# Bovine milk microbiota – methods matter

Josef Dahlberg
*Faculty of Veterinary Medicine and Animal Science*
*Department of Animal Nutrition and Management*
*Uppsala*

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2019

Acta Universitatis agriculturae Sueciae
2019:79

Cover: Two cows, and bacteria in milk
(by: Xavier and Ingrid Dahlberg)

ISSN 1652-6880
ISBN (print version) 978-91-7760-476-1
ISBN (electronic version) 978-91-7760-477-8

Print: SLU Service/Repro, Uppsala 2019

# Bovine milk microbiota – methods matter


## Abstract

The idea of a bacterial community, a microbiota, in milk with the purpose of inoculating the offspring's intestine for a better start in life is very appealing from an evolutionary perspective. The overall purpose of this thesis was to investigate if such bacterial community exists in bovine milk from healthy individuals and to describe factors that affect the microbiota.

Sequencing of the 16S rRNA gene was used for studying the microbiota. This method permits the identification of all bacteria present in a sample. Bacterial culturing according to mastitis diagnostics routines were included for reference and inflammatory status was followed by milk somatic cell count.

Three animal experiments were performed with the aims of describing variations in the milk microbiota due to milk sampling technique, milk fraction, over time and during *Escherichia coli* endotoxin induced mastitis. Additionally, the microbiota in milk samples collected directly from the udder quarter were compared to that found on the skin of the teat end and in the teat canal, as well as in udder quarter composite milk collected from the milking machine.

The results showed that udder quarter composite milk cannot be used as a representative sample to study the microbiota in the udder as the composition of the microbiota in these samples differed significantly from the microbiota in milk collected directly from the teat. Moreover, the microbiota found on the teat end and in the teat canal differed significantly from the microbiota in milk. Despite a careful and sterile handling of samples throughout the whole analytical process, contamination of the sequencing data proved to be a major contributor to the microbiota data in two of the included studies. A correlation between bacterial biomass and impact of contamination was found, showing that the problem increased with low concentration of bacteria in a sample. The degree of contamination of the sequencing data prohibited conclusions on the variation of microbiota over time and during *Escherichia coli* endotoxin induced mastitis.

The overall conclusion is that contamination, from sample processing, had such a substantial effect on the data that the presence of a microbiota in milk from healthy individuals could not be evaluated.



*Keywords:* milk microbiota, 16S rRNA, amplicon sequencing, contamination.



*Author's address:* Josef Dahlberg, SLU, Department of Animal Nutrition and Management, P.O. Box 7024, 750 07 Uppsala, Sweden

# Bovine milk microbiota – methods matter


## Sammanfattning

Idén om en bakterieflora, en mikrobiota, i mjölk som har till uppgift att inokulera avkommans tarmar för en bättre start i livet är ur ett evolutionärt perspektiv briljant. Det huvudsakliga syftet med denna avhandling var att undersöka huruvida en sådan bakterieflora finns i mjölk från friska kor och beskriva faktorer som påverkar den.

Primärt användes sekvensering av 16S rRNA genen för att studera microbiotan då denna metod gör det möjligt att identifiera alla bakterier som finns i ett prov. Bakterie odling enligt rutiner för mastit diagnostik användes som referens och inflammationsstatusen följdes genom att mäta mjölkens celltal.

Tre djurförsök utfördes i syfte att beskriva variationen in mjölkmikrobiotan till följd av provtagningsteknik av mjölk, mjölkfraktion, variation över tid och förändring under en bakterietoxin framkallad experimentell mastit. Microbiotan i mjölkprover som tagits direkt från spenen jämfördes med microbiotan som fanns på spenspetsen, i spenkanalen och i ett samlingsprov av mjölk från samma spene.

Resultaten visade att ett samlingsprov av mjölk från en juverdel inte utgör ett representativt prov för att studera mikrobiotan i juvret. Mikrobiotan i samlingsprovet skilde sig signifikant från mikrobiotan i prov tagna direkt från juverdelen, vilket sannolikt beror på att bakterier som fanns i mjölkningsmaskinen tillfördes. Vidare var mikrobiotan på spenspetsen och i spenkanalen signifikant skild från mikrobiotan i mjölk. Resultaten från experimenten som syftade till att beskriva variation över tid och förändringar vid experimentellt framkallad mastit var så påverkade av kontamination att några slutsatser inte gick att dra.

Kontaminerande bakterier från provhanteringen, bidrog väsentligt till datan i två av de inkluderade studierna. Ett samband mellan bakteriell biomassa och påverkan av kontaminering kunde påvisas, och att kontaminationsproblemet ökat med låg koncentration av bakterier i ett prov. Den övergripande slutsatsen är att kontaminering har i denna studie haft en så väsentlig effekt på mikrobiota data, att mikrobiota i mjölk från friska juverdelar inte kunde utvärderas.



*Nyckelord:* bakterier i mjölk, mikrobiota, 16S, DNA sekvensering, kontamination.



*Författarens adress:* Josef Dahlberg, SLU, Institutionen för husdjurens utfodring och vård, Box 7024, 750 07 Uppsala, Sweden

# Några ord om kor

Ost. Smör. Grädde. Filmjölk. Yoghurt. Entrecôte. Mjölkchoklad. Café au lait. Allt jag älskar att äta kommer från en ko. Jag älskar att i den svala friska vårkvällen höra fågelsången, känna lukten från slånbärsbuskarna, ljudet från betande ko-mular, ett stamp och en svansviftning. Känna glädjen i allt som vaknar till liv. Luta huvudet mot en varm ko som tagit paus för att idissla, känna det rytmiska tuggandet genom hela hennes kropp. Njuta av doft från kattfot, mandelblom och gullviva (var han månne i en kohage, Rönnerdahl?). Något som snabbt sveper förbi, en ladusvala, eller en var det en fladdermus? Jag är så glad över att mina barn får uppleva det. Om jag får barnbarn så hoppas jag innerligt att de också får göra det.

Och så älskar jag lukten av ensilage.

Så TACK Majros, Baronessa, Blomma, Bönan, Donna, Eda, Lilja, Hjärtros, Kronros, Rosa, Dagny, Brita, Fanny, Nancy, Nora, Märta, Ofelia, Perla, Prima, Sara, Stjärna, Eva, och alla ni andra 309 978 fantastiska, snälla, dumma, söta, kloka, fula, tjocka, smala,mjuka, varma, tillgivna, sparkiga, råmande, rogivande, idisslande, illa-luktande, go-luktande, gulliga svenska kor för allt ni förser oss med.

Jag är er evigt tacksam.

Elisabeth Andrée O'Hara

# Dedication

To X and I for always lightening up my life

*"I refuse to prove that I exist," says God, "for proof denies faith and without faith I am nothing."*
*"But," says Man, "the Babel fish is a dead giveaway, isn't it? It could not have evolved by chance. It proves that You exist, and so therefore, by your own arguments You don't. QED."*
*"Oh dear," says God, "I hadn't thought about that." and promptly vanishes in a puff of logic.*

Douglas Adams

# Contents

# List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Dahlberg J.*, Williams J., McGuire M., Peterson H., Östensson K., Agenäs S., Dicksved J. & Persson Waller K. Microbiota of bovine milk, teat skin and teat canal; similarity and variation due to sampling technique and milk fraction. (Submitted to Journal of Dairy Science, October 2019)

II Dahlberg J.*, Sun L., Persson Waller K., Östensson K., McGuire M., Agenäs S. & Dicksved J. (2019). Microbiota data from low biomass milk samples is markedly affected by laboratory and reagent contamination. *PLoS One*, 14 (6), pp. e0218257.

III Dahlberg J.*, Johnzon C-F., Sun L., Pelve E., Pejler G., Östensson K., & Dicksved J. Absence of changes in the milk microbiota during E. coli induced experimental bovine mastitis. (manuscript)

Paper II is reproduced with the permission of the publishers.

* Corresponding author.

The contribution of Josef Dahlberg to the papers included in this thesis was as follows:

I Performed the collection of milk samples and bacterial culturing. Performed the data interpretation and wrote the manuscript with regular input from supervisors.

II Performed the practical preparations for the animal experiment, the collection of milk samples and bacterial culturing. Performed the DNA extraction and amplicon library preparation as well as created the bacterial mock community. Performed the data analysis, presented the idea of data filtration and wrote the manuscript with regular input from supervisors. Corresponded with journal and revised the manuscript.

III Performed the collection of milk samples and prepared the amplicon library. Performed the data interpretation and wrote the majority of the manuscript.

# List of figures

# Abbreviations and definitions

| | |
|---|---|
| 16S rRNA | Component of the small subunit of the prokaryotic ribosome with the size unit 16S |
| Amplicon | Short strand of DNA that is the product of a PCR, synonym to PCR product |
| ASV | Amplicon Sequencing Variant |
| Bacterial taxonomy | Domain – Phylum – Class – Order – Family – Genus – Species – Sub-species or serovars or strain |
| DIM | Days In Milk |
| DNA | Deoxyribonucleic acid |
| Gene | Sequence of nucleotides that encodes the synthesis of a gene product |
| LPS | Lipopolysaccharides, an endotoxin from the cell wall of *Escherichia coli* |
| Microbiome | Genomes of the organisms of the microbiota |
| Microbiota | Community of microbes within a habitat. Within this thesis microbiota is referred to the bacterial community, but viruses, archaea and fungi also contribute to the microbiota |
| OTU | Operational Taxonomic Unit |
| PCoA | Principal Coordinate Analysis, a multivariate analysis displaying similarity between samples |
| PCR | Polymerase Chain Reaction, a method to amplify DNA. Polymerase, nucleotides, DNA and primers are mixed in a vial; by alternating the temperature, the DNA will be amplified |
| Polymerase | The enzyme that adds new nucleotides to DNA during PCR |
| Primer | A short strand of DNA that is complementary to the gene being amplified in PCR |

| | |
|---|---|
| RAPD-PCR | Random Amplification of Polymorphic DNA - PCR |
| Richness | Quantifies how many different types (of Genera, Families, OTUs') the dataset of interest contain |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| SCC | Somatic Cell Count |
| Sequencing | Determining the order of nucleotides in a strand of DNA |
| Sterile | Free of viable microorganisms |
| Taxonomy | The science of naming, defining and classifying groups of biological organisms based on their shared characteristics |
| V1-V8 | Variable region (1-8) of the 16S rRNA gene. There are 8 variable and 8 conserved regions on 16S rRNA gene |
| α-diversity | A calculated metric that account for the number and evenness of types (of Genera, Families, OTUs'..) within a sample |
| β-diversity | A calculated metric that account for the number and evenness of types (of Genera, Families, OTUs'..) between samples |

# 1 Background

## 1.1 Bacteria in milk - a historic perspective

Mankind has, for a long time, benefited from the effect of bacteria in milk. In the Bible (Genesis 18:8) it says that Abraham served curds and milk to his visitors. These curds are assumed to be cheese curds or possibly yoghurt. Much later, Pliny the Elder (AD 23-79) wrote about barbarous nations that were ignorant of making cheese but "yet they understand how to thicken milk and form therefrom an acrid kind of liquid with a pleasant flavour" (Pliny *et al.*, 1855). The Swedish scientist Carl Linnaeus described in "Flora Lapponica", published in 1732, how yoghurt was created by pouring milk over the leaves from the butterwort plant (*Pinguicula vulgaris)*. These are examples of written evidence that mankind has been using bacteria to process and/or preserve dairy products for a very long time. However, it was not until 1905 that Stamen Grigorov discovered *Lactobacillus bulgaricus* in yoghurt and it became evident that it is bacteria that causes the thickening and preservation of milk.

The effect of bacteria in milk has not always been beneficial. The first recorded case of mammary gland infection in women comes from the Persian Queen Atossa who lived from 550BC-475BC. Queen Atossa suffered from a boil, ulcer, swelling or abscess (depending on translation) in her breast that spread, but she was cured by the Greek physician Democêdes (Sandison, 1959). The correlation between bacteria and mastitis was discovered by Nocard and Mollereau from the Pasteur Institute who in 1887 isolated *Streptococcus agalactiae* in milk from a cows suffering from mastitis (Nocard & Mollereau, 1887).

