EPIDEMIOLOGICAL ANALYSIS AND CHARACTERIZATION OF MICROBIOME ASSOCIATED WITH BOVINE MASTITIS

DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF DHAKA
DHAKA-1000
SEPTEMBER, 2020

SUBMITTED BY
EXAMINATION ROLL NO. 02
REGISTRATION NO. 34
SESSION: 2016-2017

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A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN THE FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Dedicated to…

This dissertation is dedicated to my wife and beloved daughter, for their patience and continual support throughout my PhD degree. I also dedicate my dissertation work to my parents, and family members, for their eternal love, patience, and motivation to succeed throughout my childhood and adulthood.

Quotation…

If we knew what it was we were doing, it would not be called research.

— Albert Einstein

Certification

It is hereby certified that student bearing Reg. No. 34, Session 2016-2017 has carried out the research work entitled "**Epidemiological Analysis and Characterization of Microbiome Associated with Bovine Mastitis**" for the fulfillment of his Doctor of Philosophy degree from the University of Dhaka, Bangladesh under my academic supervision in the Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka.

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Acknowledgements

A journey is easier when we travel together. Interdependence is certainly more valuable than independence. This thesis is the result of hard work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I now have the opportunity to express my sincere gratitude to all of them. After an intensive period of three years (March, 2017- February, 2020), on the eve of my research work I thank the most Gracious, Merciful and Almighty ALLAH for giving me patience, and strength to complete this research work. Without His mercy nothing is possible.

With heartfelt and immense pleasure, I deem it my pride to acknowledge, on the veil of this dissertation, my supervisor and mentor, **Md. Anwar Hossain**, **PhD**, Professor, Department of Microbiology, University of Dhaka, for his brilliant guidance, and continuous supervision throughout the span of the present study. His dedication to research, meticulous planning, consecutive counselling, and unreserved help served as a beacon light throughout the course of study, research work and completion of this dissertation.

I am highly thankful and grateful to my joint-supervisors **Dr. AMAM Zonaed Siddiki**, Professor, Department of Pathology and Parasitology, Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Khulshi, Chattogram, and **Dr. Munawar Sultana**, Associate Professor, Department of Microbiology, University of Dhaka, for their learned counsel, innovative suggestions, and critical evaluation of my work. This dissertation would not be possible without their help and guidance.

I express my heart-felt gratitude to **Dr. Keith A. Crandall**, Professor, Department of Biostatistics & Bioinformatics, Milken Institute School of Public Health, GWU, USA who was kind enough to bringing me to the GWU, USA as a **Visiting Researcher**, and for his brilliant guidance, and continuous supervision throughout the doctoral study period. I am also grateful to **Professor Keith** for being generous to support part of my research expenses including metagenome sequencing and paying publication fees.

During my PhD study period, I came across two Chairman of the Department of Microbiology, University of Dhaka, **Professor Mrs. Humaira Akhter** and **Professor Dr. Sabita Rezwana Rahman** and all of them are highly acknowledged for their kind co-operation and support. I am also thankful to two Seminar Coordinators, **Professor Dr. Donald Jams Gomes and Professor Dr. Muhammad Manjurul Karim** for their timely help and co-operation.

I convey my cordial gratitude to **Dr. Md. Mizanur Rahaman**, Assistant Professor, Department of Microbiology, University of Dhaka, for his inspiration, valuable advice, and moral support. I also express my gratitude to all other teachers of the department who assisted me during the course of the research work.

Meticulous contributions of **Arif Istiaq**, **Md. Shaminur Rahman**, **Md. Rafiul Islam**, and **Otun Saha** towards my little task are deeply acknowledged. I also convey my gratitude to Mehedi Mahmudul Hasan, Salma Akter, Ovinu Kibria Islam, Farzana Diba, Dr. Al-Amin, Dr. Huzzat Ullah and all other members of MGBL for their supports throughout my research work. Thanks to all of the staffs of the department for their generous help during this work.

I gratefully acknowledge International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR'B) for providing me adequate number of mouse, and using their Animal Research Farm facilities to complete part of my research work. I am also thankful to the dairy farmers of Bangladesh who were kind to allowing their cows as study population, and supplying milk samples. I am also grateful to the Bangabandhu Science and Technology Fellowship Trust (BSTF), Ministry of Science and Technology, Government of the Bangladesh for selecting me as a PhD fellow and providing fellowship during the course of my PhD.

Thanks and appreciation to my family members. Their help and appreciation during this course were very supportive. Your prayer for me was what sustained me thus far.

Last, but no way the least, I am so grateful my better half Monoara Akter for her eternal support and understanding of my goals and aspirations. Her infallible love and support have always been my strength. I am also thankful to my beloved daughter Nabiha Mehjabin Hoque for giving me happiness during the last three years of my studies. Thanks a million to all concerns!

The research work was conducted in the Microbial Genetics and Bioinformatics Laboratory (MGBL), Department of Microbiology, University of Dhaka, Bangladesh, and in the Genomics Core Facility of the George Washington University (GWU), Washington DC, United States of America (USA). The research has been supported by the research grants from the Bangladesh Bureau of Educational Information and Statistics (BANBEIS), Ministry of Education, Government of Bangladesh (**Grant No. LS-3132017**).

Author

September, 2020

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Abstract

Mastitis, the inflammation of the mammary gland and/or quarters is one of the most prevalent diseases in the dairy industry worldwide with economic significance. The milk of the lactating cows presents a complex ecosystem of interconnected microbial communities which can impose a significant influence on the pathophysiology of mastitis. Bovine mastitis is caused by a wide range of apparently resident microbes including bacteria, viruses, and archaea. Our hypothesis- ***the possible dynamic shifts of microbiome compositions with the progress of different pathophysiological states of mastitis are determined by its favoring genomic potentials.*** In order to address the hypothesis, whole metagenome sequencing (WMS) is carried out to compare the microbiomes of clinical mastitis (CM = 5), recurrent clinical mastitis (RCM = 6), subclinical mastitis (SCM = 4), and healthy (H = 5) milk samples. The metagenomics data analyzed to characterize the microbiomes associated with bovine mastitis and their cross-talk in respect to disease progression, virulence factors-associated genes (VFGs), antibiotic resistance genes (AGRs), resistomes, and metabolic functional potentials. The WMS generated 416.64 million reads (with an average of 20.83 million reads/sample) from the samples of four metagenomes. PathoScope (PS) and MG-RAST (MR) analyses mapped the WMS data to 442 bacterial, 58 archaeal, and 48 viral genomes with distinct variation in microbiome composition, and abundances across these metagenomes (CM>H>RCM>SCM). Significant variations observed in species richness (i.e., alpha-diversity; P = 0.003, Kruskal–Wallis test), and microbial community structure (i.e., beta-diversity; P = 0.001, Kruskal–Wallis test) among the samples of four metagenomes. These diversities differ across the CM, RCM, SCM and H metagenomes, and numerically dominated by phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. Through PS analysis, we detected 385, 65, 80 and 144 bacterial strains in CM, RCM, SCM, and H milk, respectively, with an inclusion of 67.19% previously unreported opportunistic strains in mastitis milk metagenomes. The MR pipeline detected 56, 13, 9 and 46 archaeal, and 40, 24, 11 and 37 viral genera in CM, RCM, SCM and H-milk metagenomes, respectively. Of these, 12.06% archaeal and 20.83% viral genera found to be shared in CM, RCM, SCM, and H metagenomes. Furthermore, we identified 333, 304, 183 and 50 VFGs, and 48, 31, 11 and 6 AGRs in CM, RCM, SCM, and H-microbiomes, respectively, showing a significant correlation between the relative abundances of VFGs (P = 0.001, Pearson test), ARGs (P = 0.0001, Pearson test), and associated bacterial taxa.

We also detected correlated variations in the presence and abundance of several metabolic functional genes related to bacterial colonization, proliferation, chemotaxis, motility and invasion to mammary epithelial cells (P = 0.001, Kruskal–Wallis test) across these metagenomes. Furthermore, genes coding for oxidative stress, immune-diseases, two-component regulatory systems, regulation and cell signaling, virulence and pathogenicity, phage integration and excision, biofilm-formation, and quorum-sensing also varied significantly (P = 0.001, Kruskal–Wallis test) in different episodes of mastitis. In addition, we found a significant association between the resistomes and microbiome composition of the CM milk with no apparent association with cattle breeds, despite significant differences in microbiome diversity among the breeds. The *in vitro* investigation revealed 76.2% of six selected CM pathogens that are considered "biofilm formers", and found to be highly resistant to tetracycline, doxycycline, nalidixic acid, ampicillin, chloramphenicol while being sensitive to five heavy metals (Cr, Co, Ni, Cu, Zn) at varying concentrations. In a separate experiment, fecal microbiota transplantation (FMT) from mastitic cows to pregnant mice resulted in visible mastitis symptoms in mice mammary glands as validated through histopathologic changes in mammary glands and gut tissues, and microbiome characterization. We also observed significant (P = 0.012, Kruskal–Wallis test) microbiome dysbiosis between mastitis and healthy mice fecal samples after FMT. Therefore, profiling the dynamics of microbiomes in different states of mastitis, concurrent VFGs, ARGs, and genomic functional correlations contribute to developing microbiome-based diagnostics and therapeutics for bovine mastitis, and carries significant implications on curtailing the economic fallout from this disease. Furthermore, the cows-to-mouse FMT might shed new light on rational selection of animal models to study and interpret host-tropism to interrogate role of microbiota in this important economic disease of dairy industries.

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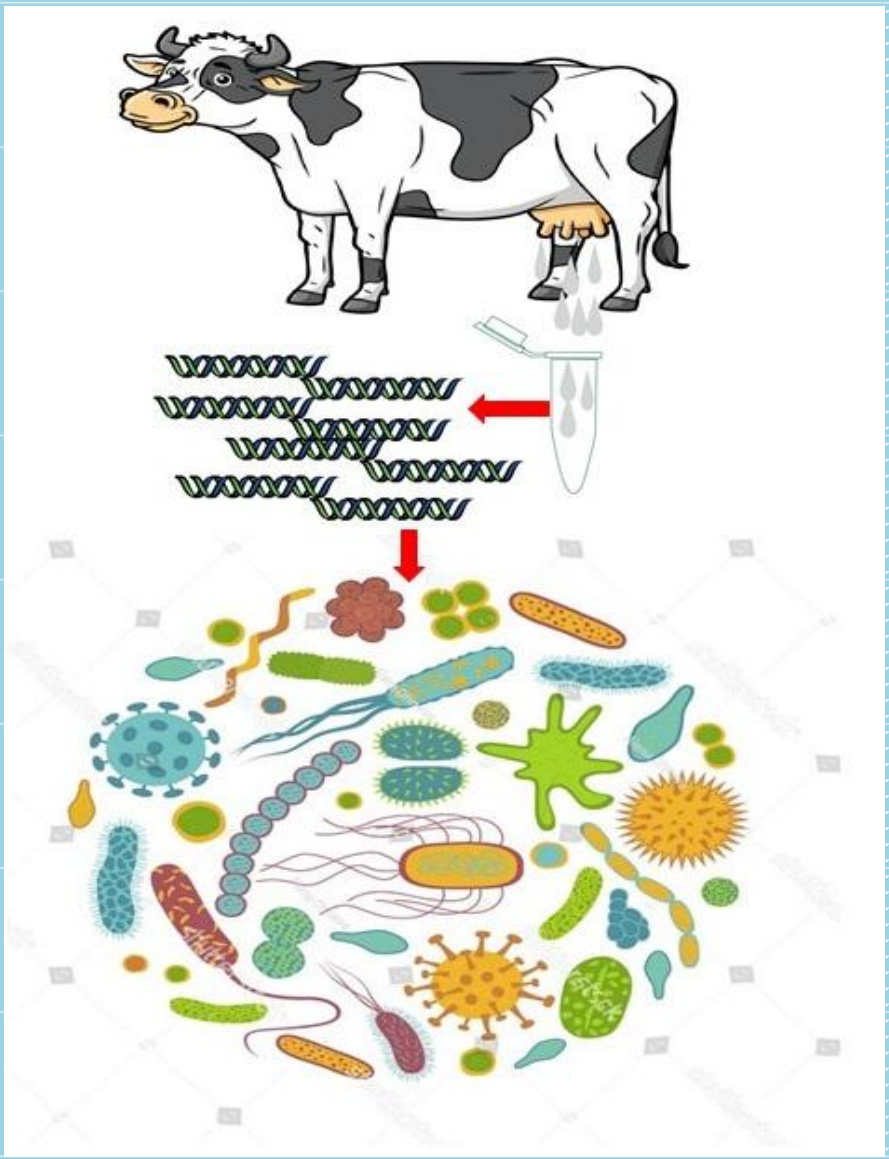
ABBREVIATIONS

AMR: Antimicrobial Resistance
ARGs: Antibiotic Resistant Genes
BF: Biofilm Formation
BTSCC: Bulk Tank Somatic Cell Count
CM: Clinical Mastitis
CMT: California Mastitis Test
ECT: Electrical Conductivity Test
ELISA: Enzyme-Linked Immunosorbent Assay
GF: Germ Free
IMI: Intra Mammary Infection
KEGG: Kyoto Encyclopedia of Genes and Genomes
LZ: Local Zebu
MALDI-TOF: Matrix-Assisted Laser Desorption Ionization Time-of-Flight
MBC: Minimum Bactericidal Concentration
MBF: Moderate Biofilm Former
MCH: Mouse Clinical Mastitis
MG-RAST: Metagenomic Rapid Annotations using Subsystems Technology
MH: Mouse Healthy
MIC: Minimal Inhibitory Concentration
NBF: Non Biofilm Former
NGS: Next Generation Sequencing
NMDS: Non-Metric Multidimensional Scaling
PCoA: Principal Coordinate Analysis
QC: Quality Control
QS: Quorum Sensing
RCC: Red Chattogram Cattle
RCM: Recurrent Clinical Mastitis
RIA: Radioimmunoassay
SBF: Strong Biofilm Former
SCC: Somatic Cell Count
SCM: Subclinical Mastitis
SFMT: Surf Field Mastitis Test
SPF: Specific-Pathogen-Free
SW: Sahiwal
VFDB: Virulence Factor Database
VFGs: Virulence Factors-associated Genes
WBF: Weak Biofilm Former
WGS: Whole Genome Sequencing
WMS: Whole Metagenome Sequencing
WST: White Side Test
XHF: Crossbred Holstein Friesian
ZOI: Zone Of Inhibition



Chapter 01:

Introduction and Literature Review

1.1 General Introduction

Mastitis in dairy animals is one of the most prevalent disease conditions that is responsible for highest clinical, and economic significance worldwide (Thompson-Crispi et al., 2014; Falentin et al., 2016). The condition usually happen when pathogenic microbes enter the germ-free environment of the mammary gland, mostly by the disruption of the physical barriers of the mammary quarters, and appropriate host defenses failed to prevent infections as aresulted subsequent disease pathology (Aitken et al., 2011; Hoque et al., 2019, this thesis). These phenomena often develop as a sequel to invasion by microorganisms, most commonly by bacteria, though other physical or chemical causes such as trauma or harmful toxins/chemicals can also lead to mastitis (Mudaliar et al., 2017). A large number of microbial species are known to colonize the mammary quarters of cow showing significant diversity and thus, evolving novel pathological mechanisms that facilitate their proliferation leading to clinical manifestations (Hoque et al., 2019, this thesis). While the etiology of mastitis always varies; it is not unusual that new microbial species or strains are being incriminated in dairy cows over different time points. Until recently, investigations of microbiome associated with bovine mastitis are mostly restricted to individual pathogen isolation and characterization (Wu et al., 2017). The involvement of the bovine mammary gland microbiota in the host-pathogen interaction has poorly been investigated mainly during the infectious episodes (Catozzi et al., 2017). There lies the need for further investigation to explore the complex nature of the microbiomes associated with bovine mastitis, and their cross-talk in order to develop different magnitudes of mastitis. Our hypothesis- ***the possible dynamic shifts of microbiome compositions with the progress of different pathophysiological states of mastitis are determined by its favoring genomic potentials*** **(Figure 1.1)**.

Mastitis in dairy animals occurs as clinical, recurrent clinical, and subclinical forms. Bovine clinical mastitis (CM) is one of the special concerns for milk producers in developing countries like Bangladesh, where dairying plays a pivotal role in the national economy. This sub-sector contributes around 1.47% to the national Gross Domestic Product with an annual growth rate of 3.4% (Datta et al., 2019), and occupy around 5.75% direct and 15.0% indirect employments (DLS, 2019).

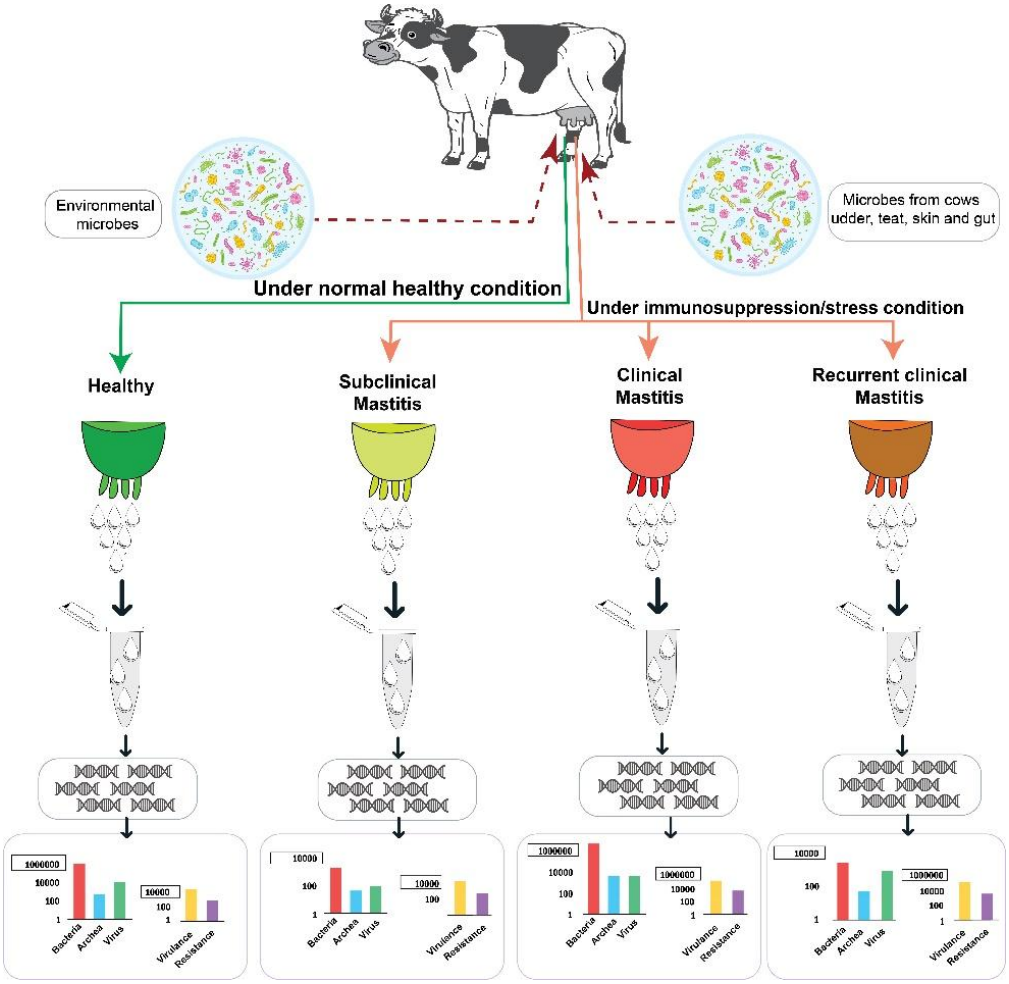


Figure 1.1: Bovine mastitis microbiome study. Bovine mastitis is caused by both environmental and comensal (from udder, teat, skin and gut) pathogens. Under immunosuppression or stress conditions (when the cow suffers from a severe negative energy balance at the onset, in-and-around lactation, and other environmental stress e.g. heat stress), environmental and commensal bacteria act as potential opportunists to manifest different episodes of clinical mastitis (CM), recurrent clinical mastitis (RCM), and subclinical mastitis (SCM). A plethora of other microbes including archaea and viruses simultaneously follow the same opportunistic pathways as bacteria. However, the possible dynamic shifts in microbiome compositions in different conditions of bovine mastitis are determined by its favoring genomic potentials including virulence, antimicrobial resistance and metabolic functions.

The disease is caused by a diverse group of microorganisms (Hoque et al., 2019), and can be epidemiologically categorized into contagious and environmental mastitis (Abebe et al., 2016). Depending on the pathogens and host responses, mastitis can manifest in clinical (CM) or subclinical (SCM) form, and present an acute or persistent/recurrent or chronic course. The CM is an inflammatory response with features of apparent changes in the color of milk that varies from pale white to dark red; change in the consistency of milk that varies from watery milk to clotted milk with flakes, clots or pus; swelling, redness and pain in the affected udder and/or quarters, systemic symptoms like fever and anorexia and sometimes death due to

toxemia (Royster and Wagner, 2015; Gomes et al., 2016). Clinical cases can be mild, moderate, or severe, depending on the presence or absence of local and systemic signs (Royster and Wagner, 2015). Moreover, diverse groups of microbial communities colonizing the mammary gland or quarters have evolved novel mechanisms to facilitate their proliferation during the pathogenesis of CM. This diversity comprises both contagious udder pathogens including *Staphylococcus aureus, Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Mycoplasma* spp., and *Corynebacterium bovis* (Oikonomou et al., 2014; Derakhshani et al., 2018, Hoque et al., 2019, this thesis), and environmental pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* (Oikonomou et al., 2014; Derakhshani et al., 2018; Hoque et al., 2019, this thesis). One of the very frustrating aspects of bovine CM is its recurrent nature. Recurrent clinical mastitis (RCM) is caused by persistent intra-mammary infections (IMI), and specific differences in the milk microbiota may also contribute to recurrence susceptibility (Döpfer et al., 1999; Jamali et al., 2018). The IMI may persist beyond the resolution of the clinical symptoms of CM, and sub-sequent RCM flare-ups may be observed (Döpfer et al., 1999; Jamali et al., 2018). Howver, RMC-associated persistence of pathogens (such as *E. coli*, *S. aureus*, *S. uberis*, and *S. dysgalactiae*) in the udder was limitedly reported (Jamali et al., 2018). Almost every dairy herd has cows with SCM, and a variety of pathogens can establish chronic infections which may only occasionally manifest the clinical signs of mastitis (Hoque et al., 2015). Predominantly identified SCM pathogens include *E. coli*, *Pseudomonas aeruginosa*, *P. mendocina*, *Shigella flexneri*, *Bacillus cereus*, *K. pneumoniae*, *S. chromogenes*, and *S. epidermidis* (Bhatt et al., 2012; Oikonomou et al., 2014; Oikonomou et al., 2020).

Over the past two decades, a wide range of phenotyping and genotyping methods have been implemented to study mastitis-causing bacteria (Hoque et al., 2018). Although culture-based techniques are still the forefront of detecting mastitis causing bacteria, these methods are time-consuming, and have some critical drawbacks like inapplicable to non-cultivable bacteria (Baron et al., 2018). Furthermore, identifying the large number of microbes are quite difficult due to their diverse culture properties and lack of relevant techniques. To overcome the challenges, advanced tools and technologies are available now. One of such techniques is metagenome analysis, which typically include the assessment of the

phylogenetic diversity of complex microbial community, and their underlying functions in any sample (Leimbach et al., 2016). Rapid advances in high-throughput next generation sequencing (NGS) technology combined with bioinformatics tools during the last decade have initiated a transition from clinical microbiology to genomic characterization of microbiomes associated with mastitis, notably in lactating women (Patel et al., 2017;) and dairy cows (Oikonomou et al., 2014; Falentin et al., 2016; Cremonesi et al., 2018). Considerably, the 16S rRNA gene sequencing has become a common method to study bovine mastitis microbiome (Oikonomou et al., 2014; Falentin et al., 2016; Lima et al., 2017; Cremonesi et al., 2018). Though this technique is highly useful to resolve more than 90.0% of the isolates at genus level, it has a number of limitations including the polymerase chain reaction (PCR) bias, inability to detect archaea and viruses, lower taxonomic resolution (typically up to genus-level), and can be less precise at the species level, and limited information on gene abundance and functional profiling (Poretsky et al., 2014; Zinicola et al., 2015). **These limitations hamper the ability to fully explore the cross-talk among the microbes present in the microbiome that leads the progression of disease, and interaction between the pathogens and the host**. The shotgun whole metagenome sequencing (WMS) produces a metagenome reflecting the total microbial genetic content in a sample, and is being used successfully to gain insights into the phylogenetic composition, species and/or strain diversity, and functional diversity for a variety of biomes (Seth et al., 2014; Oniciuc et al., 2018; Hoque et al., 2019, this thesis).

The microbiome study of both animals and humans can be influenced by *in vivo* research on laboraotry animal models. Thus, important contributions to our understanding of the bovine mastitis microbiome is possible by conducting research on mammals like mice with lower taxonomic composition (Douglas, 2019). The mouse mastitis model is a good model to study bovine mastitis compared to other laboratory animals for ease of handling, keeping them in controlled environment, low maintenance cost (as compared with other mammalian experimental models), high reproductive rates, and short life cycle (Nguyen et al., 2015). **To study the host-tropism of bovine mastitis microbiome and underlying molecular mechanisms of the mammary gland pathophysiology using mouse model, we attempted to explore whether dysbiosis of gut and milk microbiota can lead to clinical episodes of mastitis.**

1.2 Review of Literature

1.2.1 Mastitis in dairy animals

Mastitis, defined as an inflammation of the mammary gland, can have an infectious or non-infectious etiology (Abebe et al., 2016). Historical evidence suggests that cows have been milked since at least 3100 BC, and it is likely that mastitis has existed since that time. The disease is characterized by physical, chemical and usually bacteriological changes in milk, and pathological changes in glandular tissues of the udder and affects the quality and quantity of milk (Radostits et al., 2007; Sharma et al., 2015). It is also defined as an inflammation of mammary gland parenchyma which is caused by microorganisms, usually bacteria that invade the udder, multiply and produce toxins which are harmful to the mammary gland (Kee, 2012). Mastitis is one of the first observed diseases of farm animals since the domestication of cattle when cattle over 5000 years ago (Sharma et al., 2015). Bovine mastitis is the most frequent disease of dairy cows, and has well-recognized detrimental effects on animal wellbeing and dairy farm profitability.

1.2.1.1 Classification of bovine mastitis

Bovine mastitis is the persistent inflammatory reaction of the udder tissue due to physical trauma or microbial infections, that may be classified according two different criteria: either according to the clinical symptoms or depending on the mode of transmission (Radostits et al., 2007). Bovine mastitis can be classified as being either clinical mastitis (CM), whereby gross changes are seen in the milk, or subclinical mastitis (SCM) if no such changes are visible but changes in the secretion are present, such as an increase in somatic cell count (SCC) (Hoque et al., 2015). Bovine CM can be further categorized as per-acute, acute and subacute mastitis depending on severity of symptoms. Bovine CM as a frequently recurrent or persistent event can cause substantive economic loss on dairy farms. The reason for recurrent clinical mastitis (RCM) can be either a persistent infection of the bovine mammary gland by CM pathogens or a reinfection of a quarter or udder after bacteriological cure (Swinkels et al., 2013). The virulence properties of CM pathogens, and the cure odds of an individual cow determine the development of

RCM (Jamali et al., 2018). The case definition for RCM can vary according to different studies, especially regarding the minimal time required between 2 CM events, which can range from ≥1 d to ≥30 d (Jamali et al., 2018). Various factors which contribute to the level of infection include the disease-causing agent, age of the animal, its immunological health and lactation stage. The effects of RCM to the dairy producers may include increased veterinary costs, increased use of drugs, extra labor, increased number of services per conception, lower length of productive life, and decreased number of days to peak milk production (Swinkels et al., 2013; Jamali et al., 2018). Bovine subclinical mastitis (SCM), which most of the times remains undetected, and has the greatest economic consequences because of long term effects on milk yields. The treatment of a cow diagnosed with SCM is mostly discouraged (with few exceptions) considering the financial losses from the salable milk. However, presence of cows with SCM in farm or herd has some potentially negative unintended consequences as for example increased somatic cell counts (SCC) (Hoque et al., 2015). The dairy cows suffering from SCM can serve as a reservoir of infection within the farm or herd and increase exposure of healthy cows to contagious pathogens (Bhatt et al., 2012). The other indicators of SCM include increased bacterial population in milk, decreased milk production, and change in composition and quality of milk (Bian et al., 2015). Epidemiologically bovine mastitis is categorized into contagious and environmental mastitis (Kuehn et al., 2013). Contagious bacteria commonly exist within the mammary gland and are transmitted from cow to cow during the milking process. They are known to be associated with persistent infections which are reflected by a higher somatic cell counts (SCC).

1.2.1.2 Bovine clinical mastitis and its signs and symptoms

Clinical mastitis (CM) is one of the most frequent diseases affecting dairy cows worldwide. Earlier, the effect of mastitis was considered to be restricted to udder only except in severe cases where systemic illness was observed. However, there are growing evidences that mastitis is also a critical factor affecting the reproductive success of the herd (Kumar et al., 2017). Bovine CM is characterized by change in the morphology of the udder, chemical and physical changes in the milk. The udder producing this milk may become swollen, red, hot and hard and there may be also fever, rapid heart rate and loss of appetite (Sharma and Sindhu, 2007) (**Figure 1.2.1.2 a-c**). Although clinical infections are rarely apparent prior to calving, routine

observation for abnormal swelling is important. In addition to mammary glands inflammation, there might be some characteristics signs and symptoms in the quarters or teats such as laceration, necrosis, tearing and discoloration (**Figure 1.2.1.2 d**). Normal prepartum udder secretions range from a honey-like appearance to normal milk. The CM can be recognized in pre-and post-calving secretions, colostrum or milk by the presence of gargot (clots and flakes), abnormal texture or discoloration (Jamali et al., 2018) (**Figure 1.2.1.2 e**). Reduced milk production due to mastitis is associated with irreversible mammary tissue damage in majority of the cases. Mammary gland infection may result in release harmful toxins in the udder and lesions may vary from increased milk leukocytes counts with no gross changes in milk to increased vascular permeability or develop fibrosis or severe toxemia (Gogoi-Tiwari et al., 2017). The histopathological features, due to the direct action of pathogens or due to the indirect action of the inflammatory response, are also reported. Mammary tissue damage subsequently reduces the number and activity of epithelial cells and consequently contributes to decreased milk production. Mammary tissue damage has been shown to be induced by either apoptosis or necrosis (**Figure 1.2.1.2 f**). During CM infection of the mammary glands, the tissue damage can initially be caused by bacteria and their products. In addition, mastitis is characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils, into the mammary gland (Ma et al., 2018).

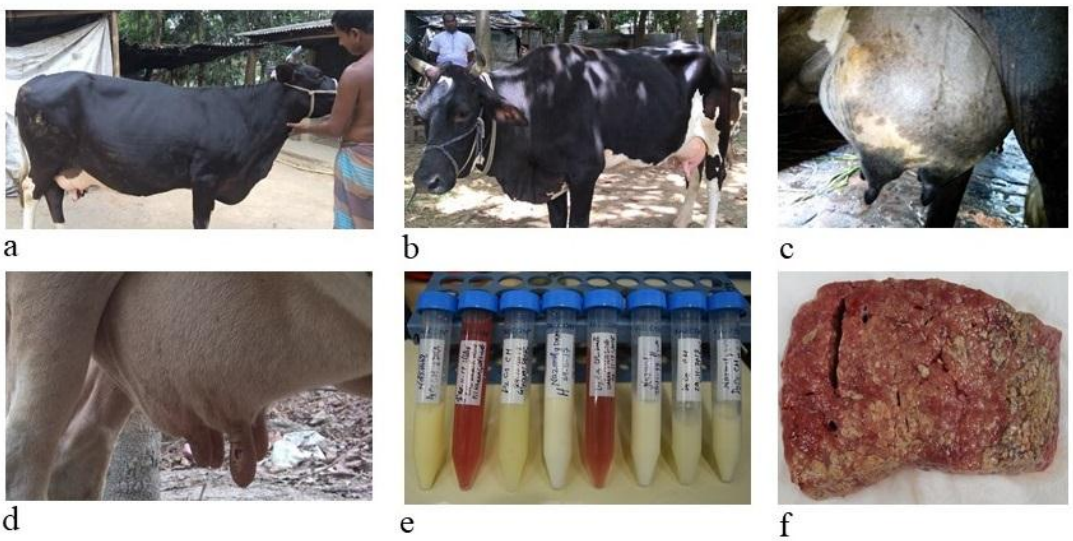


Figure 1.2.1.2: Clinical features of bovine mastitis. (a) Cows suffering from clinical mastitis (CM) with no visible signs and abnormalities, (b) CM affected cows with high fever, dullness and depression, (c) typical signs of CM; swollen, hot, hard, or painful udder, (d) teat lesions in CM, (e) characteristics changes in milk; no normal milk; watery, serum, blood and pus and (f) gross histopathological changes in mammary tissues after CM (Photographs were taken from the field during sample collection and processing).

1.2.1.3 Etiology of bovine mastitis

Diverse groups of microbes are known to colonize the mammary quarters of cows and have evolved novel mechanisms that facilitate their proliferation leading to clinical infections. Most cases of mastitis occur in response to bacterial infections of the mammary gland (Lin et al., 2016; Lima et al., 2017; Hoque et al., 2019). However non-bacterial agents are also known to cause mastitis in dairy cows that includes mycoplasmas, archaea, virus, yeasts and algae (Schukken et al., 2012; Pachauri et al., 2013; Cremonesi et al., 2018; Hoque et al., 2019). Despite the knowledge of a few of these invading microbial groups, the etiology of bovine mastitis is continuously evolving with new microbial species identified as causing disease frequently. During the progression of the mastitis, dysbiosis of the milk microbiome can occur with the increase of opportunistic pathogenic bacteria and reduction of healthy commensal bacteria (Hoque et al., 2019, this thesis).

