Milk, udder skin, and fecal microbiota and their relationships with blood metabolites and milk composition in dairy herds

March，2024

敖道胡

Graduate School of Environmental and Life Science
(Doctor’s Course)

OKAYAMA　UNIVERSITY

Contents

Introduction

1.1 Research background and motivation

Mastitis, a prevalent disease affecting the mammary glands of dairy cows globally, stands as a critical issue in dairy farming [1]. Evaluating the microbiota in milk is crucial for mastitis prevention and ensuring the overall health of the dairy herd [2-4].This inflammation of the udder, most often caused by bacterial infections, adversely affects both the volume and quality of milk produced [5]. The disease has multiple causes, with a variety of pathogens contributing to its subclinical and clinical variants [6,7]. A notable feature of subclinical mastitis (SCM) is the elevated somatic cell count (SCC) in milk, which occurs without any apparent changes in the milk or udder, nor any overt systemic symptoms [7].The incidence of mastitis in dairy cows and the somatic cell count (SCC), a measure of leukocytes in milk indicating udder health, both fluctuate with the seasons[8]. Some studies indicate that milk with high somatic cell counts (SCCs) doesn't always imply the infiltration of mastitis-causing pathogens in the mammary quarters. Conversely, it's also thought that milk with very low SCCs can contain these pathogens [9-11].

The milk from affected cows contains harmful microorganisms and white blood cells, significantly impacting both the health of the cows and the integrity of dairy products. Mastitis is among the most frequent health issues in dairy cattle, posing a considerable challenge to the industry. It manifests in symptoms like udder swelling, pain, and systemic issues such as fever, loss of appetite, and shock, all detrimental to the cows' welfare [12]. Common bacterial agents causing this condition include Streptococcus spp., Staphylococcus aureus, non-aureus staphylococci, Escherichia coli, and Bacillus spp. [13–15]. These bacteria may stem from various sources, like bedding, milking equipment, handlers' hands, or from cows themselves during cross suckling [16]. They play a vital role in both maintaining udder health and influencing susceptibility to mastitis, also affecting the quality of milk during milking [16,17].

The rate of mastitis in dairy cows is relatively lower in developed countries, attributed to effective breeding management, rigorous regulations, and advanced prevention and treatment techniques. Regions like the European Union and the United States have established strict dairy farming standards and have adopted innovative technologies such as automated milk collection and lactation control systems, improving the health and productivity of dairy cows. However, in developing countries, particularly among smaller-scale farmers, mastitis rates remain high due to a lack of knowledge in scientific breeding and management practices. The diversity and abundance of microorganisms within the mammary gland can indicate the health status of dairy cows [18]. Interestingly, research has shown that the microbial diversity in udder sections with a history of clinical mastitis is less varied than in those that are

healthy [19].

1.2 Research purpose and questions

Research purpose

This comprehensive research project is dedicated to delving into the intricate web of relationships between various microbiota present in the dairy farm environment, specifically in milk, on udder skin, in feces, and in bedding material. The focus of this study is to unravel how these diverse microbial communities interact and influence each other, and to understand their collective impact on the composition of milk, the metabolites in blood, and the overall health status of dairy cows, with a particular emphasis on the prevention of mastitis, a prevalent and significant health issue in dairy farming. Mastitis, an inflammation of the mammary gland, is often caused by bacterial infections and can severely affect milk production and quality. By examining the microbiota from different sources, this research aims to identify patterns and interactions that may contribute to the susceptibility or resistance to mastitis. The study will analyze how the microbiome of the udder skin differs from that in milk, feces, and bedding, and how these differences correlate with the cows' health. Furthermore, the research will explore the possibility that certain microbiota compositions could enhance the cows' immune response, thereby reducing the incidence of mastitis.

This investigation is crucial not only for enhancing the quality of milk and ensuring the welfare of dairy cows but also for the dairy industry. By identifying key microbial interactions and their effects on cow health and milk production, the study could lead to more effective strategies for mastitis prevention, ultimately leading to improved animal welfare, enhanced milk quality, and increased productivity in the dairy sector. Understanding these microbial ecosystems in detail will provide valuable insights into developing probiotic or other microbiome-based interventions to promote the health of dairy cows and improve milk production.

Research questions

Relationship Between Microbiota and Mastitis:

How do the compositions of milk, udder skin, feces, and bedding microbiotas relate to the incidence of mastitis in dairy cows?

Can modifications in cow management practices effectively prevent mastitis by altering these microbiotas?

Impact of Microbiota on Milk Composition:

In what ways do the microbiotas of the udder skin, feces, and bedding influence the composition of milk, particularly its protein, fat, and SNF (solids-not-fat) levels?

How do changes in these microbiotas over time (e.g., between 2018 and 2020) affect the quality and safety of milk?

Nutritional Status and Microbiota Variation:

Does the nutritional status of dairy cows influence the diversity and composition of their skin microbiota?

How does this relationship, in turn, affect the milk and udder skin microbiota, and does it have any implications for milk composition and cow health?

Transmission Dynamics of Microbiota:

What are the dynamics of microbiota transmission between different environmental components (bedding, udder skin) and the milk?

Does the microbiota transmission process involve selection and elimination mechanisms that could influence the prevalence of pathogens?

Year-to-Year Variations in Microbiota:

What is the year-to-year variations in the microbiotas of milk, udder skin, feces, and bedding in dairy cows, and how do these variations correlate with changes in milk composition and blood metabolites?

Blood Metabolites and Microbiota Correlation:

Are there specific correlations between blood metabolites and the microbiotas of milk, udder skin, or feces?

Do these correlations provide any insights into the health status of dairy cows, particularly in relation to mastitis?

1.3 Research hypothesis

Relationship Between Microbiota and Mastitis:

Hypothesis 1: The compositions of milk, udder skin, feces, and bedding microbiotas are significantly correlated with the incidence of mastitis in dairy cows.

Hypothesis 2: Modifications in cow management practices can significantly alter the microbiotas of milk, udder skin, feces, and bedding, thereby effectively preventing the incidence of mastitis.

Impact of Microbiota on Milk Composition:

Hypothesis 3: The microbiotas of the udder skin, feces, and bedding have a significant influence on the composition of milk, particularly affecting its protein, fat, and SNF levels.

Hypothesis 4: Changes in these microbiotas over time (e.g., between 2018 and 2020) significantly affect the quality and safety of milk.

Nutritional Status and Microbiota Variation:

Hypothesis 5: The nutritional status of dairy cows significantly influences the diversity and composition of their skin microbiota.

Hypothesis 6: This relationship between nutritional status and skin microbiota significantly impacts the milk and udder skin microbiota, further influencing milk composition and cow health.

Transmission Dynamics of Microbiota:

Hypothesis 7: There are specific dynamics of microbiota transmission between different environmental components (bedding, udder skin) and the milk, involving distinct selection and elimination mechanisms.

Hypothesis 8: The microbiota transmission process significantly influences the prevalence of pathogens.

Year-to-Year Variations in Microbiota:

Hypothesis 9: There are significant year-to-year variations in the microbiotas of milk, udder skin, feces, and bedding in dairy cows, which correlate with changes in milk composition and blood metabolites.

Blood Metabolites and Microbiota Correlation:

Hypothesis 10: Specific correlations exist between blood metabolites and the microbiotas of milk, udder skin, or feces, providing insights into the health status of dairy cows, particularly in relation to mastitis.

1.4 Research scope and limitations

Research scope

This research aims to explore the connection between various microbiotas—milk, udder skin, feces, and bedding—and the health of dairy cows, particularly in preventing mastitis. It involves a comparative analysis of changes in microbiota and milk composition over a two-year period (2018 to 2020), providing a temporal perspective on these relationships. The study is set in the context of advanced dairy farming, utilizing automatic milking systems in a free-stall barn to reflect modern agricultural practices. It encompasses a comprehensive approach to sampling, including the collection and analysis of milk, udder skin, feces microbiota, bedding microbiota, and blood metabolites, to provide a holistic view of the factors impacting dairy cow health.

Research limitations

Limited to Specific Farming Setup: The findings are specific to cows managed in a particular type of barn with automated milking systems, which may not be generalizable to all dairy farming setups.

Temporal Scope: The study is limited to two time points (2018 and 2020), which may not capture long-term trends or seasonal variations in microbiota and milk composition.

Focus on Specific Microbiota Families: The study primarily centers around certain microbiota families, which might overlook the role of less abundant or unexamined microbes.

Mastitis Focus: While the study provides insights into mastitis prevention, it may not comprehensively cover other health issues in dairy cows.

Chapter 1 Literature Review

1.1 Mastitis and the importance of milk quality

Bovine mastitis, a prevalent disease in dairy cows, is an inflammation of the udder tissue typically caused by infection or physical injury. It significantly impacts the dairy industry financially, primarily due to its effect on lowering milk production and quality [20]. On average, it's estimated that each case of bovine mastitis costs about $147 per cow annually. This expense, which amounts to about 11% to 18% of the annual gross margin for each cow, mainly arises from the loss of milk output and the need for culling affected cows [21]. Notably, reduced milk production due to damage in the mammary tissue constitutes approximately 70% of these financial losses [22].

Bovine mastitis, a significant issue in dairy farming, is categorized into three types based on the severity of inflammation: clinical, sub-clinical, and chronic mastitis. In clinical mastitis, symptoms are apparent and include visible signs such as a red and swollen udder, fever in the cow, and changes in the milk, which becomes watery and may contain flakes and clots [23]. This type of mastitis can be further broken down into per-acute, acute, and sub-acute categories, depending on the inflammation's intensity [24]. In severe cases, clinical mastitis can even lead to the cow's death [25]. In contrast, sub-clinical mastitis does not present visible symptoms in the udder or milk. However, it leads to a decrease in milk yield and an increase in somatic cell count (SCC) [26]. The economic impact of sub-clinical mastitis is difficult to measure precisely, but it's generally believed to cause greater financial loss in dairy herds than in clinical cases [22, 27].

Milk quality is a multifaceted concept that extends beyond mere taste and texture, encompassing a range of factors that collectively ensure its safety, nutritional value, and overall acceptability for consumption. At its core, high-quality milk is rich in essential nutrients such as proteins, vitamins (notably Vitamin D and B12), minerals like calcium and phosphorus, and beneficial fats. The balance and concentration of these nutrients are pivotal in determining the milk's nutritional value and its role in a healthy diet. Central to milk quality is the adherence to stringent sanitary standards. The absence of harmful microorganisms and contaminants, including bacteria, viruses, and residues from antibiotics or pesticides, is fundamental. Regular testing for somatic cell count (SCC) and bacterial count is a critical practice to ensure that the milk meets established health and safety standards. These measures are essential in safeguarding public health and maintaining consumer trust in dairy products.

The processing and handling of milk play a significant role in preserving its quality. Methods like pasteurization, which involves heating the milk to a specific temperature to destroy harmful pathogens, are crucial. Equally important is the handling and storage of milk, including effective refrigeration and measures to prevent contamination during

transportation, which is crucial for preserving milk's freshness and preventing spoilage. The sensory attributes of milk, such as taste, color, and consistency, are also vital indicators of its quality. High-quality milk should possess a pleasantly sweet and creamy taste, free from any off-flavors or odors. It should be devoid of discoloration or sediment, ensuring that it has been properly filtered and is free from extraneous matter. Regulatory compliance is another cornerstone of milk quality. Adherence to national and international standards and regulations, which set limits for various parameters like bacterial count, antibiotic residue, and nutrient levels, is essential. These regulations ensure that milk is not only safe but also of high quality, meeting the expectations of consumers and authorities alike.

The health and welfare of dairy animals are directly linked to the quality of milk they produce. Healthy, well-nourished, and stress-free animals are more likely to produce higher quality milk. This involves providing proper veterinary care, ensuring balanced nutrition, and maintaining humane living conditions for the animals. Lastly, environmental factors surrounding the dairy farm can significantly impact milk quality. The quality of feed, availability of clean water, and the overall climate conditions are critical elements that influence the health of dairy animals and, consequently, the quality of milk they produce. These factors must be carefully managed to ensure the production of high-quality milk.

1.2 Causes and prevention of mastitis.

To date, numerous pathogens have been identified as causes of mastitis in dairy. cows. These include bacteria like Staphylococcus aureus, Streptococcus agalactiae, and other Streptococcus species [28-32]. Specifically, Streptococcus dysgalactiae is noted as a significant contributor to sub-clinical mastitis (SCM) in bovine herds, along with other microbes like Clostridium perfringens, Mycobacterium, Mycoplasma, Prototheca, Pasteurella, Nocardia asteroids, Pseudomonas aeruginosa, Staphylococcus aureus, and various yeasts [33]. Additionally, Actinomyces spp., Staphylococcus spp., and Streptococcus spp. have been isolated from cases of bovine mastitis [34]. Among these, Staphylococcus aureus, Streptococcus agalactiae, and Mycoplasma spp. are known for causing contagious mastitis [35]. The microorganisms responsible for mastitis are generally grouped into three categories: 1) Contagious pathogens, which include Corynebacterium bovis, Staphylococcus aureus, Streptococcus agalactia, and Mycoplasma sp.; 2) Environmental pathogens, which encompass Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Streptococcus uberis, Streptococcus bovis, Streptococcus dysgalactiae, Citrobacter sp., and Serratia sp.; and 3) Other pathogens, which include Coagulase-negative Staphylococci sp., Arcanobacterium pyogenes, Candida sp., Nocardia asteroids, Pseudomonas aeruginosa, Prototheca sp., and Serratia sp. [36].

Mastitis, a prevalent and economically significant condition in dairy cows, is an inflammation of the mammary gland, typically caused by bacterial infections. Understanding its causes and implementing prevention strategies are essential for maintaining herd health and optimizing milk production. The primary cause of mastitis is the invasion of the udder tissue by pathogens, predominantly bacteria such as Staphylococcus aureus, Escherichia coli, and Streptococcus agalactiae. These bacteria can enter through the teat canal, often during milking or from environmental sources. Contributing factors include poor milking practices, such as improper use of milking machines or inadequate hand-milking techniques, which can damage the teat and facilitate bacterial entry. Additionally, environmental conditions, such as wet and dirty bedding, overcrowding, and poor ventilation, can increase the risk of infection. To prevent mastitis, dairy farmers must adopt comprehensive management practices. A key part of this is maintaining strict hygiene during milking. This includes thoroughly cleaning and disinfecting teats before and after milking, using individual towels for each cow, and ensuring that milking equipment is properly sanitized. Regular maintenance and calibration of milking machines are also crucial to prevent teat injury and ensure effective milking.

Environmental management plays a significant role in mastitis prevention. Providing clean, dry bedding, ensuring proper ventilation, and reducing overcrowding can significantly lower the risk of environmental pathogens. Nutrition also impacts mastitis rates; a balanced diet strengthens the immune system of cows, making them less susceptible to infections. Regular herd health monitoring is essential. This involves conducting routine checks for early signs of mastitis, such as changes in milk appearance or udder swelling. Implementing a systematic approach to treat and isolate infected cows can prevent the spread of infection within the herd.

Vaccination and selective breeding are additional strategies. Vaccines can boost the immune response against specific pathogens, while selective breeding can favor cows with a lower incidence of mastitis, gradually improving herd resistance.

1.3 Milk microbial and its influencing factors

Over time, the prevalent causes of mastitis in dairy cows, initially dominated by Streptococcus agalactiae and Staphylococcus aureus, shifted due to improved milking hygiene. This change led to a rise in culture-negative milk samples, partly because of the reduced prevalence of the easily cultured S. agalactiae [37, 38]. Researchers initially believed that culture-negative milk from quarters with low somatic cell counts (SCC) was sterile, and lacking normal flora [39]. However, recent inquiries using culture-independent sequencing technologies have challenged this notion of sterility. These technologies uncovered a diverse array of bacterial DNA in milk from both healthy and inflamed quarters [40, 41-43]. The source of this DNA remains uncertain, with potential

origins being external contamination during sampling, bacteria or their DNA embedded in teat canal keratin, within leukocytes in milk, or bacteria present in the milk from the mammary gland [44]. Understanding the exact source is crucial as it determines whether these bacteria are linked to mammary health. Presently, there is limited research on the milk microbiota, but knowledge in this area is expected to grow as detection and analysis techniques for milk bacteria evolve. The origin of bacteria in expressed milk remains a topic of much debate. It's believed that these bacteria might be introduced externally, either during breastfeeding or from contact between feedings, or internally through a proposed enter mammary pathway, as suggested by studies on human [45] and cow milk [46]. This notion is supported by similar findings in studies of cow milk and teat skin [47]. The milk microbiota has been frequently found to include bacteria typically found in the oral cavity [48, 49]. However, despite some similarities, there are significant differences in the bacterial compositions of milk and skin [48, 50]. Interestingly, even within the same host, Enterococcus or Lactobacillus species isolated from milk are genetically distinct from those found on the host's skin [51].

The composition of milk microbiota is subject to influence not only by sampling and methodological biases but also by various host and environmental factors. It's important to note that the factors examined for humans and cattle differ, especially since cattle factors are often associated with husbandry practices [52]. Amongst these factors, several studies highlight a connection between the milk microbiota and the health of the mother, particularly focusing on infections in the mammary gland. Initially, it might seem that the mother's health straightforwardly affects the milk microbiota composition. However, research exploring the relationship between mammary gland health and milk microbiota indicates that this interaction is more intricate and not merely unidirectional.

Host Factors

In dairy farming, the interaction between a cow and its calf is greatly reduced due to their early separation. Despite this, variations in bovine milk microbiota have been observed over time, influenced by the lactation stage and the number of lactations a cow has undergone. Notably, colostrum, the first form of milk produced after giving birth, has been identified as possessing a rich and varied microbiota. This microbial richness is notably higher in cows during their first lactation (primiparous) compared to those who have had multiple lactations (multiparous) [53]. Additionally, both the taxonomic composition and alpha diversity of the milk microbiota are influenced by the lactation stage, showing noticeable changes during the initial week of lactation [54]. Furthermore, distinct differences have been observed in clinically healthy Holstein dairy cows between the microbiota present in the teat canal and the mammary secretions (either milk or colostrum) at the cessation of lactation and immediately following

calving [55].

Environmental Factors

Although there hasn't been a specific study on the direct effect of geographical location on bovine milk microbiota, research indicates that it is influenced by the conditions and practices at the farm. [56-58]. For instance, a connection between the types of bedding materials used for cows and the composition of their milk microbiota was found [58]. Additionally, pointed out that milking procedures, especially those involving preparation of the teats before milking, have an impact on the microbiota of the milk [56]. Similarly, the diet has been noted to influence the microbiota of milk in both humans and cows, demonstrating that certain bacterial communities in human milk are associated with specific fatty acid profiles, hinting at a dietary influence on milk composition [59]. Further noted a correlation between the consumption of fats, carbohydrates, and proteins by mothers and the diversity of certain bacterial groups in their milk [60]. In the case of cows, it suggested that diet might affect milk microbiota, observing that a diet high in concentrates led to a greater presence of bacteria known to cause mastitis in milk [61].

Antibiotics are known to significantly influence and potentially reduce the diversity of microbiota due to their direct effects on microbial communities. This is particularly evident in cow milk. During the dry period, which is the interval between two lactations and a time of heightened infection risk, antibiotics, and teat sealants are commonly used in herd management. These are employed both to treat subclinical infections and to prevent new infections in the upcoming lactation [62]. The impact of such Dry Cow Therapy (DCT) on the microbiota of a healthy mammary gland was a subject of investigation. [63] researched this by examining the effect of DCT using just a teat sealant or combining it with antibiotics like ceftiofur hydrochloride on cows without mastitis. Their findings indicated that excluding antibiotics from DCT didn’t alter the milk microbiota at the start of the dry period or seven days after giving birth in the next lactation. This suggests that milk bacterial communities are capable of dynamic change and can recover from disturbances caused by antimicrobial agents by the beginning of a new lactation. Similarly, when antimicrobial DCT, involving penicillin G and novobiocin within a teat sealant, was studied by [55]. Many bacterial genera, including typical mastitis pathogens, were found to persist from before DCT to after birth. This implies that the mammary microbiota is highly resilient to antimicrobial exposure during the dry period. An alternative interpretation of these observations could be that much of what is identified as milk microbiota consists of DNA from bacteria that are already non-viable and thus unaffected by antibiotic treatment.

1.4 Udder microorganisms and their functions

The microbiota within the bovine udder has garnered increasing interest, particularly

due to advancements in sequencing technologies that offer more comprehensive insights into the microbial inhabitants of intramammary tissues than traditional culture-based methods. However, obtaining accurate representations of udder microbiota is often complicated by the risk of contamination from the teat apex and cisterns during sample collection, as noted in various studies [64, 65]. This is especially problematic since the bacterial count in a healthy udder is low, making contamination a significant concern for samples taken by milking. Typically, a noticeable bacterial population is present only during an intramammary infection, leading to the earlier belief that a healthy udder is sterile, and bacteria only appear during infections [66]. However, a recent understanding of microbiota in all body organs challenges this view, indicating that even organs once thought to be sterile have their distinct microbiota [65, 67–69]. The microorganisms present in the cow's udder, a complex and dynamic ecosystem, play a critical role in both the health of the udder and the quality of milk produced. Understanding the composition and function of these microorganisms is vital for improving dairy cow health, enhancing milk quality, and preventing udder diseases, most notably mastitis. The udder microbiome comprises various bacteria, including beneficial, commensal, and potentially pathogenic species. These microorganisms originate from different sources, such as the cow's skin, the farm environment, milking equipment, and even the feed. The composition of the udder microbiome is influenced by several factors, including the cow's genetics, age, lactation stage, hygiene practices, antibiotic use, and overall farm management. Beneficial microorganisms in the udder play several crucial roles. Firstly, they help maintain a healthy udder by competing with and inhibiting the growth of pathogenic bacteria. This is achieved through the production of antimicrobial substances, competition for nutrients, and modulation of the udder environment, making it less hospitable for harmful pathogens. These beneficial microbes also contribute to the development and function of the cow's immune system, enhancing its ability to ward off infections.

On the other hand, pathogenic microorganisms are the primary cause of mastitis, one of the most significant health issues in dairy farming. Mastitis pathogens, such as Staphylococcus aureus, Streptococcus agalactiae, and Escherichia coli, can invade the udder tissue, leading to inflammation, decreased milk production, and altered milk composition. The presence of these pathogens not only affects animal welfare and farm economics but also poses risks to milk quality and safety for human consumption.

Managing the udder microbiome involves several strategies aimed at promoting the growth of beneficial microbes while inhibiting pathogenic ones. Good hygiene practices during milking are paramount. This includes proper cleaning and disinfection of the udder and teats, using sanitized milking equipment, and ensuring that the milking environment is clean. Moreover, maintaining overall cow health through proper

nutrition, stress reduction, and regular veterinary care is essential in supporting a healthy udder microbiome. The role of antibiotics in managing the udder microbiome is a double-edged sword. While necessary for treating infections, overuse or misuse of antibiotics can disrupt the natural microbial balance, leading to the emergence of antibiotic-resistant bacteria. Therefore, the judicious use of antibiotics, coupled with alternative strategies like vaccination, selective breeding for disease resistance, and the use of probiotics, is crucial. Emerging research in udder macrobiotics is revealing the intricate interactions between different microbial species and their impact on udder health and milk production. For instance, certain microbial profiles are being linked to higher or lower risks of mastitis, suggesting potential for predictive diagnostics and targeted interventions.

The composition of the udder's commensal microbiota, which varies due to factors like housing, management practices, and bedding material, complicates the comparison of different studies [70, 71]. The bovine milk microbiota is diverse, usually comprising species from phyla like Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes. Common genera include Staphylococcus, Streptococcus, Ruminococcaceae, Lachnospiraceae, Propionibacterium, Stenotrophomonas, Corynebacterium, Pseudomonas, Fusobacterium, Lactobacillus, Enterococcus, Comamonas, and Bacteroides [64]. The commensal species found on the external teat skin, in the teat canal, and in the udder lumen may play a crucial role in protecting against pathogens, like commensal species at other body sites [64, 72, 73]. Research indicates that certain non-aureus Staphylococci and Corynebacterium species produce bacteriocins that inhibit the growth of potential pathogens, suggesting their role in defending the bovine udder from mastitis [74, 75].

1.5 Automatic milking system and farm management

Milking is a laborious and time-intensive task, particularly on smaller dairy farms where it is often carried out by the farm family. Employing outside labor for this chore is challenging due to better-paying job opportunities elsewhere with more benefits. Automatic Milking Systems (AMS) provide a solution to alleviate this demanding task from farmers [76]. This technology, while poised for further advancements, is currently well-established for commercial farm use. In Europe, over a thousand farms have adopted automatic milking, and it is also in use on several Canadian farms [77]. In the United States, it is still experimental on a few farms, pending official regulatory approval. There are two primary benefits of AMS. The first is the significant reduction of labor needed for milking. By replacing hired labor, farms can save up to $200 per cow annually [78]. When the system replaces the labor of the farm owner, the savings are less quantifiable but potentially more significant, freeing up time for managing the farm, family time, and leisure. The second benefit is the potential increase in milk

production. AMS typically allows for cows to be milked three times daily (3×), leading to an increase in milk production of 3 to 11% compared to the traditional twice-a-day (2×) milking routine [77, 79].

There are several drawbacks to Automatic Milking Systems (AMS). One of the main disadvantages is the significant initial financial outlay required. The cost of the equipment for a particular herd size can be two to three times higher than that of a conventional milking parlor. Milk quality is another area of concern [80]. Increased milking frequency can lead to a slight reduction in milk fat content compared to the standard twice-a-day milking process [80, 81]. Additionally, to support higher milk production, cows need more feed, which in turn increases feed expenses. There's also a risk of higher bacterial counts in the milk, as more frequent milking provides more opportunities for microbes to enter and proliferate during milking. However, these bacterial levels can be brought down to those comparable with traditional parlor systems through better animal management, improved udder washing techniques, and efficient milk pre-cooling practices [79, 82].

Automatic Milking Systems (AMS) seem particularly suitable for the smaller dairy farms prevalent in the northeastern and upper Midwestern states [76]. These farms are often growing in response to the pressures of low milk prices and diminishing profits. Such expansion necessitates either an increase in labor or more efficient utilization of existing labor. Furthermore, there's a need to upgrade or enlarge aging facilities.

In recent times, with the growth of dairy cattle herds and the increasing need for competitiveness, dairy farming practices have evolved significantly [83]. There is now a greater focus on disease prevention and production optimization. The use of advanced technologies to gather herd data plays a crucial role in guiding management decisions [84, 85]. Consequently, Herd Health and Production Management (HHPM) has become a critical component in many dairy farms, as indicated in studies like those by [86]. [87, 88] describes herd health management as a process aimed at enhancing health, welfare, and production of dairy cows by systematically analyzing relevant data and consistently making objective observations of the cows and their environment. This approach enables informed, ongoing adjustments and improvements in herd management.

Typically, the herd veterinarian serves as the primary advisor for delivering HHPM services. However, the role of veterinary care is evolving from primarily treating ill animals to a more proactive stance focused on disease prevention [89]. Nowadays, farmers expect veterinarians to provide guidance on broader issues, including nutrition and animal welfare [90]. In Canada, most dairy farms have HHPM farm visits scheduled one or two times per month, carried out by either clinic owners or employed veterinarians [91]. [92] noted that 22% of veterinarians serving Western Canadian dairy farms devoted more than 75% of their workload to dairy cattle, identifying as “dairy

practitioners." These practitioners reportedly spend about 85% of their time on dairy farms, engaging in activities related to both individual animal care (like abdominal examinations) and overall herd health (such as estrus synchronization). Furthermore, among veterinarians in mixed practices, 53% and 43% were reported to perform pregnancy palpations and breeding soundness exams at least monthly, respectively [92].

1.6 Summary of previous research

Our topic focuses on understanding the complex interactions between the milk, udder skin, feces, and bedding microbiota in dairy cows and how these relate to mastitis, milk composition, and cow health. Based on the detailed information you provided, your study examines various aspects, including the changes in microbiota over time, the influence of environmental factors, and the potential correlations between microbiota composition and various health indicators like blood metabolites and milk composition. Previous research has explored the following points.