Since the late 19$^{th}$ century there has been a discussion on the origin of bacteria in milk. In 1874, William Roberts (1874) presented a theory that milk was produced germ-free and performed a series of experiments to prove his point.

He experienced difficulties withdrawing milk free from "extraneous contamination" and had to develop a sampling technique that bypassed the teat canal to prove his point. According to Plastridge (1958), a theory that the udder is inhabited by a "normal flora" consisting mainly of "streptococci, micrococci and diphteroids" was presented soon after Roberts' theory. Knowledge about environmental contamination of milk samples has been available since the days of Roberts (1874) and today techniques on how to take an aseptic, non-contaminated milk sample from cows is well described (Oliver *et al.*, 2004).

For a long time, there has been agreement on the idea that milk is produced sterile and becomes contaminated at sampling (Rainard, 2017). In recent years this idea has been challenged when sequencing techniques have been used to study the bacterial composition in milk (Kuehn *et al.*, 2013; Oikonomou *et al.*, 2012).

## 1.2 Theory of a microbiota in milk

A bacterial community that always is present in an environment is referred to as a microbiota. The theory about the evolutionary benefits of a microbiota in milk is, in brief and freely interpreted, as follows: The gut microbiota is essential for the health and wellbeing of mammals, facilitating uptake of nutrients and providing the host with vitamins, etc. Consequently, establishing the gut microbiota is a highly important event. Milk is produced in order to provide the offspring with nutrients during the first period in life. A bacterial community present in milk would, thus, inoculate the intestines of the offspring during the critical phase of gut microbiota establishment and be beneficial for the offspring (Martin *et al.*, 2004).

## 1.3 Anatomy of the bovine udder

The bovine udder consists of four individual tubuloalveolar glands that are largely independent of each other. The number of glands per teat or nipple varies between species ranging from one per teat in bovines, caprines and ovines to 10-25 in humans (Sjaastad *et al.*, 2016). In bovines, the glands on the right and left side are separated from each other, and attached to the body by suspensory ligaments. The front and rear glands on each side are also separated by connective tissue.

Milk is produced by mammary epithelial cells that are organised into secretory alveoli and clusters of alveoli are organised into lobules. Milk is drained from the alveoli via a system of increasingly larger ducts which open into the gland cistern that drain into the teat cistern. As the ducts become larger,

the epithelial cell layer becomes thicker. In the alveoli and milk ducts, the epithelium is one or two-layered cuboidal and in the cisterns, the epithelium is stratified cuboidal. Within the larger ducts, the udder cistern and teat cistern milk can be stored between milkings, however the majority of milk is stored within the alveolar compartment (Dellmann *et al.*, 2006).

The mammary epithelial cells are connected to the basal lamina and to adjunct epithelial cells. Tight junctions between cells prevents the transfer of components from blood to milk and *vice versa*, forming the so-called blood-milk barrier. In the case of inflammation when immune cells are recruited to the mammary gland, the tight junctions between mammary epithelial cells are temporarily dissolved to allow active passage of immune cells by diapedesis into the milk (Rainard & Riollet, 2003). Interstitial tissue surrounding the alveoli and milk ducts provide structural support and contain blood vessels, lymph vessels, nerves and smooth muscles. The smooth muscles are involved in the milk-ejection reflex. The milk-ejection reflex is a neuro-endocrine reflex that shifts milk from the alveolar compartment to the udder cistern and thereby make milk available for removal through suckling or milking. It is initiated by sensory stimuli and leads to the release of oxytocin and when oxytocin reaches the smooth muscle cells in the udder they contract. Because of different orientation of the muscle cells, this leads to an increased pressure in the alveoli and a decreased resistance in the milk ducts and teat canal and milk is pushed towards the udder cistern and teat. Without the milk-ejection reflex, approximately only 20% of the milk stored in the udder is available for removal (Tančin & Bruckmaier, 2015).

Milk is evacuated from the gland via the teat canal. The teat canal is encircled with smooth muscle cells keep the canal closed between milkings. Stratified squamous epithelium in the teat canal produces antibacterial keratin that, together with the circular smooth muscles, prevents micro-organisms from entering the mammary gland. A schematic drawing of the bovine udder is presented in Figure 1.

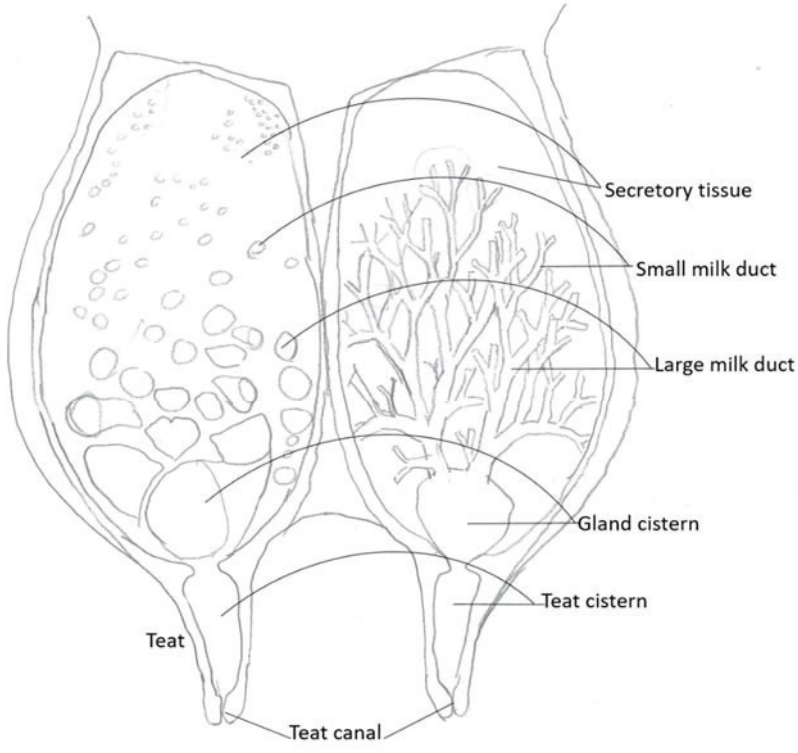


*Figure 1.* Schematic drawing of the bovine udder. Showing how milk ducts are organised on the right and a macroscopic view to the left.

## 1.4 Milk composition and synthesis

Milk is a complex emulsion consisting of water, fat, protein and carbohydrates and it is produced to meet the nutritional requirements of the offspring. It is said that milk and honey are the only foods in the human diet that are produced for the purpose of becoming feed.

In order to meet the nutrient requirements of their particular offspring, the composition of milk differs between mammal species. In dairy production, small variations in milk composition can be attributed to breed, individual, stage of lactation, diet and degree of milk removal. The typical composition of bovine milk is around 3% protein, 5% lactose, 4% fat and 87% water (Jenkins & McGuire, 2006). Approximately 80% of the protein in milk is caseins and the remaining 20% whey proteins. Caseins have the capacity to form micelles, a

conglomerate of casein, calcium, phosphorus and other substances. Lactose is the most common carbohydrate in milk, it is specific for mammalian milk and consists of galactose and glucose linked together to a disaccharide. Lactose is a carbohydrate source for the offspring and is a strong osmotic component, attracting water into the milk during milk synthesis. Milk fat is present in milk in the form of fat globules that comprise a triglyceride core surrounded by a lipid bilayer membrane. The membranes repel each other, keeping the fat droplets as an emulsion. Despite the repellent force of the membranes surrounding the lipid droplets, milk naturally separates into a lipid phase and an aqueous phase under the influence of gravity.

Most of the major organic components in milk are only synthesised in the mammary epithelial cell, and nowhere else in the body. Precursors for the synthesis, such as glucose, amino acids and fatty acids, are provided by blood and absorbed from the extracellular fluid. Other components such as vitamins, minerals and immunoglobulins are also brought to the udder by blood and are transported intact across the mammary epithelial cell into the alveoli (Sjaastad *et al.*, 2016).

## 1.5 Mastitis

Mastitis (from the Greek *mastos* = breast and *itis* = inflammation) is by definition an inflammation of the mammary tissue. Mastitis is most commonly the result of a bacterial infection, but it can also be caused by viruses, fungi, algae and trauma (Radostits *et al.*, 2007; Wellenberg *et al.*, 2002). The inflammatory status of the udder can be evaluated by measuring inflammatory indicators. Infection can only be diagnosed by bacteriological examination of the milk (see chapter 1.6). Mastitis is categorised as clinical (with visual signs of inflammation) or subclinical (without visual signs of inflammation) (IDF, 1987).

Mastitis is one of the most prevalent and costly diseases in dairy production leading to decreased milk production and, in clinical mastitis, it also impairs animal welfare and health (Hogeveen *et al.*, 2011).

There is a wide range of inflammatory indicators that can be measured in milk; somatic cell count (SCC) is the most reliable and most commonly used. The term SCC denotes the concentration of body cells, in contrast to bacterial cells, present in milk. In bovine milk it consists mainly of leucocytes (polymorphonuclear neutrophils (PMN), monocyte-macrophages and lymphocytes) and, to a smaller extent, epithelial cells from the secretory tissue. Milk from a healthy bovine mammary gland may contain up to 100 000 cells/ml (Hamann, 2002). A SCC >200 000 cells/ml is a clear indication of mammary gland inflammation. The inflammatory response of the mammary gland is swift,

complex, well studied and a detailed description is out of scope for this thesis. A comprehensible review was provided by Rainard and Riollet (2003).

A total of 137 microbial species, subspecies and serovars have been identified as mastitis-causing bacteria of the bovine mammary gland (Watts, 1988). Bacteria responsible for intramammary infections are largely separated by their main reservoir i.e. if they are coming from the environment or are spread between cows in a contagious way. Environmental bacteria are present in the surroundings of the cows and often have a faecal origin and cause infection if they enters the mammary gland. The main point of entry is through the teat canal (Figure 1). *Escherichia coli* is a typical example of an environmental bacteria; it is secreted in large amounts in faeces and causes a clinical mastitis with varying degrees of severity. *Staphylococcus aureus,* on the other hand, is a typical example of a contagiously spread bacteria; it is often isolated from skin and, if it enters the mammary gland, it is not uncommon that it causes chronic, subclinical infectious mastitis. Within the mammary gland *S. aureus* has been found in the alveoli and within the interstitial tissue (Hensen *et al.*, 2000), indicating that the bacteria has the ability to translocate within the udder and evade the immune system.

## 1.6 Methods to identify bacteria in milk

There are two main strategies to identify bacteria in milk; culture-dependent and culture-independent methods. Culture-dependent methods demand a living bacterial cell while culture-independent methods utilise the bacterial DNA. For culture-dependent methods, time until culturing and handling of samples can affect the results as the bacterial cells must be alive. For culture-independent methods, DNA from the cell must first be extracted and made accessible. When extracting DNA, cell disruption or cell lysis is a critical step; cells can be lysed mechanically, chemically or enzymatically. Often, several different lysing methods are used in the same protocol, either simultaneously or after each other. Cell lysis is often directly coupled to DNA purification in a DNA extraction protocol. Overdoing the lysis can cause DNA damage, while too little lysis can result in small or non-existing yields (Persing, 2011).

### 1.6.1 Bacterial culturing

To culture bacteria has been, and to a large extent still is, the golden standard for bacteriological examination of milk. Bacteria can be cultured in a lab if their basic requirements are met. The basic requirement may vary between different species of bacteria, but generally involve a sugar for energy, a nitrogen source

for protein production, a balanced pH, aerobic or anaerobic conditions and an optimal temperature. Media used to culture bacteria also often contain additional substances that are added for easier identification and differentiation of bacterial colonies. Typically, 10 µl of milk is spread on an agar plate containing the basic requirements, with or without additional substances, and then left to grow under aerobic conditions at 37°C for 24-48 hours. Viable bacteria will create a colony forming unit (cfu) on the agar plate that can be distinguished based on its appearance. Typing can be made based on the bacterial capacity to metabolize different sugars, Gram-staining properties or other methods (Madigan, 2012).