The udder of the dairy cows is the primary reservoir of contagious pathogens including *S. aureus, S. agalactiae, S. dysgalactiae, Mycoplasma* spp., and *Corynebacterium bovis* (Kateete et al., 2013; Falentin et al., 2016). Environmental bacteria are not adapted to survive in the host but are opportunistic invaders from the cow's environment. These are generally acquired between milking, multiply, instigate an immune response and are rapidly dealt with by the immune system resulting in a transient increase in SCC. Environmental pathogens such as *E. coli, K. pneumoniae, K. oxytoca, Enterobacter aerogenes, S. dysgalactiae* and *S. uberis* can also be implicated in disease (Kateete et al., 2013; Kuehn et al., 2013; Falentin et al., 2016). Mastitis pathogens have also historically been classified as either 'major' or 'minor' pathogens based on the inflammatory response that they engender, their virulence and the severity of damage they cause to the udder, and their propensity to cause clinical signs. The major pathogens include *Streptococcus* spp., *S. aureus, E. coli, K. pneumoniae* and *Mycoplasma bovis*, and are more virulent and their infection severely impacts on the milk quality and quantity (Pyörälä et al., 2011). The minor pathogens which are less damaging to the udder, and generally cause SCM s include coagulase-negative *Staphylococcus* species (CNS) such as *S. hyicus, S. chromogenes* and *S. xylosus* and the *Corynebacterium* species. The minor pathogens are generally associated with mild immune responses, and rarely with associated with CM (Green et al., 2008). However, with the advent of recent metagenomic sequencing

technology, more than 500 different bacterial strains have been isolated from bovine CM, RCM., and SCM milk, where the majority of infections are caused by the genera *Acinetobacter*, *Pseudomonas*, *Escherichia*, *Vibrio*, *Erwinia*, *Pantoea*, *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Bacillus*, *Chryseobacterium*, *Porphyromonas*, *Prevotella*, *Corynebacterium* etc. (Hoque et al., 2019, this thesis).

1.2.1.4 Pathogenesis of bovine mastitis

Important progress has been made in the last decades in our knowledge of bovine mastitis. However, prevention and control of bovine mastitis is still difficult because the pathogenesis of the disease, and pathogen-specific risk factors remain largely unknown (De Vliegher et al., 2012). Irrespective of whether the origin is from an environmental source or from a contagious source, infection of the mammary gland usually occurs via the teat canal. The bovine mammary gland is composed of glandular tissue, gland cistern and branching network of ducts formed of epithelial cells ending in alveolar clusters that are the sites of milk secretion (McManaman and Neville, 2003). There is only one type of secretory epithelial cell that surround each alveolus within these clusters, forming a single layer over the cells (Jahan, 2017). The apical junction complex that is composed of adherens- and tight-junctional elements connects all the secretory cells to each other (McManaman and Neville, 2003). The function of the tight-junction is to inhibit any direct exchange of substances between vascular and milk compartments during lactation (McManaman and Neville, 2003). Upon transmission to the outer edge/skin of the teat, the pathogens invade to the milk inside the teat cistern, and multiply. Depending on the nature and ability of the pathogen, they may further invade the mammary tissue. Once the pathogens penetrate the physical barrier of the teat canal, the host innate immune system detects the pathogens through the pattern-recognition receptors (PRRs), particularly via the toll-like receptors (TLRs) (Ezzat Alnakip et al., 2014). Migration of immune cells, particularly neutrophils, and desquamation of mammary epithelium accompanied with reduced milk production result in a several-fold increase in SCC per unit volume of milk (Sharma and Sindhu, 2007). Bovine neutrophils migrate to the mammary epithelium by diapedesis, and they constitute more than 90% of the total leukocytes in mammary gland during inflammation. At the site of infection, the neutrophils engulf, phagocytose, and destroy the invading pathogens via an oxygen-dependent respiratory burst system producing hydroxyl and oxygen radicals, and an oxygen-independent system using peroxidases, lysozymes, hydrolytic enzymes, and lactoferrin (Ezzat

Alnakip et al., 2014; Gogoi-Tiwari et al., 2017). If the pathogens are eliminated rapidly resulting in the removal of the inflammatory stimuli, the neutrophil recruitment ceases, and the SCC return to normal levels. However, if the pathogens survive the immediate host defense response, then the infection and inflammation continue to spread to the adjacent mammary tissues. Following pathogen invasion, and establishment in the gland, either of the two major forms of mastitis may result, namely CM or SCM.

1.2.1.5 Epidemiology and the risk factor of bovine clinical mastitis

Despite many years of research devoted to mastitis, the disease remains a serious problem. The incidence of CM is an important indicator of animal health and welfare. The incidence of CM is associated with many risk factors, and can vary from quarter level to herd level. Parity, month of lactation, season of the year, somatic cell count in previous lactation, and CM history are the cow-specific risk factors that are currently known (Kee, 2012; Hoque et al., 2015; Santman-Berends et al., 2015). The incidence of mastitis in dairy animals in Asian countries ranges from 30.0 to 78.0% in cows (Ebrahimi et al., 2007; Jha et al., 2010; Sharma and Maiti, 2010), and 27.0 to 70.0% in buffaloes (Sharma and Sindhu, 2007). The prevalence rate of CM ranges from 13 to 50 cases/100 cow years in different countries and housing types (McDougall et al., 2007; van den Borne et al., 2010). Though not studied well, the prevalence of CM in dairy cows of Bangladesh was reported 11.0 to 50.0% (Jha et al. 2010). Chandrasekaran et al. (2014) studied the prevalence of drug resistant mastitis pathogens, and their pattern of antibiotic resistance in dairy cows from Tamil Nadu. The causative resistant pathogens were *E. coli* (50.64%), *S. aureus* (44.25%), and methicillin resistant *S. aureus* (MRSA, 5.11%) (Chandrasekaran et al., 2014). In another study, Vinod Kumar et al. (2016) investigated the prevalence of bacterial pathogens from milk samples of crossbred cows affected with mastitis in and around Tirupati, and reported that *S. aureus* as the predominant etiology (83.33%) of mastitis followed by *E. coli* (7.94%), *Pseudomonas* (3.96%) and *Salmonella* species (2.38%). Furthermore, the incidence of CM has not decreased over time despite great efforts to identify management and cow factors that are associated with it (Richert et al., 2013). Thus, knowledge of the incidence of CM, and the distribution of pathogens involved is essential for development of prevention and control programs as well as treatment protocols (Gao et al., 2017).

Bovine mastitis is considered to be a typical example of complex diseases, known to be established as a result of the interactions of three bio-systems namely the causative agent (pathogen), the animal (host) and the environment in which the animal lives. Host factors include breed, parity, lactation stage, milk yield, physiological state of mammary gland, and anatomy of teat canal, sphincter tone and presence of teat lesion (Sharma and Sindhu, 2007; Kee, 2012; Hoque et al., 2015). Agent factors comprises the ability of the pathogens to survive in the immediate environment of the animal, the ability to colonize the teat duct, the ability to adhere to the mammary epithelium and not to be flushed out with milk flow. Environmental factor includes milking practice, housing system and bedding materials etc. (Makovec and Ruegg, 2003) (**Figure 1.2.1.5.1**). The environmental factors such as management, feeding, hygienic status, bedding, milking and the virulence of the organism contribute to the disease. Information related to mastitis risk factors is useful for the design and implementation of mastitis control programs. Animal-specific risk factors that could associated with CM in dairy farms include lactation stage, high somatic cell count (SCC), season and history of previous CM for both primiparous and multiparous cows (Oliveira et al. 2015). Udder edema, teat edema, blood in the milk, and milk leakage at time of calving are significant risk factors for CM occurring during the first weeks after calving (De Vliegher et al., 2012). Teat edema, blood in the milk, and milk leakage also increase the odds of clinical mastitis. Precalving milking can reduce the risk of IMI, possible by reducing udder edema or physically removing bacteria from the teat canal (Daniels et al., 2007).

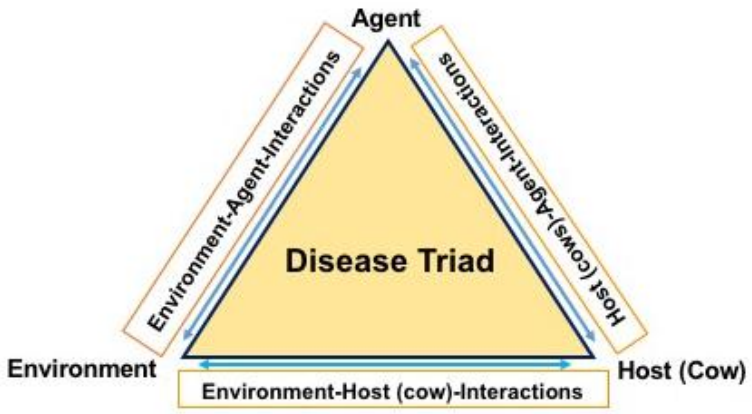


Figure 1.2.1.5.1: Bovine mastitis epidemiological triad. The triangle incudes – host (cow), mastitis-causing agent (organisms) and the environment (Adapted from Makovec and Ruegg, 2003).

In addition to cow-specific risk factors, knowledge of the pathogens involved in CM is also useful to improve the efficiency of CM control measures (Steeneveld et al., 2008). Many studies investigated risk factors for CM as well as for CM recurrence.

1.2.1.6 Impact of dairy industry in national economy of Bangladesh

Bangladesh is a densely populated agro-based developing country where livestock plays a crucial role in the national economy. Dairying is considered a strong tool to develop a village micro-economy in Bangladesh in order to alleviate rural poverty, and improve rural livelihoods. Bangladesh is endowed with 24 million cattle, out of which 10 million are milking cows, 85–90% of them are indigenous and 10–15% are crossbred (Datta et al., 2019). The crossbreds and purebreds are mostly Sindhi, Sahiwal, and Holstein Friesian breeds. In Bangladesh, cows are the main source of milk. About 90% of the produced milk in the country comes from cows, and annual milk production is 3.97 million tons which is much lower than our neighboring countries such as Pakistan and India (Hemme et al., 2007). Smallholder producers dominate the dairy sector in Bangladesh (Shamsuddin et al., 2997). More than 70% of the dairy farmers are smallholders, and produce around 70–80% of the country's total milk (Uddin et al., 2012). It is estimated that there are about 1.4 million dairy farms with an average herd size of 1–3 cows.

In recent times dairying has become an efficient tool for poverty alleviation, income generation, creation of employment of youths and women; and food security of last majority people. The country has achieved almost four-folds increase in milk production during the last decade. In terms of the regional share of milk production, the northern part of Bangladesh produces nearly half of the country's milk, as a result of the availability of fodder and the establishment of several dairy development programs (Hemme et al., 2007).

Economic consequences of mastitis, clinical or subclinical, include reduced milk yield, poorer quality milk, increased culling rate and increased cost of veterinary services and medicine. Studies to determine the economic impact of bovine mastitis have been conducted mainly in developed countries. However, the economic impact of mastitis varies in different countries, and should be calculated at the farm or herd level depending on the local, regional, epidemiological, managerial, and economic conditions. Annual economic losses from mastitis in the dairy industry of India is Rs.

7165.51 crores (Motwani and Kishore, 2011). In a recent study, Rollin et al. (2015) reported that the overall cost per case of CM in the first 30 days in milk (DIM) is $444 including $128 in direct costs and $316 in indirect costs in the United States of America. Hogeveen et al. (2011) reported that economic losses due to clinical and subclinical mastitis can ranges from €17 to €198 per cow per year in Netherlands. This is approximately 10% of the total value of farm milk sales, and about two-thirds of this loss is due to reduced milk production in subclinically infected cows (Sargeant et al., 2001).. Other losses are due to discarded abnormal milk and milk withheld from cows treated with antibiotic, costs of early replacement of affected cows, reduced sale value of culled cows, costs of drugs and veterinary services, and increased labor costs. Similarly, mastitis has been identified as one of the limiting factors in the development of a dairy industry in Pakistan (Bilal et al., 2004), and a major impediment to increasing milk production in Thailand (Boonyayatra and Chaisri, 2004). Despite having no comprehensive data on the economic lossess from mastitis in the dairy sector of Bangladesh, one would assume that economic loss associated with mastitis in Bangladesh is much more than what reported in other countries, since dairy farmers in Bangladesh are not always aware of the best practices to control mastitis.

1.2.1.7 Importance of early diagnosis of bovine mastitis

Early diagnosis is of the utmost importance due to the high management costs of mastitis in the dairy farms. Mastitis has large number of causative agents, and the rapid detection of the pathogen is crucial for the control and prevention measures of mastitis. A reliable and rapid diagnostic test is a dire need for the wellbeing of the dairy industry (Hoque et al., 2015; Hoque et al., 2018), rapid turnaround time being the key factor because the storage of perishable foods for longer period is cost-effective and a big problem in such instances. Though the classical milk culture techniques are useful for the primary identification of important bacterial pathogens in mastitic milk samples, but most of them require longer time of incubation and adequate training to perform the tests. Also, biochemical tests can be nonspecific, slow, costly, and more importantly result interpretation can be critical in relation to the diagnosis of mastitis pathogens.

1.2.1.8 Diagnosis of bovine mastitis

Mastitis is an inflammation of parenchyma of mammary gland characterized

by physical, chemical, and usually bacteriological changes in milk and pathological changes in glandular tissue. Mastitis occurs in all species but assumes major economic importance in dairy cattle and buffaloes due to its effect on quality and quantity of milk in high yielders. To avoid these economic losses due to mastitis, it is distinctly important to identify the disease in early stage. Unlike the clinical form, in subclinical form there is neither visual detection of abnormalities in milk nor in mammary gland. Therefore, knowledge of routine diagnostic tests for early detection of mastitis is desirable to treat the condition, and to avoid the subsequent economic losses. Although, most of the dairy farmers are not sentient of the different easiest and effective screening tests like California mastitis test (CMT), White side test (WST), Surf field mastitis test (SFMT), and milk somatic cell count (SCC) for the diagnosis of CM, however, these tests are routinely used by different research and academic institutes (Hoque et al., 2015). The diagnostic methods for mastitis diagnosis developed so far is represented in **Table 1**. Current trends in diagnosis of mastitis involves following routine diagnostic tests:

I. **Physical examination of udder:** Examination of mammary gland is important for successful detection of mastitis. It is emphasized to view the shape, size, consistency and contour of the udder properly. Thorough examination of the teat and teat orifices should be made to assess inflammation, hot painful swelling, and loss of function (Adkins and Middleton, 2018).

II. **Strip cup test:** In individual animals and in herds, strip cup or strip plate test is routinely used in milking parlor for detection of clinical mastitis. In herd health management practice operators of the milking machines visually examine the fore milk for gross abnormalities by squirting few stripes of milk on strip cup where the abnormalities are usually manifested in the form of blood, flakes, clots and wateriness suggestive of mastitis (Hansen, 1934; Adkins and Middleton, 2018). The use of strip cup test bears some additional benefits apart from general identification of clinical mastitis as –

    a. Stripping the first stream of milk stimulates milk letdown, resulting in faster milk letout.

    b. Fore milk is higher in bacteria than subsequent milk; removal of this milk may reduce bacterial contamination of the milking machine and udder.

III. **On-farm screening tests:** The CMT, WST, and SFMT are arguably the only reliable cow-side screening tests for clinical or subclinical mastitis that can easily be applied. The CMT is a simple, inexpensive, rapid screening test for subclinical mastitis, based upon the amount of cellular nuclear protein present in the milk sample. The CMT is also indicator test for mastitis by somatic cell count estimation of milk which allows the DNA in those cells to react with the test reagent, forming a gel. The reaction is scored on a scale of 0 (where mixture remains unchanged) to 3 (solid gel forms) with a score of 2 or 3 being considered a positive result (**Figure 1.2.1.8.1 A-D**). The electrical conductivity of milk may be defined as the property of substances in solution which can ionize and therefore can conduct an electrical current. When the concentration of sodium chloride rises in milk, the conductivity rises proportionately. Therefore, measurement of electrical conductivity is used as a simple physical method to diagnose mastitis. Electrical conductivity (EC) of milk can be determined by using a hand held (portable) electrical conductivity meter (milk checker or digital mastitis detector e.g. Draminiski) (**Figure 1.2.1.8.1 E**). The EC of milk is expressed in the unit of milk seimens/cm (Ms/cm) (Adkins and Middleton, 2018). Other methods described are Portacheck (esterase-catalysed enzymatic reaction), pH tests, and biosensors (Hoque et al., 2015; Martins et al., 2019). It is a test which is not very costly and nontechnical persons or laymen can use it as routine test for the dairy animals.

IV. **Somatic cell counting of milk:** Modern mastitis tests allow for indirect determination of the number of somatic cells (SCs) in milk. The SCs are the epithelial (25%) and leukocytes (75%) cells secreting through milk. If inflammation i.e., mastitis occurs, somatic cells number also become higher and it is due to migration of more neutrophils in the milk which is around 90%. Measurement of SCs in the milk samples are referred as Somatic Cell Count (SCC). As the inflammatory process develops in udder tissue, the number of these cells (particularly leukocytes) in milk sharply increases. SCC if is lower than $1x10^5$ cells mL^{-1}, indicate normal milk and while during infection it can rise to above $1x10^6$ cells mL^{-1} (Hoque et al., 2015). Thus, SCC can be referred as an indicative test for early diagnosis of mastitis and any

alteration in cell count can be correlated with the presence of potent pathogen. To diagnose bovine CM, SCC can be performed within 24 h of collection using an automated (e.g. NucleoCounter SCC-100) instrument (**Figure 1.2.1.8.1 F**). The count used includes the direct microscopic SCC (DMSCC), the bulk milk SCC (BMSCC) and individual cow SCC (ICSCC). Thus, SCC, CMT and intramammary infection are associated significantly; therefore, these parameters provide the necessary information to evaluate udder health status in cows.

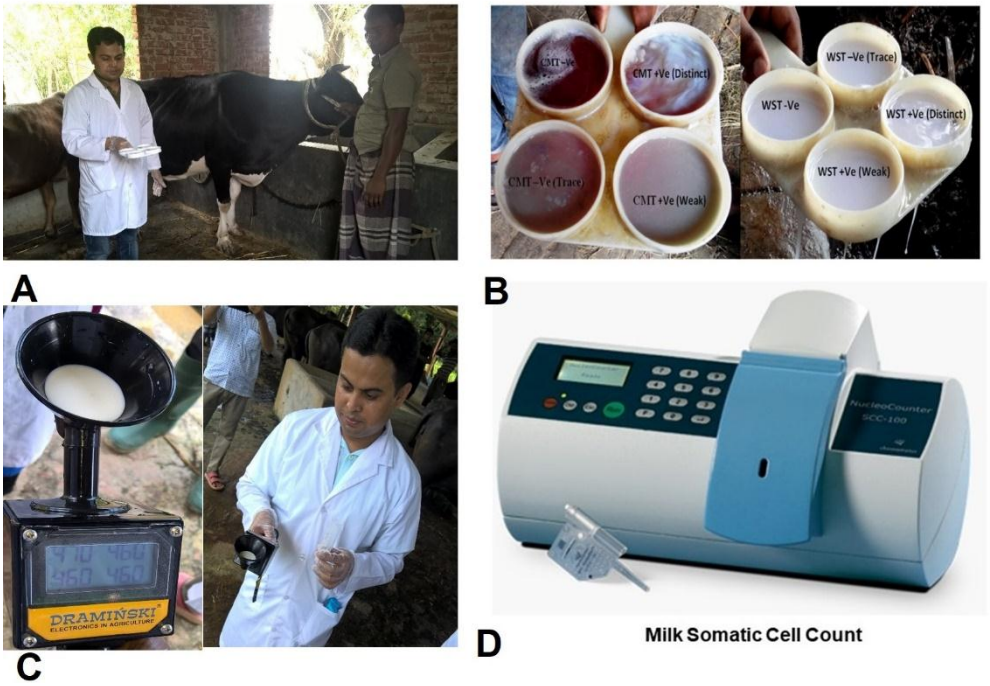


Figure 1.2.1.8.1: Screening tests for bovine mastitis. Different screening tests (A-B), electrical conductivity test (C), and milk somatic cell count (D) used for on-farm, and laboratory diagnosis of bovine mastitis.

1.2.1.9 Detection of bacterial pathogens from bovine mastitis

1.2.1.9.1 Detection through conventional methods

Bovine mastitis is a multifactorial disease, which develops as a result of the interaction between various factors associated with the host, the specific pathogens, the environment, the season, and the farm management. Numerous bacterial species have been isolated from bovine mastitis cases, but historically *Staphylococci* and *E. coli*, as well as other members of the family *Enterobacteriaceae*, are the most common agents of mastitis (Cheng et al., 2019). Bacterial pathogens of mastitis are usually detected by cultural examination of mastitis milk samples. Conventional tests for detecting and identifying the bacterial pathogens of mastitis include cultural,

morphological, biochemical, serological and molecular methods. *In vitro* culture is regarded as gold standard test for mastitis diagnosis. Swab of milk samples can be taken for bacterial, viral, archaeal and fungal culture in a specific media and further microbiological/biochemical test applied for specific detection viz. coagulase tests for *Staphylococcus* and *E. coli* (Cheng et al., 2019; Hoque et al., 2020a). The most frequently isolated pathogens are *S. aureus*, *E. coli*, *Klebsiella*, *Streptococcus*, *Mycoplasma*, *Enterobacter*, *Bacillus*, and *Corynebacterium* species (Hoque et al., 2015, Abebe et al., 2016, Gao et al., 2017, Cheng et al., 2019). Although culture-based techniques are still the forefront of detecting CM bacteria, these methods are time-consuming and have the critical drawback of not being applicable to non-cultivable bacteria (Baron et al., 2018). Moreover, the isolation and identification of viral and archaeal etiological agent is cumbersome and facilities are also a limitation. Virus and archaea isolation are very tedious from the cases of chronic infections or the cases with secondary invasions. In contrast fungal pathogens can be isolated easily in routine microbial media but it is time consuming as fungi can take weeks together to grow in laboratory media. It is important to note that culture is capable of detecting only viable cells and thus the clinical relevance of culture negative results requires further study (Deb et al., 2013).

1.2.1.9.2 Detection of through molecular techniques

Compared to bacterial culture methods, the polymerase chain reaction (PCR)-based detection from directly mastitis milk samples are less time consuming. PCR-based detection usage pathogens DNA, and thus no matter of culturable or non-culturable organisms which are crucial point for culture-based detection. Various PCR-based tools have been demonstrated for detecting microbes in mastitis milk samples as example detection of *S. aureus*, *E. coli*, and *Streptococcus* species (Amin et al., 2011). A rapid PCR test for identification of *S. agalactiae* by 16S-23S rRNA intergenic spacer region (ISR) amplification (Wu et al., 2008), and *S. aureus* genotype B (GTB) detection (Syring et al., 2012) had been documented. Mostly fastidious etiological agents remain untraced, and for all such conditions multiplex PCR (mPCR) can be a boon for the veterinarian since this can identify multiple pathogens in a single reaction and at a same time (Phuektes et al., 2001). Thus, mPCR could effectively be used for diagnosis of multiple pathogens in bovine mastitis milk samples. The main drawback with mPCR is that there is competition between

different sets of primers for PCR substances like dNTPs and Taq polymerase which might reduce the sensitivity.

Real-time PCR based assay is an alternate to *in vitro* culture for detecting bacterial pathogens in milk samples. To afford particularly in the developing countries, this method is not economically viable and needs sophisticated equipments. Enzyme-linked immunosorbent assay (ELISA) based detection of various biomarkers like heptoglobulin, NAGase, lactate dehydrogenase (LDH) (Orden et al., 1992), and acute phase protein (Eckersall, 2007) have already developed for the diagnosis of bovine mastitis pathogens (**Table 1**). Advancement in proteomics tools for examples two-dimensional gel electrophoresis (2D-GE), and mass spectroscopy (MS) (Smolenski et al., 2007) helped to identify various protein expressed during mastitis. These methods can be applied to detect the marker proteins from the cases of mastitis particularly from the acute, subacute and chronic mastitis (Boehmer, 2011). Furthermore, biochips having the capacity to use as a diagnostics are already in use for the detection of bovine mastitis (Lee et al., 2008), and biosensors also known as biological sensors which uses bio-receptors like-antibody, nucleic acid, enzymes and produce a signal after combination with transducers are nowadays being used for to discriminate between mastitic and healthy milk (Martins et al., 2019).

1.2.1.10 Microbiome characterization in bovine mastitis

The microbiome is the catalogue of microbes and their genes associated with the host organism (Ursell et al., 2012). Over the past two decades, a wide range of phenotyping and genotyping methods have been developed or implemented to study mastitis-causing bacteria. Although culture-based techniques are still the forefront of detecting clinically relevant bovine mastitis causing bacteria, these methods are time-consuming, and have the critical drawback of not being applicable to non-cultivable bacteria (Hoque et al., 2019, this thesis). It is postulated that up to 99% of the microbes in the environment cannot be readily cultivated, and culture-based tests in mastitis fail to identify pathogenic organisms in about 30% of cases (Oikonomou et al., 2014). The concept of microbiome analysis serving as a non-invasive but very important culturable and non-culturable diagnosis tool for interacting microbiomes that leads to multi-organisms associated diseases including bovine CM. There has been an increased interest in the characterization of the bovine milk microbiome during mastitis, and its comparison between healthy and disease states (this thesis,

Hoque et al., 2019; Hoque et al., 2020a).

Table 1: Diagnostic methods/protocols developed for mastitis diagnosis (indicative markers and description).

Methods/platform (technology)	Description	Reference
Conventional methods		
Physical examination of udder	To examine the shape, size, consistency, and contour of the udder, teat and teat orifices properly.	Adkins and Middleton, 2018
Strip cup test (SCT)	Indirect indicator for estimating somatic cells (SC). The test reagents react with cell's DNA to form a gel. The gel viscosity is proportional to SC present in a milk sample.	Hansen, 1934
Leukocyte counting		Halversen et al., 1934
Chlorine test		Rosell, 1936
California mastitis test (CMT)		Schalm and Norlander, 1957
Surf field mastitis test (SFMT)		Muhammed et al., 2010
pH tests	Measures increases in normal milk pH (normal milk: pH = 6.7).	Marschke and Kitchen, 1985
Somatic cell count (SCC)		
Haemocytometer method	Detects and counts SC by counter e.g. Haemocytometer, NucleoCounter® SCC-100™.	Freshney, 1993
Automated cell counter		Hoque et al., 2015
Bulk tank Somatic cell count (BTSCC)		Radostitis et al., 2000
Electronic diagnosis		
Electrical conductivity test (ECT)	Abnormal conductivity of milk.	Milner et al., 1996
Mast-O-test		Musser et al., 1998
Enzymatic methods		
Enzyme-linked immunosorbent assay (ELISA)	Measures the activity of enzymes reflecting tissue destruction such as NAGase and lactate dehydrogenase (LDH).	Orden et al., 1992
Radioimmunoassay (RIA)		Orden et al., 1992
Culture-based methods		
On-farm culture	On-farm culturing system to identify mastitis causing pathogens.	McCarron et al., 2009
Laboratory-based microbiological culture	Use of culture media to identify mastitis causing pathogens (selective and non-selective).	Hoque et al., 2015
Advanced tecniques		
PCR-based diagnosis (real-time PCR, mPCR, gradient gel electrophoresis PCR, and PCR-single-strand conformation polymorphism, SSCP)	Identification and quantification of mastitis pathogens by detecting specific nucleic acid sequences (pathogen molecular signatures).	Phuektes et al., 2001

Ribosomal (16S rRNA) gene sequencing	16S rRNA gene sequencing is used as a tool to identify mastitis causing bacteria at the species level, and assist with differentiating between closely related bacterial species.	Hoque et al., 2020a
Whole genome sequencing (WGS)	The WGS refers to the construction of the complete nucleotide sequence of a genome, and allows clinical isolates of a particular organism to be compared with each other, and with reference sequences across time and space, with an accuracy of a single nucleotide difference.	Leimbach et al., 2016
Proteomics (Biomarkers)-based diagnosis	Proteomics refer to providing an accurate and early detection of the mastitis, to establish a correct identification of causative agents and to evaluate animal–pathogen interactions and animal immune responses.	Boehmer, 2011
Chip-based diagnosis	Sedimentation microfluidic (rotational disc), exploiting the differences between fat and cell fraction in milk.	Lee et al., 2008
Biosensor	Affinity sensor to detect biological molecules in milk.	Martins et al., 2019
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry is a highly accurate technique in identifying mastitis pathogens.	Jahan, 2017
Ultrasonography	The critical thresholds for ultrasonographic dimensions of mammary glands is a rapid field test for supporting diagnosis of mastitis	Hussein et al., 2015
Metagenome-sequencing based diagnosis		
Amplicon metagenome sequencing	Amplicon sequencing or targeted metagenomics of marker-genes (e.g. 16S, 18S, ITS) involves using specific primers that target a specific gene or gene fragment of an organism.	Oikonomou et al., 2014
Whole metagenome sequencing (WMS)	The WMS comprehensively detect the total microbial community (bacteria, archaea, and viruses), and diversity in a given sample, their resistomes, virulence factors, and metablolic functions.	Hoque et al., 2019 (this thesis)

1.2.1.10.1 Metagenomics approaches for milk microbiome characterization

The term ‘Metagenomics’ was first coined by Jo Handelsman in 1998. Metagenomics is the study of genetic material recovered directly from environmental samples. The broad field may also be referred to as environmental genomics, ecogenomics or community genomics. This is a molecular tool used to analyse DNA acquired from environmental or clinical samples, in order to study the community of microorganisms present, without the necessity of obtaining pure cultures (Handelsman et al., 1998). It revolutionizing, the understanding of microbial diversity present in natural and man-made environments, and links microbial community profiles with health and disease (Flygare et al., 2016). These approaches reduce bias, improve detection of less abundant taxa, and enable discovery of novel pathogens.

Alongside, metagenomic studies of host-microbe interactions can provide useful information applicable to a wide array of disciplines including pathogen surveillance, biotechnology, host-microbe interactions, functional dysbiosis, and evolutionary biology (Hoque et al., 2019). Metagenomics projects have very vast applications, ranging from ecology and environmental sciences (Dinsdale et al., 2008), the chemical industry (Lorenz and Eck, 2005), marine environments (Zhao and Bajic, 2015), human health (Lloyd-Price et al., 2017), animal health (Kuehn et al., 2013; Ma et al., 2018) and forensic applications (Hampton- Marcell et al., 2017). However, future microbiome research regarding the molecules and mechanisms mediating interactions between members of microbial communities and their hosts should lead to discovery of exciting new biology and transformative therapeutics (**Figure 1.2.1.10.1**).

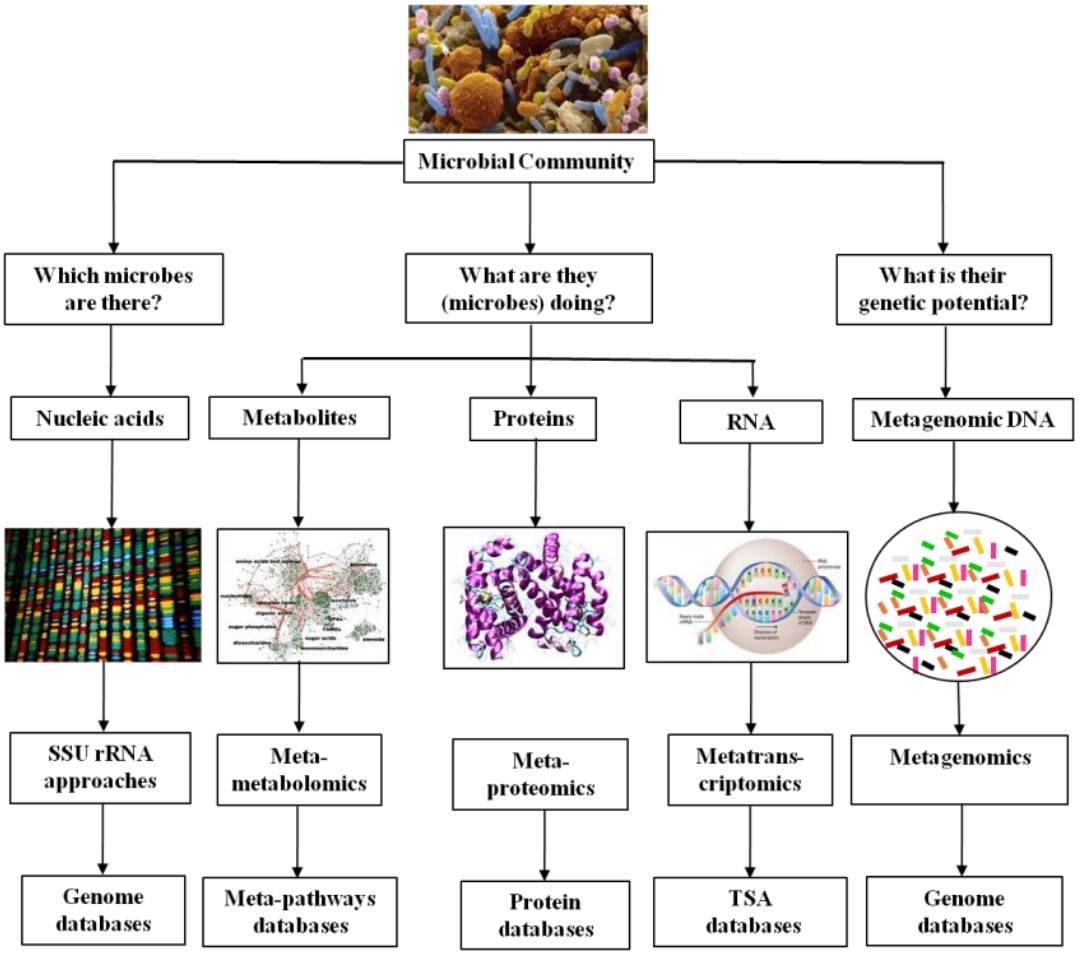


Figure 1.2.1.10.1: Schematic representation of metagenomics and bioinformatics analyses to explore microbiomes. The illustration covers topics that are already popular, that need further development, and that will become important in the future. At the bottom of the illustration, reference databases lay foundations for various bioinformatic analyses (Adapted from Morgan and Huttenhower, 2012).

1.2.1.10.2 Next-generation sequencing for microbiome characterization

The power of high–throughput DNA sequencing technologies is being harnessed by researchers to address an increasingly diverse range of biological problems. The advances of NGS technology have facilitated microbiome research, and enabled the exploration of genetic and functional diversity of microbial communities with affordable costs and sufficient throughput (Levy and Myers, 2016). There are two major paradigms in NGS technology: short-read sequencing and long-read sequencing. Short-read sequencing approaches provide lower-cost, higher-accuracy data that are useful for population-level research and clinical variant discovery. By contrast, long-read approaches provide read lengths that are well suited for *de novo* genome assembly applications and full-length isoform sequencing (Goodwin et al., 2016). Up to now, there are two main strategies implemented for the analysis of microbial communities through NGS: shotgun metagenomics and 16S rRNA sequencing.

