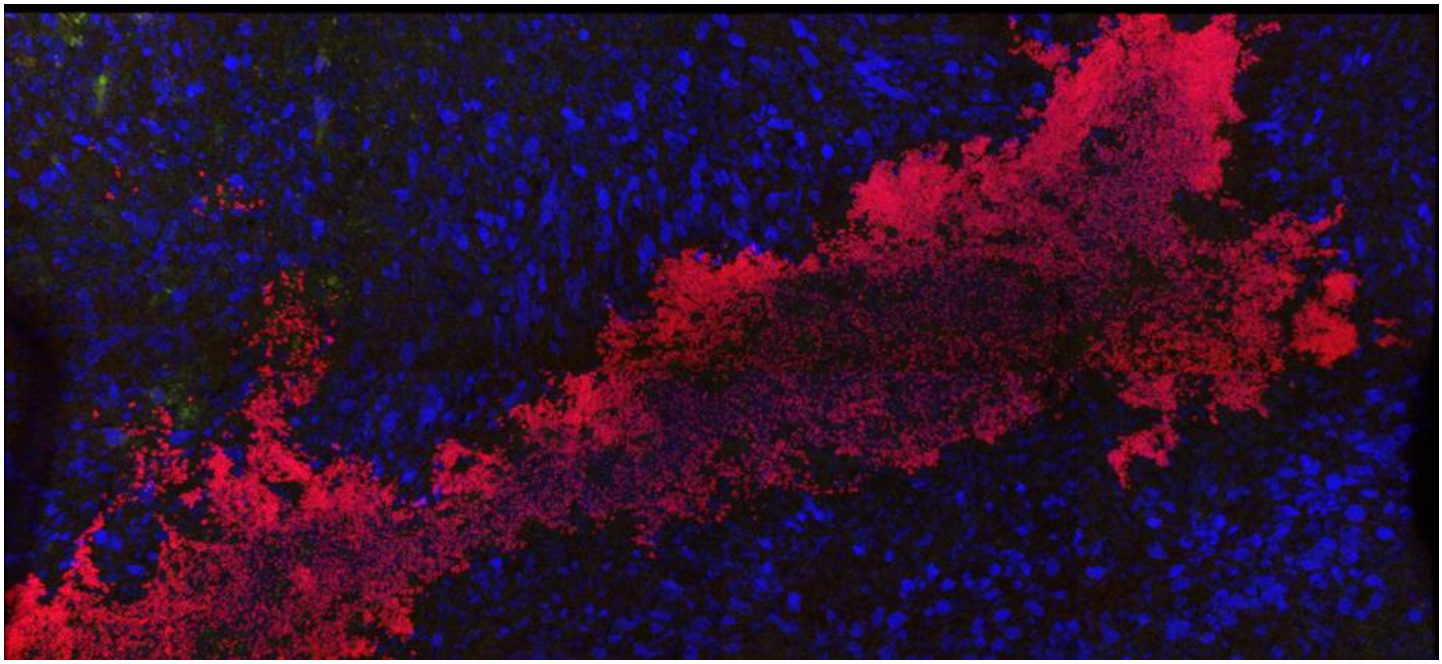
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Bacterial visualization and identification in bovine udders

PhD Thesis by Regitze Renee Pedersen



Supervisors: Rikke Buhl, Thomas Bjarnsholt, Elin Jørgensen, Kirstin Dahl-Pedersen, Volker Krömker

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Departments:	Department of Veterinary Clinical Sciences, Large Animal Teaching Hospital, University of Copenhagen & Institute for Immunology and Microbiology, Costerton Biofilm Centre, University of Copenhagen
Author:	Regitze Renee Pedersen, cand.scient of Biology, regitze.pedersen@sund.ku.dk & regitzerpedersen@gmail.com
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Supervisors:	Volker Krömker, Professor, DVM & PhD Kirstin Dahl-Pedersen, Assistant professor & DVM Rikke Buhl, Professor, DVM & PhD Elin Jørgensen, Postdoc, DVM & PhD Thomas Bjarnsholt, Professor, DmSc. &PhD
Evaluation Committee:	**Chairperson:** Associate professor Nynne Capion Department of Veterinary Clinical Sciences, University of Copenhagen, Denmark **Representative from Danish research institution**: Associate professor Trine Rolighed Thomsen, Department of Chemistry and Biosciences, Aalborg University, Denmark **Representative from research institution abroad:** Professor Paolo Moroni, Department of Population Medicine and Diagnostic Sciences Cornell University and University of Milano, United States

Front page illustration:

Aggregate from tissue section from mastitis quarter, stained with Texas Red universal PNA-FISH probe (red) and DAPI (blue). 630x magnification, obtained by CLSM. Image from Manuscript II.

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Table of Contents

ACKNOWLEDGMENTS **3**

ABBREVIATIONS **7**

ENGLISH SUMMARY **8**

DANISH SUMMARY **10**

LIST OF PUBLICATIONS **12**

BACKGROUND **13**

Bovine mastitis **13**

The microbiome of the bovine udder and milk **15**

Chronic mastitis **15**

Biofilm infections **16**

Biofilm in veterinary disease and mastitis **17**

Diagnosis and treatment of bovine mastitis **19**

OBJECTIVES **21**

METHODS **22**

METHODS IN MANUSCRIPT II **22**

Including dairy cows in the study **22**

Pilot study for collecting samples **22**

Collecting of udders from dairy cows **23**

Collection of milk samples and biopsies from udders **23**

Identification of bacterial species in milk and biopsies **27**

Somatic cell count of milk samples **29**

Visualization of bacteria and cells **29**

METHODS IN MANUSCRIPT III 30

Raman spectroscopy 31

RESULTS 32

Current research of biofilm in bovine mastitis – Manuscript I 32

Bacteria in the bovine milk and udder – Manuscript II 32

Multi-excitation Raman spectroscopy as a future diagnostic tool - Manuscript III 46

DISCUSSION 50

Dairy cows included in study – Manuscript II 50

The bacterial composition in bovine udders – Manuscript II 50

Histopathology of cells - Manuscript II 52

Biofilm in bovine mastitis - Manuscript I and II 52

Raman spectroscopy: a future diagnostic tool of bovine mastitis? Manuscript III 53

General Discussion 54

Limitations 55

CONCLUSION 57

PERSPECTIVES 59

REFERENCES 61

MANUSCRIPT I 74

MANUSCRIPT II 86

MANUSCRIPT III 121

Abbreviations

CLSM	Confocal laser scanning microscopy
CMT	California Mastitis Test
DAPI	4',6-diamidino-2-phenylindole (DAPI)
EPS	Exopolysaccharides
HE	Hematoxylin and Eosin
MALDI-TOF MS	Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry
PNA-FISH	Peptide Nucleic Acid Fluorescence *in situ* Hybridization
SCC	Somatic cell count

English summary

Bovine mastitis is affecting the dairy industry greatly. The condition has great impact on the welfare of animals and is the main reason for antibiotic treatment in the dairy industry. Mastitis is inflammation in the mammary gland and the primary reason is bacterial origin, which is often treated with antibiotics. However, for some cases of mastitis, the condition is not resolved and to optimize the treatment of these dairy cows, it is important with a deeper understanding of mastitis. The majority of the previous research is based upon milk samples.

The aim with this thesis was to contribute to the existing knowledge about mastitis by investigating the bacterial distribution in the udders, visualize the bacteria and determine the bacterial composition in the tissue and of milk samples. Tissue samples from different locations of healthy and mastitis quarters as well as milk samples were collected from 28 recently slaughtered dairy cows with increased somatic cell count. The samples were cultivated, and the bacterial isolates were identified with MALDI-TOF MS. Furthermore, the tissue samples were analyzed with 16s rDNA sequencing to identify non-cultivable bacteria. To visualize the bacteria, tissue samples were stained with PNA-FISH and examined by confocal laser scanning microscopy. The tissue samples from the deepest location in the udders were stained with HE staining and examined by light microscopy to determine the inflammatory response.

Furthermore, Raman spectroscopy was investigated as a possible diagnostic tool, by applying the method to the two pathogens *Staphylococcus aureus* and *Streptococcus uberis*, which are associated with mastitis.

There was bacteria present in tissue samples and milk samples from both healthy and mastitis quarters and bacteria was found in every location of the udder. Furthermore, bacterial species were found in either milk samples or tissue samples. Microscopy of the stained samples showed a higher abundance of aggregates in the mastitis quarters and aggregates were found in all locations of mastitis quarters. On the contrary, very few of the healthy quarters had aggregates and the aggregates were only found at the teat end and the annula ring. The histopathological investigation of the tissue samples from the deepest location of the udders showed an inflammatory response in both healthy and mastitis quarters, however a lower response in the healthy quarters.

It was possible by Raman spectroscopy to detect spectra of the two mastitis pathogens *S. aureus* and *S. uberis* as well as milk, and the method was able to differentiate between different bacterial concentrations in the milk.

The results of this thesis indicate that udders of dairy cows are an organ where bacteria exist in both healthy and mastitis and can be found in different formations. The discrepancies of species

found between milk samples and tissue samples indicate that milk samples are not always reflecting the bacteria present in the tissue. Furthermore, Raman spectroscopy was a promising method for diagnostic use in the future.

There is a need for more research with the focus on tissue samples and comparing them with milk samples, as well as the application of Raman spectroscopy as a diagnostic tool.

Danish summary

Yverbetændelse i malkekvæg påvirker mælkeindustrien i høj grad. Sygdommen har stor indvirkning på dyrenes helbred og er den største årsag til antibiotikabehandling i mælkeindustrien. Yverbetændelse er inflammation i yveret, og den primære årsag er af bakteriel oprindelse, som oftest behandles med antibiotika. Men for nogle tilfælde af yverbetændelse virker behandlingen ikke og for at optimere behandlingen af disse køer, er det vigtigt at forstå sygdommen til fulde. Hovedparten af den tidligere forskning er baseret på mælkeprøver.

Målet med denne afhandling var at bidrage til den eksisterende viden om yverbetændelse ved at undersøge hvor bakterierne var til stede i yverne, visualisere bakterierne og bestemme den bakterielle sammensætning i vævet og i mælkeprøverne. Vævsprøver fra flere forskellige områder i raske og syge yverkirtler samt mælkeprøver blev indsamlet fra 28 nyligt slagtede malkekøer med forhøjet celletal. Prøverne blev dyrket og de bakterielle isolater blev identificeret med MALDI-TOF MS. Vævsprøverne blev ydermere analyseret med 16S rDNA sekventering for at identificere bakterier som ikke kan dyrkes frem. For at visualisere bakterierne blev vævsprøver fra de forskellige lokationer farvet med PNA-FISH og undersøgt med konfokalt laser scanning mikroskopi. Vævsprøver fra den dybeste lokation i yveret blev ydermere farvet med HE farvning og undersøgt med lysmikroskopi, for at bestemme det inflammatoriske respons. Derudover blev det undersøgt om Raman spektroskopi er en mulig diagnostisk metode, ved at anvende metoden på mælk med de to patogener *Staphylococcus aureus* og *Streptococcus uberis* som er associeret med yverbetændelse.

Undersøgelserne af mælk og vævsprøverne viste at både raske og syge yverkirtler havde bakterier og at der fandtes bakterier i alle lokationer af yverkirtlerne. Der blev ydermere fundet bakterielle arter som enten blev fundet i mælk eller vævsprøver. Mikroskopering af de farvede prøver fandt at der var en højere andel af prøver fra syge yverkirtler som havde bakterielle aggregater og at de fandtes i alle lokationer i yverkirtlen. Derimod var der en meget lille andel af prøverne fra raske yverkirtler hvor bakterielle aggregater var til stede og de fandtes kun i pattespidsen og overgangen mellem pattekanalen og cisterne. Den histopatologiske undersøgelse af cellerne i prøverne fra den dybeste lokation i yverkirtlerne viste inflammatorisk respons i både syge og raske kirtler, men en mildere grad hos de raske yverkirtler.

Det var muligt med Raman spektroskopi at bestemme spektre for de to bakterier *S. aureus* og *S. uberis* samt mælk, og metoden kunne differentiere mellem forskellige bakterielle koncentrationer i mælk.

Resultaterne fra denne afhandling viser at malkekøers yvere er et organ, hvor bakterier findes både hos syge og raske og kan findes i forskellige formationer. Uoverensstemmelserne mellem mælkeprøverne og vævsprøverne indikerer at mælkeprøver ikke altid reflekterer hvilke bakterier der er tilstede i vævet. Derudover blev Raman spektroskopi fundet til at være en mulig diagnostisk metode i fremtiden.

Der er brug for mere forskning med fokus på vævsprøver og sammenligne dem med mælkeprøver samt mere forskning i Raman spektroskopi som et muligt diagnostisk værktøj.

List of publications

Articles included in thesis:

Manuscript I:

Pedersen, R. R., Krömker, V., Bjarnsholt, T., Dahl-Pedersen, K., Buhl, R., & Jørgensen, E. (2021). *Biofilm Research in Bovine Mastitis*. Frontiers in Veterinary Science, 8, 656810.

Manuscript II:

Pedersen, R. R., Hornemann, E., Meyer, B., Bay, L., Fritz, B.G., Jakobsen, T.H., Jensen, L. K., Dahl-Pedersen, K., Jørgensen, E., Buhl, R., Krömker, V., Bjarnsholt, T.
Distribution and spatial organization of bacteria in bovine mammary glands. Manuscript in preparation.

Manuscript III:

Pedersen, R. R., Highmore, C., Hanrahan, N., Mahajan, S., Krömker, V., Dahl-Pedersen, K., Buhl, R., Jørgensen, Bjarnsholt, T., E., Webb, J.S.
Detection of bovine mastitis pathogens in milk by multi-excitation Raman spectroscopy. Manuscript in preparation.

Articles not included in thesis

Pedersen, R. R., Kragh, K. N., Fritz, B. G., Ørbæk, M., Østrup Jensen, P., Lebech, A.-M., & Bjarnsholt, T. (2022).
A novel Borrelia-specific real-time PCR assay is not suitable for diagnosing Lyme neuroborreliosis. Ticks and Tick-Borne Diseases, 13(5), 101971.

Clausen, A. S., Ørbæk, M., **Pedersen, R. R.**, Jensen, P. O., Lebech, A., & Kjaer, A. (2020).
Positron Emission Tomography (PET) of Borrelia burgdorferi Infection: In Vivo Imaging of Macrophages in Experimental Model of Lyme Arthritis. 1–11.

Background

Bovine mastitis

Historically, dairy products have been an important way for humans to get nutrients for thousands of years [1]. The dairy industry is significant in Denmark, with 564.000 dairy cows producing 5644 million kilograms of milk in 2021 [2]. One of the most impactful diseases on the dairy industry is bovine mastitis, which causes decreased milk production, increases veterinary costs for dairy farmers, has a significant impact on animal welfare, and can lead to early euthanization of animals [3,4]. The disease is defined as "inflammation of the mammary gland" [5] and can be caused by physical damage or, more commonly, bacterial agents [3]. Typically, the pathogens involved in the disease are categorized as contagious or environmental, where examples of contagious bacteria are *Streptococcus agalactiae, Streptococcus dysgalactiae* and *Staphylococcus aureus*, environmental bacteria are *Escherichia coli* and some pathogens like *Streptococcus uberis*, that cannot be strictly categorized [5]. Over 120 bacterial species have been identified and linked to bovine mastitis [6].

Bovine mastitis is often categorized as subclinical or clinical mastitis. Subclinical cases of mastitis do not have any visible changes to the mammary gland or milk; however, they can be detected by an increased somatic cell count in the milk [7]. Clinical cases can vary from mild cases with changes in the milk to severe cases where the animal is visibly sick and can lead to culling [4,5,7].

The bovine mammary gland—the udder—consists of four quarters. Each quarters is a system that consist of a teat, the teat canal, the teat cistern, the gland cistern, the duct, and the alveoli (Figure 1).

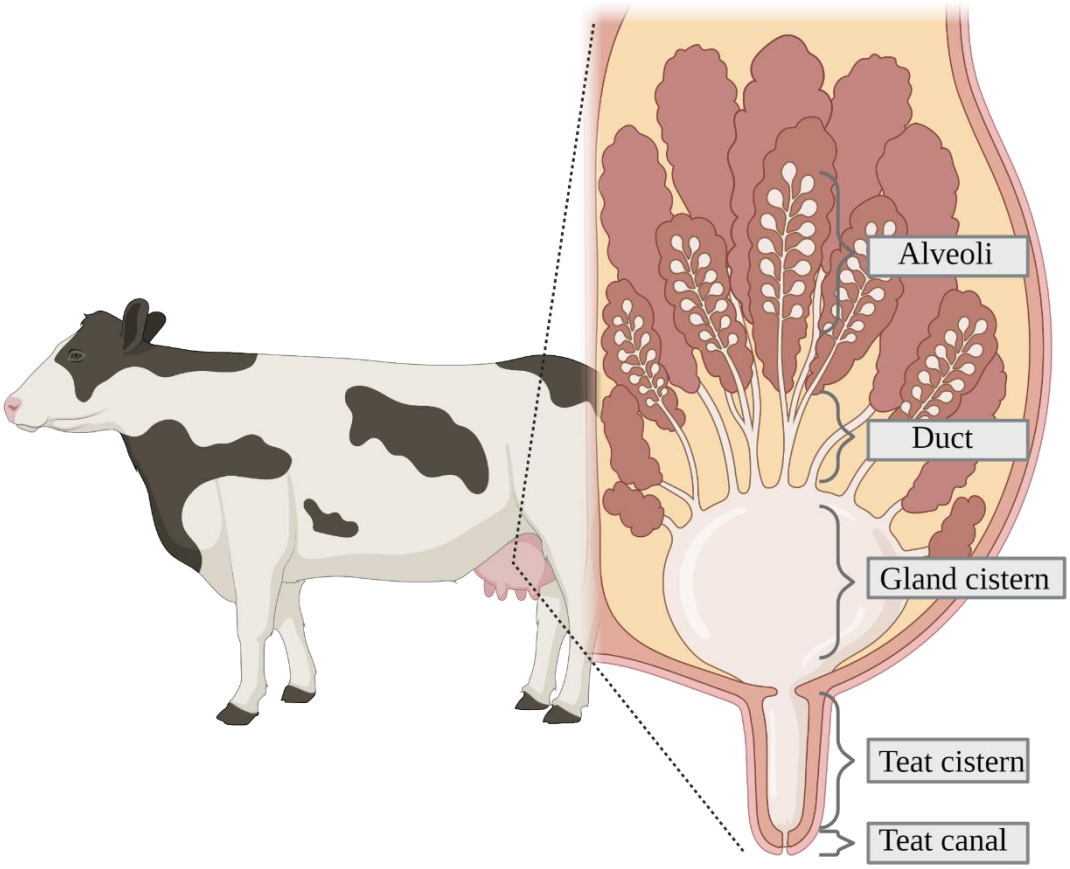

Figure 1. The anatomy of a quarter from a cow. The teat consists of the teat canal and the teat cistern. The udder consists of the gland cistern, ducts, and alveoli. Created in Biorender.com.

This combination creates an organ capable of producing 30 kg of milk per milking [8]. The udders of dairy cows are under high pressure and at risk of infection. However, the mammary gland is prepared to protect itself from invading pathogens that can enter through the teat orifice. The teat has several defenses against threats; the teat opening consists of the sphincter muscle, causing the teat to be tightly closed between milkings and thus keeping pathogens out of the mammary gland. The teat canal has a protecting lining of keratin, which acts as a physical and chemical barrier [9,10], and the cell surface has a high potential for recruiting immune cells to defeat invading bacteria. If the bacteria circumvent this barrier, they will enter the teat canal, where the immune system detects the bacteria [9,10]. The immune cells will increase in numbers to defend against the bacteria and may cause damage to the mammary gland in the process [9,10]. The milk's elevated number of somatic cells is often detected during milking and is a crucial detection method for dairy farmers [4,7].

The microbiome of the bovine udder and milk

Traditionally, the udder has been considered a sterile environment where bacteria would be present only during infection [11]. However, in recent years, the microbiome of bovine udders has been an expanding research area with a high interest in determining the bacteria in the tissue.

Studies have shown that a healthy udder contains multiple bacterial species. Molecular investigations of milk have shown that healthy udders often contain phyla such as Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria. The genera often found are *Staphylococcus, Corynebacterium, Acinetobacter, Pseudomonas,* and *Streptococcus* [12–16]. The most prevalent phyla in milk from dairy cows with mastitis are often the same as in healthy cows; however, a dysbiosis of the microbiota is proposed as mastitis has fewer bacterial species and a higher bacterial load [15–17]. Nonetheless, it is widely discussed whether a shift in microbial composition causes mastitis [18].

The risk of contamination is a concern among researchers studying the bovine udder microbiota [11,18]. The research is heavily based on milk samples, which are collected in the dirty environment of dairy farms. The dirty exterior of the cow, and if the teat is not correctly cleaned beforehand, increases the risk of contamination when collecting milk samples. Milk samples should be collected after cleaning the teats, taking the first sample from the nearest teat and working towards the farthest. When collecting the sample, the vial should not touch the teat and must be at a 45-degree angle. The first milk should be discarded, and the vial should be closed before returning to an upright position [19]. When more than three species are present in a milk sample, it is considered contaminated and should be discarded [7].

Most studies have been based on bacteria isolated from milk samples and few studies have investigated the teat canal microbiome by swabs and found the teat canal had diverse bacteria, mainly Actinobacteria, Firmicutes, and Proteobacteria [14,20,21].

Chronic mastitis

In certain bacterial mastitis cases, antibiotics do not resolve disease. Chronic cases are defined as "udder inflammation that continues over a long period of time," [22] and chronic mastitis may be subclinical or clinical [22].

It is unknown why these cases maintain a high somatic cell count (SCC) or why the infections reoccur despite antibiotic treatment for the involved pathogen, but multiple factors are proposed. Factors that could interfere with the success of antibiotic treatment include the age of the dairy cow if the somatic cell count has been elevated, and the animal's clinical history [23]. Another factor that impacts the success of the treatment is the pathogen causing the infection. *S. aureus* a

pathogen associated with chronic mastitis and tissue infected with *S. aureus* may have a high proportion of polymorphonuclear leukocyte infiltration and necrosis, causing a decrease in milk synthesis [9].

The complicated diagnosis and the recurrent and chronic infections are similar to the characteristics of biofilm infections, which indicates that biofilm could be a possible factor for chronic mastitis.

Biofilm infections

Traditionally. bacteria were thought of as single cells with no communication or cooperation.

However, J.W. Costerton's discovery of biofilm in the '70s led to a new understanding of bacteria [24,25]. Biofilm is defined as "a coherent cluster of bacterial cells embedded in a biopolymer matrix, which, compared with planktonic cells, shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defense" [26].

Rather than the bacteria being single-celled "lone wolves," the bacteria can form this community, almost like a small village.

A biofilm is often compared to a fortress [27], as the bacteria surround themselves with exopolysaccharides (EPS). This physical barrier protects them against environmental challenges such as ultraviolet light, desiccation, and other threats from their surroundings. The biofilm aids the bacteria to remain in a favorable habitat. In the biofilm, a kind of community is formed where bacteria share genes and nutrients [27,28].

Regarding infection, these characteristics can be an advantage for the bacteria.

The physical barrier in the form of EPS can shield the bacteria against the host immune cells and antibiotics, provide the ability to stay in a favorable position in the host, and allow the bacteria to share genes in the biofilm, thereby increasing the risk of sharing antibiotic-resistance genes [27].

Combined, these factors result in a perfect storm, making the conditions for a chronic infection almost impossible for the host immune response to eradicate and cure with antibiotics.

The awareness of the importance of biofilm in infections is growing. The majority of research is on chronic infections in humans. Chronic infections with biofilm as a factor range from cystic fibrosis to otitis media, and the primary investigated pathogens range from *S. aureus* to *Pseudomonas aeruginosa* [26].

Biofilm infections often share the characteristic that they are chronic diseases, either for an extended period of time or recurrent, often with persistent inflammation [29]. However, recent

research has found that biofilms are also found in acute cases of human lung infections [30]. Chronic biofilm infections are challenging to diagnose and treat; antibiotic treatment often results in failure [31].

A new focus of research on biofilm and bacterial infections is rising. Researchers have long investigated the bacteria or biofilm isolated from the environment (i.e., the host tissue) from which they originated. However, bacteria influence and are influenced by their environment [32]. During an infection, the bacteria are surrounded by immune cells, dead cells, and EPS, as well as excreted products from their metabolism, all of which change the environment around them. This can alter the host's environment, bacterial behavior, and physiology [32].

Biofilm in veterinary disease and mastitis

Biofilm in veterinary diseases is a relatively new research area. It has been investigated in several animals and diseases, such as chronic wounds in horses and dogs [33–35]; the canine ear disease, canine otitis externa [36,37]; heart diseases, such as bovine myocarditis [38]; and endometritis in several animal species [39,40]. Research has indicated that biofilm plays a role in several veterinary diseases.

Biofilm in bovine mastitis has been investigated over the past two decades. **Manuscript I,** the published review "Biofilm Research in Bovine Mastitis" [41], offers a detailed summary of the current status of the research, and I will briefly cover the highlighted aspects of the current research here.

In the recent decades, biofilm research in bovine mastitis has expanded as a possible way to better understand and treat bovine mastitis (Figure 2).

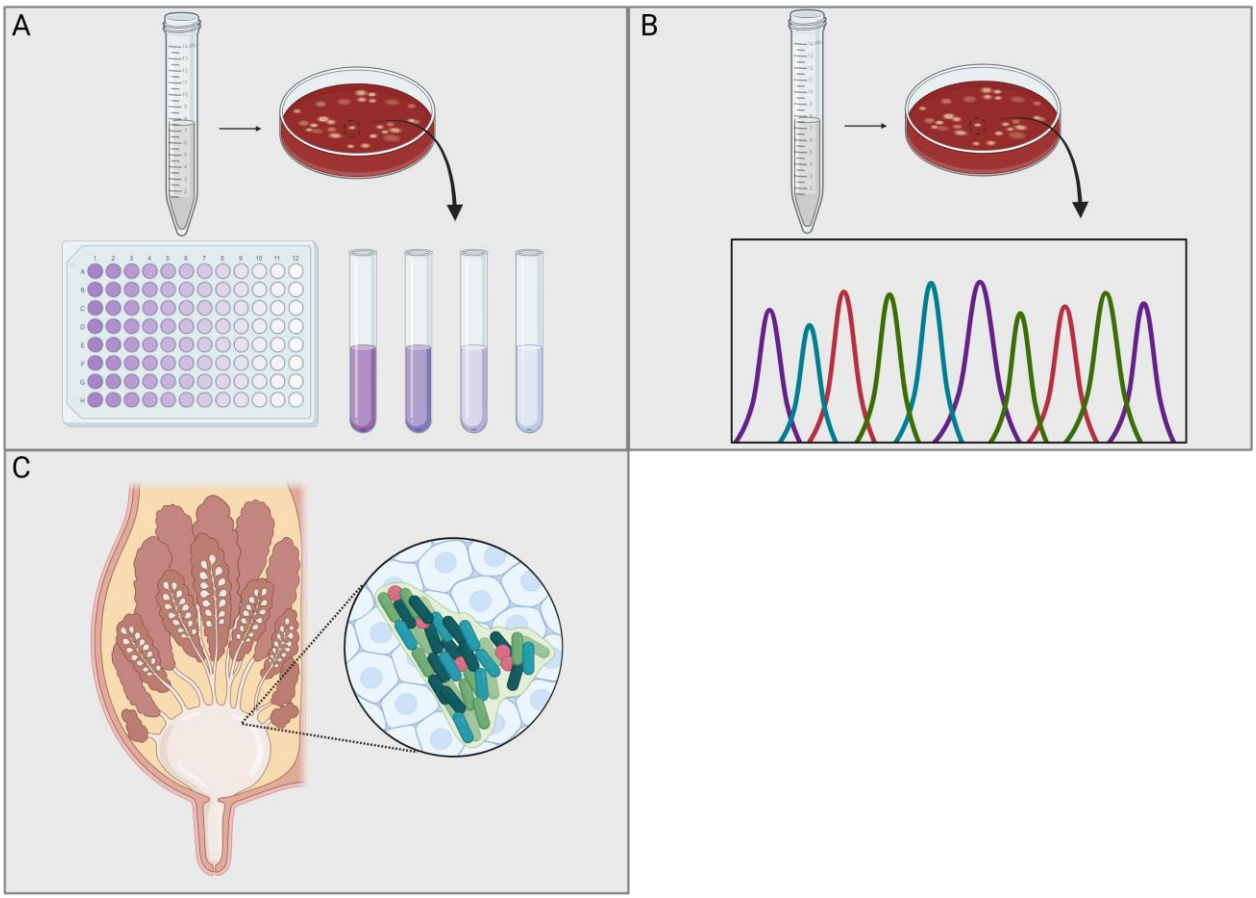

Figure 2. The research on biofilm in bovine mastitis is mainly focused on three areas: (A) the biofilm-forming abilities of bacterial species isolated from milk samples; (B) the molecular profile of bacterial pathogens involved in bovine mastitis; and (C) the visualization of biofilm in bovine mastitis. Created in Biorender.com.

The majority of the research has been based on bacteria isolated from cultured milk samples [42–47]. Hereafter, the bacteria's ability to form biofilm is tested in vitro by various biofilm methods, such as 96 well plates with crystal violet, the tube method, and LIVE/DEAD staining with confocal laser scanning microscopy (CLSM) [42–44,46,48–54]. On this basis, it was established that several mastitis pathogens, such as *S. aureus* [42,43,45,46,48,55], *Klebsiella* spp. [50], *Pseudomonas aeruginosa* [49], and *S. agalactiae* [45,46,48,56] can form biofilms in vitro.

The molecular way is another method to investigate whether mastitis pathogens can form biofilm. The genes (e.g., intercellular gene cluster adhesion operon and biofilm-associated proteins) are also being studied, especially for *S. aureus* [57–60].

Other focuses on the research of biofilm in bovine mastitis are the search for treatment and prevention. Antimicrobial compounds from plants (e.g., spur flowers and rose myrtles) are being investigated as possible treatments [61–63], and vaccines for *S. uberis* and *S. aureus* are developed and under development to prevent mastitis with biofilm infections in dairy cows [64–67].

Few studies have directly observed biofilm in tissue from dairy cows [68,69]. Hensen et al. detected clusters of *S. aureus* in both early and chronic mastitis in three dairy cows that were experimentally infected with *S. aureus* [68]. Schönborn et al. used naturally infected dairy cows with *S. aureus* and detected biofilm components by immunofluorescent staining of the component polysaccharide intercellular adhesin in seven out of 184 smears from swabs taken from the infected udders [70].

Diagnosis and treatment of bovine mastitis

An intramammary infection is detected in different ways depending on the type of mastitis. Clinical mastitis is detected by visible changes to the milk, the udder or the animal's general health [7]. Subclinical mastitis cases are detected due to elevated somatic cell counts in the milk [7].
The somatic cell count (SCC) is often detected during milking and is either estimated by microscopic methods where the cells are stained and counted, by flow cytometry, or by the more on-hand farm method, California Mastitis Test (CMT) [4,7,71,72]. The threshold for inflammation has often been defined as 200,000 cells/mL [73]; however, it is now being discussed that it might be 100,000 cells/ml [74,75]. These methods describe a change in the udder and an indication of an infection but do not provide information about the agent causing the infection.

Different bacterial identification methods are used in diagnostics. The primary methods to detect the bacterial agent is to culture bacteria from milk samples, as most mastitis pathogens grow under aerobic conditions on blood plates and on selective media to identify the pathogen [4,7,71,72]. More precise identification methods for identifying the bacteria isolated from milk samples include matrix-assisted laser desorption ionization–time of flight (MALDI-TOF), where it is possible to identify the bacteria to species level [71,72,76] and molecular methods such as real-time PCR and 16S rDNA sequencing [71,73]. A rule of thumb in diagnosing bovine mastitis is that the milk sample is contaminated when more than three species are present [73].

However, these methods are time-consuming and laborious. Fast and precise methods for diagnostics are needed, and a new and promising one is Raman spectroscopy. Raman spectroscopy is based on Raman scattering and detects the energy levels of molecules, which is measured as wave numbers (i.e., wavelengths per cm^{-1}) and creates a unique imprint of every molecule, allowing chemical bonds to be detected and distinguished. This results in a sensitive and specific method used in multiple research areas, from chemistry to biology [77–79]. In the field of microbiology, Raman spectroscopy could become a potential diagnostic tool to detect and identify

bacterial pathogens [80,81]. This method is precise and only requires minutes to few hours for bacterial identification. [78,80,82–89].

Mastitis is most commonly treated with antibiotics and nonsteroidal anti-inflammatory drugs [90]. In Denmark, it is required that:

> "A milk sample must be taken prior to antibiotic treatment of mastitis. The sample must be examined by a veterinarian or at an externally qualified laboratory as soon as possible and no later than seven days after initiating the antibiotic treatment. No later than seven days after the result of the examination are available, the veterinarian must report the result to the industry-owned register (*Kvægdatabasen;* 'the Cattle Database')" [2]

and only treated with simple penicillins unless the veterinarian starts another treatment [2]. Dry cow treatment is also possible, which is a way to treat the animals with antibiotics when they are at the end of their lactation [91]. The primary use of antibiotics in dairy cows is due to mastitis [2,92,93].