Matrix-assisted lased desorption/ionization time of flight (MALDI-TOF) mass spectrometry is a commonly used method to type different bacteria. Colonies from an agar plate are spread on a matrix and are vaporized with a laser beam; charged molecules will then be caught and conveyed in an electromagnetic field. The time it takes for the molecule to reach a sensor is recorded, giving a specific spectrum. The spectrum created from a colony is compared to a database of spectrums from known species. Although the cost per analysed sample is low, machine investment costs are high, and the technique is primarily and routinely used by larger laboratories (Fournier *et al.*, 2013).

For easily grown bacteria, culturing is an uncomplicated way to identify species. However, is it estimated that the majority of known bacterial species are not cultivable in a laboratory due to limitations in knowledge about their specific requirements (Torsvik *et al.*, 1998). Even though culturing is labour intensive, and some bacteria are hard to grow, one advantage with the method is that you have live bacteria that can be explored further with regards to their phenotypic and genotypic characteristics. For diagnostics of intra-mammary infection, culturing of bacteria has been used for a long time and the description of the methods and interpretation of results are well described by Oliver *et al.* (2004).

### 1.6.2 qPCR

Quantitative polymerase chain reaction (qPCR) is a culture-independent technique used to detect and quantify specific genes in a sample. It monitors the amplification of a targeted DNA sequence during PCR. In addition to the conventional PCR, the qPCR reaction contains a fluorophore that re-emits light upon excitation. The fluorophore will bind either non-specific to double stranded DNA or sequence-specific and emit light when the specific DNA-sequence has been amplified. The intensity of the re-emitted light will increase with every cycle in the PCR, and the abundance of DNA in the original sample can be calculated from the number of cycles needed to reach a specified light intensity threshold (Ct-value) (Arya *et al.*, 2005). For diagnostics of intra-mammary

infections, commercial qPCR-kits are available to measure the abundance of DNA from a specified number of bacteria.

### 1.6.3 16S rRNA gene sequencing

16S rRNA gene sequencing, sometimes called 16S amplicon sequencing, is a culture-independent method commonly used to identify and compare the composition of bacteria communities present in samples. The technique is primarily used for research purposes and a commercial product for diagnostics of intra-mammary infections is not yet available. Several different platforms for sequencing exist, where Illumina sequencing it the most commonly used.

In brief, bacterial DNA from a sample is amplified in a PCR reaction. In the PCR, a primer (a short nucleotide strand) binds to the flank of the 16S rRNA gene and polymerase is responsible for making a complimentary copy of the gene. Primers can contain additional nucleotides, making the produced amplicon longer than the gene. The additional nucleotides are designed to function as adaptors compatible with the selected sequencing technology. The additional nucleotides can also be designed with a specific nucleotide sequence, these tags or barcodes, make it possible to pool samples and sequence several samples in the same run. In the sequencer, additional amplification of the gene is performed before the sequencing commences. During sequencing fluorescent labelled nucleotides are incorporated and recorded. After sequencing, the data is quality controlled and low quality reads and PCR artefacts (chimeric sequences) are removed. Further, sequences with high homology are grouped together in an OTU or ASV-table that is matched against existing sequences from bacteria. The final result is a list with the bacteria discovered and their relative abundance. Disadvantages with the technique are the high dependence on PCR reactions, and PCR primers, and that results are displayed as relative abundance and not absolute numbers (Morey *et al.*, 2013; Persing, 2011; Metzker, 2010; Morozova & Marra, 2008). A schematic picture of the process is presented in Figure 2.

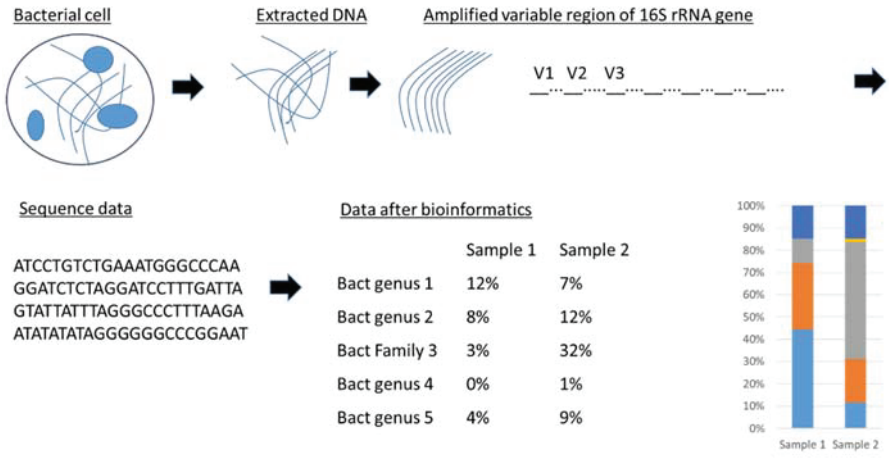


*Figure 2.* Schematic picture of the process for 16S rRNA gene sequencing.

### 1.6.4 Ribosomal RNA

The ribosome in the living cell is responsible for the translation of a nucleotide (RNA) strand to a strand of amino acids, the first step in protein synthesis. The ribosome consists of a large and a small sub-unit, and each sub-unit consists of ribosomal proteins and ribosomal RNA (rRNA). The role of rRNA in the ribosome is to give structure and to act as scaffolding for ribosomal proteins. As protein synthesis is essential for the living cell, the rate of evolution and mutations in the ribosome is low. The 16S rRNA is encoded on a gene, approximately 1500 base pairs long, that consists of eight highly conserved regions and as many variable regions where mutations have been allowed to occur. The nucleotide sequence in the variable regions is used to identify species of bacteria. The gene encoding for the 16S rRNA can exist in multiple copies in the bacterial genome. The size of rRNA is measured in the sedimentation unit Svedberg (S) (Cooper, 2000).

## 1.7 Mammalian microbiota

As the microbiota is essential for mammalian survival, the mammalian body has developed to make co-inhabitation possible. In the interphase between microbes and host, numerous immune cells are present, and strategies to balance the interactions have been developed. The strategies for co-inhabitation vary with body-site. The upper respiratory tract continuously produces mucus that captures and flushes micro-organisms away, and the skin consists of a thick layer of squamous epithelial cells that are continuously being regenerated. In the vagina,

the epithelium is thick and is continuously flushed with mucus produced by the cervix and uterus. The cervix acts as a barrier to the uterus, it has a small diameter and an epithelium that changes from stratified squamous on the vaginal side to single columnar with numerous mucus glands on the uterus side. The intestines and the microbes in the intestines are protected from influence of external bacteria by the low pH in the stomach. In the intestines there is a thick layer of mucus between the epithelial cells and bacterial community (Human Microbiome Project, 2012; Dellmann *et al.*, 2006). Thus, there is a thick barrier of cells or mucus in the interphase between the microbiota and the host.

### 1.7.1 Milk microbiota

In the early 21st century, parallel to when DNA sequencing became cheaper, faster and more available for research, the interest to study bacterial communities in various habitats was spurred. In 2007, the USA launched the Human Microbiome Project, a huge project with the aim of understanding the microbial flora involved in health and disease (Turnbaugh *et al.*, 2007). In 2008, the EU launched a similar program called Meta HIT (Dusko Ehrlich & MetaHIT consortium, 2010). During the same period, the enthusiasm to study bacteria in milk with new techniques evolved. The first publications reporting on a microbiota in bovine milk using DNA sequencing were published in 2012 and 2013 (Kuehn *et al.*, 2013; Bhatt *et al.*, 2012; Oikonomou *et al.*, 2012). Since then, there have been several publications using DNA sequencing and associating the bovine milk microbiota to SCC (Oikonomou *et al.*, 2014), intra-mammary infection (Ganda *et al.*, 2017; Ganda *et al.*, 2016), history of intra-mammary infection (Falentin *et al.*, 2016) and bedding type (Metzger *et al.*, 2018), to mention a few. A list of available publications where sequencing techniques have been used to study the bovine milk microbiota in samples collected from the udder is presented in Table 1. Latterly, doubt about the presence of a microbiota in milk has been expressed by Rainard (2017), who questions the reliability of the 16S rRNA sequencing techniques, methods to collect samples and “logical implications” of a milk microbiota. In the last year, difficulties obtaining reliable results using 16S rRNA sequencing to study the bovine milk microbiota have also been described in publications (Dahlberg *et al.*, 2019; Taponen *et al.*, 2019).

Studies in humans have covered both the existence of a microbiota in milk and how it inoculates the intestines of the offspring. It has been shown that bacteria with the same RAPD-PCR profile (Albesharat *et al.*, 2011; Martin *et al.*, 2003) or of the same strain (Martin *et al.*, 2012) are shared between milk from lactating women and their infant’s faeces. Lactic acid bacteria that *in vitro*

inhibits the growth of the known pathogen *Staphylococcus aureus* have been isolated from human milk (Heikkila & Saris, 2003). Further, oral intake of lactic acid bacteria has, in humans, been shown to diminish the presence of staphylococci in milk and reduce clinical signs of mastitis (Arroyo *et al.*, 2010; Jimenez *et al.*, 2008). Additionally, the same species of lactic acid bacteria that was given as an oral supplement was also isolated from the milk of the study subjects. The discovery that lactic acid bacteria taken orally can be isolated in milk prompted the idea of an active translocation of bacteria from the mother's intestine to the mammary gland, the so-called entero-mammary pathway (Martin *et al.*, 2004). The existence of an entero-mammary pathway would add strength to the theory of a microbiota in milk and it has been explored in humans (as mentioned), mice and dairy cows. Perez *et al.* (2007) and de Andres *et al.* (2017) found support for the existence of an entero-mammary pathway in mice and Young *et al.* (2015) also found some support in their study on dairy cows.

Even though important lessons about biology and physiology can be learned from studies of other species, differences between species must be kept in mind. A breast-fed human infant will likely ingest bacteria present on the skin and identifying the same species in the infant's faeces would not be surprising. During breast-feeding a retrograde backflush into the mammary duct has been observed (Fernandez *et al.*, 2013), providing an ideal route of inoculation of the human breast with bacteria from the infants mouth. There are also anatomical differences in the mammary gland between humans and bovines; the human nipple is connected to several mammary glands and the human glands do not contain a cisternal compartment, making it difficult to collect samples of human milk that have not been in contact with the skin.

A commonly used argument against the presence of a microbiota in the bovine mammary gland is based on the anatomical structure within the mammary gland (Rainard, 2017). The mammary gland epithelium is not protected by a barrier similar to that seen on other body sites that harbours a microbiota. The barrier for the bovine mammary gland is in the teat canal; the teat canal has a small diameter, is encircled with smooth muscle cells, is opened by a neuro-endocrine reflex and the epithelium is keratin producing and stratified squamous, making it difficult for bacteria to pass.

Arguments for and against the presence of a microbiota in milk are strong, the contribution of this thesis will be discussed in a later section.