1.2.1.10.2.1 Target or amplicon (16S rRNA) sequencing

The most widely used NGS method for the taxonomic and phylogenetic evaluation of bacterial community composition relies on amplicon or target sequencing or the 16S rRNA gene amplicon analysis. The 16S rRNA gene, which is a specific marker gene of microbes, having an essential role in protein synthesis, is present in all prokaryotes. The 16S rRNA gene sequencing relies on the polymerase chain reaction (PCR) amplification of a specific region in the 16S gene, using degenerated primers allowing amplification of ribosomal RNA from the largest possible number of species and finally PCR amplicons are then sequenced by NGS platforms (Tremblay et al., 2015) (**Figure 1.2.1.10.2.1**). Amplicon sequencing of the 16S locus has been used to characterize the biodiversity of microbes from a great range of environments including the human gut (Lloyd-Price et al., 2017), bovine *milk (Falentin et al., 2016; Catozzi et al., 2017; Lima et al., 2017), and humans milk (Patel et al., 2017; Ruiz et al., 2019).*

Though powerful, amplicon sequencing is not without limitation. The inherent limitations include the choice of primers used to amplify rRNA is critical, as some primers have been shown to exhibit a bias resulting in over- or under- representation of specific taxa, can produce widely varying estimates of diversity and the taxonomic resolution is not enough for identification at species or strain level and the functions

of these microbes also cannot be directly determined (Oniciuc et al., 2018). In addition, sequencing error and incorrectly assembled amplicons (i.e., chimeras), can produce artificial sequences that are often difficult to identify. Finally, amplicon sequencing is limited to the analysis of taxa for which taxonomically informative genetic markers are known and can be amplified. Novel or highly diverged microbes, especially viruses, archaea and fungi are difficult to study using this approach (Hoque et al., 2019, this thesis). Additionally, because the 16S locus can be transferred between distantly related taxa (i.e., horizontal gene transfer), analysis of 16S sequences can result in overestimations of the community diversity (Oniciuc et al., 2018).

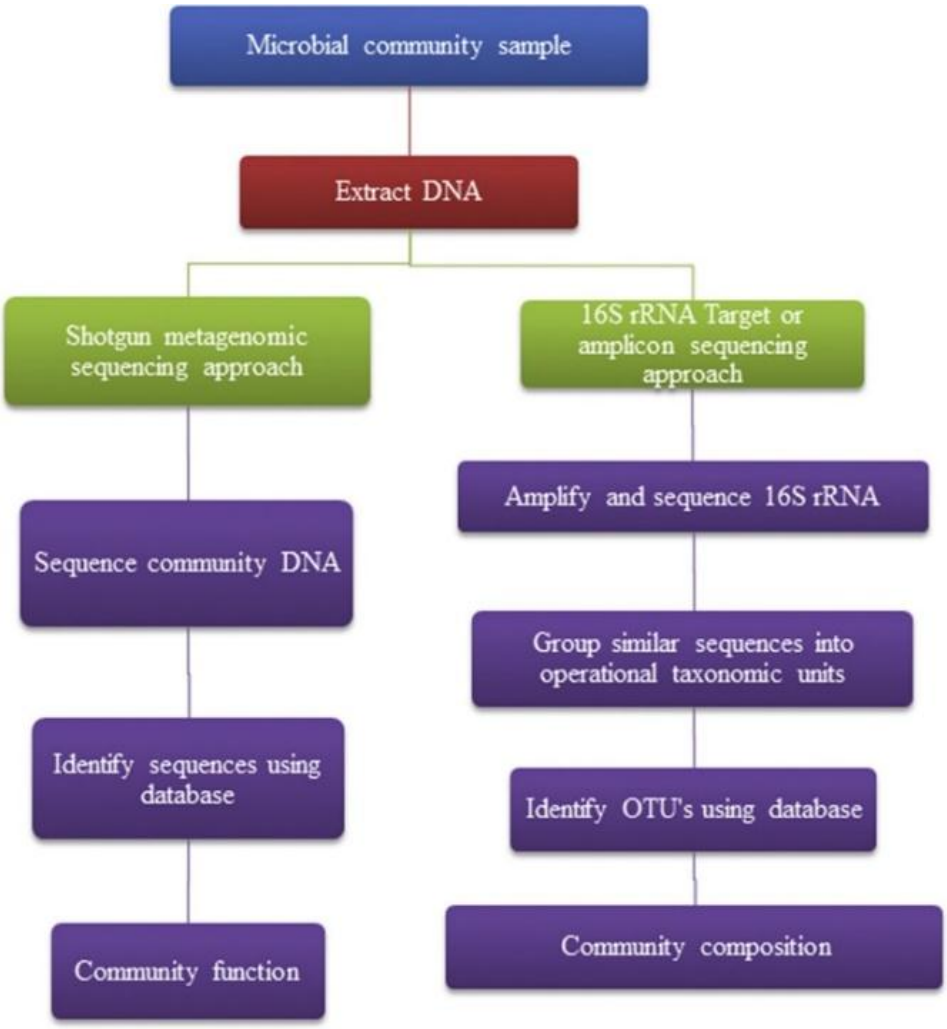


Figure 1.2.1.10.2.1: Amplicon (16S rRNA) sequencing and whole metagenome sequencing (WMS) approaches (Adapted from Tremblay et al., 2015).

1.2.1.10.2.2 Shotgun whole metagenome sequencing (WMS)

Since 2002 onwards researchers began using a shotgun WMS approach for

metagenomics. This random sampling of the DNA of an environmental sample makes it possible to characterize the entire genome of the species rather than just the 16S rRNA gene. This approach is very successful, especially when conducted on a massive scale. The WMS also known as shotgun metagenomics involves the fragmentation and subsequent sequencing, assembly and annotation of total genomic DNA isolated from a given sample (e.g., milk), and makes it possible to gain information on its entire (prokaryotic and eukaryotic) gene (**Figure 1.2.1.10.2.1**). This is an alternative approach to amplicon sequencing in exploring the microbiome of an ecological niche avoiding different known limitations (Salvetti et al., 2016). The WMS typically produces high complexity datasets with billions of short reads allowing extensive characterization of microbiome in an ecological niche, profiling their functional attributes, and gradually becoming a cost-effective metagenomic approach (Oniciuc et al., 2018; Hoque et al., 2019). Still, the application of WMS to explore bovine mastitis microbiome has not been reported elsewhere, but it has the potential to use for routine diagnostic pipeline particularly for dairy animal microbiome studies (Atkinson et al., 2017; Hoque et al., 2019). When compared to 16S-based metagenomics, it offers the advantage of identification of strain level taxonomy and the estimation of metabolic pathway activities from human, animal and environmental samples (Oniciuc et al., 2018). With the generation of vast amounts of data, the bioinformatics and computational analysis of WMS results become vital for the success of a metagenomics study. However, each step in the WMS data analysis, including metagenome assembly, gene prediction, taxonomy identification, function annotation, and pathway analysis, is complicated by the shear amount of data (Seth et al., 2014).

1.2.1.10.2.3 Strain-level microbiome profiling in bovine mastitis

Advances in metagenome bioinformatics over the last decade have refined the resolution of microbial community taxonomic profiling from the phylum to the species, but it is still difficult to characterize microbes in communities at the strain level. Investigations on microbial strains and characterizing their functional potential have been proven to be crucial for understanding of the evolutionary processes, pathogen discovery, adaptation, epidemiology, pathogenicity, drug resistance, transmission dynamics, and population genomics. Moreover, the functional capacities, interaction within host tissues, immunomodulatory homeostasis, and xenobiotic

metabolism of the microbiomes either in healthy or disease state of their associated hosts depends on their strain-level variants (Segata, 2018). Differences occurring on strain level in microbiomes of bovine milk samples can be of high relevance for disease and health status of the mammary gland or udder (this thesis, Hoque et al., 2019). The pathogenic potential of the microorganisms causing bovine mastitis always remains strain-specific, and thus, their characterization and identification in the environment as well as in individual host is of paramount importance to understanding the progression of pathogenesis (this thesis, Hoque et al., 2019). The strain-level variation of microorganisms classically comprises of single-nucleotide variants (SNVs), acquisition/loss of genomic elements including genes, operons, or plasmids (Truong et al., 2017). Strain individuality is present in most of the microbial diversity, and thus identification of conspecific bacterial strains is very important to understand the functions of associated microbiomes. The cutting-edge WMS technique can detect the strain-level taxonomic profile of different microorganisms, and also identify the resistome, virulence, and metabolic functional potentials of the microbial strains both in disease and healthy condition (Segata, 2018; Hoque et al., 2019; Hoque et al., 2020a, this thesis).

1.2.1.11 Treatment of bovine mastitis

Antimicrobial therapy is the preferred approach for treating bovine mastitis cases. For an antimicrobial to be effective, it must reach and persist at the site of infection in effective concentration. Antimicrobials are used in the dairy industry for prevention and control of mastitis, and other bacterial diseases affecting dairy cows. Therefore, dependence on antimicrobials has become a widespread phenomenon on dairy farms. Antimicrobials are commonly administered for appropriate management CM during lactation, and effective dry cow management as a part of ten points recommended by National Mastitis Council (NMC)’s mastitis control program (Hoque et al., 2020a, this thesis). Dry cow therapy is intended to cure existing infections and prevent new infections during the dry period (Gruet et al., 2001). Treatment of intramammary infection (IMI) at dry off has many advantages over treatment during the lactation such as higher dosage of an antimicrobial can be administered safely, a more uniform level of antimicrobial is maintained over longer duration, higher cure rate, lower risk of contamination of milk with antibiotic residues, and no discard of milk; incidence of new IMI during non-lactating period

and CM at freshening is also reduced (Nickerson, 2009). The bovine udder is a difficult target for antibacterial treatment. Absorption and distribution of the drugs through the gland's tissue as well as penetration into milk depends on their pharmacokinetic properties (Giguère et al., 2013). The main treatment of bovine mastitis is commonly administered by intramammary infusion of an ointment or intramuscular or intravenous injection of antibiotics, such as cephalosporins (cephapirin sodium, cephapirin benzathine, ceftiofur hydrochloride), penicillins (cloxacillin benzathine, penicillin G procaine–novobiocin combination), penicillin combinations (penicillin G procaine–dihydrostreptomycin sulfate–novobiocin sodium–polymyxin B sulfate combination), macrolides (erythromycin), and lincosamides (pirlimycin) (Cheng et al., 2019; Yang et al., 2019; this thesis, Hoque et al., 2020a,). Antimicrobials such as oxytetracycline, sulfadimethoxine, ceftiofur, ampicillin and amoxicillin have an appropriate spectrum of activity against *E. coli* and *Klebsiella* species isolates (Wagner and Erskine, 2006). In general, broad-spectrum antimicrobials are commonly used to treat cmastitis; however, routine use of antibiotics in dairy herds may increase the development of bacterial resistance against antimicrobials which is considered as one of the reasons for low cure rates in bovine mastitis pathogens (Barkema et al., 2006). However, the treatment is anticipated to become problematic in the near future owing to the rapid increase in antibiotic-resistant pathogens (Yang et al., 2019). Therefore, seeking for treatments alternative to antibiotic therapy is required.

1.2.1.12 Prevalence of antimicrobial resistance in bovine mastitis pathogens

Currently, antimicrobial treatment is indispensable to keep bovine udder health, animal welfare and economic aspects in balance. The use of antimicrobials has become a widespread phenomenon on dairy farms for mastitis management, prevention and control programs. However, efficacy of antimicrobial therapy against bovine mastitis pathogens is considerably low, and the use of antibiotics are mostly confined to selected severe CM cases only. The prevalence of antimicrobial resistance (AMR) is increasing in human and animal pathogens, becoming a concern worldwide. The use of antimicrobials during lactation and non-lactating period is hypothesized to select for AMR in bovine mastitis pathogens. The vast diversity of bacterial species in mastitis milk coupled with short generation times and horizontal gene transfer permit the rapid accumulation of countless resistance variations (Weller and Wu,

2015). Remarkably, resistance, at the genetic level, has existed for thousands of years, and is a natural process of bacteria to survive (Bengtsson-Palme et al., 2017). AMR is thought to be due to the acquisition of foreign DNA by transfer of genes encoding for resistance from one bacterial host to a new bacterial host via conjugation (sexual transfer of DNA), transduction (bacteriophage transfer), or transformation (acquisition and incorporation of DNA released into the bacteria's environment by lysis of other bacteria) or by resistance-mediating mutations (Schwarz et al., 2017). Contact between antimicrobials and susceptible bacteria represent a selective pressure where the latter want to protect themselves against being killed or inhibited by antimicrobials. As a result, susceptible bacteria are being killed or inhibited whereas resistant bacteria replicates, resulting in an increasing number of resistant bacteria. Knowing the antimicrobial susceptibilities of common mastitis pathogens can help aid veterinarians in their choice of an effective antibiotic treatment for an individual infection. Multi-drug resistance (MDR) is common in bovine coliform mastitis. In general, resistance to various antimicrobials is frequently seen in bovine mastitis isolates (Cheng et al., 2019; Qu et al., 2019).

Bacteria residing in the bovine gastrointestinal tract, and udder may become resistant to these antibiotics, and once released into the milk, they may take part in horizontal transfer of antibiotic resistance genes (ARGs) to other CM bacteria of contagious and environmental origin (Zaheer et al., 2019). Furthermore, AMR is a global health concern in both human and veterinary medicine, and thus monitoring the emergence of AMR strains is an essential component of bovine mastitis prevention and control strategies (Cheng et al., 2019). Recently, we reported that 79.3% *S. aureus* strains were resistant to at least one antimicrobial, 49.0% to two or more antimicrobials, and highest resistance rate was found to oxytetracycline (Hoque et al., 2018). Bovine CM caused by *E. coli* were resistant to ampicillin (98.4%), streptomycin (40.3%), sulfisoxazole (34.1%) and tetracycline (24.8%), and less than 20% of *E. coli* isolates were resistant to carbenicillin, gentamicin, cephalothin, trimethoprim and amikacin (Srinivasan et al., 2007). In a previous study of bovine mastitis, 41.44, 25.65, 13.81, 11.84, 3.94 and 3.28% isolates were resistant against penicillin, streptomycin, erythromycin, tetracycline, ampicillin and Cephalothin, respectively (Muhamed et al., 2012). In another study Alekish et al. reported the highest rate of resistance among the CM isolates against trimethoprim/sulfamethoxazole (87.4%), penicillin (84.5%), tetracycline (77.7%), and

erythromycin (70.4%), respectively (Alekish et al., 2013). In a recent study, Wald et al. studied *in vitro* antimicrobial sensitivity patterns of isolates of *S. aureus* recovered from CM and showed higher MIC90 (minimal inhibitory concentration) values for penicillin, ampicillin, cefoperazone, pirlimycin and marbofloxacin (Wald et al. 2019). Therefore, finding an effective alternative strategy towards the control of bovine mastitis is a significant challenge for the dairy producers.

1.2.1.13 Antibacterial effects of metals on bovine mastitis pathogens

The routine use of antibiotics is questionable because their use can generate unwanted residues not accepted in milk, and because they can spread the emergence of antimicrobial-resistant strains. Since bovine mastitis remains a worldwide problem, producers and governments continue searching for a non-antibiotic solution or technologies to reduce the prevalence of the disease (Reyes-Jara et al., 2016). The antimicrobial properties of metals have been documented throughout the history of medicine and healthcare (Vaidya et al., 2017). To reduce the transmission of potentially infectious microorganisms, there has been a revival of interest in the utilization of metals as antimicrobial/biocidal agents. The antimicrobial properties of metals have been documented in many bodies of work and continue to be the subject of investigation in an attempt to understand the mechanisms of metal toxicity and resistance (Gugala et al., 2018). While the minimal inhibitory concentrations (MIC), minimal bactericidal concentration (MBC), and minimal biofilm eradication concentrations for many metals have been determined, the lack of consistency between techniques, conditions and media have resulted in difficulties when comparing the susceptibilities of bacterial strains to metal compounds. The metal salts such as chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn) are effective in controlling bacterial transmission and infection risks (Reyes-Jara et al., 2016; Vaidya et al., 2017). Metals could be used both individually and in combination (Vaidya et al., 2017), and when used as synergistic treatments, composed of transition metals (zinc, copper, nickel, or cadmium) and silver, the combinations exhibit up to an 8-fold enhancement in their antimicrobial properties, compared to the individual treatments (Garza-Cervantes et al., 2017). However, their use is limited to dairy industries particularly as therapeutic agents against bovine CM pathogens due to their effectiveness, cost, toxicity and possible detrimental environmental effects. The use of toxic metals as a substitute to antibiotics prevent bovine mastitis appears as a novel

promising idea supported by several earlier studies (Reyes-Jara et al., 2016; Vaidya et al., 2017).

1.2.1.14 Biofilm formation in mastitis pathogens

Biofilms are structural community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface. Biofilm formation is an important virulence factor for mastitis causing bacteria, and contributes to the resistance to different classes of antimicrobials (Singh et al., 2017). Recurrent mastitis (RCM) infections are often attributable to biofilm growth of bacteria. Both gram-positive and gram-negative bacteria such as *S. aureus, E. coli, Klebsiella*, *Enterococcus*, *Enterobacter*, *Streptococcus*, *Bacillus*, *Proteus* and *Pseudomonas* species causing bovine mastitis can form biofilms (Chen et al., 2013; Gomes et al., 2016; Vaidya et al., 2017). Biofilm formation by bovine CM pathogens is accompanied by significant genetic and subsequent physiological changes in the microorganisms resulting in a loss of sensitivity to virtually all classes of antibiotics. The biofilm related infections are chronic type and caused by bacteria like *S. aureus* and *S. epidermidis* (nosocomial infections), *E. coli* (bacterial prostitis and biliary tract infections) and *Streptococcus* species (dental caries, periodontitis, endocarditis, meningitis and pneumonia). Most of these bacterial species are also involved as major pathogens in bovine mastitis and leads to difficulties in treating recurrent infections. Therefore, detection of biofilm forming ability in mastitis isolates might provide useful information for the establishment of more adequate therapeutic approach (Gogoi-Tiwari et al., 2017). Enzymes produced by biofilm polymeric substances plays a key role in protecting the bacteria organism through metabolizing of biopolymers and other substances used as antimicrobial agents (Wang et al., 2018).

1.2.1.14.1 Role of biofilm formation in antimicrobial resistance

Antimicrobial therapy is the major component and a primary tool for treating bovine mastitis. Antimicrobial resistance is usually associated with the improper use of antimicrobial agents and it is important to monitor the antimicrobial susceptibility patterns of mastitis pathogens (Li et al., 2009). Antibiotic chemotherapy is becoming increasingly ineffective in the treatment of mastitis infections because of antibiotic resistance of biofilms (Fair and Tor, 2014). Bacteria living in biofilms are better able to survive the host immune response and antimicrobial therapy by slowing their growth, reducing metabolism and reducing penetration of antimicrobial into the

biofilm structure. In the case of penicillins and cephalosporins, they were virtually ineffective on non-growing cells and the rate of bacterial killing was proportional to the growth rate (Fleitas et al., 2019). Because of the resistance of biofilms to common and otherwise effective antimicrobials, research is focusing on the ability of bacteria to form biofilms and to unravel the genetic adaptive processes that are responsible for biofilm formation. The greatest challenge posed by biofilm is thought to be its incredible ability to resist most of the currently existing antibiotics.

1.2.1.15 Mouse model for bovine mastitis microbiomes study

The study of animal microbiomes is widely perceived as a young discipline, made possible by the advent of sequencing technologies to determine the taxonomic identity and functional traits of microorganisms without cultivation. Dairy cows have been genetically selected for their unique linked or pleiotropic traits to achieve high production efficiency. The unique milk composition, the high milk production and the immune system of the cow and the possible bacterial adaptation to the milk environment of the udder imply that confirmatory mastitis studies should always be performed within the target-animal species. Several examples illustrate that the pathogenicity of some inflammatory agents and the acquired host response can be species dependent (Werling et al., 2009). Another argument in favour of the mouse mastitis model is that the use of laboratory Specific-Pathogen-Free (SPF) mice facilitates mastitis experiments and allows to downsizing of the number of animals required to achieve a statistically conclusive result (Ma et al., 2018). This is mainly due to the fact that these strains have the same genotype and are easily housed and/or managed. Last but not least, mouse models are extensively investigated for a whole array of predominantly human diseases. The use of mice opens up a wide panel of sampling, immunological reagents and research tools that are either very difficult or even impossible to perform in cows.

1.3 Background and hypothesis of the work

1.3.1 Background of the work

Bovine mastitis is an inflammatory disease caused by tripartite interaction among host, pathogens and environment. So far, more than 350 species of contagious environmental microorganisms were reported to be associated with the disease (this thesis, Hoque et al., 2019; Hoque et al., 2020a; Oikonomou et al., 2020). Globally,

clinical mastitis remains an important animal health issue and its incidence in dairy herds varies from 20.0 to 65.0% (Jha et al., 2010, Timofte et al., 2014). Antimicrobials are the only means for the treatment and prevention of clinical mastitis in dairy herds globally. In recent years, the emergence and spread of antimicrobial resistant bacterial strains have become a major public health concern and thus, it has become paramount important to determine the antimicrobial resistance patterns of mastitis pathogens (Kateete et al., 2013). This requires use of reliable methods in obtaining data on the bacterial distribution and defining the profiles of species or strains involved. However, until now, the involvement of the bovine mammary gland microbiota in the host-pathogen interaction has poorly investigated mainly during the infectious episode. The capacity for pathogens to switch host species leading to their epidemic spread in new host populations is a major veterinary and public health concern (Viana et al., 2015). The next generation sequencing (NGS) technology is an advanced diagnostic tool and can promptly be used for the diagnosis of mastitis (Oikonomou et al., 2014; Leimbach et al., 2016). Several reports have attempted to use NGS approach to explore the teat and milk microbiome and their role in clinical and subclinical mastitis (Kateete et al., 2013; Falentin et al., 2016; Hoque et al., 2019). Metagenome analysis is a powerful tool for assessing the phylogenetic diversity of complex microbial community present in diverse group of samples, and for exploring the genomic features including virulence, antimicrobial resistance, and metabolic functional properties of their dominant host populations (Coughlan et al., 2015; Oniciuc, et al., 2018). The whole metagenome sequencing can provide relatively rapid and cost-effective methods for assessing microbial diversity and their genomic functional potentials in mammary secretion of healthy and affected cows.

1.3.2 Hypothesis of the work

The application of culture-independent whole metagenome sequencing (WMS) approach would provide new insights into the identification and characterization of the microbiomes associated with bovine mastitis in their epidemiological niche; their assessment throughout infection process which will ultimately allow us to formulate most effective intervention strategies against this fearsome disease.

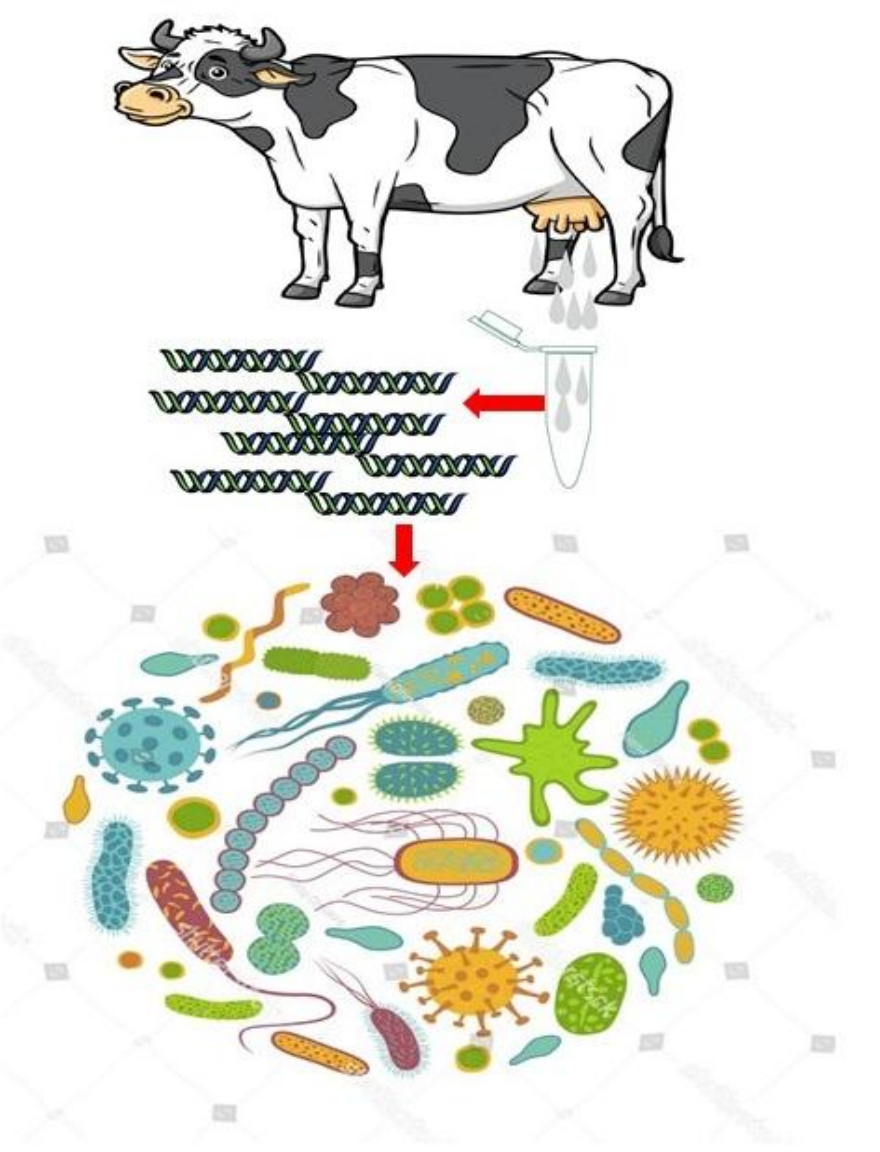
1.4 Objectives of the work

To address the hypothesis, this investigation has done highlighting the following objectives in order to explain the role of microbial interaction in the progression of mastitis and formulate a possible preventive and curative control pathway-

I. to characterize microbiome in bovine mastitis and healthy milk samples using whole metagenome sequencing (WMS);

II. to understand functional genomics and cross-talk among the pathogens involves in the progression of bovine mastitis; and

III. to study host tropism, taxonomic compositions, species diversities and functional profiling of bovine mastitis pathogens using mouse model.



Chapter 02:

Materials and Methods

2.1 Research plan

The following research plan has been followed to characterize microbiome associated with bovine clinical mastitis in some selected areas of Bangladesh.

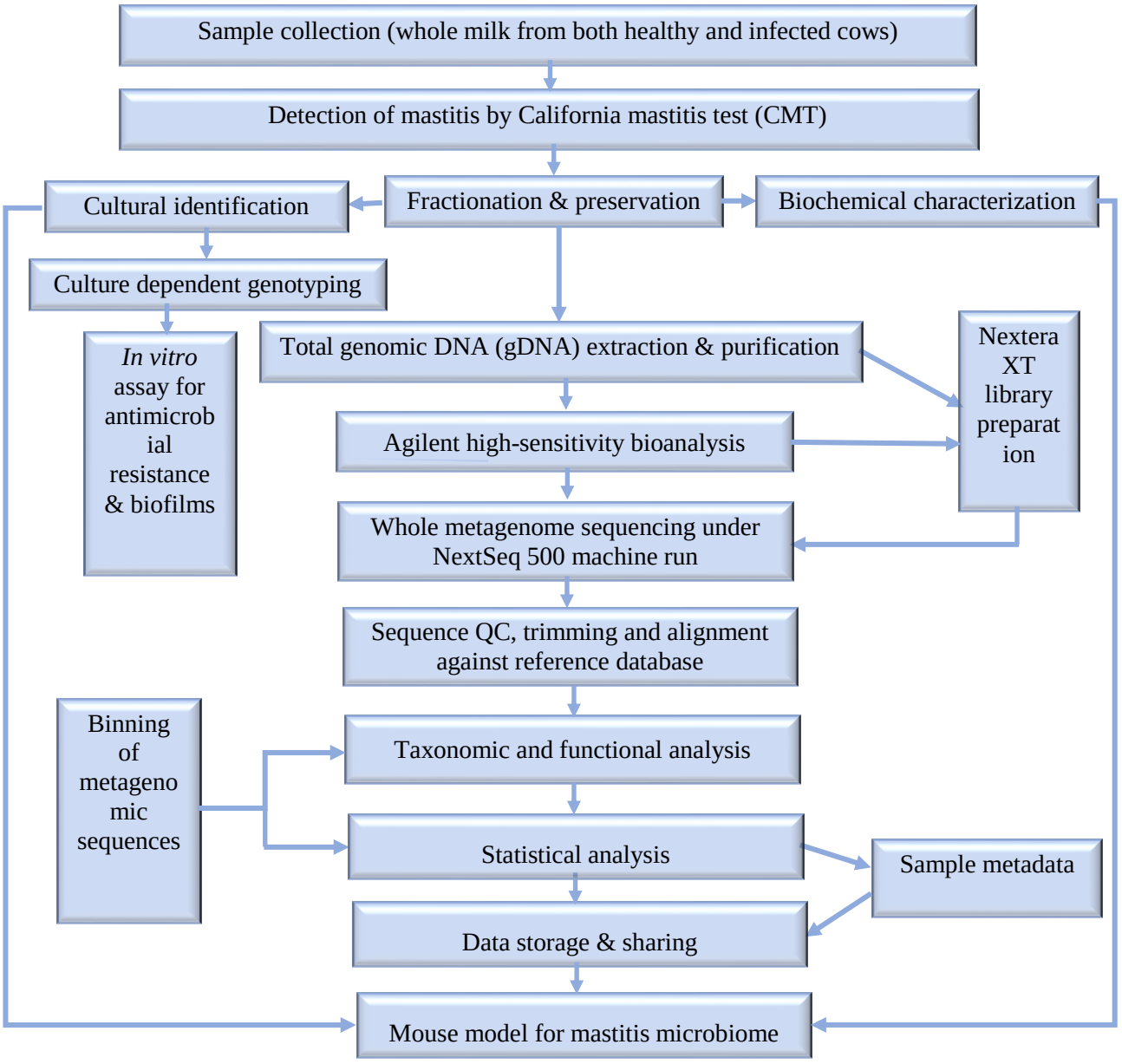


Figure 2.1: Research plan for bovine mastitis microbiome study. The study includes the steps in culture-based, biochemical, and metagenomic identification, bioinformatics analyses, antimicrobial resistance and biofilm formation assays, and mouse model selection for host-tropism of bovine mastitis microbiome.

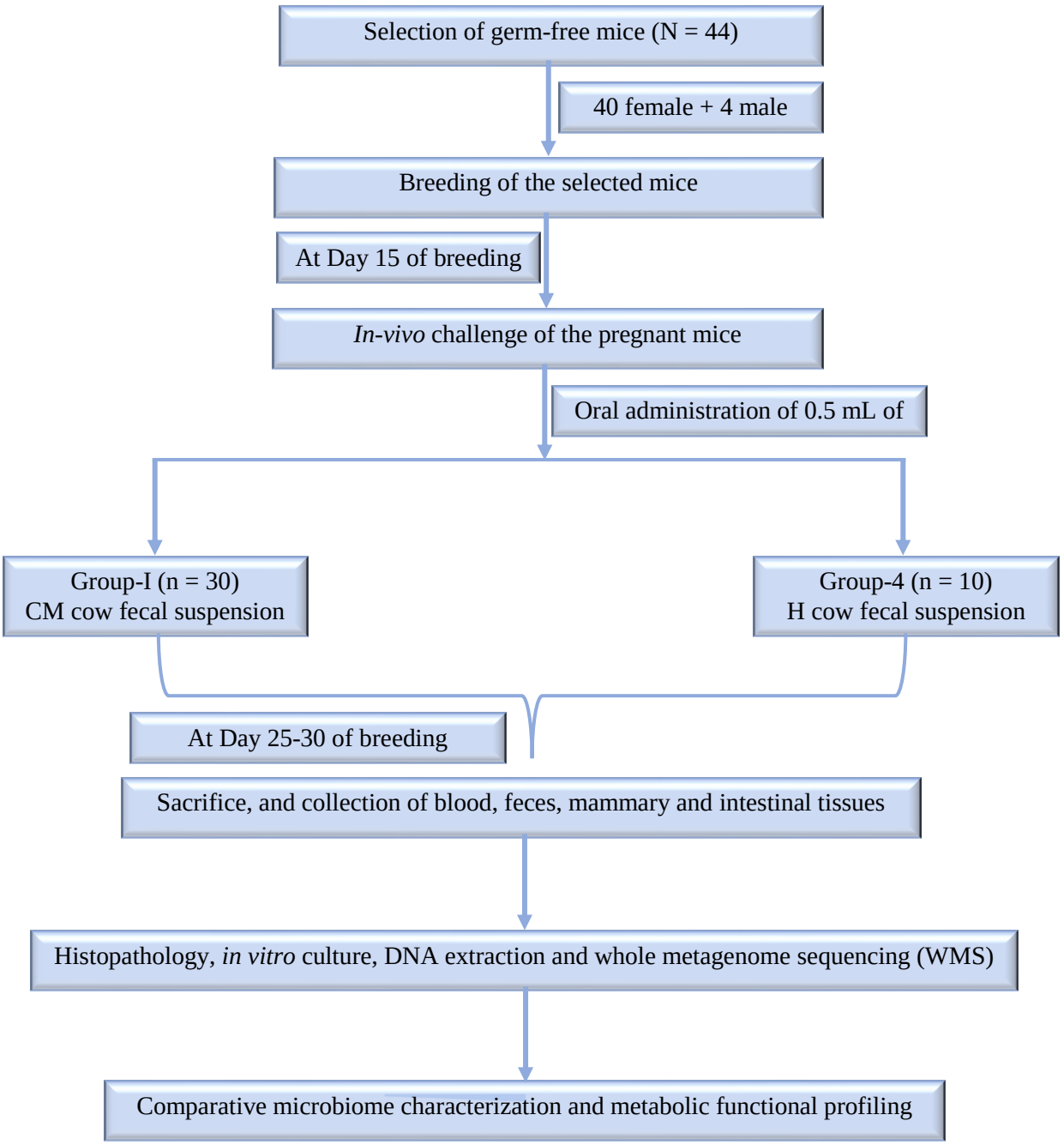


Figure 2.1.1: Layout for cows-to-mouse fecal microbiota transplantation. Research included fecal material transplantation and milk transplantation from both mastitic and healthy cows into selected pregnant mice with subsequent culture-based, biochemical and metagenomic identification for host-tropism of CM microbiome.

