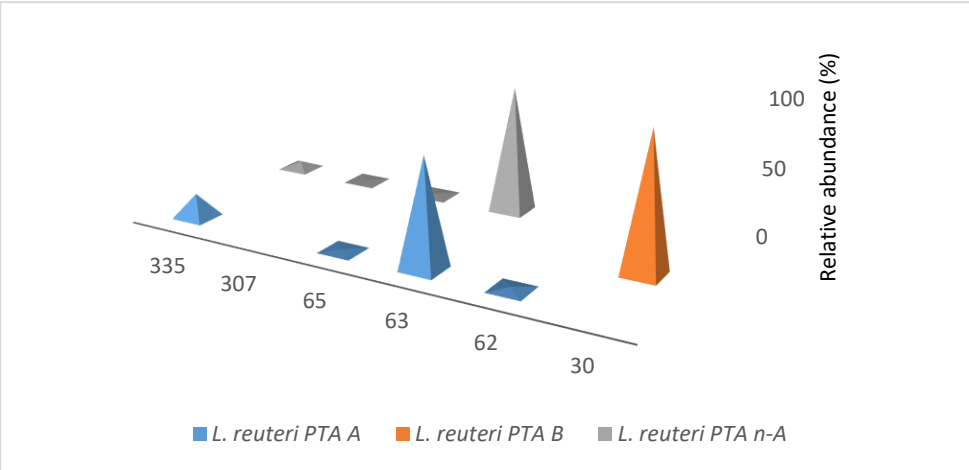

Figure 8. T-RFLP results based on three different methods (A, B, n-A) from the samples spiked with *L. reuteri* PTA.

In the analysis for *E. coli* (figure 9), there appears to be some error as well, since no T-RF of 32 BP was obtained in the samples analysed with method A. Instead there were only T-RFs 118 and 67 BP presented. In the other two methods however, there are quite equal result, with the exception of T-RF 63 BP that indicate presence of LAB and T-RF 30 BP, which probably is a fragment belonging to *E. coli*.

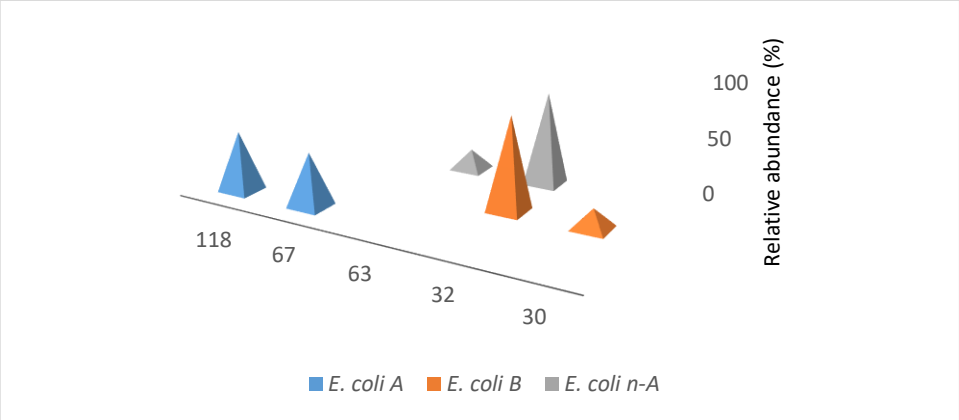

Figure 9. T-RFLP results based on three different methods (A, B, n-A) from the samples spiked with *E. coli*.

In result from the T-RFLP for the controls (figure 10) the most dominated T-RFs are especially 275 BP but also T-RFs 32, 199, 307 and 317 BP. The A and B method give equal results, but the n-A method generates more T-RFs. The T-RF indicates that the milk contains some naturally occurring *E. coli* (T-RF 32 BP) and some LAB (T-RF 307 BP).