*Table 1*. List of publications exploring the bovine udder milk microbiota using 16S rRNA sequencing methods.

| Author | Year | Journal | Title |
|---|---|---|---|
| Bhatt *et al.* | 2012 | Journal of applied microbiology | Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing |
| Oikonomou *et al.* | 2012 | PLoS One | Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA |
| Kuehn *et al.* | 2013 | PLoS One | Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis |
| Oikonomou *et al.* | 2014 | PLoS One | Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters |
| Young *et al.* | 2015 | PeerJ | Transfer of intestinal bacterial components to mammary secretions in the cow |
| Zhung *et al.* | 2015 | Journal of the science of food and agriculture | Characterization of bacterial community of raw milk from dairy cows during subacute ruminal acidosis challenge by high-throughput sequencing |
| Falentin *et al.* | 2016 | Frontiers in microbiology | Bovine teat microbiome analysis revealed reduced alpha diversity and significant changes in taxonomic profiles in quarters with a history of mastitis |
| Ganda *et al.* | 2016 | Scientific Reports | Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin |
| Bonsaglia *et al.* | 2017 | Scientific Reports | Milk microbiome and bacterial load following dry cow therapy without antibiotics in dairy cows with healthy mammary gland |
| Doyle *et al.* | 2017 | Applied environmental microbiology | Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study |
| Ganda *et al.* | 2017 | Microbiome | Normal milk microbiome is re-established following experimental infection with Escherichia coli independent of intramammary antibiotic treatment with a third-generation cephalosporin in bovines |
| Lima *et al.* | 2017 | Journal of Dairy Science | The bovine colostrum microbiome and its association with clinical mastitis. |
| Oultram *et al.* | 2017 | Frontiers in veterinary science | A metataxonomic approach could be considered for cattle clinical mastitis diagnostics |
| Cremonesi *et al.* | 2018 | PLoS One | Milk microbiome diversity and bacterial group prevalence in a comparison between healthy Holstein Friesian and Rendena cows. |

| Author | Year | Journal | Title |
|---|---|---|---|
| Derakhshani *et al.* | 2018 | Microbiome | Association of bovine major histocompatibility complex (BoLA) gene polymorphism with colostrum and milk microbiota of dairy cows during the first week of lactation |
| Derakhshani *et al.* | 2018 | Journal of Dairy Science | Composition of the teat canal and intramammary microbiota of dairy cows subjected to antimicrobial dry cow therapy and internal teat sealant |
| Lima *et al.* | 2018 | PLoS One | Evaluation of milk sample fractions for characterization of milk microbiota from healthy and clinical mastitis cows. |
| Metzger *et al.* | 2018 | Journal of Dairy Science | Influence of sampling technique and bedding type on the milk microbiota: Results of a pilot study |
| Metzger *et al.* | 2018 | Frontiers in veterinary science | A cohort study of the milk microbiota of healthy and inflamed bovine mammary glands from dry off through 150 days in milk. |
| Pang *et al.* | 2018 | Frontiers in microbioology | Insights into the bovine milk microbiota in dairy farms with different incidence rates of subclinical mastitis |
| Dahlberg *et al.* | 2019 | PLoS One | Microbiota data from low biomass milk samples is markedly affected by laboratory and reagent contamination |
| Savin *et al.* | 2019 | PLoS One | Faecalibacterium diversity in dairy cow milk. |
| Taponen *et al.* | 2019 | Veterinary research | Bovine milk microbiome: a more complex issue than expected |
| Vasquez *et al.* | 2019 | Journal of Dairy Science | The microbiome of Escherichia coli and culture-negative nonsevere clinical mastitis: Characterization and associations with linear score and milk production |

# 2 Aims

The overall aim of this thesis was to evaluate the presence of a bacterial community in milk from clinically healthy lactating dairy cows and to describe factors that affect the bacterial community. Specific aims were:

- To evaluate the effect of sampling technique and milk fraction on bovine milk microbiota data and to compare the microbiota in milk to microbiota on the teat end and in the teat canal.
- To describe the composition and temporal stability of the bacterial microbiota in bovine milk from healthy udder quarters.
- To describe the changes in milk microbiota composition during the course of LPS-induced experimental mastitis.
- To describe the effect of contamination on bovine milk microbiota data.

# 3 Materials and Methods

A summary of the materials and methods applied in this thesis is given below. Detailed descriptions are found in papers I-III.

## 3.1 Cows

In total three animal experiments were performed to generate data for papers I-III. The experiments were performed at, and all the cows were provided by, the Swedish Livestock Research Centre in Uppsala. A total number of 32 cows were included in the different experiments. Recruited cows were in mid to late lactation with days in milk ranging from 145 to 316 and were of the Swedish Red (SR) or Swedish Holstein (SH) breed. Cows included in papers I-II were in lactation 1-3 and cows included in paper III were in their first lactation. The SCC in composite milk, which is the pooled milk from all quarters, was below 100 000 cells/ml for six to eight weeks in selected animals before the onset of the experiment in papers I and II. The animals in paper III had an SCC below 65 000 cells/ml in the selected udder quarter before the intervention. At the research herd, the cows were kept in a loose housing system and fed a standard diet with *ad libitum* silage and individual concentrate rations to meet the calculated nutrient requirements for their individual milk production. The cows were milked either in a voluntary milking system or twice per day in an automatic rotary. All animal experiment protocols were approved by the Uppsala Ethics Committee.

## 3.2 Experimental design

*Paper I*

In the animal experiment for paper I, all samples were collected during one milking using a milking machine developed for experimental milkings that allows quarter level milking and separation of milk from the different quarters. Five cows were included and samples were taken from all udder quarters separately, giving samples from 20 udder quarters in total. Sampling was performed in a tie-stall unit within the research barn and cows were moved there at least one hour before sampling. Before milking, swab samples from the teat end and the teat canal were collected as well as hand stripped milk samples and milk samples taken through a teat canal cannula. After milking cows were sedated with an intramuscular injection of xylazin and post-milking samples were collected by hand stripping, through a teat canal cannula and by a trans-teat wall needle aspirate. An udder quarter composite milk sample was collected from the milking machine. The different sampling techniques are illustrated in Figure 3.

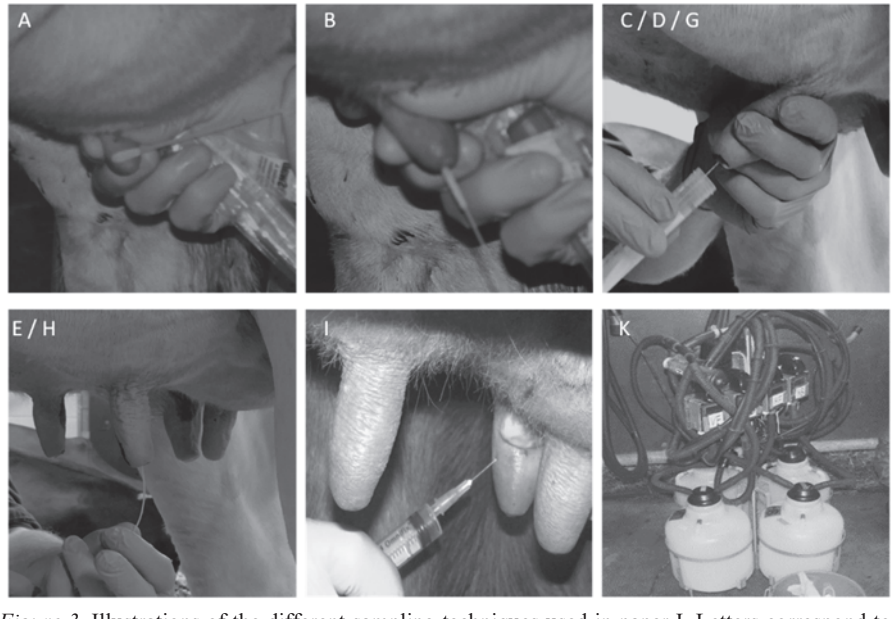


*Figure 3.* Illustrations of the different sampling techniques used in paper I. Letters correspond to the different samples taken and are in detail described in paper I. For clarity; teat end swab (A), teat canal swab (B), hand stripped milk samples (C, D, G), teat canal cannula samples (E, H), trans-teat wall needle aspirate (I), quarter milk machine sample (K). Samples A-E were taken directly before machine milking, samples G-I were taken directly after machine milking, sample K is a composite milk sample from individual quarters.

*Paper II*

In the animal experiment for paper II, hand stripped quarter level milk samples were collected from nine healthy cows directly before milking in the automatic rotary. The cows were sampled twice per week during four consecutive weeks.

*Paper III*

Two experimental rounds with identical setup generated the data for paper III; the first round included 8 cows and the second 10 cows. In this experiment, mastitis was induced by infusion of *E. coli* endotoxin (LPS) in one udder quarter on every other cow. Local inflammatory reactions were assessed by palpation and observation of the udder, and the body temperature was measured rectally. In total, mastitis was induced in one udder quarter of nine cows and the other nine cows were infused with saline solution serving as controls. For easier sampling and better control of animal welfare the cows were housed in a tie-stall unit within the research barn during the phase with frequent sampling and when pronounced clinical signs of mastitis were present. Hand stripped milk samples were collected on four specified occasions before infusion and eight specified occasions after infusion over a time period of 20 days.

## 3.3 Laboratory methods

Samples were placed in an ice filled container directly after sampling. They were transported to the laboratory where they were brought to room temperature, mixed and aliquoted. One aliquot was used for bacterial culturing and SCC measurement, the other aliquots were kept in -80°C conditions until DNA extraction. Only sterile equipment was used in contact with the samples.

Bacterial culturing was performed on all milk samples collected except from the first round of the experiment generating data to paper III. Milk was inoculated on agar plates containing 5% bovine blood and 0.05% esculine. In papers I and II 10 µl of milk was inoculated, and in paper III both 10 and 100 µl of milk were inoculated in parallel. SCC was measured on all milk samples. A DeLaval Cell counter was used in papers I and II, in paper III the SCC was measured with a Fossomatic 5000 from FOSS or a FTIR 300HP from Perten Instruments.

Before DNA from the milk samples was extracted, an extensive pilot study of nine different DNA extraction protocols from seven different providers was performed in different labs with varying amount of starting material. The DNA extraction protocol that yielded the most DNA with the smallest problem with contamination in negative controls was chosen for the milk samples. Evaluation

of DNA extraction protocols continued, and DNA was extracted using similar types of extraction kits but from different providers and with different modifications to the protocol. In paper I, a modified version of QIAamp DNA Mini Kit from Qiagen was used with mechanical and enzymatic lysis of the cell wall. In paper II, the PowerFood Microbial DNA isolation kit from MO BIO Laboratories was used with mechanical and chemical lysis of the cell walls. For paper III, the DNA extraction protocol used in paper II was no longer available on the market. Instead the DNeasy PowerFood Microbial kit from Qiagen was used with mechanical and chemical lysis of the cell wall. Before DNA extraction 1 ml of milk was centrifuged at 13 000 g for 5-10 minutes for content separation. In papers I and II bacterial DNA was extracted only from the cell pellet and in paper III from the cell pellet and fat layer.

The variable regions amplified and sequenced were V1-V3 in paper I and V3-V4 in papers II and III. Sequencing was performed on an Illumina MiSeq sequencer with v3 chemistry in all studies. Three different laboratories were engaged in sequencing; the University of Idaho Genomics Resources Core, Science for Life Laboratory in Uppsala and the Science for Life Laboratory in Solna.

Negative (blank) control samples from DNA extractions and PCR reactions were included in all papers but were only sequenced in papers II and III.

In papers II and III, a bacterial-based mock community with defined levels of bacteria were included as positive controls in the sequencing. The mock community was created from five commonly occurring mastitis causing pathogens with different characteristics regarding size, Gram-staining and number of 16S rRNA gene copies. Bacterial numbers were determined by manual counting using a Bürker counting chamber. The mock community was prepared with equal numbers of cells in three different dilutions ($10^7$, $10^5$ and $10^3$ cells of each bacterial species per ml). In paper III an additional, commercially available DNA-based mock community was included.

DNA from 44 milk samples collected from endotoxin infused cows were sequenced twice. Samples were processed differently before the two sequencing runs, either according to the protocol used in paper II or according to the protocol used in paper III. These samples were included in an analysis of repeatability in paper III.

## 3.4 Data editing and statistics

Different methods to analyse Illumina data were used in the different papers. The principal platforms for Illumina data analysis were; dbcAmplicon in python

in paper I, Quantitative Insights Into Microbial Ecology (QIIME) in paper II and QIIME2 in paper III.

Data sets with bacterial taxonomy assigned to genus level, or lowest taxonomy level possible, were used for all further analyses. Descriptive analyses on taxonomy data, statistical calculations, data filtration and multivariate analyses were performed using Microsoft Excel, PAST (Hammer *et al.*, 2001) and R (R Core Team 2019) in papers I-III.