2.2 Study areas and population

The bovine mastitis research group of the Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka collected milk samples regularly from different dairy farms of the country. During 2017 to 2019, we collected 450 quarter milk (including CM=270, RCM=30, SCM= 100, and H=50) samples from 420 lactating dairy cows belonged to 80 smallholding dairy farms. The sampling was done in two geographical regions of Bangladesh (central region= 150; southeastern region, SER = 300). The central region (CR) included four districts such as Dhaka (23.8103° N, 90.4125° E), Gazipur (23.9999° N, 90.4203° E), Manikganj (23.8644° N, 90.0047° E), and Sirajganj (24.4526° N, 89.6816° E) while the southeastern (SER) region encompassed only the Chattogram (22.3569° N, 91.7832° E) district of Bangladesh (**Figure 2.2**).

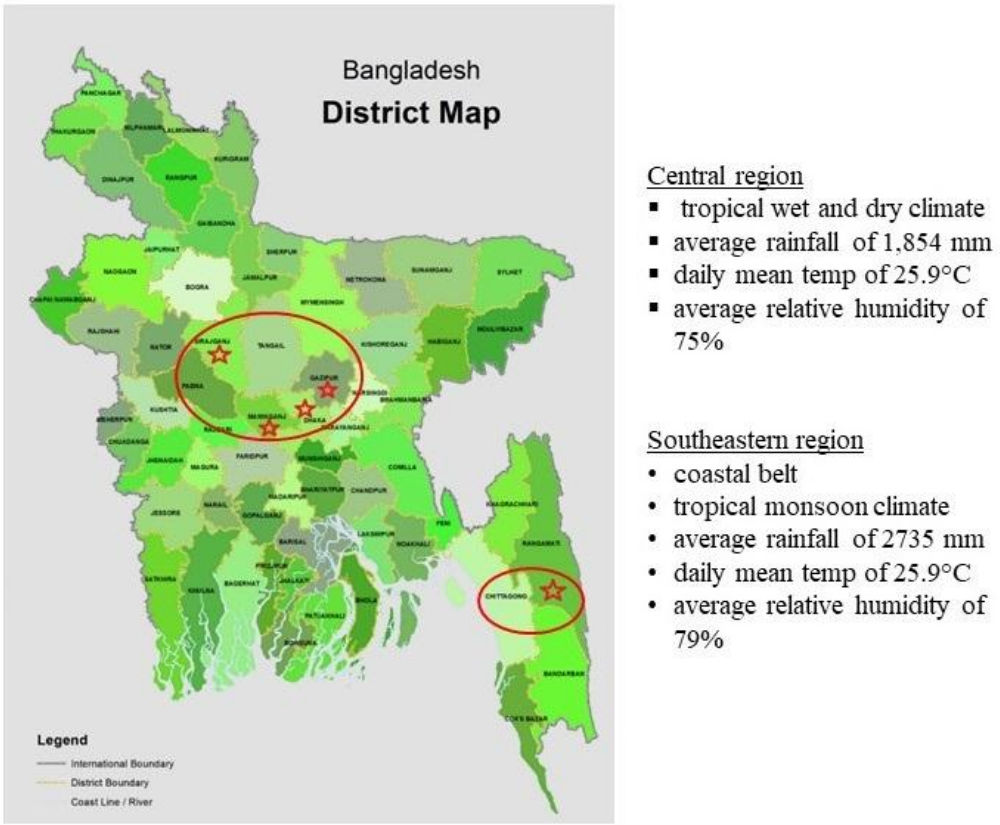


Figure 2.2: Study areas. This study was conducted in two different regions (central and southeastern) of Bangladesh (marked with red stars and circle). The lower red circle indicates the southeastern coastal region (SER); the Chattogram district, and the top red circle represents the central region (CR) which includes four (Dhaka, Gazipur, Manikganj and Sirajganj) districts of Bangladesh.

The examined cows were represented by four major dairy breeds of Bangladesh including Local Zebu (LZ), Red Chattogram Cattle (RCC), Sahiwal (SW)

and Crossbred Holstein Friesian (XHF) at their early stage of lactation (within 10-40 days post-calving) having parity of 1 to 5 (parity 1 = 2, parity 2 = 8, parity 3 = 6, parity 4 = 2, and parity 5 = 2) (**Table 2**). The cows were kept indoors in free stalls with a limited access to grazing and fed on cut and carry grass, commercial concentrates and occasionally with vitamin-mineral supplements. The cows were milked twice a day by hand milking, typically by the same person every day. Cows were considered as mastitic if they had already developed presenting clinical signs of cows (swelling, heat, hardness, redness or painful udder) and milk (watery appearance, flakes, clots or pus) as observed by the farm owners or attendants (**Figure 1.2.1.2**) and claimed it to practicing Veterinarians. Animals were considered as healthy (H) when they had never been encountered with mastitis, and have had a sound health with good milking records.

Ethical approval (Ref. 79/Biol.Scs., Dated:12-12-2019) was taken from the "Ethical Review Committee" of the Faculty of Biological Sciences, University of Dhaka (**Appendix-I**). A pretested structured questionnaire (**Appendix-II**) was used for data collection during milk sampling which describes the history of the farms and animals. The sequence data reported in this dissertation have been deposited in the submitted in the National Center for Biotechnology Information (NCBI) database (**BioProject PRJNA529353** for metagenome sequences, and NCBI accession numbers: **MN 620423–MN 620430** for 16S rRNA gene sequences) (**Appendix-III**).

2.3 Diagnosis of mastitis

2.3.1 General physical examination

Initial diagnosis of CM (and/or RCM) in affected cows was made by following the procedures of general physical examination by the registered Veterinarians based on the complaint of the respective farm personnel (owners or attendants). The general examination included assessment of the posture, behavior, body condition and general condition (respiratory rate, pulse frequency, rumen motility and body temperature). Then the udder itself was examined during milking at the milking parlor by inspection, palpation and examination of quarter milk secretion and milk appearance. Systemic and local signs were recorded and categorized using a 3-point scale as follows: (1) mild clinical mastitis: milk from a quarter had abnormal viscosity (watery or thicker than normal), color (yellow or blood-tinged), or consistency (flakes or clots), but no udder swelling or systemic signs; (2) moderate

clinical mastitis: similar to mild clinical mastitis, but with the addition of visible or palpable changes in the udder (swelling or pain) without systemic signs; and (3) severe clinical mastitis: both local and systemic signs (fever above 39.2°C) (**Figure 2.3.1**).

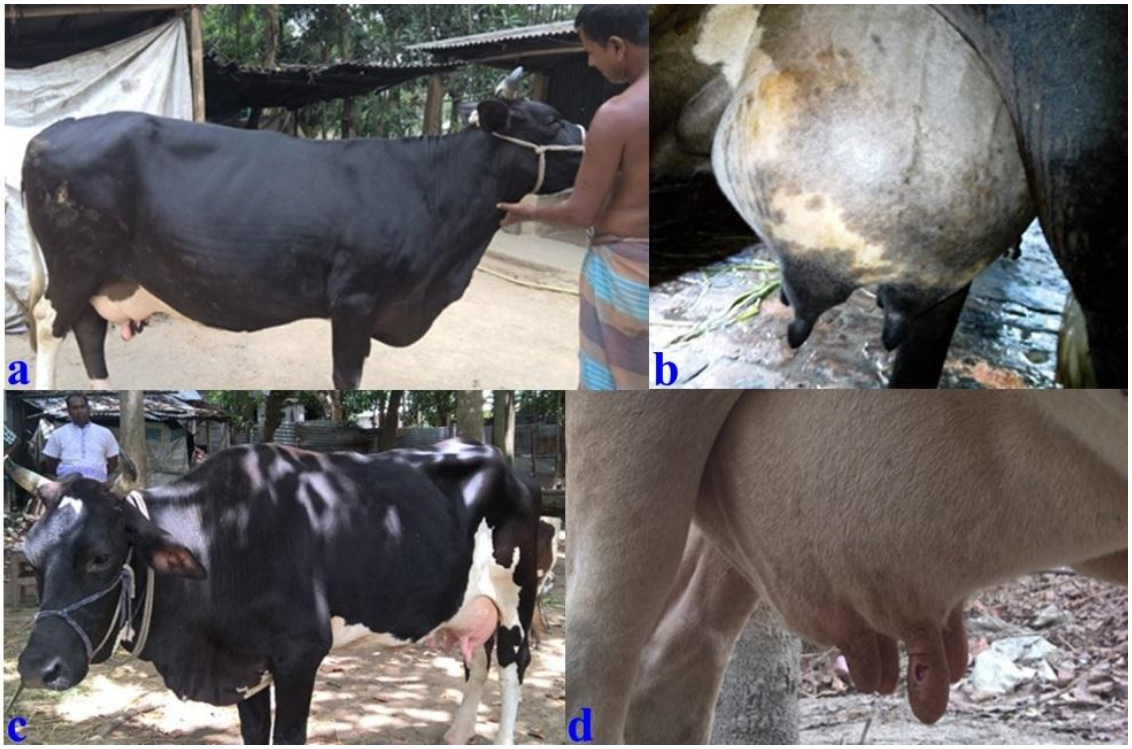


Figure 2.3.1: Dairy cows suffering from clinical mastitis. Sometimes the mastitic cows were found normal with (a) no visible signs and symptoms, (b) the udder of the CM affected cows was enlarged, swollen, hard and painful to touch, (c) the CM cows had dullness, depression, anorexia, elevated body temperature, and (d) swollen, enlarged and lacerated teats.

Table 2: Study information (farm location, population, breeds, parity, lactation and sequence data). A total of 20 lactating crossbred cows (including 5 clinical mastitis, 6 recurrent clinical mastitis, 4 subclinical mastitis and 5 healthy) were selected for the study from five different districts of Bangladesh. The cow, farm, milk sample and reads obtained from whole metagenome sequencing (WMS) of each sample related information are given in the table below. Here, CM, Clinical mastitis; RCM, recurrent clinical mastitis; SCM, subclinical mastitis; H, Healthy milk; SW, Sahiwal crossbred; RCC, Red Chattogram Cattle; LZ, Local zebu; XHF, Holstein Friesian crossbred.

Cow ID	Sample ID	Farm Location	GIS (Longi-/Latitude)	Breeds	Lactation (Days after calving)	Parity	Reads/sample (Before QC)	Reads/sample (after QC)
Cow 1	CM1	Gazipur	24.19 N, 90.47 E	SW	17	2	16784920	15532924
Cow 2	CM2	Gazipur	24.19 N, 90.47 E	RCC	34	3	18607098	17157162
Cow 3	CM3	Gazipur	24.19 N, 90.47 E	LZ	25	1	14769340	13810876
Cow 4	CM4	Gazipur	24.19 N, 90.47 E	XHF	17	2	20090020	18727286
Cow 5	CM5	Manikgonj	23.86° N, 90.00° E	XHF	9	4	39754738	37356232
Cow 6	RCM1	Gazipur	24.19 N, 90.47 E	SW	48	2	28275998	26336406
Cow 7	RCM2	Gazipur	24.19 N, 90.47 E	RCC	42	2	22676110	20364244
Cow 8	RCM3	Chattogram	22.20 N, 91.98 E	RCC	15	3	17311874	15788554
Cow 9	RCM4	Dhaka	23.81 N, 90.41 E	XHF	10	3	18497524	17110308
Cow 10	RCM5	Manikgonj	23.86° N, 90.00° E	SW	32	1	18339188	16846638
Cow 11	RCM6	Sirajgonj	24.31° N, 89.57° E	XHF	25	2	19507424	18088740
Cow 12	SCM1	Chattogram	22.20 N, 91.98 E	SW	31	2	23563460	21888704
Cow 13	SCM2	Dhaka	23.81 N, 90.41 E	XHF	13	5	19999578	18620308
Cow 14	SCM3	Gazipur	24.09 N, 90.42 E	SW	41	3	2728026	2500796
Cow 15	SCM4	Manikgonj	23.86° N, 90.00° E	RCC	22	2	34215394	31875744
Cow 16	H1	Chattogram	22.20 N, 91.98 E	XHF	7	5	28491010	26161394
Cow 17	H2	Chattogram	22.34 N, 91.87 E	RCC	32	3	19692182	18189320
Cow 18	H3	Sirajgonj	24.31° N, 89.57° E	XHF	28	4	23857586	22114902
Cow 19	H4	Dhaka	23.81 N, 90.41 E	SW	12	2	6772790	6009520
Cow 20	H5	Gazipur	24.19 N, 90.47 E	XHF	23	3	22710894	21050232

2.3.2 On-farm diagnosis of bovine mastitis

The California Mastitis Test (CMT) was used to screen different states mastitis (CM, RCM and SCM) following the modified procedure described by Hoque et al. (2015) and Lesile et al. (2002) along with the manufacturer's instruction (CMT®, Original Schalm reagent, ThechniVet, USA). In brief, about 2 mL of milk sample was squirted in each cup of mastitis paddle, and an equal volume (2 mL) of CMT reagent was added to the cups. The reactions were developed within 20 seconds in positive samples and scoring was carefully done. To mix the contents, the paddle was then rotated with a circular motion in the horizontal plane for not more than 10-20 seconds, after which the result was controlled. The CMT paddle was rinsed with water after each test. The CMT scores were categorized in to five grades based on gel formation (Scandinavian scoring system) in the reaction mixture viz. 0 (negative), T (trace, possible infections), 1 (weak positive), 2 (distinct positive), and +3 (strong positive) (Kandeel et al., 2017). The cows having ≥2 CMT score along with gross visible signs of mastitis (fibrosis, inflammatory swellings, pain, visible injury or lesion, atrophy of the tissue and teat blindness, and presence of clots, flakes, blood and changes in consistency in milk) were grouped as CM (Kandeel et al., 2017; Curone et al., 2018). The CM cows those received antibiotic treatments for 5-7 days, apparently resolved clinical signs and symptoms, and a reinfection of quarter(s) or udder 10-15 days after antimicrobial cure (with or without visible signs of CM, and ≥2 CMT score) were defined as RCM. The cows having a CMT score of T (trace) or 1 (weak positive) with no visible physical signs and symptoms, and no changes in milk were considered as positive for SCM (Hoque et al., 2015). Animals were considered as healthy (H) when they had never been encountered with mastitis, had negative CMT score, and have had a sound health with good milking records.

2.4 Milk sample collection

Once mastitis (CM, RCM, SCM) was diagnosed, quarter milk samples were taken under aseptic condition according to the method recommended by the National Mastitis Council (NMC) (NMC), and Middleton et al. (2014). The samples were collected during the summer months of Bangladesh (March to June, 2017-2018) having almost same weather condition in the study areas. The sampling patterns followed simultaneous collection both mastitis (one or more), and H (single) milk samples from the same farm. Approximately 15-20 ml of milk from all of the infected

quarters of the mastitic cows, and ≥2 quarters of the healthy (H) cows was collected in a sterile falcon tube during morning morning milking (8.00-11.00 AM, daytime) (**Figure 2.4**), with an emphasis on pre-sampling discard of first few streams, disinfection of teat-ends and hygiene during sampling. The tubes were then labelled and shaken to make a pulled sample for each corresponding category. After collection, the milk samples were placed in an ice box (at 4 °C), transported to the laboratory for subsequent processing and examination. The continued laboratory work of culturing the milk samples was processed not more than 24 hours after the milk sampling.

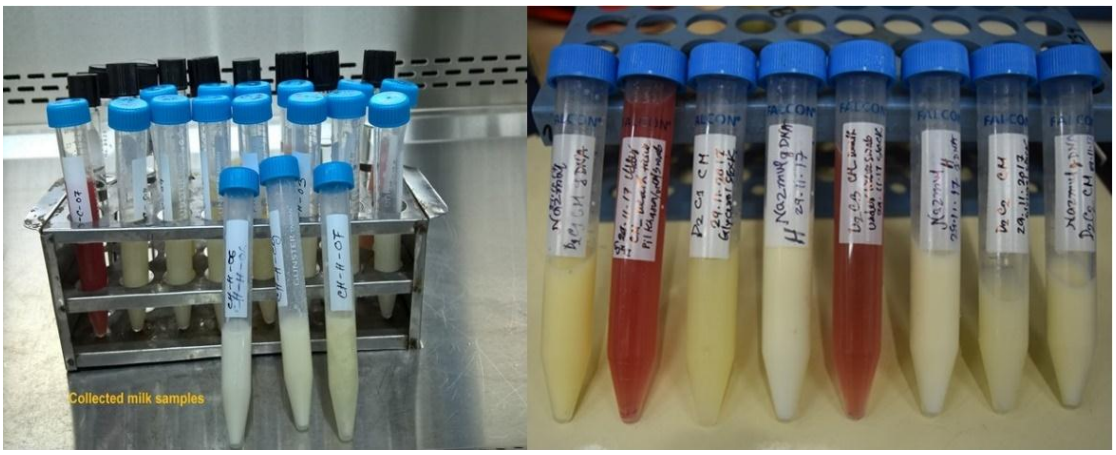


Figure 2.4: Collected milk samples. Some of the CM milk are normal in color and viscosity while others had abnormal viscosity (watery or thicker than normal), color (yellow or blood-tinged), or consistency (flakes or clots).

2.5 Microbiome characterization in bovine mastitis

2.5.1 Genomic DNA extraction and quantification

Total genomic DNA (gDNA) from 20 milk samples (CM = 5, RCM = 6, SCM = 4, and H = 5) was extracted by an automated Maxwell 16 DNA extraction platform. Two different DNA purification kits viz. the blood and cell DNA purification kits (Promega, UK) were used to extract gDNA following previously described protocols (Foley et al., 2011; Boix-Amorós et al., 2016). Briefly, 400 µL of the collected whole milk was added to well number 1 of Maxwell® 16 SEV DNA cartridge (Catalog No. AS1030). Cartridges were then loaded into the Maxwell System, seals were removed and plungers were added to well 7. After addition of 400 µL of elution buffer, the blue elution tubes were also loaded onto the platform. The correct program on the Maxwell System was selected and DNA was eluted after approximately 40 minutes. DNA quantity and purity were determined using NanoDrop ND-2000 spectrophotometer

(ThermoFisher, USA) by measuring 260/280 absorbance ratios (Oikonomou et al., 2014). The DNA samples were subsequently transferred to a microcentrifuge tube for storage at -80 °C until further use.

2.5.2 Library preparation, and whole metagenome sequencing (WMS)

Extracted gDNA samples were first quantified and normalized to 0.2 ng/µl DNA material, using a Quant-It PicoGreen dsDNA assay (Thermo Fisher Scientific), in order to use 1 ng input DNA for the library construction. Metagenomic library preparation was performed using Nextera XT DNA library preparation kit (Illumina Inc., San Diego, CA, United Sates of America) according to the manufacturer's protocol. Briefly, after normalized samples were fragmented and tagged by tagmentation of the DNA in a single-tube reaction (Head et al., 2014). The tagmented DNA was amplified through a limited-cycle PCR program using a unique combination of barcode primers, the Index 1 (i7), Index 2 (i5) and full adapter sequences (Pereira-Marques et al., 2019) required for cluster generation (**Figure 2.5.2.1)**. Amplification was followed by a cleanup step that purified the library DNA, and removed small library fragments by using Agencourt AMPure XP beads (Beckman Coulter, Inc.). The quality of the prepared libraries was checked by running 1µl of undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip, and the quantity of each library was normalized to ensure more equal library representation in the pooled libraries. Finally, libraries were loaded onto a reagent cartridge, clustered on the NextSeq 500 System, and paired-end sequencing (2×150 bp) was performed using the Illumina NextSeq 500 High-Output Kit on the NextSeq 500 desktop sequencer (**Figure 2.5.2.2**). The metagenomic DNA of this study yielded a total of 416.65 million raw reads with an average of 20.83 million (maximum = 39.75 million, minimum = 2.72 million) reads per sample (**Table 2**). Base calls generated by the NextSeq 500 System were converted to FASTQ files for further preprocessing and bioinformatic analysis.

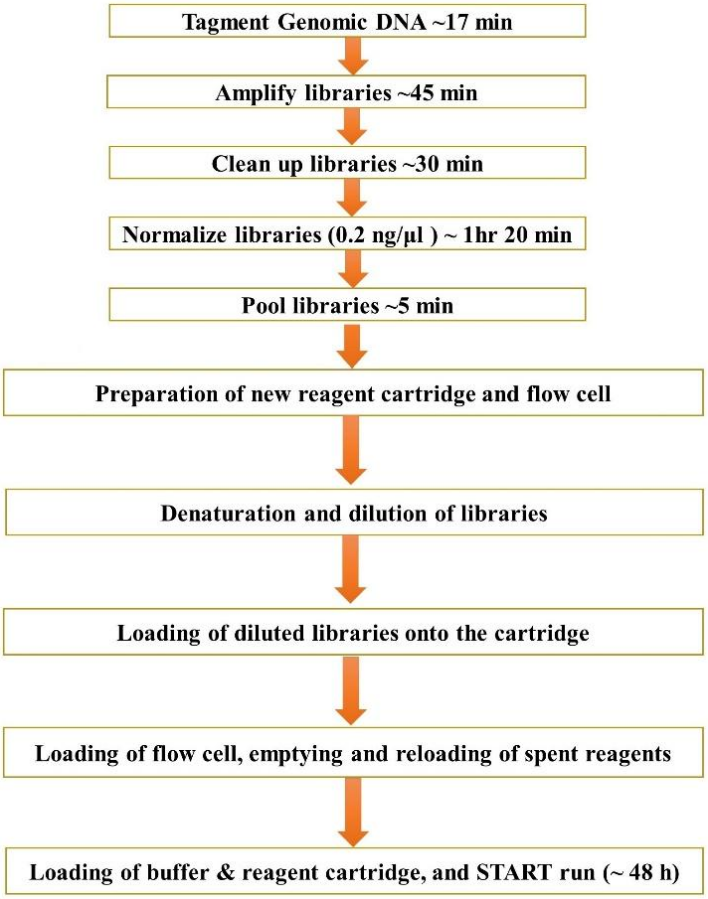


Figure 2.5.2.1: Library preparation and sequencing. In brief, the steps include- tagmentation of the genomic DNA for about 17 min, amplification of the tagmented libraries for 45 min, cleaning of the amplified libraries for 30 min, normalization of the cleaned libraries (0.2 ng/µl) for 1 hr 20 min, pooling of the normalized libraries for 5 min, preparation of the reagent cartridge and flow cell, denaturation and dilution of the pooled libraries, loading of the diluted libraries onto the cartridge, loading flow cell, emptying and reloading of the spent reagents, loading of buffer and reagent cartridge into the sequencer, and finally select and set a program to run the sequencer pressing on the start button. In our study, we selected a program that ran about 48 hr to generate desired amount of WMS reads.

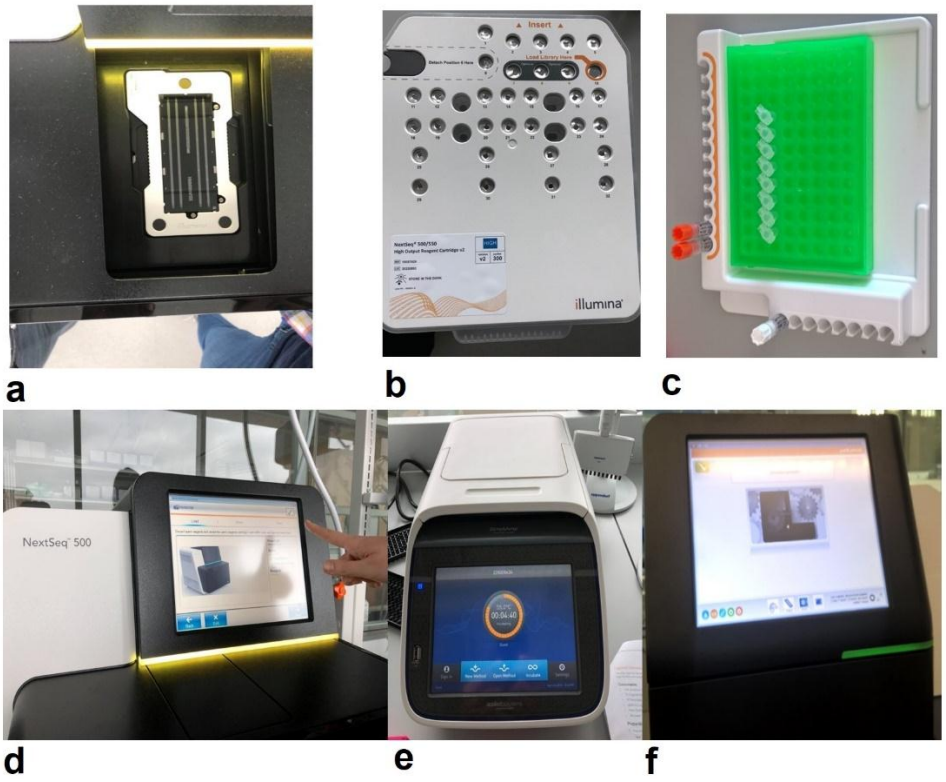


Figure 2.5.2.2: NextSeq 500 sequencer and related equipment. (a) flow cell, (b) reagent cartridge, (c) sample cartridge, (d) setting of correct program for sequencing run, (e) running of sequencing program (~ 48 h) and (f) generation of raw sequence data (FASTQ files).

2.5.3 Sequence reads preprocessing

The generated FASTQ files were concatenated and filtered through BBDuk (Hoque et al., 2019) (with options k=21, mink=6, ktrim=r, ftm=5, qtrim=rl, trimq=20, minlen=30, overwrite=true) to remove Illumina adapters, known Illumina artifacts, and phiX. Any sequence below these thresholds or reads containing more than one 'N' were discarded. A total of 385.53 million reads, averaging 19.28 million reads per sample, passed quality control 423.37 million reads with an average of 20.16 million reads per sample (maximum = 37.36 million, minimum = 2.50 million) passed these quality thresholds control steps (**Table 2**) (this thesis, Hoque et al., 2019).

2.5.4 Microbiome diversity analysis

The WMS data were analyzed using both mapping-based and assembly-based hybrid methods of PathoScope 2.0 (PS) (Hong et al., 2014) and MG-RAST 4.0 (MR) (Glass et al., 2010), respectively. In PS analysis, a 'target' genome library was constructed containing all bacterial sequences from the NCBI Database using the PathoLib module (Hong et al., 2014). The reads were then aligned against the target libraries using the very sensitive Bowtie 2 algorithm (Langmead et al., 2012), and

filtered to remove the reads aligned with the cattle genome (bosTau8) and human genome (hg38) as implemented in PathoMap (−very-sensitive-local -k 100 --score-min L,20,1.0). Finally, the PathoID (Francis et al., 2013) module was applied to obtain accurate read counts for downstream analysis. In these samples, 16.88 million reads (4.52% of total cleaned reads) mapped to the target reference genome libraries after filtering the cow and human genome. The raw sequences were simultaneously uploaded to the MR server (release 4.0) with properly embedded metadata and were subjected to quality filtering with dereplication, and host DNA removal (filtering against the set reference genome of both cattle and human), and finally screening for taxonomic and functional assignment. Alpha diversity (diversity within samples) was estimated using the observed species, Chao1, ACE, Shannon, Simpson and Fisher diversity indices (Koh et al., 2018) for both PS and MR read assignments and counts. To visualize differences in bacterial diversity, a principal coordinate analysis (PCoA) based on the Bray-Curtis distance method (Beck et al., 2013) for MR data (genus level), and non-metric multidimensional scaling (NMDS) measured by weighted-UniFrac distance on PS data (at strain level) through Phyloseq R package, version 3.5.1 (McMurdie et al., 2013) were performed. Taxonomic abundance was determined by applying the ‘‘Best Hit Classification’’ option using the NCBI database as a reference with the following settings: maximum e-value of $1x10^{-30}$; minimum identity of 95% for bacteria, 60% for archaea and viruses, and a minimum alignment length of 20 as the set parameters. The phylogenetic origin of the metagenomic sequences was projected against the NCBI taxonomic tree and determined by the lowest common ancestor (LCA) with the same cut off mentioned above.

2.5.5 Taxonomic analysis of the microbial communities

Taxonomic abundance of the WMS data was determined by applying the “Best Hit Classification” option in the PS pipeline using the NCBI database as a reference, with the following settings: maximum e-value of 1×10^{-30}, minimum identity of 95% for bacteria, and a minimum alignment length of 20 as the set parameters (this thesis, Hoque et al., 2020a). A midpoint rooted phylogenetic tree consisting of the top 200 abundant bacterial strains, identified through PS analysis from the WMS reads of the mastitis (CM, RCM and SCM) samples with >90% taxonomic identity, was constructed using the maximum-likelihood method in Clustal

W, version 2.1 (Larkin et al., 2007) and visualized using the interactive Tree Of Life (iTOL) (Letunic and Bork, 2011).

2.5.6 Virulence factors-associated genes (VFGs) analysis

The virulence factor database (VFDB) and DNA sequences with full datasets for virulence factors of pathogenic bacteria (Liu et al., 2019) were used to identify VFGs among the microbiomes of the four sample categories. Every protein included in each sample category was used as a query to search for similarities to VFGs protein-coding traits. Furthermore, we computed the representative counts for the different gene families that coded for VFGs traits from all the generated alignments between our metagenomic query cohort and the preceding databases. Hence, we aimed at retrieving the best hit (best-scored alignment) that enabled us to assign a VFG function to each of the metagenomic proteins. The VFGs which fulfilled the similarity criteria (cut off): e-value $< 1e^{-5}$, percent identity $\geq$ 80%, alignment length/subject length ≥ 0.8, and alignment length/query length ≥ 0.8 were included in the study (Liu et al., 2019). Thus, the amounts of different classes (gene families) that are present in each metagenome represent its diversity in terms of VFGs traits. We used OmicCircos (version 3.9) (Hu et al., 2014), an R package based on a Python script for circular visualization of both diversity and composition of virulence factors associated genes (VFGs) across the four sample categories under study.

2.5.7 Antibiotic resistance genes (ARGs) analysis

The WMS data were further searched for to identify the ARGs or resistomes among the microbiomes of all sample categories using the ResFinder 2.0 database (Doster et al., 2019). The ResFinder database was integrated within AMR++ pipeline to identify the respective ARGs and/or protein families. Every protein included in each of the addressed metagenomic samples was used as a query to search for similarities to ARGs protein-coding traits. Hence, we aimed at retrieving the best hit (best-scored alignment) that enabled us to assign an ARG function to each of the aforementioned metagenomic proteins. The ARGS which fulfilled the similarity criteria (cut off): e-value $< 1e\text{-}^{5}$, percent identity $\geq$ 80%, alignment length/subject length ≥ 0.8, and alignment length/query length ≥ 0.8 were included in the study (Doster et al., 2019). Thus, the amounts of different classes (gene families) that are present in each metagenome represent the diversity in terms of ARGs traits. The z-score for ARGs was calculated using the mean and standard deviation of the

abundances of all individual gene in the four sample categories.

2.5.8 Metabolic functional potential analysis

The functional profile of the microbiome was annotated according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (Kanehisa et al., 2019), and SEED subsystem (Overbeek et al., 2005) databases in MR pipeline using a "Best Hit Classification" method (Glass et al., 2010). We aligned the amino acid sequences that were translated from the gene catalogue against the proteins/domains in KEGG database. Each protein was assigned to the KEGG orthologue group (KO) by the highest scoring annotated hit (Zhang et al., 2017). For functional abundance analysis "Hierarchical Classification", was applied with a maximum e-value of $1x10^{-30}$, minimum identity of 60%, and a minimum alignment length of 20 measured in amino acids for proteins and base pairs for Refseq databases (Zinicola et al., 2015).

2.6 *In vitro* study of bovine mastitis pathogens

2.6.1 *In vitro* cultural identification of the six selected CM pathogens

Collected CM milk samples (n=100, out of 270) were subjected to selective isolation and identification of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Shigella* spp., and *Bacillus* spp. according to previously described microbiological methods (Cheng et al., 2019). The pathogens were identified based on their colony morphology, hemolytic patterns on blood agar and Gram-staining. Gram-positive bacteria were further confirmed based on their biochemical characteristics in indole, methyl red (MR), Voges-Proskauer (VP), catalase, oxidase, urease and triple sugar iron (TSI) tests, and growth on mannitol salt agar. Gram-negative bacteria were confirmed based on the results of indole, methyl red, citrate (IMViC) tests and lactose fermentation on MacConkey agar (**Figure 2.6.1**). Finally, all isolates were stored at -80 °C for further genomic identification.

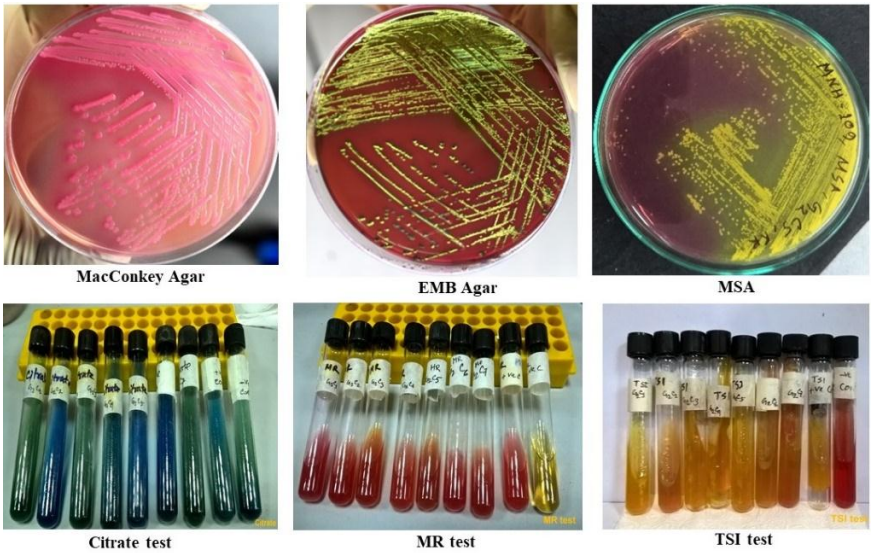


Figure 2.6.1: *In vitro* identification of mastitis pathogens. The selective culture media including MacConkey agar, Eosin methylene blue (EMB) agar, and Mannitol salt agar (MSA), and biochemical tests such as citrate, methyl red (MR), and triple sugar iron (TSI) tests were used.