With the frequent use of antibiotics to treat bovine mastitis, it is essential that their use be efficient and kept to a minimum to avoid compromising animal welfare. It is therefore crucial to understand chronic cases of mastitis. As noted, most research on the microbiome and biofilm in bovine mastitis is based on bacteria isolated from milk samples or teat swabs. However, this may not reflect the situation in the udder. There is a risk that some of the bacteria will not shed into the milk and are located so far up in the udder tissue that they are not detectable by a milk sample.

To my knowledge, few published studies have been based on biopsies on bacterial composition and biofilm formation in mastitis.
It should be investigated whether the milk samples reflect the bacteria inside the tissue. Additionally, there is a need to investigate further; by determining not only the species of bacteria found, but also the inflammation and spatial distribution of bacteria.

Objectives

As bovine mastitis is a key disease for the dairy industry, it is essential for animal welfare and the industry that it is well understood and adequately treated. Antibiotic resistance is a threat to human and animal health, and antibiotic use should be minimal without compromising the patient's health – whether it is a human being or an animal.
As antibiotic treatment can fail in chronic mastitis cases, it is crucial to understand the situation in the udder during infection and thereby optimize the treatment, perhaps even minimizing the use of antibiotics.

This study aimed to investigate the composition and distribution of bacteria directly in the udder and in the milk, as well as the histopathological changes and inflammatory response present. Furthermore, I sought to examine the use of Raman spectroscopy to detect mastitis pathogens in milk samples.

The objectives of this thesis were

- To investigate the spatial distribution of bacteria in different locations of the tissue of healthy and mastitis udder quarters;
- To determine the bacterial composition in different depths of tissue from healthy and mastitis quarters;
- To identify the bacterial composition in milk samples from healthy and mastitis quarters;
- To examine whether the bacterial composition in milk samples is similar to the bacterial composition in the biopsies;
- To determine the histopathological changes in the tissue of healthy and mastitis quarters;
- To assess whether multi-excitation Raman spectroscopy could be a possible diagnostic tool for bovine mastitis.

Methods

Methods in Manuscript II

Manuscript II was based on the collection of milk samples and biopsies from dairy cow udders. As the task revealed itself to be far more daunting than anticipated, it was thus necessary to develop a protocol. The following sections describe the rationale behind the process, as well as the analysis of the collected samples.

Including dairy cows in the study

Eight dairy farmers agreed to participate in the study. When dairy cows with elevated SCC were sent to slaughter, I was contacted and went to collect the udders either at the Danish Crown Holsted Slaughterhouse or Kjellerup Slaughterhouse. In addition, two udders were collected at the Large Animal Teaching Hospital. After sample collection, information about SCC, age, parity, and antibiotic treatment of the dairy cows were obtained from the Cattle Database.

Pilot study for collecting samples

Prior to establishing the protocol for collecting the udders, several logistical challenges had to be solved.

Dairy farmers contacted me when dairy cows were sent to slaughter with a high somatic cell count for a period, and I collected the udders from the slaughterhouses. Initially, the plan was to collect the samples from the udders at the slaughterhouse, ensuring limited damage to the samples during transportation time to the Large Animal Teaching Hospital in Taastrup. I was provided with an improvised table in the slaughterhouse to collect samples from the removed udders (Figure 3). However, during the pilot study, it became clear that this resulted in a high risk of contamination.

The original table only had room for half a quarter. The udder therefore had to be cut in half, increasing the risk of contamination. Furthermore, the udder slid off the table. The second table had a larger cutting board that prevented the udders from sliding off. This setup was more optimal for sample collection, but the high risk of contamination remained since the environment was dirty and had direct access to the outside (Figure 3). Moreover, the table was placed adjacent to where the emergency slaughter of cows took place, with blood, feces, and dirt spreading everywhere.

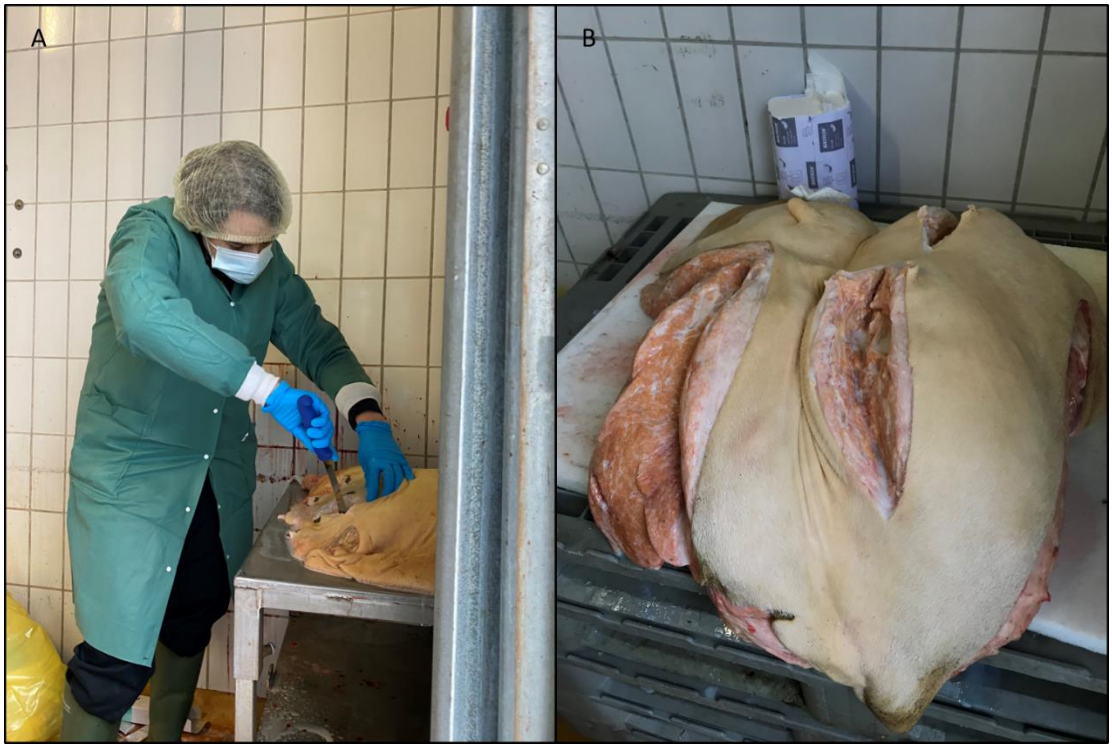

Figure 3. Collection of samples from recently removed udders at Holsted slaughterhouse during the pilot study. (A) The first table provided at the slaughterhouse. (B) The second table provided by the slaughterhouse.

Due to the high risk of contaminating the samples, I decided to remove the udders at the slaughterhouse and transport them to the Large Animal Teaching Hospital in Taastrup. A pilot study for collecting biopsies from udders was conducted at the Large Animal Teaching Hospital in Taastrup and at the Copenhagen Zoo, where the protocol for preparing the udders and collecting the biopsies was practiced.

Collecting of udders from dairy cows

The udders used in the study were collected at the slaughterhouses or the Large Animal Teaching Hospital, University of Copenhagen in Taastrup. They were transported to the Large Animal Teaching Hospital within three to four hours, where a clean dissection room was available. The udders from the dairy cows were removed by the slaughterhouse workers, while I removed the two udders from the teaching cows.

Collection of milk samples and biopsies from udders

The collection of milk samples and biopsies from the udders was conducted with as minimal a risk of contamination as possible. Various tissue sampling methods were attempted; however, some instruments (e.g., biopsy punches) could not effectively cut through the udder tissue. Single-use

scalpels and tweezers were therefore used and discarded after every biopsy. During the procedure, single-use laboratory coats, masks, hairnets, and gloves were worn at all times. Gloves were changed after cleaning the udders and before collecting samples. All sterile tools were placed on an autoclaved cutting board while sampling.

All udders were thoroughly cleaned with sterile cleaning brushes with water (Mediq) for 4 minutes. A minimum of two brushes per udder were used, according to how dirty they were. Subsequently, the entire udder was rinsed with 70% ethanol, and the gloves were changed.

For each quarter, the initial stream of milk was discarded and CMT was conducted. The quarter with the highest and lowest CMT scores was selected for sample collection. The CMT is a quick test to assess the SCC on the spot. Milk from each quarter was milked into the "paddle," an instrument with a "cup" for each quarter of an udder. The CMT solution was added to the milk, and the paddle was briefly rotated. The CMT solution consists of a purple detergent that lyses cell membranes and coagulates according to the SCC [7,73].

Milk samples were collected aseptically from the selected quarters by holding the vial at a 45-degree angle without touching the skin and closing it before returning it to an upright position [19]. Milk samples were stored at 5 ºC until further processing. Afterwards, the udder was once again cleaned with a sterile cleaning brush and sprayed with 70% ethanol that was left to evaporate.
The first incision was made with a single-use scalpel through the outermost layer of skin to avoid contaminating the udder tissue with bacteria from the skin (Figure 4). The next incision, opening up the quarter, was made by an autoclaved knife. Biopsies from five different tissue depths were collected to determine where in the udders biofilm is found (Figure 4) [94]. The biopsies were named after the location they were collected from – Location 1 from the teat end, to Location 5 in the deepest part of the udder (Figure 4B).

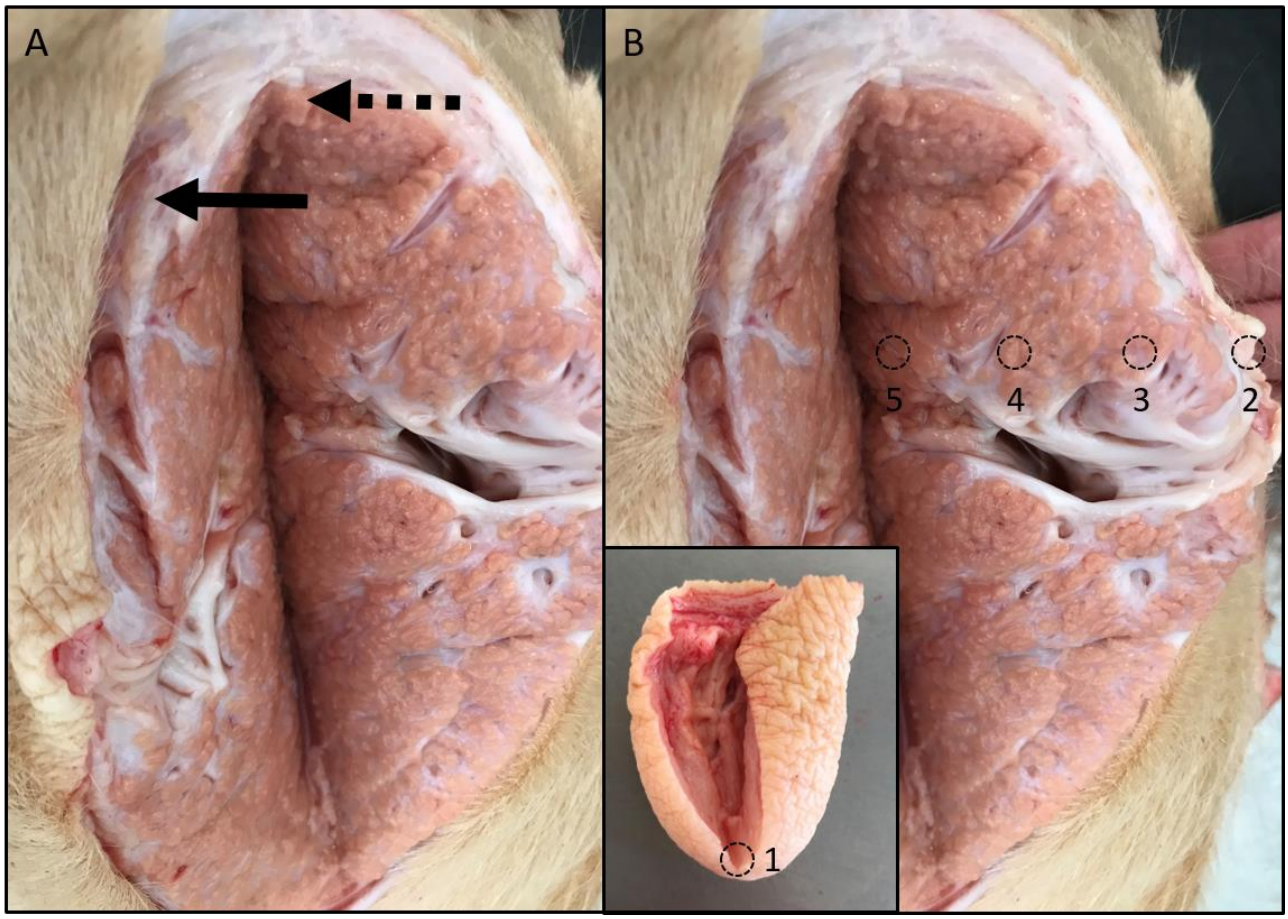

Figure 4. (A) Black arrow: The first incision was cut through the skin by a single-use scalpel to avoid relocating possible bacteria further into the udder and contaminating the biopsies. Dotted arrow: The second incision laid by an autoclaved knife opened the udder. (B) The five locations of the collected biopsies. The biopsies were named after their location from the teat end (1) to the deepest location of the udder (5). The udder shown was a part of the pilot study, and therefore, gloves were not worn when taking the photographs.

Biopsies were collected with single-use tweezers and scalpels, which were changed between each biopsy. Three biopsies were collected from each location (Figure 5). Samples for 16S rDNA sequencing were collected in 1.5ml cryotubes (TTP) and kept on dry ice until storage at -80 ºC. Samples for culturing and MALDI-TOF MS were collected in autoclaved microtubes (Sarstedt) with 0.5mm beads (MP Biomedicals, USA) and kept on cooling elements at approximately 5 ºC until further processing. The tissue samples for microscopy were preserved in Monovette tubes (Hounisen) with 4% formaldehyde on cooling elements and stored at 5 °C until further processing (Figure 6).

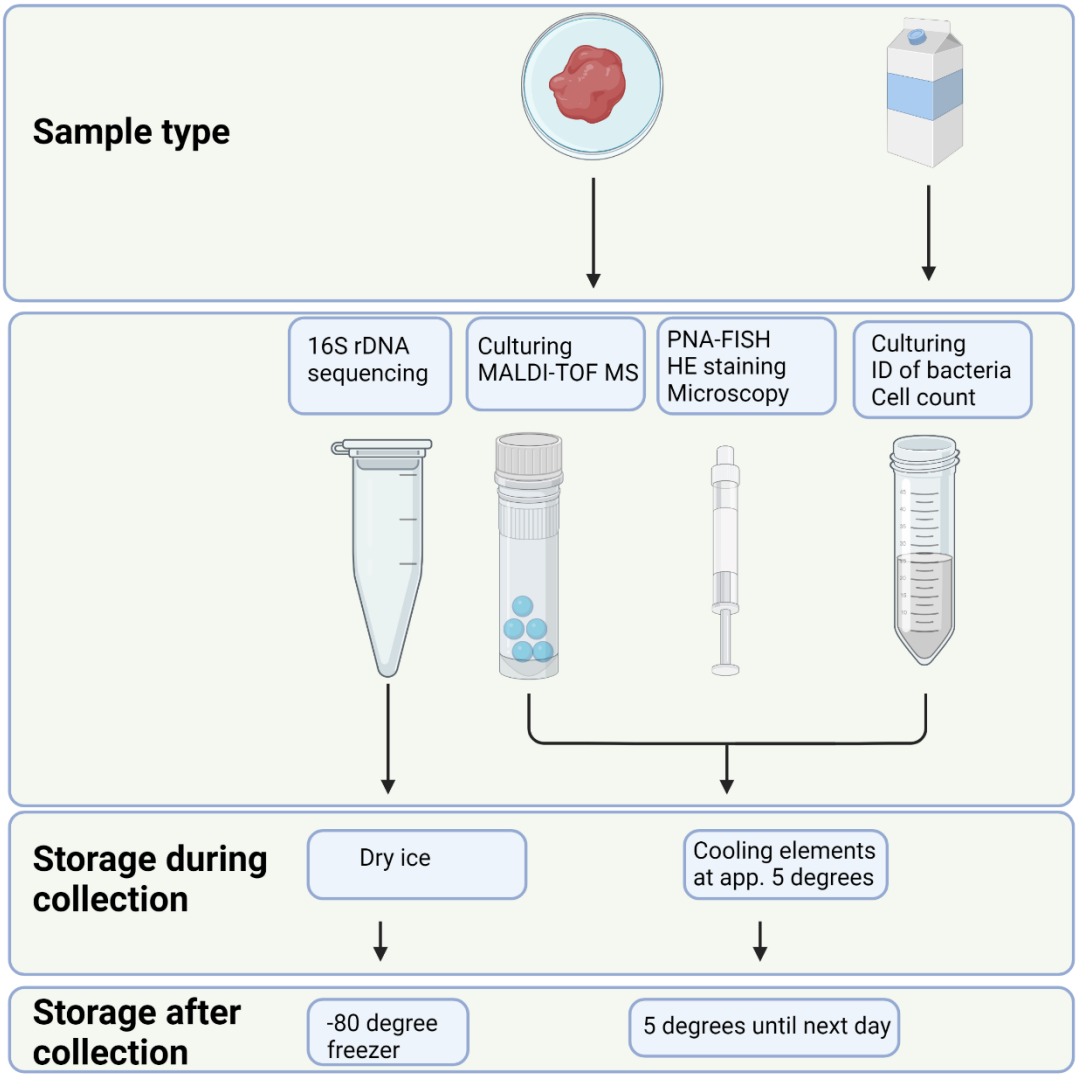

Figure 5. Overview of the collected samples for study. A milk sample and three biopsies at five locations in a quarter were collected and stored in a different tube for further analysis. The sample for 16S rDNA sequencing was stored at -80 ºC, and the remaining samples were kept at 5 ºC until further analysis the next day. Created in Biorender.com

Biopsies were collected four to eight hours after the slaughter and udder removal. Further analysis of the samples was conducted the following day.

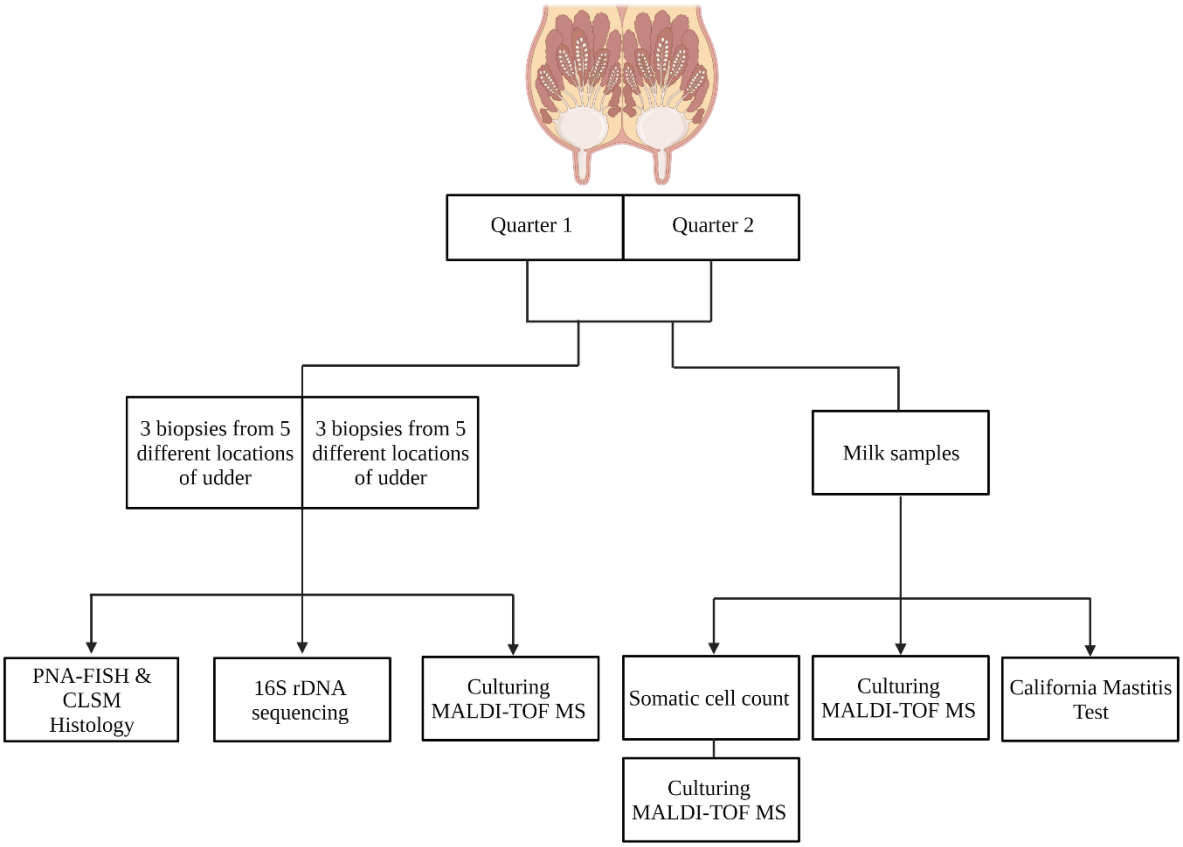

*Figure 6, modified from **Manuscript II**: Overview of the collection of samples. From each dairy cow, samples were obtained from two quarters. Three biopsies from five locations and a milk sample were collected from each quarter. Created in Biorender.com.*

Identification of bacterial species in milk and biopsies

In this study, the milk samples were cultivated twice to determine whether they had been contaminated during collection. Contamination of milk samples is a well-known issue, and I wanted to ensure that the results were not based upon contaminants from the environment, thus not reflecting the bacterial composition in the milk samples.

To minimize the risk of contamination, the handling and processing of milk samples and biopsies were conducted in a laminar flow bench (LAF) cleaned with 70% ethanol prior to use. Milk samples were cultivated by streaking 150 µl milk on 5% blood agar plates (SSI, Denmark) that were incubated at 37 ºC for up to seven days. After the milk samples were streaked onto plates, they were stored at -20 ºC. When plates were examined, the lid was kept closed to reduce the risk of contamination. Bacterial isolates were re-streaked individually on 5% blood agar plates, incubated at 37 ºC overnight and subsequently stored in 1.5 ml cryotubes in Luria Broth with 33% glycerol at -80 ºC.

The biopsies were added 500µl of sterile 0.9% NaCl, homogenized with beads and sonicated as described in Jørgensen et al. [95]. In this study, the volume for inoculation was 150 µl. The samples were cultivated, and bacteria were isolated as described for milk samples. The frozen milk samples were ultimately sent to the laboratory of the Hannover University of Applied Sciences and Arts in Germany, where they were cultivated and identified, and the somatic cell counts were determined in parallel by the recommendations of the National Mastitis Council [96].

Identification of bacterial species

In this study, MALDI-TOF MS investigated isolates from both milk samples and biopsies. Prior to the investigation, freeze isolates were grown on 5% blood agar plates at 37 ºC ON. The bacteria were re-streaked on fresh 5% blood agar plates and incubated, as experience then showed a higher positive rate. The veterinary laboratory, *Sund Vet Diagnostik*, Frederiksberg, identified the isolates using the Vitek MS RUO MALDI-TOF MS system (*bioMérieux*) and the SaramisTM version 3.5 software. For the isolates not identified, the protocol was repeated. At the Hanover laboratory, a MALDI-TOF MS system from Bruker Daltonics with the MBT Compass Library simultaneously cultivated and identified the milk samples (Revision F, MBT 84,668 MSP Library, Bruker Daltonics). For both laboratories, an accepted identification at the species level was ≥ 2.0.

For metagenomic investigations the biopsies were sent to Novogene, UK, who conducted the DNA extraction, PCR amplification, library preparation, and 16S rDNA amplicon sequencing using 16S primers for regions V3–4. The TruSeq® DNA PCR-Free was used for library preparation and Sample Preparation Kit (Illumina) and sequenced on the Illumina Hiseq platform, providing at least 50,000 raw reads per sample.

Statistics and bioinformatics

A biostatistician in the department performed the data analysis using R statistical software. For data analysis, the raw absolute amplicon sequence variant (ASV) generated by the DADA2 pipeline was used [97]. The data analysis is described in detail in **Manuscript II**.

Alpha diversity was measured by Shannon's diversity index, which identified the diversity and abundance of the mastitis versus healthy quarters (condition), locations (tissue depth), and cows. The beta diversity was similarly measured for conditions, locations, and cows. To determine the effects of the alpha and beta diversity on the conditions, locations, and cows, mixed effects models and ANOVA were used.

Somatic cell count of milk samples

In this study, mastitis quarters were defined with a SCC above 200,000 cells/ml and the quarters with a SCC below the threshold were defined as healthy. The SCC of the milk samples collected in this study was determined by the laboratory of the Hannover University of Applied Sciences and Arts in Hannover, Germany. The SCC of the milk samples was determined after cultivation and MALDI-TOF MS. The milk samples were thawed, and the SCC was determined by two methods: microscopic cell differentiation and flow cytometer.

For microscopic cell differentiation, 10µl of raw milk was smeared on a slide and fixated, defatted, and stained with Broadhurst polychromatic solution as described by Prescott et al. [98]. The stained slides were examined by light microscopy, and the different cell types were differentiated.

Visualization of bacteria and cells

Biopsies were used to visualize cells and bacteria and stored in 4% formaldehyde until the biopsies were embedded in paraffin. The biopsies were cut into tissue sections of 4µm in thickness and fixated on microscopic glass slides.
To determine the histopathological changes and evaluate the inflammation present in the tissue sections, the light microscope Leica DMLB/100 S was used.

Tissue sections from Location 5 were stained with hematoxylin and eosin (HE) and evaluated by a blinded pathologist. In collaboration with the pathologists, a scoring system within eight parameters was developed for 10 tissue sections (Table 1), the scoring system was thereafter applied to all tissue sections from Location 5. The scoring system was based on the parameters in Table 1, with the total score resulting from the presence of inflammatory lesions. The resulting groups indicated the presence of pathological changes in the tissue and were described in **Manuscript II**: none (0), mild (1–2), moderate (3–4), and severe (5–8). The neutrophilic granulocyte infiltration was semi-quantitatively categorized as low, moderate, or high.

Hemorrhagi	1
Fibrosis	1
Edema	1
Perivasculitis	1
Inflammation with neutrophilic granulocytes in glandular lumen	1
Inflammation with neutrophilic granulocytes inter/intralobular	1
Inflammation with mononuclear cells in glandular lumen	1
Inflammation with mononuclear cells inter/intralobular	1

*Table 1, **Manuscript II**. The presence of inflammatory lesions by the eight parameters. When a parameter is present, one point is given. The resulting score categorized the tissue sections into the following groups: none (0), mild (1-2), moderate (3-4) and severe (5-8).*

Furthermore, tissue sections from all locations were deparaffinated and stained with peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) and 4',6-diamidino-2-phenylindole (DAPI) according to the protocol developed by Fazli et al. [99].

The double-labeled universal Texas Red PNA-FISH bacterial probe (BacUni, AdvanDx) and DAPI were used to stain 278 tissue sections – five locations from 20 healthy and 36 mastitis quarters. The stained tissue sections were visualized by confocal laser scanning microscopy (CLSM). Two different CLSMs were used in this study; the inverted microscope LSM 880 (Zeiss) with the objectives EC Plan-Neofluar 40x/1.30 Oil DIC M27 and a Plan-Apochromat 63x/1.4 Oil DIC M27 and the upright LSM 710 (Zeiss) with the 63x/1.40 oil Plan-Apochromat objective and EC Plan-Neofluar 40x/1.30 Oil DIC M27. The tissue sections were manually scanned for bacterial aggregates using the 710 microscope, while the 880 was preferred for imaging. The software used was Zen Black version 2.1, and the images were analyzed by Imaris version 9.7.2 (Oxford Instruments).
Tissue sections containing bacterial aggregates over 5 μm were registered as positive [100].

Methods in Manuscript III

I conducted my research exchange in collaboration with Southampton University, spending a month learning the method of Raman spectroscopy. This resulted in **Manuscript III**, whose methods are described here.

Raman spectroscopy

The bacterial strains *S. aureus* and *S. uberis* for the experiments were donated by The Vale Veterinary Laboratory (Devon, UK). The bacteria were grown on 5% blood agar plates.

The bacteria were centrifuged and diluted in 10-times dilution series in semi-skimmed 1.5% fat milk from a supermarket. The diluted bacteria and controls were smeared on quartz microscope slides and air-dried. The protocol developed by Lister *et al.* [80] using a Renishaw InVia Raman microscope (Renishaw, UK) with a Leica DM 2500-M bright field microscope being used. The stage was 100 nm-encoded XYZ, and the lasers used in the study were 532 nm and 785 nm. The cosmic rays were removed by the Renishaw Wire 5.5 software.

Data analysis was performed in Mathlab. In short, the data was first imported to iRootLab and the "Support vector machine" (SVM) machine learning tool was used to classify the data.

Results

Current research of biofilm in bovine mastitis – Manuscript I

Manuscript I, the review "Biofilm Research in Bovine Mastitis," included over 170 papers to assess the current state of the research area.

The research area is still relatively new and mainly focuses on the *in vitro* biofilm-forming abilities of isolates from mastitis milk [41]. Different biofilm assays have been applied to disclose these abilities, and over 140 of the 170 papers reviewed used microtiter plates with crystal violet staining [41]. Furthermore, methods such as the tube method, the Congo red agar test, and microtiter assay with LIVE/DEAD staining and microscopy have been used to elucidate the biofilm-forming abilities of mastitis isolates [42,43,46,47,49–52,54,55,101]. The primary investigated pathogen has been *S. aureus* and other pathogens (e.g., *P. aeruginosa*, *Klebsiella*, and *S. uberis*) have been investigated [42,43,45,49,50]. The findings conclude that the pathogens can mostly form biofilms *in vitro*.

Furthermore, the development of antimicrobial compounds, primarily extracted from plants, is being investigated, and inhibitory effects on mastitis pathogens *in vitro* have been reported [61–63]. Molecular investigations of genes associated with biofilms, such as the intercellular gene cluster adhesion operon (*ica*) and biofilm-associated proteins (bap), are found in multiple *S. aureus* isolates from bovine mastitis cases [57–60]. Two studies have been published where biofilm has been visualized in bovine udders [68,69].

Bacteria in the bovine milk and udder – Manuscript II

The objectives of **Manuscript II** were to determine the bacteria in the milk and the biopsies throughout the quarters and to compare the findings. Furthermore, tissue sections were visualized to determine the spatial distribution of bacteria and the histopathological changes in the tissue. These results were obtained by collecting milk samples and biopsies from the udders of recently slaughtered dairy cows.

Dairy cows in the study

Quarters from twenty-eight dairy cows were collected in the study, and two quarters from each udder of each cow were collected. The udders were categorized according to their somatic cell count, and the results were 20 healthy quarters and 36 mastitis quarters (Figure 7, **Manuscript II**). Furthermore, a CMT score was determined for each quarter.

For 56.7% of the healthy quarters, the CMT score of 0 did not correlate with the corresponding SCC and for 26.9% of mastitis quarters, the CMT score of 2 did not correlate with the SCC.

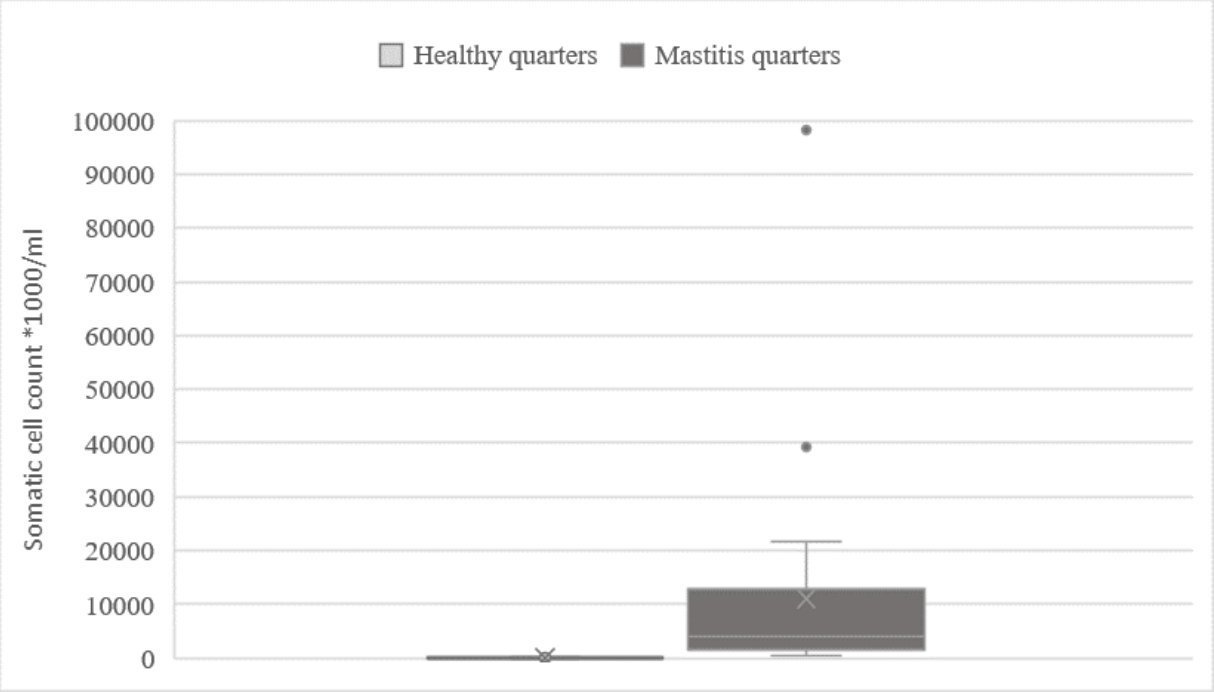

Figure 7. ***Manuscript II:*** *The somatic cell count visualized by a boxplot. The light gray on the left is the healthy quarters, and the dark gray on the right is the mastitis quarters.*

The dairy cows ranged in age from approximately two to 11 years and in parity from 1 to 6. It had been at least 17 days since they had received an antibiotic treatment, and for most dairy cows, it had been over a month since they received antibiotics (Table 2).