In papers II and III a threshold of more than 1% abundance in negative controls was used to identify contaminants. These contaminants were compared to the contaminants identified by the "decontam" R-package (Davis *et al.*, 2018) in paper II. In paper III an alternative method to identify contaminants was used based on the "decontam" R-package and unexpected presence of genera in the low biomass mock community. Identified contaminants were filtered (subtracted) from the data in papers II and III. Analyses were performed on the data before and after filtration.

# 4 Results

A summary of the most important results generated in papers I-III is given below. Detailed descriptions of the results are found in the papers.

### 4.1.1 Paper I

The microbiota on the teat end, in the teat canal and in udder quarter composite milk collected using a milking machine differed in its composition from the microbiota in milk collected directly from the udder quarter. We detected no differences in the milk microbiota composition between hand stripped milk samples and milk samples taken through a teat canal cannula before or after milking, nor between milk samples taken as a trans teat wall needle aspirate after milking. Bacteria belonging to the genera *Stenotrophomonas* and *Pseudomonas* were significantly more abundant in udder quarter composite milk samples compared to milk samples collected directly from the udder quarter, or swab samples from the teat end and teat canal. Bacteria of the genus *Dyella* was significantly more abundant in milk samples collected directly from the udder quarter compared to swab samples collected from the teat end and teat canal or udder quarter composite milk samples. The similarity between the different types of collected samples is illustrated in Figure 4.

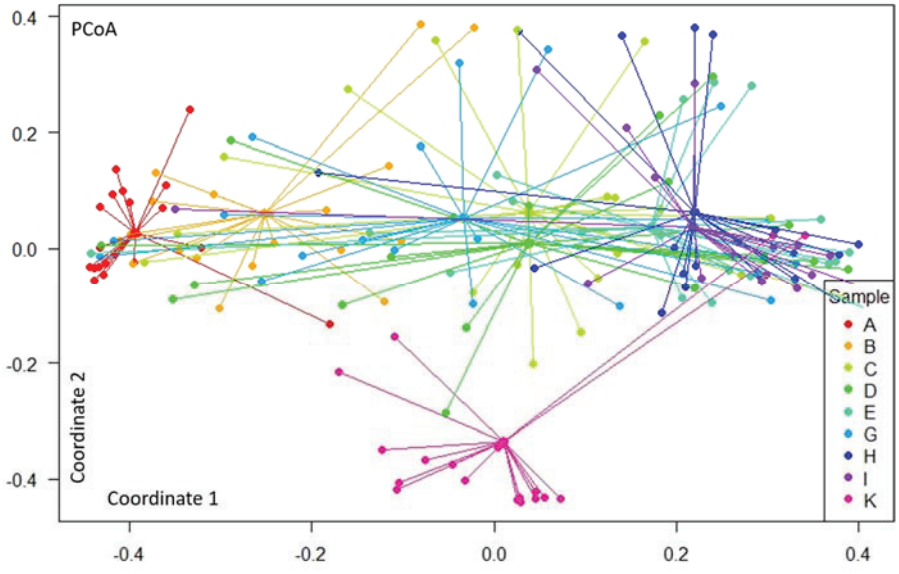


*Figure 4.* PCoA for the microbiota composition in different samples from paper I. In the figure each dot is a sample, samples with high similarity cluster together while samples with low similarity are further apart. Teat end swab (A), teat canal swab (B), hand stripped milk samples (C, D, G), teat canal cannula samples (E, H), trans-teat wall needle aspirate (I), quarter milk machine sample (K). Samples A-E were taken directly before machine milking, samples G-I were taken directly after machine milking, sample K is a composite milk sample from individual quarters.

### 4.1.2 Paper II

In total 288 milk samples with various amount of bacterial growth were collected and sequenced. Evaluation of the composition and temporal stability of the milk microbiota was hampered by background contamination, despite careful and aseptic sample processing. The primary contaminant, *Methylobacterium,* was found in all samples and had a strong effect on the data. The amount of bacterial growth in milk samples was utilised for method evaluation. We observed an increasing impact of contamination with decreasing microbial biomass, illustrated in Figure 5. The contaminating taxa became dominant in samples with less than $10^4$ bacterial cells per ml. Identification of contaminants and data filtration decreased the number of available reads by 72%, but only a minor impact on number of identified taxa was observed. After data filtration, consecutively taken samples had a larger similarity than random comparisons, suggesting that data filtration can be useful to identify biologically relevant associations in milk microbiota data.

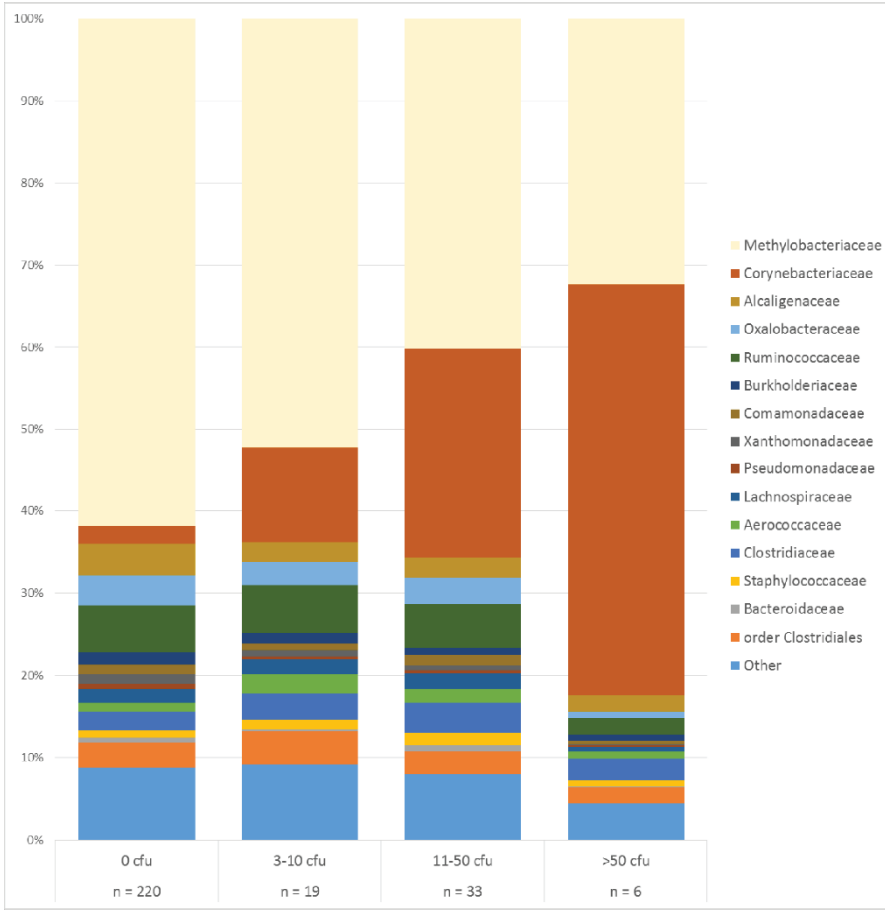


*Figure 5.* Relative abundance of the 15 most common families or order of bacteria found in the milk samples from paper II. The milk samples are grouped by number of colony forming units (cfu), where more cfu´s indicate higher bacterial biomass.

### 4.1.3 Paper III

*E. coli* endotoxin-infused cows responded with transient clinical signs of inflammation and increased SCC while no response was observed in the control cows. In the sequenced negative (blank) controls, a diverse bacterial community consisting of 129 different bacterial taxa was identified. There was a large overlap of bacterial taxa identified in the negative controls and in the milk samples. The observed increased impact of contamination with decreased microbial biomass in paper II was confirmed in the bacterial-based mock communities. In the milk microbiota data, no association with the inflammatory response was detected when analysed with multivariate methods or by Shannon

diversity, illustrated in Figure 6. A large proportion of the sequencing data (65-88% depending on the model used) was classified as contamination. Data filtration was used to clear the data from contamination, however, none of the models generated results that were associated with the degree of the inflammatory reaction. After data filtration, several samples collected during the first hours after LPS-infusion clustered separately, this effect was at large due to the presence of the genus *Stenotrophomonas*. The genus *Stenotrophomonas,* which was not classified as contamination, was abundant in milk samples collected during the first hours after LPS-infusion but absent in samples taken before the LPS-infusion, and barely detected in samples from control quarters. The measured similarity between samples from different sequencing runs was 42%. The results indicate that bacterial taxa of unknown origin constitute a major part of the data, preventing the effect of the intervention to be evaluated.

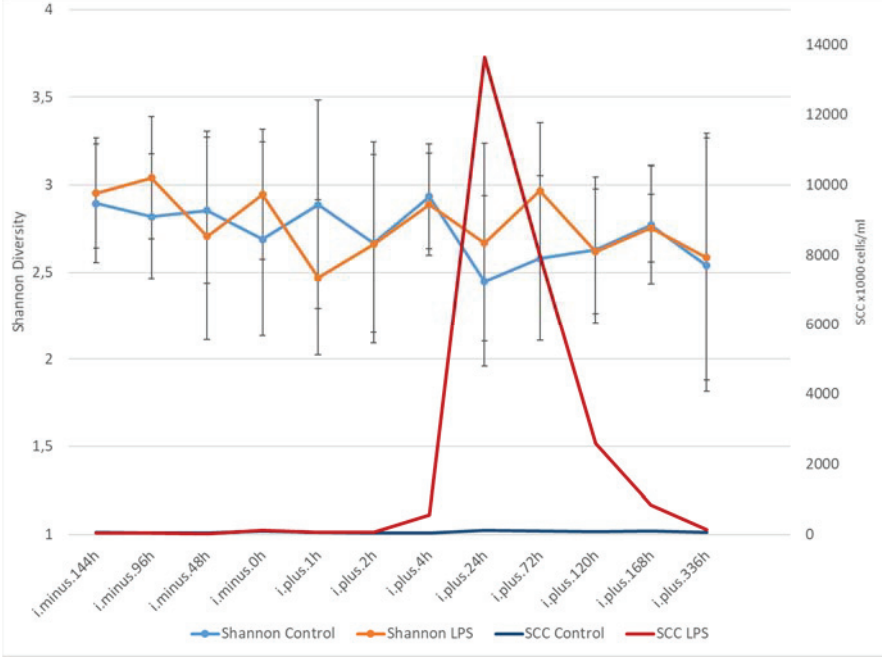


*Figure 6.* Shannon diversity for control and LPS-infused udder quarters in relation to time of infusion from paper III. Shannon diversity expressed as mean ± SD. On the x-axis, i = infusion. As a reference the mean SCC x1000 cells/ml for control and LPS-infused udder quarters is included with values on the right y-axis.

# 5 General discussion

*The effect of contamination on milk microbiota data*

Contamination can be a significant problem in studies of microbiota, especially when the bacterial biomass is low (Glassing *et al.*, 2016; Salter *et al.*, 2014). The results in papers II and III show that contamination can be a big problem in microbiota studies of milk and confirm that contamination becomes more abundant in milk samples with lower bacterial biomass.

Inclusion of negative and positive controls, as well as using more than one kit for DNA isolation and/or different batches of kits, as applies in our studies, has latterly become a recommendation for studies of the microbiota (de Goffau *et al.*, 2018). These recommendations have been developed as a consequence of unexpected discoveries about a microbiota contradicting previous knowledge in various environments. For example, inclusion of controls have resulted in publications that dismiss the presence of a microbiota in the human placenta (de Goffau *et al.*, 2019; Lauder *et al.*, 2016). Methods to identify and filter contamination have been developed and evaluated (Karstens *et al.*, 2019; Davis *et al.*, 2018; de Goffau *et al.*, 2018), although for samples with low bacterial biomass, all methods described have limitations (Karstens *et al.*, 2019).