2.6.2 *In vitro* antimicrobial susceptibility tests of the CM pathogens

The *in vitro* antibiogram profile of 221 CM isolates was determined using the disk diffusion method following the Clinical Laboratory Standards Institute (CLSI, 2017) guidelines. Antibiotics were selected for susceptibility testing corresponding to a panel of antimicrobial agents (Oxoid™, UK) of interest to the dairy industry and public health in Bangladesh. The selected groups of antibiotics were commonly used in treating CM by the dairy farmers, and included Penicillins (ampicillin, 10 µg/mL), Tetracyclines (doxycycline, 30 µg/mL; tetracycline, 30 µg/ML), Nitrofurans (nitrofurantoin, 300 µg/mL), Quinolones (ciprofloxacin, 10 µg/mL; nalidixic acid, 30 µg/mL), Cephalosporins (cefoxitin, 30 µg/mL), Penems (imipenem, 10 µg/mL), Phenols (chloramphenicol, 30 µg/mL), Aminoglycosides (gentamycin, 10 µg/mL; vancomycin, 30 µg/mL), Macrolides (erythromycin, 15 µg/mL) (**Figure 2.6.2 a-d**). Resistance was defined according to CLSI (2017) guidleines.

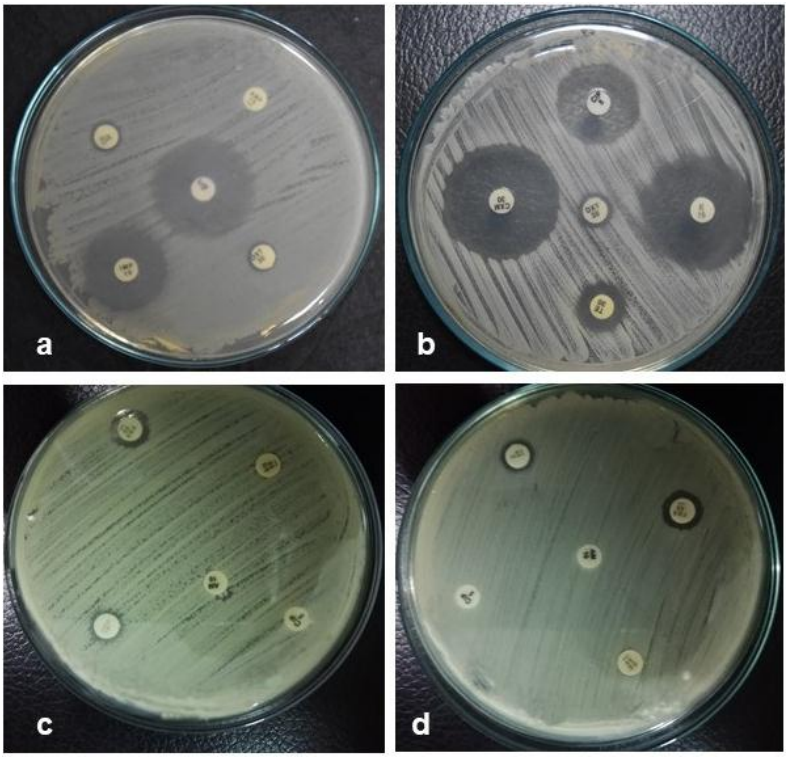


Figure 2.6.2: Disk diffusion method for antimicrobial susceptibility tests against CM pathogens: (a-b) Ampicillin (AMP), Doxycycline (DOX), Tetracycline (TCN), Ciprofloxacin (CIP), Imipenem (IMP), Chloramphenicol (CHL), and (c-d) Gentamycin (GEN), Nalidixic acid (NAL), Nitrofurantoin (NIT), Cefoxitin (CFX), Vancomycin (VAN) and Erythromycin (ERY) (Hoque et al., 2020a, this thesis).

2.6.3 *In vitro* metal susceptibility tests of the CM pathogens

The antibacterial effect of heavy metals was evaluated *in vitro* for the isolated pathogens using both agar well diffusion and tube dilution methods (Reyes-Jara et al., 2016; Vaidya et al., 2017). Five heavy metals such as copper (Cu), zinc (Zn), chromium (Cr), nickel (Ni), and cobalt (Co) were used as salts: CuSO4.5H2O, ZnSO4.7H2O, K2Cr2O7, NiCl2, and CoCl2.6H2O, respectively to study the level of zone of inhibition (ZOI). Briefly, pure culture of the isolated pathogens from nutrient agar (NA) plates were sub-cultured into Mueller-Hinton agar (Oxoid^{TM}, UK) plates, and five 7 mm wells were made, one in the center of the plate, and the other four about 20 mm away from the center. Varying concentrations of the metal solutions were prepared (2, 4, 8, 16, 32, 48 and 64 µg/mL), and 100 µl of prepared solution was inoculated into the central well of 1 cm in diameter (**Figure 2.6.3 a-e**). The plates were incubated at 37 °C for 24 h to allow diffusion of the metal into the agar, and the antibacterial activity was determined by measuring the diameter of ZOI in mm. After investigating the resistance profile of the isolates at different concentrations, the minimal inhibitory concentration (MIC) of the metals was determined by the tube

dilution method by gradually increasing or decreasing the heavy metal concentrations. Finally, growth of bacterial colonies was observed, and the concentration that showed no growth was considered as the minimum bactericidal concentration (MBC) (this thesis, Hoque et al., 2020a).

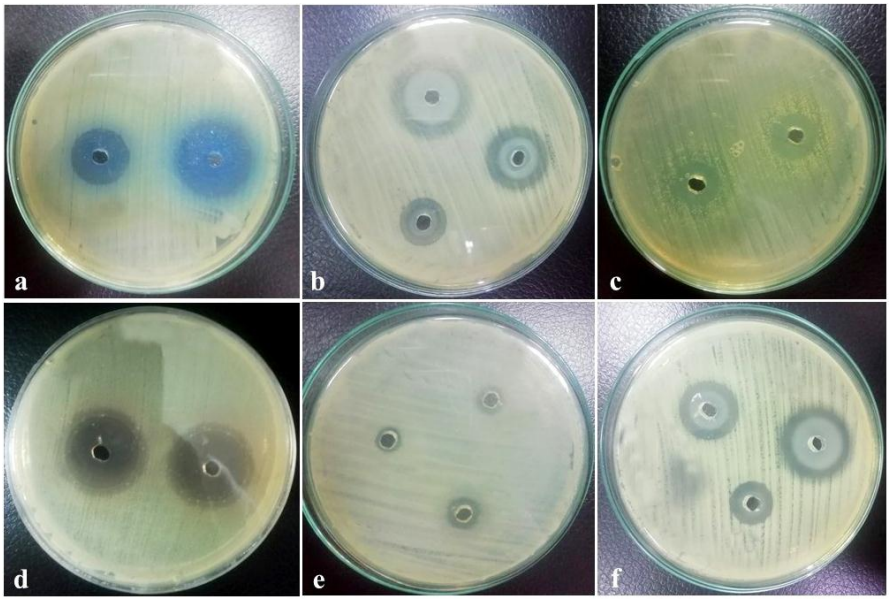


Figure 2.6.3: Agar well diffusion tests of different metals against bovine mastitis pathogens: (a) Cu (CuSO4), (b) Zn (ZnO), (c) Cr (K2Cr2O7), (d) Co (CoCl2), (e) Ni (NiCl2) and (f) Zn (ZnO) (Hoque et al., 2020a, this thesis).

2.6.4 *In vitro* biofilm assay of the six selected CM pathogens

Microtiter plate assays were performed to screen the biofilm formation (BF) ability of isolated CM pathogens (80 randomly selected isolates) using standard protocols (this thesis, Hoque et al., 2020a). We quantified the absorbance of solubilized crystal violet (CV) in a plate reader at 600 nm using 30% acetic acid in water as the blank and TSB as negative control. The solution was removed, and the absorbance measured at optical density-590 (OD590) (n = 3). To determine BF ability of strains, cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Strains were classified as: non-biofilm formers, NBF (OD $\leq$ ODc); weak biofilm formers, WBF (ODc < OD $\leq$ 2 x ODc); moderate biofilm formers, MBF (2 x ODc < OD $\leq$ 4 x ODc) and strong biofilm formers, SBF (OD > 4 x ODc). In this study, the ODc value was set as 0.045, and the mean OD of the negative control was 0.039±0.002. The biofilm surfaces were then visualized using 5% TSB as nutrient rich media and FilmTracer™ LIVE/DEAD® Biofilm Viability Kit as staining materials to observe the proportion of live or active cells (fluorescent green) under

Olympus BX51 upright microscope (40X objective), and finally images were collected using Olympus DP73 camera through cellSens entry software (Olympus Corporation, Japan) and visualized using image J software.

2.6.5 Ribosomal (16S rRNA) gene sequencing and phylogenetic analysis

Genomic DNA of the probable *S. aureus*, *E. coli*, *Klebsiella*, *Enterobacter*, *Shigella* and *Bacillus* species was extracted from overnight cultures using the boiling method. The quantity and purity of the extracted DNA was measured with NanoDrop 2000 (ThermoFisher, USA) by measuring 260/280 absorbance ratios. The ribosomal (16S rRNA) gene was amplified using two universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and U1492R (5′-CTACGGCTACCTTGTTACGA-3′) (this thesis, Hoque et al., 2020a). Agarose gel electrophoresis (1.2% wt/vol) was used to verify the presence of PCR products. DNA sequencing was carried out at First Base Laboratories Sdn Bhd (Malaysia) using Applied Biosystems highest capacity-based genetic analyzer (ABI PRISM® 377 DNA Sequencer) platforms with the BigDye® Terminator v3.1 cycle sequencing kit chemistry.

The ribosomal gene sequence-based phylogenetic tree consisting of 40 strains corresponding to the six CM bacteria identified *in vitro* was constructed using the maximum-likelihood method and the Tamura-Nei evolutionary model (Kumar et al., 2016), with >90% taxonomic identity. Using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 for the larger datasets (Kumar et al., 2016), the 16S rRNA gene sequences were aligned with each other, and with relevant reference sequences obtained from the NCBI database. Nodal confidence in the resulting phylogenetic relationships was assessed using the bootstrap test (1000 replicates) (Pattengale et al., 2010).

2.7 *In vivo* mouse model for bovine mastitis microbiome study

2.7.1 Selection of experimental mice

A total of forty (N=40) timed pregnant (Day 15 of breeding) Swiss albino mice were procured from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR'B). The mice were randomly divided into two groups: Group-I (CM fecal microbiota transplantation, n = 30) and Group- II (healthy fecal material transplantation, n = 10). The animals were individually housed in the germ-free

animal research facilities of the Department of Microbiology, University of Dhaka (Dhaka 1000, Bangladesh). All procedures involving animals were approved by the "Ethical Review Committee" of the faculty of Biological Sciences, University of Dhaka (**Appendix-I**) (Reference number: 79/Biol.Scs, Dataed: 12-12-2019). Animal care and treatment were conducted in accordance with the institutional guidelines and international laws and policies (Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.7.2 Challenge test in experimental mice

To carry out fecal microbiota transplantation (FMT), fecal samples were collected from both clinical mastitis (CM) and healthy (H) cows under aseptic, and anaerobic conditions (Ma et al., 2018). For either the CM or H group, fecal samples were freshly collected, transported to the laboratory, the content was thereafter divided into aliquots, and stored at −20 °C. The mice were challenged with FMT on the same day of sampling. At the day of challenge (Day 15 after mating), 0.5 g fecal sample obtained from each of the CM and H cow was mixed together, and then suspended with twice the fecal volume of sterile physiological saline. After thorough mixing and resting (to minimize the amount of bacteria lost), the supernatant was collected, and FMT was performed by a single oral administration of 1 g/kg fecal suspension to each mouse of both groups. Mice were then observed for up to Day 25-30 post-challenge for mastitis development (the experiment had to be terminated at this point, due to time constraints) (**Figure 2.7.2 a-d**).

2.7.3 Sampling from the experimented mice

During the incubation period, all of the mice in two groups (Group-I and Group-II) were fed normal saline and commercially available mice feed. In order to prevent cross-contamination of gut microbiota, the two groups of mice were physically separated into different germ-free (GF) isolators after inoculation. Moreover, each mouse was housed in a separate cage with safe distance apart within each of the individual GF isolator, so as to prevent any island effects (Koren et al., 2012; Ma et al., 2018). At the end of Day 25-30 of mating (10-15 days of challenge), the mice were sacrificed, and fresh fecal samples, mammary tissues, blood, and gut (jejunum and colon) were collected. The collected fecal samples were then mixed with freshly prepared phosphate buffered saline (PBS), and finally stored at −20 °C

until further processing and DNA extraction (Ma et al., 2018).

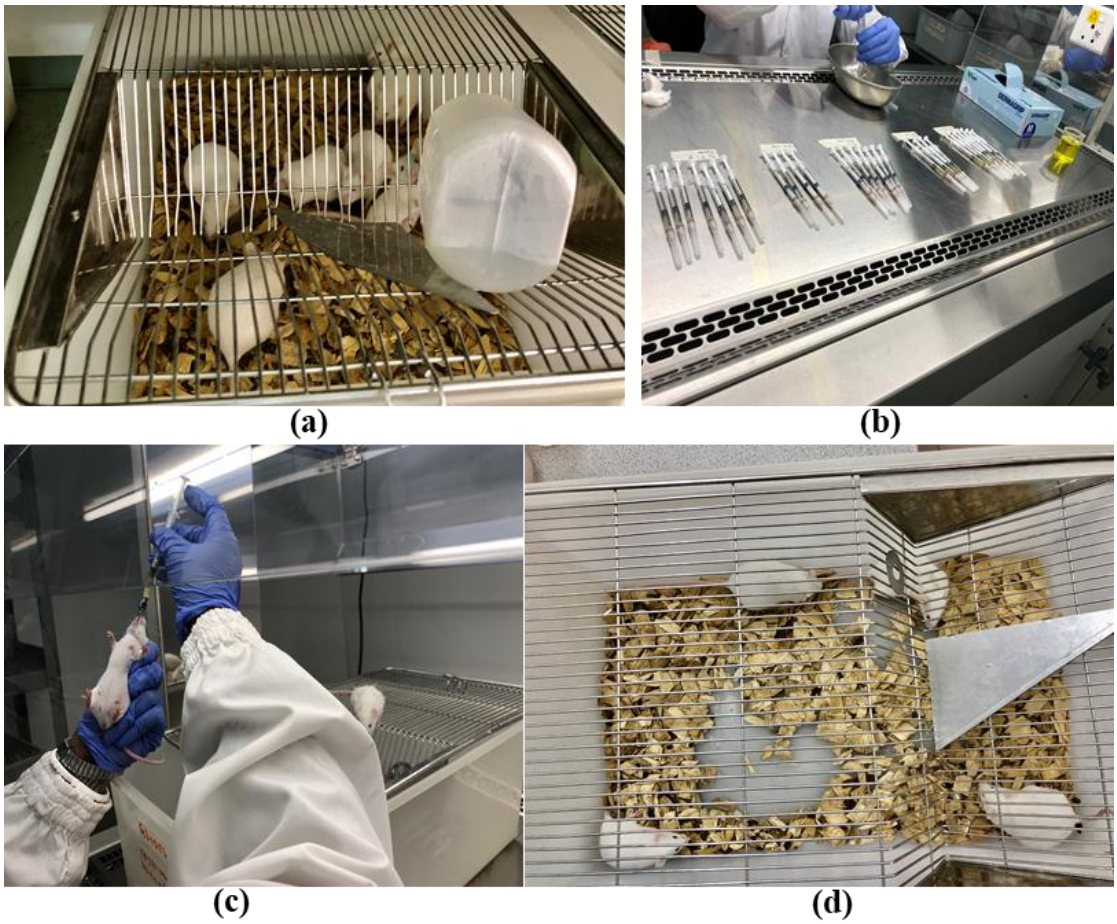


Figure 2.7.2: The mouse model for microbiome study: (a) Adaptation and selective breeding of mice, (b) prepared fecal suspension for challenge, (c) oral administration of fecal suspension to pregnant mice (at Day 15 of breeding), and (d) post-challenge rearing and management of mice.

2.7.4 Histopathological examination of mammary glands and gut tissues

For mice, milk somatic cell count (SCC) was not feasible, therefore histopathological examination was employed to assess the alterations, and inflammatory changes of mammary gland and gut (jejunum and colon) tissues during mastitis. Mammary gland, jejunum and colon tissues were fixed in 4% paraformaldehyde for at least 48 h, embedded in paraffin wax, and sectioned (Guhad et al., 2020). Sections were deparaffinized with xylene, and gradually rehydrated through graded alcohols for staining. Sections were stained with hematoxylin and eosin (i.e., HE staining), and then examined under a light microscope. Lesions observed based on the characterization of the inflammatory cell infiltrate, specifically by accumulation of polymorphonuclear cells (PMNs). The slides were observed for severe, diffuse interstitial and/or alveolar infiltrate of inflammatory cells, focal to multifocal areas of tissue damage and extensive necrotic areas in mastitic mice

(Guhad et al., 2020). All the slides were assessed under Olympus BX51 upright microscope (40X objective), and finally images were collected using Olympus DP73 camera through cellSens entry software (Olympus Corporation, Japan), and visualized using image J software.

2.7.5 *In vitro* cultural identification of the six selected mouse clinical mastitis (MCM) pathogens

Fecal samples and mammary tissues from each mouse were weighed, homogenized, and cultured. In summary, mammary tissue and fecal samples were homogenized (1:1) in PBS, and then serially diluted (1:10). Dilutions were plated onto both nutrient broth agar plates-nonselective medium, and subsequently in selective and differential media for identification of *S. aureus*, *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Shigella* spp., and *Bacillus* spp. according to previously described *in vitro* cultural identification methods in **section 2.6.1** (Cheng et al., 2019).

2.7.6 Genomic DNA extraction, library preparation and WMS of mouse fecal samples

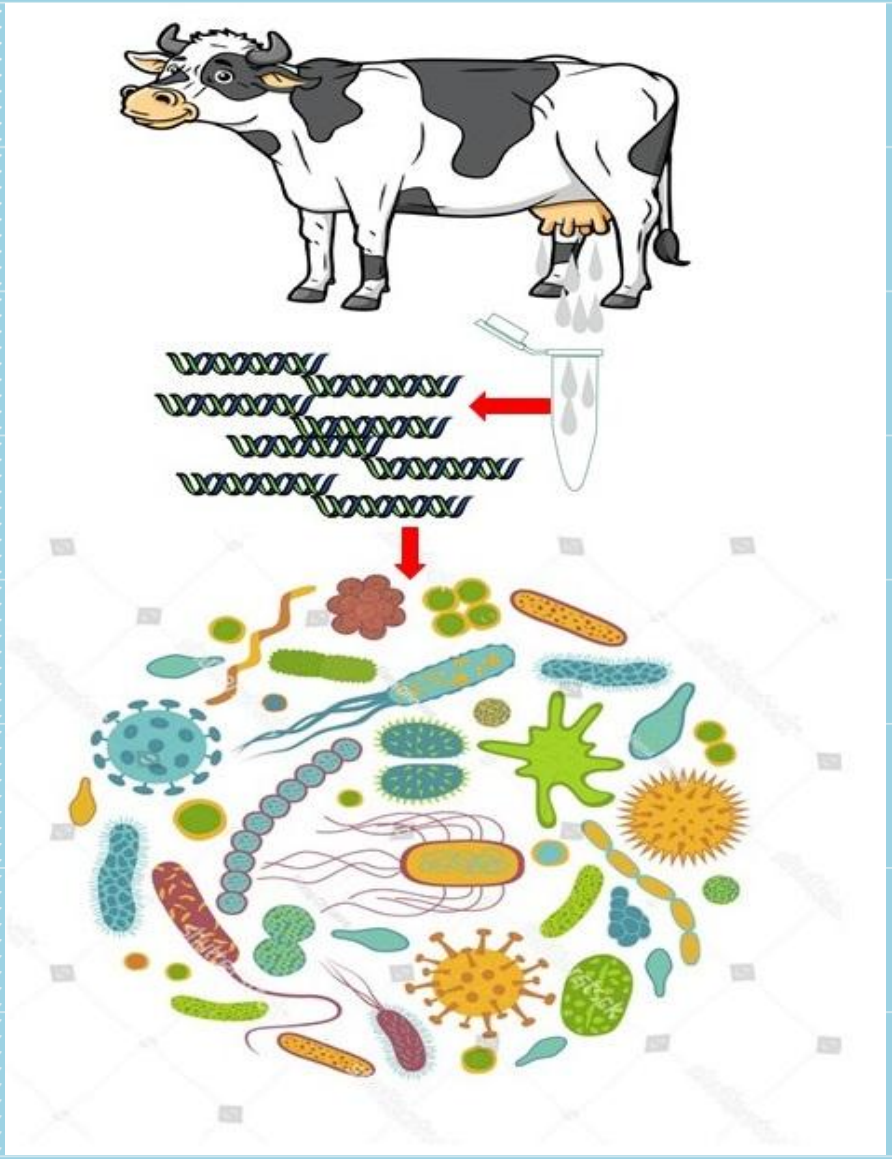
The total genomic DNA (gDNA) from twenty-four (N=24) mice including mouse clinical mastitis (MCM, n = 12), and mouse healthy (MH, n = 12) fecal samples were extracted by an automated Maxwell 16 DNA extraction platform as described previously in **section 2.5.1**. For DNA extraction from fecal content, Qiagen stool DNA extraction kits were used (Ma et al., 2018). Quantification and storage of the extracted DNA samples were made in a similar procedure described earlier in **section 2.5.1**. Metagenomic library was prepared using Nextera XT DNA library preparation kit (Illumina Inc., San Diego, CA, United Sates of America) according to the manufacturer's protocol, and paired-end (2×150) sequencing was done with NovaSeq 6000 sequencer under Illumina platform. For taxonomic identification, the assembly-based hybrid methods of MG-RAST 4.0 (MR) was used as we described previously in **section 2.5.4**.

2.7.7 Sequence reads preprocessing and microbiomes identification

The generated FASTQ files from the mouse model investigation were concatenated and filtered through BBDuk (Hoque et al., 2019) using the cutoff parameters described in **section 2.5.3**. The WMS data were analyzed using assembly-based hybrid methods of MG-RAST 4.0 (MR) following the same methods discussed in **section 2.5.4**.

2.8 Statistical analyses

The characteristics of cows with and without mastitis were compared using Fisher's exact test for categorical variables, and Mann-Whitney U test for quantitative variables. The Shapiro-Wilk test was used to check normality of the data, and the non-parametric test Kruskal–Wallis rank sum test was used to evaluate differences in the relative percent abundance of the microbial taxa in mastitis (CM, RCM, SCM) and H metagenome groups, and in different breeds (Gonzalez-Recio et al., 2018). The statistical analyses for the MR data were initially performed by embedded calls to statistical tests in the pipeline, and validated further using IBM SPSS (SPSS, Version 23.0, IBM Corp., NY USA) using the above-mentioned statistical tests. For the functional abundance profiling, the statistical (Kruskal–Wallis test, and Pearson correlation) tests were applied at different KEGG and SEED subsystem levels in the MR pipeline (Glass et al., 2010). We observed significant differences ($P = 0.001$, Kruskal–Wallis test) in the relative abundance of virulence factors associated genes (VFGs), antibiotics resistance genes (ARGs), RATC functional groups, and microbial functional genomic potentials across the four metagenomes. Differences between the pipelines were evaluated using ANOVA, and the Friedman rank sum test. A significance level of alpha=0.05 was used for all tests. To evaluate the significant relationships between identified bacterial species and the study region, we used the two-sample proportions test using SPSS. Results were considered statistically significant when $P < 0.05$ and highly significant when $P < 0.01$. Mean values were used to compare the antimicrobial efficacy results of the tested antibiotics, and heavy metals at varying concentrations. Standard error means were calculated to analyze the distributions of the data from the mean value and confidence intervals of 95% were calculated for the MIC and MBC tests results to plot error bars. We also performed Pearson correlation tests to test for relationships between taxonomic abundance of the pathogens and antimicrobial resistance both for cultural and metagenomic data. A post-hoc Bonferroni test was used to compare the biofilm OD600 mean values (Vaidya et al., 2017).



Chapter 03:

Results

3.0 Results

Bovine mastitis is one of the most prevalent diseases in the dairy industry worldwide. Bovine milk is a complex ecosystem comprising large varieties of microbial community and therefore mastitis-associated microbiomes apparently changes during different states and/or progression of the disease. To characterize the microbiomes associated with bovine mastitis, and their potential roles including virulence, antimicrobial resistance, and metabolic functions in different states of bovine mastitis, we compared the microbiomes of clinical mastitis (CM), recurrent clinical mastitis (RCM), subclinical mastitis (SCM), and healthy (H) milk samples through deep shotgun whole metagenome sequencing (WMS). Furthermore, mastitis cows-to mouse fecal microbiota transplantation (FMT) germ-free pregnant mice resulted in mastitis symptoms in mammary gland, and inflammations in both mammary and gut tissues by shifting the murine intestinal microbiota to a state that is distinct from either healthy or diseased microbiota.

3.1 Metagenomic (WMS) sequence analysis

To characterize the microbiomes associated with bovine mastitis (clinical mastitis, CM; recurrent clinical mastitis, RCM; subclinical mastitis, SCM), and in healthy (H) milk samples, we used high-throughput whole metagenome sequencing (WMS) approach. Total genomic DNA was extracted from 21milk samples obtained from four major dairy breeds of Bangladesh, and sequenced on an Illumina NextSeq 500 platform, resulting in 416.65 million raw reads with an average of 20.83 million reads per sample (range 2.72–39.75 million). After quality control with BBDuk, 385.53 million reads with an average of 19.28 million reads/sample passed the quality control steps (**Table 2**). The rarefaction curves based on observed species/strains richness reached a plateau after, on average, 19.28 million reads (**Figure 3.1**)-suggesting that the depth of coverage for most samples was sufficient to capture the entire microbial diversity within each sample.

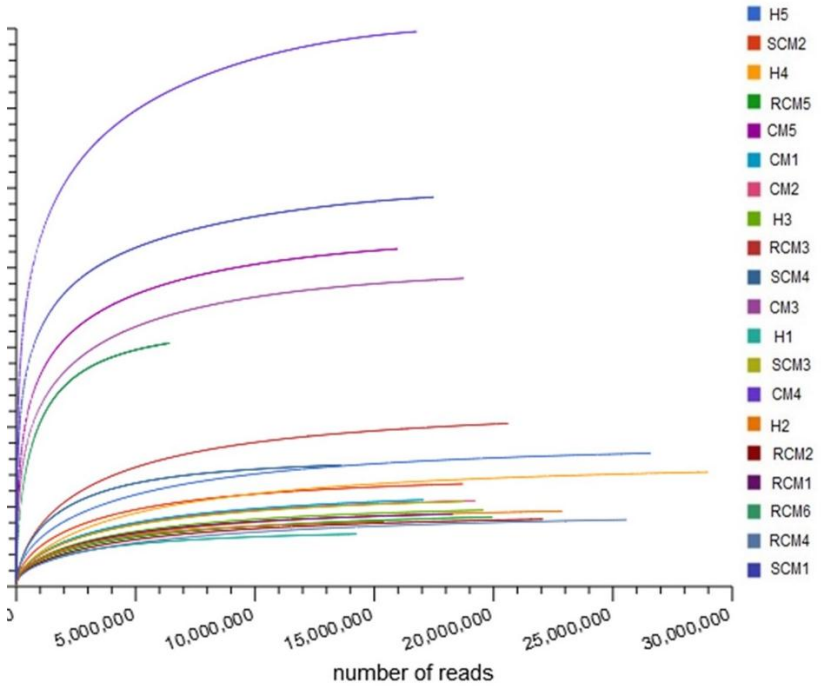


Figure 3.1: Rarefaction estimation. Taxonomic richness in bovine mastitis and healthy milk samples using MG-RAST (MR) tool. Rarefaction curves are made for all samples to evaluate the species richness, depth of sampling and sequencing coverage. Rarefaction curves representing the number of detected species (y axis) per sample (x axis). The rarefaction curves indicated that the sequencing depth was sufficient enough to fully capture the microbial diversity as existed.

3.2 Mastitis-associated changes in the milk microbiome diversity

To evaluate the effect of bovine mastitis (CM, RCM, SCM) on the milk microbial alpha diversity (i.e., within-sample diversity), we computed the number of species and/or strains observed in each sample (i.e., richness), and Chao1, ACE, Shannon, Simpson and Fisher indices (i.e., a diversity index accounting both evenness and richness) at the strain level.

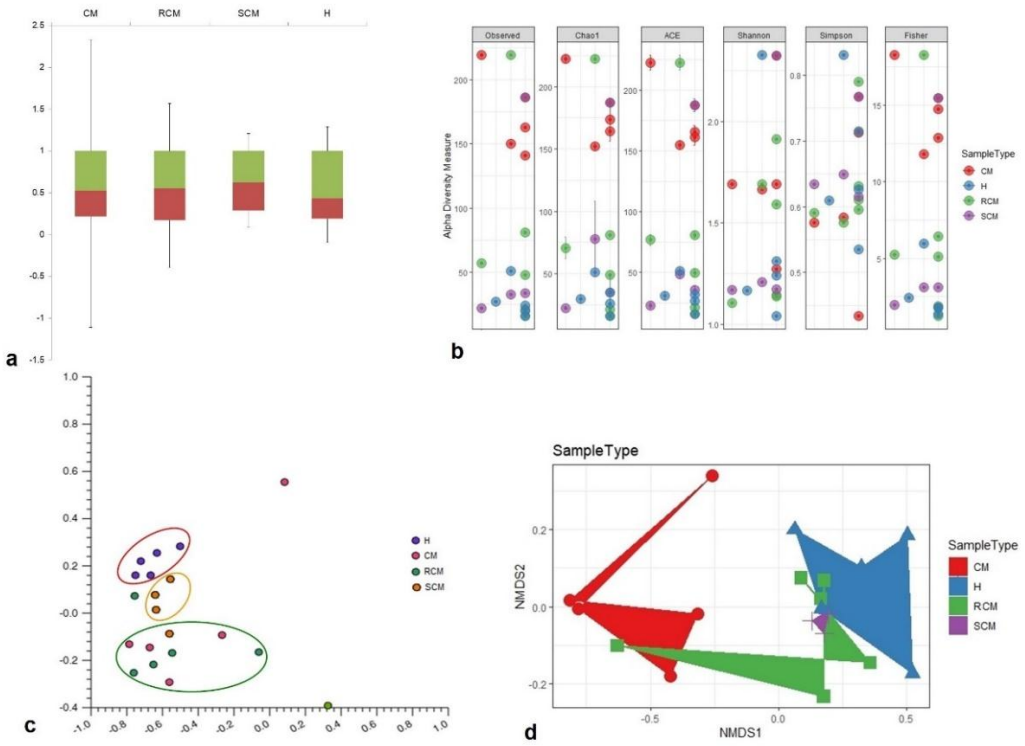


Figure 3.2 Microbiome diversity in bovine mastitis and healthy milk. (a) Box plots showing significant ($P_{Observed}$ = 0.032) differences in observed species and/or strains richness in clinical mastitis (CM), recurrent clinical mastitis (RCM), subclinical mastitis (SCM) and healthy (H) milk samples. (b) Alpha diversity measured using the observed species, Chao 1, ACE, Shannon, Simpson and Fisher diversity indices through PathoScope (PS) analysis revealed distinct microbiome diversity across the samples of four metagenomes (P = 0.003, Kruskal–Wallis test). (c) Principal coordinates analysis (PCoA) measured on the Bray-Curtis distance method using MG-RAST (MR) tool (genus-level) colored samples by populations. Each dot represents an individual, and colors indicate the populations in four metagenomes. (d) Non-metric multidimensional scaling (NMDS) ordination plots showing the clear separation between CM, RCM, SCM and H milk samples as measured by weighted-UniFrac distance on PS data at strain level. Statistical analysis using Kruskal–Wallis tests showed significant microbial diversity variations across the four metagenomes (P = 0.001, Kruskal–Wallis test) (this thesis, Hoque et al., 2020b).

The results of the present study showed that both the number of observed species/strains, Chao1, ACE, Shannon, Simpson and Fisher estimated diversity significantly varied across the four sample groups (CM, RCM, SCM and H) (P = 0.003, Kruskal–Wallis test), and were higher in CM and RCM groups relative to SCM and H groups (**Figures 3.2 a, b**). The presenting results revealed significant differences in the milk microbial community structure among CM, RCM, SCM and H milk groups (i.e., beta diversity analysis). The principal coordinate analysis (PCoA) measured on the Bray-Curtis distance method using MR generated genus-level data (**Figure 3.2 c**), and the non-metric multidimensional scaling (NMDS) ordination plots as measured by weighted-UniFrac distance on PS data at strain level (**Figure 3.2 d**)

showed clear segregation of samples represented by the microbiomes composition across the studied metagenomes. Therefore, significant variation in microbiome diversity across these four categories (P = 0.001, Kruskal–Wallis test, Beta diversity test **Figures 3.2 c, d**) was also evident.

3.3 Mastitis-associated changes in microbiomes composition

The composition of bovine milk microbial community at domain level was numerically dominated by bacteria, with a relative abundance of 99.58%, followed by archaea (0.24%), and viruses (0.18%). In this metagenome study, the relative abundance of microbiome always remained higher in mastitis (CM, RCM and SCM) milk samples compared to H milk, however, the abundance fluctuated more in mastitis samples (CV = 338.45 vs 107.21; PS, CV = 1011.12 vs 742.32; MR). The unique and shared distribution of microbiome found in both mastitis and healthy milk samples has been represented using Venn diagrams. In the present investigation, we detected 18 bacterial phyla using both PS and MR pipelines with 16 in CM milk, 11 in RCM milk, 4 in SCM milk and 13 in H milk samples, and of them 4 phyla were found to be common across the four metagenomes (**Figure 3.3.1 a**). In this study, 78 orders of bacteria were identified with highest number of bacterial orders in the CM milk (72) metagenome followed by H (61), RCM (48) and SCM (17) milk samples. Among these orders, 19.23% were shared across the all metagenomes under study (**Figure 3.3.1 b**). The MR pipeline simultaneously detected bacteria, archaea and viruses across these metagenomes.