Cow ID	Age (months)	Parity	Days from AB treatment to slaughter
1	23	1	21
2	52	3	36
3	72	4	611
4	47	2	316
5	44	2	356
6	71	5	70
7	73	4	33
8	88	5	173
9	141	6	898
10	66	3	-
11	60	3	779
12	45	2	-
13	31	1	17
15	36	1	-
17	65	3	29
18	40	1	117
19	62	3	139
20	47	2	266
21	89	6	20
22	30	1	20
23	44	2	41
24	70	4	41
25	54	3	41
26	78	4	1196
27	99	6	260
28	56	3	119

*Table 2. Supplementary table from **Manuscript II.** Data of the dairy cows included in the study (data obtained from the Cattle Database). The table contains information about the age, parity (number of calves), and days from antibiotic treatment to slaughter. Information of two dairy cows were not accessible.*

Identification of bacteria in milk and biopsies

Milk samples and biopsies from the collected udders were investigated to identify the bacteria in the samples.

The milk samples and biopsies were cultivated, and MALDI-TOF MS identified the isolates. Bacteria were identified in both milk and all locations for healthy and mastitis quarters. There was no growth in 10% of the healthy milk samples, and bacterial growth were observed in all mastitis milk samples. The biopsies had a higher occurrence of no growth, with no growth for 24.2% of the healthy biopsies and 19.5% of the mastitis biopsies. Identifying the bacterial isolates from the milk samples was

mainly possible, with 9.2% of the healthy and 10.3% of the isolates from mastitis milk samples not identified.

E. coli, *Trueperella pyogenes* and *Staphylococcus sciuri* were found to be the most predominant species in healthy milk samples. In contrast, the predominant species found in milk samples from mastitis quarters were *S. aureus*, *Bacillus licheniformis*, and *S. uberis*.

The biopsies had a similar identification success rate, with no identification of 9.94% of healthy biopsy isolates and 8.24% of the mastitis biopsies. The most frequent species of the healthy biopsies were *B. licheniformis* (45%), *Staphylococcus haemolyticius* (35%), and *S. uberis* (25%), whereas the most common species for the mastitis biopsies were *B. licheniformis* (52.7%), *S. aureus* (30.6%) and *S. uberis* (30.6%). Different species dominated the locations throughout the udder; *B. licheniformis* dominated in Location 1, *Streptococcus equinus* in Location 2, *Corynebacterium bovis* in Location 3 and 5, and *S. aureus* in Location 4 in the healthy quarters.

For the different locations in the mastitis quarters, *B. licheniformis* dominated Location 1; *S. uberis* dominated Location 3; and *S. aureus* dominated Locations 2, 4, and 5.

Furthermore, I assessed whether there was a difference in the milk and biopsy composition to reflect on whether milk samples represent the bacterial composition in the udders. I found species that were only present in either the milk or the biopsies. For the healthy quarters, 16 samples were only found in milk samples, and 21 were only found in the biopsies, which indicates that both milk and biopsies were underrepresenting the bacterial composition (Table 3).

Healthy quarters			
Species only found in milk	**Number of quarters**	**Species only found in biopsies**	**Number of quarters**
Acinetobacter johnsonii	1	*Bacillus circulans*	1
Bacillus atrophaeus/subtilis	1	*Brevibacillus species*	1
Bacillus horneckiae	1	*Brevibacter sp.*	1
Bacillus subtilis	1	*Brevibacterium sp.*	1
Brachybacterium sp.	1	*Corynebacterium amycolatum/xerosis*	1
Corynebacterium tuberculostearicum	1	*Corynebacterium freneyi*	1
Enterococcus faecalis	1	*Corynebacterium jeikeium*	1
Gordonia polyisoprenivorans	1	*Corynebacterium mucifaciens*	1
Psychrobacter faecalis	1	*Corynebacterium pilosum*	1
Psychrobacter phenylpyruvicus	1	*Enterococcus faecium*	1
Sphingobacterium daejeonense	1	*Globicatella sanguinis*	1
Staphylococcus arlettae	1	*Kocuria rhizophila*	1
Staphylococcus cohnii	1	*Lactococcus raffinolactis*	1
Staphylococcus hyicus	1	*Microbacterium sp.*	1
Staphylococcus succinus	1	*Sphingomonas parapaucimobilis*	1
Tsukamurella paurometabola	1	*Staphylococcus equorum*	1
		Staphylococcus saprophyticus	1
		Streptococcus gallolyticus	1
		Streptococcus parauberis	1
		Streptococcus sp.	1
		Streptococcus uberis	1

Table 3. ***Supplementary from Manuscript II.*** *The bacterial species were exclusively found in milk samples or biopsies from healthy quarters and identified by MALDI-TOF MS.*

For the mastitis quarters, species were again only found in milk and biopsies; however, the number of species found only in biopsies was much higher than in milk—33 species were only found in the biopsies, whereas only five species were unique to milk (Table 4).

Mastitis quarters			
Species only found in milk	**Number of quarters**	**Species only found in biopsies**	**Number of quarters**
Brevibacterium sp.	1	*Acinetobacter calcoaceticus*	1
Corynebacterium amycolatum/xerosis	1	*Arthrobacter agilis*	1
Paracoccus denitrificans	1	*Bacillus altitudinis/pumilus*	1
Psychrobacter phenylpyruvicus	1	*Bacillus cereus group*	1
Rothia mucilaginosa	1	*Bacillus clausii*	1
		Corynebacterium afermentans	1
		Corynebacterium amycolatum	1
		Corynebacterium confusum	1
		Corynebacterium glutamicum	1
		Corynebacterium mucifaciens	1
		Corynebacterium sp.	1
		Corynebacterium stationis	1
		Corynebacterium ulcerans	1
		Enterococcus cecorum	1
		Gordonia polyisoprenivorans	1
		Klebsiella oxytoca	1
		Kocuria palustris	1
		Lichtheimia corymbifera	1
		Listeria sp.	1
		Microbacterium sp.	1
		Paenibacillus	1
		Ralstonia picketii	1
		Sphingomonas parapaucimobilis	1
		Sphingomonas paucimobilis	1
		Staphylococcus auricularis	1
		Staphylococcus capitis	1
		Staphylococcus cohnii	1
		Staphylococcus epidermidis	1
		Staphylococcus hominis	1
		Staphylococcus lugdunensis	1
		Staphylococcus piscifermentans	1
		Streptococcus agalactiae	1
		Streptococcus pluranimalium	1

Table 4. ***Supplementary from Manuscript II.*** *The bacterial species from mastitis quarters that were exclusively found in milk samples or biopsies and identified by MALDI-TOF MS.*

Some mastitis pathogens are among the species only found in one type of samples; however they were found in both healthy and mastitis quarters. These species were only found once in all quarters.

For the metagenomics analysis of the biopsies, the alpha and beta diversity of the 16S rDNA sequencing data of the biopsies were determined to further identify the bacteria present in the tissue. There was a significant effect of location on alpha diversity (p-value $7.092*10^{6}$). When compared to

Location 1, all depths were less diverse (*p*-values: Location 2: 0.043011, Location 3: 0.000431, Location 4: 0.018043, and Location 5: 0.016813; Figure 8). There was no significant effect on mastitis or healthy alpha diversity, nor interaction between the locations and healthy versus mastitis alpha diversity.

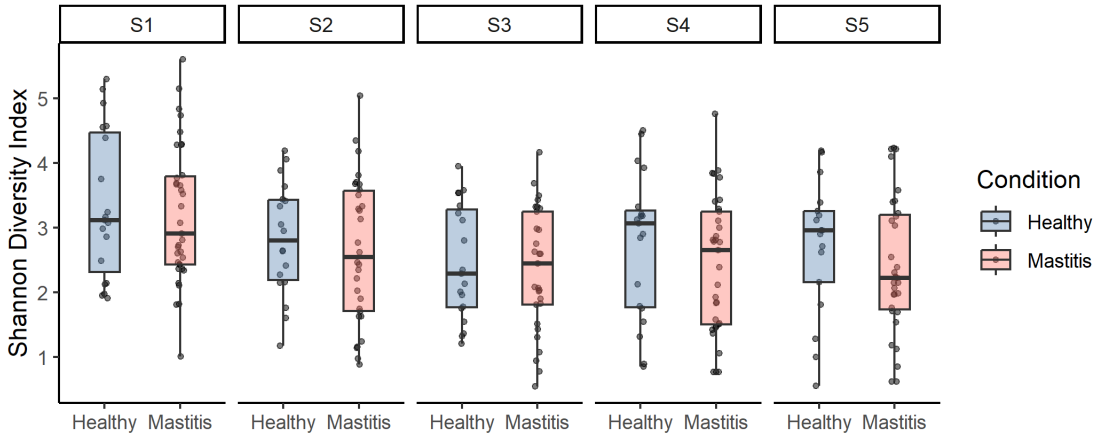

Figure 8. ***Manuscript II.*** *Alpha diversity for the different locations for the healthy and mastitis quarters.*

Acinetobacter dominated throughout the healthy quarters and was observed in four out of five locations, with *Staphylococcus* dominating Location 1. The results were mainly the same for the mastitis quarters; Locations 1, 2 and 5 were primarily dominated by *Staphylococcus,* while *Acinetobacter* prevailed in Locations 3 and 4 (Figure 9).

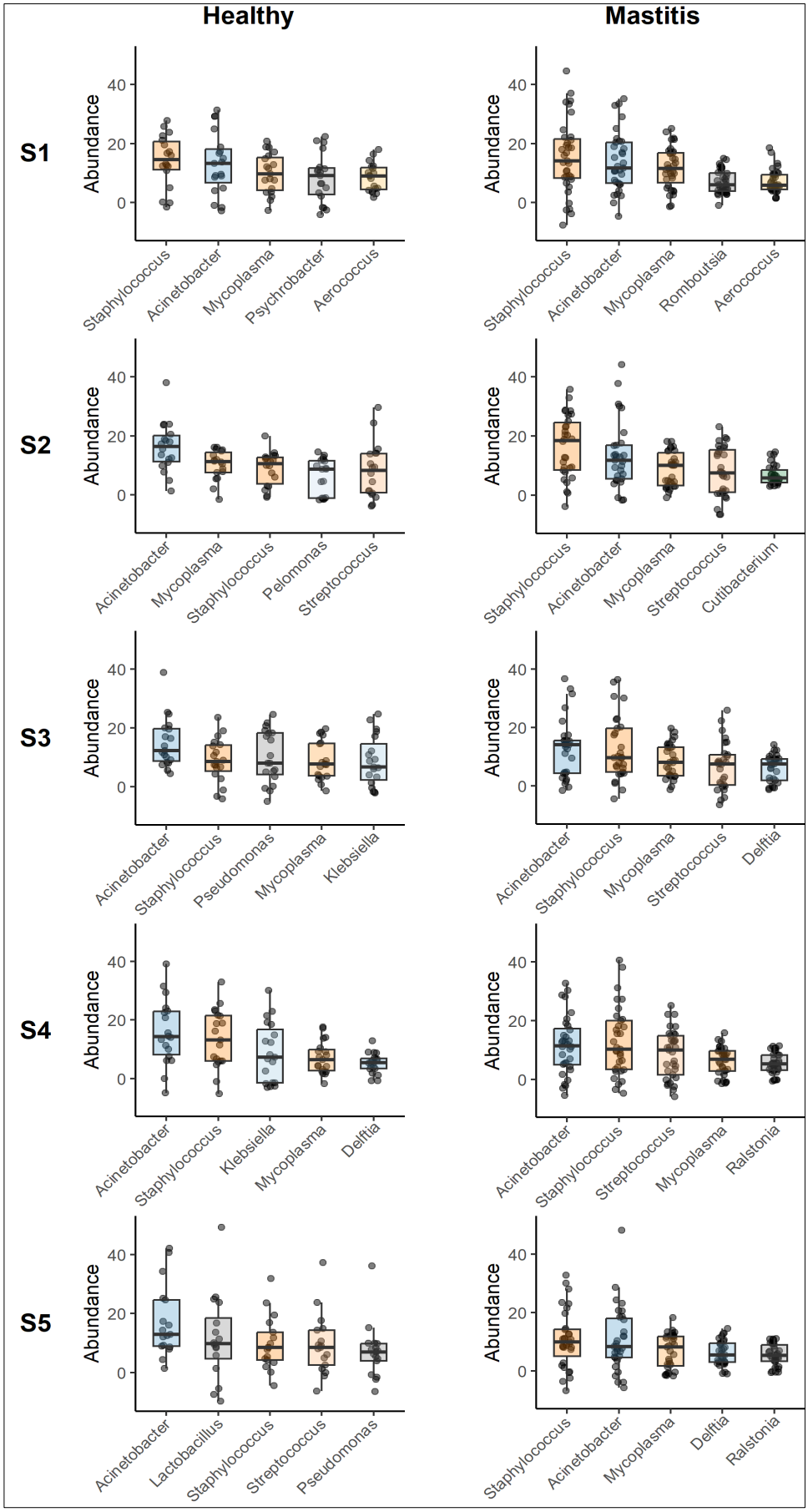

Figure 9. ***Manuscript II.*** *Most common species for the healthy and mastitis quarters for the five locations throughout the udder.*

The beta diversity showed that between-sample variability varied greatly among the dairy cows (Figure 10). When the high variability between the dairy cows was accounted for, there was a significant effect of healthy/mastitis and locations ($p < 0.001$) and the locations ($p < 0.001$) on community composition. There were not observed any significant interactions between healthy/mastitis and location. Location 1, the teat end, presented a different community composition than the other locations in the udder, as visualized by the slightly left-shifted centroid of Group S1 in Figure 10A.

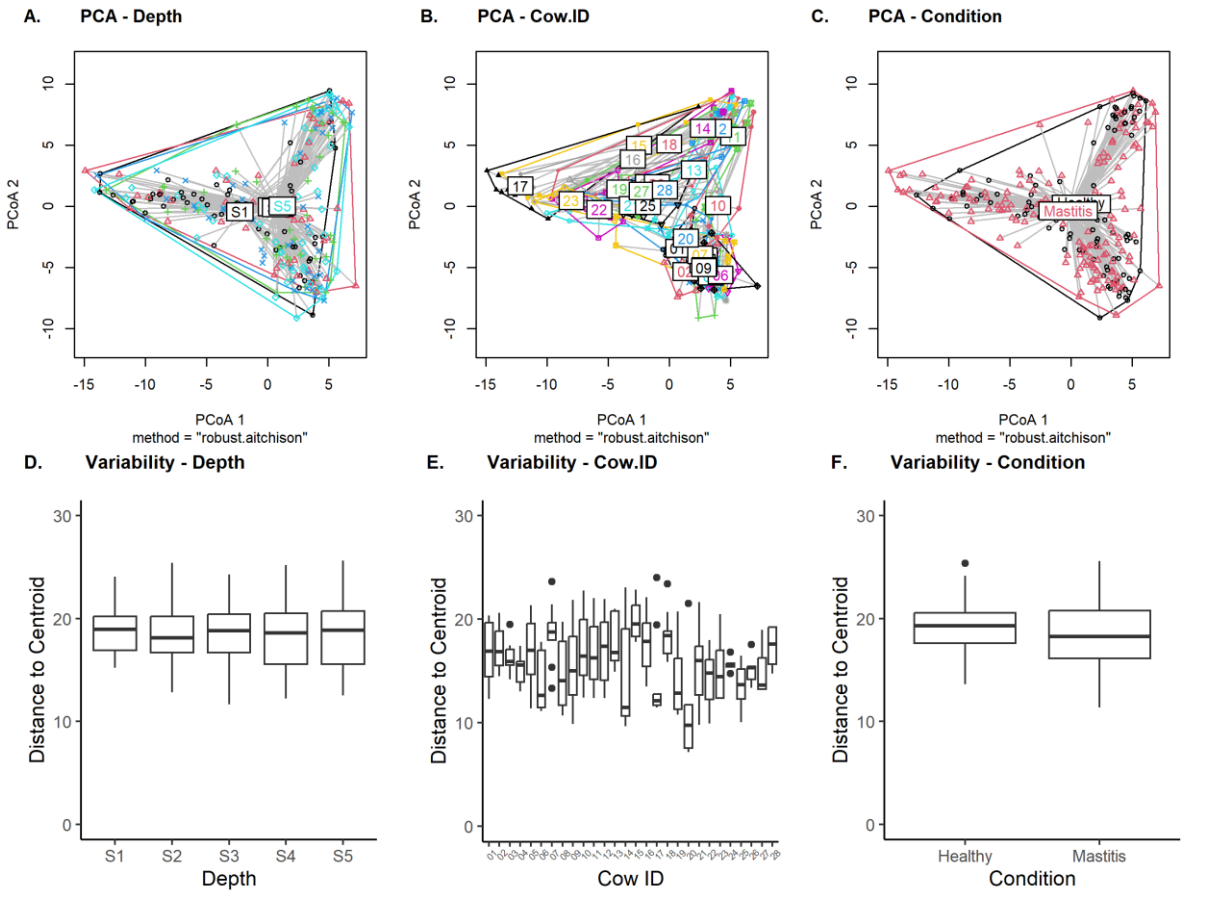

Figure 10. ***Manuscript II.*** **(***A-C) PCA plots of locations (depth), dairy cows (cow ID), and healthy/mastitis (condition). The PCA plots show the groups and the distance to the centroid lines. (D-F) Boxplots over the distance to the centroid for the locations (depth), dairy cows (cow ID), and healthy/mastitis (condition).*

Visualization of bacteria and cells

The cells in the tissue sections from Location 5 from the healthy and mastitis quarters were stained with HE staining and evaluated according the factors described in the methods section (Figure 11).

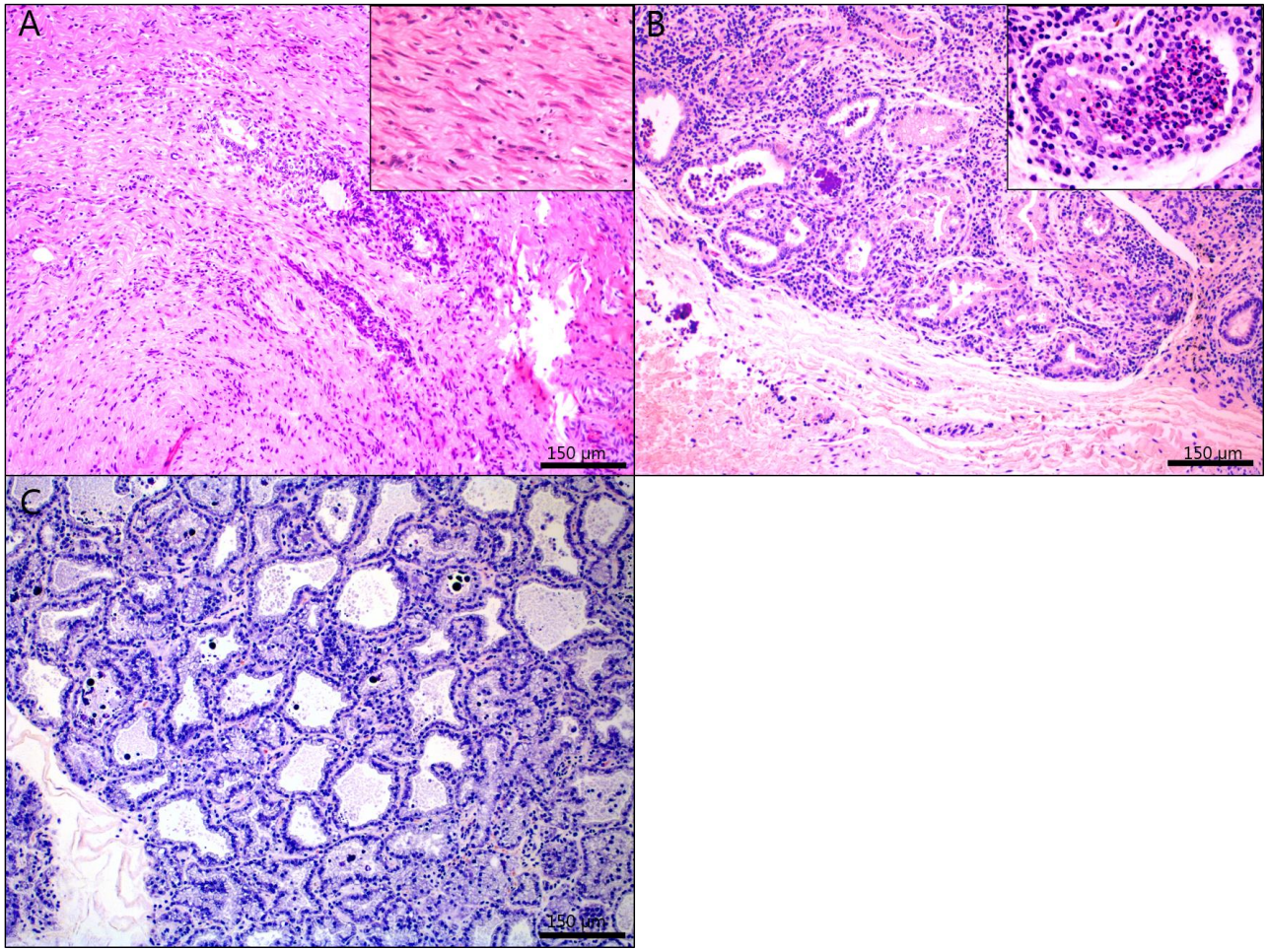

Figure 11. ***Manuscript II****. Representative images of HE-stained tissue sections included in the study. (A) Tissue section from the mastitis quarter with a histopathological score of 6 (severe) and a moderate neutrophilic score, 100–200x magnification. (B) Tissue section from the mastitis quarter with a histopathological score of 5 (severe) and a high neutrophilic score, 100–200x magnification. (C) Tissue section from a healthy quarter with a histopathological score of 0 (none) and a low neutrophilic score of 100x magnification.*

The majority of the tissue sections from the healthy quarters had a mild inflammation score (13), and two had no inflammation. However, five samples had a moderate or severe inflammation score. Surprisingly, 16 of the mastitis quarters only had a mild inflammation score, and two had none while 18 had either a moderate or severe inflammation score (Table 5).

Inflammation in the quarters	Healthy quarters	Mastitis quarters
None ***Histological score: 0***	2	2
Mild ***Histological score 1–2***	13	16
Moderate ***Histological score 3–4***	3	9
Severe ***Histological score 5–6***	2	9

Table 5. ***Manuscript II.*** *Histopathological analysis of tissue sections from Location 5. The tissue sections were scored based on the pathological change in the tissue.*

Furthermore, the neutrophilic granulocyte score was semi-quantitative (Table 6). The majority of healthy quarters had a low neutrophilic granulocyte score, and the majority of mastitis had a moderate score.

Neutrophilic granulocyte score	Healthy	Mastitis
Low	17	4
Moderate	0	10
High	3	6

Table 6. The neutrophilic granulocyte score of tissue sections from Location 5.

The spatial distribution of bacteria in biopsies from healthy and mastitis quarters was visualized by staining 278 slides with tissue sections with PNA-FISH and DAPI (Figures 12-14).

In healthy quarters, aggregates were rarely observed (i.e., in 3%). These aggregates were observed in Locations 1 and 2 of the quarters—the teat end and the annula ring. The morphology of the bacteria in these was cocci and rods. An example of an aggregate from a healthy sample is in Figure 14.B (**Manuscript II**).

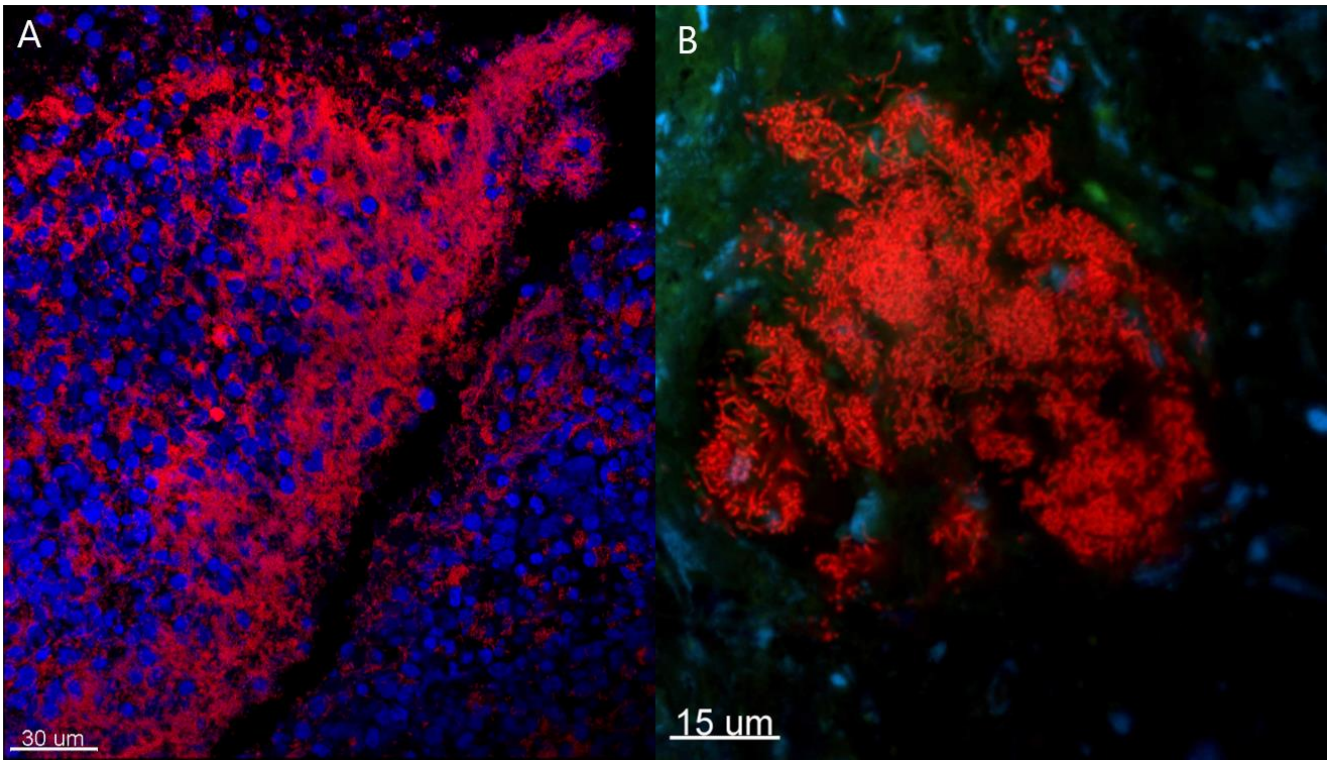

Figure 12. Tissue sections from dairy cows were stained with the Texas Red universal PNA-FISH probe (red) and DAPI (blue). (A) Aggregate with rods from Location 3 in the mastitis quarter, 630x magnification. (B) Aggregate with rods from Location 1 in the mastitis quarter, 630x magnification. All images were taken by CLSM.

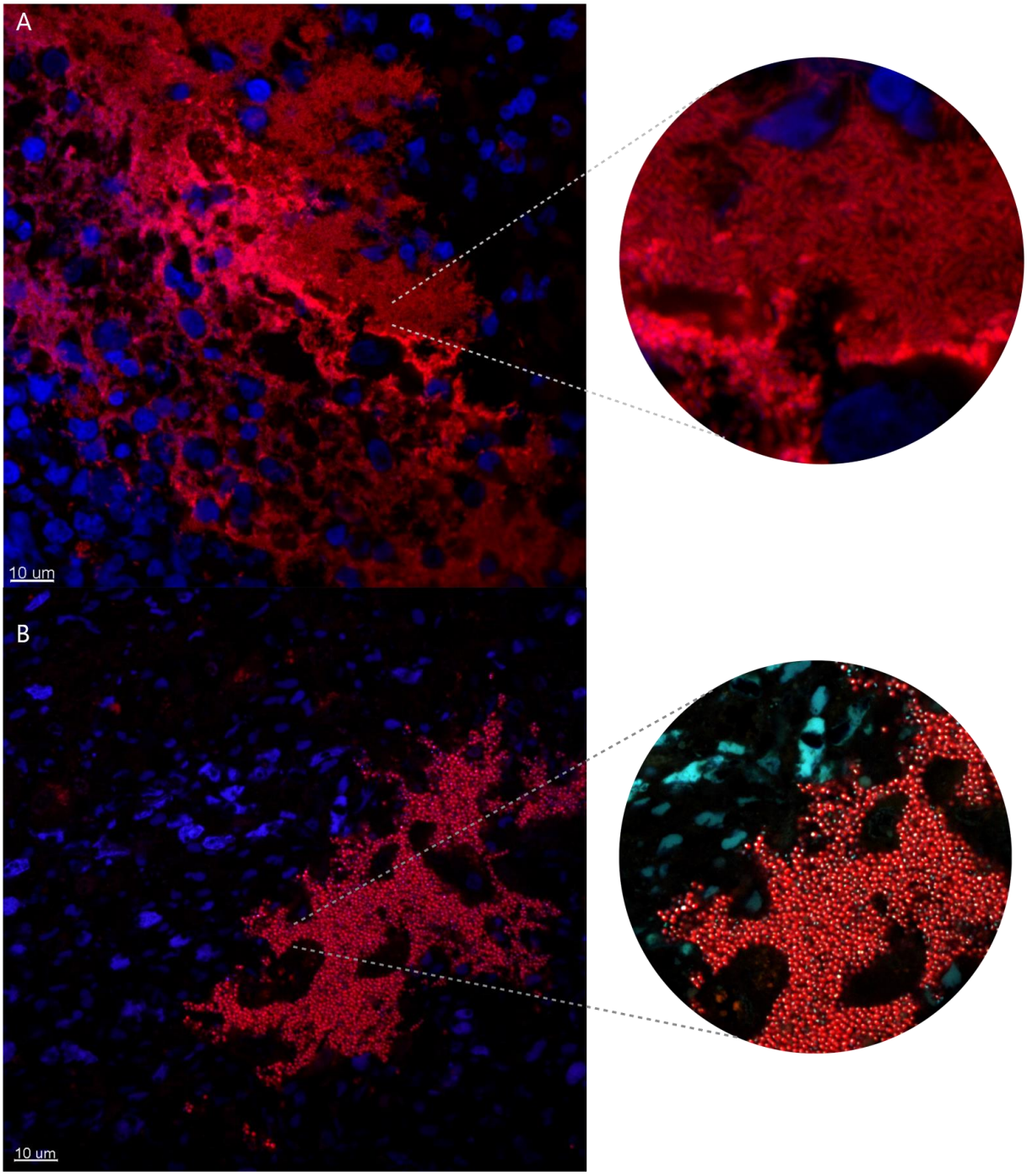

Figure 13. Tissue sections from dairy cows were stained with the Texas Red universal PNA-FISH probe (red) and DAPI (blue). (A) Aggregate with rods from Location 3 in the mastitis quarter, 630x magnification (B) Aggregate with cocci from Location 4 in the mastitis quarter, 630x magnification. All images were taken by CLSM.