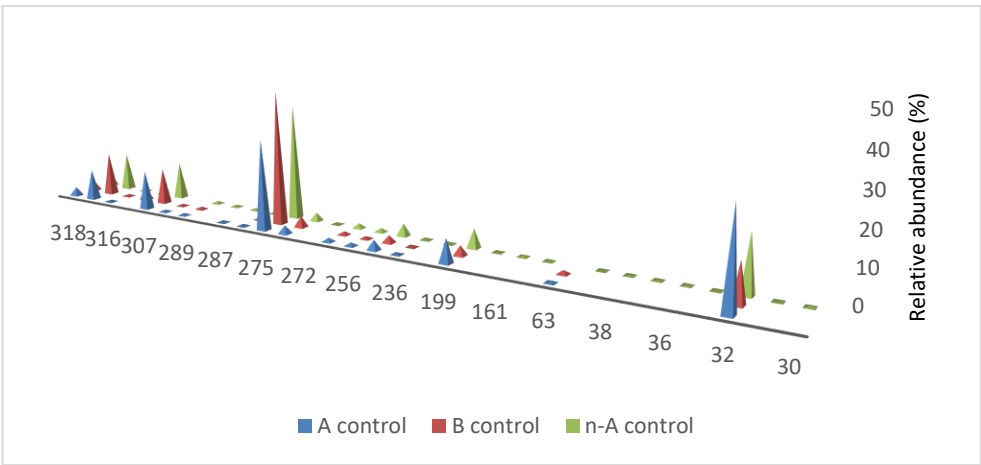

Figure 10. The results for controls based on three different methods (A, B, n-A) of the T-RFLP.

4.4 qPCR

The results from the qPCR for specific bacteria and total bacteria are shown in figure 11 and 12. The reaction efficiency (E) varied between 97.1% and 98.7% (except for *L. reuteri* PTA: 78.9%) and the linear correlation (R^2) is between 0.914 and 0.997. The melt curves (not shown) did not show only single melt peaks for all PCR runs, which indicate multiple PCR products in some samples. For the *E. coli* samples, the melt curve showed products at the same T_m for all samples, including NTC, even though the samples unspiked with *E. coli* showed results at Cq around 30. *L. reuteri* DSM showed a sharp melt curve for the spiked samples with *L. reuteri* DSM. All the other spiked samples and controls (but not the NTC), resulted in a melt curve earlier than *L. reuteri* DSM samples and standards. This indicates that another gene was amplified and would probably show shorter fragments if they would be run in a gel electrophoresis. *L. reuteri* PTA showed results with a melt curve at the expected peak for the samples spiked with *L. reuteri* PTA and the NTC were clean. A few of the samples gave results with high Cq values around 36-45, which probably can be distinguished. The samples spiked with *S. aureus* gave results for every sample including the controls but not the NTC. However, the other samples showed a very low content. Therefore, the qPCR results should be interpreted by caution. The procedure was not repeated because of too low amount of DNA template left. Nevertheless, the A-method resulted in highest DNA copies/ml for samples spiked with LAB, followed by B- and n-A-method (figure 11). For *E. coli* and *S. aureus* however, the n-A-method is equal or better compare to the A-method. When comparing these results with the Qubit results in figure 2, there are similarities, except for *E. coli*, where the A-method is better than the B-method.

The results for the total bacteria (figure 12) are based on a standard curve of E value 98.1% and R^2 0.914 which therefore is not very reliable. The R^2 value should be ≥0.985 and E value should be between 90-110%, where 100% implies perfect doubling of amplicon each cycle (Agilent Technologies, n.d.). The Cq values were around 30 for both spiked samples and controls. qPCR for total bacteria were therefore lower than the runs for specific bacteria but the controls showed relatively high values in comparison, even though they were unspiked. The total bacteria amount show higher results in both spiked samples and controls for A-method. In contrast to the specific qPCR, the B-method is in between the other two methods. Looking at the total bacteria (figure 12), there are almost equal results within the methods and also for the controls.