The Composition of Microbiota in Different Cow Environments:

Researching the types of bacteria present in the milk, on the udder skin, in feces, and in the bedding, and how these populations change over time or with different farming practices.

Correlations Between Microbiota and Cow Health:

Investigating how changes in the microbiome might relate to the overall health of the cows, including the incidence of diseases like mastitis. This might involve examining how different microbial populations affect the cow's immune response or susceptibility to infections.

Impact of Microbiota on Milk Quality and Composition:

Exploring how the presence of certain microorganisms in the milk or on the udder skin can influence milk composition, including factors like protein, fat, and somatic cell count (SCC). This aspect would be particularly relevant for understanding the implications for milk quality and safety for human consumption.

Environmental and Nutritional Influences:

Assessing how external factors, like the quality of bedding and feed, as well as the cow's nutritional status, impact the microbiota composition in different parts of the cow's environment and body.

Temporal and Spatial Variability:

Looking at how the microbiota varies not just over time but also across different parts of the cow's body and environment could reveal insights into how these microorganisms are transmitted and interact with each other.

Technological and Methodological Approaches: Utilizing advanced techniques such as next-generation sequencing to analyze the microbiota and employing statistical

methods like principal component analysis (PCA) and network analysis to understand the complex relationships between different variables.

1.7 Innovation points of this study

This research presents several innovative points in the study of dairy cow microbiota, focusing on the interrelationships between different microbiomes (milk, udder skin, feces, and bedding) and their impact on dairy cow health, particularly relating to mastitis and milk composition. A key innovation lies in the comprehensive approach of analyzing multiple microbiota types within the same study, providing a holistic view of the microbial environment in a dairy setting.

Firstly, the research breaks new ground by examining the temporal changes in the microbiota over a two-year period. This longitudinal aspect is significant as it offers insights into how microbiota compositions shift over time, an area that has not been extensively explored in previous studies. The findings that milk and udder skin microbiota vary considerably over time, while feces and bedding microbiota remain relatively stable, are particularly revealing. These results suggest that certain microbiota is more susceptible to environmental changes or farm management practices than others.

Another innovative aspect is the study's focus on the relationship between the cowshed environment and the milk microbiota. The research delves into how udder skin, which contacts the barn and bedding, may play a role in defending against pathogens and contaminants. This area of study is relatively unexplored and could lead to new understandings of how environmental factors influence milk safety and quality.

The research also provides new insights into the complex interactions between microbiota and cow health. Through network analysis, the study examines the relationships between various bacterial taxa and key health indicators like blood metabolites and milk composition. The finding that certain microbiota is related to these health indicators, while others are not, challenges previous assumptions and opens new questions about the role of microbiota in dairy cow health.

Additionally, the study addresses the important issue of mastitis, a major concern in dairy farming. By analyzing microbiota in cows without clinical symptoms of mastitis but with varying somatic cell counts, the research offers new perspectives on the subclinical stages of this disease. This could have significant implications for early diagnosis and prevention strategies.

Overall, this research stands out for its comprehensive and integrative approach, combining microbiological analysis with health and environmental data. The findings provide valuable contributions to our understanding of the dairy cow microbiome and its relationship to animal health and milk production. This could potentially lead to improved farm management practices, better prevention strategies for mastitis, and enhanced milk quality and safety.

Chapter 2 Research Methods

2.1 Experimental design

The sampling process for this study, conducted in two distinct phases – early September 2018 and late August 2020 – was meticulously designed to provide comprehensive insights into the factors contributing to mastitis in dairy cows. The subjects of this study were 10 Holstein cows, managed under an automated milking system, ensuring consistent and controlled milking conditions. These cows were housed in a free-stall barn at the Okayama Prefecture Livestock Research Institute, offering them the freedom to move and rest, thereby replicating a natural living environment. Sampling in both 2018 and 2020 was strategically carried out between 10:00–12:00, a time chosen to standardize the conditions under which samples were collected, minimizing variability due to external factors. The cows were fed a well-formulated diet, ensuring their nutritional needs were met, which is crucial as nutrition can influence both the incidence and severity of mastitis.

2.1.1 Sample collection for Experiment 1 (2018)

With the cooperation of the Okayama Prefecture Livestock Research Institute, we sampled 10 Holsteins on September 6, 2018 (Table1). Each cow was milked daily using an automatic milking machine (Astronaut A4, LELY, made in the Netherlands). The cows are fed fermented feed (fermented TMR, which is a mixture of concentrated feed and roughage), and the cows are housed in a free stall structure that allows them to freely roam around the barn. In addition, the milk yield, milk composition, parity, somatic cell count, etc. of these 10 cows are recorded at the end of every month in the cattle county inspection. Milk, feces, udder skin bacteria, and blood were collected from all 10 Holsteins. In addition, bacteria were collected from five locations on the cow bedding. Each collection method is shown below.

After sterilization with alcohol cotton, approximately 15 ml of milk was collected by hand. After wearing gloves for direct examination of feces, rectal feces were collected artificially. The skin surface was collected from five locations around the nipple: the front, back, right side, left side, and near the center of the breast, using a sterile cotton swab (swab) at least 5 times each. After sterilization with alcohol cotton, blood was collected from the median caudal vein with a 21G blood collection needle using a vacuum blood collection tube containing an anticoagulant (heparin sodium) and a vacuum blood collection tube without an anticoagulant. Cow bedding was randomly sampled from five locations in the free stall barn.

Table1. Sampling summary for 2018&2020.

Sampling date	Maximum temperature	Minimum temperature	Label			
			milk	udder skin	feces	bedding
2018/9/6	24℃	17℃	M1-M10	S1-S10	F1-F10	B1-B2

2.1.2 Sample collection for Experiment 2 (2020)

The sampling procedure encompassed collecting blood, milk, udder skin, and feces samples(Table 2) – each playing a unique role in understanding the etiology of mastitis. Blood samples were essential for assessing the systemic health and immune response of the cows. Milk samples, obtained after careful cleaning of the teat apex and barrel and discarding the foremilk, were critical for analyzing milk composition changes due to mastitis. These samples were taken from all four udders to form a composite sample, providing a holistic view of the mammary health of each cow. Udder skin samples, collected using sterile cotton swabs, were instrumental in identifying skin-borne pathogens that could contribute to udder infections. Feces samples, taken directly from the rectum, offered insights into the gut microbiota and its potential role in the overall health of the cows, including their susceptibility to mastitis. Additionally, bedding samples were gathered from three different locations within the cowshed to account for environmental variables. In the free-stall barn setting, where cows have the freedom to choose their resting places, pinpointing specific resting areas was challenging. Therefore, a composite sample of the bedding was prepared by mixing samples from these locations, providing a representative overview of the environmental conditions the cows were exposed to. All sampling procedures and protocols were conducted in strict adherence to ethical guidelines, with approval from the Animal Care and Use Committee, Okayama University (OKU-2020856), Japan. This careful and systematic approach to sampling in 2020 was crucial for ensuring the reliability and validity of the study, providing a robust foundation for the subsequent analysis, and understanding of mastitis in dairy cows.

Table2. Sampling summary for 2020.

Sampling date	Maximum temperature	Minimum temperature	Label			
			milk	udder skin	feces	bedding
2020/8/13	33℃	25℃	M11-M20	S11-S20	F11-F19	B3

2.2 Data analysis methods

2.2.1 Analysis of milk microbial community

The microbiological testing methods employed in this study were designed to meticulously analyze the bacterial communities present in the milk, feces, udder skin, and bedding samples from the dairy cows. The process began with the extraction and purification of bacterial DNA, a crucial step in identifying the specific bacteria associated with mastitis. This was achieved using the protocol described by Gathinji et al., which involved centrifuging 0.5 mL of milk at 16,000 × g for 15 minutes at 4 °C to separate the fat and supernatant from the DNA pellets. The pellets were then washed and resuspended in phosphate-buffered saline (PBS) before being homogenized with zirconia beads in a lysis buffer (Table 3), ensuring a thorough breakdown of cell walls for optimal DNA release. The DNA extraction was completed using a DNeasy Stool Mini Kit, known for its efficacy in purifying DNA from challenging sample types. The next critical step was the 16S rRNA gene amplicon sequencing, a powerful technique for microbial community analysis. This process involved two rounds of PCR using primers specifically targeting the V4 region of the 16S rRNA genes – a highly variable region that allows for the differentiation of bacterial species. The first-round PCR protocol included an initial denaturation at 94°C for two minutes, followed by 35 cycles of temperature variations for denaturation, annealing, and elongation, ensuring the amplification of the target DNA region. The resulting PCR products were then run on a 1% agarose gel, allowing for the visualization and subsequent purification using a gel extraction kit. The second round of PCR, critical for preparing the DNA for sequencing, utilized adapter-attached primers. This step was crucial for adding necessary sequences to the amplicons, facilitating their proper alignment and sequencing in the MiSeq system. The same temperature protocol was followed as in the first round, but with a reduced number of cycles to prevent over-amplification. Following the PCR, the purified amplicons were subjected to MiSeq sequencing at FASMAC Co., Ltd., a process that allows for high-throughput, accurate sequencing of the bacterial DNA. This sequencing provided an in-depth view of the bacterial composition in the samples, enabling the identification of potential mastitis-causing pathogens and their relative abundances.

Analyzing microbiota using QIIME 2 begins with importing the MiSeq sequenced bacterial DNA data, typically in FASTQ format, into the QIIME 2 environment. This is followed by quality control to assess the sequence quality, typically visualized through summary plots. Next, the sequences are filtered and denoised, with methods such as DADA2 for paired-end reads or Deblur for single-end reads, to remove noise and chimeras, yielding high-quality representative sequences. These sequences are then used to create a feature table and a phylogenetic tree, essential for diversity analysis.

Subsequently, taxonomic analysis is performed, often using a classifier like the Naive Bayes classifier against a pre-trained reference database like SILVA or Greengenes, to assign taxonomy to each feature. The core of the analysis involves alpha and beta diversity analysis, examining within-sample diversity and between-sample diversity, respectively, using various metrics and visualizations. This is complemented by differential abundance testing to identify significantly varying features across sample categories. Lastly, the results are interpreted in a biological context, which may involve additional statistical analyses and integration with metadata, providing insights into the microbial community structure and function.

Table3 . The composition of lysis buffer.

Lysis buffer	Stock solution	Working solution	Configure the buffer according to the number of samples, each sample requires 1ml.
	2.5M Nacl	500mM Nacl	
	0.5M Tris-Hcl	50mM Tris-Hcl	
	0.5M EDTA	50 mM EDTA	
	10% SDS	4% SDS	

2.2.2 Analysis of udder skin microbial community

The swab samples, which were collected from five different locations, were combined into a single container that was filled with 5 milliliters of sterile Phosphate-Buffered Saline (PBS) at a pH of 7.3. These samples were then thoroughly mixed for three minutes using a vortex mixer. Following this, 3 milliliters of the mixture were evenly distributed into two separate 1.5-milliliter tubes. Each of these tubes was then subjected to centrifugation at a speed of 15,000 revolutions per minute (rpm) for two minutes, after which the supernatant was carefully removed from both tubes. The remaining material in each tube was then refilled with 500 microliters of PBS, and the contents of the two tubes were combined into one. Subsequently, 0.5 milliliters of this combined sample were dispensed into tubes containing beads for further processing. The bead beating method in DNA extraction is a mechanical technique used to lyse cells and release DNA, particularly effective for tough-to-lyse samples like environmental samples, stool, or tissues. This method involves the use of tiny beads and a high-speed agitation system to physically break down the cell walls. In a typical process, the sample is mixed with beads and a lysis buffer in a tube. The size and material of the beads can vary depending on the sample type, with smaller beads often used for bacterial cells and larger, harder beads for plant or tissue samples. The tube is then vigorously shaken in a bead beater or vortex, allowing the beads to collide with the cells at high speed, resulting in mechanical disruption of the cell walls. This forceful agitation ensures thorough breakdown of the cells, releasing the DNA into the solution.

After bead beating, the mixture is usually centrifuged to separate the lysed cell debris, beads, and other insoluble from the supernatant, which contains the extracted DNA. The DNA is then further purified and concentrated through various downstream processes, such as ethanol precipitation or spin column purification.

After the extraction of bacterial DNA, a two-step Polymerase Chain Reaction (PCR) process is typically employed to prepare the samples for sequencing on the MiSeq platform. The first step of this PCR is aimed at amplifying the target regions of the bacterial DNA, often focusing on specific variable regions of the 16S rRNA gene, which are highly informative for bacterial identification and phylogenetic analysis. In this initial amplification, specific primers are used to ensure the selective enhancement of these regions. Following the initial PCR, a second PCR step is conducted, which is crucial for adding index sequences and adaptors to the amplified DNA fragments. These adaptors and indices are necessary for the MiSeq sequencing process, as they facilitate the proper binding of the DNA fragments to the flow cell and enable multiplexing, which allows for the sequencing of multiple samples in a single MiSeq run. This two-step PCR approach is essential for preparing the DNA in a way that is compatible with the MiSeq system, ensuring efficient and accurate high-throughput sequencing of the bacterial DNA.

After obtaining the bacterial sequence data through MiSeq, the analysis of the bacterial community structure is conducted using QIIME2, a sophisticated bioinformatics tool designed for microbial community analysis. This process begins with importing the MiSeq-generated sequence data into QIIME 2, followed by rigorous quality control checks to ensure data integrity. Subsequent steps include sequence filtering, denoising, and chimera checking to refine the dataset, often using tools like DADA2 or Deblur within QIIME2. This results in a feature table representing the abundance of each unique sequence variant across the samples. Taxonomic classification is then performed using a pre-trained classifier against a comprehensive reference database, such as SILVA or Greengenes, to assign taxonomy to these sequence variants. The core of the analysis involves evaluating the diversity within and between microbial communities, known as alpha and beta diversity analysis, using various metrics and visualizations. These analyses provide a detailed view of the bacterial community structure, including the abundance, diversity, and relational dynamics of different bacterial taxa within the samples.

2.2.3 Feces microbial community analysis

Sample Preparation:

A 0.2g feces sample is carefully weighed and placed into a bead-beating tube. This tube contains small beads that facilitate the mechanical lysis of microbial cells when agitated.

DNA Extraction:

The bead-beating method is employed for DNA extraction. The stool sample in the bead tube is subjected to high-speed agitation, which causes the beads to physically disrupt the cell walls of the microbes, thereby releasing the DNA. The mixture then undergoes centrifugation to separate the extracted DNA from other cellular debris.

PCR Amplification:

The extracted DNA is then amplified using a two-step Polymerase Chain Reaction (PCR) process:

First PCR: Targets specific regions of the microbial DNA, often the 16S rRNA gene for bacterial identification. This step uses primers that anneal to common regions flanking the highly variable regions of the 16S rRNA gene, facilitating the amplification of these regions from a diverse range of bacteria.

Second PCR: Adds sequencing adapters and unique barcodes to the amplified products. This step is crucial for sequencing, as the adapters allow the DNA to bind to the sequencing platform, and the barcodes enable the identification of sequences corresponding to each sample.

Sequencing:

The barcoded DNA fragments are sequenced using a high-throughput sequencing platform, such as MiSeq. This step generates a large volume of data, consisting of short DNA sequences from the amplified regions.

Data Analysis:

Importing Data into QIIME 2: The sequencing data is imported into QIIME 2, a comprehensive microbial analysis tool.

Quality Control and Filtering: The raw sequencing data undergoes quality checks and filtering to remove low-quality or ambiguous reads.

OTU Picking or ASV Definition: Sequences are clustered into Operational Taxonomic Units (OTUs) or defined as Amplicon Sequence Variants (ASVs) based on similarity, which helps in the identification and comparison of microbial taxa.

Taxonomic Classification: The OTUs/ASVs are then classified taxonomically using reference databases like SILVA or Greengenes.

Diversity Analysis: The software conducts alpha and beta diversity analyses to explore the complexity and differences in microbial communities across samples.

Statistical Analysis: Additional statistical methods may be applied to identify significant trends or associations in the data.

2.2.4 Analysis of microbial communities in bedding

Sample Preparation:

A 0.2g sample is carefully collected from bedding material.

DNA Extraction Using Bead Beating:

The bedding sample is placed in a bead beating tube, which contains small beads. The tube is then subjected to vigorous shaking in a bead-beating machine. This mechanical process effectively breaks down the cell walls of the microorganisms present in the bedding, releasing their DNA into the solution. Post bead-beating, the sample is centrifuged to separate the DNA from other particulates.

PCR Amplification:

The extracted DNA undergoes a two-step PCR amplification process.

First PCR: This step targets specific regions of the microbial DNA that are commonly used for microbial identification, like the 16S rRNA gene for bacteria. Primers specific to these regions are used to amplify the DNA of interest.

Second PCR: In this round, sequencing adaptors and barcodes are added to the PCR products. These adaptors are necessary for sequencing, and the barcodes allow for the identification of sequences from each specific sample in the sequencing process.

Sequencing:

The PCR-amplified DNA is sequenced using a high-throughput sequencing platform, typically Illumina MiSeq. This technology generates a large volume of sequence data from the amplified DNA fragments.

Data Analysis:

Importing and Quality Control: The sequencing data is imported into a bioinformatics tool like QIIME 2. The data first undergoes quality control to remove low-quality or ambiguous sequences.

OTU/ASV Picking: The high-quality sequences are then clustered into Operational Taxonomic Units (OTUs) or Amplicon Sequence Variants (ASVs), representing groups of similar sequences.

Taxonomic Classification: These OTUs or ASVs are classified at different taxonomic levels using databases such as SILVA or Greengenes, which helps identify the types of microorganisms present in the bedding.

Diversity Analysis:

Both alpha (within-sample) and beta (between-sample) diversity analyses are conducted to understand the complexity and comparative differences of the microbial communities.

Statistical Analysis: Additional statistical methods may be applied to draw significant conclusions from the data.

2.2.5 Blood metabolite analysis

providing important insights into an individual's metabolic and physiological status. several key metabolites and enzymes were analyzed using plasma samples with the aid of specialized kits. BUN (Blood Urea Nitrogen) reflects protein metabolism and kidney function. Albumin, a major plasma protein, is indicative of nutritional status and liver

function. NEFA (Non-Esterified Fatty Acids) are crucial for understanding lipid metabolism and energy balance. HP (Haptoglobin) is a marker of inflammation and can indicate hemolysis. GPT (Glutamic-Pyruvic Transaminase), also known as ALT (Alanine Aminotransferase), and GOT (Glutamic-Oxaloacetic Transaminase), also known as AST (Aspartate Aminotransferase), are enzymes that are key indicators of liver health. Each of these components is measured quantitatively using specific assay kits, which provide reliable and standardized results. This comprehensive analysis is essential for diagnosing, monitoring, and managing various health conditions, including liver diseases, kidney dysfunction, nutritional deficiencies, and metabolic disorders.

Kits used for measuring blood metabolites like BUN, NEFA, GOT, GPT, and Albumin are specialized tools designed for accurate and efficient biochemical analysis. These kits typically include reagents, standards, and protocols specific to the metabolite being tested. For instance:

BUN (Blood Urea Nitrogen) Kit: This kit measures the amount of nitrogen in the blood that comes from urea, a waste product of protein metabolism. It usually involves an enzymatic or colorimetric assay, where the urea is converted to a measurable product.

NEFA (Non-Esterified Fatty Acids) Kit: This kit quantifies the level of free fatty acids in the blood. It often uses an enzymatic method where NEFAs are converted into compounds that can be measured spectrophotometrically.

GOT (AST) and GPT (ALT) Kits: These kits measure the activities of the enzymes Glutamic-Oxaloacetic Transaminase (GOT, also known as AST) and Glutamic-Pyruvic Transaminase (GPT, also known as ALT). They typically work through enzymatic assays where the conversion of specific substrates is measured, indicating enzyme activity.

Albumin Kit: This kit determines the concentration of albumin in blood plasma, usually through colorimetric assays or immunoassays. The methods might involve binding the albumin to a dye and measuring the color change or using antibodies specific to albumin.

Each kit comes with a detailed manual outlining the procedure, which generally includes sample preparation, reagent addition, incubation, and then measurement using a spectrophotometer or similar instrument. The results are compared to standards provided within the kit to quantify the levels of each metabolite or enzyme activity in the blood sample. These kits are designed for ease of use, accuracy, and reproducibility, making them essential tools in clinical diagnostics and research. Please see Table 4 for details of blood metabolite.

Table4. Blood Metabolites Summary (2018&2020).

2018		2020		NEFA (μ Eq/L)		T-Cho (mg/dL)		Albumin (g/dL)		BUN (mg/dL)		GPT (IU/L)		GOT (IU/L)		Hp (mg/dl)	
COW. No	SCC (10^3/mL)	COW. No	SCC (10^3/mL)	2018	2020	2018	2020	2018	2020	2018	2020	2018	2020	2018	2020	2018	2020
M1	36	M11	19	435	123	126	238	4.9	3.9	15.2	9.9	3.8	5.2	18.8	38.3	352	0.32
M2	191	M12	95	248	100	98	229	4.3	4.2	15.8	9.5	1.4	10.6	15.9	61.6	144	0.38
M3	419	M13	63	217	60	117	236	4.5	4.1	12.8	7.5	2.7	6.5	14.1	59.7	115	0.31
M4	371	M14	21	199	87	137	185	4.1	4.4	11.3	10.9	2.5	5.7	25.6	28.1	210	0.34
M5	13	M15	78	304	89	144	251	4.0	4.0	10.6	11.6	4.2	7.8	37.9	38.6	46	0.84
M6	25	M16	27	248	51	139	224	4.0	3.9	11.7	6.6	2.4	8.7	15.1	52.7	128	0.32
M7	71	M17	15	320	157	152	172	4.0	3.9	11.5	5.4	3.5	4.7	21.7	23.3	99	0.32
M8	15	M18	10	281	130	154	252	4.0	4.2	16.4	7.9	3.4	6.3	15.1	39.4	89	0.33
M9	17	M19	13	241	161	162	147	4.2	3.7	11.1	5.4	2.5	4.5	21.7	25.6	98	0.42
M10	31	M20	15	504	105	73	204	4.2	4.1	11.6	8.4	3.0	5.4	17.6	28.4	980	0.58

This table provides an extensive overview of the metabolic health of a dairy herd through the analysis of various blood metabolites. Each row corresponds to an individual cow, and the data includes key indicators of energy metabolism (NEFA), liver function (GPT, GOT), kidney function (BUN), nutritional status (Albumin), and overall health (Hp, T-Cho). Such a comprehensive metabolic profile is crucial for effective herd management, allowing for early detection of health issues, optimization of feeding strategies, and overall improvement in milk production and quality. This detailed assessment aids in ensuring the well-being of the cows and the economic efficiency of dairy operations.

2.2.6 Milk composition analysis

Milk composition analysis, conducted monthly on milk obtained directly from pasture-fed livestock, provides crucial insights into the quality and nutritional value of the milk. This comprehensive evaluation includes several key parameters. Firstly, the lactation period of dairy animals is considered, as it significantly influences milk composition; milk nutrient content typically varies at different stages of lactation. Protein content, a vital component for assessing milk's nutritional profile, is measured to ensure it meets the required standards for dietary proteins. Fat content is another critical component affecting the milk's energy value, taste, and processing qualities. Somatic cell count (SCC) is also analyzed as an indicator of milk quality and udder health; higher SCC often signifies mastitis or other infections in the herd. Milk Urea Nitrogen (MUN) levels provide insights into the nitrogen efficiency in the animal's diet and its metabolic status. Lastly, Solids-Non-Fat (SNF) content, encompassing all solids other than fat, like sugars, proteins, and minerals, is measured to assess the milk's overall quality and suitability for various dairy products. Together, these parameters form a comprehensive profile of the milk's composition, reflecting the health and diet of the livestock, as well as the quality of the milk produced. Please see Table 5 for details of milk nutrients.

Table5. Milk Nutritional Content Summary (2018&2020).

cow No.		SCC（10^3/mL）		milk Yield （kg/day）		fat(%)		protein(%)		not-fat milk solid(%)		MUN(mg/dl)	
2018	2020	2018	2020	2018	2020	2018	2020	2018	2020	2018	2020	2018	2020
M1	M11	36	19	38.1	36.3	2.9	4.1	2.9	3.4	8.1	9.2	15	11.6
M2	M12	191	95	24.2	45.9	3.8	3.25	2.8	3.0	8.4	8.7	15.25	14.1
M3	M13	419	63	29.3	37.3	3.5	3.68	3.4	3.2	8.8	9.1	7.35	15.5
M4	M14	370.5	21	36.3	30.5	2.7	4.55	2.9	3.4	8.5	9.2	13.65	14.8
M5	M15	12.5	78	43.4	26.8	3.0	4.1	3.1	3.1	8.9	8.6	13.95	11
M6	M16	25	27	35.7	21.2	3.6	4.82	2.7	3.2	8.3	8.8	16.55	9.4
M7	M17	71	15	34.6	39.6	3.3	3.99	3.2	3.2	9.1	9.2	13.3	12.1
M8	M18	14.5	10	32.3	47.3	2.9	3.44	2.8	3.4	8.5	9.3	15.6	14.1
M9	M19	16.5	13	33.4	39.2	3.8	4.12	3.1	3.1	8.9	8.9	61.05	13.1
M10	M20	31	15	26.9	33.2	3.2	4.28	3.1	3.3	8.7	9.2	12.1	14

This table provides a detailed analysis of the milk's composition from different cows over a period in 2018. Each row represents data from an individual cow. The parameters measured include somatic cell count, milk yield, and the percentages of key nutritional components like fat, protein, and non-fat solids. This information is critical for assessing the quality of milk, the health of the dairy cows, and the efficiency of their diet and metabolism. The data can be used to make informed decisions about dairy herd management, milk processing, and ensuring the overall quality of dairy products.

2.2.7 Analysis of total bacterial count

Quantitative PCR (qPCR), also known as real-time PCR, is a powerful molecular technique used for the quantification of DNA in a sample. In the context of determining the total number of bacteria in samples such as milk, feces, udder skin, and cow bedding, qPCR serves two main purposes:

Quantification: It allows for the precise quantification of the bacterial load in a sample, providing insights into the microbial population's size and density.

Sensitivity and Specificity: qPCR is highly sensitive and can detect very low levels of bacterial DNA, making it suitable for samples where bacteria might be present in small quantities. It also offers high specificity, meaning it can target and quantify specific bacterial groups or species if designed with appropriate primers.

The process of qPCR for total bacterial count involves several key steps:

Sample Preparation: Samples from milk, feces, udder skin, and cow bedding. These samples are then processed to extract DNA. This involves breaking down the cell walls and membranes to release DNA, which is then purified to remove proteins, lipids, and other cellular components.

Primer Design: Primers specific to bacterial DNA are designed. For total bacterial count, primers are often targeted towards conserved regions of the bacterial genome, such as the 16S rRNA gene, which is present in all bacteria.

Setting Up the qPCR Reaction: The qPCR reaction mix includes the extracted DNA, the designed primers, a DNA polymerase enzyme, nucleotides, and a buffer. Additionally, a fluorescent dye or probe is included, which binds to the double-stranded DNA or emits fluorescence upon excitation when incorporated into the DNA.

Thermocycling and Amplification: The qPCR machine performs thermocycling, which involves repeated cycles of heating and cooling to:

Denature the DNA (separate the strands).

Anneal the primers to the target sequences.

Extend the primers to synthesize new DNA strands.

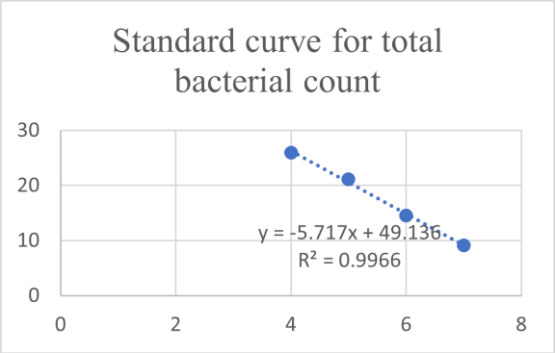
Fluorescence Detection: During the amplification, the fluorescent dye or probe intercalates with the double-stranded DNA or releases fluorescence upon probe degradation. The qPCR machine measures the fluorescence intensity after each cycle, which increases as more DNA is amplified.