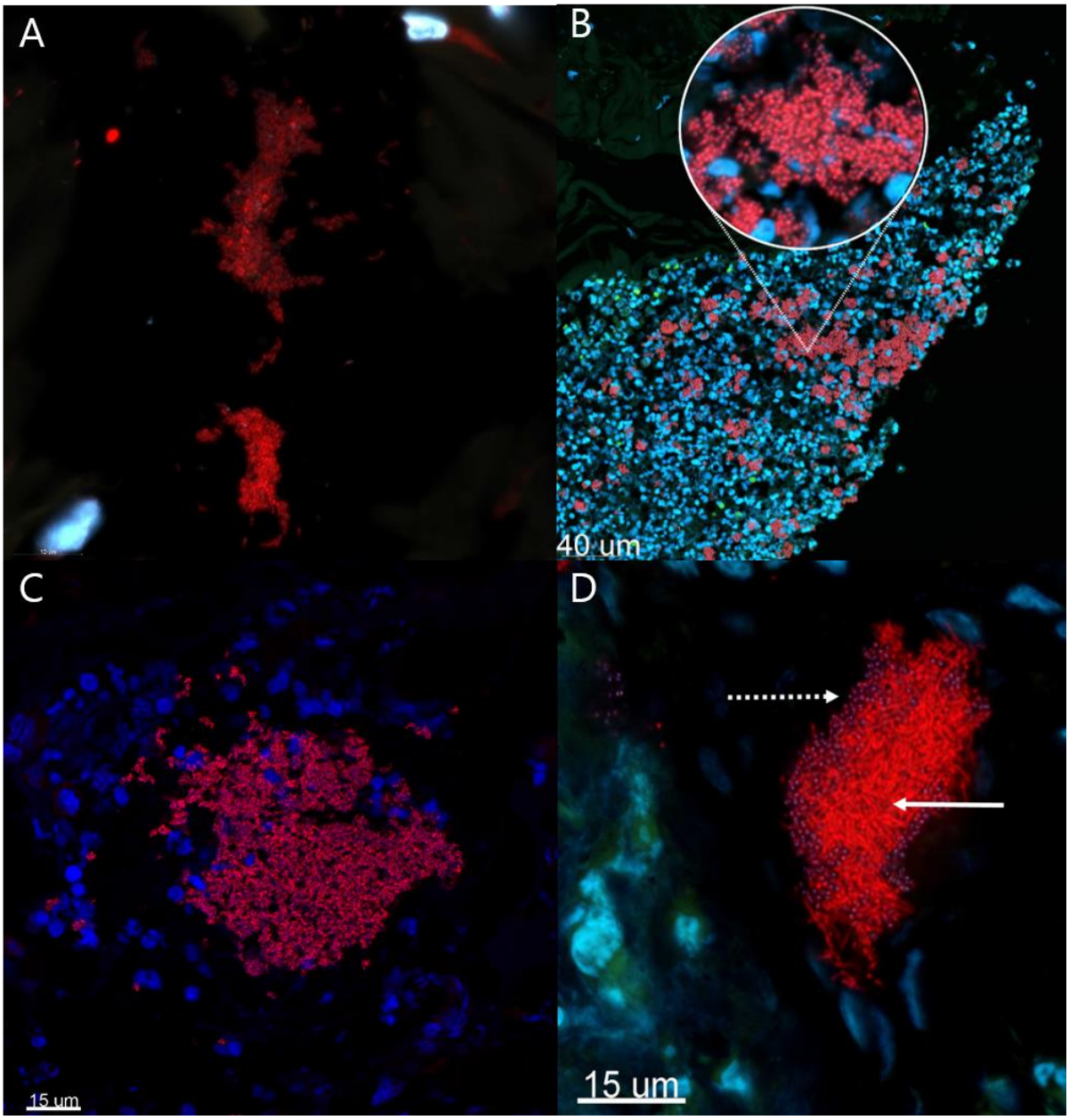

Figure 14. ***Manuscript II.*** *Tissue sections from dairy cows stained with Texas Red universal PNA-FISH probe (red) and DAPI (blue). Green is tissue autofluorescence. (A) Aggregate with cocci from Location 2 in the mastitis quarter, 630x magnification. (B) Aggregate with zoom on cocci from Location 2 in the healthy quarter, 630x magnification. (C) Aggregate with cocci from Location 4 in the mastitis quarter, 400x magnification. (D) Aggregate with both cocci (dotted arrow) and rod (filled arrow) from Location 1 in the mastitis quarter, 630x magnification. All images were taken by CLSM.*

The aggregates in the healthy quarters were observed in different quarters, meaning no healthy quarters had aggregates in more than one location.

Aggregates were observed in 18.4% of the mastitis tissue sections and found in all five locations. Location 2 had a slightly higher proportion of aggregates than the others. In half of the quarters, there were only aggregates in one location, and the exact location differed between the quarters. For the other half of the mastitis quarters, aggregates were found in multiple locations. Both cocci and rods

were observed, and one quarter had both cocci and rods in one aggregate (Figure 12.D; **Manuscript II**).

Multi-excitation Raman spectroscopy as a future diagnostic tool - Manuscript III

The objective of **Manuscript III** was to examine the potential use of multi-excitation Raman spectroscopy to detect bacteria in bovine mastitis milk.

It was possible to detect spectra for both *S. aureus* and *S. uberis* at 532 nm and 785 nm, although distinct peaks were observed at 785 nm (Figure 15).

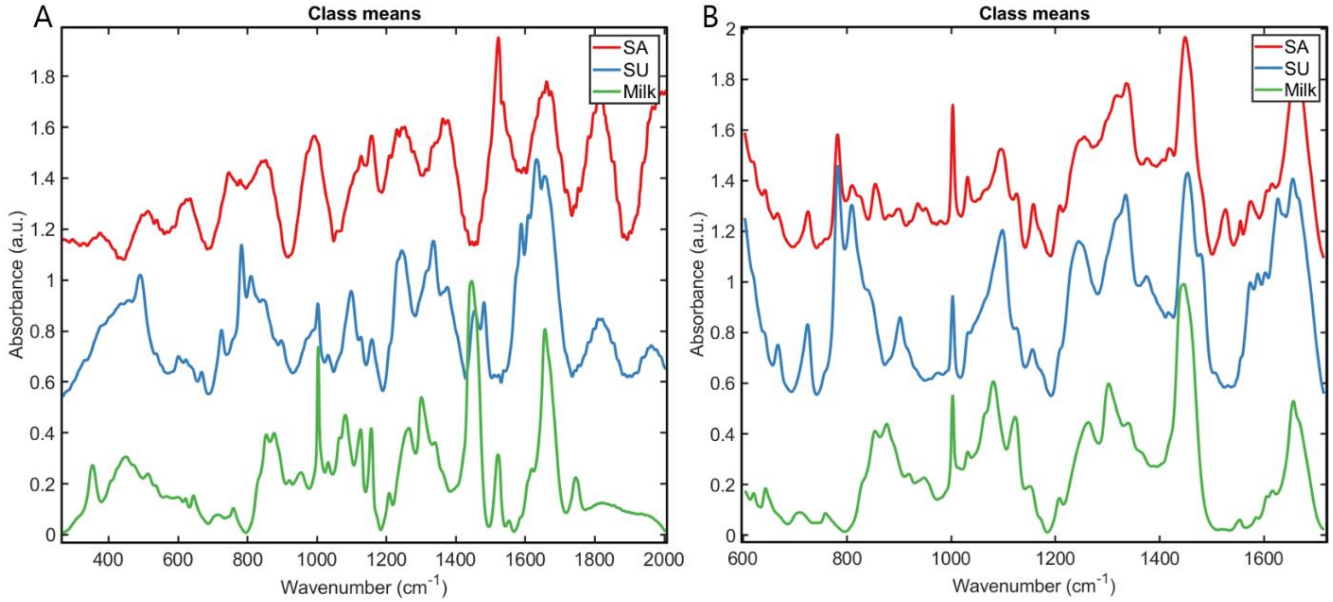

Figure 15. ***Manuscript III****. (A) Class means for the two bacterial species* S. aureus *and* S. uberis *and milk at 532 nm. (B) Class means for the two bacterial species* S. aureus *and* S. uberis *and milk at 785 nm.*

Table 7 depicts the distinct peaks for the two bacterial species and the milk for the two wavelengths, 532 nm and 785 nm. The bacterial strains and the milk had unique and shared peaks.

	Wavenumbers cm^{-1}
532	
S. aureus	372, 508, 630, 750, 837, 847, 987, 1155, 1229, 1249, 1360, 1372, 1522, 1659, 1806, 1816
S. uberis	487, 723, 784, 808, 1004, 1096, 1155, 1240, 1333, 1372, 1451, 1482, 1587, 1628, 1660, 1806, 1816, 1957
Milk	351, 450, 641, 756, 851, 872, 1003, 1079, 1123, 1157, 1259, 1298, 1441, 1517, 1654, 1740
785	
S. aureus	603, 618, 665, 782, 851, 900, 934, 948, 1003, 1030, 1095, 1130, 1252, 1315, 1333, 1447, 1524, 1556, 1572
S. uberis	603, 665, 782, 808, 900, 1003, 1095, 1130, 1241, 1333, 1371, 1451, 1475, 1572, 1586, 1600, 1625, 1654
Milk	618, 702, 756, 851, 874, 1003, 1078, 1120, 1205, 1259, 1300, 1333, 1443, 1654

Table 7. ***Manuscript III****. The spectral peaks for the two bacterial strains* S. aureus *and* S. uberis *and milk at the two wavelengths of 532 nm and 785 nm.*

The SVM machine learning model was used to analyze the method's capability by classifying the data (Figure 16) [81]. The classification accuracies of *S. aureus* were high for 532 and 785 nm, with the lowest accuracy being 80.21% for 532 nm and 83.53% for 785 nm. The highest accuracies for *S. aureus* were 97.8% for 532 nm and 99.29% for 785 nm, indicating that 785 nm was the most optimal for *S. aureus*.

S. uberis had a lower classification accuracy of 532 with 72.74% and 78.72% for 785 nm, indicating that the two wavelengths were less optimal for this bacterial species (Figure 16). However, the highest accuracies were 98.82% and 98.29%. In general, the method could differentiate between the different bacterial concentrations, and the few concentrations the method had difficulty classifying were closely related to concentrations. The concentrations that were challenging to classify for *S. aureus* were $5.5*10^4$ and $5.5*10^3$ for 785 nm. *S. uberis* had more misclassifications of $4*10^4$ and $4*10^3$ for 532 nm and $4*10^4$ and $4*10^6$ for 785 nm.

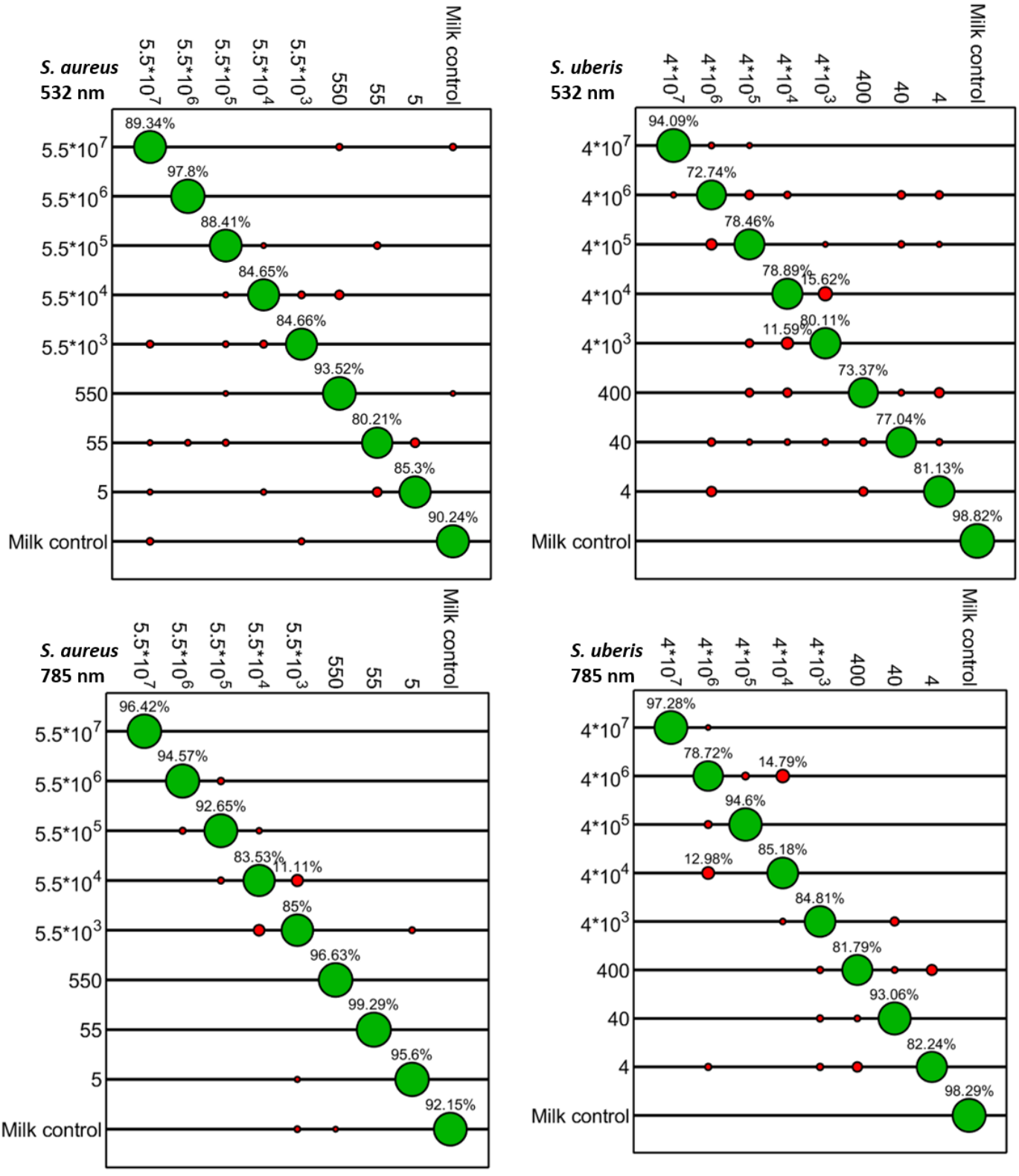

Figure 16. ***Manuscript III.*** *Support vector machine learning was used to determine the classification accuracies for* S. aureus *and* S. uberis *in different concentrations at 532 nm and 785 nm excitations. The green balls refer to the correct identification of the sample, and the red balls refer to the incorrect identification.*

When the two excitations of 532 nm and 785 nm were combined, the SVM had higher success in classifying the samples (Figure 17). Here, the lowest accuracy was 91.41% for *S. aureus* and 90.4% for *S. uberis*.

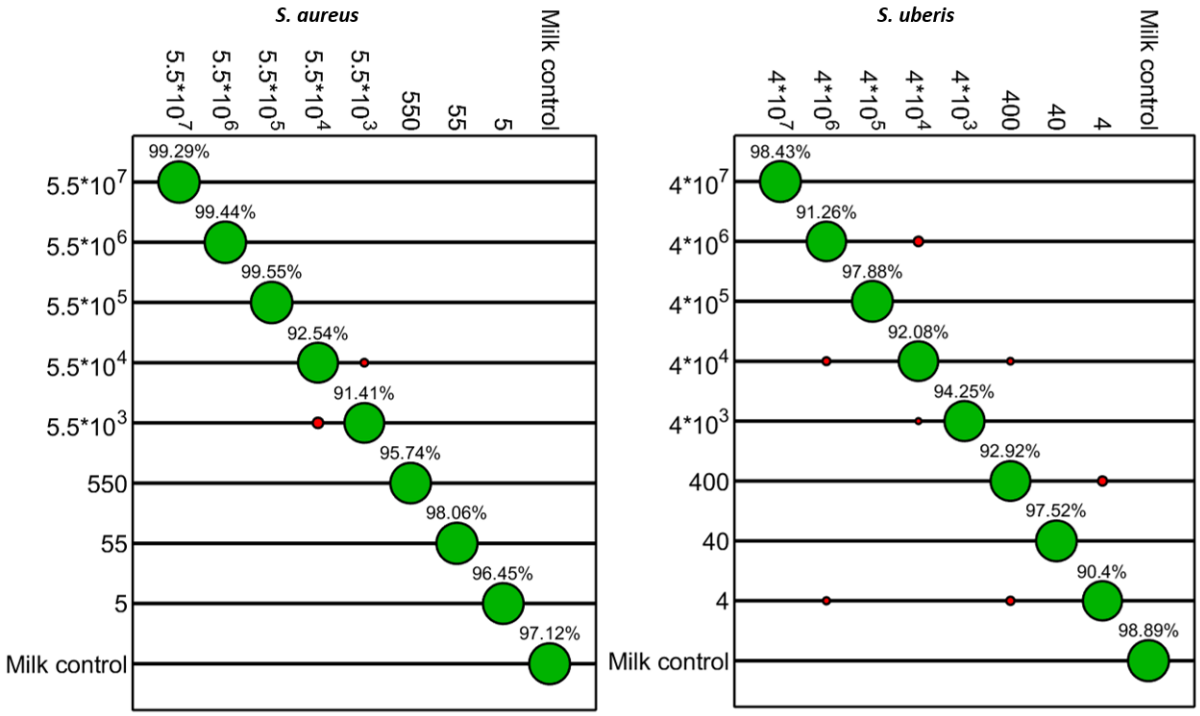

Figure 17. ***Manuscript III.*** *Support vector machine learning was used to determine the classification accuracies for* S. aureus *and* S. uberis *in different concentrations at the combined excitations of 532 nm and 785 nm. The green balls refer to the correct identification of the sample, and the red balls refer to the incorrect identification.*

Discussion

Dairy cows included in study – Manuscript II

Quarters from dairy cows with elevated somatic cell counts were collected for **Manuscript II.** The quarters were divided into the categories "Healthy" or "Mastitis" according to their SCC. This resulted in 20 healthy quarters and 36 mastitis quarters from 28 dairy cows. The corresponding SCC results did not correlate with the CMT results for all quarters. In general, the CMT is considered a reliable analysis to identify intramammary inflammation on the cow-side [7,72]. Studies have found that CMT and SCC typically correspond well [102,103]. A reason for the two methods not correlating well in this study could be the subjective nature of CMT. I conducted CMT on milk during sample collection. I followed the protocol carefully; however, it is possible that my limited experience with the method and the subjective nature of the test led to the results of the two methods are not correlating. This did not influence the study's results, as the quarters were categorized by SCC and not CMT, despite the two dairy cows having no available SCC data. However, the quarters were selected due to the CMT, and it would have been most optimal to have selected a healthy and a mastitis quarter for every cow, resulting in a paired internal control for each mastitis quarter in the study.

The bacterial composition in bovine udders – Manuscript II

The human microbiome conveyed that bacteria are a vital part of the body that maintain many essential functions [104,105]. The importance of bacteria as beneficial commensals and dysbiosis is evolving in many areas [106,107]. When a shift occurs in the commensal microbiome – antibiotic treatment, or other factors, the microbial composition changes to a higher prevalence of a few opportunistic pathogens, causing disease in the host [106,107].

The idea of a commensal microbiome in dairy cows' udders is also evolving. The healthy udder was initially thought to be a sterile environment [11], but in the recent decades, studies have shown that bacteria are present in healthy udders. The most common phylum detected in milk samples include Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes [14,15,21]

In the study, a discrepancy was found of the count of bacterial species between milk samples and biopsies by cultivating and MALDI-TOF MS. To my knowledge, no studies have compared milk samples and biopsies. The bovine mastitis pathogens *S. uberis*, *E. faecium*, and multiple

Corynebacterium species were found only in biopsies. The results were similar for the mastitis quarters; however, more species were only detected in the biopsies and not in the milk. Here the *B. cereus, Corynebacterium, Listeria,* and *S. agalactiae* pathogens were identified in the biopsies but not in the milk samples. The presence of the pathogens only in the tissue and not detected in milk samples could indicate a risk of underdiagnosis of mastitis pathogens.
Another explanation for why the milk samples are not reflecting the bacterial composition in the tissue samples, could be that the number of bacteria is so low in the tissue that they are not shedding into the milk. However, it is essential to remember that all the bacteria, unique for either milk samples or biopsies, were only detected in either one quarter or milk sample.

The most common species by cultivation and MALDI-TOF for biopsies from the healthy quarters were *B. licheniformis*, a soil bacteria [108] that is not associated with mastitis. In the mastitis quarters, the known mastitis pathogen *S. aureus* dominated the samples [109,110]. Another mastitis pathogen, *S. uberis*, was found to be one of the most common bacteria in both healthy and mastitis quarters. The findings of *S. uberis* in healthy and mastitis quarters correlate with other studies that have found the bacteria in healthy udders [111].

The results of published studies investigating the commensal microbiome of bovine udders and the microbiome during mastitis differ. Some studies have found a higher diversity in healthy udders [17,21], and others have noted that both healthy and mastitis udders have a high diversity but with different compositions [12,15,112]. Acinetobacteria, Firmicutes, Proteobacteria, and Bacterioidetes are phyla often detected in healthy udders [15,20,21,113], whereas *Bacilli, Staphylococcus, Streptococcus,* and *Chlamydiia* are associated with mastitis cases [12,15,21,111].

In this study, *Acinetobacter* was one of the most dominant species in both healthy and mastitis quarters found by the metagenomics analysis. The bacteria typically found in soil [114] are not associated with mastitis; however, other studies reported it as one of the most common, indicating that the bacteria could be part of a core microbiota [13–15,115–117]. However, Wang et al. found an association between *Acinetobacter* and subclinical mastitis [114].

When the metagenome of the biopsies was analyzed, the biopsies from Location 1, the teat end, had significantly different alpha diversity than those from the other locations, which could be explained by the teat end being exposed to the environment.

Multiple factors could influence the bacterial composition of the udders. As Ruegg states in her review of the bovine milk microbiome, many factors could influence bacterial composition, such as the animal's age, diet, lactation status, country of origin, bedding and environment, and medical history [18]. Ruegg also identifies a relevant point – that the methods used are not standardized and that contamination is at significant risk when collecting milk samples [18].

This study also demonstrated that more than three bacterial species could be present in a milk sample, challenging the rule that a milk sample is contaminated when more than three bacterial morphologies are present on a plate [7]. These results show that milk and udder composition comparisons should be investigated further to determine whether milk samples truly reflect the bacterial composition in the udders. Moreover, the data presented suggests that more research is needed to understand the role of other species that cannot be cultured.

Histopathology of cells - Manuscript II

In **Manuscript II**, quarters in the healthy category underwent histopathological changes, indicating inflammation. The majority had a low score of neutrophilic granulocytes, which could be due to lactation. When a dairy cow is entering into lactation, local inflammation prepares the udder for the possibility of incoming bacteria during milking. The mastitis quarters varied in histopathological and neutrophilic results. Only the tissue sections from the deepest location of the quarters, Location 5, were analyzed. A small sample from a large organ such as the udder might not reflect the inflammation in the whole quarter.

Biofilm in bovine mastitis - Manuscript I and II

Manuscript I describes how bovine mastitis and biofilm research has mainly focused on the *in vitro* abilities of bacteria to form biofilm in the last few decades [41]. The current research has laid a solid foundation, as we know that some of the most common mastitis pathogens form biofilms *in vitro* [41,118,119]. However, it is suggested that most bacteria can form biofilm and may prefer the biofilm form [27]. It is therefore essential to examine whether mastitis pathogens form biofilm *in vivo* – and to determine whether they are in biofilm form inside the udders of dairy cows. Few studies have visualized the bacteria in the udders of dairy cows [68,70], and this study is one of them. In **Manuscript II**, aggregates were found in approximately 18% of mastitis tissue sections and in 3% of the healthy tissue sections. In the healthy quarters, aggregates were found exclusively in the first two locations. In the mastitis quarters, the aggregates were found in all locations – from

the teat end to the parenchyma. These findings correspond with the two other studies that have investigated biofilm in bovine mastitis *in vivo*. Hensen et al. found "clusters of bacteria" in dairy cows' udder cistern and parenchyma experimentally infected with *S. aureus* [68]. Schönborn et al. detected the polysaccharide intercellular adhesin (PIA) biofilm component for *S. aureus* in multiple locations (e.g. the teat cistern, the gland cistern, and the parenchyma), and found PIA in seven out of 184 swabs [70].

Bacteria can be visualized using several methods. Gram-staining is a common method and used by Hensen et al. [9]. Specific stains can be used to visualize the polysaccharides of EPS and the biofilm matrix [10–12] In this study, I used PNA-FISH to visualize the bacteria present in the tissue sections, as PNA-FISH is a recognized staining technique for visualizing bacteria in tissue and is used both in clinical settings and for research purposes [100,120–123]. The advantage of PNA-FISH is the "uncharged backbone", that enables the probes to hybridize targets less accessible than other probes [124]. The protocol for staining samples with PNA-FISH is simple, and the probes easily bind to both Gram-negative and positive bacteria [99,124]. PNA-FISH is very suitable for visualizing bacteria within tissue in human diseases, due to great resolution [26].

Nonetheless, bacterial aggregates was not found in all samples or all dairy cows. When tissue biopsies are collected from udders, a very small sample is collected from a very big organ, thus resulting in the risk of missing possible aggregated bacteria. Bacteria are often heterogeneously distributed in the tissue, and the placement of the aggregates in the tissue may depend on stochastic events [94]. As few studies have investigated biofilm in the tissue of bovine mastitis udders, future research should aim to investigate the bacteria in the udders by visualizing them to fully understand aggregates and biofilm's role in bovine mastitis.

Raman spectroscopy: a future diagnostic tool of bovine mastitis? Manuscript III

The current diagnostic methods to identify bacterial agents in bovine mastitis rely on cultures or molecular analysis [7,72]. These methods may take at least 24 hours to identify bacteria. In **Manuscript III**, the potential use of multi-excitation Raman spectroscopy was investigated with strains of *S. aureus* and *S. uberis*. It was possible to detect the different spectra of the pathogens and the milk as well as the various concentrations for both species. Some peaks for the two species correlated with other studies on *S. aureus* and *Streptococci* [77,80,82]; however, no other studies with *S. aureus* and *S. uberis* in milk have been published. A few studies have examined surface-

enhanced Raman and Raman spectroscopy to detect antibiotic residues and inflammatory markers in milk [125–127].

This study used milk from a supermarket rather than of directly from dairy cows, to ensure that there no pathogens were present in the milk. However, this does not resemble the milk collected directly from a dairy cow, especially not from a cow with mastitis [7,72,128,129]. Further research should examine whether the changed composition of milk during mastitis and milk from a healthy dairy cow could affect the results of Raman spectroscopy.

A small area is being analyzed when using Raman spectroscopy, resulting in the risk of missing bacteria. The risk can be avoided by concentrating the bacteria in the sample while preparing for Raman spectroscopy or scanning a larger area. However, this technique requires an extended turn-around time.

No studies have examined the use of Raman spectroscopy to detect bacterial agents in milk, and the results of this study are a promising foundation for further research. The analysis is fast, and relatively easy to use, and the materials are inexpensive. One study investigating the method for detecting antibiotic residues in milk from dairy cows used on-site [125] has suggested that an on-farm use of the method could be possible.
This method could deliver results in minutes rather than days, making it possible to treat dairy cows efficiently and quickly. One main limitation of the method is reference libraries. Commercial and free online libraries exist [130–133], but they are still limited, and with the limited research on Raman spectroscopy on bovine mastitis and milk, there is still some way before the method can be implemented in daily diagnostics.

General Discussion

The results from **Manuscript II** demonstrated that bacterial species are found in all udder locations, regardless of whether the dairy cow is healthy or has mastitis. They may be found as single cells or aggregates. The idea of the udder as a sterile environment is being disproved by researchers, and this study contributes to this notion. This study indicates the need for more research on the bacterial composition and spatial distribution in the udder tissue and whether or not they are in biofilm formation. The knowledge of whether mastitis is underdiagnosed or if

biofilm causes chronic disease could affect the treatment outcome and gaining an understanding of biofilm in bovine mastitis is crucial.

Furthermore, the whole environment of the udder should be investigated, as the bacteria and the environment around them act differently in the original environment than in *in vitro* settings in a laboratory [32].
However, collecting biopsies from udders are not simple. Collecting biopsies deep in the udder from living animals is difficult; therefore researchers mainly rely on recently euthanized animals. Relying on euthanized animals limits the knowledge that is possible to gain, as it is only possible to gain information about a single time point. Collecting information over time, (e.g. during the development of an infection), is challenging. Swabs, as described by Hensen et al. a possible way to collect samples from living dairy cows; however they are limited how far they can be inserted [68].

The risk of contamination is necessarily present when collecting samples. In this study, it was an ever-present concern and consideration, especially with 16S rDNA sequencing, where only a small amount of DNA is required for the analysis, increasing the risk of contaminants being included as part of the microbiome.

I discovered that some species only detected in either milk samples or biopsies. Faster and more precise diagnostics could improve the outcome. A method that could be applied in the future could be Raman spectroscopy, as examined in **Manuscript III**. The study results indicated that the method could be implemented as a diagnostic tool in the future – however, more research is needed before the method is ready for diagnostic purposes.

Limitations

A primary limitation of the study of **Manuscript II** was the lack of information about the dairy cows before collection. The only information available from the dairy farmers prior to collecting was that the dairy cows had elevated SCC for a longer period. To ensure a more homogeneous group, it would have been optimal to select dairy cows in advance, culture their milk, and study their medical history and SCC before slaughter. This method also would have eliminated the study's other main limitations: the lack of a "control" quarter and the uneven distribution of healthy versus mastitis quarters. It would have been ideal to have a healthy quarter as internal control and

a mastitis quarter from each dairy cow and to have healthy cows as external controls. This was impossible due to time restrictions.

Only the biopsies were analyzed by 16S rDNA sequencing to determine the bacterial composition due to budget limitations. In the current study of **Manuscript II**, the milk samples were only analyzed by cultivation and MALDI-TOF MS, thus limiting the results to bacteria that could be cultivated. To truly compare the results of the milk samples and biopsies, 16S rDNA sequencing would have been optimal. Furthermore, estimating the bacterial load of the bacterial species would have provided interesting information. The biopsies and milk samples were only grown on blood agar plates under aerobic conditions. There is a possibility that more species would be detected by cultivation and MALDI-TOF MS if multiple conditions and plates were used.

In **Manuscript II,** aggregates were found in nearly one out of five tissue sections from mastitis quarters. As mentioned previously, it is possible to miss areas with biofilm when collecting tissue samples. In this study, five biopsies were collected from the quarters, and the size of the biopsies was small compared to the large size of the udders. The visual examination of the 278 tissue sections in this study was time-consuming, and it would not have been possible to collect and examine additional samples. Future studies could circumvent this issue by selecting a few quarters and collecting more biopsies from them.

Manuscript III was a smaller study based on a one-month exchange stay at Southampton University, United Kingdom. The study could have benefited from more replications to strengthen the results. Furthermore, the milk used in the study was supermarket milk, rather than the milk collected from dairy cows on a dairy farm. Experiments with milk from healthy cows and milk from a dairy cow with mastitis with visible changes in the milk composition are essential to investigate. There is a risk that the increase of cells and various milk components in mastitis milk could interfere with Raman spectroscopy, so this possibility should be investigated.

Conclusion

As one of the most common diseases in the dairy industry and with the highest use of antibiotics, it is vital to understand the dynamics of bovine mastitis. Although bovine mastitis has been extensively researched, certain research areas are new and under development. This thesis contributed to the knowledge of bacteria in bovine udders.

Manuscript I reviewed the current status of biofilm in bovines. The last decades of research have mainly focused on bacteria isolated from milk and whether they could form biofilm in laboratory *in vitro* settings. The main studied species is *S. aureus,* and most studies show that the investigated isolates could form biofilm by examining them with standard biofilm assays. However, only two studies investigated whether biofilm was formed in the tissue of udders [68,70]. While the current research has laid a foundation, further research should focus on the visualization of bacteria to assess whether biofilm plays a role in bovine mastitis.

The spatial distribution of bacteria in the quarters was determined by visualizing the bacteria in tissue sections by PNA-FISH and CLSM in **Manuscript II**. Bacterial aggregates were mainly found in mastitis quarters and in all locations of the quarters, where the healthy quarters had limited aggregates. This finding indicated that aggregates are mainly associated with mastitis; however, more conclusive research is needed.

In Manuscript II, bacteria were found in all locations of healthy and mastitis quarters. The bacterial composition at the teat end was different from the biopsies obtained deeper inside the udder. The most common species in milk samples and biopsies were similar for healthy and mastitis quarters, with a few bacteria that differed between healthy and mastitis, which adds to the knowledge that the bovine udder is not a sterile environment. Some species were only found in either milk samples or biopsies, indicating that milk samples do not detect some species in the quarters. As this factor could have implications for the diagnostics of bovine mastitis, it should be investigated further.

The histopathological changes in the tissue samples indicated that there may be a low grade of inflammation in the healthy quarters and that the inflammation varied among the mastitis quarters.

In **Manuscript III,** multi-excitation Raman spectroscopy was investigated to detect bovine mastitis pathogens in milk. The method was able to detect the spectra of *S. aureus, S. uberis,* and milk, as well as spectra for different concentrations of *S. aureus* and *S. uberis* in milk. These

promising results lay a foundation for future research on multi-excitation Raman spectroscopy as a diagnostic tool.

The findings in this thesis contribute to the knowledge that the bovine udder is not a sterile environment. As one of the few studies focusing on biopsies collected from udders, the results indicate the need for future research on biopsies obtained from udders. Furthermore, the promising aspects of Raman spectroscopy as a diagnostic tool should be investigated further.

Perspectives

There is room for continued research in the areas investigated in this PhD thesis. In this section, I propose suggestions for future research.

The bovine udder microbiome

An aspect not investigated in **Manuscript II** was the 16S rDNA sequencing of the milk samples collected. A study analyzing both milk and biopsies with 16S rDNA sequencing would be fascinating to compare the findings. Furthermore, estimating the bacterial load of these samples would give valuable information about the bacteria's significance. RNA sequencing of both the bacteria and the dairy cow cells to estimate up- and down-regulation of genes during infection, as well as in healthy dairy cows, could also be interesting to attain more profound knowledge of the interactions during infection in the dairy cow. Healthy dairy cows with no prior mastitis history could be interesting as a control group.