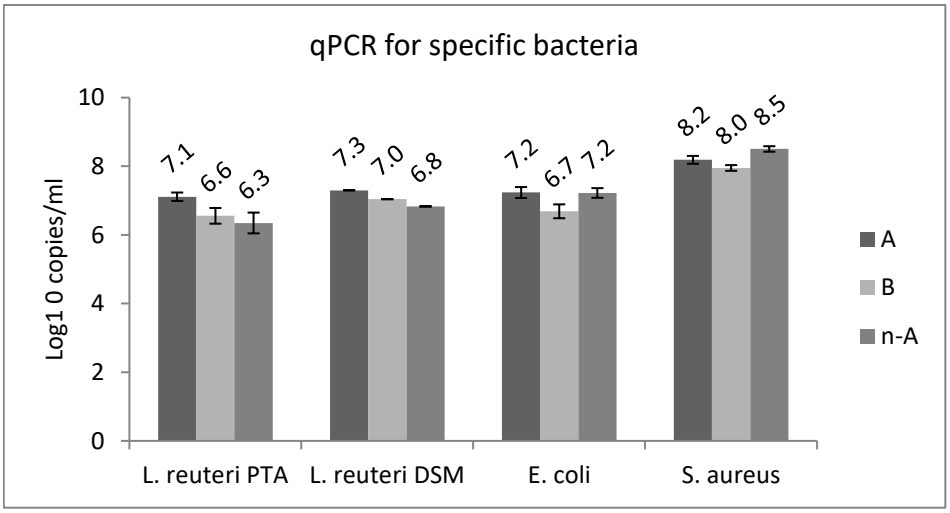

Figure 11. Mean values of the spiked bacteria obtained after three different extraction methods (A,B and n-A), using primers for each specific bacterium.

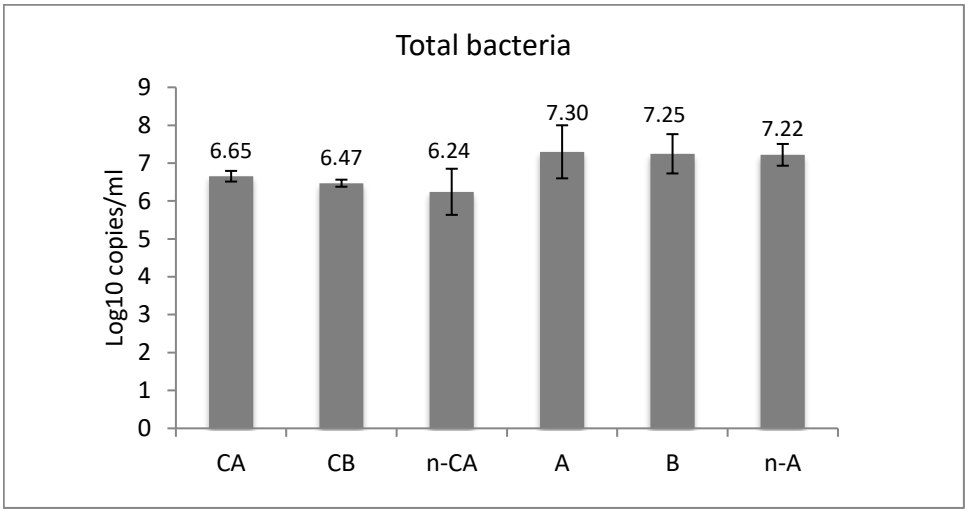

Figure 12. Mean values of the controls and spiked bacteria obtained after three different extraction methods (A, B and n-A).

5 Discussion

The aim of this project was to explore the proportion of the microbiota which is connected to the milk fat fraction and also to investigate if an already existing method for DNA based analyses of microbiota in defatted milk is suitable for detection of microbiota in whole milk. This work is important since bacteria that bind to the fat can be underestimated or not detected at all in the currently used methods.

5.1 Culturing of bacteria

The first task during the laboratory part was to examine by cultivation the distribution of four spiked bacteria species in three different milk fractions. The results showed that all of the tested bacteria were in higher amounts in the fat fraction, which is in line with previous findings (Anderson, 1909; Poms, Glössl & Foissy, 2001). Between 43-68% of the cultured bacteria originated from the fat, 17-19% originated from the supernatant and 15-40% from the pellet. Unfortunately, the only results from the culturing were obtained from the samples of the dilution 10^{-2}. A possible reason for this can be that there were lower amounts of spiked bacteria than expected. Based on the CFU, the amounts of bacteria in 5 ml (table 4) showed lower values than 10^7. This was probably due to the difficulties to calculate the exact number of bacteria using a counting chamber. Counting bacteria in broth and thereafter spiking milk is not a fully reliable method in order to get the exact amount of 10^7 bacteria cells in the milk samples. Both *S. aureus* and *E.coli* were difficult to count in the heamocytometer and no staining was used in order to separate dead cells from alive. There is a risk that the spiked amount of bacteria varied between the samples. There is an even higher risk for differences in bacteria numbers for *L. reuteri* PTA, since samples analysed using the n-A-method were spiked on a different day than the samples spiked with *L. reuteri* PTA. Tables 5 and 6 (Appendix 1) show the results from the controls; unspiked milk cultured on the agar plates used after enrichment in broth. In the original milk (concentration 10^0)

LAB, but not *E. coli* or *S. aureus* were detected. This is in agreement with the fact that LAB can naturally be found in fresh milk (von Neubeck *et al*., 2015). Since there was no colony of *S. aureus* in unspiked milk (10^0), but 61 colonies from milk dilution 10^2, it might be due to contamination caused by bad handling of equipment and materials or that the spreading of 10^0 concentration failed. The cultivation could benefit from including multiple agar plates for each bacterium species to strengthen the results.