A total of 373 bacterial genera including 314, 187, 66 and 272 in CM, RCM, SCM and H milk samples, respectively were detected. Out of these genera, 51.8%, 18.0% and 66.5% detected in RCM, SCM and H samples, respectively shared with the bacterial genera found in CM samples (**Figure 3.3.2 a**). However, only 12.06% (45) bacterial genera were found common across all the four categories samples revealed by metagenomes analysis.

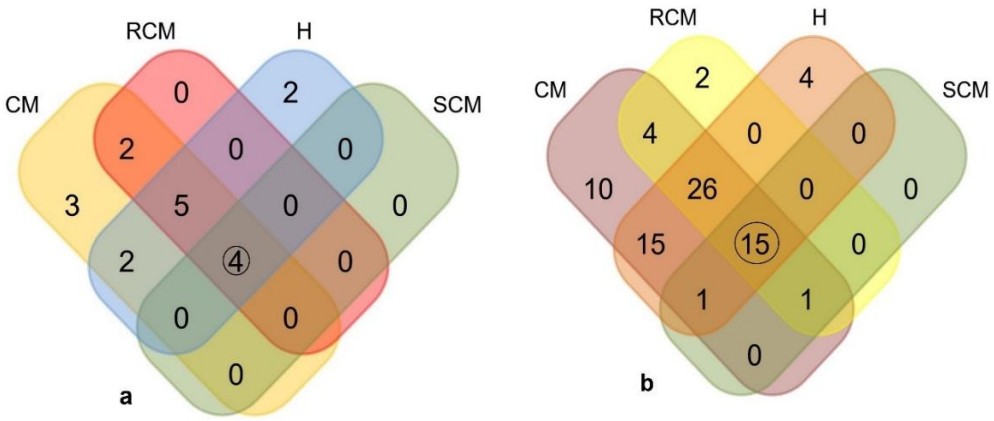


Figure 3.3.1: Taxonomic composition of bovine mastitis and healthy milk microbiome (at phylum and order level). (a) Venn diagram representing the core unique and shared bacterial phyla in CM, RCM, SCM and H milk samples. (b) Venn diagram representing the core unique and shared bacterial orders in CM, RCM, SCM and H milk samples. Microbiome sharing between the conditions are indicated by black circles.

Through PS analysis, 442 bacterial strains were mapped across the sequences four metagenomes, of which 385, 65, 80 and 144 strains were found in CM, RCM, SCM and H milk, respectively. Of these strains, only 4.75% were found to be shared across all of the milk (mastitis and healthy) samples. The CM milk microbiomes however had 13.1%, 14.8% and 27.6% bacterial strains shared with RCM, SCM and H milk microbiomes, respectively (**Figure 3.3.2 b**).

The MR pipeline detected 58 archaeal and 48 viral genera in mastitis and healthy milk samples, of which 56, 11, 9 and 46 archaeal genera were found in CM, RCM, SCM and H milk metagenomes, respectively, and 12.06% of the archaeal genera were shared among catagories of metagenomes (**Figure 3.3.2 c**). The CM milk metagenome had highest number (40) of viral genera followed by H (37), RCM (24), and SCM (11) milk metagenomes, and among these viral genera, 20.83% were found to be shared across the four metagenomes (**Figure 3.3.2 d**). By comparing the archaeal and viral genera in the sample categories, it was revealed that 23.2%, 16.1% and 75.9% of the detected archaeal genera in RCM, SCM and H milk, respectively shared with the archaeal genera of CM samples (**Figure 3.3.2 c**); while 60.0%, 27.5% and 45.3% of the identified viral genera in RCM, SCM and H milk samples, respectively found to be common with those detected in CM samples ((**Figure 3.3.2 d**).

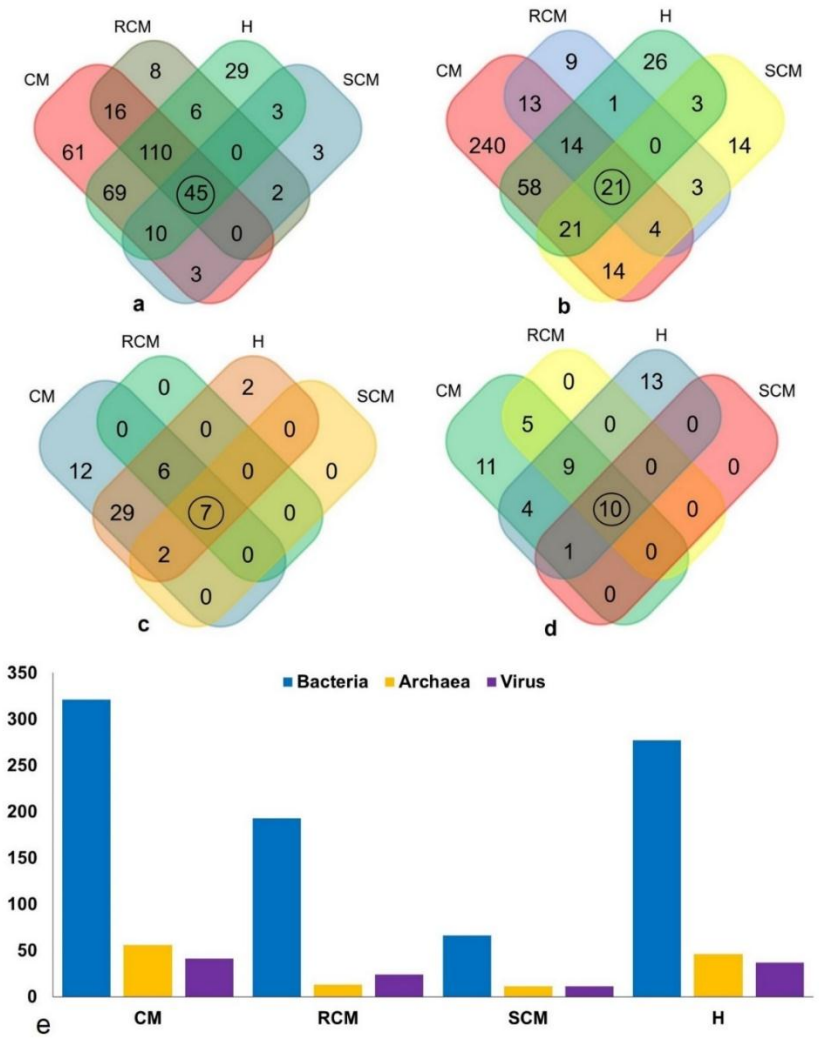


Figure 3.3.2: Taxonomic composition of bovine mastitis and healthy milk microbiome. Venn diagrams representing the core unique and shared microbiomes in CM, RCM, SCM and H milk samples. (a) Venn diagram showing unique and shared bacterial genera by MR analysis, (b) Venn diagram comparison of bacteria at strain level as measured through PS analysis, (c & d) Venn diagrams representing unique and shared archaeal and viral genera, respectively found in mastitis (CM, RCM, SCM) and H milk samples as analyzed with MR pipeline. Microbiome sharing between the conditions are indicated by black circles. (e) Dynamic changes in the composition of bacteria, archaea and viruses in four metagenomes at genus level. Each bar plot represents total genera of bacteria, archaea and viruses detected in the respective metagenome (this thesis, Hoque et al., 2020b).

3.4 Mastitis-associated shifts in bacteriome composition

To identify changes in microbial community that were specific to different states of mastitis (CM, RCM, SCM), and healthy condition of the mammary gland, we found that the composition and the relative abundance of bacterial phyla, orders, genera and strains differed in both bioinformatics tools used. The composition of the bacterial communities at phylum level was numerically dominated by *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (comprising >99% of relative

abundance) in the niche of both mastitis and health milk in both PS and MR analyses. Additionally, *Proteobacteria* was found as the single most dominating phylum in CM, RCM and H milk samples (>99.0%) relative abundance, but the SCM milk metagenome had highest relative abundance of *Firmicutes* (42.95%) followed by *Bacteroidetes* (33.95%), *Proteobacteria* (22.15%) and *Actinobacteria* (0.95%). *Pseudomonadales* was the predominantly abundant bacterial order in CM (86.25%), RCM (90.93%) and H (98.51%) milk samples whereas *Lactobacillales* (41.59%) and *Flavobacteriales* (31.01%) were the most abundant orders in SCM milk samples (**Figure 3.4.1**). The relative abundance of *Enterobacteriales* also varied among the sample categories, and remained significantly higher in mastitis milk (CM=12.81%, RCM=5.42%, SCM=12.11%) compared to H milk (0.23%) sample group. Rest of the bacterial orders also had comparatively higher relative abundance in mastitis milk than H milk samples.

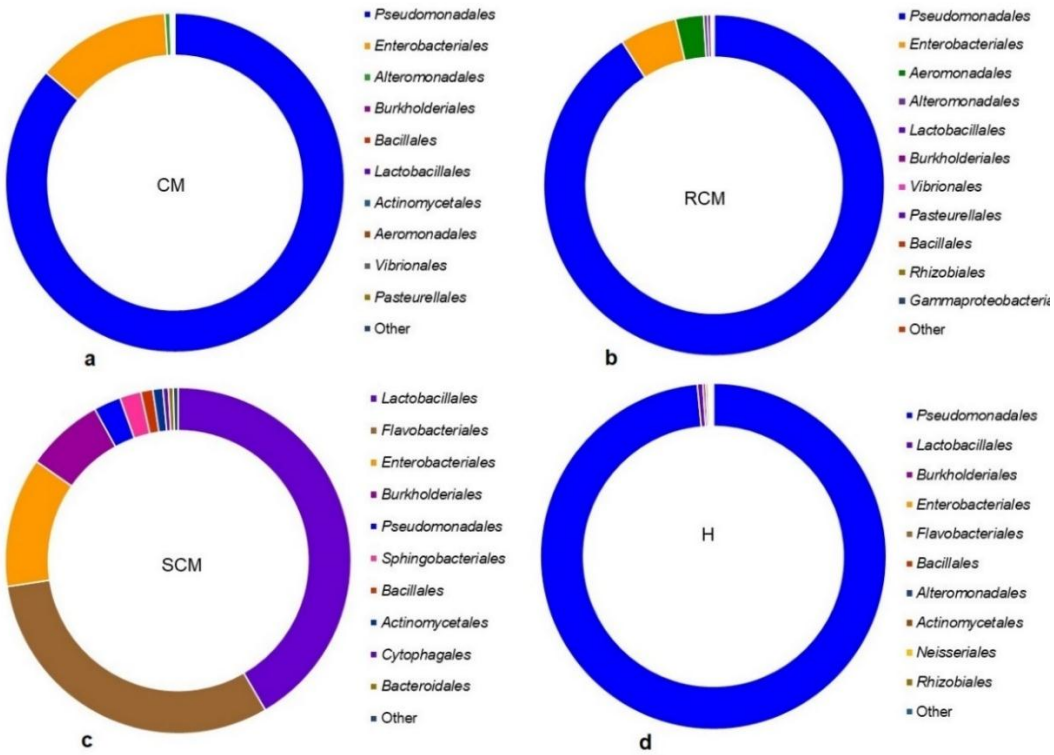


Figure 3.4.1: Taxonomic profile of bacteria (at order level) in bovine mastitis and healthy milk. The circular plots showing top abundant bacterial orders in (a) clinical mastitis (CM), (b) recurrent clinical mastitis (RCM), (c) subclinical mastitis (SCM) and (d) healthy (H) milk samples. Different color codes indicate the relative abundance of the respective orders in all metagenome groups.

We also found significant differences (P = 0.001, Kruskal–Wallis test) in the

composition and the relative abundance of bacteria at the genus level. The bacterial community of the phylum *Proteobacteria* was mainly dominated by *Acinetobacter*, *Pseudomonas*, *Escherichia*, *Shigella*, *Klebsiella*, *Enterobacter*, *Salmonella*, *Shewanella*, *Serratia*, *Ralstonia*, *Citrobacter*, *Psychrobacter*, *Yersinia*, *Pantoea* and *Aeromonas* genera. On the other hand, the phylum *Firmicutes* was dominated by *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Staphylococcus* and *Bacillus* genera; *Bacteroidetes* by the genera of *Chryseobacterium*, *Porphyromonas* and *Prevotella*; and *Actinobacteria* by the *Propionibacterium* and *Corynebacterium* genera across the four metagenomes (**Figure 3.4.2**).

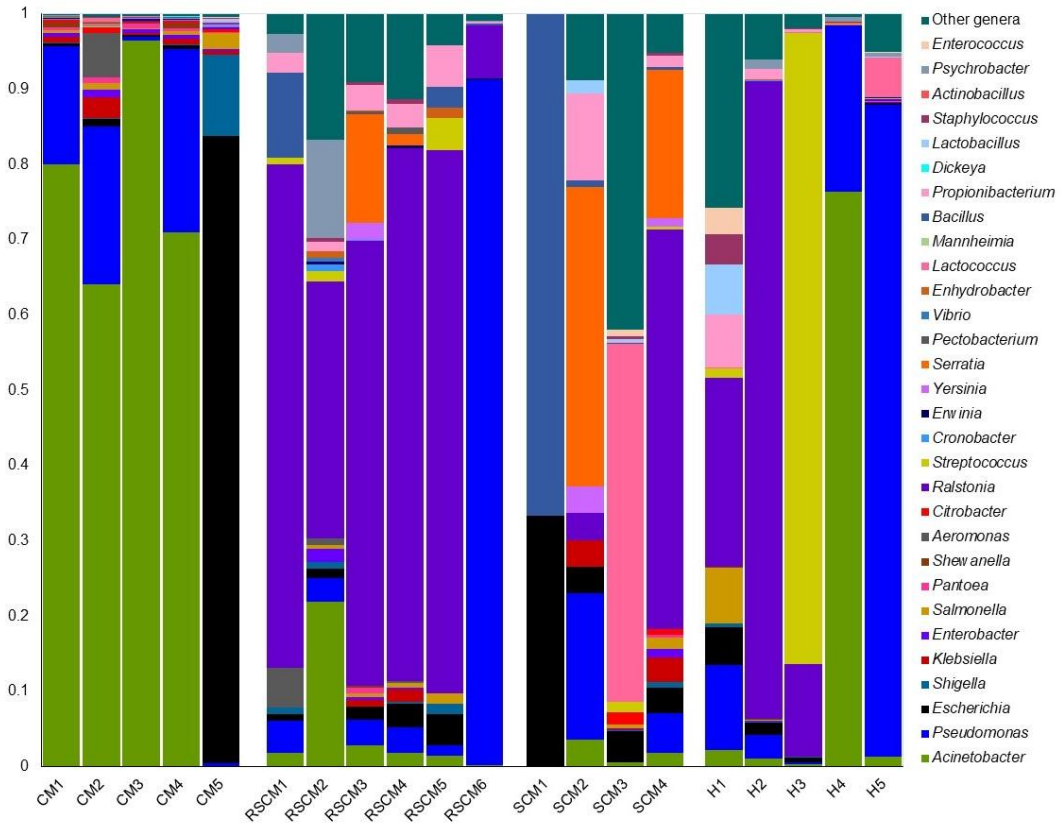


Figure 3.4.2: The genus level taxonomic profile bacteria in bovine mastitis and healthy milk. Stacked bar plots showing the relative abundance and distribution of the 30 most abundant genera, with ranks ordered from bottom to top by their increasing proportion among the CM, RCM, SCM and H milk samples. Only the 29 most abundant genera are shown in the legend, with the remaining genera grouped as 'Other genera'. Each stacked bar plot represents the abundance of bacteria in each sample of the corresponding category (this thesis, Hoque et al., 2020b).

In CM and RCM samples, *Acinetobacter* was the most abundant bacterial pathogen with a relative abundance of 68.59% and 72.61%, respectively, but remained much lower (0.81%) in SCM-milk samples. The CM milk microbiome was

also predominated by *Pseudomonas* (17.69%), *Escherichia* (8.62%), *Shigella* (1.12%), *Klebsiella* (0.66%), *Salmonella* (0.52%), *Enterobacter* (0.50%), *Shewanella* (0.46%) *Pantoea* (0.46%), and *Citrobacter* (0.32%), and rest of the genera had < 0.1% relative abundance (**Figure 3.4.2**). Simultaneously, *Pseudomonas* (18.21%), *Aeromonas* (2.64%), *Klebsiella* (1.68%), *Enterobacter* (0.79%), *Escherichia* (0.68%), *Pantoea* (0.61%), *Citrobacter* (0.54%), *Salmonella* (0.52%), *Shewanella* (0.35%), *Lactococcus* (0.24%), and *Cronobacter* (0.13%) were the most abundant genera in the RCM (**Figure 3.4.2**). Likewise, top abundant bacterial genera in SCM were *Lactococcus* (39.20%), *Chryseobacterium* (23.55%), *Ralstonia* (6.65%), *Serratia* (4.43%), *Escherichia* (4.98%), *Riemerella* (2.58%), *Pseudomonas* (1.67), *Citrobacter* (1.40%), *Streptococcus* (1.13%), *Pedobacter* (1.08%). Conversely, the H-milk was mostly dominated by two bacterial genera, *Acinetobacter* (73.66%) and *Pseudomonas* (24.01%), with relatively lower abundances of *Psychrobacter* (0.62%), *Streptococcus* (0.25%), *Lactococcus* (0.22%), and *Ralstonia* (0.18%) (**Figure 3.4.2**). Despite having substantially lower (< 0.5) relative abundances, 60, 7, 3 and 27 exclusively associated bacterial genera were found in CM, RCM, SCM and H-milk samples (**Figure 3.3.2a**). These findings suggest that mastitis-associated microbiomes have higher relative abundances of the detected bacterial genera compared to the H milk microbiome, with the inclusion of new or the same genera of opportunistic bacteria. However, the relative abundances of mastitis causing bacteria (for example: *Escherichia*, *Klebsiella*, *Shigella*, *Salmonella*, *Enterobacter*, *Shewanella*, *Pantoea*, *Citrobacter Aeromonas*, *Cronobacter*, *Chryseobacterium*, *Serratia*, *Riemerella*, and *Pedobacter*) remain more inconsistent according to the different categories of mastitis across CM, RCM and SCM (**Figure 3.4.2**).

Profiling of the strain-level differences of microbial communities across these four levels of mastitis through PS analysis revealed significant variations (P = 0.002, Kruskal–Wallis test) in microbiome composition, diversity and relative abundances. The strain-level microbiome shift was also evident within three mastitis conditions, with a significantly (P = 0.001, Kruskal–Wallis test) higher number of bacterial strains in the CM-microbiome over the RCM and SCM. Of the detected strains, 62.33%, 13.85% and 17.50% had a sole association with bovine CM, RCM and SCM, respectively (**Figure 3.3.2b**). The mastitis associated microbiomes was dominated by 29 different strains of *Pseudomonas* species, while *Acinetobacter*, *Streptococcus*, *Lactobacillus*, *Corynebacterium*, *Staphylococcus* and *Enterococcus* species

represented by 27, 27, 18, 17, 15 and 10 different strains, respectively (**Figure 3.4.3**).

The most prevalent bacterial strain, *Acinetobacter johnsonii* XBB1 had almost 1.7-fold higher relative abundance in CM (59.92%) milk over H (36.2%) milk but remained much lower (< 0.5%) in the other two microbiomes (RCM, SCM) (**Figure 3.4.4**). Moreover, *Pseudomonas putida* KT2440 (11.81%), *Escherichia coli* O104:H4 str. 2011C (0.036%), *Aeromonas veronii* B565 (1.79%), *Pantoea dispersa* EGD-AAK13 (1.74%), *Klebsiella oxytoca* KA-2 (1.09%), *Pseudomonas entomophila* L48 (0.80%), *Kluyvera ascorbata* ATCC 33433 (0.68%), *P. japonica* NBRC 103040 (0.64%), *Aeromonas hydrophila* YL17 (0.61%) and *Acinetobacter tandoii* DSM 14970 (0.56%) were the predominant strains found in CM milk (**Figure 3.4.4**). *Micromonospora* sp. HK10 (54.11%), *Campylobacter mucosalis* CCUG 21559 (26.52%), *Catenibacterium mitsuokai* DSM 15897 (11.15%), and *Anaerobutyricum hallii* DSM 3353 (7.09%) were the predominant strains in RCM. The most abundant strains in SCM were *Micromonospora* sp. HK10 (38.29%), *Campylobacter mucosalis* CCUG 21559 (26.25%), *Anaerobutyricum hallii* DSM 3353 (25.74%), and *Catenibacterium mitsuokai* DSM 15897 (8.62%). In contrast, the H-milk microbiome was mostly dominated by *Acinetobacter johnsonii* XBB1 (36.20%), *Micromonospora* sp. HK10 (15.16%), *Anaerobutyricum hallii* DSM 3353 (11.60%), *Campylobacter mucosalis* CCUG 21559 (7.42%), and different strains of *Pseudomonas* such as *Pseudomonas fragi* str. A22 (16.04%), *Pseudomonas fluorescens* SBW25 (2.43%), *Pseudomonas protegens* CHA0 (1.02%), *Pseudomonas fluorescens* SBW25 (0.70%), *Pseudomonas alkylphenolica* KL28 (0.65%), and *Pseudomonas resinovorans* NBRC 106553 (0.52%). The rest of the bacterial strains identified in these samples had much lower (< 0.50%) relative abundances (**Figure 3.4.4**).

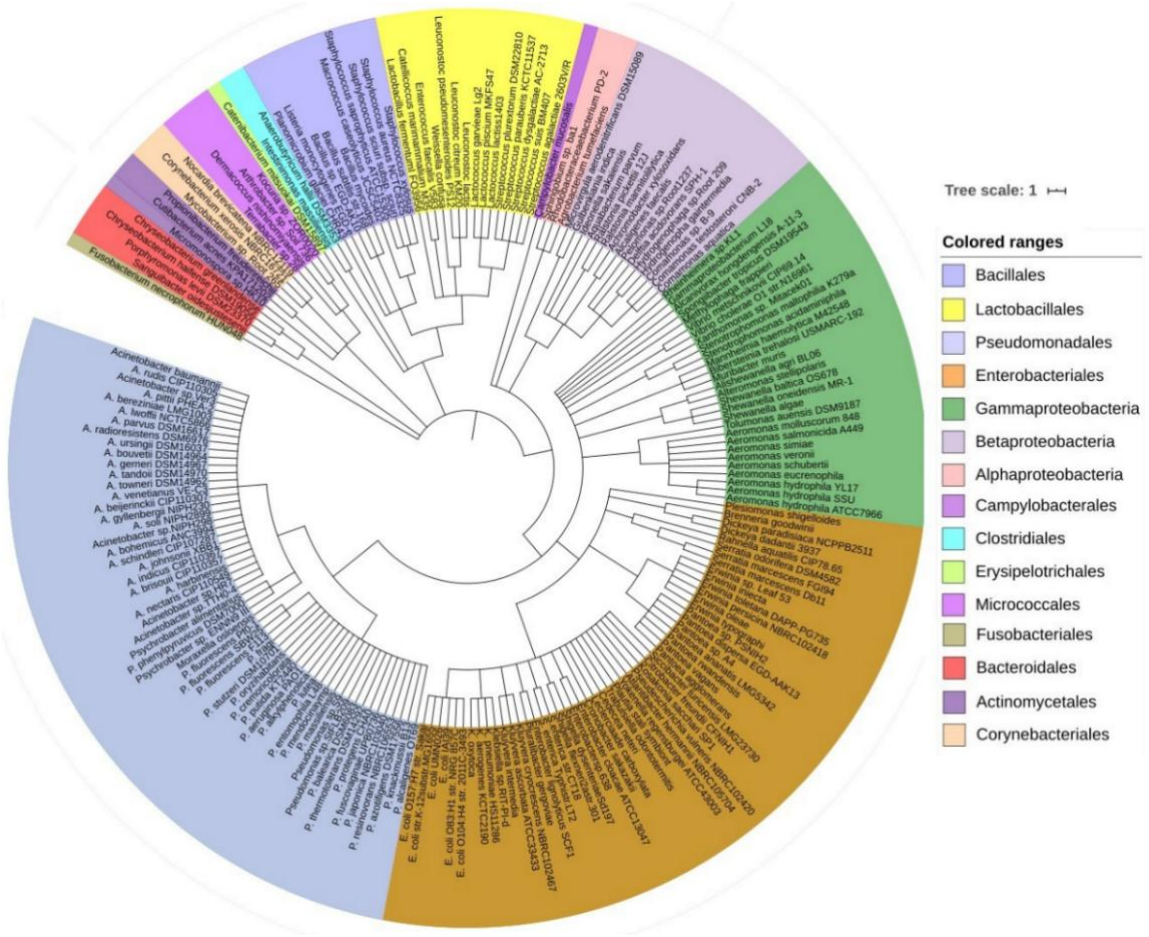


Figure 3.4.3: The strain-level taxonomic profile of bacteria in bovine mastitis (CM, RCM, SCM). Taxonomic dendrogram showing the top bacterial microbiome of bovine mastitis milk. Color ranges identify different strains within the tree. Taxonomic dendrogram was generated with the top 200 abundant unique strains of bacteria in mastitis milk metagenomes based on the maximum likelihood method in ClustalW and displayed with iTOL. Each node represents a single strain shared among more than 50 % of the samples at a relative abundance of >0.0006% of the total bacterial community. The inner circle represents the root of the microbiome defined as bacteria mastitis milk samples. The outer circle shows the strains and/or species colored by different order of bacteria present in >80% of samples (this thesis, Hoque et al., 2020a).

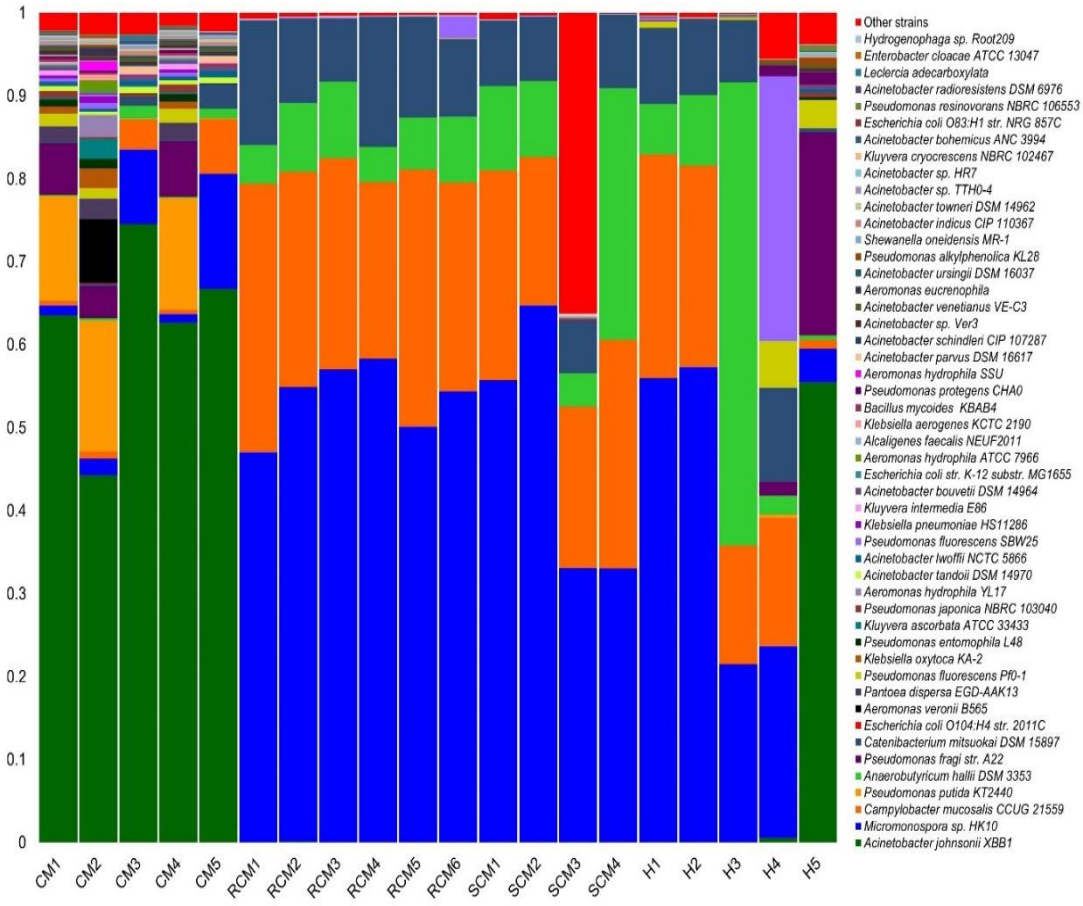


Figure 3.4.4: The strain level taxonomic profile of bacteria in bovine mastitis and healthy milk. Stacked bar plots showing the relative abundance and distribution of the 50 most abundant strains, with ranks ordered from bottom to top by their increasing proportion among the CM, RCM, SCM and H milk samples. Only the 49 most abundant strains are shown in the legend, with the remaining strains grouped as 'Other strains'. Each stacked bar plot represents the abundance of bacteria in each sample of the corresponding category.

The literature on the strain-level microbiome composition in bovine mastitis through shotgun metagenomics is scarce, and most of the microbiome studies carried out in bovine mastitis are restricted only up to genus-level detection of bacteria using the amplicon sequencing. Therefore, most bacterial strains identified in this study are unreported. Interestingly, among the total bacterial strains identified using WMS method in the present work, 67.19% strains (of which 70.13% in CM, 44.62% in RCM and 43.75% in SCM), were unreported in bovine mastitis (**Table 3)**. The top abundant opportunistic strains in CM-milk were *Aeromonas veronii* B565 (19.16%), *Pantoea dispersa* EGD-AAK13 (18.65%), *Klebsiella oxytoca* KA-2 (11.70%), *Kluyvera ascorbata* ATCC 33433 (7.27%), *Aeromonas hydrophila* YL17 (6.54%). In RCM-milk, the most abundant strains were *Nocardia pseudobrasiliensis* (48.06%), *Serratia marcescens* subsp. marcescens Db11 (16.22%), *Nocardia mikamii* NBRC 108933 (15.19%), *Corynebacterium bovis* DSM 20582 (4.66%), and *Aeromonas*

hydrophila SSU (3.11%) (**Table 3)**. Different strains of the *Chryseobacterium* genus such as *Chryseobacterium* sp. Leaf405 (19.06%), *Chryseobacterium* sp. CF299 (13.68%), *Chryseobacterium haifense* DSM 19056 (13.19), *Chryseobacterium greenlandense* (13.04%), and *Chryseobacterium* sp. YR460 (12.56%), and *Serratia marcescens* subsp. marcescens Db11 (8.30%) and *Citrobacter freundii* CFNIH1 (6.65%) were the most abundant opportunistic strains in SCM (**Table 3**).

3.5 Breed-associated changes of bacterial community in bovine mastitis

The current metagenome study provided the advantage of examining mastitis-associated microbiomes in four different dairy breeds Local Zebu, LZ; Red Chattogram Cattle, RCC; Sahiwal, SW; Crossbred Holstein Friesian; XHF) of Bangladesh kept in the similar farm management, environmental conditions and diets. Moreover, significant diversity (alpha and beta) differences were observed among the mastitis-associated microbial communities across the four cattle breeds (LZ, RCC, SW, XHF) regardless of the method (i.e., either PS or MR) used to tabulate microbial abundances (PS; P = 0.005, MR; P = 0.001, Kruskal–Wallis test). The breed specific microbiomes distribution revealed that the XHF cows suffering from mastitis had highest number of bacterial strains (331) in their milk followed by SW (231), RCC (110), and LZ (56), and among these strains, 22 strains were found to be common across these four dairy breeds (**Figure 3.5 a**). Our analysis also revealed that 37.4, 17.9 and 10.9% of the detected bacterial strains in mastitis milk samples of SW, RCC, and LZ cows, respectively, were also found in the mastitis-associated microbiomes of XHF cows (**Figure 3.5 b**).

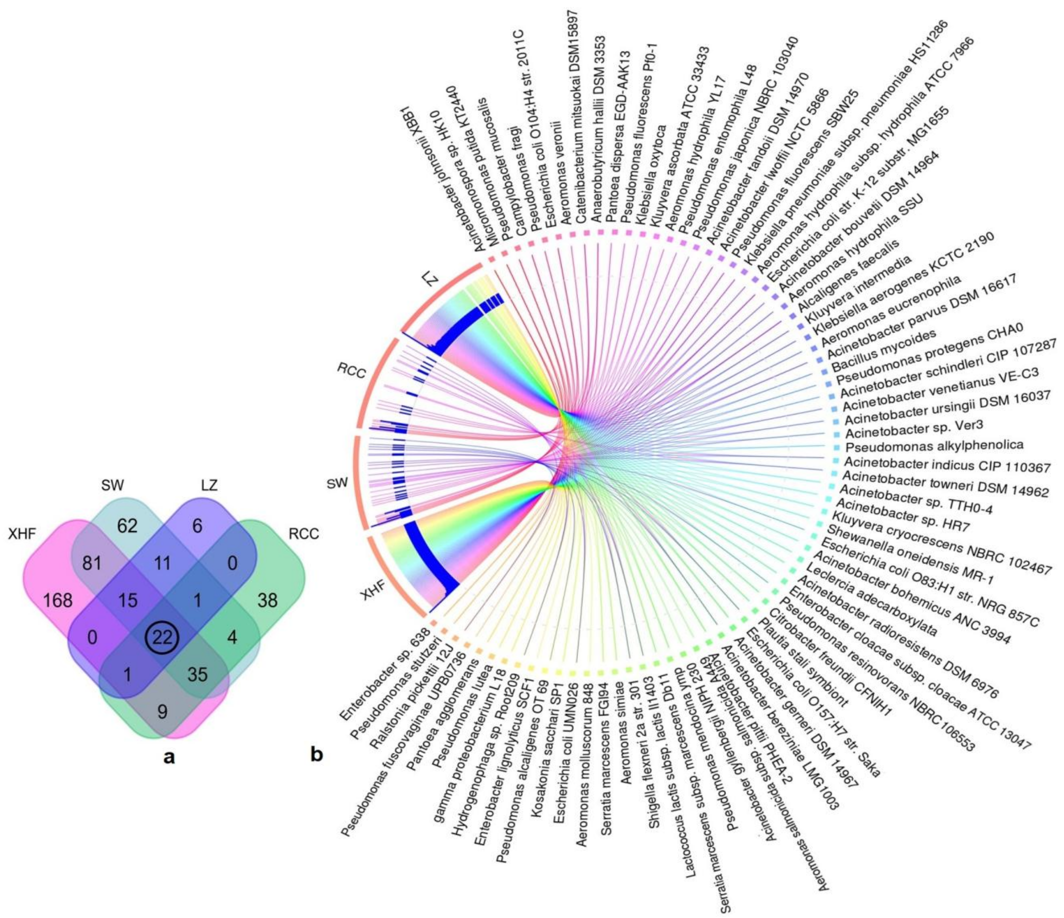


Figure 3.5: The strain-level microbiome composition in four different breeds of cows. (a) Venn diagrams representing the core unique and shared microbiomes of bovine mastitis in XHF, RCC, SW and LZ breeds. (b) The circular plot illustrates the relative abundance of the top 75 mastitis causing bacterial strains in milk samples obtained from XHF, LZ, SW and RCC dairy breeds. Taxa in the respective breed of cows are represented by different colored ribbons, and the inner blue bars indicate their respective relative abundances. The XHF cows had the highest number of microbial strains followed by LZ, SW and RCC. This breed specific association revealed that 45.66, 22.58 and 19.11% of the detected bacterial strains in CM milk collected from LZ, SW and RCC cows, respectively, were also seen in the CM milk microbiome of XHF cows (this thesis, Hoque et al., 2020a).