Biofilm in bovine mastitis

As noted, it is possible to miss bacteria both single cells and aggregates in an organ as large as the bovine udder. *S. aureus* are associated with low bacteriological cure rates [23], and a future study could investigate dairy cows with *S. aureus* infection and a prolonged period of high SCC. Milk samples and biopsies could be collected as in **Manuscript II**; however, more biopsies would be collected from each quarter to increase the possibility of finding the biofilm. Combining the universal bacteria probe used in **Manuscript II** and a specific probe for *S. aureus* could elucidate whether the biofilm was an *S. aureus* biofilm to determine whether *S. aureus* are, in fact, forming a biofilm during infection.

Raman spectroscopy

The study in **Manuscript III** was on a small scale and in a relatively sparsely investigated area. Many aspects could be interesting to study further. In **Manuscript III,** the two bacterial species were examined individually. Further analysis of mixed cultures in milk could be interesting—both with multiple pathogens and with commensals with a pathogen in different concentrations. As mentioned previously, **Manuscript III** only used milk from a supermarket, which may not resemble milk collected on a dairy farm. An experiment with milk from healthy udders and udders with mastitis in different degrees could be interesting to explore to determine whether the change in milk composition will mask the bacteria in the milk. The presence of biofilm in milk samples

could be relevant to explore to assess whether the method could detect biofilm and differentiate between single cells and biofilm in milk samples. Another possible application for the method could be Raman spectroscopy on biopsies. My ambition was to investigate this during my research exchange; however, due to time restraints, it was only possible to conduct a few pilot studies. If Raman spectroscopy could detect bacteria or biofilm in biopsies, its application to diagnostics would be highly valuable.

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Biofilm Research in Bovine Mastitis

*Regitze Renee Pedersen[1], Volker Krömker[2], Thomas Bjarnsholt[3,4], Kirstin Dahl-Pedersen[1], Rikke Buhl[1] and Elin Jørgensen[3]**

[1] Department of Veterinary Clinical Sciences, University of Copenhagen, Copenhagen, Denmark, [2] Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark, [3] Department Immunology and Microbiology, Costerton Biofilm Center, University of Copenhagen, Copenhagen, Denmark, [4] Department of Clinical Microbiology, Copenhagen University Hospital, Copenhagen, Denmark

Bovine mastitis is one of the most important diseases in the dairy industry and has detrimental impact on the economy and welfare of the animals. Further, treatment failure results in increased antibiotic use in the dairy industry, as some of these mastitis cases for unknown reasons are not resolved despite standard antibiotic treatment. Chronic biofilm infections are notoriously known to be difficult to eradicate with antibiotics and biofilm formation could be a possible explanation for mastitis cases that are not resolved by standard treatment. This paper reviews the current literature on biofilm in bovine mastitis research to evaluate the status and methods used in the literature. Focus of the current research has been on isolates from milk samples and investigation of their biofilm forming properties *in vitro*. However, *in vitro* observations of biofilm formation are not easily comparable with the *in vivo* situation inside the udder. Only two papers investigate the location and distribution of bacterial biofilms inside udders of dairy cows with mastitis. Based on the current knowledge, the role of biofilm in bovine mastitis is still unclear and more *in vivo* investigations are needed to uncover the actual role of biofilm formation in the pathogenesis of bovine mastitis.

Keywords: biofilm, bovine mastitis, chronic infections, antimicrobial resistance, antimicrobial therapy

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***Correspondence:**
Elin Jørgensen
elin.jorgensen@sund.ku.dk

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INTRODUCTION

Bovine mastitis is an important disease in the dairy industry with severe consequences for the welfare of dairy cows and the economy of the industry (1). Antibiotic treatment of bovine mastitis account for the highest antibiotic use in the dairy industry (2).

Bovine mastitis is defined as inflammation of the mammary gland and is most commonly caused by bacterial infection (3). Bovine mastitis occurs in two different clinical manifestations; subclinical and clinical mastitis, and ranges from mild, moderate to severe cases. Subclinical mastitis can be diagnosed by tests, e.g., the somatic cell count in milk, however, no clinical signs are apparent (4). Clinical mastitis manifests with visible changes to the milk in the form of clots or flakes and clinical signs of infection and inflammation, such as fever, redness, pain, and swelling of udder and lymph nodes (4). Some cases of bovine mastitis resolve themselves and most cases resolve after standard antibiotic treatment (2), however, some cases can progress to a detrimental point where the cow is culled, and in severe cases, spontaneous death may even occur (1, 4).

The most common infectious agents of bovine mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae* (2), *Escherichia coli* (5), and *Streptococcus uberis* (6). *S. aureus* is a common and challenging mastitis pathogen, as *S. aureus* has a high persistence rate (7, 8) and a low bacteriological cure rate in clinical mastitis cases (9). During bovine mastitis, bacteria potentially upregulate expression of virulence factors that can lead to higher resistance to phagocytosis (10)

and upregulation of genes that destruct host tissue and the ability of the host cells to capture iron, e.g., lactoferrin (11). The pathogens are adapted to infection of the tissue in the mammary gland by a broad variety of virulence factors, e.g., the propensity to invade and escape host cell defenses by hemolysins (12), adhesion to host cells and production of leukotoxins to destroy monocytes and polymorphonuclear cells (12). Further, some of the pathogens are low shedders (13) and some form biofilm (12). All resulting in pathogens capable of causing long-lasting infections.

Bovine mastitis is normally treated with antibiotics, however, in some cases, the antibiotics are not resolving the disease and the infection becomes chronic. Continued antibiotic treatment in these cases where antibiotics do not eradicate the microbial agents increases the risk of developing antibiotic resistance, which is one of the greatest threats to human and animal health (14).

Chronic and recurrent cases of bovine mastitis share similar characteristics with chronic biofilm infections observed in humans and other animals. Biofilm is defined as "a coherent cluster of bacterial cells imbedded in a biopolymer matrix, which, compared with planktonic cells, shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defense" (15), however the role of matrix is unclear *in vivo* (16). Biofilm is suggested to be the default mode of growth for bacteria (17). Antibiotic treatment of biofilm infections is often unsuccessful and thus the infections are difficult to eradicate (18, 19). Being part of a biofilm can provide protection for the bacteria against threats from the environment, including antibiotics and host defenses (20).

The role of biofilm in human infections has been an expanding research field since bacterial aggregates were observed in 1977 in the lungs of patients with cystic fibrosis (21), and since 1982 where the first report on a medical biofilm causing recurrent infection (bacteremia) was described (22). However, in veterinary medicine, few reports exist on biofilms' direct role in infections, as most literature focuses on *in vitro* characteristics of pathogenic bacteria/biofilms and not their role *in vivo*.

In human medicine, biofilms are known to contribute to a wide variety of infections and diseases including wound infections, implant related infections, lung infections, osteomyelitis, chronic otitis media, urinary tract infections, chronic sinusitis, dental plaque, endocarditis, etc. (23). *Pseudomonas aeruginosa* biofilm infection in cystic fibrosis patients is one of the most well-studied biofilm infections to date, and intense research has revealed both pathogenetic, diagnostic, and therapeutic breakthroughs, and has increased the life expectancy of these patients dramatically (24–26). Biofilms are found in the majority of human chronic wounds and are considered to play a consistent role in the pathogenesis of impaired wound healing (27–29). Major biofilm pathogens in chronic wounds are *S. aureus*, *P. aeruginosa*, and *Enterobacteriaceae* (27, 30, 31). Implant related infections are often also driven by biofilms that cause low grade, difficult to detect infections with delayed onset (32, 33). Biofilm infections are thus important and are estimated to account for 550,000 deaths and 17 million infections yearly in the USA (34).

Understanding the role of biofilm in bovine mastitis will potentially unlock new treatment options and avoid unnecessary antibiotic treatment. If thereby being able to cure these chronic and recurrent bovine mastitis cases, the economy of the dairy industry, as well as animal welfare will improve and use of antibiotics will decrease.

In this paper, we review the literature on the development of bovine mastitis biofilm research with focus on the last two decades. In addition, we review the methods applied in published research and propose new methods for future research of biofilms' role in bovine mastitis.

DEVELOPMENT OF RESEARCH OF BOVINE MASTITIS

The first studies investigating biofilm forming abilities of bovine mastitis pathogens emerged in the early 1990s. In 1993, "slime production" (exopolysaccharide matrix) was observed in bovine coagulase-negative staphylococci (CNS) strains. This slime production was observed *in vitro* by using the tube method together with Congo Red Agar plates and suggested that the slime-production functioned as a virulence factor (35). Later, strains of *S. aureus* isolated from bovine mastitis cases were found to bind to milk fat globules. This suggested that the bacteria were in a biofilm mode of growth *in vitro* (36). During the first decade of 2000, most papers concentrated on investigating the *in vitro* biofilm forming abilities of *S. aureus* and *Staphylococcus epidermidis* isolates from bovine mastitis cases (37–39), the genes that were associated with biofilm formation (39–41), the susceptibility to antimicrobial agents (42), and potential treatment options against biofilm infections (43–45).

After 2010, the research of biofilm in bovine mastitis accelerated, and during the last decade, over 170 studies have been published. The focus of the research is still the *in vitro* biofilm forming abilities of bovine mastitis pathogens but also investigations of antibiotic resistance, molecular investigations of biofilm related genes, and the search for potential treatments and vaccines; the majority of these paper have focused on *S. aureus.* In only two *in vivo* studies, bacterial biofilms have been directly identified in bovine udders with mastitis (46, 47).

BIOFILM METHODS APPLIED TO THE RESEARCH OF BIOFILM IN BOVINE MASTITIS

Methods Used for Investigation of Biofilm Forming Abilities of Bovine Mastitis Pathogens

The biofilm forming abilities of bovine mastitis pathogens *in vitro* have been investigated by multiple traditional biofilm methods. Most studies have focused on bacterial isolates from milk samples of bovine mastitis cases and the main focus has been on *S. aureus*, a well-known *in vitro* biofilm producer (48) and one of the most common pathogens in chronic bovine mastitis (49). The majority of the studies, i.e., more than 140 papers, have been using microtiter plates with crystal violet staining for

quantification of the bacterial biomass. When using this biofilm assay, the bacteria are grown in polystyrene microtiter plates. The wells are emptied and washed at different time points, whereby the remaining biofilm biomass can be stained and quantified with crystal violet (50, 51). The crystal violet stain is used to quantify the total biomass in these system, as the stain binds to negatively charged molecules, which means to both the bacteria and exopolysaccharides (50). *S. aureus* is the most common species investigated using the microtiter assay in biofilm and bovine mastitis research to investigate its ability to form biofilm *in vitro*. Multiple studies found that majorities of *S. aureus* isolates from bovine mastitis cases can form biofilm *in vitro* by this assay (52–54). Applying the same method, 20–30% of *S. agalactiae* mastitis isolates also showed biofilm forming abilities *in vitro* when cultivated in different atmospheric conditions and growth media (55–57). This assay was also used to investigate the biofilm forming abilities of 53 mastitis isolates of *Klebsiella* spp. and 17 *Pseudomonas aeruginosa* mastitis isolates, all isolates were able to form biofilm (58, 59).

Although not as common as the crystal violet assay, several studies use the Congo Red Agar (CRA) test. The CRA method was developed by Freeman et al. (60) in 1989 for "detecting the production of slime by coagulase-negative staphylococci." The "slime-forming" strains are black and the strains not capable of forming slime appear red on the agar (60). The CRA test is a qualitative method to estimate whether staphylococci isolates are able to produce biofilm *in vitro* and is often followed by a quantitative assay—such as the tube method or the microtiter assay. Half of *S. aureus* isolates from dairy cows with subclinical mastitis were able to produce biofilm by the CRA method (61, 62).

In the standard tube method, bacteria are cultivated in culture tubes, washed and then stained with crystal violet, safranine, or other stains. Biofilm production is observed by color on the sides and bottom of the tube (63). When the biofilm forming ability of *S. aureus* isolates from bovine mastitis cases was investigated by the tube method using safranine stain, 25–70% of the isolates were able to form biofilm (61, 64, 65).

Using yet another staining method, ∼85% of CNS isolates from mastitis milk samples were able to form biofilm when their biofilm forming ability was investigated by the microtiter assay and stained using the LIVE/DEAD technique with subsequent confocal laser scanning microscopy (CLSM) to study the composition of the matrix (66). Confocal laser scanning microscopy is widely used in the visualization of medical biofilm, as some of the advantages of this technique are the possibility to visualize 3D and spatial structures of biofilms (51). Furthermore, it is possible to quantify volume and other parameters of the biofilm and to apply different fluorescent probes (51).

Quantitative and qualitative assays for investigating the biofilm forming abilities of bovine mastitis pathogens *in vitro* are inexpensive, fairly simple and fast. In the last years, microscope techniques have become more accessible and would facilitate more detailed investigations of the biofilm phenotype and interactions between antimicrobial compounds and biofilms.

Investigations of Antimicrobial Compounds Against Biofilm Forming Mastitis Pathogens

Different antimicrobial compounds and antibiotics have been tested on bovine mastitis isolates' ability to form biofilm. The biofilm forming ability of *E. coli* in the presence of different antibiotics was investigated using CLSM and revealed increased adhesion of the isolates (67) and a greater biofilm formation of *E. coli* bovine mastitis isolates in the presence of enrofloxacin (68). When grown as biofilms, *S. aureus* bovine mastitis isolates are highly resistant to antimicrobial agents (42). The antibacterial use of the traditional medicinal plant *Plectranthus ornatus* (spur flowers) used in Brazil for treatment of skin infections was investigated for its anti-biofilm properties by using the plant as a herbal soap on gloves contaminated with *S. aureus* from dairy cows with bovine mastitis. There was no microbial growth after the gloves were submerged in the herbal soap and when the biofilm inhibitory concentration by microtiter plates and crystal violet staining was investigated, the plant was able to inhibit biofilm formation (69). Anti-biofilm agents against *S. aureus* have also been investigated *in vivo*. Ethanolic extracts from the leaves of *Rhodomyrtus tomentosa* (*rose myrtle*) were investigated as a possible antimicrobial agent against biofilm producing *S. aureus* in combination with the antibiotic pirlimycin. When extracts were used alone, there was no significant reduction in the bacterial load in a murine mastitis model. In combination with the antibiotic, a significant antibacterial effect was observed, but there was no significant difference between the antibiotic used alone compared with the combination of antibiotics and extract (70). The possible inhibitory effect of the Argentinian medicinal plant *Minthostachys verticillata* was tested on *Escherichia coli*, *Bacillus pumilus*, and *Enterococcus faecium* isolated from mastitis milk. The essential oil of the plant had inhibitory effect on the production of biofilm of all isolates in 96 well-microtiter plates (71). The naturally occurring signaling molecule of bacteria, cyclic dinucleotide 3′,5′-cyclic diguanylic acid (c-di-GMP), has been investigated to inhibit biofilm formation of *S. aureus*, and a decrease in the colonization of the pathogen in the mammary glands was shown in a murine mastitis model (44). The alternative drug, 1-hydroxyanthraquinone, was found to have a significant inhibitory action against *Staphylococcus xylosus in vitro* as well as a reduction in inflammation in the mammary glands of murine models (72).

Biofilm-Associated Genes in Bovine Mastitis Pathogens

The molecular identification of pathogens is another direction in the research of biofilm in bovine mastitis and several studies have investigated different biofilm-associated genes of bovine mastitis isolates. The intercellular gene cluster adhesion operon (*ica*) is one of the genes that has been investigated for its role in biofilm formation and has been found in 40% of *S. aureus* isolates from bovine mastitis by analyzing their biofilm forming abilities within the microtiter assay and then sequencing the isolates (73). However, whether the isolates carrying the *ica* genes actually produce biofilm *in vitro*, depends on the biofilm assay. Some

studies found that even if the isolates carried the *ica* genes, not all of the isolates produced biofilm in the microtiter plate (74) and that some isolates would form black colonies (indicating slime-formation) when grown on CRA plates but not necessarily form biofilm in the microtiter assay (41). Biofilm-associated proteins (bap) has been researched by several bovine mastitis studies and *S. aureus* isolates have been investigated for the presence of *bap* genes and their biofilm forming ability (40). A study found that over 90% of isolates carried *icaADBC* genes and of these 25% carried the *bap* genes. When the isolates were positive for both *icaADBC* and *bap*, they were strong biofilm producers *in vitro*, however, when only positive for *icaADBC*, they produced less biofilm. The role of *bap* was investigated by constructing a mutant only positive for *bap* and found that the mutant had the same biofilm forming capacity as the wild type (40). However, in other studies, the *bap* gene was not found at all in *S. aureus* isolates from bovine mastitis cases (39, 74).

OTHER TOPICS ADDRESSED IN THE RESEARCH OF BIOFILM IN BOVINE MASTITIS

Multispecies Biofilm

The research of bovine mastitis and biofilm often focuses on one specific pathogen and its ability to form biofilm *in vitro*. When only single species are investigated, there is a risk of overseeing keystone species (75) or possible interactions between commensals and pathogens or amongst pathogens, which might be important in the understanding of biofilms' role in bovine mastitis. However, the majority of studies investigating the role of biofilm in bovine mastitis focuses solely on *S. aureus*. In the environment, there is often more than one bacterial strain present and multispecies biofilms are commonly observed (76). Bovine mastitis infections can have multiple bacterial agents (9) and it is also important to consider the possible role of commensal bacteria in udders. Lactic acid bacteria (LAB) are commonly isolated from the teat canals and milk of dairy cows (77). Wallis et al. investigated the effect of growing two probiotic LAB strains together with a challenge between biofilm of probiotic LAB and *S. aureus* biofilm. They observed that when two LAB strains were co-cultured with *S. aureus*, it resulted in no growth of *S. aureus*, suggesting the beneficial use of probiotic bacteria against pathogenic biofilms in bovine mastitis (78). The presence of specific bacteria can either promote or decrease growth of other bacteria (79, 80) and the competition between these bacteria can cause damage to the surrounding environment or tissue (81). Immune responses toward bacteria and biofilm may similarly cause collateral damage to the surrounding tissue (82). Therefore, it is important to consider possible interactions between other bacteria and bovine mastitis pathogens as well as between pathogens and the immune response.

Potential Vaccines Against Biofilm Forming Mastitis Pathogens

Two mastitis vaccine candidates against *S. uberis* have shown a significant reduction in the mortality of mice infected with the pathogen (83). Different candidates for a *S. aureus* vaccine is currently being investigated; in one study, live-attenuated small-colony variants have shown promising results compared to inactivated bacteria in murine models (84). However, in another study, a formalin-killed whole-cell vaccine candidate of *S. aureus* biofilm showed a significant reduction in the colonization of *S. aureus* in the udder in vaccinated mice compared to mice vaccinated with a vaccine candidate from planktonic *S. aureus* (85). A killed bacterin vaccine candidate against *S. aureus* was tested in primiparous gestating cows. There were no observations of any prevention of intramammary infection by *S. aureus* but a reduced multiplication of *S. aureus* in the mammary glands was observed (86). *S. aureus*' protein A has also been investigated as a possible vaccine target and a vaccine candidate has shown a significant reduction in bacterial load of the mammary glands of pregnant mice. However, the immunized mice were not protected when they subsequently were infected with biofilm producing encapsulated *S. aureus* (87). Currently, two mastitis vaccines are available on the market against *S. aureus* and *S. uberis* from the company HIPRA (Amer, Spain).

In vivo Investigations of Biofilm in Bovine Mastitis

Most of the so-called *in vivo* investigations of biofilm in bovine mastitis have used experimental models (mice and sheep), and the majority of these studies focused on anti-biofilm treatment or vaccines against biofilm udder infections (**Table 1**) (44, 69, 70, 72, 83–85). Only a few studies investigated and confirmed biofilm *in vivo* within udder tissue of dairy cows with bovine mastitis. Two studies directly detected biofilm inside udders of dairy cows with mastitis. Clustering of *S. aureus* bacteria in udders of dairy cows with bovine mastitis were observed by microscopy to be located in the lumen of the alveoli and lactiferous ducts of the udders of experimentally infected dairy cows (47). In another study, the presence of biofilm was investigated directly in the udders of dairy cows by collecting swabs from the udders of slaughtered dairy cows with *S. aureus* infection. Swabs were obtained from the teat cistern, gland cistern, and parenchyma and were subsequently stained using immunofluorescence staining of polysaccharide intercellular adhesions (PIA), which is a component of the *S. aureus* biofilm matrix. The samples were investigated by fluorescence microscopy and PIA was found in 71 out of 184 swabs (46).

The Bovine Mammary Immune Response to Biofilm Infection

The response to infections is crucial for the survival of mammals. The response mechanisms to bacterial and viral infections are widely investigated, however much less is known about the immune response toward biofilm. As per definition, host immune responses are tolerated by biofilms, and no specific anti-biofilm immune responses have been identified (82).

The protection against infectious agents in the bovine mammary gland has been recently reviewed by Sordillo (93). As for biofilm infections in general, the mammary gland response toward bacterial biofilms is not fully understood yet, and as

TABLE 1 | Studies investigating biofilm forming bovine mastitis pathogens in animal models or the effect of possible antibacterial agents and vaccines in animal models.

References	Year	Sample type	Focus of study	Pathogen	Experimental animal
Cucarella et al. (88)	2001	Bovine subclinical mastitis and human isolates	Molecular basis of biofilm	*S. aureus*	Mice
Brouillette et al. (44)	2005	Clinical bovine mastitis isolates	Antibacterial treatment	*S. aureus*	Mice
Gogoi-Tiwari et al. (85)	2015	Bovine mastitis isolates	Vaccine	*S. aureus*	Mice
Collado et al. (83)	2016	Clinical bovine mastitis isolates	Vaccine	*S. uberis*	Mice
Gogoi-Tiwari et al. (87)	2016	Bovine mastitis isolates	Vaccine	*S. aureus*	Mice
Mordmuang et al. (70)	2019	Bovine mastitis isolates	Antibacterial treatment	*S. aureus*	Mice
Montironi et al. (89)	2019	Subclinical bovine mastitis isolates, milk samples	Investigation of phenotype, genotype and virulence	*Enterococcus faecium*	Mice
Côté-Gravel et al. (84)	2019	Bovine mastitis isolates	Vaccine	*S. aureus*	Mice
Marbach et al. (90)	2019	Subclinical bovine mastitis isolates, milk samples	Interactions between host and bacteria	*S. aureus*	Mice
Wang et al. (72)	2020	Isolates (Purchased strains)	Antibacterial treatment	*S. xylosus*	Mice
Prenafeta et al. (86)	2010	Ruminant mastitis isolates	Vaccine	*S. aureus*	Heifers, cows
Savijoki et al. (91)	2014	Bovine mastitis isolates	Genomics and proteomics	*S. epidermidis*	Cows
Seroussi et al. (92)	2018	Bovine mastitis isolates	Antibacterial treatment	*E. coli*, *S. aureus*	Cows
Cucarella et al. (40)	2004	Bovine subclinical mastitis isolates	Molecular basis of biofilm	*S. aureus*	Sheep

described above very few *in vivo* investigations of mammary biofilm infections exist. Some studies have investigated the response of mammary cells to biofilm-producing strains of known mastitis-causing pathogens *in vitro*. The ability of *S. aureus* biofilm forming strains to adhere and invade the mammary cells is especially investigated. A study found that *S. aureus* biofilm showed lower invasion ability into mammary epithelial cells compared to planktonic *S. aureus* cultures and that the biofilm culture induced less cellular activation than the planktonic cultures. Both planktonic culture and *S. aureus* biofilm culture induced expression of interleukin 6 by mammary alveolar cells, which could be an anti-inflammatory response (94). This corresponds well to human research of biofilm infections and immune response, where biofilms do not trigger any specific immune responses (82) and downregulates specific virulence genes when the cell density is low to "fly under the radar" so the immune system does not detect the bacteria. Whenever the cell density is high enough, the bacteria can upregulate the virulence factors (95). However, other *in vitro* studies found no difference in the ability to invade host cells by non-biofilm producing mastitis strains compared to biofilm producing mastitis strains (96, 97). The role of the Bap protein expressed by *S. aureus* has been investigated in a lactating mouse model, where the surface protein Bap, involved in biofilm matrix, adhered to epithelial cells and bound to host receptor Gp96. The bacteria expressing the surface protein Bap did not invade the cells and had increased persistence in the mammary glands of the lactating mice, indicating that the protein promotes adhesion to the cells and limits invasion of the host cells (98).

The main question still not resolved is how the biofilms go undetected and survive the immune response (99) and more research is needed to answer that question both for mastitis and for all other biofilm infections. The current research is based on *in vitro* experiments and as discussed earlier in this review, more *in vivo* research is needed to fully understand the role of biofilm in mastitis.

DISCUSSION

The role of biofilm in the pathogenesis of bovine mastitis infection is still unclear. To the authors' best knowledge, only two papers investigate and detect the presence of biofilms inside udders from dairy cows with mastitis (46, 47). Plenty of *in vitro* studies investigate the biofilm forming abilities of mastitis pathogens isolated from milk samples. Similar to swabs and wound fluid samples, analyzing milk samples is a great, easy and quick way to investigate and culture the bacteria present in the samples and to determine, e.g., the genetic composition and antibiotic susceptibility. However, disadvantages are the risk of contamination from the environment and that it is only possible to detect bacteria present in or released into the milk, however bacteria embedded in the tissue, encapsulated bacteria, low-shedding bacteria, and potential biofilms might not be detectable in milk samples (100). Even if bacteria are isolated from milk samples and are able to form biofilm *in vitro*, this does not provide any information on the bacteria's phenotype *in vivo* in the infected udder. *In vitro* biofilms of *P. aeruginosa* have a markedly different genetic expression profile than *in vivo* biofilms during human infections (101). This is due to, e.g., the environment in the host tissue, interactions with the immune system, and antibiotic treatment that are impossible to fully mimic *in vitro*. Further, major physical differences exist between *in vitro*

and *in vivo* biofilms; for example, *in vitro* biofilms normally form large mushroom-shaped structures, which are never observed *in vivo*, where the biofilms are markedly smaller in size (16).

Hence to find the actual role of biofilm in bovine mastitis, the approach needs to change from *in vitro* to *in vivo* investigations of biofilms in infected udders. When diagnosing biofilm infections in human medicine, the gold standard is to directly visualize the biofilms and concurrent immune response in the tissue. This can be done with, e.g., CLSM or scanning electron microscopy (15, 18, 102). Sample collection for biofilm diagnosis naturally varies for different diseases/tissue, e.g., from cystic fibrosis patients, expectorated sputum samples, bronchoalveolar lavage, or biopsies from removed lung tissues during lung transplantation can be collected (18, 102), and from chronic wounds, biopsies or debrided tissue can be investigated (103). Especially in wound infections, the spatial distribution of different bacterial biofilms within the tissue can be observed using microscopic examination; this method has further found the pathogen *P. aeruginosa* to be underestimated when performing culture of standard wound swabs (104, 105). A good technique to detect bacterial biofilms in tissue is peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH), with probes that hybridize to bacterial ribosomal RNA, which can subsequently be detected using CLSM. This is a sensitive method that is well-established in the research of biofilm infections in humans (102, 104, 106–108). This method would be applicable to udder tissue samples as well.

If biofilms are found present in mastitis udders, e.g., by use of the methods just described, the next question is whether the biofilms are part of the pathogenesis of bovine mastitis? Therefore, the immune response to the biofilms is also important to investigate. The cytological cure of mastitis is delayed compared to the bacteriological cure, meaning that when the infection appears cleared, the inflammation can continue in the udder (9). The cytological cure rate can be as low as around 20% and therefore it should be considered that chronic mastitis cases could be due to long-lasting inflammation, potentially driven by biofilms, after the apparent bacterial cure (9). The treatment of especially *S. aureus* mastitis cases is difficult, and therefore the connection between these cases and *S. aureus* biofilm presence and many virulence factors that are upregulated during mastitis infections should be further investigated (10, 11). We propose that udder cell and tissue models could potentially be applied to investigate how biofilms affect bovine udder tissue, however, studies of natural or experimentally induced mastitis will provide more information as a competent immune system would respond to the infection.

Collecting udder tissue biopsies from live dairy cows with mastitis for microscopy is difficult, if not impossible. However, biopsies can easily be obtained after euthanasia and by applying relevant staining and microscopy techniques, a more accurate view of biofilms' potential location and distribution as well as the related host immune response during mastitis can be revealed. Only a few papers provide information on biofilms' presence in udders from dairy cows with mastitis (46, 47) and more research is needed to elucidate biofilms' role in mastitis pathogenesis. Therefore, the collection of biopsies from euthanized animals might not have any direct clinical relevance, as the animals would be dead, but has important scientific relevance to better understand the disease and relate this to findings in milk samples. If biofilms play a role in bovine mastitis, diagnostic methods to detect biofilm in milk samples could be a possible way to easily diagnose the biofilms. However, for now, no such biofilm marker, specific biofilm product, or specific biofilm immune response have been identified that would be usable for quick and simple biofilm diagnostics neither in human or veterinary medicine. This is naturally the topic and aim of many human research groups' intense work, as biofilm infections play an important part of many human infections, and whenever found this would hopefully also be applicable to milk samples from bovine mastitis. By understanding the bacteria and biofilms including their interactions with the host immune system during mastitis infections, potentially new possible diagnostic methods could be developed as well as new optimized treatment options.

CONCLUSIONS

Bovine mastitis is one of the most important diseases in the dairy industry and a better understanding of the role of biofilm in the disease is of high importance to achieve more successful treatments. Chronic biofilm infections are recognized as serious and difficult-to-treat diseases in human medicine. The majority of the research on biofilm and bovine mastitis has so far focused on *in vitro* studies; however, to uncover the presence of biofilm in udders of dairy cows suffering from mastitis, direct methods need to be applied. Some of the methods used in the diagnosis and research of biofilms in human infections could be applied to the research of biofilm in bovine mastitis. There is a need for *in vivo* research where the location and distribution of biofilms are investigated directly in the udder of dairy cows with mastitis and where these findings are related to findings in milk samples. The continuous unsuccessful antibiotic treatment of potential biofilm mastitis infections can increase the risk of antibiotic resistance, which is one of the biggest threats to human and animal health. The role of biofilm infections in bovine mastitis therefore seems a key to unlock the required knowledge to develop new diagnostic methods and treat the persistent and chronic cases of bovine mastitis.

METHODS

This review has included studies that examine biofilm in relation to mastitis in dairy cows. We have included studies investigating biofilm abilities, molecular properties, treatment options, prevention and interactions of bovine mastitis related pathogens. Studies published since 2000 were included. Reviews and manuscripts in other languages than English have not been included in this review.

The literature search was carried out using the database Pubmed on October 1st 2020 with the search words "bovine mastitis + biofilm." Over 170 papers investigated the role of

biofilm in bovine mastitis by *in vitro* methods and 16 papers used *in vivo* methods.

AUTHOR CONTRIBUTIONS

RP: original draft preparation, methodology, and writing. EJ: writing, editing, and supervision. VK, TB, KD-P, and RB: editing and supervision. All authors have read the manuscript and agreed to the published version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

NOMENCLATURE

CNS, Coagulase-negative staphylococci
CRA, Congo Red Agar
CLSM, confocal laser scanning microscopy
LAB, lactic acid bacteria
PIA, polysaccharide interstellar adhesions
PNA-FISH, peptide nucleic acid fluorescence *in situ* hybridization.

Manuscript II

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"Distribution and spatial organization of bacteria in bovine mammary glands."

Manuscript in preparation.

Distribution and spatial organization of bacteria in bovine mammary glands

Regitze Renee Pedersen[1,3], Emma Hornemann[1], Benjamin Meyer[2], Lene Bay[3], Blaine Gabriel Fritz[3], Tim Holm Jakobsen[3], Louise Kruse Jensen[2], Kirstin Dahl-Pedersen[1], Elin Jørgensen[3], Rikke Buhl[1], Volker Krömker[2] and Thomas Bjarnsholt[3,4]

[1] Department of Veterinary Clinical Sciences, University of Copenhagen, Copenhagen, Denmark,
[2] Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark,
[3] Department Immunology and Microbiology, Costerton Biofilm Center, University of Copenhagen, Copenhagen, Denmark,
[4] Department of Clinical Microbiology, Copenhagen University Hospital, Copenhagen, Denmark

Abstract:

In mastitis control, the detection of pathogenic microorganisms in milk is used to describe microbial dysbiosis on the milk-producing epithelium. However, there are few studies comparing the microbiological and cytological findings of the milk with those of the milk-giving epithelium. This study aims to determine the microbial composition and spatial distribution in healthy and mastitis quarters by analyzing milk samples and biopsies from different locations within the udder. Cultivation, MALDI-TOF MS, and 16S rDNA sequencing were used to determine the bacterial composition. HE-staining was used to evaluate the histopathology of the biopsies. Furthermore, this study is the first to apply PNA-FISH and CLSM to visualize the spatial distribution of bacteria in the udders. This study's findings included diverse microbial compositions for both the healthy and mastitis quarters and the presence of bacteria throughout the udders. Bacterial aggregates were found in 18% of samples from mastitis quarters and 3% samples from healthy quarters. We conclude that bacteria are present throughout the bovine udder, and aggregates are found in some cases of bovine mastitis.