Furthermore, it was difficult to remove all of the fat from the tube and it requires training to use a spatula with a good technique. The fat readily sticks to plastic materials, whereas a plastic spatula is difficult to use. A metallic spatula is to prefer, but since every sample needs a sterile one, there is important to have sterile spatulas prepared for every sample. The results obtained for *S. aureus* spiked samples showed that there was even more bacteria in the fat in contrast to the results obtained for the other spiked bacteria samples, probably due to better technique when the fat was removed in the *S. aureus* sample (table 4). Therefore, a good standardized method needs to be practised before determination and by keeping the sample on ice the fat will be more solid and improve the conditions to succeed.

It is interesting that distribution of bacteria in the different milk fractions has been known for more than a century (Anderson, 1909) but surprisingly few seems to take this into account when analysing bacteria in whole milk. Brewster & Moushumi (2016) who was unaware of this until they observed it, tried to overcome it by developing an agitation treatment. They added this step before centrifugation of the milk in the extraction procedure and it took 2-8 minutes, depending on the amount of milk. The aim was to reproduce the effects of homogenization in the laboratory by agitation of the raw milk with a high-shear mixer. This was done since homogenised samples give a higher recovery of bacteria in the pellet. The treatment with agitation disrupted the bacteria binding to the fat layer and resulted in more than 95% recovery, without affecting the bacteria viability. This could be an additional step for optimizing the protocol used in this thesis.

Since fat interfere with most of the DNA extracting methods (Mertens *et al*., 2014), it is understandable that discarding of supernatant containing fat is recommended. But considering the fact that it contains high levels of bacteria, it might be time to take this into account during analyses and develop existing methods.

5.2 DNA extraction

In this project, the protocol obtained with MoBio PowerFood kit was used with some additional steps, resulting in methods called A, B, n-A and m-A, were B was the same as the manufacturer´s protocol. Quigley *et al*. (2012) investigated seven different methods for DNA extractions and concluded that the PowerFood microbial DNA kit (MoBio Laboratories Inc.) was the best for the extraction of total DNA from both raw skim milk and raw milk cheese. Furthermore, Thomas *et al*. (2013) evaluated nine commercial DNA extraction kits for *Bacillus anthracis* spores from different media, including milk, and concluded that the PowerFood kit was as one of three kits that yielded the most consistent DNA extraction from the spores. The Pre-treating of fat and pellet with hexane in this projects, the n-A method, was based on the findings of Poms, Glössl & Foissey (2001). Hexane removes fat, allowing dispersion in an aqueous phase, and can therefore improve the extraction in the samples containing fat (Terry *et al*., 2002). The method called m-A, where methanol was used, was not successful, since the fat became very difficult to transfer into new tubes and got stuck into the pipette tips. When using as small amounts as 1 ml of each sample, it is important to be able to get as much of the fat as possible. In the beginning of the project, the plan was to remove the fat after centrifugation, add solvents (hexane or methanol) only to the fat and then transfer back to the tube with the pellet. Since the problem was almost the same for the n-A as with m-A method, the decision to treat both pellet and fat fraction was decided, to minimize the risk of loosing fat and thereby bacteria. It is favourable to use methods that are manageable and easily worked since the milk fat readily stick to the plastic surface of materials. A good sample preparation technique for routine analysis should be simple, safe, inexpensive and have a good reproducibility that results in sufficient DNA quality and yield (Terry *et al*., 2002).

The results for the DNA concentration (figure 2) are in general low (between 14.7 ng/ml and 76.5 ng/ml), compare to Quingley *et al*. (2012) using the same PowerFood kit for raw milk and raw milk cheese with the results of 909.53 ± 6.0 ng/ml and 6756.14 ± 16.47 ng/ml respectively. Furthermore, Usman *et al*. (2014) compared three methods how to extract genomic DNA in bovine milk but with the use of 50 ml for each method. The tested methods were Nucleospin Blood Kit, TianGen Kit and the classical and cheaper Phenol-Chloroform method. The results were 170.5 ± 113.9 ng/µl, 139.9 ± 92.7 ng/µl and 105.9 ± 55.6 ng/µl respectively.