The inclusion of controls in papers II and III led to the discovery that a large proportion of available data was due to contamination (72% in paper II, 65-88% in paper III, depending on method used). While the contamination largely was dominated by one genus in paper II, it was more complex and diverse in paper III. In paper III, two different kits were used for DNA extraction and utilised to compare the results from the double-sequenced samples. The average similarity between different sequencing runs was found to be 42% (based on Dice similarity index). Interestingly, the similarity between negative controls in paper III ranged from 26-32%, these results demonstrate the diversity of the contamination and indicate that it comes from multiple sources. We concluded

that the majority of data available in paper III has an unknown origin making conclusions about the microbiota in milk difficult to draw.

Data filtration was used as a method to clear the data set from contamination. After filtration, data was re-analysed with the purpose of finding biologically relevant results that were comparable with previously published studies. In paper II, the increased similarity between consecutively taken samples that was observed after data filtration was comparable with previously published results (Derakhshani *et al.*, 2018) and apparently improved the data set. In paper III, data filtration did not lead to results that were in line with the gradual development of the inflammatory reaction nor expected compared to previous publications (Ganda *et al.*, 2017; Ganda *et al.*, 2016). Whether the different results of data filtration in papers II and III were due to, a) limitations in the method to identify contamination or b) the pattern of contamination requires further investigation.

Determining the origin of contamination in milk samples can be difficult. Roberts (1874) was the first to show that milk samples can easily be contaminated at sampling. In 2014, Salter *et al*. (2014) showed that the contamination can be introduced during sample preparation and patterns vary between different DNA extraction kits. In paper II *Methylobacterium* was found in all samples, a pattern that would be expected for a contamination that is introduced during sample processing. In paper III *Stenotrophomonas* was not identified as a contamination, but was only present in milk samples collected after endotoxin infusion and barely detected in milk samples from control cows. This pattern of appearance would be expected from a contaminant that was infused together with the endotoxin. Further, in paper I *Stenotrophomonas* was more abundant in milk samples collected from the milking machine. Based on the natural habitat of *Stenotrophomonas,* water, it is not impossible that this genus was introduced from water residues after washing of the milking machine.

In paper I, *Dyella* was unexpectedly one of the most abundant genera in milk samples collected directly from the udder quarter. *Dyella* has, to the best of our knowledge, never been discovered in milk and the presence of this rare and soil dwelling bacteria in milk was highly surprising. Until the presence can be confirmed with other methods, it cannot be excluded that the *Dyella* is a contaminant.

### *Evaluation of sampling technique and milk fraction*

Studies on how sampling technique affects the microbiota in milk are rare, although the subject has received attention in studies assessing discoveries of specific bacteria (Friman *et al.*, 2017; Hiitio *et al.*, 2016; Bexiga *et al.*, 2011). In theory, the mammary gland microbiota could differ between the secretory tissue

and the cisternal compartments. Such difference would be possible to identify by collecting milk from different milk fractions, as the milk that has been stored in the secretory tissue would be evacuated last. Our data does not support the theory of different microbiota within the mammary gland. Metzger *et al.* (2018) used 16S rRNA gene sequencing to compare the microbiota in udder composite, udder quarter and gland cisternal milk samples. In accordance with our results, they did not find a difference in the microbiota between udder quarter milk samples and gland cisternal milk. An important difference in the sampling protocol for the composite milk sample was that Metzger et al (2018) hand stripped milk aseptically from each quarter and pooled the milk to get a whole-udder composite sample while we used a special milking machine that allowed for quarter level milking and collection of an udder quarter composite milk sample. Thus, the studies used different types of composite milk sample. Nevertheless both groups found a difference between composite milk samples and samples taken from one quarter.

### *Comparison of the microbiota in milk to the microbiota on the teat end and teat canal*

Studies of the microbiota on the bovine teat skin or teat canal have been performed before (Falentin *et al.*, 2016; Braem *et al.*, 2012; Verdier-Metz *et al.*, 2012; Gill *et al.*, 2006), but they have rarely included a comparison between the microbiota on the teat and the microbiota in milk. Doyle *et al.* (2017) compared the microbiota on the teat skin to the microbiota of whole-udder composite milk samples. In contrast to our results, they found a large overlap between the microbiota on the skin and in the milk. An important difference in the sampling protocol between our study and the study of Doyle *et al.* (2017) is that they pooled the teat swab samples from one cow and compared with a whole-udder composite milk sample from the four quarters while we compared the microbiota from one teat to a composite milk sample of the same quarter.

In our study the microbiota on the teat end and in the teat canal differed from the microbiota found in milk samples aseptically collected directly from the udder quarter. Milk sampling techniques have been shown to affect findings of specific bacteria in milk (Friman *et al.*, 2017; Hiitio *et al.*, 2016; Bexiga *et al.*, 2011), supporting the theory that milk gets contaminated when passing through the teat canal. Our results indicate that such contamination has a minor impact on the milk microbiota.

*The composition and temporal stability of milk microbiota*

The design of the experiment generating the milk samples in paper II was created in order to increase the knowledge on temporal stability of milk microbiota from healthy cows. Analyses of the milk microbiota from repeated samplings from the same quarter have been performed previously (Derakhshani *et al.*, 2018; Ganda *et al.*, 2017; Ganda *et al.*, 2016), although those studies included an intervention (mastitis or dry period) that theoretically would have affected the results. Unfortunately, the degree of the sequencing data in this study made it impossible to draw conclusions on temporal stability. Derakhshani *et al.* (2018) observed a high degree of individuality in the samples, meaning that the microbiota profile from udder quarters of the same cow were more similar to each other than udder quarters of other cows. In paper II, similar results were only found in the data after filtration. After data filtration, consecutively taken samples became more similar than random comparisons of samples, indicating that data filtration can be useful to find biologically relevant associations in a contaminated data set.

*Changes in milk microbiota during experimentally induced mastitis*

In paper III, infusion of *E. coli* endotoxin was used to study the effects of inflammation on the milk microbiota. Infusion of live *E. coli* in the bovine mammary gland has been used to study changes in the milk microbiota by Ganda *et al.* (2017). In contrast to Ganda *et al.* (2017) we could not identify an effect on the microbiota due to the intervention. This could, however, be due to the different strategies used to induce mastitis, *E. coli* endotoxins do not contain any DNA that will be detected in the analyses of the microbiota, but is largely responsible for the inflammatory reaction.

We did not observe a correlation between SCC and milk microbiota, this is in line with previous reports from Derakhshani *et al.* (2018) but in contrast to Ganda *et al.* (2016 and 2017). Further, we did not observe a response in Shannon diversity over the course of inflammation, this too is in contrast to Ganda *et al.* (2016 and 2017). A reasonable conclusion for these results is that the bacterial source for inflammation, but not LPS or the inflammation per se, is the driving force for alteration of the microbiota diversity.

# 6 Conclusions

In this thesis, sequencing of the 16S rRNA gene was used as a method to study the microbiota in bovine milk. The overall aim was to evaluate the presence of a microbiota in milk and describe factors that affect the microbiota.

For milk samples taken aseptically directly from the udder quarter, no differences in the microbiota were detected regardless of whether the samples were collected by hand stripping, through a teat canal cannula or by trans-teat wall needle aspirate. Likewise, no differences in the microbiota were detected for milk samples taken aseptically directly from the udder quarter by hand stripping or through a teat canal cannula before or after milking. However, the microbiota on the skin of the teat end and in the teat canal differed in its composition from the microbiota in milk. Similarly, the microbiota in udder quarter composite milk samples differed from the microbiota found in milk samples collected aseptically directly from the udder quarter and from the microbiota found on the skin of the teat end and in the teat canal.

No conclusions about the temporal stability of the milk microbiota or changes in the microbiota during endotoxin induced mastitis could be drawn due to the large impact of contamination on the data from these studies.

The results in this thesis show that contamination has a large impact on the microbiota data, and points out the importance of including control samples at relevant stages of the sample processing in the microbiota analysis. Moreover, a negative correlation between bacterial biomass and impact of contamination was observed, pointing out the importance of assessing the bacterial biomass in studies of the microbiota in milk.

The overall conclusion from the results presented in this thesis is that inclusion of controls is highly important when assessing the microbiota in milk. Considering the large proportion of contamination and low similarity between duplicate samples, conclusions about a resilient microbiota in milk cannot be confirmed. Consequently, the question about the existence of a bacterial community always present in the bovine mammary gland is yet to be answered.

# References


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 Applied Microbiology*, 34(2), pp. 148-55.

Arroyo, R., Martin, V., Maldonado, A., Jimenez, E., Fernandez, L. & Rodriguez, J.M. (2010). Treatment of infectious mastitis during lactation: antibiotics versus oral administration of Lactobacilli isolated from breast milk. *Clinical Infectious Disease*, 50(12), pp. 1551-8.

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N. & Patel, H.R. (2005). Basic principles of real-time quantitative PCR. *Expert review of molecular diagnostics*, 5(2), pp. 209-19.

Bexiga, R., Pereira, H., Pereira, O., Leitao, A., Carneiro, C., Ellis, K.A. & Vilela, C.L. (2011). Observed reduction in recovery of Corynebacterium spp. from bovine milk samples by use of a teat cannula. *Journal of Dairy Research*, 78(1), pp. 9-14.

Bhatt, V.D., Ahir, V.B., Koringa, P.G., Jakhesara, S.J., Rank, D.N., Nauriyal, D.S., Kunjadia, A.P. & Joshi, C.G. (2012). Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing. *Journal of Applied Microbiology*, 112(4), pp. 639-50.

Braem, G., De Vliegher, S., Verbist, B., Heyndrickx, M., Leroy, F. & De Vuyst, L. (2012). Culture-independent exploration of the teat apex microbiota of dairy cows reveals a wide bacterial species diversity. *Veterinary Microbiology*, 157(3-4), pp. 383-90.

Cooper, G.M. (2000). *Cell: A molecular approach,* Sunderland (MA), Sinauer Associates Inc.

Dahlberg, J., Sun, L., Persson Waller, K., Ostensson, K., McGuire, M., Agenas, S. & Dicksved, J. (2019). Microbiota data from low biomass milk samples is markedly affected by laboratory and reagent contamination. *PLoS One*, 14(6), p. e0218257.

Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A. & Callahan, B.J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, 6(1), p. 226.

de Andres, J., Jimenez, E., Chico-Calero, I., Fresno, M., Fernandez, L. & Rodriguez, J.M. (2017). Physiological translocation of lactic acid bacteria during pregnancy contributes to the composition of the milk microbiota in mice. *Nutrients*, 10(1).

de Goffau, M.C., Lager, S., Salter, S.J., Wagner, J., Kronbichler, A., Charnock-Jones, D.S., Peacock, S.J., Smith, G.C.S. & Parkhill, J. (2018). Recognizing the reagent microbiome. *Nature Microbiology*, 3(8), pp. 851-853.

de Goffau, M.C., Lager, S., Sovio, U., Gaccioli, F., Cook, E., Peacock, S.J., Parkhill, J., Charnock-Jones, D.S. & Smith, G.C.S. (2019). Human placenta has no microbiome but can contain potential pathogens. *Nature*, 572(7769), pp. 329-334.

Dellmann, H.-D., Eurell, J.A.C. & Frappier, B.L. (2006). *Dellmann's textbook of veterinary histology*. 6th ed. ed. Ames, IA: Blackwell Publishing.

Derakhshani, H., Plaizier, J.C., De Buck, J., Barkema, H.W. & Khafipour, E. (2018). Composition of the teat canal and intramammary microbiota of dairy cows subjected to antimicrobial dry cow therapy and internal teat sealant. *Journal of Dairy Science*, 101(11), pp. 10191-10205.

Doyle, C.J., Gleeson, D., O'Toole, P.W. & Cotter, P.D. (2017). Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study. *Applied and Environmental Microbiology*, 83(2).

Dusko Ehrlich, S. & MetaHIT consortium. (2010). Metagenomics of the intestinal microbiota: potential applications. *Gastroentérologie Clinique et Biologique*, 34 Suppl 1, pp. S23-8.

Falentin, H., Rault, L., Nicolas, A., Bouchard, D.S., Lassalas, J., Lamberton, P., Aubry, J.M., Marnet, P.G., Le Loir, Y. & Even, S. (2016). Bovine teat microbiome analysis revealed reduced alpha diversity and significant changes in taxonomic profiles in quarters with a history of mastitis. *Frontiers in Microbiology*, 7, p. 480.