Keywords: Mastitis, milk microbiota, chronic infections, biofilm, PNA-FISH

Background

Bovine mastitis is one of the most crucial diseases that the dairy industry is facing – affecting the economy and animal welfare [1]. Mastitis is inflammation in the udder and is caused mainly by bacteria [2]. Mastitis can present itself in several aspects, including subclinical and clinical, environmental, and contagious [1]. Various bacterial species can be involved in the bovine mastitis, such as *Streptococcus uberis*, *Staphylococcus aureus*, and *Streptococcus dysgalactiae* [3]. Bovine mastitis is the primary reason for antibiotic treatment in dairy cows [4–6].

Bovine mastitis can be treated in various ways, depending on the disease's clinical aspects and origin. However, in some cases, the disease is not resolved despite antibiotic treatment and becomes chronic. Chronic mastitis is characterized by udder inflammation over a long period of time [7] with an elevated somatic cell count for over three weeks [8]. The reason why some cases of mastitis do not resolve, despite antibiotic treatment, is unknown, although, some factors have been shown to affect the success of a bacteriological cure. For example, the animal's age, the somatic cell count, the pathogen causing the infection, or whether the animal had an intramammary infection earlier in the lactation are factors that impact the bacteriological cure [9]. Another possible explanation for the unsuccessful antibiotic treatment could be bacterial biofilm formation. Biofilm has been defined as "a coherent cluster of bacterial cells embedded in a biopolymer matrix, which, compared with planktonic cells, shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defence" [10]. Biofilm infections can be challenging to eradicate and are associated with chronic infections in several human diseases [10]. The role of biofilm in veterinary diseases has been sparsely investigated. Nevertheless, research on this topic [11–13] as well as bovine mastitis [14] is evolving. Most research demonstrates that bacterial strains isolated from the milk of mastitis cases can form biofilm *in vitro* [15–17], while only a few research papers have investigated biofilm in bovine mastitis *in vivo* [18,19].

Current diagnostic methods for bovine mastitis of bacterial origin often rely on culturing milk samples and analyzing them with morphological and biochemical methods [20,21]. Identifying the bacterial agents by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) or 16S rDNA sequencing has been increasingly used in diagnostics [1,20–23]. However, these methods only disclose the bacterial agent in the samples but not whether the bacteria are in fact embedded in biofilms [14]. The importance of visualizing biofilm *in situ* has been a focus in research on biofilm infections in humans for a long time [24–28]. Visualization methods such as peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) are one of the most common ways of diagnosing biofilm infections [10,24,29].

Previous studies of biofilm and the bacterial composition of bovine mastitis have mainly been based on milk samples. To the best of our knowledge, few studies have investigated the bacterial composition in udder tissues and compared to that of milk samples. This study aims to describe the distribution of bacteria in various locations in dairy cows' udders and teat canals to better understand chronic mastitis. Additionally, to determine the bacterial composition and visualize the spatial distribution of bacteria as well as the histopathology in the infected and healthy udders using biopsies and milk.

Materials and methods

Dairy cows

All udder quarters included in the study were from dairy cows sent to slaughter. The dairy cows had elevated cell counts over a longer period of time. All dairy cows were either Holsteins or Jerseys from

Danish dairy farms. Twenty-eight dairy cows were included in the study, with ages ranging from 2 to 11 years and parity from 1 to 6 (Table 1, supplementary).

Collection of udders

The udders were collected from slaughterhouses or the University Hospital for Large Animals, (University of Copenhagen, Denmark) and transported to the University Hospital for Large Animals to be prepared for biopsy collection. Biopsies were collected within 4–8 hours of slaughter.

Sample collection from the quarters

To minimize the risk of contamination, the investigator wore a hairnet, gloves, a mask, and a single-use laboratory coat during the collection of samples.

The udders were cleaned with water (Mediq) using sterile cleaning brushes for 4 minutes, after which 70% ethanol was applied. Milk samples were collected from the selected quarters in sterile tubes and stored at 5°C until the following day. The first milk from the quarters was discarded. Analysis was conducted only on the milk that followed thereafter was used.

Milk samples were analyzed with a California Mastitis Test (Kruuse) [30] before the udders were opened. The cleaning steps were then repeated for the udder. Biopsies were obtained from two quarters of each udder: the quarters with the highest and lowest CMT score, respectively. A sterile scalpel was used for the first incision, while an autoclaved knife was used for the remaining sectioning of the quarter.

Biopsies were collected at five locations (Figure 1); three biopsies were collected from each of the locations using sterile surgical single-use tweezers and scalpels. For each biopsy collected, a new sterile scalpel and tweezer was used to minimize the risk of cross-contamination.

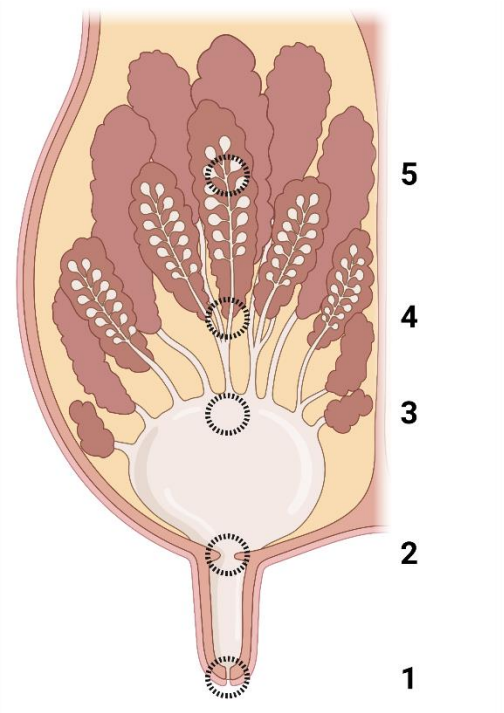

Figure 1. Locations (1 to 5) of collected biopsies from the udders of dairy cows included in the study. At each location, three biopsies were collected. Created in Biorender.com

Biopsies for cultivation were stored in microtubes (Sarstedt) at 5°C until the next day. Prior to collecting biopsies, the microtubes were prefilled with two 0.5 mm beads (MP Biomedicals) and autoclaved. The samples for microscopy were stored in Monovette (Hounisen) tubes with 4% formaldehyde at 5°C for a maximum of three weeks (Figure 2). The biopsies for 16S rDNA sequencing were stored at −80°C in 1.5 ml cryotubes (TTP) (Figure 2).

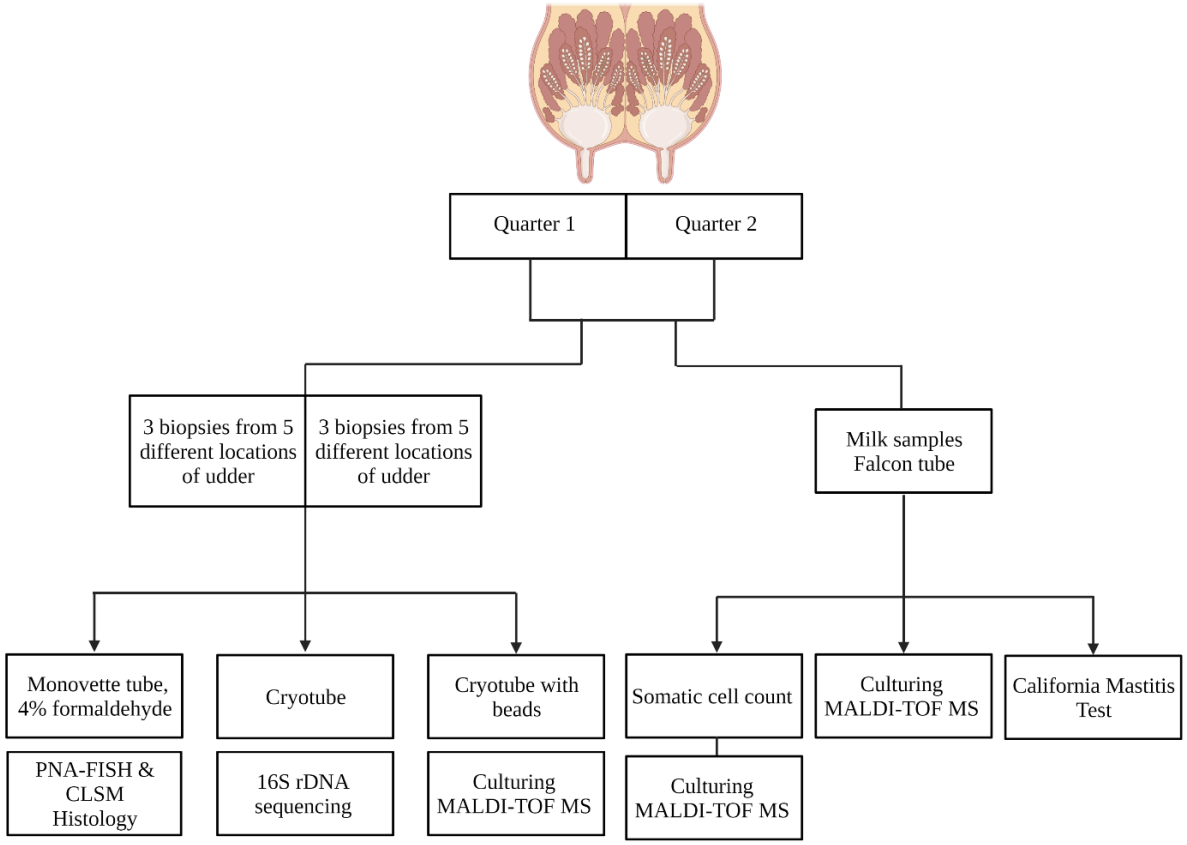

Figure 2. Overview of samples taken from the quarters. Milk samples were collected from two quarters of each dairy cow and analyzed with the California Mastitis Test. Furthermore, microscopy determined the somatic cell counts. The milk samples were cultivated, and the isolates were identified by cytomicrobiological tests and MALDI-TOF MS. Three biopsies at five locations were collected from the quarters and analyzed with 16S rDNA sequencing and MALDI-TOF MS (after culturing) for identification of bacteria present in the tissue and PNA-FISH and CLSM for visualization of the bacteria. Abbreviations in the figure: PNA-FISH: peptide nucleic acid fluorescent in situ hybridization; CLSM: confocal light scanning microscopy; 16S rDNA sequencing: 16S ribosomal DNA sequencing; MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Created in Biorender.com

Cultivation of biopsies and milk samples:

The handling and processing of biopsies and milk samples was performed in sterilized laminar flow benches. Sterile water with 0.9% NaCl (500 µl) was added to the biopsies that were homogenized with a Magnalyzer (Roche) three times in 20 seconds at 6,000 RPM. Between cycles, the samples were kept on ice. The samples were degassed (5 minutes), sonicated (5 minutes) (Branson 2510 Ultrasonic Cleaner, Marshall Scientific), vortexed, and 150 µl of the liquid was spread on a 5% horse blood agar plate (SSI). The plates were incubated at 37°C for seven days, examining the plates daily without opening the lid. The milk samples were thoroughly vortexed, and 100 µl of the milk was spread on 5% horse blood agar plates and incubated for one to seven days. The remaining milk samples were stored at −20°C until further analysis.

Single bacterial colonies were streaked on 5% blood agar plates and incubated at 37°C ON. The isolates were transfered to 1.5 ml cryotubes (TTP) with Luria broth with 33% glycerol and stored at −80°C.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry of isolates from biopsies and milk samples:

Bacterial isolates were incubated consecutively twice on 5% blood agar plates at 37°C ON. The bacterial identification was performed using the MALDI-TOF MS system Vitek MS RUO (*bioMérieux*) with the software used SaramisTM v3.5. The cut-off level for species identification was defined as ≥2.0. All negative isolates were analyzed twice.

Cytomicrobiological diagnostics of milk samples, somatic cell count, and microscopic cell differentiation:

The milk samples were analyzed by conventional diagnostics at the laboratory of Hannover University of Applied Sciences and Arts, Hannover, Germany, as described by Fredebeul-Krein *et al.* [31]. The bacteria were further analyzed by MALDI-TOF MS (Bruker Daltonics) without acid extraction, and the species were identified by the MBT Compass Library (Revision F, MBT 84,668 MSP Library, Bruker Daltonics). Isolates were stored in brain-heart broth (Merck KGaA) with 25% glycerol at −80°C.

The cell count of the milk samples was determined by somatic cell count and microscopic cell differentiation. A flow cytometer was used to determine the somatic cell count (SomaScope Smart & Delta Instruments). Milk (10 µl) was smeared on an area of 1 cm^2 on a clean slide. Fixation, defatting, and staining of smears with Broadhurst polychromatic solution were done as described in Prescott *et al.* [32]. Smears were examined microscopically with oil immersion at 100x magnification. Fifty cells in each sample were used to calculate a percentage ratio of cell populations, size, and granularity.

The quarters were categorized as either "Healthy Quarter" or "Mastitis Quarter" according to a microscopic somatic cell count below or above 200,000 cells/ml, respectively. For one cow, no milk was available, and the quarters were categorized according to the CMT results.

16S rDNA amplicon sequencing and data analysis

The DNA extraction, PCR amplification, library preparation, and 16S rDNA amplicon sequencing were performed by Novogene Co. Ltd, UK. The PCR was performed with the primer 16SV34, the library preparation was performed with the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina) and the libraries were sequenced with Illumina platforms with a minimum of 50,000 raw reads per sample.

The raw, absolute amplicon sequence variant (ASV) feature tables were used for the data analysis. ASVs with zero counts across all samples were removed. Alpha diversity was calculated by the Shannon Diversity Index. A mixed-effects model was fitted to the alpha-diversity data using Condition (mastitis/healthy) and Location (S1-S5) and the interaction (Condition:Depth) as fixed effects, including nested random effects of Quarter within Cow.ID, to determine the effects of mastitis and depth on alpha diversity. Statistical analysis was performed with a type III ANOVA implemented in the lmerTest package in R.

To analyze beta diversity, ASVs, which were observed in less than 5% of samples, were first removed. The count data were normalized using a centered-log ratio transformation implemented in the decostand

function in the vegan R package. A sample distance matrix was then created using the robust aitchinson distance method implemented in the vegdist function of the vegan R package. For plotting PCAs and distances to the centroid for the groups, the betadisper function of vegan was used to calculate the group dispersions and distances to the centroid. The adonis2 function in the vegan package was used to assess the effect of Condition and Depth on composition. The model included Condition and Depth as well as the interaction as fixed effects and was stratified by Cow.ID.

The abundance-filtered ASV table was summarized at the genus level using the tax_glom function in phyloseq to identify genera that were differentially abundant due to condition. Differential abundance testing was performed with DESeq2 using default settings with ~Condition as the design formula. Results were filtered to only include differentially abundant ASVs with a base mean expression of 100, adjusted *p*-value less than 0.05 and an absolute log2 fold change >2.

PNA-FISH and HE staining of tissue section

The biopsies stored in 4% formaldehyde were embedded in paraffin. From each biopsy tissue sections (4 µm) were cut and deparaffinized; one was stained with an universal bacterial, double-labeled PNA-FISH probe (Panagene) described by Fazli *et al.* [26] and one with hematoxylin and eosin staining for histology. The slides were incubated at 55°C for 90 minutes in the dark; coverslips were removed, and slides were inserted into the wash buffer at 55°C consisting of 4 ml of washing buffer (AdvanDx) and 240 ml of MilliQ water for 30 minutes. The slides were air-dried in the dark and then counterstained with 0.3 mM DAPI (Sigma) and incubated for 15 minutes in the dark. Slides were rinsed with sterile PBS buffer and air-dried in the dark. One drop of Pro-long Gold (ThermoFisher) was added to the slides and covered with a cover glass. Slides were air-dried in the dark until the next day and the edges were sealed with clear nail polish.

Aggregate detection by CLSM and image analysis:

Two confocal laser scanning microscopes were used in this study: the inverted microscopes 880 and the upright 710 (Zeiss). The objectives EC Plan-Neofluar 40x/1.30 Oil DIC M27 and Plan-Apochromat 63x/1.4 Oil DIC M27 objective were used. Image analysis was performed by Zen Black version 2.1, and the software used for image analysis was Imaris version 9.7.2 (Oxford Instruments). A sample was positive for aggregate if a minimum of a 5 µm aggregate was observed in the sample, as established by Bay *et al.* [33].

Microscopic evaluation of histological slides

The histological slides from location five were evaluated using a light microscope (Leica DMLB /100 S microscope). Initially, biopsies from 10 cases were used to select the parameters for a scoring system developed to evaluate the presence of inflammatory lesions. Each slide was scored "yes/no" on the individual inflammatory parameters listed in Table 1. Four of the parameters were based on the dominant immune cell type (neutrophilic granulocytes, mononuclear cells, or both) within well-defined mammalian tissue structures. Depending on the number of observed pathological changes, an overall histopathological score was given (0–8). The biopsies were divided into four groups according to their scoring: none (0), mild (1–2), moderate (3–4), and severe (5–8). According to this approach, if the score values increase, more inflammatory change is observed. The amount of neutrophilic granulocyte infiltration within the udder tissue was evaluated and semi-quantitatively categorized as "low," "moderate," or "high." Histology was performed by a pathologist blinded to the group allocation. The

scoring system is based on comparable scoring systems used in previous experimental studies of bacterial-induced inflammatory lesions [34,35].

Table 1. Scoring system to evaluate histological slides from location 5 from biopsies collected from bovine mastitis and healthy quarters. Indication of pathological change in the tissue was based upon the total score of the following parameters.

Hemorrhagi	1
Fibrosis	1
Edema	1
Perivasculitis	1
Inflammation with neutrophilic granulocytes in glandular lumen	1
Inflammation with neutrophilic granulocytes inter/intralobular	1
Inflammation with mononuclear cells in glandular lumen	1
Inflammation with mononuclear cells inter/intralobular	1

Results

Milk samples

Based on the somatic cell count determined by microscopy. If no microscopic somatic cell count was available, the cell count by flow cytometry (SomaScope) was used. One cow did not have any milk available for analysis, so the CMT score was used (Table 2, supplementary).

This resulted in 20 "healthy quarters" and 36 "mastitis quarters" (Figure 3).

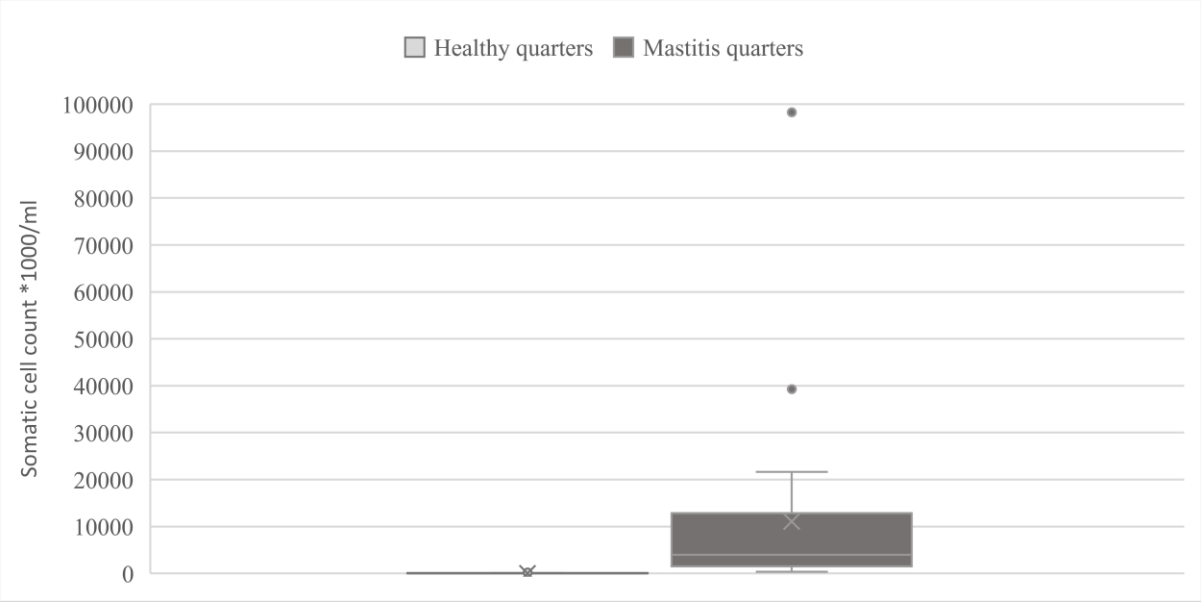

Figure 3. Boxplot for the somatic cell count for the healthy and mastitis quarters. The quarters are categorized by the microscopic cell count. If the data is not available, the somatic cell count is used. For one cow, neither data nor results were available, so the results from the California Mastitis Test were used. The healthy quarters are defined by a somatic cell count under 200,000 cells/ml and the mastitis quarters with a somatic cell count over 200,000 cells/ml.

Cultivation and MALDI-TOF MS of milk samples and biopsies

In the healthy quarters, 40 bacterial species were identified in the milk samples and 46 in the biopsies. There were more species in location 1 (teat) of the quarters compared to the other locations (2–5) deeper in the quarter (Figure 4). Both the healthy and mastitis quarters had more species in the biopsies than the milk samples (Figure 4). For mastitis quarters, 33 unique species were identified in the milk samples and 62 in the biopsies. For both milk and biopsy samples, and more bacterial species were identified in the mastitis quarters than in the healthy quarters. However, it should be noted that there were more quarters in the mastitis group (36) than in the healthy group (20).

Figure 4. The number of species in milk and five locations in the quarters for the healthy and mastitis quarters. The species were cultivated on blood agar plates, and the isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Identification of bacterial isolates from healthy milk samples and biopsies was unsuccessful for 9.21% and 9.94%, respectively (Table 2). For the mastitis quarters, it was not possible to identify 10.34% of the isolates in the milk samples and 8.24% of the biopsies (Table 2). The milk samples from healthy quarters had no bacterial growth in 10% of the samples, and 24.2% of the biopsies had no growth. All milk samples from mastitis quarters had bacterial growth, and 19.5% of the biopsies had no growth.

Table 2. The percentage of bacterial isolates from milk samples and biopsies from different locations in healthy and mastitis dairy cow quarters that could not be identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

	Milk	Location 1	Location 2	Location 3	Location 4	Location 5	All biopsies
Healthy							
No match	9.21	10.64	3.70	14.29	6.25	13.51	9.94
Mastitis							
No match	10.34	9.57	9.23	6.15	7.14	8.33	8.24

Unique species identified in milk samples and biopsies

Some species were unique for the milk and for the biopsies, respectively. In the healthy quarters, we found 16 unique species for milk and 21 unique species for the biopsies. However, these species were only found once. For the mastitis quarters, we found five unique species in the milk samples and 33 unique species in the biopsies. All the species were found only once.

Most common species found in milk samples and the udder biopsies

The most common species in the healthy quarters are shown in Table 3. In healthy quarters, the most common species in milk were *E. coli*, *S. sciuiri*, and *T. pyogenes*. At the different biopsy locations in the quarter, the most common species were *B. licheniformis*, *S. equinus*, *C. bovis*, and *S. aureus.*

Table 3. The most common bacterial species found by cultivation in healthy quarters for the different locations in the udder. Only species found in more than three quarters are included in the table. The species were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Milk	Location 1	Location 2	Location 3	Location 4	Location 5
Escherichia coli	*Bacillus licheniformis*	*Streptococcus equinus*	*Corynebacterium bovis*	*Staphylococcus aureus*	*Corynebacterium bovis*
Staphylococcus sciuri	*Staphylococcus haemolyticus*				*Trueperella pyogenes*
Trueperella pyogenes					
Bacillus licheniformis					
Corynebacterium bovis					
Staphylococcus aureus					
Staphylococcus haemolyticus					
Streptococcus equinus					

Bacillus licheniformis was the most common species found in healthy quarter biopsies (45%) when accounting for the total number of species throughout the healthy quarters. *S. haemolyticus* (35%), *S. uberis* (25%) and *C. bovis* (20%) (Table 4) were the next most common species.

Table 4. The most common bacterial species for all biopsy locations from quarters. Only species observed in more than three quarters are included. The bacterial species were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and are shown in percentage of quarters.

Healthy quarters	%	Mastitis quarters	%
Bacillus licheniformis	45	*Bacillus licheniformis*	52.7
Staphylococcus haemolyticus	35	*Staphylococcus aureus*	30.6
Streptococcus uberis	25	*Streptococcus uberis*	30.6
Corynebacterium bovis	20	*Bacillus pumilus*	19.4
Paenibacillus durus	20	*Staphylococcus hominis*	19.4
Streptococcus equinus	20	*Corynebacterium bovis*	16.7
Corynebacterium xerosis	15	*Paenibacillus durus*	16.7

Staphylococcus haemolyticus	16.7
Lactococcus lactis	13.9
Sphingomonas parapaucimobilis	13.9
Staphylococcus chromogenes	13.9
Trueperella pyogenes	13.9
Aerococcus viridans	11.1
Micrococcus luteus	11.1
Staphylococcus sciuri	11.1

The most common species in the milk and biopsies from mastitis quarters were *S. aureus*, *B. licheniformis*, and *S. uberis* (Table 5).

Table 5. The bacterial species found by cultivation in most quarters with mastitis at the different locations in the udder, with the most common species listed first. Only species found in more than three quarters are included in the table. The species were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Milk	Location 1	Location 2	Location 3	Location 4	Location 5
Staphylococcus aureus	*Bacillus licheniformis*	*Staphylococcus aureus*	*Streptococcus uberis*	*Staphylococcus aureus*	*Staphylococcus aureus*
Bacillus licheniformis	*Staphylococcus aureus*	*Streptococcus uberis*	*Staphylococcus aureus*	*Streptococcus uberis*	*Streptococcus uberis*
Streptococcus uberis	*Bacillus pumilus*	*Lactococcus lactis*	*Trueperella pyogenes*	*Lactococcus lactis*	*Paenibacillus durus*
Staphylococcus haemolyticus	*Lactococcus lactis*	*Staphylococcus chromogenes*	*Corynebacterium bovis*	*Trueperella pyogenes*	*Lactococcus lactis*
Aerococcus viridans	*Trueperella pyogenes*	*Trueperella pyogenes*			*Ralstonia picketii*
Staphylococcus sciuri	*Streptococcus uberis*				
Corynebacterium bovis	*Staphylococcus haemolyticus*				
Paenibacillus durus					
Staphylococcus chromogenes					
Staphylococcus equorum					

16S rDNA sequencing of biopsies

Alpha-diversity of the samples

To determine whether condition or location affects the microbial diversity, we analyzed alpha diversity with the Shannon Diversity Index. We identified a significant effect of depth on the alpha diversity (p-value: $7.092*10^{6}$), which generally decreased in deeper udder locations. No significant effect on condition or interaction between depth and condition was found. All depths had a lower alpha diversity than location 1 (p-values: Location 2: 0.043011, Location 3: 0.000431, Location 4: 0.018043, Location 5: 0.016813) (Figure 5).

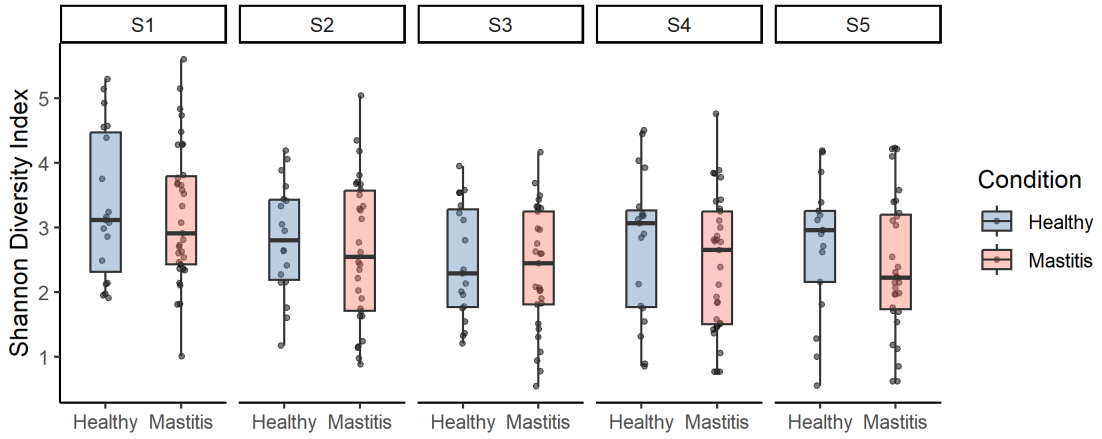

Figure 5. Alpha diversity of the biopsies collected from bovine udders in the two groups, healthy and mastitis. S1–S5 refers to the different locations in the quarter (Figure 1), S1 being location 1 at the teat end.

Beta-diversity of samples

To analyze differences in bacterial community between conditions and location, beta diversity analysis was performed. The largest source of community variability was due to the cow. We observed large differences in the inter-sample variability, depending on from which cow the samples were collected. When accounting for this in our model, we identified a significant effect of both condition ($p<0.001$) and depth ($p<0.001$) on community composition. There was no significant interactions between these two effects and the effect of mastitis was consistent regardless of the sample depth. We also identified that samples from S1 displayed an overall different community composition than samples deeper in the udder (Figure 6A).

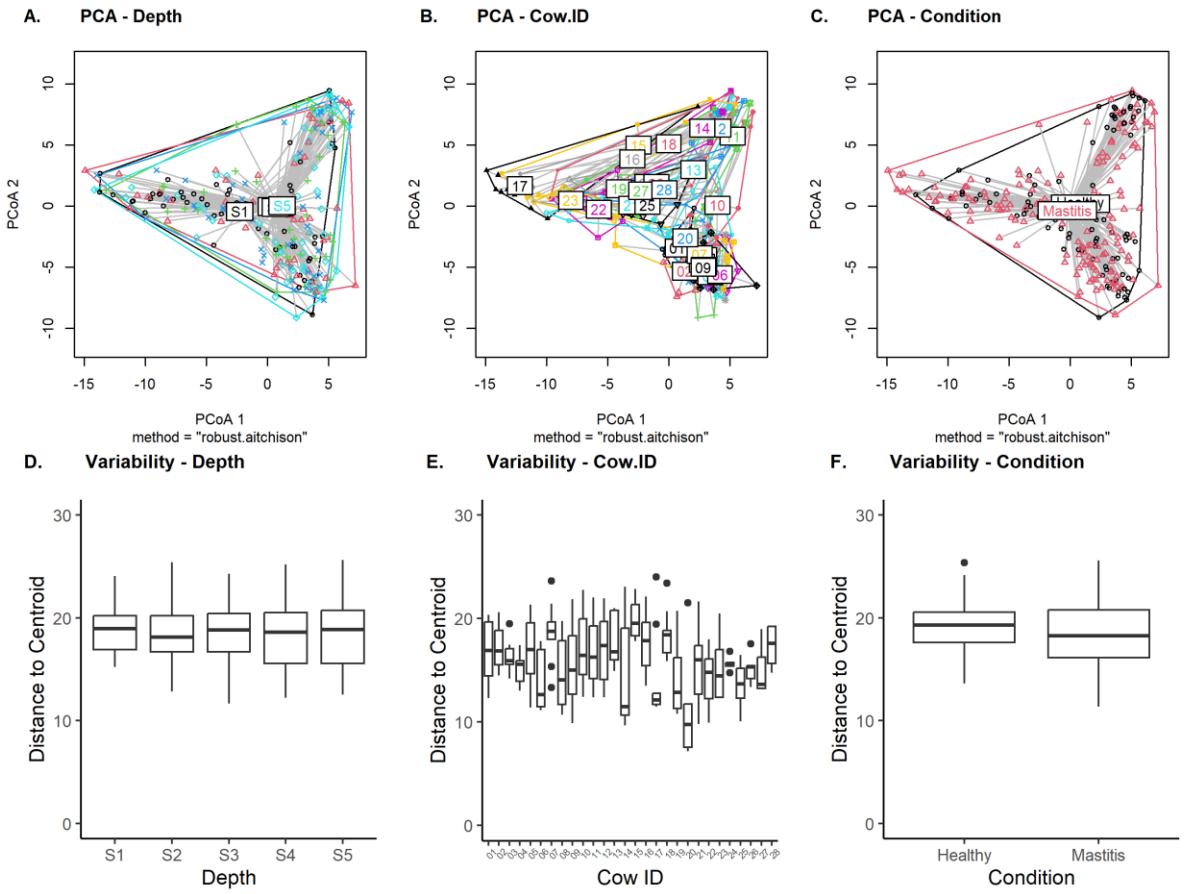

Figure 6. A–C: PCA plot of the groups and distance to centroid lines for the groups over the first two principal components for the following: A) location of the biopsies, B) dairy cows, and C) condition (healthy or mastitis quarter). D–F: Boxplots showing the distance to the centroid for the following: D) depth (location of the biopsies), E) the dairy cows, and F) condition (healthy or mastitis quarter).

Figure 7 depicts the five most common species at the different locations of the udder. For healthy quarters, the dominating genera were *Acinetobacter* and *Staphylococcus*, with *Acinetobacter* dominating in locations 2–5 and *Staphylococcus* in location 1. For the mastitis quarters, the same two genera dominated; however, *Staphylococcus* dominated locations 1, 2, and 5, and *Acinetobacter* dominated locations 3 and 4.