Data Analysis and Interpretation: The machine generates an amplification curve, and the point at which the fluorescence surpasses a certain threshold (Ct value) is used to quantify the amount of DNA. The Ct value is inversely proportional to the amount of target DNA in the sample. A standard curve, created using known concentrations of bacterial DNA, allows for the quantification of the bacterial load in the samples. The results are shown in Table 6.

Table6. Summary of Total Bacterial Count in Various Samples (2018&2020).

	log10(x)			log10(x)	
	Quantity (copies) Per 1g feces			Quantity (copies) Per 1 mL milk	
Feces	F3	10	Milk	M3	4
	F4	8		M4	4
	F5	10		M5	4
	F6	8		M6	7
	F7	8		M7	4
	F8	10		M8	7
	F9	8		M11	3
	F11	11		M12	3
	F12	8		M13	3
	F13	8		M14	3
	F14	8		M15	6
	F15	11		M16	3
	F16	11		M17	3
	F17	8		M18	6
	F18	8		M19	3
	F19	8		M20	3

Bedding	log10(x)	
	Quantity (copies) Per 1g bedding	
	B1	8
	B2	8
	B3	8



The table is divided into three main sections, each representing a different sample type: Feces, Milk, and Udder Skin. Within each section, the bacterial count is reported in log10 values. Using a logarithmic scale is common in microbiology to manage the wide range of bacterial counts, as it simplifies data interpretation and comparison.

2.2.8 mRNA analysis

The analysis of mRNA, particularly for measuring the gene expression of inflammatory factors like IL-8, TNF-α, and others, is a critical process in molecular biology. However, the success of this analysis heavily depends on the quality of the mRNA extracted from the samples. Here's an explanation of the process and the factors that can affect its quality:

Sample Preservation: The first step involves collecting milk samples. It's crucial to handle these samples carefully to prevent RNA degradation. Since RNA is highly susceptible to degradation by RNases (enzymes that degrade RNA), the samples must be kept at low temperatures or treated with RNA stabilization solutions immediately after collection.

RNA Extraction: This step involves separating RNA from other cellular components. The process typically includes cell lysis, separation of RNA from DNA and proteins, and purification. Various kits and protocols are available for RNA extraction, each designed to maximize RNA yield and purity while minimizing contamination and degradation.

Quality Check of RNA: Before proceeding with gene expression analysis, it's vital to assess the quality and quantity of the extracted RNA. This is usually done using spectrophotometry or more sophisticated methods like agarose gel electrophoresis and capillary electrophoresis (e.g., using a Bioanalyzer). High-quality RNA should have intact bands (for 18S and 28S rRNA in eukaryotes) and a high purity ratio (A260/A280 ratio close to 2.0).

cDNA Synthesis: mRNA is transcribed into complementary DNA (cDNA) using reverse transcriptase. The quality of mRNA directly affects the efficiency and fidelity of this step. Poor quality mRNA may lead to incomplete or biased cDNA synthesis, affecting downstream results.

Quantitative PCR (qPCR) or Other Gene Expression Analysis: The cDNA is then used as a template for qPCR to quantify the expression of target genes like IL-8, TNF-α, etc. The amplification data from qPCR provides insights into the gene expression levels in the original milk samples.

If issues with gene expression analysis in milk are encountered, it might be due to compromised mRNA quality. This can occur due to several factors:

Improper Sample Handling: If the milk samples were not promptly processed or stored at incorrect temperatures, RNA degradation could occur.

Inefficient RNA Extraction: The extraction method might not be fully effective in isolating intact RNA from milk, which contains fat and other components that can interfere with the process.

Contamination: The presence of RNases or other contaminants in reagents or during

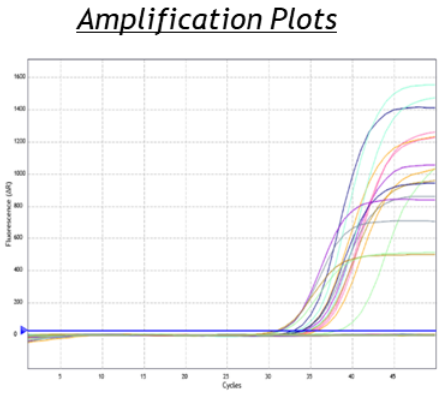
the handling process can degrade RNA.

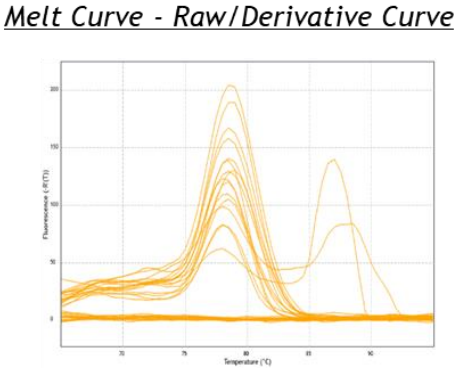
To address these issues, might consider optimizing the sample collection and storage methods, using more effective RNA extraction protocols or kits designed specifically for challenging samples like milk, and ensuring a contamination-free environment during RNA handling and processing. Additionally, it might be beneficial to include a step for RNA integrity assessment before proceeding to cDNA synthesis and qPCR.

For RT-PCR, the RNA concentration was 500 ng, and the PCR product (cDNA) was diluted 20 times and 1 μL was used for qPCR. The qPCR reaction solution was carried out in 10 μL, and the final concentration of primers was 0.5 μM. See Table 7 for specific procedures.

Table7. Quantitative PCR Gene Expression Analysis Summary.

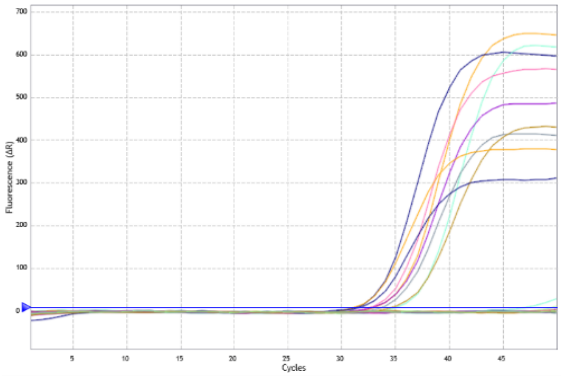
gene	No.	Cq (ΔR)	Tm 1	gene	Well	Cq (ΔR)	Tm 1
β-actin	2	34.26	87	TNF-α	2	No Cq	95
	2	33.24	78.5		2	No Cq	95
	10	33.8	78.5		10	No Cq	65
	10	35.47	78.5		10	No Cq	68.5
	18	34.68	78.5		18	No Cq	73.5
	18	34.27	78.5		18	No Cq	71.5
	24	35.21	78.5		24	No Cq	65
	24	38.66	78		24	No Cq	65
	28	36.17	78.5		28	No Cq	95
	28	34.26	79		28	No Cq	65
	31	35	78		31	No Cq	75.5
	31	35.77	78.5		31	No Cq	68.5



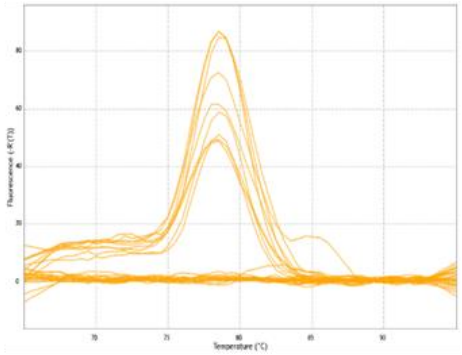


gene	No.	Cq (ΔR)	Tm 1	gene	No.	Cq (ΔR)	Tm 1
β-actin	2	33.27	78.5	IL-8	2	No Cq	65
	2	31.53	78.5		2	No Cq	65
	10	35.29	78.5		10	No Cq	95
	10	32.88	78.5		10	No Cq	65
	18	32.96	78.5		18	No Cq	95
	18	33.88	78.5		18	No Cq	79.5
	24	34.88	78.5		24	No Cq	65
	24	49.82	95		24	No Cq	95
	28	31.36	78.5		28	No Cq	88
	28	31.84	78.5		28	No Cq	85.5
	31	47.47	83		31	No Cq	65
	31	No Cq	95		31	No Cq	65

Amplification Plots



Melt Curve - Raw/Derivative Curve



2.2.9 Statistical analysis methods

The application of diverse statistical analysis methods showcases a comprehensive approach to data interpretation. The T-test, a fundamental tool, is adeptly used to ascertain significant differences between the means of two groups. This is essential in evaluating whether any observed differences in your data are statistically significant or could have occurred by chance. The one-way ANOVA, a more complex technique, is employed to assess the influence of certain factors. Specifically, it examines if there are any statistically significant differences between the means of three or more independent (unrelated) groups. This is particularly useful for understanding the impact of a single independent variable on a dependent variable across multiple groups. Finally, Principal Component Analysis (PCA) is strategically utilized for data classification purposes. PCA reduces the dimensionality of large data sets, simplifying the complexity while retaining the essential patterns and relationships. By transforming the data into principal components, it allows for the identification of the most meaningful bases to distinguish between different classes of data. These statistical methods combined offer a robust framework for analysis, enabling a detailed and nuanced understanding of the data under study.

2.3 Experimental instruments and equipment

2.3.1 DNA sequencer

The Illumina MiSeq Sequencer is a versatile, compact, and highly accurate system suitable for a variety of sequencing applications, especially where smaller-scale, yet precise sequencing is required. Its ease of use and comprehensive data analysis capabilities make it a valuable tool in both research and clinical settings.

Key Features of MiSeq Sequencer:

Sequencing Technology:

The MiSeq uses Illumina's sequencing by synthesis (SBS) technology. This method involves the sequential addition of fluorescently labeled deoxynucleotide triphosphates (dNTPs) as the DNA polymerase synthesizes a complementary strand from the template DNA. Each labeled dNTP emits a specific wavelength of light when incorporated, allowing the sequencer to determine the sequence of the DNA strand.

Read Length and Throughput:

The MiSeq offers various read length options, typically ranging from 50 to 600 base pairs per read, with a mode for paired-end sequencing where both ends of the DNA fragments are sequenced. This flexibility allows it to adapt to different types of sequencing projects. Its throughput can reach up to 15 Gb per run, with millions of reads generated.

Application Range:

It's suitable for a wide range of applications, including small genome sequencing, targeted DNA sequencing, metagenomics, gene expression profiling, and amplicon sequencing. The MiSeq is particularly favored in clinical and diagnostic settings due to its reliability and relatively quick turnaround time.

Sample Preparation and Workflow:

Sample preparation for the MiSeq involves DNA extraction and library preparation, where DNA is fragmented, and adaptors are attached. The prepared libraries are then loaded onto a flow cell for sequencing. Illumina offers various library preparation kits tailored for different applications.

Data Analysis and Software:

The MiSeq System includes onboard data analysis software, which processes the raw sequencing data to generate reads, aligns them to a reference genome if necessary, and performs basic data quality checks. Further analysis can be conducted using external bioinformatics tools.

Size and Usability:

The MiSeq is compact and user-friendly, designed for benchtop use with a relatively small footprint. This makes it an attractive option for laboratories with limited space or those that do not require the high throughput of larger sequencers.

Accuracy and Quality:

One of the strengths of the MiSeq is its high data accuracy, with a very low error rate. This high-quality data is crucial for applications where precision is key, such as in clinical diagnostics.

2.3.2 Bioinformatics tools

In this study, two key bioinformatics tools were used: the Illumina MiSeq platform for next-generation sequencing and QIIME2 for microbial community analysis. MiSeq is specialized for small-scale, high-quality sequencing projects, which is ideal for detailed profiling of microbial DNA. QIIME2, an open-source pipeline, then processes this sequencing data, offering robust functions for quality control, taxonomic analysis, and diversity assessment of microbial communities. Together, these tools provide a comprehensive approach for analyzing microbial DNA, allowing for the in-depth understanding of microbial community composition and dynamics.

2.3.3 Blood metabolite analysis equipment

In this study, the experimental setup for measuring blood metabolites focused on using specialized detection kits and microplate readers to create an efficient and accurate system to analyze various metabolites in blood samples. Assay kits are designed for specific metabolites and contain all reagents and standards required for detection. They work by producing a measurable color or fluorescence change when

reacting with a target metabolite, allowing quantification based on the intensity of this color change. After the blood sample is prepared using the kit reagents, it is placed into the wells of a microplate, which is a flat plate with 96 small wells that serve as individual test tubes. A microplate reader plays a vital role; it is an advanced instrument equipped with a light source and detector that measures the absorbance of light of a specific wavelength. When a metabolite in the sample reacts with the detection reagent, the absorbance of the solution changes, and the reader detects it. The intensity of this absorbance is directly proportional to the concentration of the metabolite in the blood sample. This setup combines the specificity of an assay kit with the precision of a microplate reader, enabling accurate and high-throughput analysis of blood metabolites, which is critical for understanding metabolic functions and disorders.

Chapter 3 Results and Discussion of Experiment 1 (2018)

3.1 Results of milk microbial community analysis

The 2018 data on milk microbiome (Figure 1) composition reveals a complex community where Moraxellaceae is the most dominant bacterial family, accounting for 35.3% of the total identified microbes. This is particularly significant given that certain species within the Moraxellaceae family are associated with bovine mastitis. The Lactobacillaceae family, comprising 8.5%, is well-regarded for its beneficial lactobacilli, which are pivotal in probiotic formulations and fermentation processes. Notably, the Bacillaceae family, making up 13.1% of the microbiome, includes species renowned for their enzyme production, beneficial in various industrial applications.

The presence of Streptococcaceae at 4.7% is also noteworthy; while many species within this family are essential for dairy fermentations, some can be pathogenic, leading to mastitis, a concern for dairy health and milk quality. Lachnospiraceae, which accounts for 3.7%, is involved in the breakdown of complex carbohydrates, indicating its role in the bovine digestive process.

The analysis also uncovers less abundant but still significant families such as Oscillospiraceae and Erysipelotrichaceae, each constituting over 2% of the milk's microbiota. The Enterobacteriaceae family, though less common at 2.3%, is important to note due to its potential impact on milk quality and safety. The diversity within the milk microbiome is underscored by the presence of several other families in smaller proportions, like Micrococcaceae, Ruminococcaceae, and Sphingomonadaceae, each contributing to the intricate bacterial ecosystem in milk.

This detailed assessment of the milk microbiome highlights the balance between beneficial bacteria, essential for dairy processing, and potential pathogens, which pose a risk to animal health and milk quality.

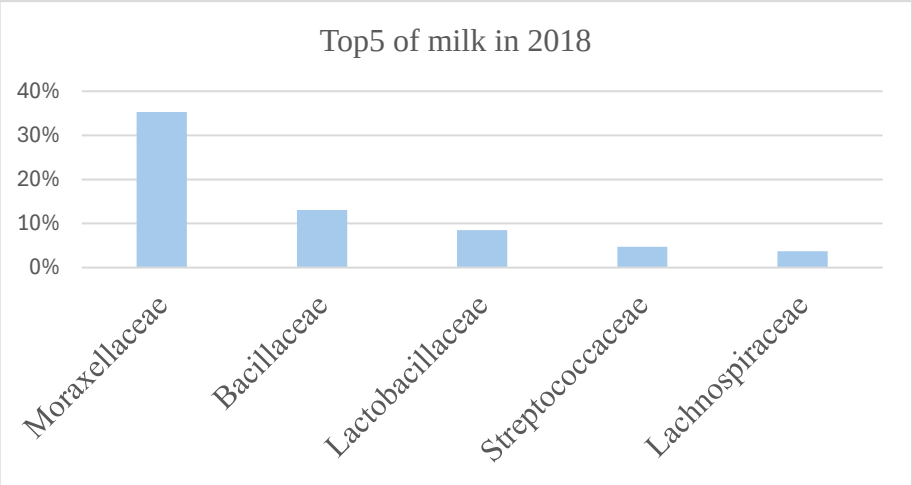


Figure1. Top 5 Predominant Bacterial Families in Milk – 2018.

3.2 Results of udder skin microbial community analysis

The microbial community on udder skin from 2018. The most prominent bacterial group is the Oscillospiraceae, which constitutes a substantial 15.8% of the identified bacteria, indicating its significant presence on the udder skin microbiome. Notably, Lachnospiraceae also makes up a considerable proportion at 9.0%, suggesting its potential role in the skin's microbial ecosystem. Ruminococcaceae and Christensenellaceae are present at 5.2% and 5.9%, respectively, reflecting a diverse bacterial composition that may influence the health and hygiene of the udder skin. The presence of RF39 at 7.3% adds to the complexity of the microbial community, possibly contributing to the metabolic processes on the skin surface. The data also shows a noteworthy presence of smaller populations, such as Aerococcaceae at 6.6%, highlighting the varied nature of the skin microbiome. Other families, including Micrococcaceae and Planococcaceae, though found in lesser quantities, contribute to the overall diversity of the udder skin microbiota.

This analysis underscores the dynamic and multifaceted nature of the udder skin's microbial community, with a range of bacteria that could potentially impact both the health of the dairy cattle and the quality of milk production. The diversity observed in this profile points to a complex interplay between different bacterial species residing on the udder skin, which can have important implications for animal health management and mastitis prevention strategies.

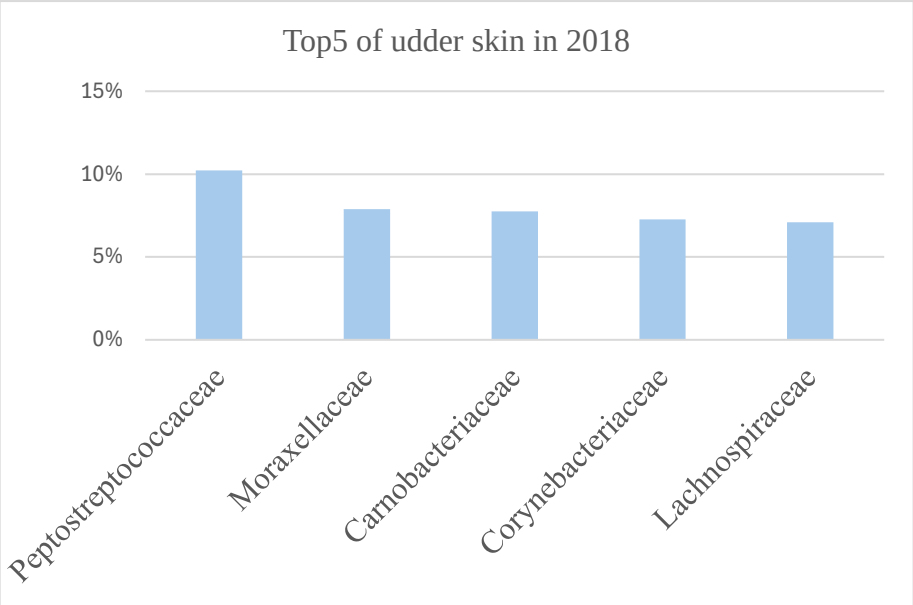


Figure2. Top 5 Bacterial Families Found on Udder Skin - 2018.

3.3 Results of Feces microbial community analysis

The bacterial community profile in the feces samples (Figure 3) from 2018 reveals a diverse and complex microbiota. Notably, Oscillospiraceae is the most abundant group, representing 15.8% of the microbiome, suggesting its significant role in the gastrointestinal environment. Lachnospiraceae follows with a considerable 9% presence, known for its involvement in fermenting plant-based fibers in the gut.

Ruminococcaceae and Christensenellaceae are also prevalent, constituting 5.2% and 5.9%, respectively, indicating their potential contribution to the gut's fermentative processes. RF39 stands out with 7.3%, which might be indicative of its specific role in the bovine digestive tract. Rikenellaceae, which accounts for 8.4%, adds to the diversity of the functional microbial ecosystem within the fecal matter.

In contrast, Moraxellaceae, which is significantly present in milk microbiota, shows a mere 0.1% in feces samples, underscoring the different microbial landscapes between the two environments. Similarly, Lactobacillaceae, which is prominent in milk, is almost negligible in feces matter, highlighting the specificity of microbial niches.

The presence of other families such as Bacillaceae and Erysipelotrichaceae in lesser amounts further illustrates the complexity of the fecal microbiota. The profile is rounded out with smaller populations of bacteria such as Aerococcaceae, Peptostreptococcaceae, and Methanobacteriaceae, each contributing to the ecological balance within the bovine gastrointestinal system.

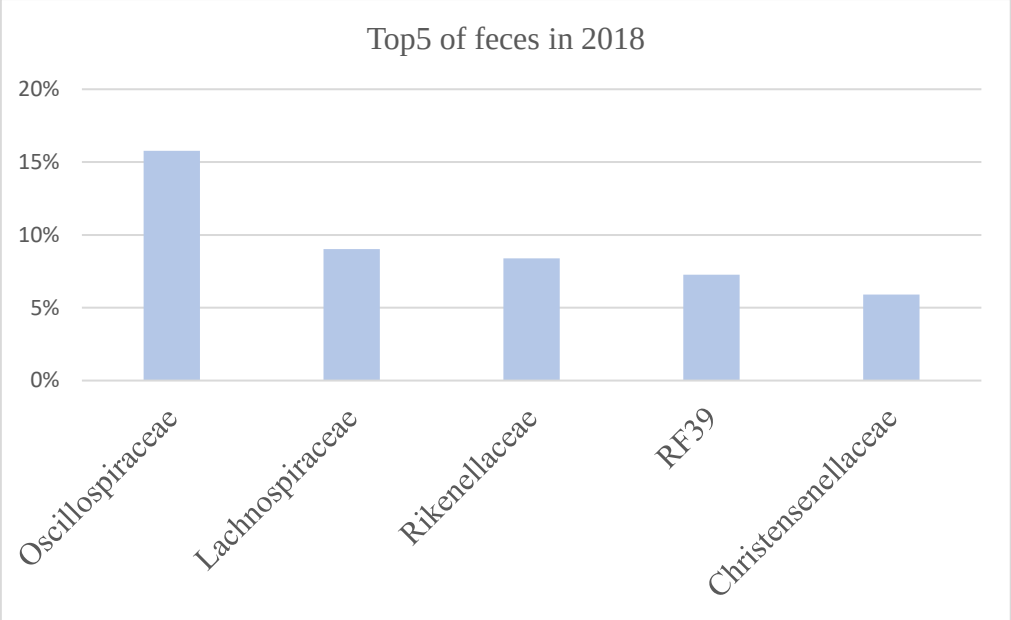


Figure3. Top 5 Bacterial Families in Feces Samples -2018.

3.4 Results of bedding microbial community analysis

The microbial composition of cow bedding (Figure 4) in 2018 was characterized by a significant diversity of bacterial families, with certain groups predominating the ecological niche. Notably, the Corynebacteriaceae family was the most abundant, comprising 16.1% of the total microbiota, which could be indicative of their role in the degradation of organic material within the bedding. Following this, Carnobacteriaceae represented a substantial 13.6%, suggesting that the conditions in the bedding support the growth of this family. Another considerable presence was the Peptostreptococcales-Tissierellales group, accounting for 7.7%, and the Staphylococcaceae family at 7.1%, both of which include species that can be opportunistic pathogens, raising concerns for animal health. Oscillospiraceae also made up a notable proportion at 5.5%, which may reflect the fermentative activity occurring in the bedding environment.Lachnospiraceae, known for its role in cellulose breakdown, was also present in a significant amount (3.9%), suggesting the bedding is rich in plant fibers. Other notable presences included the Planococcaceae at 4.5% and the Aerococcaceae at 4.0%, adding to the complexity of the bacterial ecosystem.

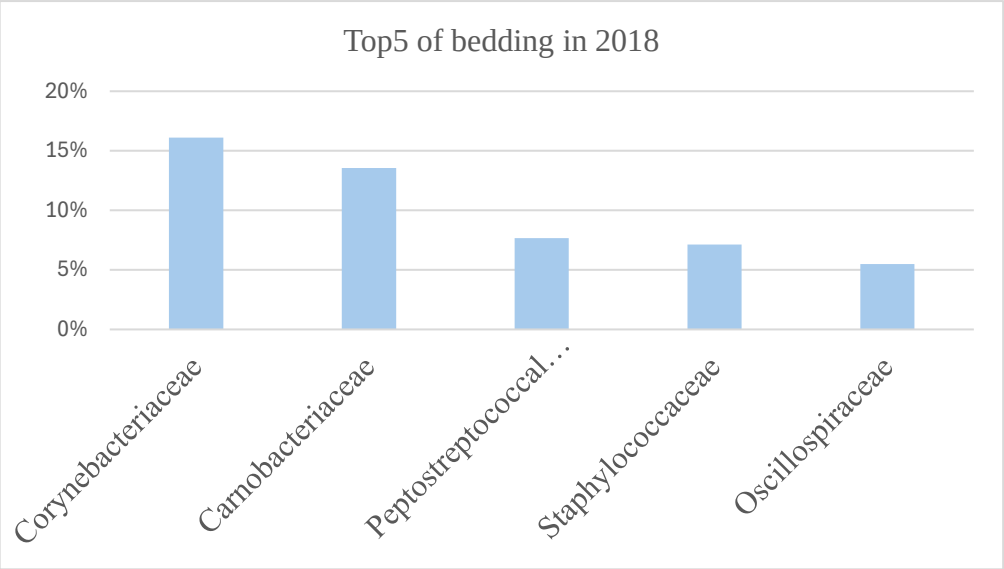


Figure4. Top 5 Bacterial Families in Bedding Materials - 2018.

3.5 Principal component analysis results

The principal component analysis (PCA) (Figure 5) shows One of the udder skin samples grouped with the milk microbiome, suggesting a close relationship or crossover in the bacterial populations between the skin surface where milk is expressed and the milk itself.In contrast, the bedding samples showed a microbial community composition that grouped with the majority of the udder skin samples, indicating a shared microbiota between the skin of the cows and their immediate environment, which includes the material they come into contact with while lying down.

Feces samples, however, formed a separate cluster in the PCA, signifying a distinct microbial community that is markedly different from those found in the milk, udder skin, and bedding. This separation suggests that the bacterial populations in the feces do not significantly influence or are not reflected in the milk microbiome.

Overall, the PCA results suggest that while there is some overlap between the microbiota of the udder skin and the milk, the milk's bacterial community seems to be insulated from direct influence by the microbes present in the bedding and fecal material. This could imply that milk production processes or the mammary glands intrinsic factors help maintain the milk microbiome's unique composition, which is crucial for milk quality and safety.

3.6 Blood metabolite analysis results(See Table 4 for specific data)

NEFA (Non-Esterified Fatty Acids) are at 300 μ Eq/L, indicating the level of lipid mobilization, which can reflect energy balance in the body. Total cholesterol (T-Cho) is measured at 130 mg/dL, which falls within the normal range and is indicative of lipid metabolism and overall cardiovascular risk factors. Albumin, a key protein in blood

plasma that maintains oncotic pressure and transports hormones, vitamins, and drugs, is at 4 g/dL, which is within the standard reference range and suggests adequate nutritional status and liver function. BUN (Blood Urea Nitrogen), at 13 mg/dL, provides information on protein metabolism and renal function. Liver enzymes GPT (Glutamic Pyruvic Transaminase) and GOT (Glutamic Oxaloacetic Transaminase) are at 3 IU/L and 20 IU/L, respectively; these values suggest normal liver health as they are within typical limits. The phosphorus level is 6 mg/dL, essential for bone health and cellular function, which appears to be within the normal range. Lastly, Haptoglobin (Hp) is notably high at 226 mg/dl; such an elevated level can be associated with an inflammatory response or tissue damage. Overall, the metabolic profile suggests a relatively stable state with a potential indication of an acute phase reaction given the raised haptoglobin level.