Fernandez, L., Langa, S., Martin, V., Maldonado, A., Jimenez, E., Martin, R. & Rodriguez, J.M. (2013). The human milk microbiota: origin and potential roles in health and disease. *Pharmacological Research*, 69(1), pp. 1-10.

Fournier, P.-E., Drancourt, M., Colson, P., Rolain, J.-M., Scola, B.L. & Raoult, D. (2013). Modern clinical microbiology: new challenges and solutions. *Nature Reviews Microbiology*, 11(8), pp. 574-585.

Friman, M., Hiitio, H., Niemi, M., Holopainen, J., Pyorala, S. & Simojoki, H. (2017). The effect of a cannula milk sampling technique on the microbiological diagnosis of bovine mastitis. *The Veterinary Journal*, 226, pp. 57-61.

Ganda, E.K., Bisinotto, R.S., Lima, S.F., Kronauer, K., Decter, D.H., Oikonomou, G., Schukken, Y.H. & Bicalho, R.C. (2016). Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin. *Scientific Reports*, 6, p. 37565.

Ganda, E.K., Gaeta, N., Sipka, A., Pomeroy, B., Oikonomou, G., Schukken, Y.H. & Bicalho, R.C. (2017). Normal milk microbiome is reestablished following experimental infection with Escherichia coli independent of intramammary antibiotic treatment with a third-generation cephalosporin in bovines. *Microbiome*, 5(1), p. 74.

Gill, J.J., Sabour, P.M., Gong, J., Yu, H., Leslie, K.E. & Griffiths, M.W. (2006). Characterization of bacterial populations recovered from the teat canals of lactating dairy and beef cattle by 16S rRNA gene sequence analysis. *FEMS (Federation of European Microbiological Societies) Microbiology Ecology*, 56(3), pp. 471-81.

Glassing, A., Dowd, S.E., Galandiuk, S., Davis, B. & Chiodini, R.J. (2016). Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples. *Gut Pathogens*, 8, p. 24.

Hamann, J. (2002). Relationships between somatic cell count and milk composition. *Bulletin of the International Dairy Federation* (372), pp. 56-59.

Hammer, O., Harper, D. & Ryan, P. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, 4(1).

Heikkila, M.P. & Saris, P.E.J. (2003). Inhibition of Staphylococcus aureus by the commensal bacteria of human milk. *Journal of Applied Microbiology*, 95(3), pp. 471-478.

Hensen, S.M., Pavičić, M.J.A.M.P., Lohuis, J.A.C.M., de Hoog, J.A.M. & Poutrel, B. (2000). Location of Staphylococcus aureus within the experimentally infected bovine udder and the expression of capsular polysaccharide type 5 in Situ. *Journal of Dairy Science*, 83(9), pp. 1966-1975.

Hiitio, H., Simojoki, H., Kalmus, P., Holopainen, J., Pyorala, S. & Taponen, S. (2016). The effect of sampling technique on PCR-based bacteriological results of bovine milk samples. *Journal of Dairy Science*, 99(8), pp. 6532-6541.

Hogeveen, H., Huijps, K. & Lam, T.J. (2011). Economic aspects of mastitis: new developments. *New Zealand Veterinary Journal*, 59(1), pp. 16-23.

Human Microbiome Project, C. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, 486(7402), pp. 207-14.

IDF, (1987). Bovine mastitis. Definition and guidelines for diagnosis. *Bulletin of the International Dairy Federation*, No 211.

Jenkins, T.C. & McGuire, M.A. (2006). Major advances in nutrition: Impact on milk composition. *Journal of Dairy Science*, 89(4), pp. 1302-1310.

Jimenez, E., Fernandez, L., Maldonado, A., Martin, R., Olivares, M., Xaus, J. & Rodriguez, J.M. (2008). Oral administration of Lactobacillus strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. *Journal of applied & environmental microbiology*, 74(15), pp. 4650-5.

Karstens, L., Asquith, M., Davin, S., Fair, D., Gregory, W.T., Wolfe, A.J., Braun, J. & McWeeney, S. (2019). Controlling for contaminants in low-biomass 16S rRNA gene sequencing experiments. *mSystems*, 4(4).

Kuehn, J.S., Gorden, P.J., Munro, D., Rong, R., Dong, Q., Plummer, P.J., Wang, C. & Phillips, G.J. (2013). Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. *PLoS One*, 8(4), p. e61959.

Lauder, A.P., Roche, A.M., Sherrill-Mix, S., Bailey, A., Laughlin, A.L., Bittinger, K., Leite, R., Elovitz, M.A., Parry, S. & Bushman, F.D. (2016). Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. *Microbiome*, 4(1), p. 29.

Madigan, M.T. (2012). *Brock biology of microorganisms*. 13th. ed. San Francisco: Benjamin Cummings.

Martin, R., Langa, S., Reviriego, C., Jimenez, E., Marin, M.L., Olivares, M., Boza, J., Jimenez, J., Fernandez, L., Xaus, J. & Rodriguez, J.M. (2004). The commensal microflora of human milk: new perspectives for food bacteriotherapy and probiotics. *Trends in Food Science & Technology*, 15(3-4), pp. 121-127.

Martin, R., Langa, S., Reviriego, C., Jiminez, E., Marin, M.L., Xaus, J., Fernandez, L. & Rodriguez, J.M. (2003). Human milk is a source of lactic acid bacteria for the infant gut. *Journal of Pediatrics*, 143(6), pp. 754-8.

Martin, V., Maldonado-Barragan, A., Moles, L., Rodriguez-Banos, M., Campo, R.D., Fernandez, L., Rodriguez, J.M. & Jimenez, E. (2012). Sharing of bacterial strains between breast milk and infant feces. *Journal of human lactation : official journal of International Lactation Consultant Association*, 28(1), pp. 36-44.

Metzger, S.A., Hernandez, L.L., Skarlupka, J.H., Suen, G., Walker, T.M. & Ruegg, P.L. (2018). Influence of sampling technique and bedding type on the milk microbiota: Results of a pilot study. *Journal of Dairy Science*, 101(7), pp. 6346-6356.

Metzker, M.L. (2010). Sequencing technologies - the next generation. *Nature Reviews Genetics*, 11(1), pp. 31-46.

Morey, M., Fernandez-Marmiesse, A., Castineiras, D., Fraga, J.M., Couce, M.L. & Cocho, J.A. (2013). A glimpse into past, present, and future DNA sequencing. *Molecular Genetics and Metabolism*, 110(1-2), pp. 3-24.

Morozova, O. & Marra, M.A. (2008). Applications of next-generation sequencing technologies in functional genomics. *Genomics*, 92(5), pp. 255-64.

Nocard, E. & Mollereau, R. (1887). Sur une mammite contagieuse de vaches laitières. *Annales de l'institut Pasteur*, 1:109-126.

Oikonomou, G., Bicalho, M.L., Meira, E., Rossi, R.E., Foditsch, C., Machado, V.S., Teixeira, A.G., Santisteban, C., Schukken, Y.H. & Bicalho, R.C. (2014). Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. *PLoS One*, 9(1), p. e85904.

Oikonomou, G., Machado, V.S., Santisteban, C., Schukken, Y.H. & Bicalho, R.C. (2012). Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. *PLoS One*, 7(10), p. e47671.

Oliver, S.P., Gonzalez, R.N., Hogan, J.S., Jayarao, B.M. & Owens, W.E. (2004). *Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality*. 4. ed. NMC Inc, Verona, WI, USA, The National Mastitis Council.

Perez, P.F., Dore, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., Segura-Roggero, I., Schiffrin, E.J. & Donnet-Hughes, A. (2007). Bacterial imprinting of the neonatal immune system: lessons from maternal cells? *Pediatrics*, 119(3), pp. e724-32.

Persing, D. (2011). *Molecular Microbiology. Diagnostic Principles and Practice.* Washington, DC, ASM Press.

Plastridge, W.N. (1958). Bovine mastitis: A review. *Journal of Dairy Science*, 41(9), pp. 1141-1181.

Pliny, Bostock, J. & Riley, H.T. (1855). *The natural history of Pliny*. George Bohn and sons. London.

R Core Team (2019). R: A language and environment for statistical computing. [Computer Program]. Vienna, Austria: R Foundation for Statistical Computing. Available from: https://www.R-project.org/.

Radostits, O.M., Gay, C.C., Hinchcliff, K.W., Constable, P.D. & Done, S.H. (2007). *Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs and goats.* 10. ed. Edinburgh: Saunders Elsevier.

Rainard, P. (2017). Mammary microbiota of dairy ruminants: fact or fiction? *Veterinary Research*, 48(1), p. 25.

Rainard, P. & Riollet, C. (2003). Mobilization of neutrophils and defense of the bovine mammary gland. *Reproduction Nutrition Development, EDP Sciences,* 43(5), pp. 439-457.

Roberts, W. (1874). Studies on biogenesis. *Philosophical Transactions of the Royal Society of London*, 164, pp. 457-477.

Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F., Turner, P., Parkhill, J., Loman, N.J. & Walker, A.W. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*, 12, p. 87.

Sandison, A.T. (1959). The first recorded case of inflammatory mastitis - Queen Atossa of Persia and the physician Democedes. *Medical History*, 3, pp. 317-22.

Sjaastad, O., Sand, O. & Hove, k. (2016). *Physiology of domestic animals*. Scandinavian Veterinary Press, Oslo (753 pp).

Tančin, V. & Bruckmaier, R.M. (2015). Factors affecting milk ejection and removal during milking and suckling of dairy cows. *Veterinární Medicína*, 46(No. 4), pp. 108-118.

Taponen, S., McGuinness, D., Hiitio, H., Simojoki, H., Zadoks, R. & Pyorala, S. (2019). Bovine milk microbiome: a more complex issue than expected. *Veterinary Research*, 50(1), p. 44.

Torsvik, V., Daae, F.L., Sandaa, R.-A. & Øvreås, L. (1998). Novel techniques for analysing microbial diversity in natural and perturbed environments. *Journal of Biotechnology*, 64(1), pp. 53-62.

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R. & Gordon, J.I. (2007). The human microbiome project. *Nature*, 449(7164), pp. 804-10.

Watts, J.L. (1988). Etiological agents of bovine mastitis. *Veterinary Microbiology*, 16(1), pp. 41-66.

Wellenberg, G.J., van der Poel, W.H.M. & Van Oirschot, J.T. (2002). Viral infections and bovine mastitis: a review. *Veterinary Microbiology*, 88(1), pp. 27-45.

Verdier-Metz, I., Gagne, G., Bornes, S., Monsallier, F., Veisseire, P., Delbes-Paus, C. & Montel, M.C. (2012). Cow teat skin, a potential source of diverse microbial populations for cheese production. *Journal of applied & environmental microbiology*, 78(2), pp. 326-33.

Young, W., Hine, B.C., Wallace, O.A., Callaghan, M. & Bibiloni, R. (2015). Transfer of intestinal bacterial components to mammary secretions in the cow. *PeerJ*, 3, p. e888.

# Popular science summary

*Background milk microbiota*

The theory about the evolutionary benefits of a bacterial community (a microbiota always present) in milk is, in brief and freely interpreted, as follows: The gut microbiota is essential for the health and wellbeing of mammals, facilitating uptake of nutrients and providing the host with vitamins, etc. Consequently, establishment of the gut microbiota is a highly important event. Milk is produced in order to provide the offspring with nutrients during the first period in life. A bacterial community present in milk would, thus, inoculate the intestines of the offspring during the critical phase of gut microbiota establishment and be beneficial for the offspring.