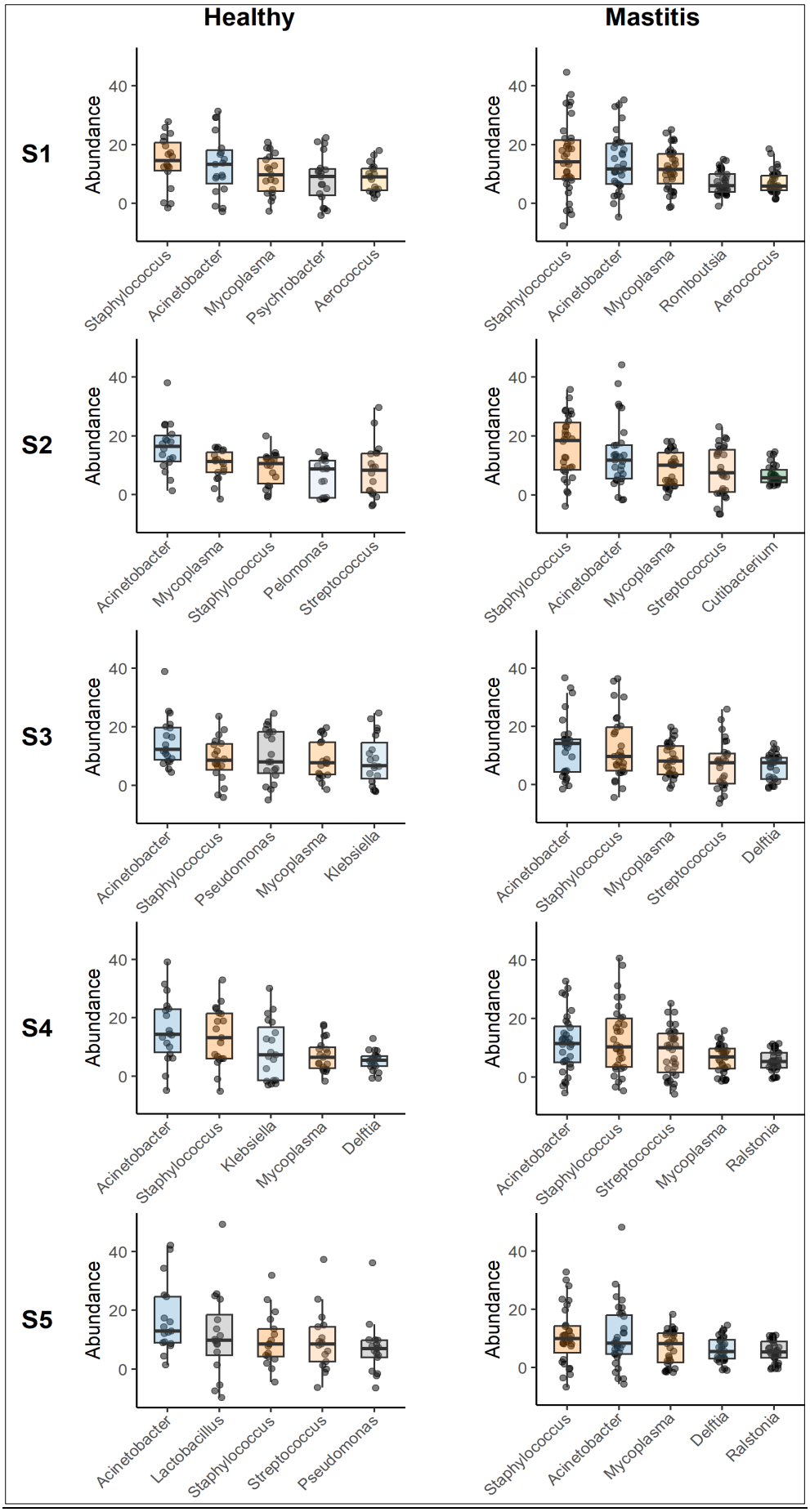
Healthy
Mastitis
S1
S2
S3
S4
S5
Abundance
0
20
40
Staphylococcus
Acinetobacter
Mycoplasma
Psychrobacter
Aerococcus
Romboutsia
Pelomonas
Streptococcus
Cutibacterium
Pseudomonas
Klebsiella
Delftia
Ralstonia
Lactobacillus

Figure 7. The five most dominant species at the five locations in healthy and mastitis quarters. S1–S5 refers to the different locations in the quarter (Figure 1), S1 being location 1 at the teat end.

PNA-FISH and CLSM of tissue sections

Bacterial aggregates were found in 18.4% of the tissue sections from mastitis quarters, while 97% of the tissue sections from healthy quarters had no bacterial aggregates (Table 6).

Table 6. Percentage of bacterial aggregates found in tissue sections from dairy cows by PNA-FISH and CLSM. A sample is defined as positive if an aggregate of a minimum of 5 µm was observed in the sample. Otherwise, the sample was negative. The results are shown in percentages.

	Negative	Positive
Healthy quarters	97%	3%
Mastitis quarters	81.6%	18.4%

Positive aggregate samples in healthy quarters were mainly found in locations 1 and 2, while positive aggregate samples in mastitis quarters were found in all locations (Table 7).

Table 7. Percentage of bacterial aggregates for the different locations in the tissue sections from dairy cows by PNA-FISH and CLSM. Samples with aggregates >5 µm were defined as positive.

	Location 1	Location 2	Location 3	Location 4	Location 5
Healthy quarters					
Negative	94.7%	90%	100%	100%	100%
Positive	5.3%	10%	0%	0%	0%
Mastitis quarters					
Negative	82.9%	77.8%	83.3%	83.3%	80.6%
Positive	17.1%	22.2%	16.7%	16.7%	19.4%

We observed bacterial aggregates of either cocci (Figure 8.A–C,9) or rods. In one quarter, an aggregate with both cocci and rod morphology was observed (Figure 8.D).

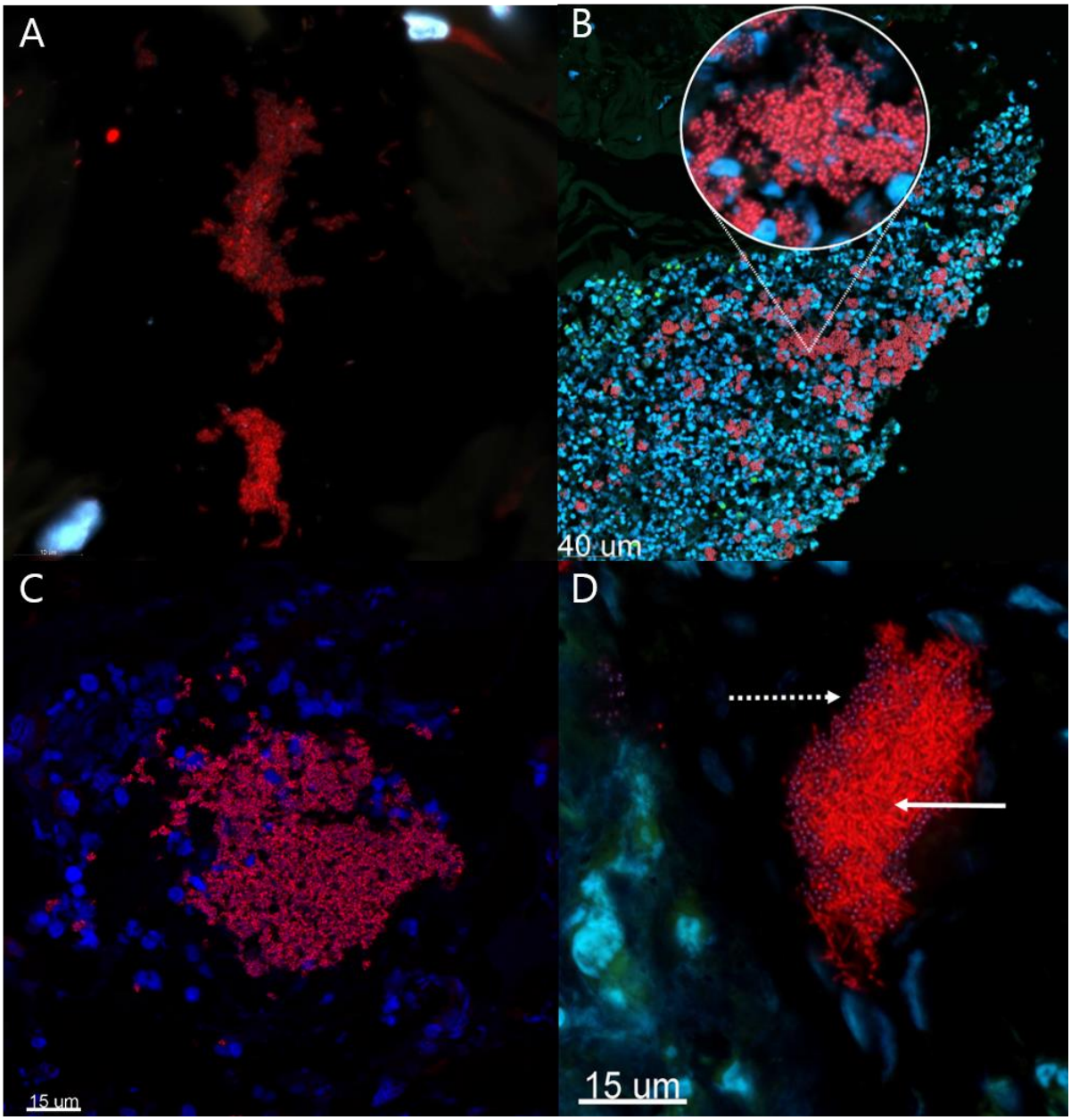

Figure 8. PNA-FISH and DAPI-stained mammary gland tissue from slaughtered dairy cows. Red is bacteria stained with the universal bacterial probe PNA-FISH, and blue is the dairy cow cell nuclei stained with DAPI. Green is autofluorescence from tissue. A) Cocci observed in aggregates in tissue section from location 2 from mastitis quarter, 630x objective; B) Cocci observed in aggregates in tissue section from location 2 from healthy quarter, 630x objective; C) Cocci observed in aggregates in tissue section from location 4 from mastitis quarter, 400x objective; D) Cocci (dotted arrow) and rods (filled arrow) observed in aggregates in tissue section from location 1 from mastitis quarter, 630x magnification. Images obtained with CLSM.

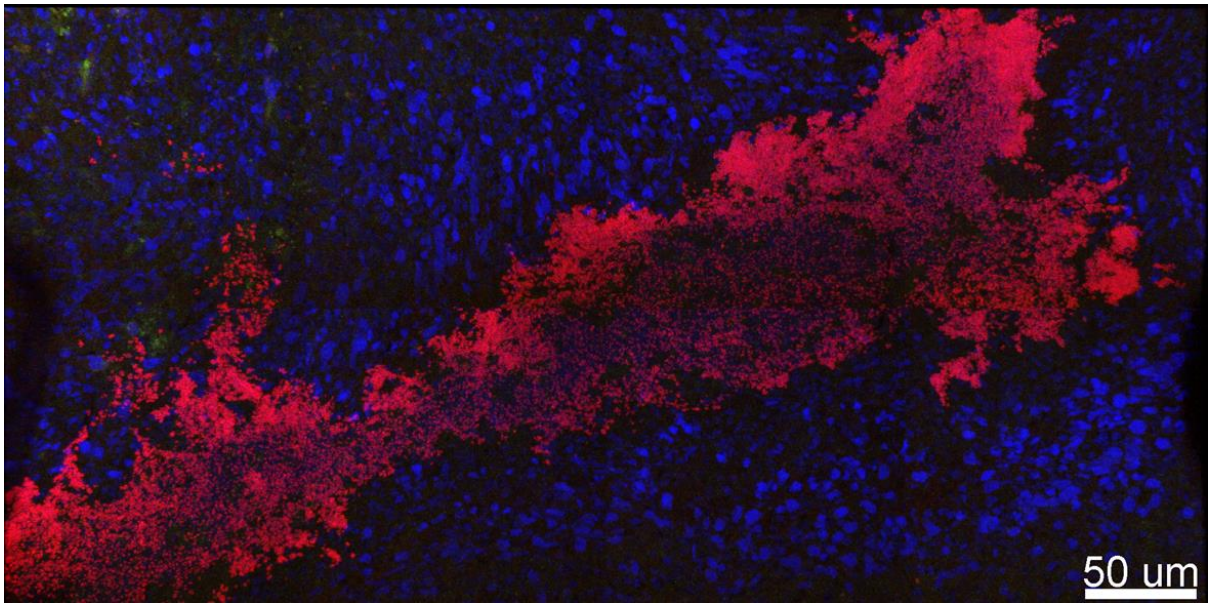

Figure 9. Cocci, as observed in the aggregate in the tissue section from location 4 of the mastitis quarter. The mammary gland tissue was stained with the universal bacterial probe (red) and counterstained with DAPI, showing the nuclei of the dairy cow cells in blue. The images were taken with a 400x magnification by CLSM.

Histopathology of biopsies

To determine the degree of inflammation in the tissue sections, HE-stained biopsies from location 5 from healthy and mastitis samples were investigated. The majority of healthy quarters and mastitis quarters were mild. Furthermore, nine mastitis quarters were moderate, and nine were severe.

Table 8. Histological analysis of the biopsies obtained from location 5 (Figure 1). The inflammation score is based upon the pathological changes of the tissue.

Inflammation in the quarters	Healthy quarters	Mastitis quarters
None *Histological score: 0*	2	2
Mild *Histological score 1–2*	13	16
Moderate *Histological score 3–4*	3	9
Severe *Histological score 5–6*	2	9

Representative images of the biopsies are shown in Figure 10:

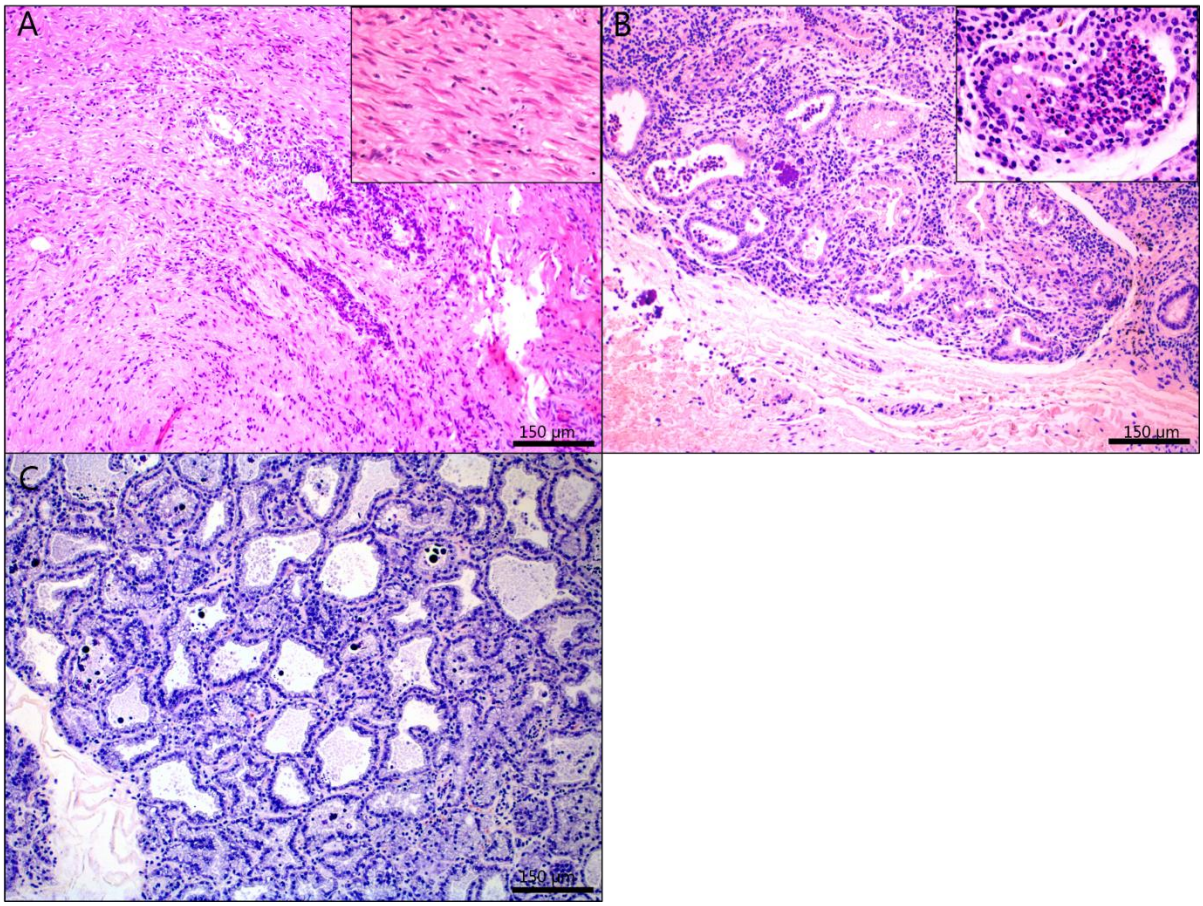

Figure 10. HE-stained tissue sections from location 5 from dairy cows. A) Chronic mastitis with intralobular fibrosis and infiltration with neutrophilic granulocytes and monocytes. Furthermore, inflammatory cells in the glandular lumen, primarily neutrophils. Edema can also be seen in the interlobular connective tissue. Overall histopathological score: 5 (severe inflammation), neutrophilic score: High. Magnification 100x and 200x; B) Normal active mammae tissue: Fat and protein in glandular lumen. A few glands have corpus amylacea (black debris in glandular lumen). Overall histopathological score: 0 (no inflammation). Neutrophilic score: Low. Magnification 100x; and C) Chronic mastitis tissue with massive fibrosis, fibroblast hyperplasia, loss of glandular epithelium, infiltration with neutrophils, and mononuclear cells. Overall histopathological score: 6 (severe inflammation). Neutrophilic score: Moderate. Magnification 100–200x.

Discussion

The research areas of the microbiome and biofilm in bovine mastitis are new and evolving. This study contributes to the knowledge of bacterial composition and spatial distribution in milk samples and biopsies throughout the udder.

Using culturing and MALDI-TOF MS, we found a difference in the number of bacterial species between the milk and the biopsies for healthy and mastitis quarters, indicating that some species were only present in one sample type. For the healthy quarters, mastitis pathogens, such as *S. uberis, E. faecium*, and different *Corynebacterium* species, were detected in the biopsies but not in the milk samples. However, the quarters were defined as healthy in terms of the somatic cell count. Thus, one explanation for this finding could be that the bacterial load of the pathogens was low and not shedding into the milk from the udder tissue and perhaps not causing an infection.

For the mastitis quarters, only a few species were detected in the milk but not in the biopsies. However, a high number of species were detected only in the biopsies. This included mastitis pathogens such as *Bacillus cereus, Corynebacterium* species, *Listeria* spp., and *S. agalactiae.* The difference in the detection of bacteria between milk and biopsies could indicate that some mastitis infections are

underdiagnosed, as the milk is not reflecting the microbial composition in the udders. It could also indicate that the bacteria are located in the udder, where they would not shed into the milk and, therefore, not be detectable by the milk samples.

It was not possible to identify all bacteria with MALDI-TOF MS. However, this outcome was expected since the bacteria needed to be in the database to identify them. Although the database has a veterinary focus, it does not contain all environmental bacteria. Furthermore, there was no bacterial growth on the plates for some samples. In this study, we inoculated 100 µl of the milk samples on blood plates. We chose to inoculate with a higher volume than standard methods to increase the possibility of detecting all bacteria present in the milk.

While *B. licheniformis* was the most common bacteria found by cultivation and MALDI-TOF MS, *S. aureus* was one of the most common bacteria in the mastitis quarters. These results correlates with *S. aureus* being a known mastitis pathogen [16,36]. Corresponding with other studies, we found *S. uberis* in both the healthy and mastitis quarters [37].

We found no significant effect on the alpha diversity between mastitis and healthy quarters using 16S sequencing. However, we did find a significant effect on the alpha diversity and the location of the biopsies, with a higher alpha diversity in location 1 compared to all other locations in the udder. This makes sense because the teat end is exposed to the environment, and the bacterial composition here could be affected by this environmental exposure.

A higher diversity in healthy samples has been reported in several studies [38,39]; however, healthy and mastitis quarters also had diverse bacterial compositions [40–42]. Many phyla have been reported in healthy quarters, especially Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes [38,42–44]. In mastitis quarters, the genera *Bacilli* [38], *Chlamydiia* [38][40], *Staphylococcus* [37,42], and *Streptococcus* [37] have been observed.

Acinetobacter was the dominant genus in the biopsies from healthy and mastitis quarters via 16S rDNA sequencing. This result is in alignment with other studies, which found *Acinetobacter* among the most common genera [42,45–49]. Furthermore, in some studies, *Acinetobacter* has been associated with subclinical mastitis cases [50]. *Acinetobacter* is often associated with the environment (soil) [50]and is not known as a mastitis pathogen but could be a part of the core microbiota of the mammary gland.

The visualization of the bacteria present in the biopsies provided a better understanding of the bacteria in the udders. We found nearly no bacterial aggregates in the samples from healthy quarters but aggregates in almost one out of five samples from mastitis quarters. One of the methods for diagnosing biofilm infections is visualizing the biofilm [10,24,29]. Most of the research on biofilm in bovine mastitis is based on sampling milk, isolating the bacteria in the milk, and analyzing the isolates for biofilm-forming abilities in the laboratory [14]. However, this process leads to the perspective that isolated bacteria can form biofilm in a laboratory setting. Nonetheless, the laboratory setting often has under-optimized conditions for the organism and does not reflect the environment in the clinical setting of the disease [14]. Only a few published studies have investigated whether biofilm was present in the udder by examining biopsies. For example, Hensen *et al.* experimentally infected three dairy cows with *S. aureus* and investigated HE and Gram-stained tissue sections by light microscopy after 24 to 96 hours. They found *S. aureus* in clusters in the alveoli and lactiferous ducts [18]. Schönborn *et al.* obtained swabs from udders with *S. aureus* infection and investigated the swabs by performing immunofluorescence staining too look for polysaccharide intercellular adhesin, a marker for the biofilm matrix by *S. aureus*. They found that the biofilm adhesin was present in seven out of 184 samples [19].

The results in this study and the *in vivo* studies of biofilm in bovine mastitis [18,19] indicate that biofilm might not play such a significant role in all chronic bovine mastitis cases, as only a few of the samples had aggregates. Cases with biofilm could be limited to certain pathogens, such as *S. aureus*. However, it is important to consider that the biopsies obtained from the udders are from a small area compared to the entire quarter, resulting in a risk of missing areas with biofilm within the quarters. In this study, we obtained biopsies from five different depths of the quarters to increase the possibility of detecting the biofilms. Despite this, we only observed aggregates in approximately one fifth of the samples. Another possible reason why we did not find more aggregates, could be that only specific pathogens, such as *S. aureus*, are relevant in biofilm formation in bovine mastitis. To gain a better understanding of the possible role of biofilm in bovine mastitis, future studies should focus on visualizing the bacteria in the tissue.

Mastitis pathogens are associated with tissue damage, either from the release of cellular products or the immune system reacting toward the invading pathogens [51,52]. Therefore, in addition to bacterial detection, we performed a histopathological evaluation of the tissue samples. Most mastitis quarters had an inflammation score ranging from moderate to severe; however, a substantial part had only mild inflammation. The histopathology of location 5 from the quarters showed that most healthy quarters had mild inflammation, while some had moderate to severe inflammation. The inflammatory response of increased immune cells in the mammary gland can cause damage to the tissue [52]. We observed healthy quarters with histological changes, although these might be from an earlier infection. Some of these had mastitis pathogens such as *S. uberis*, *E. faecium* and *Corynebacterium* species present in the biopsies. The tissue sections that were examined for the histopathology were limited to only one location on the udders, and therefore there is no information on the inflammation status on the rest of the udders.

The findings from the culturing, MALDI-TOF MS, and 16S rDNA sequencing in this study differ for the most common species. The 16S rDNA sequencing data demonstrates that *Acinetobacter* and *Staphylococcus* are the most common genera in healthy and mastitis quarters. *Acinetobacter* is the most dominant genus for healthy quarters in four out of five locations. *Staphylococcus* is the most dominant in the mastitis quarters in three out of five locations. Noticeably, the most common species by culturing and MALDI-TOF MS, *Bacillus licheniformis*, is not among the most common genus by 16S rDNA sequencing. This can be due to the differences between the two methods – for culturing and MALDI-TOF MS, we only detect the bacteria able to grow on the selected plates, resulting in the risk of not detecting all bacteria in the samples. 16S rDNA sequencing relies on the presence of DNA; therefore, a more sensitive method could detect more bacterial species.

We expected to find a high diversity among the healthy quarters and a lower diversity in the mastitis quarters. However, we found that the alpha diversity was not significantly different between the two groups. Surprisingly, the most common species was the same between the two groups. However, in this study, we did not quantify the bacterial load. It is possible that the same bacteria are present in the two groups, but the bacterial load was different, meaning that a genus such as *Staphylococcus* could then dominate.

A possible limitation of the study was that we sampled from slaughtered animals. However, since we collected biopsies from the udder, it would not have been possible to carry out the study on living animals. The udders from the dairy cows included in this study might not fully represent the living dairy cow population as the sample size was small, and the results only reflect a single time point. The milk samples showed that most of the included quarters had a higher somatic cell count than 200,000 cells/ml, indicating inflammation in the quarter. Interestingly, not all CMT results corresponded with somatic cell counts. Because we could not obtain any milk samples from the dairy cows or obtain

information about the cell counts before slaughter, the CMT test was the most optimal test to apply when sampling from the udders. Cell counting is the most optimal way to group the quarters as healthy or mastitis quarters; however, the somatic cell count was done after collecting the quarters, and it was used to categorize the quarters as either healthy or mastitis.

The research on the milk microbiome shows that the milk and udders are not sterile environments, as traditionally thought. The results from different studies vary significantly; some studies propose dysbiosis as a cause of bovine mastitis [38,39,42], and some studies report a high diversity in both healthy and mastitis udders [40,43,46]. Different factors can influence the microbiota of the dairy cows, such as an earlier infection; environmental factors, such as bedding types; time of sampling; and the risk of contamination of the milk samples [53]. More research on tissue samples is needed to fully understand the microbiome and how the bacteria are distributed in the mammary glands.

Conclusion

This study contributes to understanding biofilm and bacterial composition in bovine mastitis by studying the bacterial composition and spatial distribution directly in biopsies collected from different locations in the udder. There was a higher occurrence of bacterial aggregates in mastitis quarters than in healthy quarters. The bacterial composition was diverse in healthy and mastitis quarters, and bacteria were found throughout the entire udder. These results contribute to the research, by showing that bovine udders are not sterile but microbial diverse environments with a specific composition and, in some cases, bacteria organized in aggregates.

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Supplementary data Manuscript II

Cow ID	Age (months)	Parity	Days from AB treatment to slaughter
1	23	1	21
2	52	3	36
3	72	4	611
4	47	2	316
5	44	2	356
6	71	5	70
7	73	4	33
8	88	5	173
9	141	6	898
10	66	3	NA
11	60	3	779
12	45	2	NA
13	31	1	17
15	36	1	NA
17	65	3	29
18	40	1	117
19	62	3	139
20	47	2	266
21	89	6	20
22	30	1	20
23	44	2	41
24	70	4	41
25	54	3	41
26	78	4	1196
27	99	6	260
28	56	3	119

Table 1. *Information about dairy cows included in the study. It was not possible to obtain information of two cows. Data obtained from the Cattle Database.*

ID	Hemorrhagi	Hyperaemia	Fibrosis	Edema	Perivascular	NG/Mono in mammary gland lumen	NG/mono in inter/intralobular conective tissue	Histological score	Comment	Neutrophilic granulocytes score
H04	no	no	no	yes	no	no	no	1	Enlarged lymphvessels	low
H13	no	no	no	Yes	no	no, corpus amylacea	no	1		low
H01	no	no	no	yes	no	no	Yes (focal mono infil.)	1	Active mammae tissue	low
H19	no	no	yes	yes	no	no	no	2		low
H16	no	no	no	yes	no	yes (mild) mono infil. and Copus mylacea	no	2		low
H06	no	no	yes	yes	no	no	no	2	Slide with few glands and interlobular fibrosis and edema	low
M18	no	no	no	yes (mild)	no	no	yes (mono infil.)	2	focal intralobular mono infil.	low
M05	no	no	yes	no	no	no	yes(NG and Mono)	3	focal acccumulation (mono and NG) at the intralobular tissue. Increased connective tissue intralobular.	low
H07	no	no	no	no	no	yes (Mono and NG infil.). Corpus amylacea	yes (Mono infil. intralobular)	3	Active mammary gland tissue	high
M19	no	no	no	no	no	yes (Mono and NG)	yes (NG and Mono)	4	Areas of glandular epithialial detachment.	high
M25	yes	no	yes	yes	no	yes (mono infil.)	yes (mono infil.)	5	Fibroblast hyperplasia and fibrosis intra and interlobular. With infiltration of mononuclear immuncells	low
H03	no	no	no	Yes	no	yes (Mono and NG infil.). Corpus amylacea	yes (Mono and NG infil.) intralobular	5		high
M26	no	no	yes	yes	no	no	no	2		low
H05	no	no	no	yes	no	no	no	1	Fragmented slide.	low
H18	no	no	no	no	no	no	no (few plasmacells intra/interlobular)	0		low

H17	no	no	no	no	no	no , corpus amylacea	yes (Mono mild)	1	Detatched glandular epthilium	low
M14	no	no	yes	no	no	no	Yes (Mono mild)	2	Lymphocytes in intralobular interstitium	low
H12	no	no	no	no	no	no	no	0		low
M13	no	no	no	yes	no	no	no	1	Edema of the intralobular conective tissue	low
H08	no	no	yes	yes	no	no, corpus amylacea	no	2		low

Table 2. *Histological analysis of tissue section from Location 5 in healthy quarters.*

ID	Hemorrhagi	Hyperaemia	Fibrosis	Edema	Perivascular	NG/Mono in mammary gland lumen	NG/mono in inter/intralobular conective tissue	Histological score	Comment	Neutrophilic granulocytes score
M01	yes	yes	yes	no	no	no glandular tissue	yes(but no lobuli), mono and NG	5	Slide with no glandular tissue and very small. With fibrosis and Mono and NG infiltration in granulation tissue. Not able to detemine if mammary tissue.	high
H11	no	no	no	no	no	Yes (single gland infiltrated by NG), corpus amylacea	yes (Single area with mono infil.)	2		moderate
H20	yes	no	no	yes	no	no, corpus amylacea	no	2	intralobular edema and focal hemorrage	low
M27	no	no	no	no	no	yes (mild mono infil.)	yes (mild mono)	2		low
H10	no	no	no	no	no	no	no	0		low
M12	no	no	yes	no	no	no	yes (Mono mild)	2	Lymphocytes in intralobular interstitium	low
M06	no	no	no	yes	no	yes (NG and Mono)	Yes (mono)	4	Very fragmented slide. Edema and focal infiltration of immune cells.	moderate
H26	Yes	no	no	yes	no	no, corpus amylacea	no	2		low
M08	no	no	yes	yes	no	yes (Mono and NG)	yes (Mono)	5	Intra and interlobular ifiltration. And focal inflammation in interlobular septae. Edema dialated lymphvessels	high
M16	no	no	yes	no	no	no, corpus amylacea	no (few plasmacells intra/interlobular)	1		low
M23	no	no	yes	no	yes	No glands left	Yes (massive mono and NG)	4	Slide with skin tissue and no mammary tissue. Necrosis, demarcation line, NG and	high

									mono. Infiltration.	
H21	no	no	yes	yes	no	no	yes (mild mono infil.)	3	Intra and interlobular fibrosis, Hyperplasia of fibroblast. Interlobular edema. Mild mononuclear infiltration of intralobular tissue.	low
H15	no	no	no	no	no	no	no	0	Slide with more muscle tissue than other and less glandular tissue	low
H27	no	no	no	no	no	Yes (Mono, NG and cellular debris)	yes (Mono intralobular)	3	Mono, NG and debris in mammary gland lumen. Destruction/detachment of mammary epithilial cell	high
H28	no	no	yes	yes	no	no	no	2		moderate
M03	no	no	yes	yes	no	yes (Mono mild). Corpus amylacea	yes (Mono mild)	4		low
M24	no	no	no	yes	no	no	yes intralobular interstitial infil. NG	2	Edema and enlarged lymphvessels	low
H24	no	no	yes	yes	no	no	yes (mono and NG)	4	Mono and NG in intralobular interstitium	moderate
H09	no	no	no	yes	no	no	yes (mild mono)	2		low
M15	no	no	yes	no	no	yes (mild mono infil.)	yes (mild mono infil.)	3		low
M28	no	no	no	yes	no	yes (NG and Mono)	yes (NG and mono)	5	Generel NG infiltration and area with a larger accumulation of focal infiltrations, interlobular edema and epthilial destruction.	high
M09	no	no	no	yes	no	no	no	1		low
H25	no	no	no	yes enlarged lymphvessel	no	yes (mild) mono infil. and Corpus amylacea	no	2	Edema in inter and intralobular conective tissue with enlarged lymphvessels	low
M10	no	no	yes	no	no	yes (mono)	yes(mono infil.)	3	Very light slide difficult to determine structures	moderate