3.7 Milk composition analysis results(See Table 5 for specific data)

The Somatic Cell Count (SCC), which is an indicator of milk quality and udder health, is reported as 118.7 x 10^3 cells per milliliter, a value that suggests good udder health as it is below the threshold commonly associated with mastitis. The milk yield is 33.4 kg per day, which is a measure of productivity. The fat content of the milk is 3.3%, and the protein content is 3.0%, both important factors for the nutritional value and processing qualities of the milk. Non-fat milk solids, which include proteins, vitamins, lactose, and minerals, are 8.6%, indicating the proportion of milk constituents other than fat. Finally, the Milk Urea Nitrogen (MUN) level is 18.4 mg/dl, which can be used to assess the protein metabolism and nutritional management of the dairy cows, with this level suggesting a balanced protein diet. Overall, these figures indicate milk of good quality and nutritional content.

3.8 Discussion of results

The comprehensive data from 2018 (Figure 6) presents an intricate picture of the dairy ecosystem, where the interrelationship between milk microorganisms, blood metabolites, and milk components becomes evident. These findings are instrumental in piecing together the multifaceted nature of dairy production, where each element not only holds its significance but also influences the others in a dynamic interplay.

Analyzing the bacterial populations in milk through the sequencing of the 16S rRNA gene is a complex task, with the potential for bias and contamination issues, as highlighted by [93], [94], and [95]. While there have been reports characterizing the microbiota present in cow's milk [94], some researchers, including [96], have cast doubt on the presence of microbiota in the milk of healthy animals. The aim of this research was to assess the effect of environmental microbes on the milk's microbial community and to investigate the influence of blood metabolites and milk's nutritional content on

this microbiome.

The milk microbial community analysis unveils a complex bacterial milieu with a significant presence of both beneficial and potentially pathogenic bacteria. The dominance of Moraxellaceae, alongside notable quantities of Lactobacillaceae and Bacillaceae, suggests a balanced microbial environment conducive to quality milk production. However, the presence of potential pathogens also implicates these microorganisms in the health challenges faced by dairy herds, particularly mastitis, which can profoundly impact milk quality and yield. The beneficial bacteria play a crucial role in the fermentation process and contribute positively to the nutritional profile of the milk, enhancing its value for both direct consumption and dairy product formulation.

There is increasing evidence to suggest that clinical mastitis (CM) is linked to a reduction in diversity and a change in the makeup (i.e., dysbiosis) of the microbiota within the mammary gland. The question, however, remains whether this dysbiosis of the microbiota is a precursor to or a result of infectious mastitis. The pathogens responsible for mastitis often possess a range of virulence factors, which aid them in bypassing the immune defenses during the colonization of the udder, as noted by [97] and [98]. The udder skin and feces microbiota analyses further underscore the importance of environmental management in maintaining herd health. The skin serves as a barrier to infection and its microbial profile directly impacts milk safety. Meanwhile, the fecal microbial composition suggests that diet and digestive health are well-managed, but it also points to the risk of contamination, stressing the importance of maintaining hygiene to prevent the transfer of pathogens from feces to the udder and, consequently, to the milk.

The microbial community within bedding material is another critical factor, as it can serve as a reservoir for pathogens. The presence of bacteria such as Staphylococcus aureus in the bedding underlines the necessity of rigorous bedding management to prevent the onset and spread of infections like mastitis.

The relationship between blood metabolites and milk components is exemplified by the NEFA and cholesterol levels, which provide insights into the energy metabolism and nutritional status of dairy cows. The blood metabolite profiles, including the acute-phase protein haptoglobin, serve as indicators of physiological stress and health status, reflecting how systemic health can affect milk composition and quality. Elevated haptoglobin levels, for example, might suggest a response to infection, potentially linking back to the microbial data where pathogens were present.

The milk composition analysis, particularly the SCC, supports the notion of a healthy herd, yet it also brings to light the need for constant vigilance in monitoring udder health to maintain milk quality. The protein and fat percentages, along with the non-fat milk

solids, offer a window into the animals' diet and metabolism, corroborated by the MUN levels, which is unbalanced, could indicate nutritional excesses or deficiencies affecting milk composition.

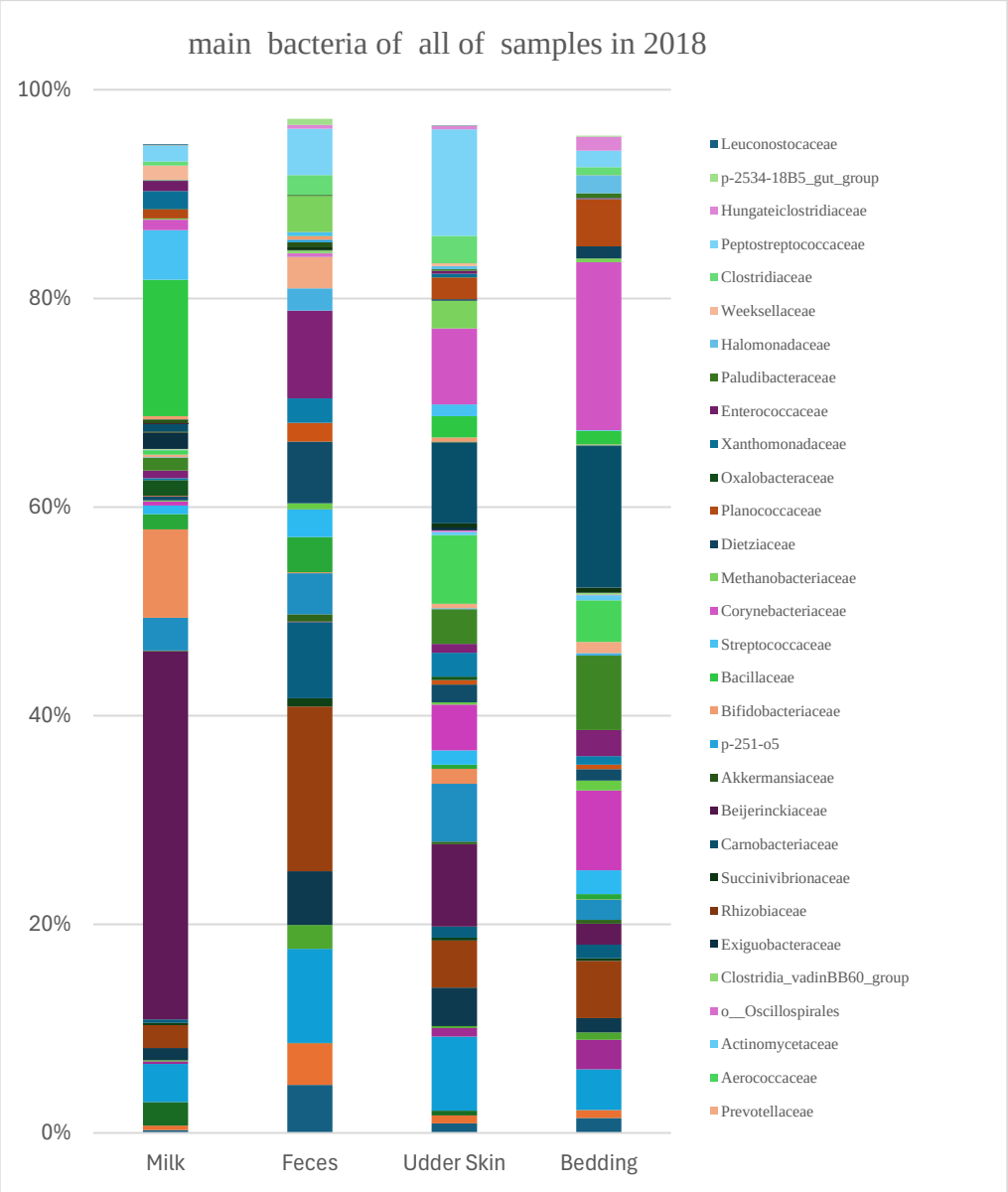


Figure 6. Bacterial Community Composition in Milk, Feces, Udder Skin, and Bedding Samples in 2018.

Chapter 4 Results and Discussion of Experiment 2 (2020)

4.1 Results of milk microbial community analysis

The 2020 milk microbiome composition (Figure 7)is characterized by a predominance of the Muribaculaceae family, which accounts for 16.0% of the total bacterial population, suggesting a significant role in the milk's microbial ecosystem. Following closely, the Lactobacillaceae family, which comprises 15.9%, is well-known for its importance in dairy processes. The Lachnospiraceae family also constitutes a substantial portion at 14.1%, indicative of its role in the complex microbial interplay within milk. Other notable bacteria include the [Eubacterium]_coprostanoligenes group at 9.5% and the Rhizobiaceae family at 8.6%, which may have specific functional roles in the milk matrix. The presence of Sphingomonadaceae at 6.0% and Clostridia_UCG-014 at 6.3% adds to the microbial diversity, with each group potentially playing a distinct role in the milk microbiota.

Lesser yet significant contributors include Oscillospiraceae at 3.4% and Rikenellaceae at 3.1%, alongside the Bacteroidaceae family at 2.9%. Oxalobacteraceae represent 5.8% of the microbiome, potentially contributing to metabolic activities. The data exhibits a minimal presence of other bacterial families, such as Streptococcaceae and Erysipelotrichaceae, each below 2%, suggesting a lower prevalence within the milk microbiome.

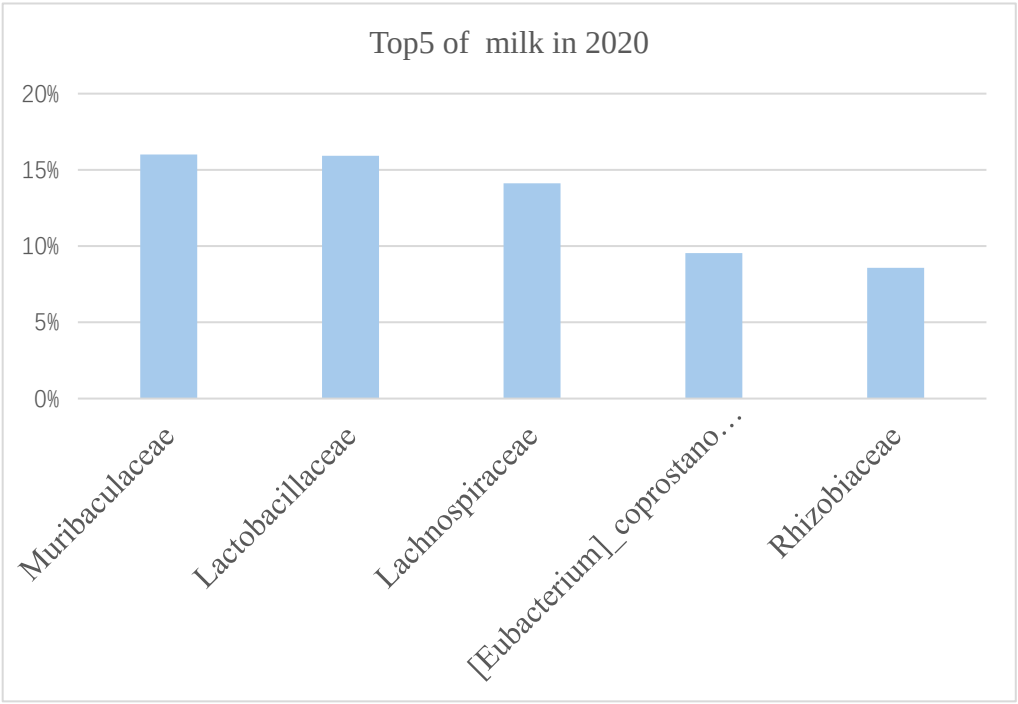


Figure7. Prevalence Top 5 Bacterial of Families in Milk - 2020.

4.2 Results of udder skin microbial community analysis

The microbial profile of bovine udder skin (Figure 8)sampled in 2020 reveals a distinct set of dominant bacterial families, with the top five being Lachnospiraceae at 9.2%, Lactobacillaceae at 8.1%, Muribaculaceae at 7.3%, Rhizobiaceae at 7.2%, and Sphingomonadaceae at 6.1%. Lachnospiraceae are known for their role in cellulose breakdown and are common in the rumen, potentially playing a part in skin health as well. Lactobacillaceae, often associated with a healthy microbiome, could contribute to a protective role on the skin surface. Muribaculaceae, a relatively recently described family, along with Rhizobiaceae, known for nitrogen fixation, suggest diverse microbial functions present on the udder skin. Sphingomonadaceae, recognized for their resilience and presence in various environments, including water and soil, may reflect environmental exposure.

Within this profile, Corynebacteriaceae stands out at 5.6%, which is noteworthy since it includes Corynebacterium species, some of which are opportunistic pathogens and could be a concern for animal health. Although not in the top five, Staphylococcaceae at 2.5% is also significant due to the potential presence of Staphylococcus species, which are commonly implicated in mastitis.

Overall, while the top families in this profile are not typically associated with infectious diseases and may contribute to a balanced microbial ecosystem, the presence of Corynebacteriaceae and Staphylococcaceae indicates the need for ongoing surveillance and management to prevent potential infection risks. This diverse microbial community reflects the complex interactions between the host and its environment, influencing udder health and milk safety.

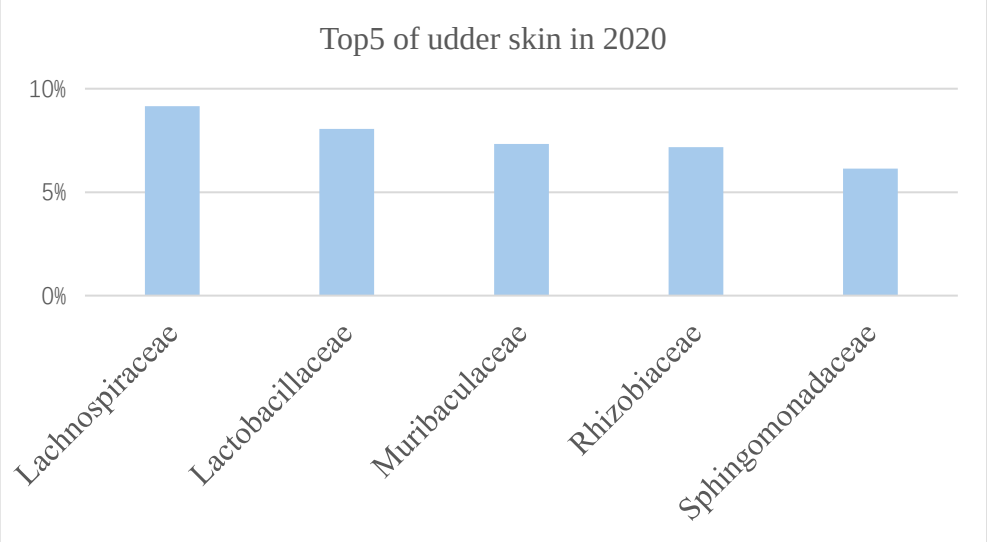


Figure 8. Top 5 Bacterial Families on Udder Skin - 2020.

4.3 Results of Feces microbial community analysis

The 2020 fecal microbial (Figure 9) composition dominated by the Oscillospiraceae family at 19.0%, which is known for its role in the fermentation of complex carbohydrates in the gut. The Lachnospiraceae family is the second most prevalent at 9.2%, also contributing to fiber degradation and overall gut health. UCG-010, a less characterized group, is notable at 4.6%, suggesting unique microbial activities or environmental influences in this bovine gut ecosystem. The Bacteroidaceae family, making up 6.0% of the profile, includes bacteria that are important for the breakdown of polysaccharides. Rikenellaceae, at 16.2%, is comprised of bacteria that are typically associated with a healthy gut microbiome in herbivores.

These top families reflect a fecal microbiome that is well-adapted for the digestion of a high-fiber diet typical of cattle. The presence of diverse fiber-digesting bacteria is indicative of a healthy rumen function and efficient nutrient absorption. Pathogenic bacteria typically associated with disease are not prominently represented in the top families, which is a positive sign for the overall health of the animals. The absence of significant levels of Enterobacteriaceae, which can include harmful species such as E. coli and Salmonella, suggests that the sampled animals were not shedding high levels of these potential pathogens in their feces at the time of sampling. However, while the dominant microbial families in this fecal sample are not commonly associated with pathogens, the presence of any pathogenic species within these or other minor families cannot be entirely ruled out without further specific analysis. Regular monitoring for potential pathogens is essential for maintaining animal health and ensuring the safety of farm environments.

The feces microbiome from this 2020 sample appears to be healthy and typical for cattle, with a rich diversity of fiber-digesting bacteria and a low representation of potential pathogens. This is indicative of good animal health and effective feed utilization.

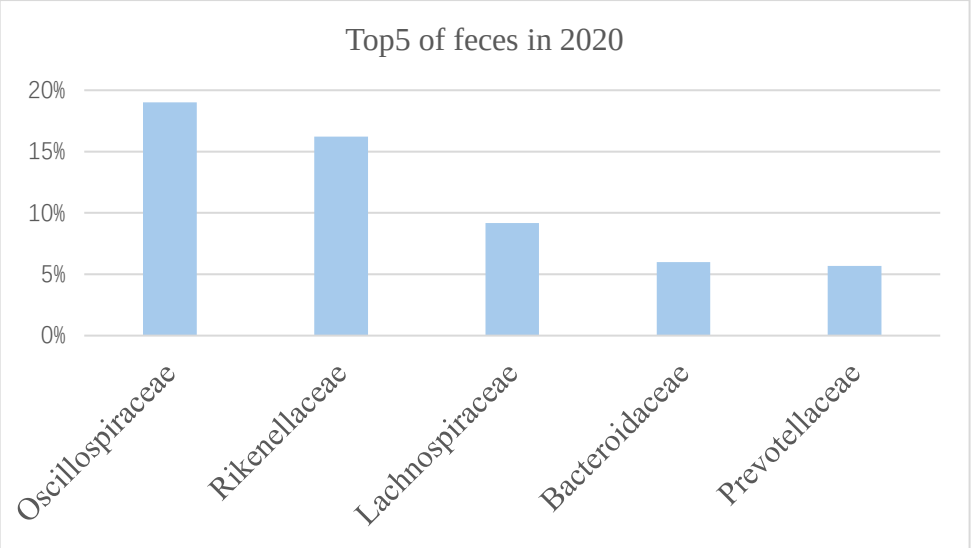


Figure9. Top 5 Bacterial Families Found in feces -2020.

4.4 Results of bedding microbial community analysis

The microbial profile from the 2020 bovine bedding sample (Figure 10) reflects a diverse microbiota, with the top five most abundant families being Carnobacteriaceae (15.5%), Corynebacteriaceae (9.7%), Oscillospiraceae (8.8%), Lachnospiraceae (4.9%), and Moraxellaceae (4.3%). Carnobacteriaceae are typically lactic acid bacteria found in various environments and are known for their fermentative abilities, which can contribute to the bedding's microbial balance. Corynebacteriaceae is significant due to the inclusion of Corynebacterium species, some of which can be opportunistic pathogens. However, many species within this family are also benign and can be part of a normal microbial community. Oscillospiraceae, a family recently reclassified and gaining attention, consists of bacteria that may play a role in the fermentation process within the gut of herbivores. Their presence in bedding could be a result of fecal contamination or could indicate their role in the decomposition of organic matter within the bedding material. Lachnospiraceae are known for their role in breaking down plant material in the gut and might also contribute to the breakdown of organic matter in the bedding. Their presence could also suggest fecal contamination or a natural part of the bedding microbial ecosystem. Moraxellaceae, while lower in abundance compared to the other leading families, could potentially include pathogenic species, but generally, they are not considered a significant concern in bedding materials.

The presence of these families indicates a complex ecosystem within the bedding that can affect animal health and milk quality. For instance, the high abundance of fermentative bacteria could have benefits in terms of breaking down organic matter and potentially suppressing pathogen growth. However, the presence of families that include potential pathogens warrants good bedding management practices to minimize

the risk of disease transmission.

Overall, this microbial composition points to the importance of maintaining clean and dry bedding to ensure a healthy environment for dairy cattle, which ultimately impacts milk safety and quality. Regular monitoring and management of bedding microbiota are crucial for preventing the proliferation of pathogenic bacteria and maintaining the health and productivity of dairy herds.

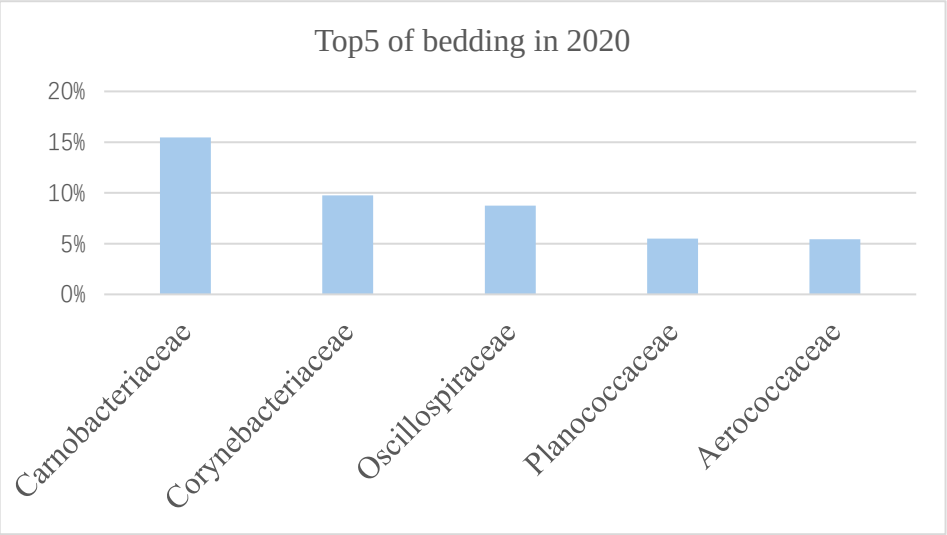


Figure 10. Top 5 Bacterial Families Found in Cow Bedding Material - 2020.

4.5 Principal component analysis results

The principal component analysis (PCA) (Figure 11) of sample types from 2020 indicates a clear demarcation in the microbial communities across different sources within the dairy farm environment. Each sample type—milk, bedding, udder skin, and feces—formed distinct and separate clusters in the PCA space, suggesting that each harbors a unique microbiome with little to no overlap between them.For the milk samples, the PCA results point to a specialized bacterial community that is characteristic of the milk environment alone. This suggests that factors intrinsic to the milk or the mammary gland itself maintain a distinct microbiota, potentially beneficial for milk quality and hygiene.

The bedding samples also displayed a unique microbial signature, indicating that the bacteria present are specialized for the bedding environment, which is likely influenced by the materials used in the bedding and the conditions of the barn.

Similarly, the udder skin microbiome formed its own cluster, separate from milk and bedding. This could be due to the skin's specific conditions and the role of the skin as a barrier, which may select a particular set of microorganisms adapted to the skin

environment.

Fecal samples were distinctly grouped away from the other sample types, reflecting a microbiome composition that is highly specialized for the gastrointestinal tract of the cows and not transferred to the milk, bedding, or skin in measurable ways.

Overall, the PCA results from 2020 reinforce the concept of compartmentalization within the microbial ecosystems of a dairy farm. Each sample type's microbiome is adapted to its specific environment and is maintained independently of the others, which has important implications for understanding the transmission of bacteria in dairy farm systems and for managing animal and milk hygiene.

4.6 Blood metabolite analysis results (See Table 4 for specific data)

The provided data from the 2020 blood metabolite analysis depicts a singular profile within normal physiological ranges, indicating a healthy metabolic state for the individual from which the sample was taken. Non-Esterified Fatty Acids (NEFA) are at 106.3 μ Eq/L, suggesting adequate energy mobilization, which is crucial for meeting the metabolic demands of the body, particularly in lactating animals. The cholesterol level is 213.7 mg/dL, which is within a normal range for bovines and indicates healthy lipid metabolism essential for various biological processes. The albumin concentration, at 4.0 g/dL, is typical for healthy adult cattle, signifying sufficient protein synthesis and balanced nutrition. Blood Urea Nitrogen (BUN) at 8.3 mg/dL is within the expected range, reflecting normal protein digestion and renal function. The liver enzymes Glutamic Pyruvic Transaminase (GPT) and Glutamic Oxaloacetic Transaminase (GOT), are measured at 6.5 IU/L and 39.6 IU/L, respectively, values that are within normal ranges and do not indicate any hepatic injury. Lastly, Haptoglobin (Hp), a marker for inflammation, shows a low value of 0.4 mg/dl, which is typical in the absence of acute inflammatory processes. This profile is indicative of an animal in good health with no evident metabolic or inflammatory distress.

4.7 Milk composition analysis results (See Table 5 for specific data)

The 2020 milk composition data indicates a healthy dairy profile with all parameters within normal ranges for quality milk production. The Somatic Cell Count (SCC) is 36 x 10^3 cells/mL, which is well below the threshold that would indicate mastitis, suggesting good udder health. A daily milk yield of 36 kg is typical for a productive dairy cow and can vary based on breed and lactation stage. The fat percentage is at 4%, which is within the ideal range for standard milk and contributes to the milk's energy value and flavor. Protein content is at 3%, indicating good nutritional quality, essential for cheese-making and milk's overall nutritive value. Non-fat milk solids are at 9%, which includes other components such as lactose and minerals, important for the milk's physical properties and nutritional content. The Milk Urea Nitrogen (MUN) level is 13

mg/dl, reflecting a balanced protein metabolism and an adequate protein intake from the diet. This milk composition profile suggests that the dairy cows are well-managed in terms of diet, health, and milking practices.

4.8 Discussion of results

The 2020 data presents an integrated view of dairy herd health by connecting the dots between milk microorganisms, blood metabolites, and milk components. This holistic approach offers deeper insights into how these factors interrelate and collectively impact dairy cattle's well-being and milk production quality.

Starting with milk microorganisms, the prevalence of beneficial bacteria like Lactobacillaceae, associated with probiotic properties, and fiber-degrading families such as Muribaculaceae and Lachnospiraceae, suggests a robust microbial environment that could positively influence the fermentation quality of milk and potentially enhance the gut health of calves and consumers alike. The relative scarcity of pathogenic species within the milk microbiome is indicative of sound herd health management practices. However, the presence of any pathogens, even in small quantities, underscores the need for vigilant monitoring to ensure milk safety. The analysis of the udder skin microbiota reveals a complex ecosystem where beneficial bacteria are prevalent, but the presence of opportunistic pathogens necessitates careful hygiene practices to prevent infections such as mastitis. A healthy skin microbiota serves as a crucial barrier to infection, protecting the integrity of the milk. When examining the fecal microbiome, the high occurrence of Oscillospiraceae and Lachnospiraceae reflects a diet rich in fibers and effective digestive processes. This could indirectly influence milk composition by ensuring efficient nutrient absorption and overall animal health, which are vital for high-quality milk production. The bedding microbiota, rich in fermentative bacteria, may benefit the breakdown of organic matter, potentially creating a healthier living environment for the cattle. However, the need to manage bedding to prevent disease spread is evident, as pathogenic bacteria can readily transfer from bedding to udder skin and into the milk supply.

Blood metabolite analysis provides a direct window into the metabolic status of the animals, with NEFA, cholesterol, and BUN levels indicating a balance between energy intake and metabolic demand. The liver enzyme levels within normal ranges suggest healthy liver function. Low Haptoglobin levels across the majority of the samples point to an absence of systemic inflammation, which can affect milk composition and yield.

Milk composition data, with an SCC indicating good udder health and balanced fat and protein levels, reflects the culmination of the interplay between diet, metabolism, and microbial environment. The correlation between a balanced diet, as shown by MUN levels, and the milk's fat and protein content highlights the importance of nutritional management.