Advances in microbiology made DNA sequencing cheaper and more available for scientists in the early 21st century. At the same time, two large research projects were initiated from the USA and EU with the aim of increasing the understanding of the microbiota and how it affects human health. This spurred an increased interest to study the microbiota in milk. The first studies that used new, modern techniques to study the microbiota in bovine milk were published in 2012 and 2013. Since then, the interest and number of publications have increased. Studies have been published that associate bovine milk microbiota to somatic cell count (an inflammatory marker in milk), mastitis and the cows' environment. In one publication, a shift in the milk microbiota was seen for cows that had suffered from mastitis earlier in the same lactation. In recent years, the presence of a microbiota in milk has been questioned based on the inconsistency to previous knowledge. Studies that express difficulties retrieving trustworthy results have also been published. Arguments used against the presence of a microbiota in milk are based on udder anatomy and the fact that cows are housed in a bacterial-rich environment and the difficulties of taking an un-contaminated milk sample.

*Aim with studies*

The overall aim of the studies that are presented in this thesis was to evaluate the presence of a microbiota in milk coming from healthy lactating cows and describe factors that affect the milk microbiota. The specific objectives were: 1) To evaluate the effect of sampling technique and milk fraction on bovine milk microbiota data, and to compare the microbiota in milk to the microbiota on the skin of the teat end and in the teat canal. 2) To describe the composition and stability over time for the bacterial microbiota in bovine milk from healthy udder quarters. 3) To describe the changes in milk microbiota composition during the course of experimentally induced mastitis. 4) To describe the effect of contamination on bovine milk microbiota data.

*Performed studies*

This thesis is based on three papers that consisted of three experimental animal studies. To assess the first objective, a study on five cows was performed where samples were taken with different techniques from each udder quarter during one milking. Milk samples were taken by hand stripping or through a teat canal cannula before and after milking. Further, swab samples from the skin of the teat end and teat canal before milking, a needle aspirate of milk taken through the teat wall was taken after milking and an udder quarter composite milk sample was taken from the milk machine. To assess the second objective, an experiment was performed where milk samples were collected from nine healthy cows twice per week for four weeks. To assess the third objective, mastitis was induced in nine healthy lactating animals with bacterial endotoxin, equally many cows were included as controls and infused with a saline solution. Somatic cell count and bacterial culturing were included for reference in all experiments. The microbiota was assessed by sequencing of regions of the 16S rRNA gene. To assess the forth objective, blank controls and controls with a known bacterial composition were included in the sequencing in paper II and III. Contaminations were identified and data filtration was used to assess the effect on microbiota data.

*Results*

Sequencing results showed that the microbiota found on the skin of the teat end, in the teat canal and in the udder quarter composite milk samples differed significantly from the microbiota found in milk samples collected directly from the teat. For milk samples taken aseptically with different techniques directly from the teat before or after milking, no significant differences in the microbiota was identified. Data from the experiment that was designed with the purpose of

assessing microbiota stability over time was so affected by contamination that no conclusions could be drawn. The same problem occurred in the study with the objective to assess change during induced mastitis. An association between the impact of contamination and bacterial biomass was observed in paper II, and was partly confirmed in paper III. Contamination was found to contribute with a majority (65-88%) of the data in paper II and III. An unexpected finding of bacteria belonging to the genus *Dyella* in paper I implies that contamination cannot be excluded as a contributing factor in that study.

The overall conclusion from this thesis is that contamination has such a substantial effect on the data that the presence of a microbiota in milk cannot be evaluated.

# Populärvetenskaplig sammanfattning

*Bakgrund mjölkmikrobiota*

Teorin om de evolutionära fördelarna med en alltid närvarande bakterieflora (en mikrobiota) i mjölk är kort sammanfattat: Tarm-mikrobiotan är essentiell för hälsa och välmående hos däggdjur, den underlättar upptaget av näringsämnen och förser värden med bland annat vitaminer. Följaktligen är etableringen av tarm-mikrobiotan en mycket viktigt händelse. Mjölk produceras exklusivt för att förse avkomman med näring under dess första tid i livet. En mikrobiota i mjölk skulle således bidra till att förse avkommans tarmar med goda och nödvändiga bakterier tidigt i livet och på så sätt vara fördelaktigt för avkomman.

Framsteg inom mikrobiologisk metodik gjorde att DNA-sekvenseringstekniker blev billigare och mer tillgängliga för forskare i början på 2000-talet. Samtidigt startade två stora projekt i USA och EU som syftade till att öka förståelsen för mikrobiota och hur den påverkar människor. Detta ledde till att intresset för att studera mikrobiota i mjölk ökade. De första studierna som använde de nya teknikerna för att studera mikrobiota i komjölk publicerades 2012 och 2013. Sedan dess har intresset och antalet publikationer ökat. Artiklar som beskriver mjölkmikrobiotans förändring i förhållande till celltal (en inflammationsmarkör i mjölk), juverinflammation och kornas miljö har till exempel publicerats. I en av publikationerna sågs förändringar i mjölkmikrobiotan om kon haft juverinflammation tidigare i laktationen. På senare år har förekomsten av en mjölkmikrobiota ifrågasatts på grund av avsaknaden av överenstämmelse mot tidigare kunskaper, och artiklar som uttrycker svårigheter med trovärdiga resultat har publicerats. Argument som används emot förekomsten av en mikrobiota i mjölk är att kor befinner sig i en bakterierik miljö och att det är svårt att ta prover som inte blir kontaminerade i samband med provtagningen. Ett annat vanligt argument är baserat på den anatomiska uppbyggnaden av juvret.

*Syftet med studierna*

Det övergripande målet med studierna som presenteras i denna avhandling var att utvärdera om det finns en mikrobiota i mjölk från friska lakterande kor och beskriva faktorer som i så fall påverkar mjölkmikrobiotan. De specifika delmålen var: 1) att utvärdera effekten av provtagningsteknik och mjölkfraktion på mjölkmikrobiotan och jämföra mjölkmikrobiotan mot mikrobiotan på huden på spenspetsen och i spenkanalen. 2) att beskriva mjölkmikrobiotans sammansättning och förändring över tid hos friska lakterande kor. 3) att beskriva förändringar i mjölkmikrobiotan i samband med experimentellt framkallad juverinflammation. 4) att beskriva vilken effekt kontamination har på mjölkmikrobiota-data.

*Studiernas genomförande*

Avhandlingen består av tre delstudier baserade på tre experimentella studier. För att undersöka det första delmålet genomfördes en studie på fem kor där mjölkprover togs med olika tekniker från en och samma juverdel under en mjölkning. Mjölkprover togs som handmjölkade prov och med en kateter införd i spenkanalen före och efter mjölkning. Vidare togs svabbprov från spenspetsens hud och från spenkanalen före mjölkning. Ett mjölkprov togs genom spenkanalsväggen efter mjölkning och ett samplingsprov togs från den mjölk som samplats upp i mjölkmaskinen. För att undersöka delmål 2 genomfördes en studie där mjölkprov togs från 9 friska kor två gånger per vecka under fyra veckor i samband med mjölkning. För att undersöka delmål 3 framkallades juverinflammation med hjälp av bakteriellt endotoxin i 9 friska kor, lika många djur var inkluderade som kontroll och fick istället koksaltlösning. Undersökning av celltalet och bakterieväxten inkluderades i alla studierna. Mikrobiotan i proverna undersöktes genom sekvensering av delar av 16S rRNA genen. För att undersöka delmål 4 inkluderades blanka kontroller och kontroller med en känd bakterie-flora i sekvenseringen i delstudie II och III. Kontaminationer identifierades och datafiltrering användes för att undersöka effekten på mikrobiotadata.

*Resultat*

Sekvenseringsresultaten visade att mikrobiotan som fanns på spenspetsen, i spenkanalen och i samlingsmjölkprovet skilde sig signifikant från mikrobiotan som fanns i mjölkproven tagna direkt från spenen. För mjölkprover tagna med olika aseptiska tekniker direkt från spenen, före eller efter mjölkning, hittades inga skillnader i mjölkmikrobiotan. Data från studien som syftade till att undersöka mjölkmikrobiotans stabilitet över tid var till så stor grad påverkad av

kontamination att slutsatser om stabilitet över tid inte gick att dra. Detsamma gällde för studien som syftade till att studera mikrobiotans förändring i samband med juverinflammation. Ett samband mellan genomslag av kontamination och bakteriell biomassa noterades i delstudie II och kunde delvis bekräftas i delstudie III. Kontamination konstaterades bidra med majoriteten (65-88%) av data i delstudie II och III. Ett oväntat fynd av bakterier tillhörande genuset *Dyella* i delstudie I gör att kontamination inte kan uteslutas som bidragande orsak även i denna studie.

Sammanfattningsvis konstateras att kontamination är så starkt bidragande till datan att slutsatser om förekomst av en mikrobiota i mjölk inte går att dra.

# Acknowledgements

This project was carried out at the **Department of Animal Management and Nutrition** (**HUV**) at the **Swedish University of Agricultural Sciences** (**SLU**), Uppsala Sweden that are thankfully acknowledged. Animal experiments were performed at the **Swedish Livestock Research Centre** in **Lövsta** that together with all the personnel is gratefully acknowledged. Financial support was provided by the **Formas a Swedish research council for sustainable development** and **Olof Claesson fund.**

Over these years, a number of people have contributed to the making of this thesis and to my life. I would like to thank you all, in particular I would like to acknowledge:

My supervisors: **Sigrid Agenäs**, **Johan Dicksved**, **Karin Persson Waller**, **Karin Östensson** and **Mark McGuire**. Your idea generated a grant that generated me a PhD. I think we have all learned a great deal along the way. A project that seemed so straight forward turned out to include more hurdles than expected. A special thanks to Johan for coping with all my questions, listening to all my ideas and explaining everything about laboratory methods.

My co-authors: **Sigrid, Johan, Karin, Karin, Mark, Li**, **Janet**, **Haley**, **Erik**, **Carl-Fredrik** and **Gunnar**. Writing would not have been as evolving if you weren't involved.

The **head of department** for hiring me. And the **administration at HUV** for trying to answer all of my questions. I gave you the blame for doings that were out of your hands, but I think you found me equally difficult. ;)

My **colleagues** at HUV, fika is more fun when you are around. My present and former **PhD-colleagues**, you know who you are. Doing a PhD wouldn't be half as fun if you weren't around.

**Emma**, **Sabine**, **Rebecca**, **Andreas** and **David** for showing me the way and proving that it is possible.

**Lisa** and **Johanna** for always letting me drink my coffee and ventilate big and small questions about life in your office.

**Jorge** and **Kamyar** for all the good laughs.

**Maja ♥**, **Delal** and **Guo** for all the outstanding help in the lab.

**Carl-Fredrik** and **Gunnar** for inviting and involving me in your project on mastitis.
**Daiana** for involving me in your projects on cow and calf brushing.

My colleagues at the Ambulatory Clinic; **Håkan**, **Anders**, **Ann**, **Eva**, **Ann-Marie**, **Inga**, **Jenny**, **Laki** and **Theo**. It is a pure pleasure to be working with you, my summers would be much more boring if it was only vacation. My bosses at the Ambulatory Clinic (**Arne** and **Jenny**) for hiring me under special requirements.

The **IME gang**, **Gustav**, **Ester**, **Jossan** and all the rest of you. I really enjoyed getting out of the office/lab to meet the patients, students and cows.

My family. **Dad**, if you were still around I would thank you for the insight about life. Strunt är strunt om än i gyll´ne dosor, men rosor i ett sprucket krus är ändå alltid rosor. **Mom** for all your outstanding support. **Jenny**, **Rikard** and **Ida** for shaping me to who I am, and **Fredrik**, **Ulle** and **Fredrik** for helping them out.

**Mireil** for being the mother to my children and the times that were.
**Ullis** for the support when times were difficult and lessons about life
**Gustav**, **Astrid**, **Martinsson**, **Helena**, **Emma**, **Håkan**, **Lisa, Anna, Bosse** for being as good friends as friends can be.
**Lena** for the time that is and the chapter that has just started. Renovating a bathroom, moving and writing a thesis might not have been optimal, but life is life and I'm thrilled that you're a part of my life.

Last but not the least, **Xavier** and **Ingrid**. Nothing brings more delight to my life than you. Je vous aimerai toujours, la nuit comme le jour. Et tant que je vivrai, vous serez mes bébés.