In the DNA extraction, the A-method give a higher amount of DNA for the two samples spiked with LAB (figure 2), but for *E. coli* and *S. aureus* it was the n-A method that yielded the highest DNA concentration. This is similar to the results obtained from the qPCR (figure 11) and also to the results obtained from

the gel electrophoresis, even though there is bigger variation between the methods for each bacteria species in figure 2. When comparing the methods for the controls (figure 3), B-method resulted in the lowest results. Undoubtedly, methods A and n-A give the highest extracted DNA.

What the low amounts of extracted DNA in this project depend on is not known, also considering that the B-method was performed according to the protocol provided with the PowerFood kit. However, since the extracted DNA can stem also from SC, results can vary largely between the milk used in different projects and Swedish milk is known for its low levels of SC compared to other countries[1]. Another contributing reason can be that the quantity of DNA was measured with different methods; Qubit in this project instead of a nanodrop flourospectrometer as in the studies made by Quigley *et al*. (2012) and Usman *et al*. (2014). One option to improve the yield of DNA is to add a second elution as a last step in the DNA extraction method. According to Thomas *et al*. (2013), kits using silica columns (e.g. PowerFood) or magnetic/ silica-based magnetic beads often requires the use of a large buffer volume (50-100 µl) for efficient elution of DNA. Therefore, a second elution could be incorporated in the protocol. Even though it is said that fat interfere with most extraction methods (Mertens *et al*., 2014), there were higher amounts of DNA in almost all of the whole milk samples compared to the skim milk samples (figure 2). This can be seen in both in spiked and unspiked samples except for *S. aureus.*

5.3 T-RFLP

The results obtained from the T-RFLP showed that the spiked bacteria could be identified through the analysis, but the microbial traces from other types of bacteria varied between the methods. The higher amounts of bacterial traces in the controls for the n-A method (figure 10) could depend on that the results were based on more replicates than the controls for the other two extraction methods. The replicates also varied within method and all traces of bacteria were not present in each replicate. This means that the controls for the other methods might have shown more microbial traces, more equal to the result of the control for the n-A method, if they would have been performed in the same amounts of replicates. The differences between replicates can also be applied to the spiked samples. It can be difficult to determine which type of extraction that is the best and most correct when looking at the T-RFLP profiles, since the DNA extraction methods can give different results without saying that one them is wrong. According to other studies, the use of a single DNA extraction can underestimate the total number of bacteria, whereas a combination of DNA extractions can be used for a more accurate evalu-

[1]Monica Johansson, Researcher at SLU, meeting 2:nd of may 2017

ation of bacterial communities (LaMontagne *et al*., 2002; Inceoglu *et al*., 2010; Willner *et a*l., 2012; Prakash *et al*., 2014). However, results observed in this project indicate that there were no big differences between the samples (including the controls), suggesting that the microbiota in the whole milk samples can be studied regardless extraction method used in the this work. Nevertheless, the A and n-A methods are to prefer for the analysis due to their higher amounts of extracted DNA compared to B-method.

5.4 qPCR

In the cultivation part, there were high amounts of bacteria that originated from the fat fraction. Figure 2 showed that method A for LAB and n-A for *E. coli* and *S. aureus* gave higher amounts of DNA compared to method B. These results gave an expectation that it could be bigger differences between whole milk and the skim milk samples also in the molecular determination. However, since this was not clearly seen in T-RFLP or qPCR, it is difficult to determine how big part of the microbiota that originated from the milk fat fraction. qPCR was performed in order to quantify the bacteria and compare the extraction methods (A, B and n-A) and if there were higher DNA yield in the whole milk samples (A and n-A). The results varied between the qPCR assays. Fully optimized assays should contain only single melting products in samples with the targeted sequence, showing one melting temperature (T_m), which not all of the runs in this project did. Another problem during the qPCR analysis in this project was that the standard curves did not turn out well for all five qPCR runs. Standard curved were run in duplicates but should favourably be run in triplicates in order determine precision of pipetting, reproducibility and the overall sensitivity of the assay (Agilent Technologies, n.d.).