Chapter 5 Comprehensive analysis and comparison

5.1 Summary of the main findings of Experiment 1 and Experiment 2

Summary of Main Findings from Experiment 1 (2018):

The principal component analysis for 2018 samples from different environments within a dairy farm revealed some overlap between microbial communities. Notably, one udder skin sample was found to share a microbial profile with the milk microbiome, indicating a possible transfer or shared environment between the udder skin and the milk itself. However, the bedding and most udder skin samples were grouped together, suggesting a shared microbial community likely due to the close contact between the cows and their bedding. Fecal samples were distinct, forming a separate cluster, indicating a unique fecal microbiome not influencing or reflected in the milk microbiome. This suggests a level of microbial segregation between the feces and other sampled environments, particularly the milk.

Summary of Main Findings from Experiment 2 (2020):

In 2020, the principal component analysis demonstrated a clear separation of microbial communities among all sampled environments. Each sample type—milk, bedding, udder skin, and feces—was distinct and segregated into its own group without overlap. This suggests that by 2020, the microbiomes of each environment were independent and exhibited specialized bacterial communities with no significant cross-contamination or influence detectable between them. This could be indicative of either improved farm management practices leading to better hygiene and separation of different farm areas or the inherent robustness of the microbial communities in resisting cross-over.

The details of the results of the principal coordinate analysis are shown in (Figure 12).

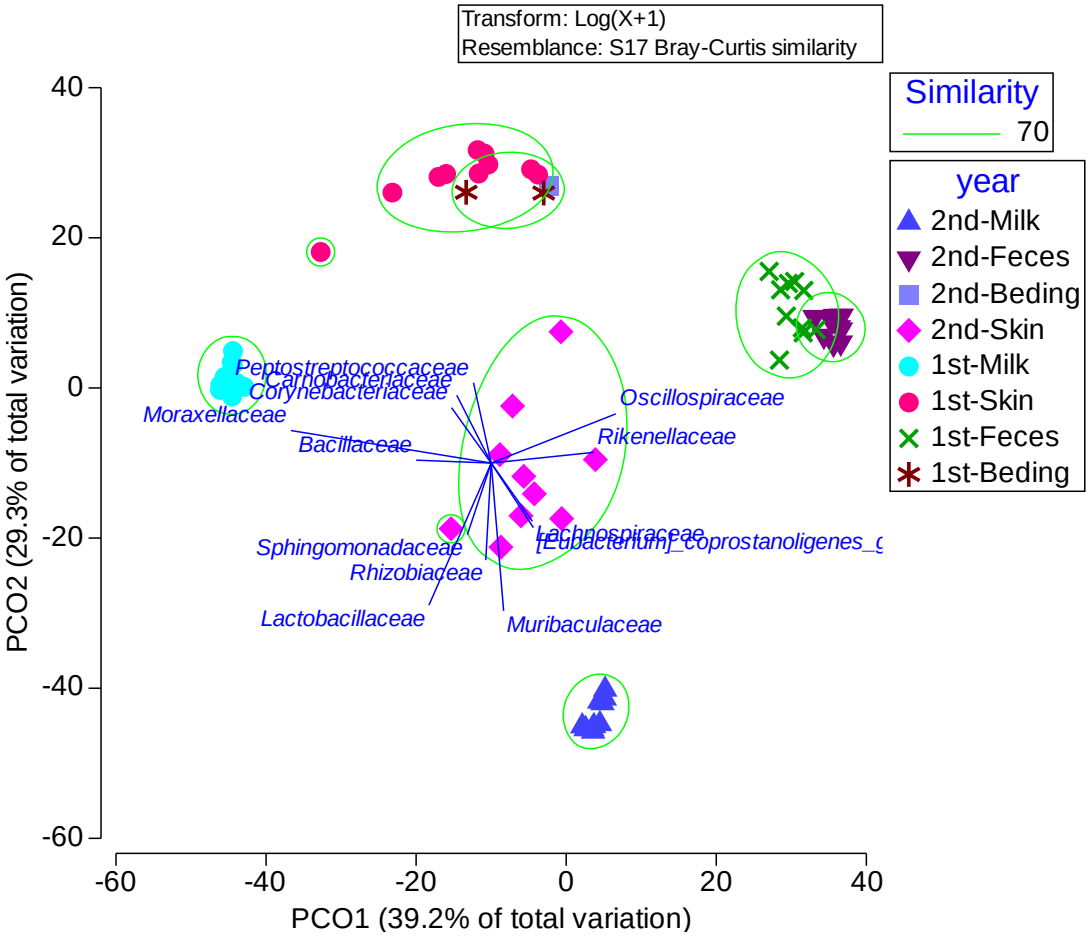


Figure 12. Comparative Principal Coordinate Analysis of Various Sample Types from 2018 and 2020.

5.2 Relationship between milk microorganisms and udder skin microorganisms

The relationship between milk microorganisms and udder skin microorganisms is intricate and significant in dairy science. The udder skin microbiota serves as one of the primary sources of microorganisms found in milk. This relationship is due to the close physical proximity of the udder skin to the milk ejection site, which allows for the transfer of bacteria from the skin into the milk during the milking process.

Udder skin microorganisms include a diverse array of bacteria, yeasts, and other microorganisms that inhabit the skin's surface and glands. These microorganisms can be influenced by factors such as the environment, hygiene practices, the health of the skin, and the cow's overall health. The composition of the udder skin microbiota can directly affect the quality and safety of the milk, as some skin bacteria may be benign or even beneficial, while others can be pathogenic, leading to milk spoilage or diseases such as mastitis.

In milk, the microbial population comprises both the resident microflora of the mammary gland and the transient flora from the udder skin, the environment, and the milking equipment. Beneficial milk microorganisms are often involved in fermentation

and can enhance the nutritional and sensory properties of dairy products. However, the presence of pathogens or spoilage microorganisms originating from the udder skin can lead to milk contamination, affecting its quality and shelf life.

The dynamic interaction between the milk and udder skin microbiomes is critical for dairy farming, milk production, and product development. Maintaining good udder health and hygiene is essential for managing the transfer of microorganisms and ensuring the production of high-quality milk.

5.3 Association of microorganisms with blood metabolites and milk composition

This study, conducted in two separate experiments in 2018 and 2020, employed advanced techniques to profile the microbiota in milk, udder skin, feces, and bedding, alongside the analysis of blood metabolites.

Microbiota Profiles:

The milk microbiota in both years was dominated by different bacterial families, with Moraxellaceae being most prevalent in 2018, and a shift towards Muribaculaceae and Lactobacillaceae in 2020. Notably, potentially pathogenic families like Enterobacteriaceae and Staphylococcaceae were present in lower abundances in 2020.

Milk Quality:

There was an improvement in milk quality over the two years, with increases in protein, fat, and solids-not-fat (SNF) levels, although somatic cell count (SCC) and milk urea nitrogen (MUN) levels did not change significantly, indicating a stable health status of the cows.

Blood Metabolites:

Blood metabolites differed between the two sample years, with reductions in blood urea nitrogen (BUN) and non-esterified fatty acids (NEFA), and increases in total cholesterol (T-Cho), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and haptoglobin (HP). These changes may reflect shifts in the cows' metabolic state and health.

Microbiota and Health Indicators:

The study also observed that while there were prevalent taxa shared between the bedding and udder skin microbiota, as well as between the udder skin and milk microbiota, selection and elimination processes during transmission appeared to prevent pathogen transfer. The separate grouping of milk, udder skin, and fecal microbiota in the principal coordinate analysis (PCoA) underscored their distinct natures.

5.4 Influence of farm management, diet

Farm Management:

The study emphasizes that advancements in milking technology and procedures have

significantly reduced milk contamination with pathogens known to cause contagious mastitis and foodborne diseases. However, challenges persist with environmental pathogens that contribute to mastitis, signaling a need for further improvements in farm hygiene and diagnostic methods to prevent the condition. The management of cowsheds plays a crucial role as the microbiota here can influence the microbiota of the milk. Yet, the transmission of microbes is not direct; the udder skin interacting with the environment could serve as a barrier, defending against pathogen invasion and contamination.

Dietary Impact:

Variations in blood metabolites and milk quality between the two study years suggest that dietary factors may have shifted, affecting the cows' metabolic profiles and potentially the microbial populations within the milk. Nevertheless, the direct correlation between diet and the skin microbiota and its impact on milk composition is not clearly established, leaving room for further investigation.

Microbiota Analysis:

The abstract reports on the comparative analysis of milk, udder skin, and fecal microbiota in cows over two separate years, using automated milking systems. The findings indicate that although there are shared microbial taxa between the bedding and udder skin, and between the udder skin and milk, there's a selection process during microbial transmission. This selection is crucial in understanding how management practices and the cow's innate defenses work together to shape the microbial landscape of the milk and udder skin.

5.5 Paper contribution and research limitations

Paper Contributions:

This research significantly contributes to the understanding of milk microbiota and its relationship with the occurrence of mastitis in dairy cows. It presents a detailed survey of milk microbiota across different years, analyzing how advancements in milking technology have reduced contamination with pathogens causing mastitis. The study also delves into the connection between cowshed microbiota and milk microbiota, suggesting a complex transmission process where udder skin might play a defensive role.

The examination of milk, udder skin, and feces microbiota in conjunction with bedding microbiota and blood metabolites provides a holistic view of the factors influencing milk composition. This comprehensive approach offers insights into the herd-level variability of milk and udder skin microbiota and their independence from nutritional status and milk composition. The study advances the understanding of microbial communities in dairy farms and highlights the importance of hygiene management in preventing mastitis.

Research Limitations:

While this research provides extensive data on microbiota and metabolite profiles, it is unable to link milk composition directly to the microbiota.

The year-to-year comparison offers valuable data but also suggests that further longitudinal studies might be required to understand the full impact of farm management practices on microbiota. The abstract does not mention any controlled interventions or changes in farm management between the two sampling years, which could have provided more concrete evidence of the factors affecting microbiota composition.Furthermore, the lack of a significant relationship between certain blood metabolites and milk microbiota could indicate the need for more targeted research, possibly at a molecular level, to unravel the complex interactions within the dairy cow's ecosystem.

Chapter 6 Conclusion

6.1 Research summary

Advancements in the dairy industry have significantly ameliorated the risk of milk contamination by pathogens responsible for mastitis, a persistent challenge impacting milk quality and safety. Despite technological strides in milking procedures that have mitigated the threat of contagious mastitis and foodborne illnesses, environmental mastitis pathogens still slip through the cracks of control measures. The complex interplay between the cowshed environment and milk quality underscores an ecosystem where udder skin not only comes into contact with potential contaminants but also serves as a barrier, highlighting the nuanced relationship between environmental microbiota and milk microbiota.

In a novel approach to understanding these dynamics, this study scrutinized the microbiota present in milk, udder skin, and feces from healthy dairy cows managed in a free stall barn equipped with an automatic milking system. The inquiry was staged twice, first in September 2018 and subsequently in August 2020, embracing a comprehensive methodology that included the analysis of bedding microbiota and blood metabolites.

The investigations revealed that certain parameters, such as milk yield, remained stable across years, while constituents like milk protein, fat, and solids-not-fat (SNF) levels saw an increase in the 2020 cohort. Interestingly, somatic cell count (SCC) and milk urea nitrogen (MUN), indicators of milk quality and cow health, did not exhibit statistically significant shifts despite apparent reductions in 2020.

A pivotal part of this research was the application of principal component analysis (PCA), which unveiled discernible shifts in milk's compositional and metabolic fingerprints between the two sampled years. However, the presence of high SCC in some cows did not correlate with noticeable changes in the overall milk quality or

metabolite concentrations.

Diving into the microbial realms, the study identified the most populous bacterial families within milk, highlighting a shift from Moraxellaceae dominance in 2018 to a rise in Muribaculaceae and Lactobacillaceae in 2020. The skin microbiota of the udder also evolved, with Lachnospiraceae topping the charts in 2020, replacing the previous frontrunners like Peptostreptococcaceae. The feces and bedding microbiota presented a consistency over the years with minor fluctuations, suggesting a relatively stable environmental microbial community within the barn. However, the PCA indicated that each microbiota—milk, udder skin, and fecal—formed distinct clusters, with year-to-year differences more pronounced in milk and udder skin microbiota.

The research broke new ground by using network analysis to explore the associations between microbiota and blood metabolites, alongside milk composition. Although a web of connections was illuminated, most lacked a consistent pattern across the two years, underscoring the complexity of these relationships and the multitude of factors influencing them.A striking conclusion of the study is the disconnection between the microbiota found in milk and on udder skin and the cows' nutritional status and milk composition. It appears that the cows' diets or health conditions do not necessarily dictate the microbial profile of their milk. Moreover, while common microorganisms were identified between the bedding and udder skin, as well as between udder skin and milk, the process of transmission seemed to involve a selective filter, allowing some microbes to pass while others were repelled, irrespective of their pathogenicity.

In essence, this research delivers vital insights into the milk microbiota, contributing to the ongoing discourse on enhancing dairy farm management to preempt mastitis. It underscores the autonomous nature of microbial communities within the dairy cow milieu and the intricate connections—or lack thereof—between them, the farm environment, and the cow's diet and health. As such, this work paves the way for further scientific inquiry to better understand the determinants of milk quality and safety in the face of environmental challenges.

6.2 Suggestions for future research

Investigate Environmental Mastitis Pathogens:

Despite advances in milking technology, environmental mastitis remains a challenge. Future research should focus on identifying and controlling these pathogens, possibly exploring new antimicrobial agents or alternative therapies.

Examine the Transmission Dynamics:

There is a need to understand the transmission dynamics between cowshed microbiota and milk microbiota. Research could delve deeper into how environmental factors and cow interactions influence microbial transfer.

Explore Nutritional Influence:

Investigate the relationship between cow diet, nutritional status, and the microbiota of milk and udder skin. This could include controlled dietary interventions to ascertain the direct impact of nutrition on microbial composition.

Study the Role of Bedding Material:

Examine how different bedding materials affect the microbiota of the bedding, udder skin, and milk. This could include comparing various bedding types and their influence on microbial diversity and mastitis incidence.

Investigate Microbiota-Blood Metabolite Relationships:

Future studies could explore the complex relationships between microbial taxa and blood metabolites more comprehensively, using advanced analytical tools to unravel these connections.

Understand Herd-Level Variability:

Research should also focus on understanding herd-level variability in microbiota and its implications for milk quality and cow health. This could involve genetic profiling and herd management analysis.

Assess Pathogen and Non-pathogen Transmission:

Investigate the selective transmission of pathogens and non-pathogens from the environment to the udder skin and milk, to understand how different microorganisms are filtered during transmission.

References

1. Hagnestam-Nielsen C, Ostergaard S. Economic impact of clinical mastitis in a dairy herd assessed by stochastic simulation using different methods to model yield losses. Animal. 2009; 3(2):315–28. https:// doi.org/10.1017/S1751731108003352 PMID: 22444235.
2. Jayarao BM, Pillai SR, Sawant AA, Wolfgang DR, Hegde NV. Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. J Dairy Sci 2004;87:3561-73. https://doi.org/10.3168/jds.S0022-0302(04)73493-1 .
3. Olde Riekerink RGM, Barkema HW, Stryhn H. The effect of season on somatic cell counts and the incidence of clinical mastitis. J Dairy Sci 2007;90:1704-15. https://doi.org/10.3168/ jds.2006-567 .
4. Kim IS, Hur YK, Kim EJ, et al. Comparative analysis of the microbial communities in raw milk produced in different regions of Korea. Asian-Australas J Anim Sci 2017;30:1643- 50. https://doi.org/10.5713/ajas.17.0689.
5. Gao, J., Barkema, H. W., Zhang, L., Liu, G., Deng, Z., Cai, L., et al. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. J. Dairy Sci. (2017);100, 4797–4806. doi: 10.3168/jds.2016-12334.
6. Kibebew, K. Bovine mastitis: A review of causes and epidemiological point of view. 2017.JBAH, 7(2): 1-14.
7. Kumari, T., Bhakat, C. and Choudhary, R. A review on subclinical mastitis in dairy cattle. 2018.Int. J. Pure Appl. Biosci., 6(2): 1291-1299.
8. Thuong Thi Nguyen , Haoming Wu , and Naoki Nishino et al. An investigation of seasonal variations in the microbiota of milk, feces, bedding, and airborne dust. Asian-Australas J Anim Sci Vol. 33, No. 11:1858-1865 November 2020 https://doi.org/10.5713/ajas.19.0506 pISSN 1011-2367 eISSN 1976-5517.
9. Petzer, I.M., Karzis, J., Donkin, E.F., Webb, E.C. and Etter, E. (2017) Somatic cell count thresholds in composite and quarter milk samples as indicator of bovine intramammary infection status. Onderstepoort J. Vet. Res., 84(1): 1-10.
10. Oliveira, L., Hulland, C. and Ruegg, P. (2013) Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. J. Dairy Sci., 96(12): 7538-7549.
11. Alekish, M. (2015) The association between the somatic cell count and isolated microorganisms during subclinical mastitis in heifers in Jordan. Vet. Med. Czech, 60(2): 71-76.
12. Cheng L, Reddy V, Solmos G, Watkins L, Cimbaluk D, Bitterman P, et al. Mastitis, a radiographic, clinical, and histopathologic review. Breast J. 2015;21(4):403–9.
13. Levison LJ, Miller-Cushon EK, Tucker AL, Bergeron R, Leslie KE, Barkema HW, et al. Incidence rate of pathogen-specific clinical mastitis on conventional and

organic Canadian dairy farms. J Dairy Sci. 2016; 99(2):1341–50. Epub 2015/12/10. https://doi.org/10.3168/jds.2015-9809 PMID: 26686728.

14. Oliveira L, Hulland C, Ruegg PL. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. J Dairy Sci. 2013; 96(12):7538–49. Epub 2013/10/11. https://doi.org/10.3168/jds.2012-6078 PMID: 24119795.
15. National Mastitis Council. Laboratory Handbook on Bovine Mastitis: National Mastitis Council, Madison, WI. 1999. p. 222.
16. Derakhshani H, Fehr KB, Sepehri S, Francoz D, De Buck J, Barkema HW, Plaizier JC, Khafipour E. Microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. 2018. J Dairy Sci 101:10605–10625. https://doi.org/10.3168/jds.2018-14860.
17. Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD. The complex microbiota of raw milk. 2013. F.
18. Guan L, Oikonomou G, Bicalho M, Meira E, Rossi R, Foditsch C, Machado V, Teixeira A, Santisteban C, Schukken Y, Bicalho R . Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters.2014.PLoS ONE 9(1):e85904. https://doi.org/10.1371/journal.pone.0085904.
19. Hélène F, Lucie R, Aurélie N, Bouchard D, Jacques L, Philippe L, Jean-Marc A, Pierre-Guy M, Le L, Sergine E . Bovine teat microbiome analysis revealed reduced alpha diversity and significant changes in taxonomic profiles in quarters with a history of mastitis. 2016. Front Microbiol. https://doi.org/10.3389/fmicb.2016.00480.
20. Gomes F, Henriques M. Control of bovine mastitis: old and recent therapeutic approaches. Curr Microbiol 2016;72:377-82. https://doi.org/10.1007/s00284-015-0958-8.
21. Hogeveen H, Steeneveld W, Wolf CA. Production diseases reduce the efficiency of dairy production: A review of the results, methods, and approaches regarding the economics of mastitis. Annu Rev Resour Economics 2019;11:289-312. https://doi.org/10.1146/annurev-resource-100518-093954.
22. Zhao X, Lacasse P. Mammary tissue damage during bovine mastitis: causes and control. J Anim Sci 2008; 86:57-65. https:// doi.org/10.2527/jas.2007-0302.
23. Khan M, Khan A. Basic facts of mastitis in dairy animals: a review. Pak Vet J 2006;26:204-8.
24. Kibebew K. Bovine mastitis: A review of causes and epidemiological point of view. J Biol Agric Healthc 2017;7:1-14.
25. Gruet P, Maincent P, Berthelot X, Kaltsatos V. Bovine mastitis and intramammary drug delivery: review and perspectives. Adv Drug Deliv Rev 2001;50:245-59. https://doi.org/10.1016/ S0169-409X(01)00160-0.

26. Abebe R, Hatiya H, Abera M, Megersa B, Asmare K. Bovine mastitis: prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed, South Ethiopia. BMC Vet Res 2016;12:270. https://doi.org/10.1186/s12917-016-0905-3.
27. Romero J, Benavides E, Meza C. Assessing financial impacts of subclinical mastitis on Colombian dairy farms. Front Vet. Sci. 2018;5:273. http://doi.org/10.3389/fvets.2018.00273.
28. Chahar A, Gahlot AK, Tanwar RK, Fakhruddin. Evaluation of different screening tests for diagnosis of subclinical mastitis in cattle. Indian J Vet Med. 2008;28:91-93.
29. Kader MA, Samad MA, Saha S, Taleb MA. Prevalence and etiology of subclinical mastitis with antibiotic sensitivity to isolated organisms among milch cows in Bangladesh. Ind J Dairy Sci. 2002;55:218-223.
30. Sharma N. Foot and mouth disease mastitis cascade in dairy cattle: A field study. Int J Zool Res. 2008;4(1):64-67. doi: 10.3923/ijzr.2008.64.67.
31. Sudhan NA, Singh R, Singh M, Soodan JS. Studies on prevalence, etiology and diagnosis of sub clinical mastitis among cross breed cows. Indian J Anim Res. 2005;39(2):127-130.
32. Young ZJ, Fang X, Mei Y, et al. Isolation and identification of pathogens from mastitis cow and drug sensitivity test. China Anim: Husb. Vet Med. 2009;36:136-140.
33. Kumar M, Goel P, Sharma A, Kumar A. Prevalence of subclinical mastitis in cow at a Goshala. Proceeding of compendium of 27th ISVM International summit and convention at Chennai, February, Tamilnadu, India. 2009:4.
34. Sharma N. Economically important production diseases of dairy animals. Sarva Manav Vikash Samiti, Gurgaon, India. 2010.
35. Garcia A. Contagious vs. Environmental Mastitis. Extension Extra. 2004. http://openprairie.sdstate.edu/extension_extra/126.
36. Sudhan NA, Sharma N. Mastitis. An important production disease of dairy animals' 1st Ed. Sarva Manav Vikash Samiti, Gurgaon, India.2010.
37. Ruegg PL. A 100-year review: mastitis detection, management, and prevention. J Dairy Sci 2017;100(12):10381–97.
38. Neave FK, Dodd FH, Kingwill RG, et al. Control of mastitis in the dairy herd by hygiene and management. J Dairy Sci 1969;52(5):696–707.
39. Dodd FH, Neave FK, Kingwill RG. Control of udder infection by management. J Dairy Sci 1964; 47 (10):1109–14.
40. Oikonomou G, Machado VS, Santisteban C, et al. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomics 16s rDNA.

PLoS One 2012;7(10):e47671.

41. Kuehn JS, Gorden PJ, Munro D, et al. Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. PLoS One 2013;8(4):e61959.
42. Oikonomou G, Bicalho ML, Meira E, et al. Microbiota of cow's milk: distinguishing healthy, sub-clinically, and clinically diseased quarters. PLoS One 2014;9(1):e85904.
43. Metzger SA, Hernandez LL, Skarlupka JH, et al. Influence of sampling technique and bedding type on the milk microbiota: results of a pilot study. J Dairy Sci 2018; 101:1–11.
44. Rainard P. Mammary microbiota of dairy ruminants: fact or fiction? Vet Res 2017; 48(1):25.
45. Jost, T., Lacroix, C., Braegger, C., and Chassard, C. (2015). Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. Nutr. Rev. 73, 426–437. doi: 10.1093/nutrit/nuu016.
46. Addis, M. F., Tanca, A., Uzzau, S., Oikonomou, G., Bicalho, R. C., and Moroni, P. (2016). The bovine milk microbiota: insights and perspectives from -omics studies. Mol. Biosyst. 12, 2359–2372. doi: 10.1039/C6MB00217J.
47. Doyle, C. J., Gleeson, D., O'Toole, P. W., and Cotter, P. D. (2017). Impacts of seasonal housing and teat preparation on raw milk microbiota: a high throughput sequencing study. Appl. Environ. Microbiol. 83:e02694-16. doi:10.1128/AEM.02694-16.
48. Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., and Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. Am. J. Clin. Nutr. 96, 544–551. doi:10.3945/ajcn.112.037382.
49. Murphy, K., Curley, D., O'Callaghan, T. F., O'Shea, C.-A., Dempsey, E. M., O'Toole, P. W., et al. (2017). The composition of human milk and infant faecal microbiota over the first three months of life: a pilot study. Sci. Rep. 7:40597. doi: 10.1038/srep40597.
50. Hunt, K. M., Foster, J. A., Forney, L. J., Schutte, U. M. E., Beck, D. L., Abdo, Z., et al. (2011). Characterization of the diversity and temporal stability of bacterial communities in human milk. PLoS One 6:e21313. doi: 10.1371/journal.pone.0021313.
51. Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M. L., Xaus, J., et al. (2003). Human milk is a source of lactic acid bacteria for the infant gut. J. Pediatr. 143, 754–758. doi: 10.1016/j.jpeds.2003.09.028.
52. Derakhshani, H., Fehr, K. B., Sepehri, S., Francoz, D., De Buck, J., Barkema,

H.W., et al. (2018). Invited review: microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. J. Dairy Sci. 101, 10605–10625. doi: 10.3168/jds.2018-14860.

53. Lima, S. F., Bicalho, M. L., de, S., and Bicalho, R. C. (2018). Evaluation of milk sample fractions for characterization of milk microbiota from healthy and clinical mastitis cows. PLoS One 13:e0193671. doi: 10.1371/journal.pone. 0193671.
54. Derakhshani, H., Plaizier, J. C., De Buck, J., Barkema, H. W., and Khafipour, E. (2018). Association of bovine major histocompatibility complex (BoLA) gene polymorphism with colostrum and milk microbiota of dairy cows during the first week of lactation. Microbiome 6:203. doi: 10.1186/s40168-018-0586-1.
55. Derakhshani, H., Plaizier, J. C., De Buck, J., Barkema, H. W., and Khafipour, E. (2018). Composition of the teat canal and intramammary microbiota of dairy cows subjected to antimicrobial dry cow therapy and internal teat sealant. J. Dairy Sci. 101, 10191–10205. doi: 10.3168/jds.2018-14858.
56. Doyle, C. J., Gleeson, D., O'Toole, P. W., and Cotter, P. D. (2017). Impacts of seasonal housing and teat preparation on raw milk microbiota: a high throughput sequencing study. Appl. Environ. Microbiol. 83:e02694-16. doi: 10.1128/AEM.02694-16.
57. Frétin, M., Martin, B., Rifa, E., Isabelle, V.-M., Pomiès, D., Ferlay, A., et al. (2018). Bacterial community assembly from cow teat skin to ripened cheeses is influenced by grazing systems. Sci. Rep. 8:200. doi: 10.1038/s41598-017-18447-y.
58. Metzger, S. A., Hernandez, L. L., Skarlupka, J. H., Suen, G., Walker, T. M., and Ruegg, P. L. (2018). Influence of sampling technique and bedding type on the milk microbiota: results of a pilot study. J. Dairy Sci. 101, 6346–6356. doi:10.3168/jds.2017-14212.
59. Kumar, H., du Toit, E., Kulkarni, A., Aakko, J., Linderborg, K. M., Zhang, Y., et al. (2016). Distinct patterns in human milk microbiota and fatty acid profiles across specific geographic locations. Front.Microbiol.7:1619. doi: 10.3389/fmicb.2016.01619.
60. Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., York, M. A., Brooker, S. L., et al. (2017). Human milk microbial community structure is relatively stable and related to variations in macronutrient and micronutrient intakes in healthy lactating women. J. Nutr. 147, 1739–1748. doi: 10.3945/jn.117.248864.
61. Zhang, R., Huo, W., Zhu, W., and Mao, S. (2015). Characterization of bacterial community of raw milk from dairy cows during subacute ruminal acidosis challenge by high-throughput sequencing. J. Sci. Food Agric. 95, 1072–1079. doi:

10.1002/jsfa.6800.