Table 3: Opportunistic and/or unreported strains in bovine mastitis milk metagenomes.

SL. No.	Opportunistic strains	Relative abundance
Clinical mastitis (CM) milk		
1	*Aeromonas veronii B565*	19.16
2	*Pantoea dispersa EGD-AAK13*	18.65
3	*Klebsiella oxytoca KA-2*	11.7
4	*Kluyvera ascorbata ATCC 33433*	7.27
5	*Aeromonas hydrophila YL17*	6.54
6	*Kluyvera intermedia E86*	4.53
7	*Aeromonas hydrophila subsp. hydrophila ATCC 7966*	3.32
8	*Klebsiella aerogenes KCTC 2190*	3.09
9	*Aeromonas hydrophila SSU*	2.81
10	*Aeromonas eucrenophila*	2.39
11	*Shewanella oneidensis MR-1*	2.08
12	*Kluyvera cryocrescens NBRC 102467*	1.67
13	*Enterobacter cloacae subsp. cloacae ATCC 13047*	1.06
14	*Plautia stali symbiont*	0.93
15	*Citrobacter freundii CFNIH1*	0.86
16	*Acinetobacter gyllenbergii NIPH 230*	0.72
17	*gamma proteobacterium L18*	0.71
18	*Serratia marcescens FGI94*	0.68
19	*Kosakonia sacchari SP1*	0.62
20	*Serratia marcescens subsp. marcescens Db11*	0.59
21	*Pseudomonas alcaligenes OT 69*	0.56
22	*Aeromonas salmonicida subsp. salmonicida A449*	0.56
23	*Enterobacter lignolyticus SCF1*	0.56
Recurrent clinical mastitis (RCM) milk		
1	*Nocardia pseudobrasiliensis*	48.06
2	*Serratia marcescens subsp. marcescens Db11*	16.22
3	*Nocardia mikamii NBRC 108933*	15.19
4	*Corynebacterium bovis DSM 20582 = CIP 54.80*	4.66
5	*Aeromonas hydrophila SSU*	3.11
6	*Aeromonas veronii B565*	1.81
7	*Aeromonas hydrophila YL17*	1.73
8	*Streptococcus salivarius*	1.64
9	*Bradyrhizobium japonicum 22*	1.64
10	*Cupriavidus metallidurans CH34*	1.55
11	*Glutamicibacter arilaitensis Re117*	1.04
12	*Methylobacterium radiotolerans JCM 2831*	0.78
13	*Nocardia veterana NBRC 100344*	0.69
Subclinical mastitis (SCM) milk		
1	*Chryseobacterium sp. Leaf405*	19.06
2	*Chryseobacterium sp. CF299*	13.68
3	*Chryseobacterium haifense DSM 19056*	13.19

4	*Chryseobacterium greenlandense*	13.04
5	*Chryseobacterium sp. YR460*	12.56
6	*Serratia marcescens subsp. marcescens Db11*	8.3
7	*Citrobacter freundii CFNIH1*	6.65
8	*Sediminibacterium salmoneum NBRC 103935*	2.35
9	*Elizabethkingia anophelis NUHP1*	2.2
10	*Streptococcus salivarius*	1.49
11	*Chryseobacterium koreense CCUG 49689*	1.27
12	*Sphingobacterium spiritivorum ATCC 33861*	1.23
13	*Empedobacter brevis NBRC 14943 = ATCC 43319*	1.12
14	*Methyloglobulus morosus KoM1*	0.75
15	*Lactobacillus algidus DSM 15638*	0.75

3.6 Mastitis-associated shift in archaeal and viral fraction of the microbiome

Another noteworthy feature of the current study is the concomitant detection of archaeal and viral fractions of microbiomes in both mastitis and H-milk samples through MR analysis. Interestingly, the structure and relative abundance of several microbial clades of these two domains were decreased in both RCM (11 genera) and SCM (9 genera) milk and remained enriched in CM-milk (56 genera) and healthy controls (46 genera) (**Figures 3.3.2 c, d**). Among the identified archaeal components of the microbiome, *Methanosarcina* was the most abundant genus both in CM (41.78%) and RCM (34.04%) milk samples. Besides this genus, *Methanococcoides* (21.25%), *Methanocaldococcus* (2.43%), *Thermococcus* (1.59%), *Haloterrigena* (0.95%), *Sulfolobus* (0.80%), *Methanothermococcus* (0.71%) and *Methanocorpusculum* (0.59%) in CM-milk, *Methanobrevibacter* (25.53%), *Halogeometricum* (10.64%) and *Pyrococcus* (6.38) in RCM-milk, and *Methanococcus* (24.39%), *Methanosphaera* (19.51%), *Methanoregula* (7.31%), *Methanospirillum* and *Methanothermobacter* (4.88% each) in SCM-milk were the predominantly abundant archaeal genera (**Figure 3.6.1**). On the other hand, *Haloarcula* (12.94%), *Methanoplanus* (11.95%), *Euryarchaeota* (4.12%), *Methanoculleus* (3.84%), *Methanosaeta* (1.28%), *Archaeoglobus* (1.0%), *Natrialba* and *Halogeometricum* (1.13%, each) were the most abundant archaeal genera in H-milk (**Figure 3.6.1**). Remarkably, the 81.03% and 77.59% of the detected archaeal genera were absent from the RCM and SCM microbiomes, respectively (**Figures 3.3.2 c, d**). The relative abundance of *Haloquadratum* and *Natronomonas* remained more than two-fold higher in SCM and RCM samples than CM and H milk samples

(**Figure 3.6.1**). The relative abundance of the rest of the genera remained much lower (< 0.05%) but varied significantly across the four sample types (**Figure 3.6.1**). Moreover, 13 (23.21%) and 2 (4.35%) archaeal genera in CM and H milk samples, respectively, had unique associations while none were found to be unique in RCM and SCM milk microbiomes. Among these unique genera, *Acidilobus*, *Aciduliprofundum*, *Candidatus*, *Pyrobaculum*, *Halobacterium*, *Thermosphaera* etc. were found in CM while only *Ferroglobus* and *Methanopyrus* were identified in H-milk samples (**Figure 3.6.1**).

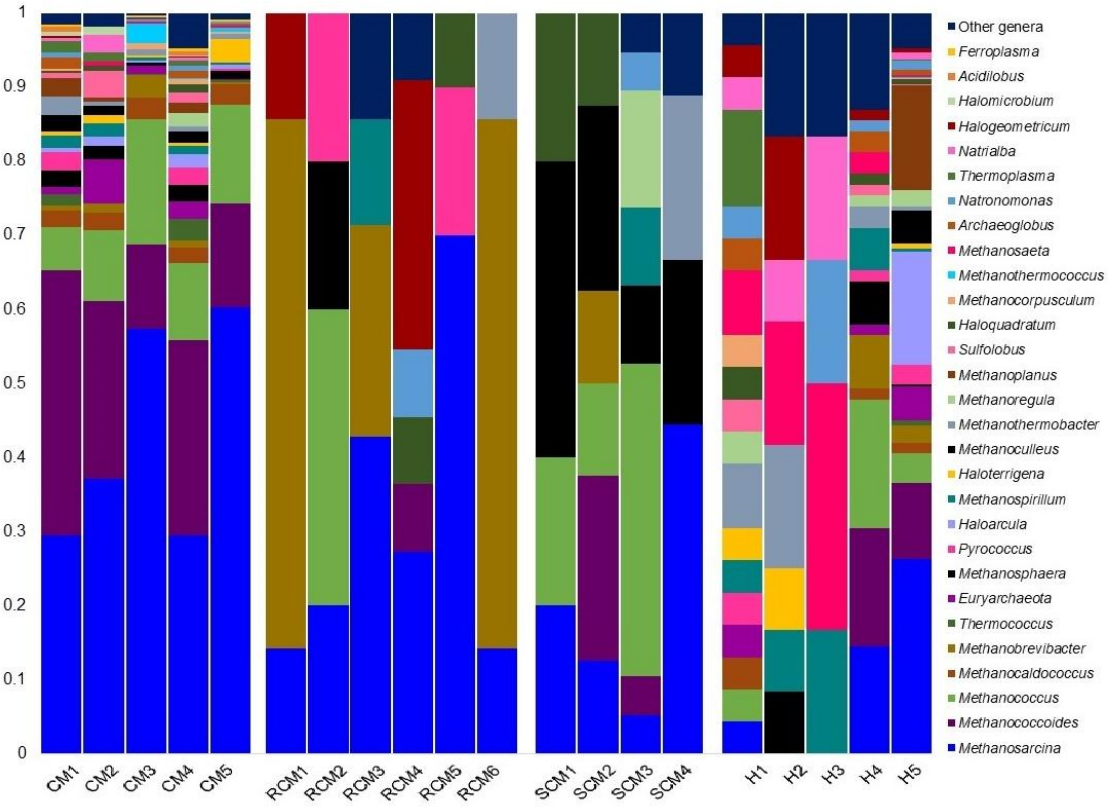


Figure 3.6.1: The genus level taxonomic profile archaea in bovine mastitis and healthy milk. Bar plots showing the distribution and relative abundance of top 30 most abundant archaeal genera, with ranks ordered from bottom to top by their increasing proportion among the CM, RCM, SCM and H milk samples. Only the 29 most abundant genera are shown in the legend, with the remaining strains grouped as 'Other genera'. Each stacked bar plot represents the abundance of archaea in each sample of the corresponding category, and notable differences in the archaeal populations are those where the taxon is abundant in one sample category and effectively undetected in the other sample types (this thesis, Hoque et al., 2020b).

The viral fraction of the microbiome was largely dominated by the members of the orders *Ortervirales* and *Caudovirales* (>90.0% of the total abundance), represented mostly by *Retroviridae* (52.84%), *Siphoviridae* (21.87%), *Podoviridae*

(12.96%), *Myoviridae* (11.21%); the remaining families had relatively lower abundances (< 0.5%) (**Figure 3.6.2**). The CM milk samples had the highest number (40) of viral genera followed by H (37), RCM (24) and SCM (11) milk samples, and of them, only 27.50% and 35.13% viral genera had sole association in CM and H-milk, respectively. Besides, 60.0%, 27.50% and 45.3% genera in RCM, SCM and H-milk, respectively were found to be shared with CM-milk microbiome (**Figure 3.6.2**). In this study, *Siphovirus* was the predominantly identified viral genus across the mastitis samples, with a relative abundance of 50.42%, 24.76%, and 20.33% in SCM, RCM, and CM, respectively. In H-milk samples the relative abundance of this genus was only 13.67% (**Figure 3.6.2**). In addition to *Siphovirus*, the most abundant viral genera in CM were *P2-like viruses* (13.67%), *Podovirus* (11.57%), *Myovirus* (7.78%) *Bpp-1-like viruses* (6.60%), phiKZ-like viruses (4.06%) and *T1-like viruses* (0.99%). Similarly, *Epsilon15-like viruses* (23.78%), *P1-like viruses* (2.53%), *P22-like viruses* (1.85%), *T4-like viruses* (1.75%), *Caudovirus* (0.97%) and *N15-like viruses* (0.88%) in RCM-milk, and *Lambda-like viruses* (10.08%), *Inovirus* (5.88%), *Molluscipoxvirus* (5.88%) and *phiKZ-like viruses* (4.20%) in SCM-milk were the predominant viral genera (**Figure 3.6.2**). The H-milk, however, had a relatively higher abundance of *Betaretrovirus* (46.42%) and *Gammaretrovirus* (30.07%) genera. The rest of the viral genera detected in both mastitis and H-milk had much lower (< 0.5%) relative abundances but their abundances always remained higher in CM (**Figure 3.6.2**). Despite having lower relative abundances (< 0.5%), *Epsilonpapillomavirus*, *Gammapapillomavirus*, *Lymphocryptovirus*, *Mastadenovirus*, *Microvirus* etc. in CM-milk, and *AHJD*-like viruses, *Alpharetrovirus*, *Avipoxvirus* c2-like viruses, *Cervidpoxvirus*, *Epsilonretrovirus*, *Lentivirus*, *Parapoxvirus*, *Retrovirus* in H-milk were found as the unique viral genera (**Figure 3.6.2**).

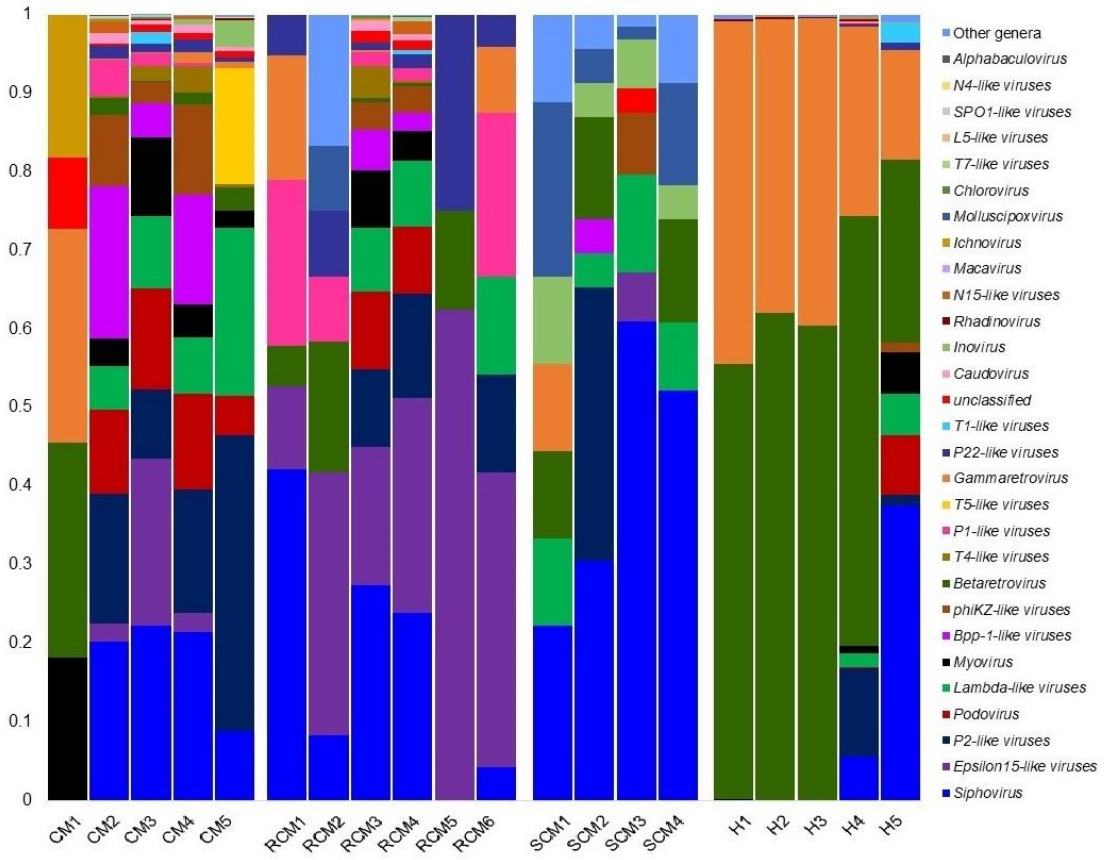


Figure 3.6.2: The genus level taxonomic profile viruses in bovine mastitis and healthy milk. Taxonomic distribution of 30 viral genera detected in all of the 20 samples of CM, RCM, SCM and H milk metagenomes. The most abundant viral genera are sorted by descending order of the relative abundance. Each stacked bar plot represents the abundance of viruses in each sample of the corresponding category, and notable differences in the viral populations are those where the taxon is abundant in one sample category and effectively undetected in the other sample types (this thesis, Hoque et al., 2020b).

3.7 *In vitro* identification of the mastitis-associated pathogens

Through *in vitro* cultural analysis, a total of 452 isolates belonged to six bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Bacillus* spp., and *Shigella* spp.) were identified in 100 CM samples collected from central (CR=60) and southeastern (SER=40) regions of Bangladesh. The number of isolated species per sample varied between 6 (Dhaka, *Shigella* spp.) and 40 (Chattogram, *S. aureus*). The overall prevalence of *S. aureus*, *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Bacillus* spp., and *Shigella* spp. were 23.46, 18.46, 19.23, 12.30, 9.23 and 17.30% CM samples, respectively (**Table 4**). The distribution of different bacterial pathogens identified related to the origin of the samples is shown in **Figure 3.7**. In cows from the SER (Chattogram), *S. aureus*, *Klebsiella* spp., and *Bacillus* spp. were the most frequent etiologies of CM as detected in 22.86%, 20.57%, and 18.29% isolates, respectively. The other bacteria detected in this region were *E. coli* (16.57%),

Enterobacter spp. (12.57%), and *Shigella* spp. (9.14%) (**Figure 3.7. a**). The frequency of the detected CM pathogens significantly (P = 0.01) varied within four different districts of CR (**Figure 3.7 b-e**), and thus results of the current study demonstrated *S. aureus* as the dominating etiology of bovine CM in Bangladesh while *Shigella* spp. remained as the least frequently detected bacterial pathogen causing mammary gland infections in dairy cows.

Analyzing the distribution of these bacterial pathogens according to the origin of the samples (SER and CR), we found significant differences (P = 0.01, Kruskal–Wallis test) in their prevalence. The culture-based findings of the current study demonstrated *S. aureus* as the dominating etiology of bovine CM in Bangladesh while *Shigella* spp. remained as the least frequently detected CM pathogen. This finding of *in vitro* study (**Figure 3.7**) corroborates with the metagenomic results of the genus-level taxonomic composition and abundance of the corresponding bacteria investigated through the state of the art WMS approach (**Figure 3.4.2**).

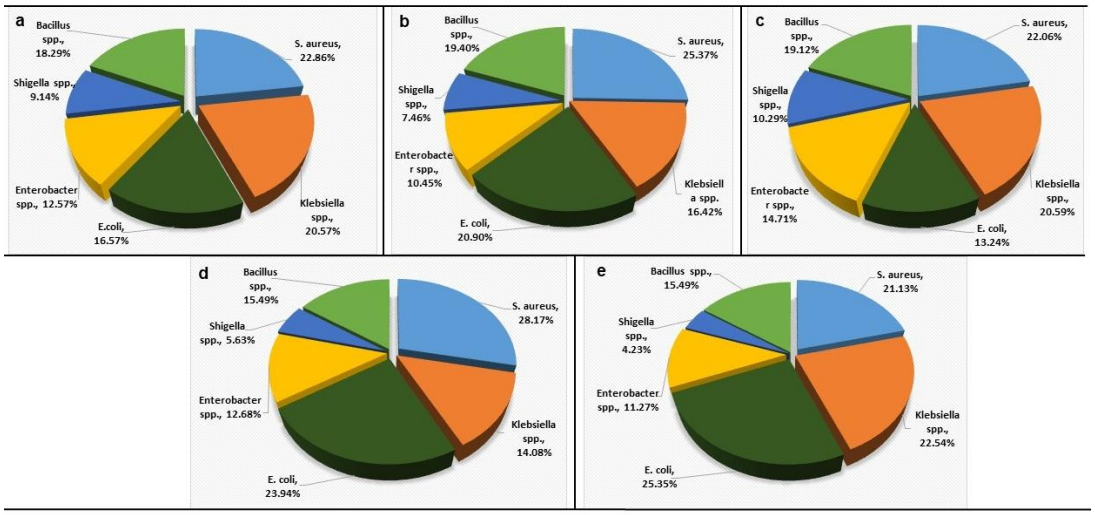


Figure 3.7: Prevalence and distribution of six selected bacteria isolated from bovine CM. Microorganisms identified in bovine CM samples originated from two different regions; (a) southeastern region and (b-e) central region of Bangladesh. The southeastern region (SER) included 175 isolates and the central region (CR) included 277 isolates (this thesis, Hoque et al., 2020a).

Table 4: Distribution of 452 isolates of six selected bacteria obtained from bovine clinical mastitis (CM) milk.

Farm location	*Staph. aureus*		*E. coli*		*Klebsiella* spp.		*Enterobacter* spp.		*Shigella* spp.		*Bacillus* spp.	
	Sample	Isol.[1]	Sample	Isol.[1]	Sample	Isol.[1]	Sample	Isol.[1]	Sample	Isol.[1]	Sample	Isol.[1]
Chattogram	24	40	16	29	20	36	12	22	10	16	18	32
Dhaka	10	17	8	14	7	11	4	7	4	5	7	13
Gazipur	8	15	5	9	8	14	6	10	5	7	8	13
Manikgonj	11	20	9	17	6	10	5	9	3	4	6	11
Sirajgonj	8	15	10	18	9	16	5	8	2	3	6	11
Total	61	107	48	87	50	87	32	56	24	35	45	80

3.8 Ribosomal gene (16S rRNA) sequencing-based identification of the mastitis-associated pathogens

Furthermore, we also identified these six bacteria at species level using ribosomal gene (16S rRNA) sequencing. The CM associated bacterial species identified through 16S rRNA gene sequencing had 100% similarity with the species identified in WMS (**Figure 3.8**). According to ribosomal gene sequencing, the isolated bacteria were divided into two subgroups, Gram-positive (*Staphylococcus aureus*, *Bacillus paramycoides* and *Bacillus altitudinis*) and Gram-negative (*Enterobacter cancerogenus*, *Klebsiella pneumoniae*, *Shigella flexneri*, and *E. coli*). The Gram-positive subgroup was further divided into two distinct clades (Clade A and B), while the Gram-negative subgroup had four different clades (Clade C, D, E and F) (**Figure 3.8 b**). The clade A contained *S. aureus* while *B. paramycoides* and *B. altitudinis* grouped into clade B. On the other hand, *E. cancerogenous aerogenes*, *K. pneumoniae*, *S. flexneri*, and *E. coli* were placed in clades C, D, E and F, respectively (**Figure 3.8 b**). The same bacterial species and/strains identified in both WMS and 16S sequencing have presented with same color codes such as *S. aureus* (black), *Bacillus* species (deep yellow) *Enterobacter* species (green), *Klebsiella* species (paste), *Shigella* species (pink) and *E. coli* (blue) (**Figure 3.8 b**).

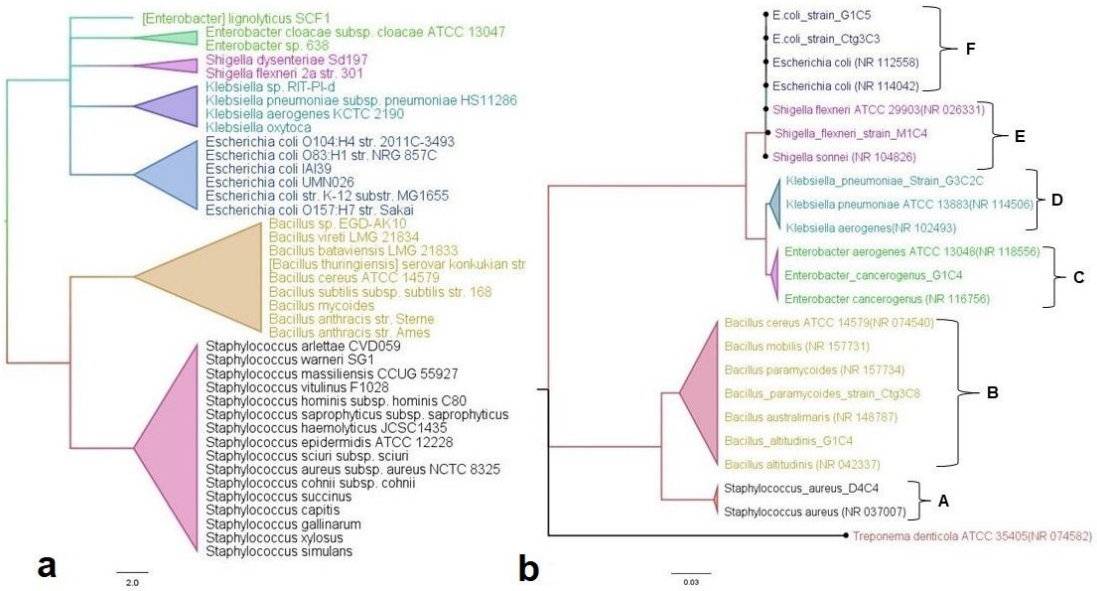


Figure 3.8: Phylogenetic diversity of the bovine clinical mastitis (CM) pathogens. (a) consensus tree based on alignment of the whole metagenome sequencing (WMS) data. (b) consensus tree based on alignment of the ribosomal (16SrRNA) gene sequencing. The first phylogenetic tree was constructed with >80% taxonomic identity using the neighbour-joining method in Clustal W while the second one in MEGA, and both trees were visualized through FigTree. The same bacterial species and/strains identified in both WMS and 16S sequencing have presented with same color codes such as *S. aureus* in black, *Bacillus* spp. in deep yellow, *Enterobacter* spp. in green, *Klebsiella* spp. in paste, *Shigella* spp. in pink and *E. coli* in blue colors. The scale bars indicate 2.0 and 0.02 substitutions per nucleotide position respectively in WMS and 16S gene sequences.

3.9 Virulence factors-associated genes (VFGs) in mastitis and healthy milk microbiomes

We next sought to identify virulence factors associated genes (VFGs) among the microbiomes of the four metagenomes using the virulence factor database (VFDB), and found a clear, significant enrichment of predicted VFGs in the association of bovine mastitis. The bovine milk microbiome harbored 494 VFGs families which revealed significant diversity (P = 0.001, Kruskal–Wallis test) both in composition and relative abundances among the four sample categories. The CM milk microbiome possessed highest number of VFGs (333) followed by RCM (304), SCM (183) and H (50) milk microbes, and only 3.24% of the VFGs were shared among the microbiomes of four metagenomes (**Figure 3.9 a**). However, 77.4%, 10.3% and 13.3% of the detected VFGs in RCM, SCM and H milk microbiota, respectively had association (shared) with CM microbiomes. By comparing the relative abundance of the detected VFGs among the microbiomes of four metagenomes, we found that innate immune response associated capsular genes (*ABZJ_00085* and *ABZJ_00086*) of *Acinetobacter* genus remained higher in H milk microbiomes (10.05% and 6.87%,

respectively) followed by CM (5.58% and 5.26%, respectively) and RCM (4.07% and 3.55%, respectively)-causing pathobiomes corroborating the higher taxonomic abundance of this genus in H milk samples.

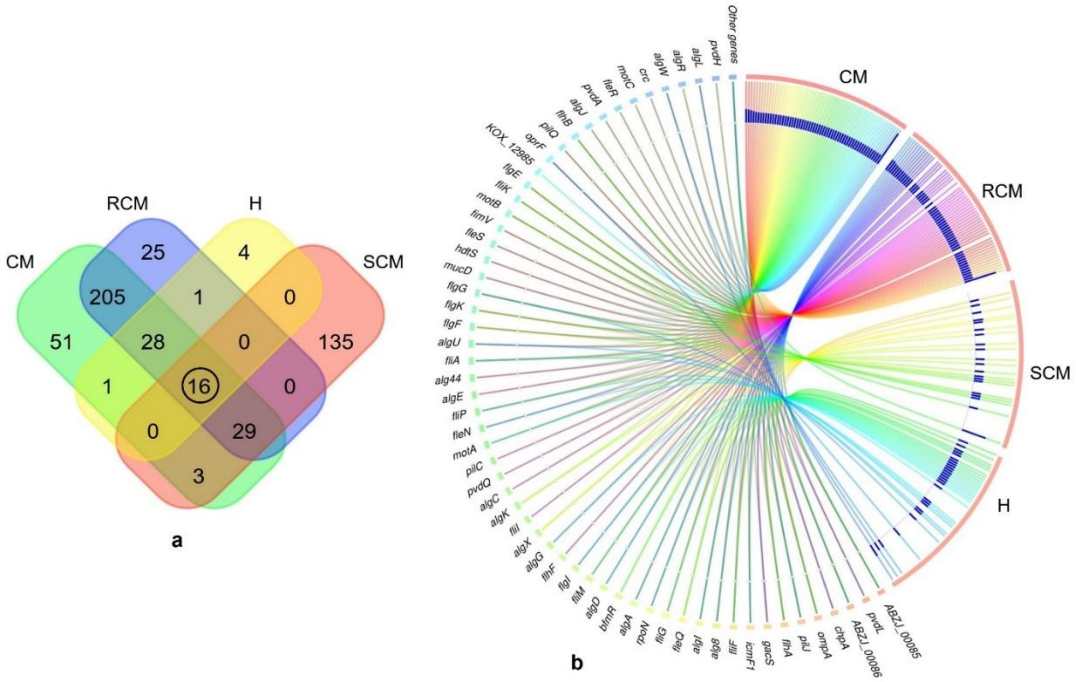


Figure 3.9: Virulence factors associated genes (VFGs) detected in bovine mastitis and healthy milk metagenomes. Metagenome sequencing data was used to search for open reading frames (ORFs) compared against the VFDB database to identify the VFGs with over 95% sequence identity. (a) Venn diagram showing unique and shared VFGs detected in CM, RCM, SCM and H milk metagenomes. VFGs sharing between the conditions are indicated by black circle. (b) The circular plot illustrates the distribution of top abundant 50 VFGs found across the four metagenomes. VFGs in the respective metagenome group are represented by different colored ribbons, and the inner blue bars indicate their respective relative abundances. The CM associated microbiomes had the highest number of VFGs followed by RCM, SCM and H metagenomes (this thesis, Hoque et al., 2020b).

Surprisingly, these two genes were not detected in SCM milk microbiomes. Moreover, the CM and RCM-causing microbiomes had sole association of VFGs coding for crucial nutrient (iron) uptake and/or regulation gene, *pvdL* (5.11% and 5.28%, respectively), twitching motility and/or chemotaxis, *chpA* (3.17% and 2.90%, respectively) and *motC* (0.55% and 0.44%, respectively), bacterial adhesion, invasion, or intracellular survival, *motB*/*ompA* (2.1% and 2.34%, respectively), biofilm formation, DNA uptake, adhesion to host cells and adherence, *pilJ* (1.83% and 1.81%, respectively) and *pvdA* (0.56% and 0.60%, respectively), two-component system, *gacS* (1.55% and 1.45%, respectively) and *gacA* (0.24% and 0.21%, respectively), intracellular multiplication factor, *icmF1* (1.51% in each), master regulator of biofilm initiation, *bfmR* (1.57% and 1.24%, respectively), quorum sensing/antibiotics

susceptibility altering, *pvdQ* (0.88% and 0.96%, respectively) and *pvdH* (0.50 in each), cellular adherence, *pilC* (0.88% and 0.91%, respectively), heat shock stress, *mucD* (0.65% and 0.55%, respectively), bacterial communication, *hdtS* (0.65% and 0.55%, respectively), and bacterial pathogenesis, *crc* (0.54% and 0.24%, respectively), which were not detected in both SCM and H milk microbes (**Figure 3.9 b**).

The SCM microbiomes possessed higher number of genes coding for bacteriocins production and cytotoxic activity in *E. coli*, *EcSMS35_B0007* (5.28%), capsular proteins in pathogenic *Klebsiella* species, *cpsB* (2.60%) and *rfb* (1.77%), fimbrial proteins, *stgC* (2.10%), and adhesin proteins, *upaG*/*ehaG* (2.09%) in pathogenic *E. coli* (Fig. 6b, Supplementary Data 2). On the contrary, genes coding for bacterial chemotaxis, *ompA* (3.43%) and *motA* (1.63%), flagellar assembly, *fliM* (3.08%), *flhA* (2.90%), *flgI* (2.18%), *fliG* (2.13%), *flgG* (1.87%), *fliF* (1.86%), fliA (1.47%) and *fliP* (1.41%), biofilm formation (BF), *alg*8 (3.93%), *algI* (2.82%), and *algD* (2.76%), alginate biosynthesis, *algG* (4.28%) and *algU* (1.62%), pili and flagella expression/adherence factors, *rpoN* (4.87%), *fleQ* (3.30%) and *fleN* (1.69%), and outer membrane proteins, *oprF* (3.33%) had relatively over expression in H milk microbiomes compared to mastitis-causing microbes. Although, the rest of the VFGs detected had relatively lower abundances (<0.5), they remained predominantly identified in both CM and RCM-associated microbiomes.

3.10 Resistomes composition in mastitis-associated microbiomes

The resistomes composition in mastitis-associated microbiomes provided a comprehensive picture. Using SEED module, 188,844 reads aligned to 30 resistance markers to antibiotics and toxic compounds (RATC) functional groups across the CM, RCM, SCM and H-milk microbiomes with different abundances. This WMS analysis showed significant correlation (Pearson correlation, P = 0.001; Nonparametric Spearman's Correlation, P = 0.003) between the number of reads aligned to bacterial genomes and number of reads mapped to RATC genes. Of the detected RATC functional groups, the CM metagenome had highest number (30) of RATC group followed by RCM (25), H (23) and SCM (14) milk metagenomes, and across these metagenomes, 40.0% of the RATC were found to be common (**Figure 3.10.1 a**). Among the RATC functional groups, the multidrug resistance to efflux pumps (*MREP*) had higher relative abundance in RCM (40.84%) and CM (38.54%)-

pathogens followed by H (16.55%), and SCM (13.77%) milk microbes.