Even though qPCR is supposed to have a low contamination risk (Wang et al., 2014) this appears to be the reason for some of the results in this project. The high values of the controls in the run for total bacteria could be due to contamination or false positive results. The products in NTC for the *E. coli* samples could also be due to contamination but also due to non-specific PCR product formation or non-specific probe degradation (Agilent Technologies, n.d.). Even though the qPCR results are uncertain, the results can be compared with the results in the previous sections. The whole milk samples resulted in higher yield of DNA, indicating that there are bacteria in the fat fraction. qPCR results also confirmed that the samples spiked with *L. reuteri* PTA analysed with method B, and samples spiked with *E. coli* analysed with method A, contained DNA from these bacteria, even though they were not seen in the T-RFLP analyses (figure 8 and 9).

5.5 Conclusion

This project investigated the proportion of the microbiota in whole milk associated to the milk fat fraction and if the existing method for DNA based analyses on milk microbiota is suitable also for detection of microbiota in whole milk. The cultivation part showed that highest numbers of CFU originated from the fat fraction. This means that existing methods risks that some bacteria can be ignored during analyses. During the molecular part of the project, the extracted DNA yield rose in the whole milk samples compared to the skim milk samples. The T-RFLP analysis showed that it can be used for investigation of the milk microbiota, and that the extraction method had limited impact on that type of analysis. Since the fat was difficult to transfer after addition of hexane and it was easy to loose fat within pipette tips, method A was recommended for the analysis of the microbiota in whole milk.

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Appendix 1. Culture controls of unspiked milk

Table 5. *Results from culturing unspiked milk on agar plates used for the tested bacterium species. Controls for LAB were spread on MRS, S. aureus on MS and E.coli on VRB*

Dilution of milk control	Amounts of CFU on controls			
	L. reuteri PTA (CFU)	*L. reuteri* DSM (CFU)	*S. aureus* (CFU)	*E. coli* (CFU)
10^{0}	15	22	0	0
10^{-1}	9	147	7	0 (1)
10^{-2}	0	15	61	0 (1)

(1) = Colony that probably originates from another bacterium species.
CFU = Colony forming units

Table 6. *Results from culturing the broths containing the bacteria on agar plates. LAB were spread on MRS, S. aureus on MS and E.coli on VRB*

Bacteria cells	*L. reuteri* PTA (CFU)	*L. reuteri* DSM (CFU)	*S. aureus* (CFU)	*E. coli* (CFU)
10^{1}	0	0	0	0
10^{3}	1	3	2	7

CFU = Colony forming units

Appendix 2. Popular summary

Microbiota in the milk fat of whole milk – do we mistakenly overlook bacteria species and if so, how can they be detected?

Whole milk and its complex microbiota have a large impact on the quality of cheese and the bacteria can be both positive and negative for the final product. The analytical methods to study these bacteria have during the years developed. However, there are still question marks regarding the identity of the existing bacteria and their association with different fractions of the whole milk. Current analytical methods are based on defatted milk samples, meaning that there is a risk that some of the bacteria binding to the fat fraction will not be identified.

The microbiota was in the early analyses studied through culture-dependent methods using mediums like agar and broths. Today, these techniques have developed; giving new possibilities through molecular methods like Polymerase Chain Reaction (PCR), Gel electrophoresis and Terminal Restriction Fragment Length Polymorphism (T-RFLP). However, there are still methods that need further development since the molecular methods can be sensitive to the presence of interfering substances and results can for different reasons give the wrong picture. Substances in milk that may interfere with the analysis include proteins, calcium and fat. To overcome the problem with the inhibitors, different methods have been applied. Centrifugation is a common technique during the extraction of DNA that is used to get rid of the fat. A problem with this though, is that some of the bacteria in the milk prefer to stick to the fat and will therefore be discarded, resulting in underestimation of the bacteria content.

This project was carried out to investigate if the existing methods for bacteria in de-fatted milk also can be used for analysing bacteria in the whole milk and how big part of the microbiota that is connected to the milk fat fraction. Milk was spiked with four different bacteria that normally occur in milk; two types of *Lactobacillus reuteri*, *Escherichia coli* and *Staphylococcus aureus*. With the use of a DNA extraction kit, modifications of the original protocol were evaluated and the microbiota was studied through culturing, T-RFLP and qPCR. This resulted in an overview of the microbiota in the samples of whole milk and how well the extraction methods had proceeded. The project is a part of a big on-going survey where

the overall objective is to increase the long-term profitability of the Swedish dairy sector by improving quality management in the dairy value chain. It will contribute to the investigation of the best-suited milk for the huge varieties of cheeses.