62. Bradley, A. J., and Green, M. J. (2004). The importance of the nonlactating period in the epidemiology of intramammary infection and strategies for prevention. Vet. Clin. North Am. Food Anim. Pract. 20, 547–568. doi: 10.1016/j.cvfa.2004. 06.010.
63. Bonsaglia, E. C. R., Gomes, M. S., Canisso, I. F., Zhou, Z., Lima, S. F., Rall, V. L. M., et al. (2017). Milk microbiome and bacterial load following dry cow therapy without antibiotics in dairy cows with healthy mammary gland. Sci. Rep. 7:8067. doi: 10.1038/s41598-017-08790-5.
64. Derakhshani H, Fehr KB, Sepehri S, Francoz D, De Buck J, Barkema HW, Plaizier JC, Khafpour E. Invited review: microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. J Dairy Sci. 2018;101:10605–25.
65. Porcellato D, Meisal R, Bombelli A, Narvhus JA. A core microbiota dominates a rich microbial diversity in the bovine udder and may indicate presence of dysbiosis. Sci Rep. 2020;10:1–14.
66. Rainard P. Mammary microbiota of dairy ruminants: fact or fiction? Vet Res. 2017;48:1–10.
67. Oikonomou G, Machado VS, Santisteban C, Schukken YH, Bicalho RC. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. 2012.
68. Dickson RP, Hufnagle GB. The lung microbiome: new principles for respiratory bacteriology in health and disease. PLoS Pathog. 2015;11:e1004923.
69. Moossavi S, Azad MB. Origins of human milk microbiota: new evidence and arising questions. Gut Microbes. 2020;12:1667722.
70. Doyle CJ, Gleeson D, O'Toole PW, Cotter PD. Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study. Appl Environ Microbiol. 2017;83:e02694-e12616.
71. Metzger S, Hernandez L, Skarlupka J, Suen G, Walker T, Ruegg P. Infuence of sampling technique and bedding type on the milk microbiota: results of a pilot study. J Dairy Sci. 2018;101:6346–56.
72. Adnane M, Chapwanya A. A Review of the diversity of the genital tract microbiome and implications for fertility of cattle. Animals. 2022;12:460.
73. Bomar L, Brugger SD, Yost BH, Davies SS, Lemon KP. Corynebacterium accolens releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. mBio 7(1):e01725-15.
74. Braem G, Stijlemans B, Van Haken W, De Vliegher S, De Vuyst L, Leroy F. Antibacterial activities of coagulase-negative staphylococci from bovine teat

apex skin and their inhibitory effect on mastitis-related pathogens. J Appl Microbiol. 2014;116:1084–93.

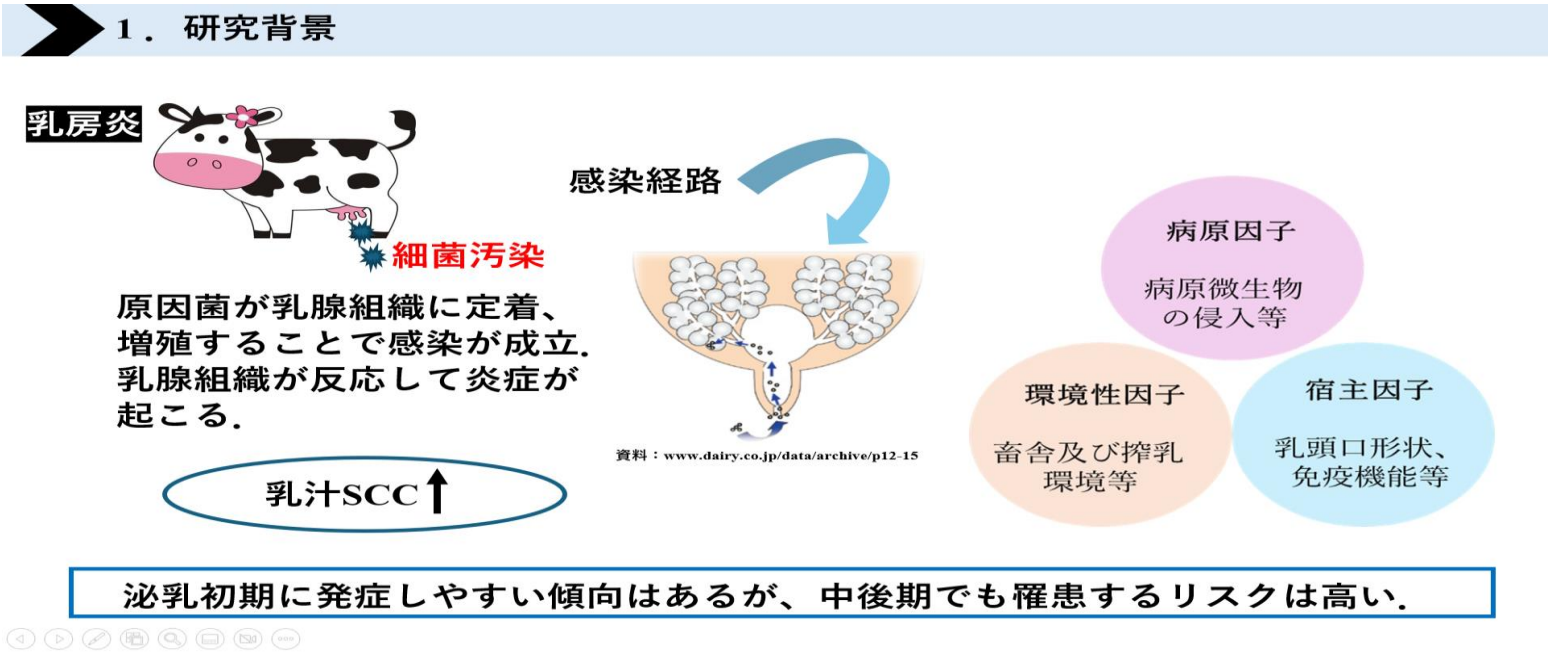
75. Woodward W, Besser T, Ward A, Corbeil L. In vitro growth inhibition of mastitis pathogens by bovine teat skin normal fora. Can J Vet Res. 1987;51:27.
76. Reinemann, D. J., and D. J. Smith. Evaluation of automatic milking systems for the United States. 2001. Pages 232–238 in Robotic Milking. H. Hogevee and A. Meijering, eds. Wageningen Pers, Wageningen, The Netherlands.
77. de Koning, K., Y. van der Vorst, and A. Meijering. Automatic milking experience and development in Europe. 2002. Pages I-1–I-11 in The First North American Conference on Robotic Milking, Wageningen Pers, Wageningen, The Netherlands.
78. Dijkhuizen, A. A., R. B. M. Huirne, S. B. Harsh, and R. W. Gardner. Economics of robotic application. Computers Electronics Agric. 1997.17:111–121.
79. Baines, J. Managing the change to a robotic milking system. 2002. Pages III-9III 17 in The First North American Conference on RoboticMilking, Wageningen Press, Wageningen, The Netherlands.
80. Klungel, G. H., B. A. Slaghuis, and H. Hogeveen, The effect of the introduction of automatic milking systems on milk quality. J. 2000. Dairy Sci. 83:1998–2003.
81. Shoshani, E., and M. Chaffer. Robotic milking: A report of a field trial in Israel. 2002.Pages III-56–III-63 in The First North American Conference on Robotic Milking, Wageningen Pers, Wageningen, The Netherlands.
82. Rasmussen, M. D., M. Bjerring, P. Justesen, and L. Jepsen. Milk quality on Danish farms with automatic milking systems. J. 2002.Dairy Sci. 85:2869–2878.
83. Barkema, H. W., M. A. G. von Keyserlingk, J. P. Kastelic, T. J. G. M. Lam, C. Luby, J.-P. Roy, S. J. LeBlanc, G. P. Keefe, and D. F. Kelton. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. 2015. J. Dairy Sci. 98:7426–7445. https://doi.org/10.3168/jds.2015-9377.
84. LeBlanc, S. J., K. D. Lissemore, D. F. Kelton, T. F. Duffield, and K. E. Leslie. Major advances in disease prevention in dairy cattle. 2006. J. Dairy Sci. 89:1267 1279. https://doi.org/10.3168/jds.S0022 -0302(06)72195-6.
85. Rutten, C. J., A. G. J. Velthuis, W. Steeneveld, and H. Hogeveen. Invited review: Sensors to support health management on dairy farms. 2013. J. Dairy Sci. 96:1928 1952. https://doi.org/10.3168/ jds.2012-6107.
86. Lind, A. K., P. Thomsen, S. Rintakoski, M. Espetvedt, C. Wolff, and H. Houe. The association between farmers' participation in herd health programmers and them behavior concerning treatment of mild clinical mastitis. 2012. Acta Vet. Scand. 54:62. https://doi.org/ 10.1186/1751-0147-54-62.
87. Derks, M., T. van Werven, H. Hogeveen, and W. D. J. Kremer. Veterinary herd health management programs on dairy farms in the Netherlands: Use, execution,

and relations to farmer characteristics. 2013. J. Dairy Sci. 96:1623–1637. https://doi.org/10.3168/jds .2012-6106.

88. Green, M., L. Green, J. Huxley, J. Statham, and S. Statham. Concepts in dairy herd health. 2012. Pages 1–9 in Dairy Herd Health. Hall, J., and W. W M. Green, ed. CABI.
89. Lam, T. J. G. M., J. Jansen, B. H. P. Van den Borne, R. J. Renes, and H. Hogeveen. What veterinarians need to know about communication to optimise their role as advisor on udder health in dairy herds. 2011. N. Z. Vet. J. 59:8–15. https://doi.org/10.1080/ 00480169.2011.547163.
90. Cannas da Silva, J., J. P. T. M. Noordhuizen, M. Vagneur, R. Bexiga, C. C. Gelfert, and W. Baumgartner. Veterinary dairy herd health management in Europe. Constraints and perspectives. 2006. Vet. Q. 28:23–32. https://doi.org/10.1080/01652176.2006.9695203.
91. Denis-Robichaud, J., R. L. A. Cerri, A. Jones-Bitton, and S. J. LeBlanc. Survey of reproduction management on Canadian dairy farms. 2016. J. Dairy Sci. 99:9339–9351. https://doi.org/10.3168/jds .2016-11445.
92. Luby, C. D., K. McIntyre, and M. D. Jelinski. Skills required of dairy veterinarians in western Canada: A survey of practicing veterinarians. 2013. Can. Vet. J. 54:267–270.
93. Metzger, S. A., L. L. Hernandez, G. Suen, and P. L. Ruegg. Understanding the milk microbiota. 2018. Vet. Clin. North Am. Food Anim. Pract. 34:427438. https://doi.org/10.1016/j.cvfa.2018.06.003.
94. Salter, S. J., M. J. Cox, E. M. Turek, S. T. Calus, W. O. Cookson, M. F. Moffatt, P. Turner, J. Parkhill, N. J. Loman, and A. W. Walker. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. 2014. BMC Biol. 12:87. https://doi.org/10.1186/s12915-014-0087-z.
95. Pollock, J., L. Glendinning, T. Wisedchanwet, and M. Watson. The madness of microbiome: Attempting to find consensus “best practice” for 16S microbiome studies. 2018. Appl. Environ. Microbiol.84:e0262717. https:// doi.org/10. 1128/AEM.0262717.
96. Rainard, P. Mammary microbiota of dairy ruminants: Fact or fiction? 2017.Vet. Res. 48:25. https://doi.org/10.1186/s13567-017-0429-2.
97. Barkema, H. W., Y. H. Schukken, and R. N. Zadoks. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis. 2006.J. Dairy Sci. 89:1877–1895.
98. Melchior, M. B., H. Vaarkamp, and J. Fink-Gremmels. Biofilms: A role in recurrent mastitis infections? 2006. Vet. J. 171:398–407.

Appendix

Appendix A: Research background



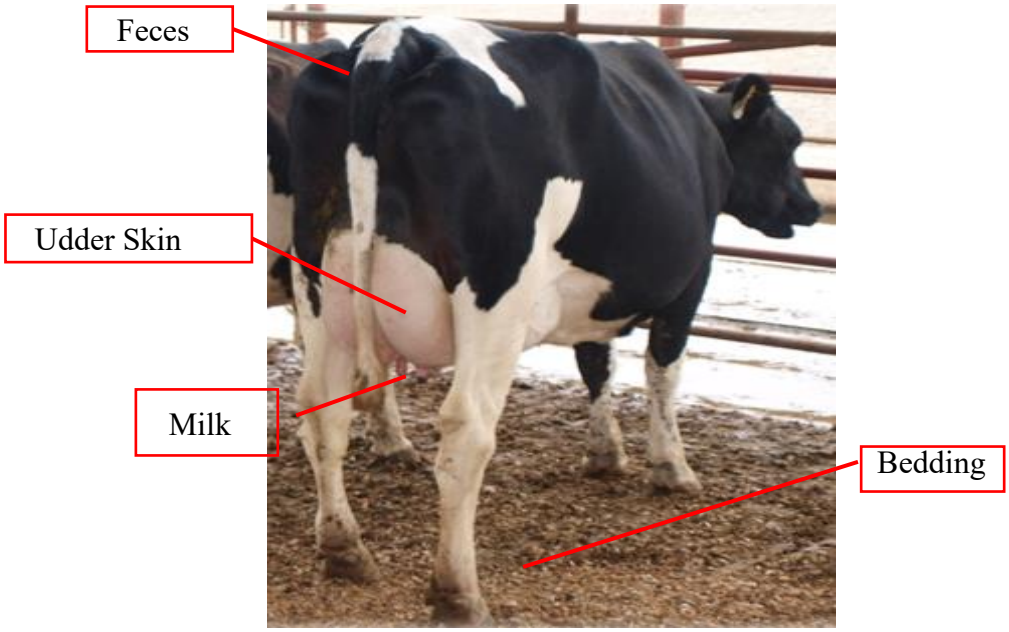
mastitis, which is an inflammation of the mammary gland and udder tissue. It begins with bacterial contamination through the teat canal into the breast. The infection is established as bacteria settle and proliferate within the mammary tissue, causing an inflammatory reaction that increases somatic cells in the milk. The primary cause of mastitis is the invasion of pathogens into the breast, but other factors such as the barn and milking environment, as well as the cow's immune function and the shape of the teat, also contribute to the development of the condition.

Appendix B: Causes and prevention of mastitis.

Mastitis pathogens and the infection routes

	Pathogens	Source of infection	Route of infection	Control measures
Contagious mastitis	*Staphylococcus aureus* (SA)	Infected udder milk, Injured teat	Cow (quarter) to cow (quarter) via hand, teat, cleaning towel, and milker during milking	Proper milking procedure, Milking order (infected cows come later), Teat dip, Treatment at dry-off period
	Streptococcus agalactiae (SAG)	Infected udder milk, Injured teat	Cow (quarter) to cow (quarter) via hand, teat, cleaning towel, and milker during milking	Proper milking procedure, Milking order (infected cows come later), Teat dip, Treatment at dry-off period
	Mycoplasma bovis (MB)	Infected udder milk (partially respiratory)	Cow (quarter) to cow (quarter) via hand, teat, cleaning towel, and milker during milking (partially hematogenous)	Isolation, Culling, Management of introduced cows and herd health
Environmental mastitis	Other Streptococci (OS)	Environment (bedding, feces), Infected udder milk	Environment to teats	Maintenance and management of cowsheds and barn surroundings, Teat dip, Treatment at dry-off period, Maintenance of the milker
	Coliforms (CO)	Environment (bedding, feces), Infected udder milk	Environment to teats	Maintenance and management of cowsheds and barn surroundings
	Coagulase-negative Staphylococci (CNS)	Environment (bedding, feces), Infected udder milk, Indigenous in udder and teats	Environment to teats	Proper milking procedure and hygiene management
	Pseudomonas aeruginosa (PA)	Contaminated water, Environment (bedding, feces)	Environment to teats	Proper milking procedure and hygiene management
	Prototheca	Contaminated water, Environment (bedding, feces)	Environment to teats	Hygienic management inside and outside of cowsheds

Appendix C: Sample collection.



Samples were collected from 10 Holstein cows, Sampling was Sep in 2018 and in Aug 2020. which operated automatic milking systems for management.

Milk:

Protein, fat, MUN, SNF, SCC.

Blood metabolism:

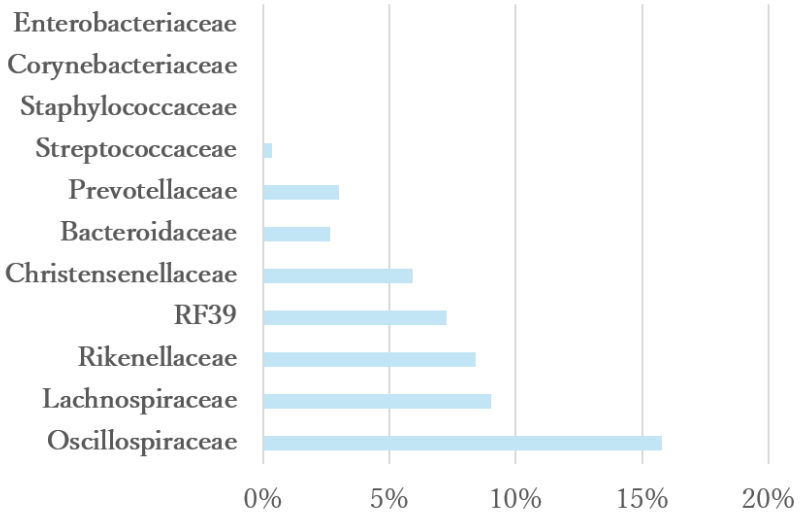
cholesterol, NEFA, albumin,BUN, ALT, AST, haptoglobin.

Bacterial flora (16S amplicon analysis):

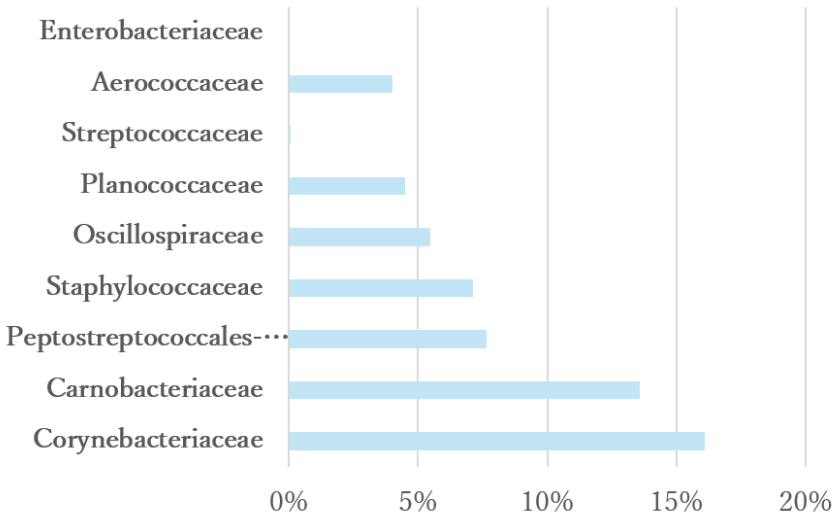
Feces, cow bedding, milk, udder skin.

Appendix D: Result of Experiment 1.

Top 5 species of fecal flora and causative bacteria

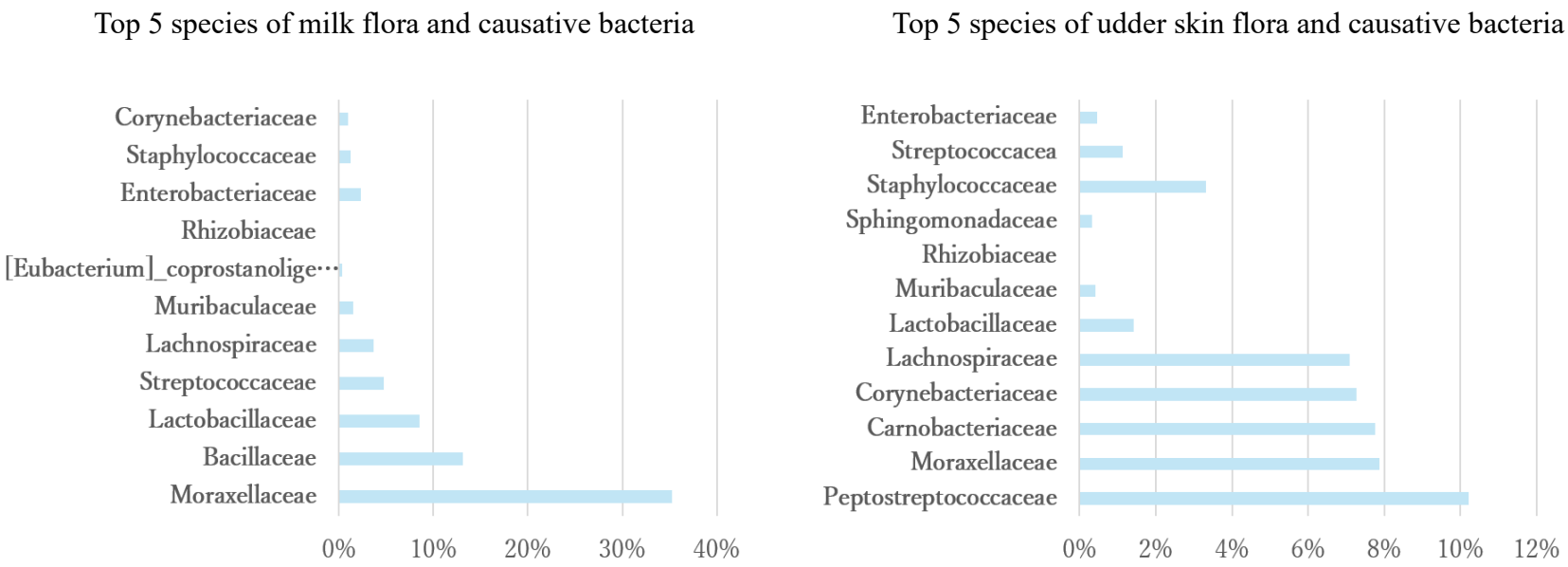


Top 5 species of bedding flora and causative bacteria



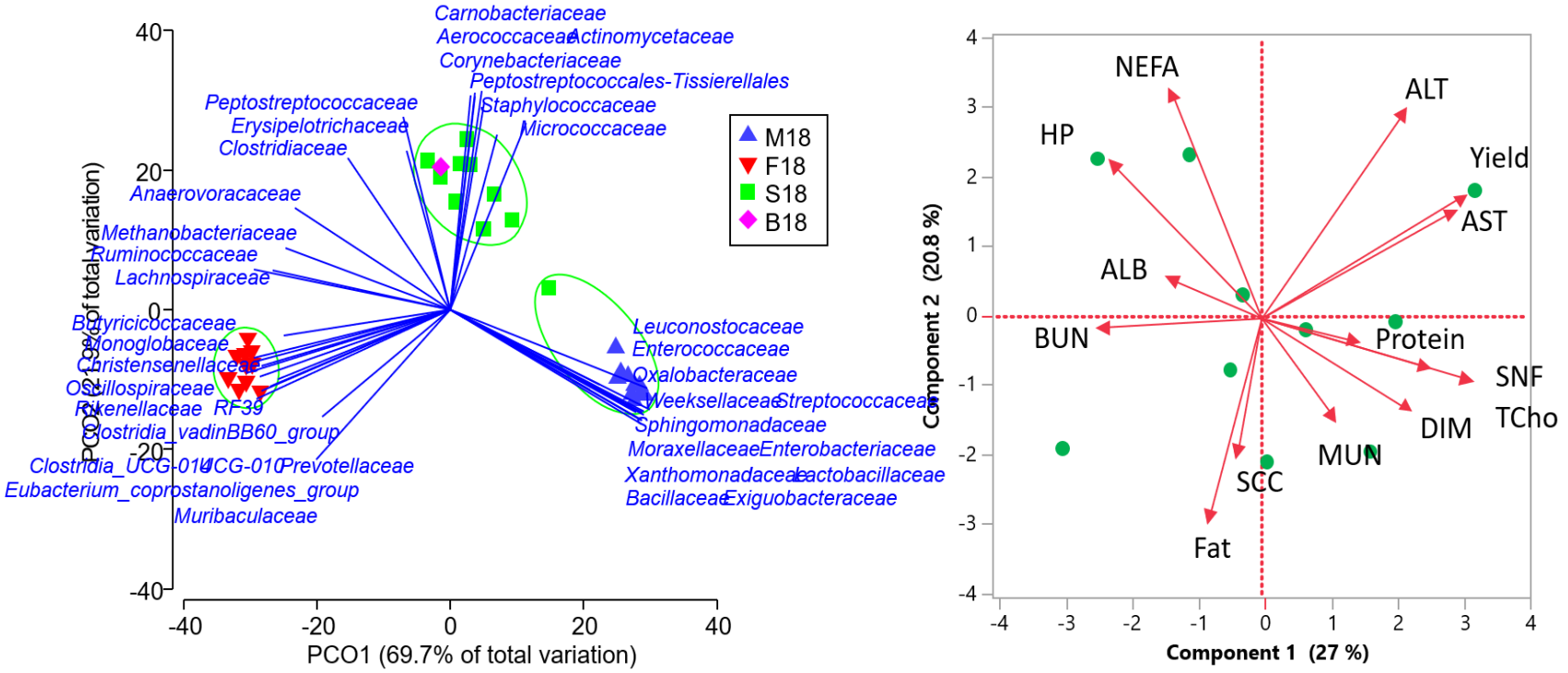
Top 5 bacterial species and causative bacteria in feces and bedding flora.

The top five bacterial species in the fecal flora are Oscillospiraceae (16%);Lachnospiraceae (9%), Rikenellaceae (8%),RF39 (7%), Christensenellaceae (6%);Corynebacteriaceae (16%) in cow beds;Carnobacteriaceae (14%),Peptostreptococales-Tischierellales (8%),Staphylococcalaceae (7%), Oscillospiraceae (6%).



Top5 bacterial species and causative bacteria in milk and udder skin flora.

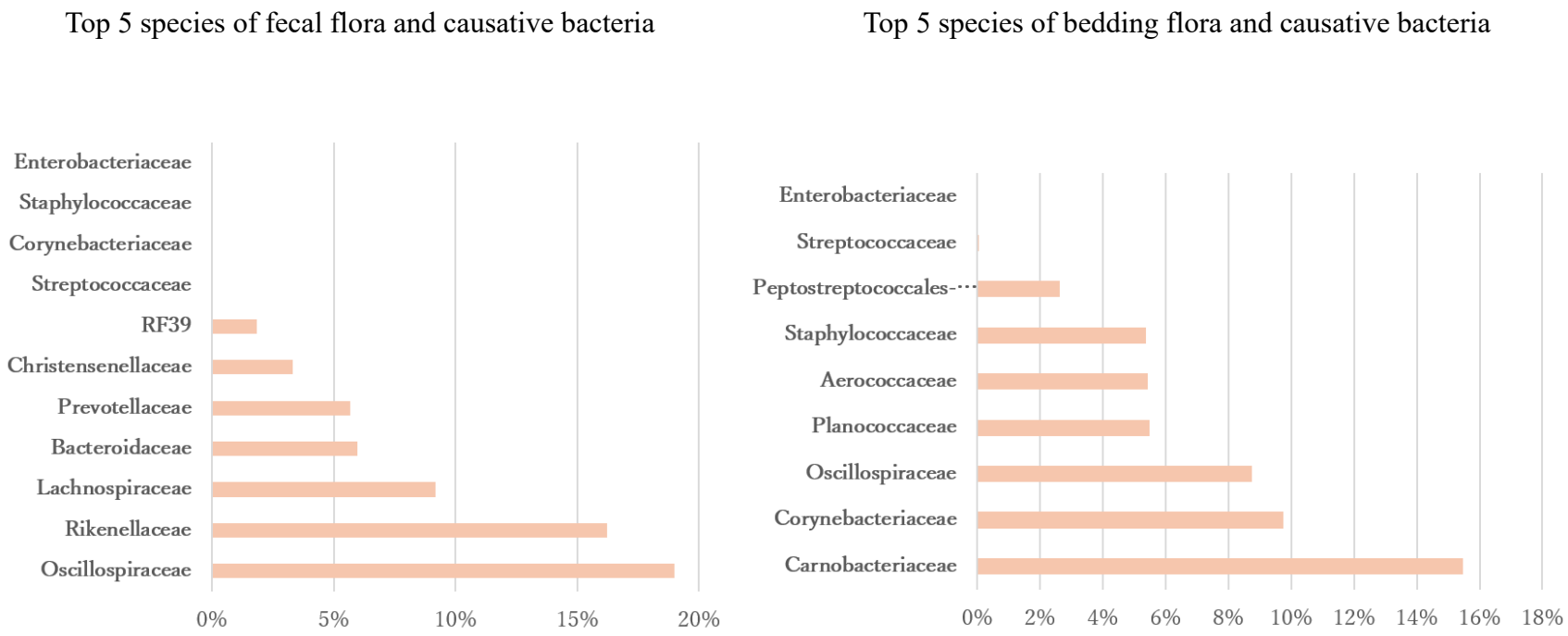
The five most abundant families of milk microbiota were Moraxellaceae (35.3%), Bacillaceae (13.1%), Lactobacillaceae (8.5%), Streptococcaceae (4.7%), and Lachnospiraceae (3.7%), and those of udder skin microbiota were Peptostreptococcaceae (10.2%), Moraxellaceae (7.9%), Carnobacteriaceae (7.8%), Corynebacteriaceae (7.3%),and Lachnospiraceae (7.1%).