H14	no	no	no	yes	no	no, corpus amylacea	no	1	Interlobular edema and enlarged lymph vessels	low
H02	no	no	no	no	no	yes (few NG)	Yes (focal mono infil.)	2		moderate
M11	no	no	no	yes	no	no	yes (mono infil.)	2	Dialated lymph vessels and interlobular mono infil.	moderate
H22	yes	yes	yes	no	no	no	Yes(NG and Mono)	5	Massive fibrosis and dissiminated infiltration of immune cells (NG/mono) Destruction of normal mammary tissue (epithilium, intra and inter lobular tissue). Fibroblast hyperplasia. Chronic mastitis	moderate
H23	no	no	yes (mild)	no	no	no	yes (Mono infil. intralobular)	2		moderate
M07	no	no	no	yes	no	yes (Mono and NG infil.). Corpus amylacea	yes (Mono and NG)	5		high
M02	no	no	yes	yes	no	yes (Mono and NG infil.).	yes mono and NG	6	Both NG and mono in lumen and connective tissue	high
M04	no	yes	yes	no	no	no	yes(mono infil.)	3		low
M22	no	no	yes	no	no	yes (NG and Mono)	yes (NG and mono)	5	Infiltration of glandular lumen and intralobular infiltration and loss of lobular structures. Hyperplasia of fibroblast. Destruction of epthialium	high
M17	yes	no	yes	no	yes	No glands left	Yes (massive mono and NG)	5	Slide with skin tissue and no mammary tissue. Necrosis, demarcation line, NG and mono. Infiltration. And hemorrhagia underneath dermal	high

									epithilial layer.	
M21	no	no	yes	no	yes	yes (few glands left with NG and Mono)	yes (NG and mono)	6	Massive fibrosis and dissiminated infiltration of immune cells (NG/mono). Destruction of normal mammary tissue (epithilium, intra and inter lobular tissue). Fibroblast hyperplasia. Chronic mastitis	moderate
M20	no	yes	no	no	no	no, corpus amylacea	no	1	Active mammary gland tissue	low

Table 3. *Histological analysis tissue sections of Location 5 of mastitis quarters.*

Cow ID	*Quarter*	*California Mastitis Test*	*Microscopic SCC *1000/ml*	*Somascope SCC *1000/ ml*	*MALDI-TOF MS result*		
1	Quarter 1	Negative			-		
1	Quarter 2	>2			-		
2	Quarter 1	Negative	8724.6		No growth		
2	Quarter 2	2	15720		*S. uberis*		
3	Quarter 1	Negative	10		*S. epidermidis*		
3	Quarter 2	1	3144		*S. aureus*		
4	Quarter 1	Negative	10		*C. bovis*		
4	Quarter 2	2	21615		*S. uberis*		
5	Quarter 1	Negative	49.125		No growth		
5	Quarter 2	2	10		No growth		
6	Quarter 1	Negative	10		No growth		
6	Quarter 2	>2	982.5		No growth		
7	Quarter 1	Negative	10		*S. aureus*	*E. faecalis*	*A. viridans*
7	Quarter 2	2	15720		*C. bovis*		
8	Quarter 1	Negative	196.5	206	No growth		
8	Quarter 2	>2	1454.1		No growth		
9	Quarter 1	Negative	3949.6		*S. aureus*	*L. lactis*	
9	Quarter 2	>2	5895		No growth		
10	Quarter 1	Negative	982.5		*L. garviae*		
10	Quarter 2	2	6484.5		No growth		
11	Quarter 1	Negative	393	931	No growth		
11	Quarter 2	>2	10611		No growth		
12	Quarter 1	Negative		197	No growth		
12	Quarter 2	>2	982.5		No growth		
13	Quarter 1	Negative	196.5	1021	No growth		
13	Quarter 2	2	10		No growth		
14	Quarter 1	Negative	6905.01		*S. aureus*		
14	Quarter 2	2	78.6		*S. aureus*		

15	Quarter 1	Negative	2593.8		No growth		
15	Quarter 2	2	4637.4		*S. aureus*		
16	Quarter 1	Negative	10		No growth		
16	Quarter 2	2		1527	No growth		
17	Quarter 1	Negative	68.775		No growth		
17	Quarter 2	2	39300		*P. mirabilis*		
18	Quarter 1	Negative	58.95		No growth		
18	Quarter 2	2	10		No growth		
19	Quarter 1	Negative	10		No growth		
19	Quarter 2	2	10		*S. aureus*		
20	Quarter 1	Negative	589.5	1002	No growth		
20	Quarter 2	2	98250		*S. uberis*		
21	Quarter 1	Trace	2456.25		No growth		
21	Quarter 2	>2	39300		*T. pyogenes*		
22	Quarter 1	1	12811.8		*L. monocytogenes*		
22	Quarter 2	2	39300		*S. aureus*		
23	Quarter 1	Not possible	15720		No growth		
23	Quarter 2	2	1572		*H. kunzii*		
24	Quarter 1	Trace	3478.05		No growth		
24	Quarter 2	>2	3261.9		No growth		
25	Quarter 1	Trace	6130.8		*S. aureus*		
25	Quarter 2	>2	10		No growth		
26	Quarter 1	Negative	1375.5	1135	No growth		
26	Quarter 2	2	39.3		*L. lactis*		
27	Quarter 1	Negative	2751	3456	No growth		
27	Quarter 2	2	668.1		*L. lactis*		
28	Quarter 1	Negative	3144		*S. aureus*		
28	Quarter 2	2	5207.25		*S. uberis*		

Table 4. *Results of milk samples – identification of bacteria present in milk samples, CMT score and somatic cell count. The somatic cell count were investigated by flow cytometry and microscopy. Furthermore, the milk samples were analyzed with the California Mastitis test, and the bacterial species of isolates from the milk samples were identified by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). SCC: Somatic cell count.*

Healthy quarters			
Species only found in milk	**Number of quarters**	**Species only found in biopsies**	**Number of quarters**
Acinetobacter johnsonii	1	*Bacillus circulans*	1
Bacillus atrophaeus/subtilis	1	*Brevibacillus species*	1
Bacillus horneckiae	1	*Brevibacter sp.*	1
Bacillus subtilis	1	*Brevibacterium sp.*	1
Brachybacterium sp.	1	*Corynebacterium amycolatum/xerosis*	1
Corynebacterium tuberculostearicum	1	*Corynebacterium freneyi*	1
Enterococcus faecalis	1	*Corynebacterium jeikeium*	1
Gordonia polyisoprenivorans	1	*Corynebacterium mucifaciens*	1
Psychrobacter faecalis	1	*Corynebacterium pilosum*	1
Psychrobacter phenylpyruvicus	1	*Enterococcus faecium*	1
Sphingobacterium daejeonense	1	*Globicatella sanguinis*	1
Staphylococcus arlettae	1	*Kocuria rhizophila*	1
Staphylococcus cohnii	1	*Lactococcus raffinolactis*	1
Staphylococcus hyicus	1	*Microbacterium sp.*	1
Staphylococcus succinus	1	*Sphingomonas parapaucimobilis*	1
Tsukamurella paurometabola	1	*Staphylococcus equorum*	1
		Staphylococcus saprophyticus	1
		Streptococcus gallolyticus	1
		Streptococcus parauberis	1
		Streptococcus sp.	1
		Streptococcus uberis	1

Table 5. *Bacterial species only found in either milk samples or biopsies in healthy quarters. The species are identified by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry*

Mastitis quarters			
Species only found in milk	**Number of quarters**	**Species only found in biopsies**	**Number of quarters**
Brevibacterium sp.	1	*Acinetobacter calcoaceticus*	1
Corynebacterium amycolatum/xerosis	1	*Arthrobacter agilis*	1
Paracoccus denitrificans	1	*Bacillus altitudinis/pumilus*	1
Psychrobacter phenylpyruvicus	1	*Bacillus cereus group*	1
Rothia mucilaginosa	1	*Bacillus clausii*	1
		Corynebacterium afermentans	1
		Corynebacterium amycolatum	1
		Corynebacterium confusum	1
		Corynebacterium glutamicum	1
		Corynebacterium mucifaciens	1
		Corynebacterium sp.	1
		Corynebacterium stationis	1
		Corynebacterium ulcerans	1
		Enterococcus cecorum	1
		Gordonia polyisoprenivorans	1
		Klebsiella oxytoca	1
		Kocuria palustris	1
		Lichtheimia corymbifera	1
		Listeria sp.	1
		Microbacterium sp.	1
		Paenibacillus	1
		Ralstonia picketii	1
		Sphingomonas parapaucimobilis	1
		Sphingomonas paucimobilis	1
		Staphylococcus auricularis	1
		Staphylococcus capitis	1
		Staphylococcus cohnii	1
		Staphylococcus epidermidis	1
		Staphylococcus hominis	1
		Staphylococcus lugdunensis	1
		Staphylococcus piscifermentans	1
		Streptococcus agalactiae	1
		Streptococcus pluranimalium	1

Table 6. *Bacterial species which are only found in either milk samples or biopsies in mastitis quarters. The species are identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.*

Manuscript III

Pedersen, R. R., Highmore, C., Hanrahan, N., Mahajan, S., Krömker, V., Dahl-Pedersen, K., Buhl, R., Jørgensen, Bjarnsholt, T., E., Webb, J.S.

"**Detection of bovine mastitis pathogens in milk by multi-excitation Raman spectroscopy.**"

Manuscript in preparation.

Detection of bovine mastitis pathogens in milk by multi-excitation Raman spectroscopy

Regitze Renee Pedersen[1, 2], Callum Highmore[3,4]*, Niall Hanrahan[5,6], Sumeet Mahajan[5,6], Volker Krömker[7], Kirstin Dahl-Pedersen[1], Rikke Buhl[1], Elin Jørgensen[2], Thomas Bjarnsholt[2,8] and Jeremy S. Webb[3,6]

[1] Department of Veterinary Clinical Sciences, University of Copenhagen, Copenhagen, Denmark,
[2] Department Immunology and Microbiology, Costerton Biofilm Center, University of Copenhagen, Copenhagen,
[3] School of Biological Sciences, Faculty of Environmental and Life Sciences, University of Southampton, United Kingdom,
[4] Faculty of Medicine and Institute for Life Sciences, University of Southampton, United Kingdom,
[5] School of Chemistry, Faculty of Engineering and Physical Sciences, University of Southampton, United Kingdom,
[6] National Biofilms Innovation Centre (NBIC) and Institute for Life Sciences, University of Southampton, United Kingdom,
[7] Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark,
[8] Department of Clinical Microbiology, Copenhagen University Hospital, Copenhagen, Denmark

*Corresponding author

Abstract:

Bovine mastitis is a significant disease in the dairy industry that affects all dairy farms. Its leading cause is bacterial in origin, and its diagnosis often relies on slow methods like cultivation, morphological and biochemical methods, MALDI-TOF MS, or sequencing. In this study, we investigated whether multi-excitation Raman spectroscopy could be applied to detect the bacteria in milk samples with the mastitis pathogens *Staphylococcus aureus* and *Streptococcus uberis* in different concentrations. Multi-excitation Raman spectroscopy is able to detect the unique fingerprint of each bacterium, and sample analysis and bacterial detection can occur within minutes. We found that this method could distinguish between the control and milk samples with added bacteria in different concentrations, down to 4 CFU/ml. The support vector machine's accuracy was 91.4% for *Staphylococcus aureus* and 90.4% for *Streptococcus uberis*. More research is needed on the milk composition of infected animals, and a database needs to be established before applying the method to day-to-day diagnostics at dairy farms. However, the results of this study show that multi-excitation Raman spectroscopy is a potential method for the rapid diagnosis of bovine mastitis.

Keywords: multi-excitation Raman spectroscopy, bovine mastitis, diagnostics, intramammary infection, milk

Introduction

In the dairy industry, bovine mastitis is one of the diseases that have the most significant implications for animal health, the economy, and the use of antibiotics [1]. The disease is an intramammary infection mainly caused by a bacterial infection, with some of the most common bacterial agents being *Staphylococcus aureus, Streptococcus uberis,* and *Streptococcus dysgalactiae* [2]. Common current diagnostic methods for bacterial identification in bovine mastitis are cultivation, morphological and biochemical methods, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), and 16 rDNA sequencing [3,4]. However, one of the most significant flaws of these methods is that it can take 24–48 hours for test results to become available.

Raman spectroscopy is a promising method for rapid bacterial identification. It does not require a cultivation step prior to analysis, and sample preparation is simple, non-destructive, and fast compared to methods such as mass spectroscopy and molecular methods [5,6]. Where these methods can take days, Raman spectroscopy can provide results in a matter of minutes. The simplicity of the technique adds to its benefits, as Raman spectroscopy is label-free and does not require the addition of specific reagents or dyes. Raman spectroscopy uses the principle of the Raman effect, which is the scattering of light that causes the molecules in a sample to vibrate, to create unique fingerprints for the samples [7]. The use of Raman spectroscopy for the diagnosis of bacterial infections is being investigated for its potential use with different human diseases such as cystic fibrosis [5], oral diseases [8], spontaneous bacterial peritonitis [9], and pneumonia [10], but to the authors' knowledge, the technique has not been applied to the diagnosis of bacterial pathogens that cause bovine mastitis.

In this study, we investigated the possible application of Raman spectroscopy to diagnose bacterial bovine mastitis infections by detecting the commonly associated mastitis pathogens *S. aureus* and *S. uberis* in milk samples. Furthermore, we evaluated the ability of the method to detect different concentrations of the two bacterial species in milk samples.

Materials and Methods

Bacterial strains

The bacteria *S. aureus* and *S. uberis* used in the study were kindly donated by The Vale Veterinary Laboratory (Devon, UK) and were isolated from bovine mastitis cases. The bacteria were stored on 5% blood agar plates at 5ºC between experiments.

Preparation of bacteria for spectroscopy

Bacteria were grown from single colonies of blood agar plates overnight in Brain Heart Infusion broth (Sigma-Aldrich, USA). The number of cells was determined by optical density (Jenway, UK). The overnight cultures were centrifuged at 4000 RPM for 10 minutes, the

supernatant was discarded, and 5 ml of nuclease-free water (Sigma-Alrich, Germany) was added to the pellet. The pellets were dissolved by vortexing and centrifuged at 4000 RPM for 10 minutes. The supernatants were discarded, and pasteurized semi-skimmed 1.5% fat milk from the supermarket was added to the pellet to dilute the two strains in a 10-times dilution series. Quartz microscope slides (UQG Optics, UK) were prepared using 30µl of the milk with each bacterium and a sterile milk control and dried at 48ºC for 15 minutes. Experiments with both strains were carried out in three biological replicates.

Raman micro spectroscopy

The spectroscopy of the samples was carried out as described in the work of Lister et al. [5]. In short, a Renishaw InVia Raman microscope (Renishaw, UK) containing a Leica DM2500 M bright field microscope with a 100 nm encoded XYZ stage was used with 532 nm and 785 nm lasers. The two lasers were used to excite samples with a Leica 50x long working distance air objective (NA = 0.50). Spectra were acquired with 3 accumulations of 5s, and 50 spectra of each sample were obtained.

Data analysis

Cosmic rays were removed using the Renishaw Wire 5.5 software, imported to iRootLab version 0.17.8.22 [11], and analyzed by Matlab 2020a. Data analysis was carried out as described in the work of Lister et al. [5]. In short, a support vector machine (SVM) was used to classify the data, and to validate these results, a 10-fold cross-validation was performed with iRootLab. Furthermore, the SVM and k-fold cross-validation functionality were used for the classification of the milk and the different concentrations of bacteria.

Results

Two different laser wavelengths were investigated in this study: 785 and 532 nm. Stronger peaks were observed at the 785 nm wavelength for both strains. The spectral information for the strains and the milk at the two different wavelengths is shown in Figure 1.

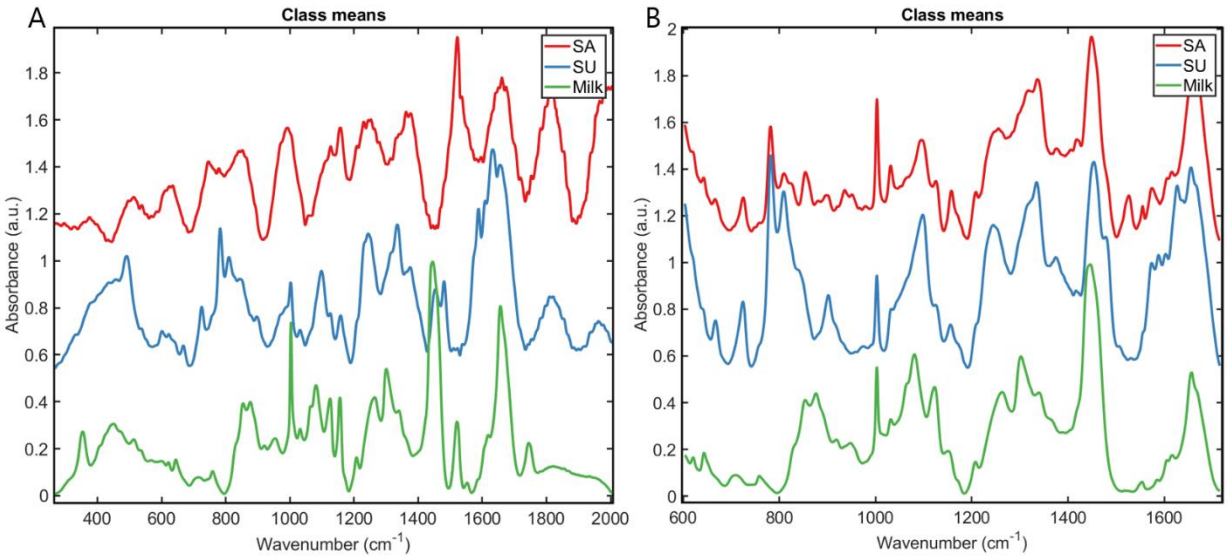

Figure 1. Class means of A) S. aureus *(SA),* S. uberis *(SU), and milk at 532 nm wavelength and B)* S. aureus *(SA),* S. uberis *(SU), and milk at 785 nm wavelength.*

The specific peaks for the wavelengths, bacteria, and milk are listed in Table 1.

	Wavenumbers cm^{-1}
532	
S. aureus	372, 508, 630, 750, 837, 847, 987, 1155, 1229, 1249, 1360, 1372, 1522, 1659, 1806, 1816
S. uberis	487, 723, 784, 808, 1004, 1096, 1155, 1240, 1333, 1372, 1451, 1482, 1587, 1628, 1660, 1806, 1816, 1957
Milk	351, 450, 641, 756, 851, 872, 1003, 1079, 1123, 1157, 1259, 1298, 1441, 1517, 1654, 1740
785	
S. aureus	603, 618, 665, 782, 851, 900, 934, 948, 1003, 1030, 1095, 1130, 1252, 1315, 1333, 1447, 1524, 1556, 1572
S. uberis	603, 665, 782, 808, 900, 1003, 1095, 1130, 1241, 1333, 1371, 1451, 1475, 1572, 1586, 1600, 1625, 1654
Milk	618, 702, 756, 851, 874, 1003, 1078, 1120, 1205, 1259, 1300, 1333, 1443, 1654

Table 1. Peaks for S. aureus, S. uberis, *and milk for wavelength 532 and 785 measured in wavenumbers in* cm^{-1}.

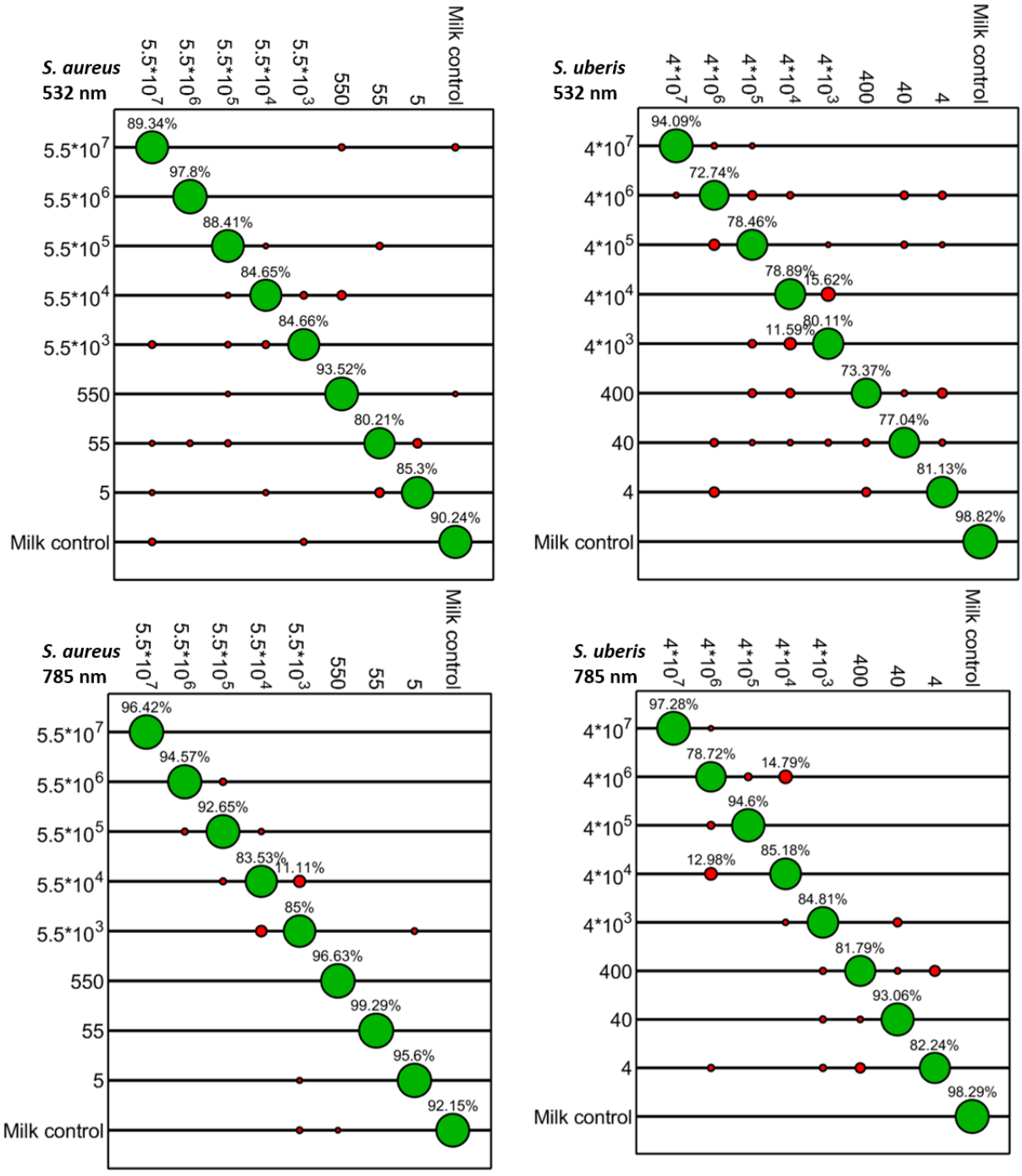

Figure 2. Classification accuracies by SVM for the excitations 532 nm and 785 nm for S. aureus *and* S. uberis. *Green balls represent the correct identifications and the red balls the incorrect by SVM.*

The machine learning algorithm SVM was used to detect the performance of the Raman spectroscopy on the samples. The SVM was used on both excitations and on the concatenated spectra. For *S. aureus*, there was a minimum of 80.21% classification accuracy on both spectra (Figure 2), and the combined spectra had a minimum of 91.41% (Figure 3). There was a misclassification of 11% between the two concentrations, $5.5*10^4$ and $5.5*10^3$ CFU/ml, for the excitation at 785 nm.

For *S. uberis*, the minimum classification accuracy was lower, around 72.74% (Figure 2). There was some misclassification for the excitation at 532 nm between $4*10^4$ and $4*10^3$ CFU/ml at

15.62% and 11.59% for 523 nm, and for 785 nm, there were some misclassifications at $4*10^4$ and $4*10^6$ CFU/ml at 12.98% and 14.79% (Figure 3). However, the combined spectra resulted in a classification accuracy of at least 90.4% (Figure 3).

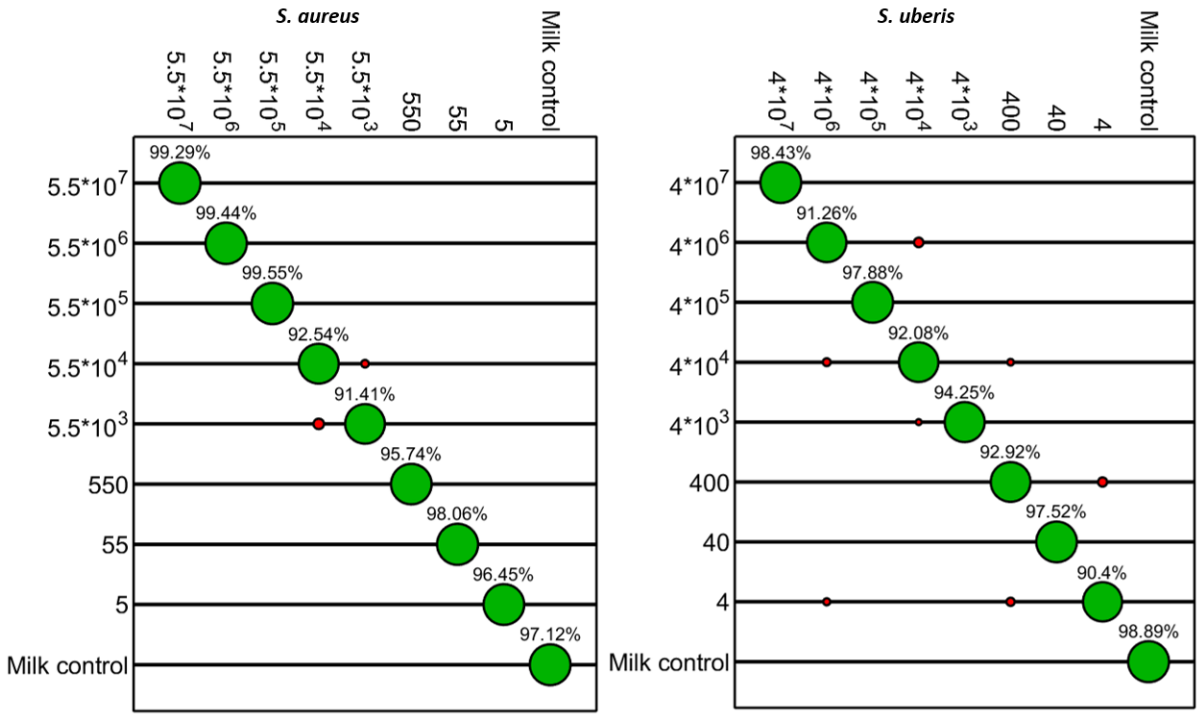

Figure 3. Classification accuracies by SVM for the concatenated spectra for 532 nm and 785 nm for S. aureus *and* S. uberis. *Green balls represent the correct identifications, and the red balls the incorrect by SVM.*

Discussion

To the authors' knowledge, this is the first study to apply Raman spectroscopy to detect *S. aureus* and *S. uberis* in bovine milk. We showed that the method, with high accuracy, could detect spectra for two isolates of the bacterial species and milk. Furthermore, the method detected, with high accuracy, different concentrations of the bacteria in milk and differences between the bacterial milk samples and the control milk samples. Comparing the two spectra, 785 nm was better for detecting both bacteria. However, it should be noted that the method could detect a difference between 4 CFU/ml and 0 CFU/ml in a very small imaging area of 5x10 µm. This should be investigated further to ensure that the SVMs can actually detect such a small difference.

Additionally, to the authors' knowledge, this is the first study applying multi-excitation Raman spectroscopy specifically to *S. uberis*. Dahms et al. investigated whether Raman spectroscopy could be used to differentiate various *Streptococci* species and found different specific peaks for the distinct strains at 532 nm [12]. We found that *S. uberis* shared the peaks with other *Streptococci* at 784 cm^{-1}, 1,004 cm^{-1}, 1,096 cm^{-1}, and 1,660 cm^{-1}, which were not found with either *S. aureus* or the milk [12]. Lister et al. found peaks for *S. aureus* at 750 cm^{1} and 1,120 cm^{1} at 532 nm [5], whereas we, in this study, found a peak at 750 cm^{-1}.

For the 785 nm wavelength, we found that most peaks for *S. aureus* were either carotenoids or DNA, as described by Movasaghi et al. [13]. For *S. uberis*, it was not possible to find any reference for *Streptococci* at this wavelength, but the found peaks correlated with DNA and amino acids [13]. For the milk samples, many of the peaks corresponded to fatty acids, lipids, and phospholipids [13].

The most common current diagnostic methods for bovine mastitis of bacterial origin are culturing from milk samples, phenotypic characteristics, selective media, bi- and triplate systems, MALDI-TOF MS, and PCR [1,3,4,14,15]. All of these methods share the same limitation – time. It can take 24–48 hours for these methods to produce results for diagnostics. For the method used in this study, it took approximately 1 hour to prepare the sample and obtain both spectra. The faster turnaround time, combined with the simplicity of the method due to its label-free and reagent-less nature, makes Raman spectroscopy a promising tool for future diagnostics of bacterial infections in both human and veterinary clinical settings [7].

The use of Raman spectroscopy as a diagnostic tool for bovine mastitis is an unexplored research area. The possible application of the method of surface-enhanced Raman spectroscopy (SERS) has been investigated by a few research groups focusing on screening milk. However, SERS is a more complicated version of Raman spectroscopy where materials such as nanoparticles are added to the sample to enhance the Raman scattering signal [5,16]. One study applied SERS to screen milk at dairy farms for antibiotic residues [17], while two studies investigated whether SERS could be used to identify inflammatory markers in milk to detect bovine mastitis [18,19]. In the latter studies, the inflammatory biomarker N-acetyl-β-D-glucosaminidase was identifiable using the SERS technique, differentiating between healthy, clinical, and subclinical mastitis [18], and IL-6 was found to have a detection limit much lower than the current threshold value [19]. However, the use of Raman spectroscopy for diagnosing mastitis is still an unexplored area of research.

Raman spectroscopy has been investigated for several human bacterial infections and found to be effective in the detection of pathogens such as *S. aureus* in artificial sputum media mimicking cystic fibrosis [5] and different pathogens from sputum samples [10]. Virulent factors, biofilm phenotypes, and antibiotic-resistant profiles are also being investigated using this method [6,20–23].

While our findings are promising, this study is small and has some limitations. The milk used in this study was pasteurized semi-skimmed 1.5% fat milk from a supermarket, meaning the texture of the milk was liquid and homogenous. However, the milk from a dairy cow with bovine mastitis can differ significantly. Milk from a dairy cow with subclinical mastitis can have a different composition than that of a healthy cow [3]; the texture of the milk can be clumpy, and the milk can change color [4]. Healthy milk is composed of different molecules such as proteins, fatty acids, and lactose [24], whereas the milk from infected animals can consist of immune cells; a different composition of lactose, fatty acids, and oligosaccharides; and the possibility of chemical components from the metabolism of the bacteria [25]. There is a possibility that the texture of the milk can impact the ability of Raman spectroscopy to detect the spectra of the bacteria in the milk sample, which needs to be investigated. Nicolaou et al. applied the method of Fourier transform infrared spectroscopy to assess whether it could detect

bacteria in spoiled milk and found the method successful at this [26]. In some cases of bovine mastitis, more than one bacterium can be present [3], and a co-infection scenario should be investigated. Raman spectroscopy of multiple species is still scarcely studied, but Raman imaging could be a place to begin. Kriem et al. found that confocal Raman spectroscopy could be used for differentiating multispecies biofilm, and this method could be investigated for possible application to multispecies detection in bovine mastitis milk [27].

Raman spectroscopy reference libraries for biosolids and biomaterials remain limited; they are often commercial and highly application specific [28–32], so material identification methods rely on technical expertise for the construction of such libraries before large-scale material identification can take place. For the use of Raman spectroscopy in the microbiological diagnostic routine, a commercial database must be available to identify the bacteria; while this does not yet exist, some research groups are mapping Raman peaks for different chemical bonds [13]. Furthermore, only a small area of the sample is typically scanned. This results in the risk of missing bacteria in a sample with a low bacterial count. A possible way to circumvent the risk of missing bacteria in a sample with low bacterial count could be to investigate larger areas or add a sample processing step, such as filtration, to concentrate the bacteria to increase the possibility of detecting them. However, this comes at the cost of a longer processing time.

Conclusion

In this study, we detected specific spectra through Raman spectroscopy of *S. aureus* and *S. uberis* in milk and differentiated between various concentrations of the bacterial species. These findings provide a foundation for the use of Raman spectroscopy as a fast, precise, and simple diagnostic tool for bovine mastitis. Rapid diagnostics are crucial for the treatment and recovery of mastitis in dairy cows, which has great implications for the economy and welfare of the dairy industry.

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