We then performed principal component analysis on the bacterial groups of each sample. The graph on the left is a principal component analysis (PCA) showing the classification of microbiota.is a graph.

These results indicate that the bacterial flora of cow bedding and udder skin are one. You can see that you belong to a group, Milk and feces form separate groups. This is because the milk flora is influenced by the environment and the breast skin flora. Suggesting that it is unaffected and has its own characteristics and the reason why cow bedding and udder skin are classified into the same group is as follows.

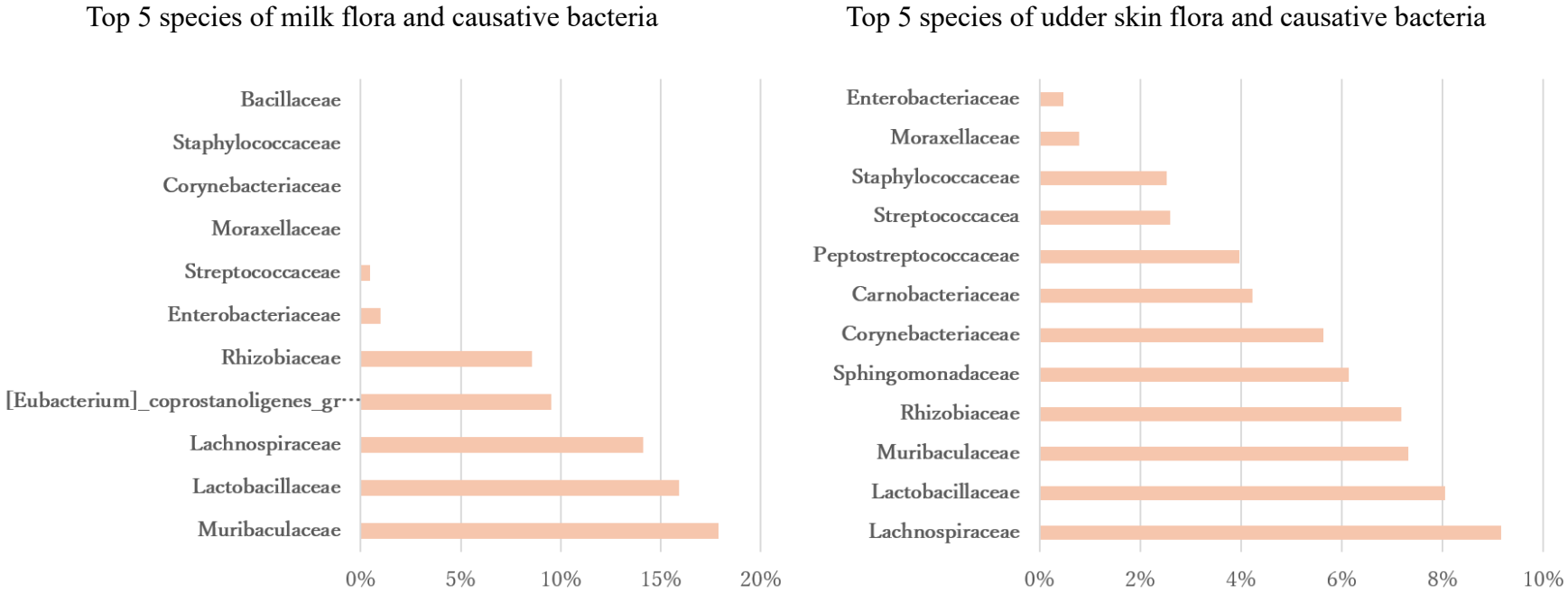
Appendix D: Result of Experiment 2.



Top 5 bacterial species and causative bacteria in feces and bedding flora.

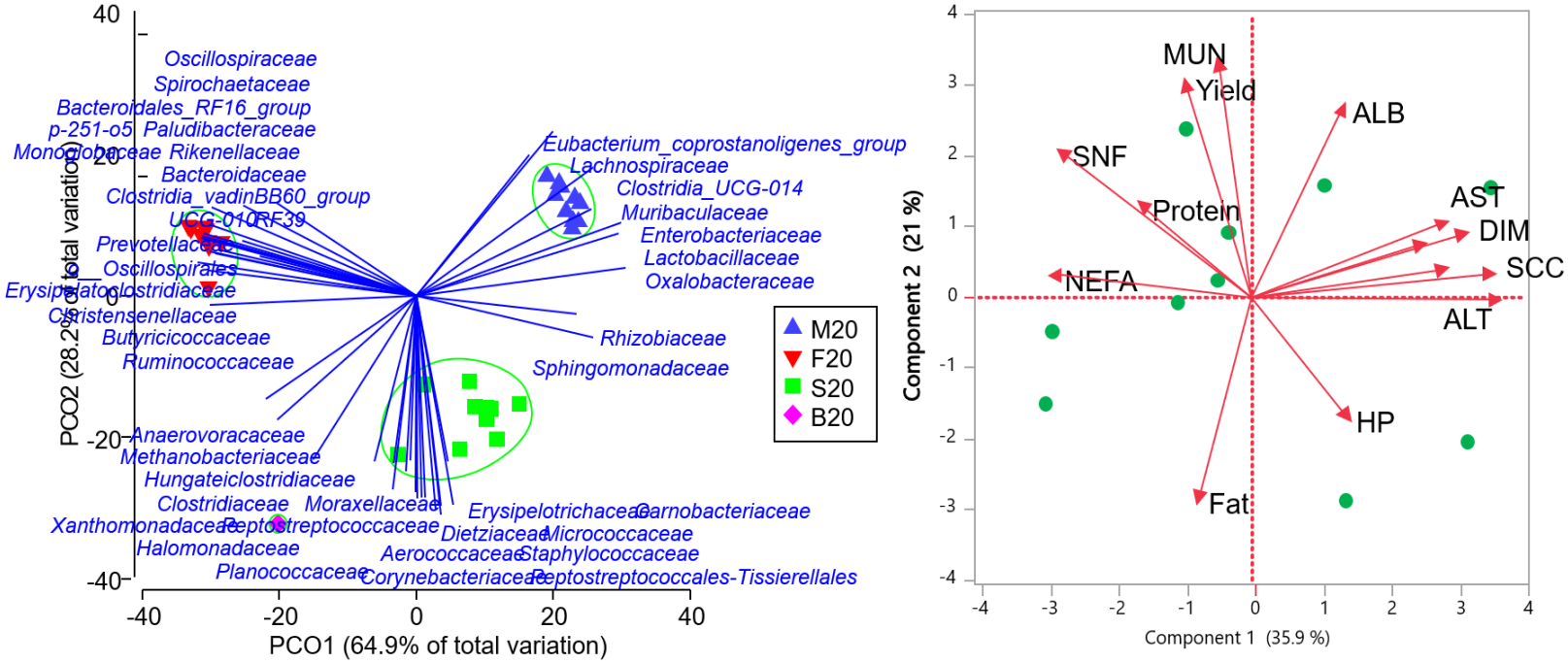
sampled in 2020, i.e. in Experiment 2.The results for fecal and cow bedding microbiota are as follows:

The bacterial flora of feces had the highest proportion Oscillospiraceae (19.0%), Rikenellaceae (16.2%),Lachnospiracea (9.2%), Bacteroidetaceae (6.0%),It was Prevotella care (5.7%).On the other hand, the bacterial flora of the cow bedding included Carnobacteriaceae (15.5%);Corynebacterium (9.7%), Oscillospiracea (8.8%),Planococcalaceae (5.5%), Aerococcaceae (5.4%).



Top5 bacterial species and causative bacteria in milk and udder skin flora.

The most abundant bacterial flora in milk and breast skin the bacterial species are as follows. In milk, Muribakuracareae was the most common at 17.9%.Lactobacillaceae followed with 15.9%;Lachnospiraceae family 14.1%, Eubacterium coprostanoligenes group 9.5%,Rhizobia care department accounted for 8.6%.On the other hand, in the breast skin bacterial flora, Lachnospiraceae is the most abundant at 9.2%.Lactobacillaceae accounted for 8.1%, Muribacillaceae accounted for 7.3%;Rhizobiaceae accounted for 7.2%, and Sphingomonadaceae accounted for 6.1%.



In Experiment 2, we also performed principal component analysis on the bacterial groups of each sample. The graph on the left shows the results of principal component analysis (PCA) of the bacterial flora. The four different sample bacterial flora formed different groups. The results of this PCA indicate that each sample group has a unique microbial composition.is showing.From this result, cow bedding, udder skin, milk and feces are they each form an independent group.

Appendix E: Changes in microbial flora between 2018 and 2020.

Phylum-Level Relative Proportions of Bacterial Communities in Bedding, Feces, and Milk Samples (a) (2018 vs. 2020)

#OTU ID	bedding（2018）	bedding（2020）	feces（2018）	feces (2020)	milk（2018）	milk（2020）
Firmicutes	63.05%	66.73%	72.67%	58.23%	48.23%	53.64%
Proteobacteria	5.20%	7.07%	0.42%	0.26%	41.81%	21.90%
Actinobacteriota	21.54%	12.21%	0.67%	0.29%	1.83%	0.07%
Bacteroidota	8.93%	12.52%	19.65%	38.54%	5.56%	24.29%
Patescibacteria	0.01%	0.00%	0.17%	0.00%	0.01%	0.00%
Spirochaetota	0.26%	0.39%	2.15%	1.73%	0.06%	0.01%
Planctomycetota	0.01%	0.00%	0.05%	0.00%	0.02%	0.00%
Verrucomicrobiota	0.12%	0.15%	0.53%	0.03%	0.43%	0.00%
Deinococcota	0.00%	0.00%	0.00%	0.00%	0.46%	0.00%
WPS-2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria	0.06%	0.05%	0.16%	0.03%	0.67%	0.01%
Euryarchaeota	0.36%	0.40%	3.45%	0.80%	0.12%	0.02%
Desulfobacterota	0.26%	0.21%	0.01%	0.03%	0.30%	0.00%
Campilobacterota	0.18%	0.23%	0.00%	0.00%	0.05%	0.00%
Thermoplasmatota	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synergistota	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chloroflexi	0.00%	0.00%	0.04%	0.00%	0.02%	0.00%
Bacteria	0.00%	0.00%	0.00%	0.01%	0.03%	0.00%
Unassigned	0.00%	0.00%	0.00%	0.00%	0.13%	0.06%

Phylum-Level Relative Proportions of Bacterial Communities in Bedding, Feces, and Milk Samples (b) (2018 vs. 2020)

Bdellovibrionota	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
Nanoarchaeota	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
Fibrobacterota	0.02%	0.02%	0.02%	0.03%	0.00%	0.00%
Fusobacteriota	0.00%	0.01%	0.00%	0.00%	0.21%	0.00%
Deferribacterota	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
Elusimicrobiota	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%
Halobacterota	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%
Thermotogota	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cloacimonadota	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Arthropoda	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Vertebrata	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteriota	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
RCP2-54	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
SAR324_clade(MarinegroupB)	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Dependentiae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Myxococcota	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

This table presents the comparative analysis of bacterial community composition at the phylum level in bedding, feces, and milk samples collected in the years 2018 and 2020. The data is expressed as a percentage, representing the relative abundance of each bacterial phylum within the samples. The analysis highlights the changes and trends in microbial populations over the two-year period, offering insights into the dynamics of bacterial ecosystems in agricultural settings. Such information is crucial for understanding the impact of microbiota on animal health, product quality, and environmental interactions.

Family-Level Relative Proportions of Bacterial Communities in Bedding, Feces, and Milk Samples(a) (2018 vs. 2020)

family	Milk (2018)	Milk (2020)	Feces (2018)	Feces (2020)	Skin (2018)	skin (2020)	Bedding (2018)	Bedding (2020)
[Eubacterium]_coprostanoligenes_group	0.3%	9.5%	4.6%	4.5%	0.9%	4.1%	1.4%	1.8%
Clostridia_UCG-014	0.4%	6.3%	4.0%	1.6%	0.7%	2.5%	0.8%	0.8%
Enterobacteriaceae	2.3%	1.0%	0.0%	0.0%	0.5%	0.5%	0.0%	0.0%
Lachnospiraceae	3.7%	14.1%	9.0%	9.2%	7.1%	9.2%	3.9%	4.9%
Micrococcaceae	0.2%	0.0%	0.0%	0.0%	0.8%	0.4%	2.8%	1.3%
UCG-010	0.1%	0.0%	2.3%	4.6%	0.2%	0.3%	0.7%	1.3%
Ruminococcaceae	1.1%	0.6%	5.2%	3.9%	3.7%	1.9%	1.4%	1.9%
Oscillospiraceae	2.2%	3.4%	15.8%	19.0%	4.5%	4.3%	5.5%	8.8%
Erysipelatoclostridiaceae	0.2%	0.0%	0.8%	0.5%	0.3%	0.1%	0.2%	0.2%
RF39	0.3%	1.0%	7.3%	1.9%	1.1%	0.7%	1.3%	1.2%
Moraxellaceae	35.3%	0.1%	0.1%	0.0%	7.9%	0.8%	2.0%	4.3%
Butyricicoccaceae	0.1%	0.0%	0.7%	0.8%	0.3%	0.1%	0.4%	0.4%
Erysipelotrichaceae	3.1%	1.2%	3.9%	1.1%	5.5%	2.3%	1.9%	1.9%
Lactobacillaceae	8.5%	15.9%	0.1%	0.0%	1.4%	8.1%	0.0%	0.0%
Muribaculaceae	1.5%	16.0%	3.4%	2.4%	0.4%	7.3%	0.5%	0.8%
Bacteroidaceae	0.8%	2.9%	2.7%	6.0%	1.4%	2.4%	2.3%	3.0%
Peptostreptococcales-Tissierellales	0.4%	0.0%	0.0%	0.0%	4.4%	1.4%	7.7%	2.6%
Bacteroidales_RF16_group	0.1%	0.0%	0.6%	4.7%	0.2%	0.3%	1.0%	1.6%
Christensenellaceae	0.4%	0.0%	5.9%	3.3%	1.7%	0.7%	1.1%	1.2%

Family-Level Relative Proportions of Bacterial Communities in Bedding, Feces, and Milk Samples(b) (2018 vs. 2020)

Monoglobaceae	0.1%	0.0%	1.8%	2.4%	0.4%	0.2%	0.4%	0.4%
Sphingomonadaceae	1.5%	6.0%	0.0%	0.0%	0.3%	6.1%	0.0%	0.0%
Anaerovoracaceae	0.2%	0.3%	2.4%	1.0%	2.3%	0.9%	0.8%	0.9%
Rikenellaceae	0.7%	3.1%	8.4%	16.2%	0.8%	2.3%	2.5%	3.7%
Staphylococcaceae	1.2%	0.0%	0.0%	0.0%	3.3%	2.5%	7.1%	5.4%
Spirochaetaceae	0.1%	0.0%	2.2%	1.7%	0.1%	0.1%	0.3%	0.4%
Prevotellaceae	0.2%	0.4%	3.0%	5.7%	0.4%	0.8%	1.0%	1.7%
Aerococcaceae	0.4%	0.0%	0.0%	0.0%	6.6%	4.5%	4.0%	5.4%
Actinomycetaceae	0.0%	0.0%	0.0%	0.0%	0.3%	0.4%	0.5%	0.2%
o__Oscillospirales	0.0%	0.0%	0.3%	0.2%	0.1%	0.0%	0.1%	0.1%
Clostridia_vadinBB60_group	0.1%	0.0%	0.3%	0.9%	0.0%	0.0%	0.1%	0.1%
Exiguobacteraceae	1.5%	0.0%	0.0%	0.0%	0.3%	0.0%	0.0%	0.0%
Rhizobiaceae	0.0%	8.6%	0.0%	0.0%	0.0%	7.2%	0.0%	0.0%
Succinivibrionaceae	0.1%	0.0%	0.3%	0.1%	0.4%	0.1%	0.5%	0.5%
Carnobacteriaceae	0.8%	0.0%	0.0%	0.0%	7.8%	4.2%	13.6%	15.5%
Beijerinckiaceae	0.0%	0.4%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%
Akkermansiaceae	0.4%	0.0%	0.5%	0.0%	0.1%	0.0%	0.1%	0.0%
p-251-o5	0.0%	0.0%	0.2%	0.1%	0.0%	0.0%	0.0%	0.1%
Bifidobacteriaceae	0.3%	0.0%	0.3%	0.2%	0.4%	0.8%	0.1%	0.2%
Bacillaceae	13.1%	0.0%	0.0%	0.0%	2.0%	0.1%	1.3%	1.5%
Streptococcaceae	4.7%	0.5%	0.4%	0.0%	1.1%	2.6%	0.1%	0.1%

Family-Level Relative Proportions of Bacterial Communities in Bedding, Feces, and Milk Samples (c) (2018 vs. 2020)

Corynebacteriaceae	1.0%	0.1%	0.0%	0.0%	7.3%	5.6%	16.1%	9.7%
Methanobacteriaceae	0.1%	0.0%	3.4%	0.8%	2.6%	0.8%	0.4%	0.4%
Dietziaceae	0.0%	0.0%	0.0%	0.0%	0.2%	0.1%	1.2%	0.4%
Planococcaceae	0.8%	0.0%	0.0%	0.0%	2.1%	1.7%	4.5%	5.5%
Oxalobacteraceae	0.1%	5.8%	0.0%	0.0%	0.0%	3.7%	0.0%	0.0%
Xanthomonadaceae	1.7%	0.0%	0.0%	0.0%	0.3%	0.0%	0.1%	0.1%
Enterococcaceae	1.0%	0.0%	0.0%	0.0%	0.3%	0.0%	0.1%	0.1%
Paludibacteraceae	0.0%	0.0%	0.1%	2.4%	0.1%	0.2%	0.5%	0.9%
Halomonadaceae	0.0%	0.0%	0.0%	0.0%	0.3%	0.2%	1.7%	1.1%
Weeksellaceae	1.4%	0.0%	0.0%	0.0%	0.3%	0.0%	0.0%	0.0%
Clostridiaceae	0.4%	0.4%	1.9%	0.7%	2.6%	1.1%	0.7%	0.9%
Peptostreptococcaceae	1.5%	0.0%	4.4%	1.2%	10.2%	4.0%	1.6%	2.4%
Hungateiclostridiaceae	0.0%	0.0%	0.4%	0.1%	0.3%	0.1%	1.3%	0.5%
p-2534-18B5_gut_group	0.0%	0.0%	0.6%	0.2%	0.0%	0.0%	0.1%	0.1%
Leuconostocaceae	0.1%	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%

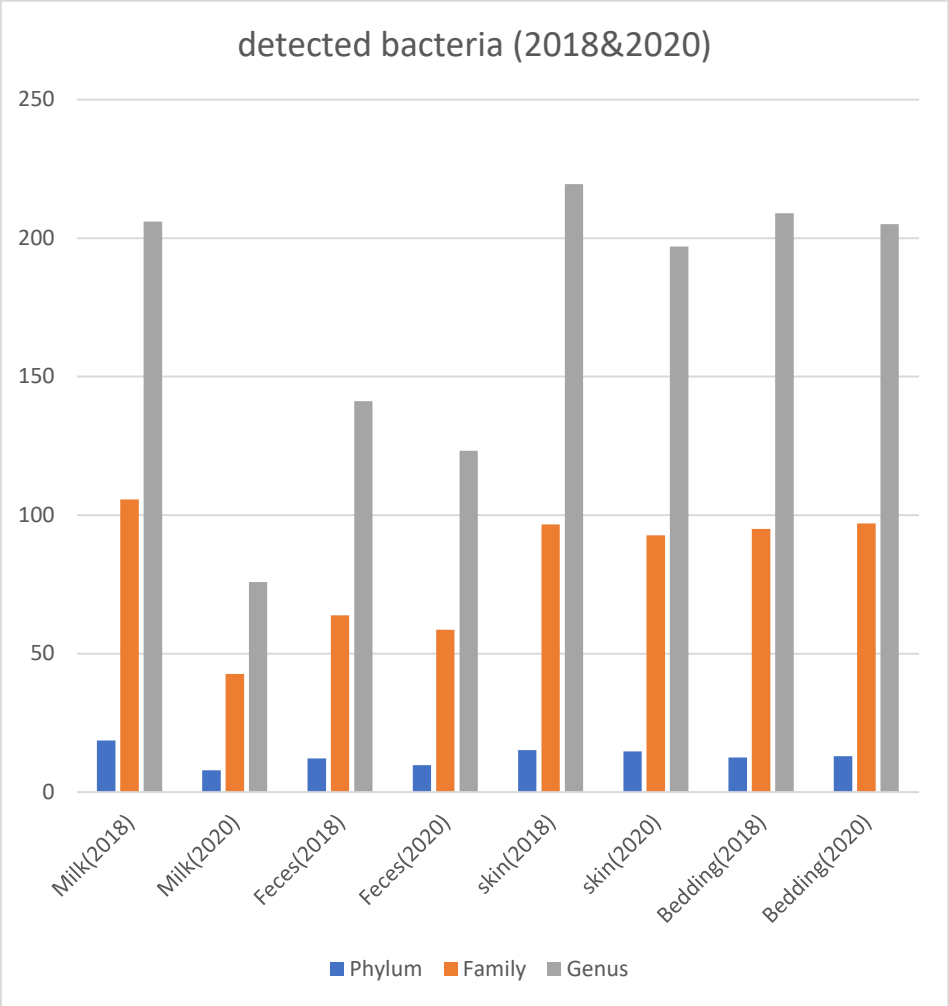
The percentages reflect the distribution of each microbial family within the sample types across the two years. Notably, this data provides insight into the microbial dynamics and possible shifts in the microbiota due to environmental changes, management practices, or other factors over the two-year span. The table facilitates a comparative understanding of the microbiome's complexity and its temporal variations, which could have implications for animal health, hygiene practices, and overall microbial ecology within an agricultural setting.

milk top5 (2018&2020、family level)			
family	**2018 year**	**2020 year**	**P-value**
Moraxellaceae	35.3%	0.1%	**
Bacillaceae	13.1%	0.0%	**
Lactobacillaceae	8.5%	15.9%	**
Streptococcaceae	4.7%	0.5%	**
Lachnospiraceae	3.7%	14.1%	**
Muribaculaceae	1.5%	17.9%	**
[Eubacterium]_coprostanoligenes_group	0.3%	9.5%	**
Rhizobiaceae	0.02%	8.6%	**
Enterobacteriaceae	2.3%	1.0%	**
Staphylococcaceae	1.2%	0.03%	**
Corynebacteriaceae	1.0%	0.1%	**

The table provides a comparative analysis of the top microbial families present in milk samples from 2018 to 2020, revealing significant shifts in their relative abundances. In 2018, the most predominant family was Moraxellaceae, constituting 35.3% of the microbial population, which dramatically decreased to only 0.1% in 2020.

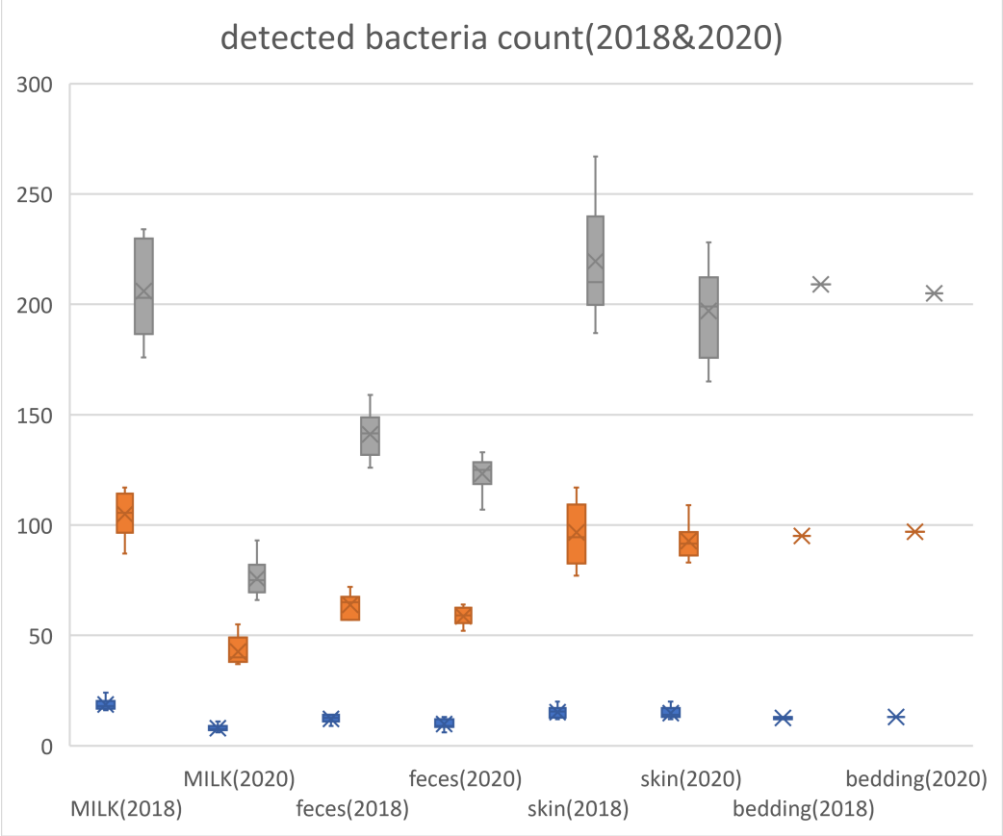
feces top5 (2018&2020 、family level)			
family	**2018 year**	**2020 year**	**P-value**
Oscillospiraceae	15.8%	19%	*
Lachnospiraceae	9.0%	9.2%	NS
Rikenellaceae	8.4%	16.2%	**
RF39	7.3%	1.9%	**
Christensenellaceae	5.9%	3.3%	**
Ruminococcaceae	5.2%	3.9%	**
Bacteroidaceae	2.7%	6.0%	**
Prevotellaceae	3.0%	5.7%	**
Streptococcaceae	0.35%	0.03%	NS

The table shows a comparison of the top microbial families found in feces samples from 2018 to 2020 with an emphasis on the top five by relative abundance. Oscillospiraceae remained the most abundant family, increasing from 15.8% to 19%, with the change being statistically significant (denoted by a single asterisk). Lachnospiraceae's presence remained stable, showing no significant difference (NS) between the two years. Rikenellaceae more than doubled, jumping from 8.4% to 16.2%, indicating a major change in the fecal microbiota, marked as highly significant with double asterisks.



Comparison of Bacterial Detection Levels by Taxonomic Rank in Various Samples (2018 vs. 2020)"

The chart visualizes the quantity of detected bacteria across different taxonomic ranks—phylum, family, and genus—in milk, feces, skin, and bedding samples collected in the years 2018 and 2020. In each category, the data are displayed in pairs to directly compare the two years. It is evident that, regardless of the sample source, the number of detected bacterial taxa at the phylum level tends to be the lowest, with an increase in numbers at the family level, and the highest diversity observed at the genus level. This pattern holds true for both years across all sample types. Notable trends include a general increase in bacterial diversity from 2018 to 2020 in most sample types and taxonomic ranks, except for a decrease in genera detected in feces. This comprehensive overview suggests a dynamic microbial environment with shifting population structures over the two-year period, which may reflect changes in environmental conditions, sampling techniques, or analytical sensitivity.

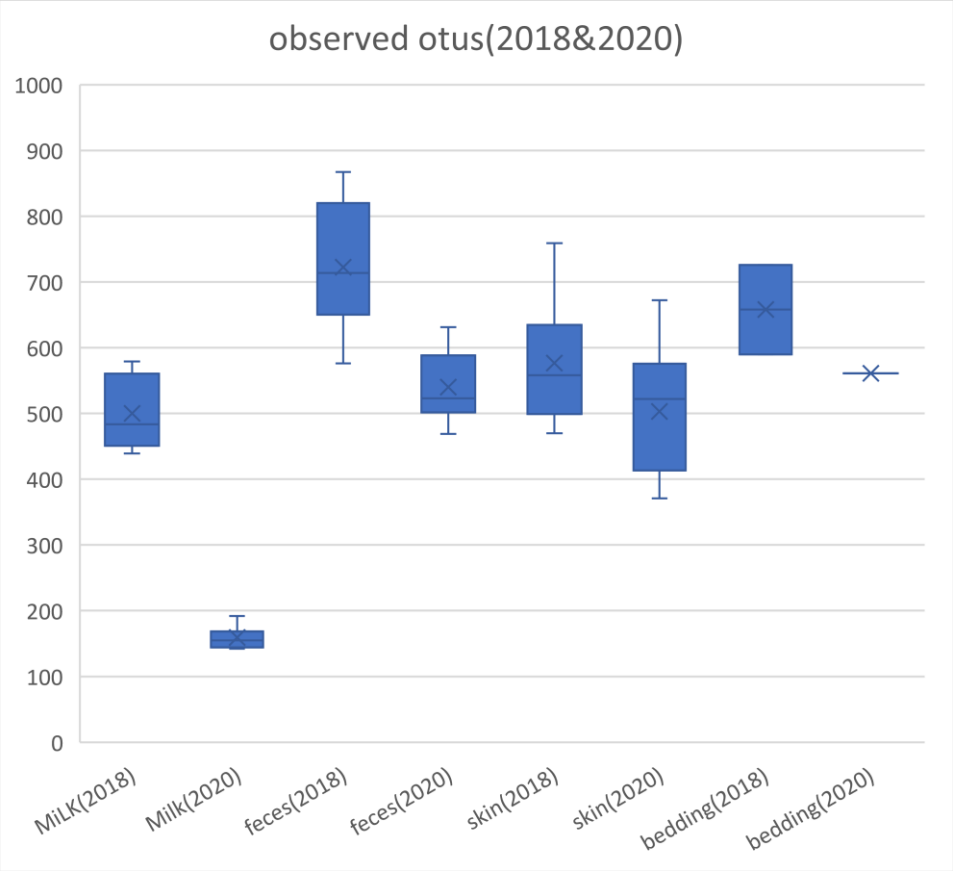


Detected Bacterial Count in Various Samples (2018 vs. 2020)

The graph presents a boxplot comparison of the bacterial count detected in milk, feces, skin, and bedding samples for the years 2018 and 2020. Each boxplot shows the range of variability (the vertical lines or "whiskers" indicating the extent of the data), the median of the dataset (the horizontal line within the box), and potential outliers (indicated by "X" symbols).

In 2018, milk samples exhibit a wide range of bacterial counts, significantly decreasing in 2020, as shown by the lower median and reduced variability. Feces samples show a similar trend with a notable decrease in bacterial variety. For skin samples, the data suggest an increase in bacterial counts from 2018 to 2020, indicated by higher medians and variability. Bedding samples also show an increased range of bacterial counts in 2020 compared to 2018, albeit with a lower median count.

These visual representations indicate that while there is a decrease in detected bacteria in milk and feces from 2018 to 2020, there's an increase in skin and bedding samples. These trends may be indicative of shifts in microbial populations, changes in sampling methods, or differences in environmental conditions between the two years. The outliers suggest the presence of samples with bacterial counts significantly different from the general data set, which may warrant further investigation.



Observed Operational Taxonomic Units (OTUs) in Various Samples (2018 vs. 2020)

The chart displays the number of observed operational taxonomic units (OTUs) within milk, feces, skin, and bedding samples, comparing data from 2018 and 2020. OTUs are a measure of microbial diversity, used to classify groups of closely related individuals.

In 2018, milk samples had significantly fewer OTUs as depicted by the narrow boxplot, which drastically expanded in 2020, indicating a substantial increase in microbial diversity. Fecal samples show a moderate increase in OTUs, with higher medians and wider ranges in 2020, suggesting increased diversity. Skin samples demonstrate a slight increase in diversity from 2018 to 2020, while bedding samples exhibit a notable increase in OTUs, with the 2020 data showing a wider range and higher median.

The boxplots illustrate the interquartile range (IQR) of the data, with the top and bottom of the boxes representing the third and first quartiles, respectively, and the horizontal line within the box representing the median. Whiskers extend to 1.5 times the IQR, and outliers are represented by 'X' markers.

Overall, the observed OTUs suggest that the microbial diversity in all sampled environments increased from 2018 to 2020, with the most pronounced changes seen in milk and bedding samples. This may be attributed to variations in environmental conditions, microbial ecosystem changes, or improvements in detection and sequencing methods.

Summary table of alpha diversity（a）

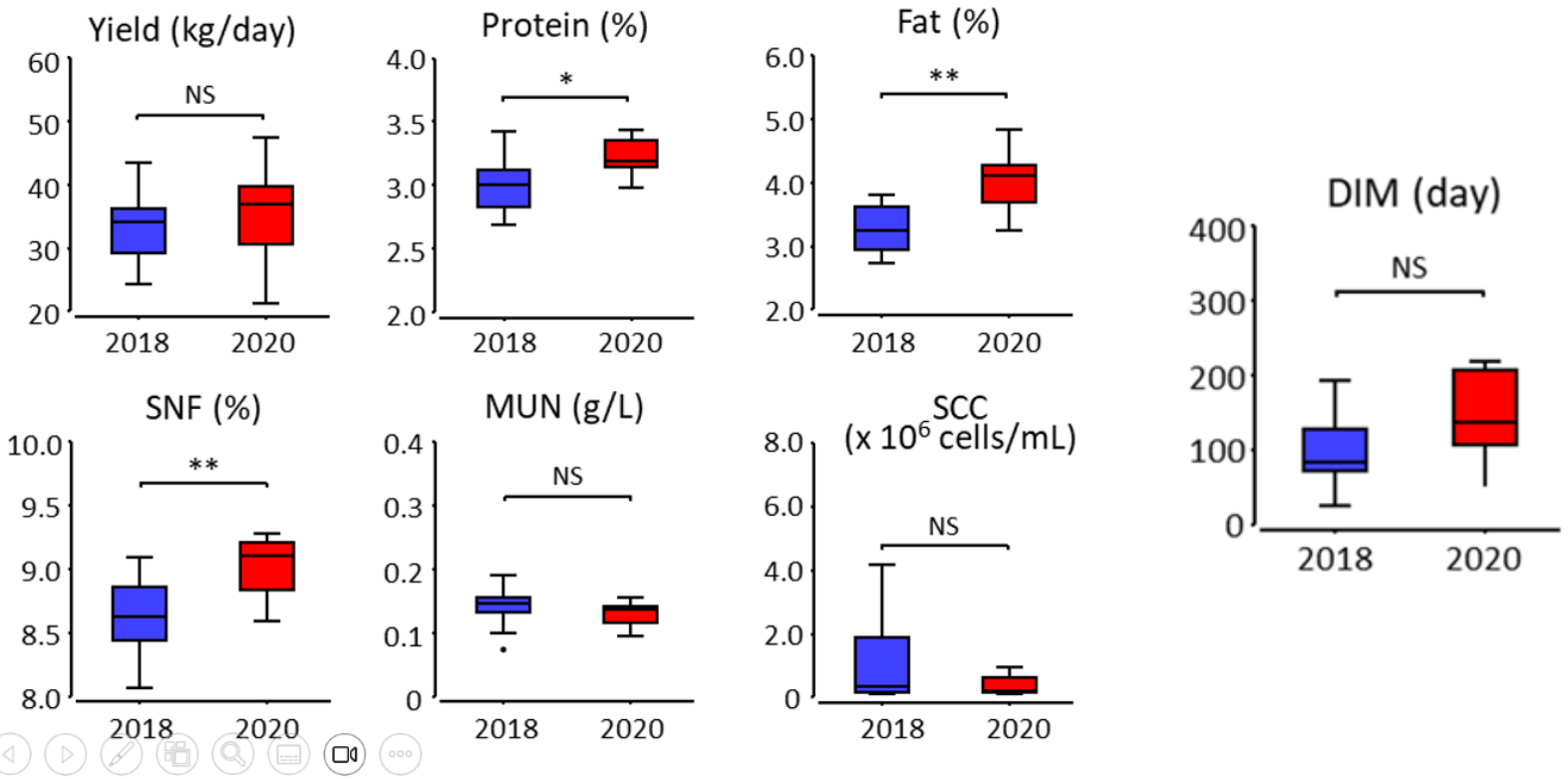
id	Source	Sample	Source sample	Sampling time	observed features	Shannon entropy	pielou_evenness	faith_pd
M11	milk	2nd-M1	2nd-milk	2nd	151	4.918098	0.679445	18.13872
M12	milk	2nd-M2	2nd-milk	2nd	144	4.977186	0.694176	18.04872
M13	milk	2nd-M3	2nd-milk	2nd	142	4.975388	0.695883	17.30022
M14	milk	2nd-M4	2nd-milk	2nd	154	4.897922	0.674015	18.28459
M15	milk	2nd-M5	2nd-milk	2nd	162	4.83008	0.658062	19.8843
M16	milk	2nd-M6	2nd-milk	2nd	183	5.193137	0.690972	20.74749
M17	milk	2nd-M7	2nd-milk	2nd	192	4.745172	0.625603	22.98223
M18	milk	2nd-M8	2nd-milk	2nd	144	5.10601	0.712143	16.52682
M19	milk	2nd-M9	2nd-milk	2nd	156	5.147442	0.706542	16.70692
M20	milk	2ndM10	2nd-milk	2nd	164	5.018951	0.68215	18.09741
F11	feces	2nd-F1	2nd-feces	2nd	523	7.801222	0.863859	27.53596
F12	feces	2nd-F2	2nd-feces	2nd	498	7.702609	0.859666	27.5881
F13	feces	2nd-F3	2nd-feces	2nd	599	8.126933	0.880833	31.42124
F17	feces	2nd-F7	2nd-feces	2nd	551	7.70301	0.845935	28.95479
F18	feces	2nd-F8	2nd-feces	2nd	631	8.048548	0.865296	30.51277
F19	feces	2nd-F9	2nd-feces	2nd	469	7.709377	0.868814	28.11289
B3	bedding	2nd-B	2nd-bedding	2nd	561	7.455722	0.816452	35.55031
S11	skin	2nd-S1	2nd-skin	2nd	437	6.675112	0.761001	34.56267
S12	skin	2nd-S2	2nd-skin	2nd	541	6.786736	0.74748	37.04535
F17	feces	2nd-F7	2nd-feces	2nd	551	7.70301	0.845935	28.95479

Summary table of alpha diversity（b）

F18	feces	2nd-F8	2nd-feces	2nd	631	8.048548	0.865296	30.51277
F19	feces	2nd-F9	2nd-feces	2nd	469	7.709377	0.868814	28.11289
B3	bedding	2nd-B	2nd-bedding	2nd	561	7.455722	0.816452	35.55031
S11	skin	2nd-S1	2nd-skin	2nd	437	6.675112	0.761001	34.56267
S12	skin	2nd-S2	2nd-skin	2nd	541	6.786736	0.74748	37.04535
S13	skin	2nd-S3	2nd-skin	2nd	547	6.90977	0.7597	36.56553
S14	skin	2nd-S4	2nd-skin	2nd	371	5.748822	0.673537	34.64931
S15	skin	2nd-S5	2nd-skin	2nd	372	5.984764	0.700861	29.01359
S16	skin	2nd-S6	2nd-skin	2nd	427	6.355948	0.727384	35.76548
S17	skin	2nd-S7	2nd-skin	2nd	672	7.63476	0.812873	42.85865
S18	skin	2nd-S8	2nd-skin	2nd	589	7.474028	0.812207	38.35887
S19	skin	2nd-S9	2nd-skin	2nd	571	7.144205	0.780161	43.52726
S20	skin	2nd-S10	2nd-skin	2nd	503	6.779879	0.755468	38.13434
M1	milk	1st-M1	1st-milk	1st	566	5.921332	0.647518	44.06361
M2	milk	1st-M2	1st-milk	1st	579	5.589456	0.609044	39.47601
M3	milk	1st-M3	1st-milk	1st	439	5.075937	0.578252	34.00352
M4	milk	1st-M4	1st-milk	1st	452	5.364617	0.608221	42.54711
M5	milk	1st-M5	1st-milk	1st	539	5.709574	0.629214	39.70535
M6	milk	1st-M6	1st-milk	1st	447	5.191489	0.589665	40.23967
M7	milk	1st-M7	1st-milk	1st	499	5.486234	0.612105	39.68396
M8	milk	1st-M8	1st-milk	1st	559	5.835798	0.63942	42.86552
M9	milk	1st-M9	1st-milk	1st	468	5.319073	0.599645	34.13763

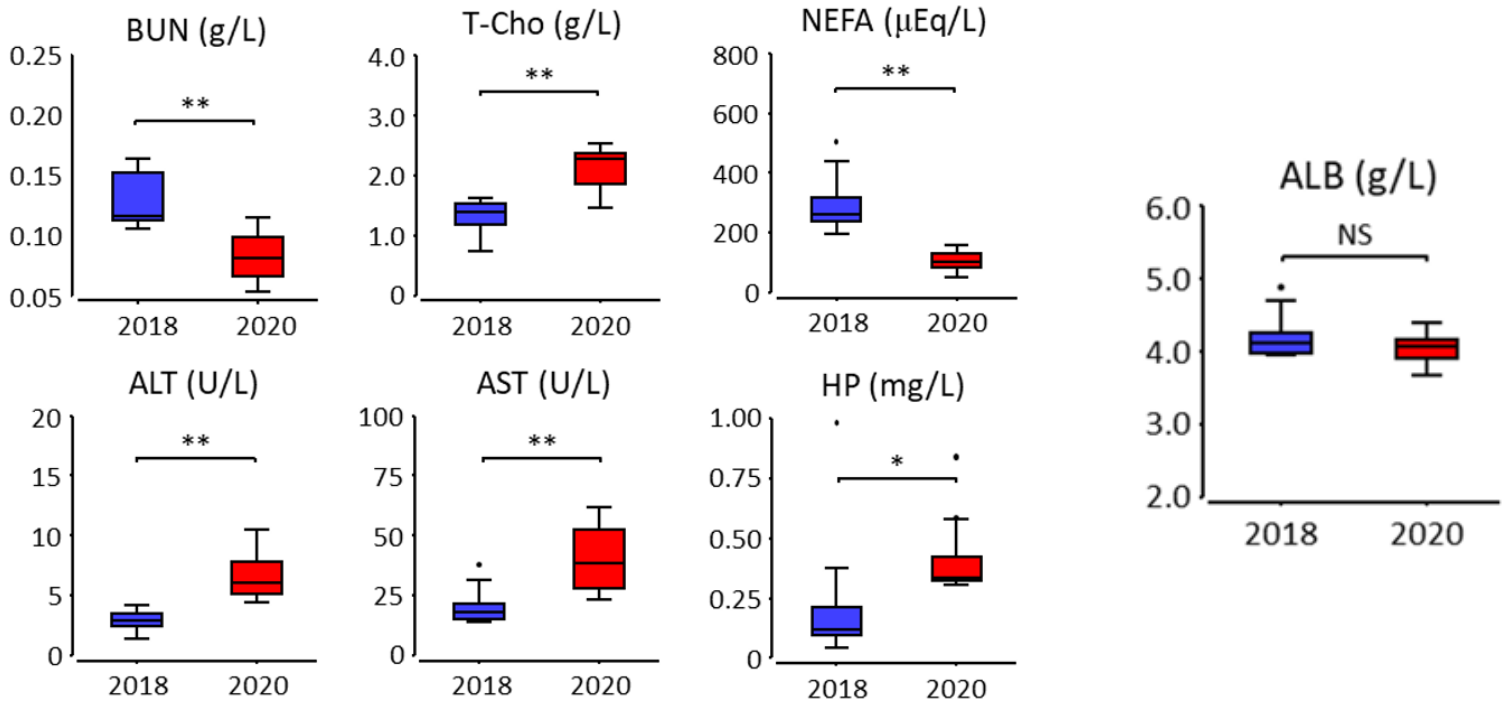
Summary table of alpha diversity（c）

M10	milk	1st-M10	1st-milk	1st	453	5.440757	0.61663	33.54685
S1	skin	1st-S1	1st-skin	1st	576	6.791202	0.740595	37.57793
S2	skin	1st-S2	1st-skin	1st	497	7.148031	0.798029	33.16463
S3	skin	1st-S3	1st-skin	1st	500	7.396323	0.82495	29.66085
S4	skin	1st-S4	1st-skin	1st	539	7.256368	0.799675	32.45929
S5	skin	1st-S5	1st-skin	1st	470	7.144545	0.804882	28.65909
S6	skin	1st-S6	1st-skin	1st	540	7.546728	0.831429	30.51286
S7	skin	1st-S7	1st-skin	1st	649	7.611937	0.814802	38.25588
S8	skin	1st-S8	1st-skin	1st	759	7.791909	0.814375	44.17553
S9	skin	1st-S9	1st-skin	1st	606	7.587223	0.820846	32.64414
S10	skin	1st-S10	1st-skin	1st	630	7.372528	0.792813	40.60628
F1	feces	1st-F1	1st-feces	1st	867	8.583249	0.879441	37.08842
F2	feces	1st-F2	1st-feces	1st	757	8.304659	0.868311	34.29394
F3	feces	1st-F3	1st-feces	1st	844	8.359973	0.859982	37.45354
F4	feces	1st-F4	1st-feces	1st	576	7.38548	0.805402	28.07923
F5	feces	1st-F5	1st-feces	1st	696	8.075147	0.855151	33.6003
F6	feces	1st-F6	1st-feces	1st	668	8.039505	0.856752	30.87233
F7	feces	1st-F7	1st-feces	1st	608	7.920944	0.85651	28.77979
F8	feces	1st-F8	1st-feces	1st	731	8.028252	0.84386	32.8418
F9	feces	1st-F9	1st-feces	1st	812	8.520725	0.881576	35.05481
B1	bedding	1st-B1	1st-beding	1st	590	7.216386	0.784	33.1243
B2	bedding	1st-B2	1st-beding	1st	726	7.571728	0.796703	38.07294



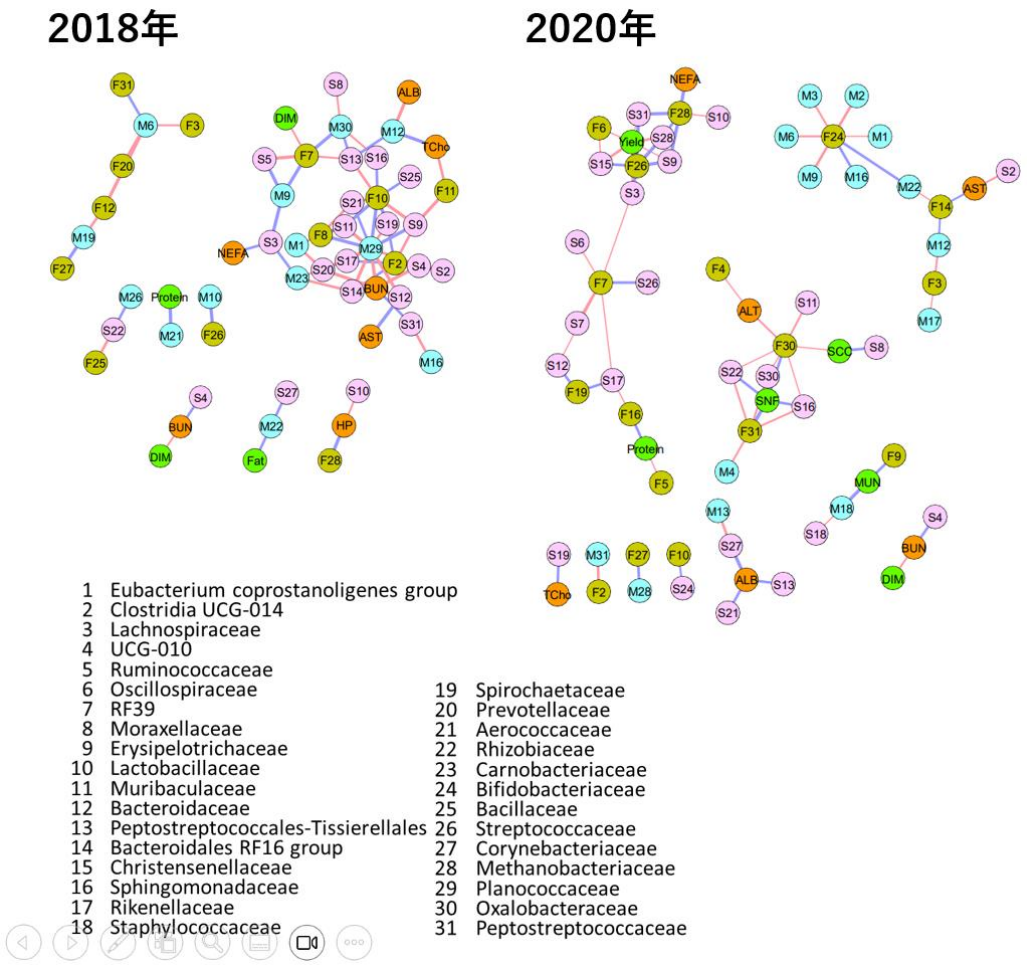
Dairy Cow Production Parameters: 2018 vs. 2020 Comparison.

The provided graphic illustrates a comparison of dairy cow production parameters between the years 2018 and 2020 using boxplots for each parameter. The parameters analyzed include milk yield (kg/day), milk composition (protein and fat percentage), solids-not-fat (SNF) percentage, milk urea nitrogen (MUN) concentration (g/L), and somatic cell count (SCC) (x10^6 cells/mL).



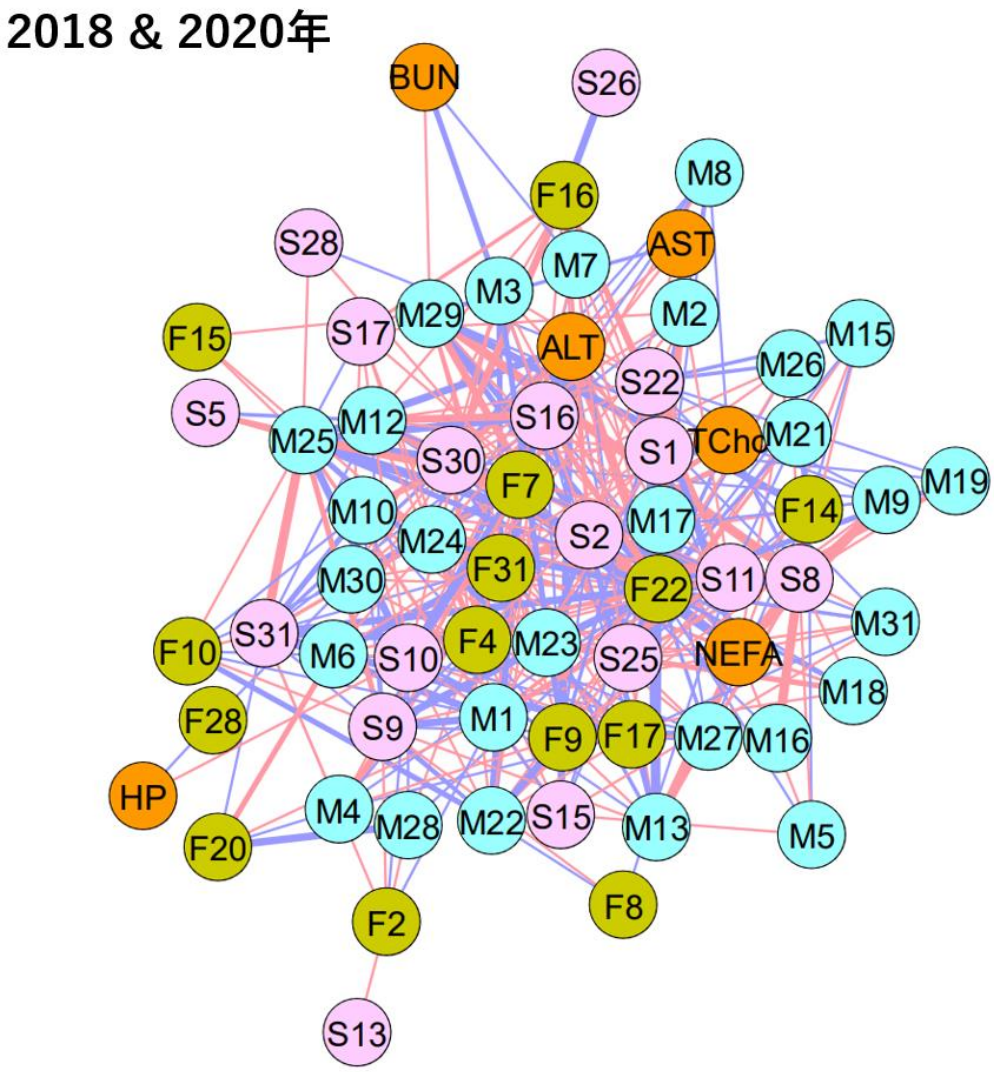
Comparative Analysis of Blood Biochemistry Parameters (2018 vs. 2020)

The graph displays boxplots representing the changes in blood biochemistry parameters of a population over two years, 2018 and 2020. The parameters shown are Blood Urea Nitrogen (BUN), Total Cholesterol (T-Cho), Non-Esterified Fatty Acids (NEFA), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Haptoglobin (HP).



Microbial Community and Metabolic Parameter Network Analysis (2018 vs. 2020)

In the 2018 network, connections between microbial families and metabolic parameters appear to be less dense compared to 2020, suggesting a change in the relationships between the microbiota and the metabolic state of the host over the two years. The 2020 network shows a more complex interplay, with a higher degree of connectivity between different microbial families and metabolic parameters. This could indicate a more dynamic or interactive microbial environment in 2020, possibly due to changes in management practices, animal health, or environmental conditions affecting the microbiome.



Integrated Network Analysis of Microbial Families and Metabolic Parameters (2018 & 2020).

The image illustrates an integrated network analysis that merges data from 2018 and 2020, mapping the interactions between microbial families (denoted by F followed by a number) and metabolic parameters (denoted by their abbreviations, e.g., BUN for Blood Urea Nitrogen). Each node represents either a microbial family or a metabolic parameter, with lines indicating relationships or correlations.

Nodes are color-coded to differentiate between the various microbial families, and distinct shapes are assigned to metabolic parameters for easy identification. The network's complexity, shown by the interconnections, suggests a multifaceted relationship where certain metabolic parameters are influenced by multiple microbial families. The thickness of the lines may indicate the strength of the relationship, with thicker lines representing stronger associations.