Moreover, the CM microbiomes had higher abundance of genes coding for *CmeABC* operon (20.94%), *RFL* (11.24%), *mdtABCD* (9.82%), *MRS* (4.98), *AR* (3.77%), *CHCT* (2.41%), *OprN* (2.36%) and *RVAN* (0.52%) while the genes coding for *BlaR1* (19.57%), *CZCR* (9.71%), *mdtABCD* (5.80%), *CDR* (4.35%), *MRS* (4.04%), *CmeABC* operon (3.72%), *OprN* (3.66%), *AR* (3.51%), *BLAC* (2.37%), and *RCHC* (1.58%) in RCM, *CZCR* (30.0%), *CH* (15.0%), *BlaR1* (8.57%), *RFL* (7.1%), *MRS* (5.7%), *CmeABC* operon and *CHCT* (4.29%, each), and *BLAC* (3.11%) in SCM, and *CZCR* (28.59%), *CH* (12.38%), *CmeABC* operon (8.3%), RFL (6.62), *OprM* (5.49%), and *BLAC* (3.99%) in H-milk microbiomes had higher relative abundances (**Figure 3.10.1 b**).

Although the relative abundance of these RATC genes varied among the microbiomes of the four breeds (XHF, SW, RCC and LZ), but their resistomes composition did not vary significantly (P = 0.692) by taxonomic diversity of respective breeds. The *MREP* (35.45%), *CmeABC* (11.21%), RFL (7.18%), *mdtABCD* (5.95%), *MRS* (4.29%), *RCHC* (1.77%) and *RVAN* (0.71%) functional groups had higher relative abundance in XHF cow's milk microbiomes whereas the genes encoding for *OprN* (3.37%) and *BH* (1.02%) in SW, *CZCR* (30.00%), *CH* (15.00%), *BlaR1* (8.57%), *CHCT* (4.29%), *BLAC* (2.86%), *ZR* (1.43%) and *MROP* (0.71%) in RCC, and *AR* (3.47%), *AADNYL* and *LI* (0.52%, each) in H cow's milk microbiomes had relatively higher abundances (**Figure 3.10.2**).

The resistance potentials of RATC functional groups also varied significantly (P = 0.029, Kruskal–Wallis test) in *in vitro* selected six pathogens isolated and identified from different sources of mastitis samples (breed and study areas) under almost same farming management system (**Figure 3.10.3 a**). Among the RATC groups, the predominant antibiotic resistant genes were *MRS* (*S. aureus*, 37.0%), *RFL* (*S. aureus*, 14.8%; *Shigella*, 7.8%), *MREP* (*E. coli*, 28.5%; *Klebsiella*, 28.4%), *BlaR1* (*E. coli*, 6.0%; *Shigella*, 8.5%), *mdtABCD* cluster (*E. coli*, 17.5%; *Klebsiella*,18.9%; *Enterobacter*, 21.4%; *Shigella*, 11.7%), *MAR* locus (*E. coli*, 2.4%; *Enterobacter*, 2.6%), *CmeABC* operon (*E. coli*, 9.1%; *Enterobacter*, 11.0%; *Shigella*, 25.6%), and *ADCYS* (*Bacillus*, 5.5%) (**Figure 3.10.3 b**).

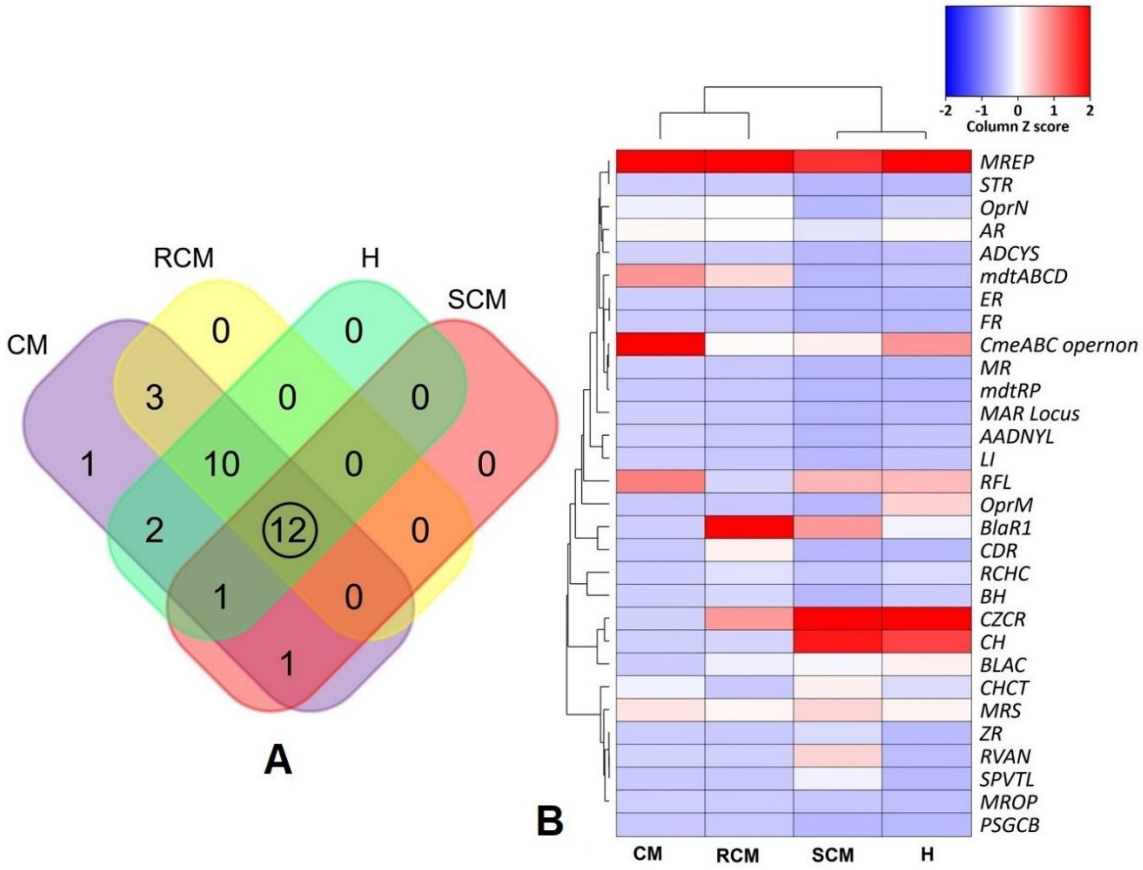


Figure 3.10.1: Projection of the resistomes in bovine mastitis and healthy-milk microbiomes. (A) Venn diagram representing unique and shared RATC detected in CM, RCM, SCM and H-milk metagenomes. RATC sharing between the conditions is indicated by black circle. (B) The clustered heatmap showing the hierarchical clustering of 30 different RATC genes detected in CM, RCM, SCM and H-milk associated microbiomes as measured at level-3 of SEED subsystems in MG-RAST pipeline. The color bar at the top represents the relative abundance of putative genes, and expressed as a value between -2 (low abundance) and 2 (high abundance). The red color indicates the more abundant patterns, while blue cells for less abundant RATC genes in that particular sample. Abbreviations- *MREP*: multidrug resistance efflux pumps, *CZCR*: cobalt-zinc-cadmium resistance, *BlaR1*: *BlaR1* family regulatory sensor-transducer disambiguation, *BLAC*: beta-lactamase resistance, *AR*: arsenic resistance, *RFL*: resistance to fluoroquinolones, *CH*: copper homeostasis, *CHCT*: copper homeostasis-copper tolerance, *RCHC*: resistance to chromium compounds, *mdtABCD*: the mdtABCD multidrug resistance cluster, *OprN*: mexe-mexf-oprN multidrug efflux system, *MROP*: mercury resistance to operon, *MRS*: methicillin resistance in *Staphylococci*, *CmeABC*: operon, multidrug efflux pump in *Campylobacter jejuni*, *ZR*: zinc resistance, *BH*: bile hydrolysis, *ER*: erythromycin resistance, *ADCYS*: adaptation to d-cysteine, *SPVTL*: *Streptococcus pneumoniae* vancomycin tolerance locus, *STR*: streptothricin resistance, *MAR*: multiple antibiotic resistance to locus, *RVAN*: resistance to vancomycin, *MRD*: mercuric reductase, *LI*: lysozyme inhibitors, *AADNYL*: aminoglycoside adenylyltransferases, *mdtRP*: multidrug resistance operon *mdtRP* of *Bacillus*, *FR*: fosfomycin resistance, *PSGCB*: polymyxin synthetase gene cluster in *Bacillus*, *OprM*: mexA-mexB-oprm multidrug efflux system, and *CDR*: cadmium resistance.

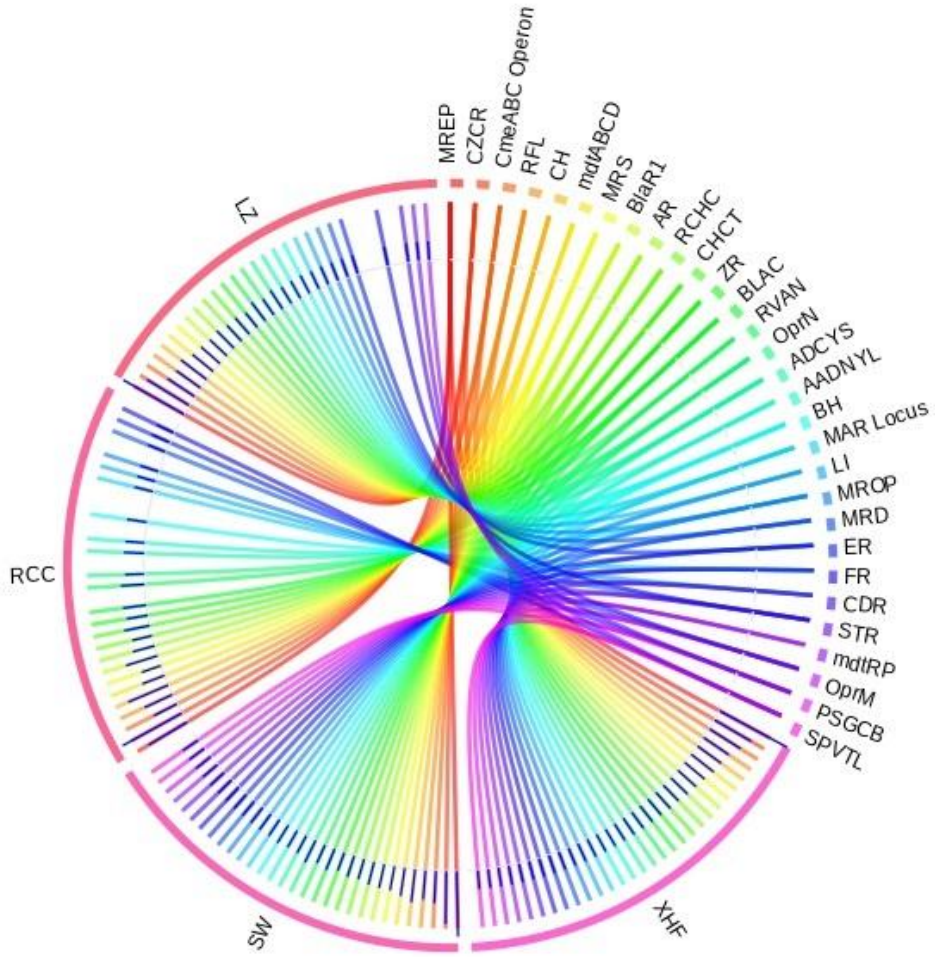
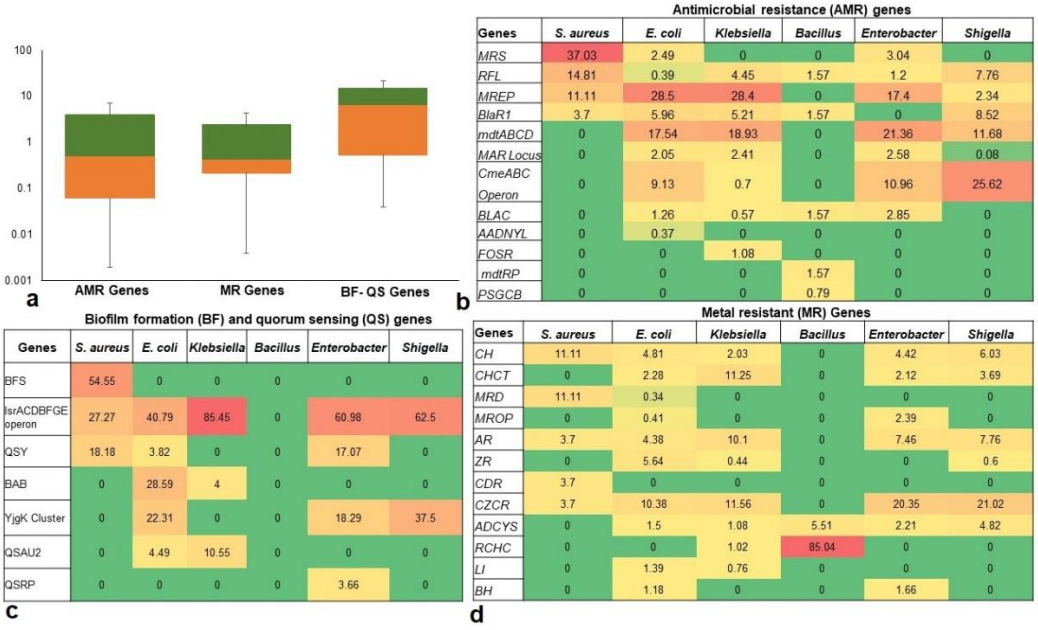


Figure 3.10.2: Resistomes diversity and composition in different dairy breeds. The circular plot illustrates the diversity and relative abundance of the RATC genes detected among the microbiomes of the four different breeds (Crossbred Holstein Friesian, XHF; Sahiwal, SW; Red Chattogram Cattle, RCC; Local Zebu, LZ) of cows. The association of the RATC genes according to breeds is shown by different colored ribbons and the relative abundances these genes are represented by inner blue colored bars. Part of the RATC functional groups are shared among microbes of the four breeds, and some are effectively undetected in the microbiomes of the other breeds (this thesis, Hoque et al., 2020a).



Antimicrobial resistance (AMR) genes

Genes	S. aureus	E. coli	Klebsiella	Bacillus	Enterobacter	Shigella
MRS	37.03	2.49	0	0	3.04	0
RFL	14.81	0.39	4.45	1.57	1.2	7.76
MREP	11.11	28.5	28.4	0	17.4	2.34
BlaR1	3.7	5.96	5.21	1.57	0	8.52
mdtABCD	0	17.54	18.93	0	21.36	11.68
MAR Locus	0	2.05	2.41	0	2.58	0.08
CmeABC Operon	0	9.13	0.7	0	10.96	25.62
BLAC	0	1.26	0.57	1.57	2.85	0
AADNYL	0	0.37	0	0	0	0
FOSR	0	0	1.08	0	0	0
mdtRP	0	0	0	1.57	0	0
PSGCB	0	0	0	0.79	0	0

Biofilm formation (BF) and quorum sensing (QS) genes

Genes	S. aureus	E. coli	Klebsiella	Bacillus	Enterobacter	Shigella
BFS	54.55	0	0	0	0	0
lsrACDBFGE operon	27.27	40.79	85.45	0	60.98	62.5
QSY	18.18	3.82	0	0	17.07	0
BAB	0	28.59	4	0	0	0
YjgK Cluster	0	22.31	0	0	18.29	37.5
QSAU2	0	4.49	10.55	0	0	0
QSRP	0	0	0	0	3.66	0

Metal resistant (MR) Genes

Genes	S. aureus	E. coli	Klebsiella	Bacillus	Enterobacter	Shigella
CH	11.11	4.81	2.03	0	4.42	6.03
CHCT	0	2.28	11.25	0	2.12	3.69
MRD	11.11	0.34	0	0	0	0
MROP	0	0.41	0	0	2.39	0
AR	3.7	4.38	10.1	0	7.46	7.76
ZR	0	5.64	0.44	0	0	0.6
CDR	3.7	0	0	0	0	0
CZCR	3.7	10.38	11.56	0	20.35	21.02
ADCYS	0	1.5	1.08	5.51	2.21	4.82
RCHC	0	0	1.02	85.04	0	0
LI	0	1.39	0.76	0	0	0
BH	0	1.18	0	0	1.66	0

Figure 3.10.3: Comparison of antibiotics, metals, biofilm formation and quorum sensing genes. Heatmap showing the associated genes found in the metagenome sequences (WMS) of six *in vitro* identified mastitis causing bacteria through SEED subsystems analysis in MG-RAST pipeline. (a) Diversity and relative abundance of the antimicrobial resistance (AMR), metal resistance (MR), and biofilm formation (BF) and quorum sensing (QS) genes in the study bacteria. (b) Relative abundance of AMR genes, (c) Relative abundance of MR genes (d) Relative abundance of BF-QS genes. Values are colored in shades of green to yellow to red, indicating low (absent), medium and high abundance, respectively (this thesis, Hoque et al., 2020a).

It was noted that, genes encoding *CH* (copper homeostasis) in *S. aureus* (11.1%), *E. coli* (4.8%), *Enterobacter* spp. (4.4%), and *Shigella* spp. (6.0%), *CHCT* (copper homeostasis-copper tolerance) in *Klebsiella* spp. (11.2%) and *Shigella* spp. (3.7%), mercuric reductase (*MRD*) in *S. aureus* (11.1%), mercury resistance to operon (*MROP*) in *Enterobacter* spp. (2.4%), *AR* (arsenic resistance) in *S. aureus* (3.7%), *E. coli* (4.4%), *Klebsiella* spp. (10.1%), *Enterobacter* spp. (7.5%) and *Shigella* spp. (7.8%), *ZR* (zinc resistance) in *E. coli* (5.6%), cadmium resistance (*CDR*) in *S. aureus* (3.7%), *CZCR* (cobalt-zinc-cadmium resistance) in *S. aureus* (3.7%), *E. coli* (10.4%), *Klebsiella* spp. (11.6%), *Enterobacter* spp. (20.3%) and *Shigella* spp. (21.0%) and *RCHC* (resistance to chromium compounds) in *Bacillus* spp. (85.0%) were the most abundant toxic metals resistant RATC functional groups among the six selected pathogens (**Figure 3.10.3 c**). In addition, assessment of the biofilm formation and quorum sensing (BF-QS) ability of the CM microbiomes revealed that autoinducer 2 (AI-2) transport and processing (*lsr*ACDBFGE operon, 33.7%), biofilm adhesion biosynthesis (*BAB*, 24.2%), protein *Yjg*K cluster linked to biofilm formation (*Yjg*K cluster, 15.5%), quorum sensing: autoinducer-2 synthesis (QSAU2, 9.4%) were

the most abundant genes among CM associated pathogens. However, by comparing the association of these BF-QS genes among the selected six bacterial pathogens, we found significant variation (P = 0.017, Kruskal–Wallis test) in their diversity, composition and relative abundances (**Figure 3.10.3**).

3.11 Antibiotics resistance genes (ARGs) in mastitis and healthy milk microbiomes

Attempt was made to explore the extent of different antibiotic resistance genes (ARGs) present in the selected metagenomes. We measured the total number and classes of ARGs detected in bovine milk microbial genomes using ResFinder database (Zankari et al., 2012). In our current WMS data set, there was a broad variation in ARGs diversity. ResFinder identified a total of 58 ARGs distributed in 442 bacterial genomes (**Figure 3.11 a**) and across 18 antibiotic classes (**Table 5**). Of the ARGs detected, the CM associated microbiomes harbored highest number genes (48 genes) followed by RCM (31 genes), SCM (11 genes) and H (6 genes) milk microbes. The RCM-causing bacteria possessed highest number (12 genes) ARGs classes followed by CM (11 genes), SCM (9 genes) and H (4 genes) milk bacteria. In addition, 36.2%, 20.4% and 12.5% of the ARGs detected in RCM, SCM and H milk microbes, respectively shared with the ARGs of CM-pathogens (**Figure 3.11 a**).

The most abundant ARGs detected during this study were associated with resistance to beta-lactams (13 genes), tetracyclines (5 genes), quinolones (5 genes), macrolides (10 genes), and trimethoprim (3 genes). Moreover, of the detected four broad-spectrum beta-lactams resistant genes (*blaOXA*, *blaROB*, *blaPLA1a*, *blaCARB*-2), *blaOXA* was found to be a common gene across the microbiomes of four metagenomes with highest relative abundance (97.63%) in H milk microbiota followed by RCM (46.17%), SCM (42.54%) and CM (40.80%)-causing bacteria (**Figure 3.11 b**). However, the rest of the beta-lactams resistant genes were found only in mastitis associated microbiomes with greatly varied relative abundances, and the *blaROB* and *blaPLA1a* genes had higher abundance in CM (3.73%) and SCM (1.16%)-pathogens, respectively. The tetracyclines (doxycycline and tetracycline) resistant genes (*tet*, *tet*A), however, remained most abundant (37.62%) in SCM associated bacteria compared to RCM (25.84%) and CM (13.33%) microbiota, and not detected in H milk microbiomes.

Resistance Classes	Gene name	Resistance mechanism	Relative abundances
Beta-lactam	*blaOXA*	Enzymatic inactivation	42.396
Doxycycline/Tetracycline	*tet*	Translational GTPase	19.249
Quinolone	*oqxB*	Antibiotic efflux	11.582
Macrolides	*mdf*	Efflux pump systems	3.285
Beta-lactam	*blaROB*	Class A β-lactamase	3.634
Aminoglycoside	*aph*	Enzymatic modification	8.510
Macrolide/Streptogramin B	*msr(E)*	Antibiotic target protection	1.702

Table 5: Relative abundance of major antibiotic resistance genes and classes with their respective mechanisms.

Trimethoprim	*dfrA*	Folate pathway antagonist	0.868
Sulfamethoxazole	*sul*	Folate pathway antagonist	4.195
Macrolides	*mph*	Antibiotic inactivation	0.979
Beta-lactam	*blaPLA1a*	beta-lactamase	0.340
Fosfomycin	*fosA*	Antibiotic inactivation	0.638
Chloramphenicol/Florfenicol	*floR*	Antibiotic efflux	0.996
Tetracycline	*Bla/ampS*	Class D β-lactamase (OXA)	0.885
Chloramphenicol	*cmlA*	Catalyze β - hydroxylation of L-PAPA	0.374
Aminoglycoside	*aadA*	Enzymatic modification	0.213
Beta-lactam	*blaCARB*	Unknown	0.077
Quinolone	*qnrS*	Inhibit DNA synthesis	0.077

Though, aminoglycoside (*aph*) and sulfonamide (*sul*) resistant genes were found in the microbes of four metagenomes, the relative abundance of these two genes remained several-folds (> 5) higher in CM pathogens compared to the microbes of RCM, SCM and H milk groups. Macrolides (erythromycin and streptogramin B) resistant (*msrE*) and fosfomycin resistant (*fosA*) genes had higher relative abundance in RCM pathogens than CM and SCM-causing bacteria. The multidrug-resistant genes, *oqxB* and *mdf(A)* were found only in mastitis causing pathogens, with highest relative abundance of *oqxB* gene in RCM (15.11%) and *mdf(A)* gene in CM (3.31%)-pathogens (**Figure 3.11 b**).

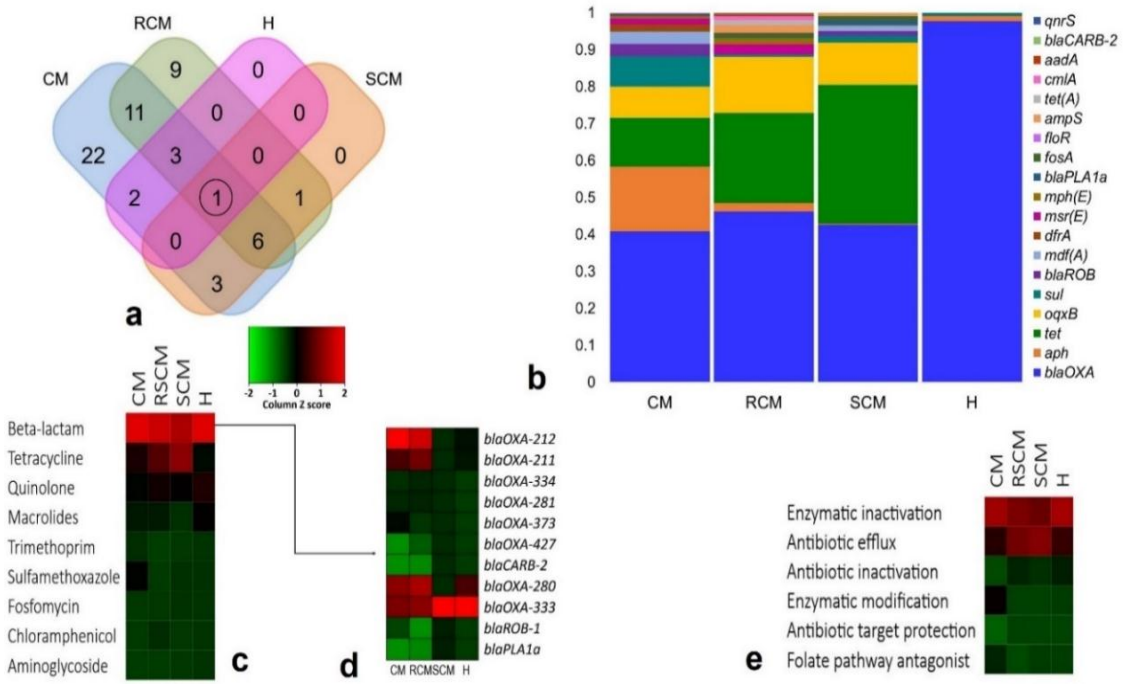


Figure 3.11: Antibiotic resistance genes (ARGs) in bovine mastitis and healthy milk metagenomes. Metagenome sequencing data was used to search for open reading frames (ORFs) compared against the ResFinder database to identify ARGs with over 95% sequence identity. (a) Venn diagram showing unique and shared ARGs detected in CM, RCM, SCM and H milk metagenomes. ARGs sharing between the conditions are indicated by black circle. (b) Stacked bar plots showing the relative abundance and distribution of the ARGs across the four metagenomes. (c) Heatmap showing the classes of antibiotics associated with the identified ARGs in bovine mastitis and healthy milk microbiomes. (d) Different beta-lactam genes resistant genes found in four metagenomes. (e) The possible mechanisms of actions/pathways of antimicrobial resistance found in this metagenome sequences. The color code indicates the presence and completeness of each gene, expressed as a value (Z score) between -2 (low abundance), and 2 (high abundance). The red color indicates the highest abundance whilst light green cells accounts for lower abundance of the respective genes in each metagenome (this thesis, Hoque et al., 2020b).

Notable that, some of the ARGs identified only in the CM associated microbial genomes, such as *dfrA* (trimethoprim resistant, 2.01%) and *floR* (florfenicol resistant, 0.24%) genes. Similarly, the RCM-causing pathogens had unique association four ARGs such as *cmlA* (chloramphenicol resistant), *aadA* (aminoglycoside resistant), *blaCARB-2* (beta-lactam resistant), and *qnrS* (quinolone resistant). In general, beta-lactam resistant genes (*blaOXA* particularly) presented the highest level of expression followed by tetracycline resistant (*tet* and *tetA*), quinolone resistant (*oqxB*, *mdfA* and *qnrS*), and macrolide resistant (*mphE*, *msrE* and *mdfA*) genes (**Figure 3.11 c, d)**.

Further analyses indicate that genes varied in their expression levels across the four metagenomes, being more prevalent in RCM associated microbial genomes. By

investigating the possible mechanisms of the detected ARGs, it appears that enzymatic inactivation associated resistance had the highest level of expression followed by antibiotic efflux pumps, antibiotic inactivation, enzymatic modification, antibiotic target protection, and folate pathway antagonist attributed resistance (**Figure 3.11 e**).

3.12 *In vitro* antibiogram profile of the isolated mastitis pathogens

The *in vitro* antibiogram profiling of 221 individual isolates of the six bacteria (56 isolates from *S. aureus*; 42 isolates from *E. coli*; 56 isolates from *Klebsiella* spp.; 26 isolates from *Enterobacter* spp.; 31 isolates from *Bacillus* spp. and 12 isolates from *Shigella* spp.) was attempted for twelve commonly used antibiotics from nine different groups (**Table 6**). It was revealed that *S. aureus* isolates had significant resistance to doxycycline, ampicillin, tetracycline and erythromycin (73.0 to 88.0%) and moderate resistance to chloramphenicol, ciprofloxacin and nitrofurantoin (50.0 to 58.0%) (**Figure 3.12**, **Table 6**). The isolates of *Klebsiella* spp., *Enterobacter* spp., and *Shigella* spp. displayed highest resistance to doxycycline, nalidixic acid, tetracycline and ampicillin (70.0 to 100.0%) and moderate resistance to ciprofloxacin, gentamicin, nitrofurantoin and chloramphenicol (30.0 to 70.0%) (**Table 6**). Study also indicates that imipenem and cefoxitin are the most sensitive antibiotics against four Gram-negative bacteria (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Shigella* spp.), while the two Gram-positive (*S. aureus* and *Bacillus*) species were surprisingly sensitive to imipenem, cefoxitin and vancomycin. The isolates of *Klebsiella* spp., *Enterobacter* spp., and *Shigella* spp. displayed highest resistance to doxycycline, nalidixic acid, tetracycline and ampicillin (70.0 to 100.0%) and moderate resistance to ciprofloxacin, gentamicin, nitrofurantoin and chloramphenicol (30.0 to 70.0%) (**Table 6**).

Table 6: Antibiotic resistance pattern of bacteria [n (%) of isolates] associated with bovine clinical mastitis.

Antibiotic	Content per disk	Breakpoint to declare resistance (≤)	*S. aureus* (n=56)	*E. coli* (n=54)	*Klebsiella* spp. (n=42)	*Enterobacter* spp. (n=26)	*Bacillus* spp. (n=31)	*Shigella* spp. (n=12)
AMP	10 µg	28 mm	48 (85.71)	42 (77.78)	36 (85.71)	24 (92.30)	25 (80.64)	10 (83.33)
DOX	30 µg	23 mm	49 (87.50)	46 (85.18)	39 (92.86)	22 (84.61)	26 (83.87)	10 (83.33)
TCN	30 µg	23 mm	46 (82.14)	50 (92.59)	38 (90.48)	24 (92.30)	11 (35.48)	12 (100)
CIP	10 µg	20 mm	28 (50.0)	22 (40.74)	18 (42.86)	8 (30.77)	13 (41.94)	4 (33.33)
IMP	10 µg	22 mm	10 (17.86)	12 (22.22)	11 (26.19)	5 (19.23)	2 (6.45)	3 (25.0)
CHL	30 µg	12 mm	32 (57.14)	34 (62.96)	23 (54.76)	18 (69.23)	6 (19.35)	6 (50.00)
GEN	10 µg	12 mm	22 (39.28)	23 (42.60)	21 (50.0)	4 (15.38)	23 (74.19)	5 (41.67)
NAL	30 µg	16 mm	ND	46 (85.18)	36 (85.71)	20 (76.92)	23 (74.19)	12 (100)
NIT	10 µg	64 mm	28 (50.0)	32 (59.25)	30 (71.42)	12 (46.15)	ND	4 (33.33)
CFX	30 µg	24 mm	14 (25.0)	14 (25.0)	12 (28.57)	8 (30.77)	ND	2 (16.67)
VAN	30 µg	20 mm	12 (21.42)	ND	ND	ND	6 (19.35)	ND
ERY	15 µg	20 mm	41 (73.21)	ND	ND	ND	19 (61.29)	ND

n: total number of isolates tested; ND: Not done; AMP: Ampicillin; DOX: Doxycycline; TCN: Tetracycline; CIP: Ciprofloxacin; IMP: Imipenem; CHL: Chloramphenicol; GEN: Gentamycin; NAL: Nalidixic acid; NIT: Nitrofurantoin; CFX: Cefoxitin; VAN: Vancomycin; ERY: Erythromycin.

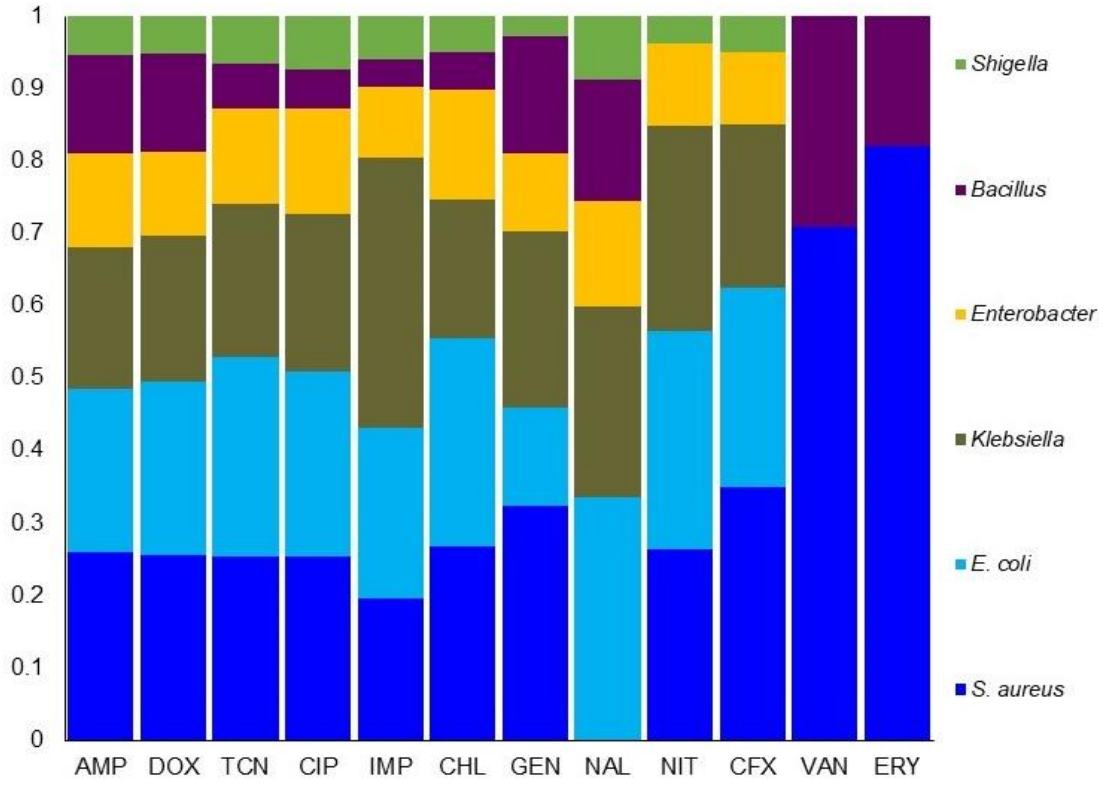


Figure 3.12: Antibiotic resistance pattern of bovine mastitis pathogens by disk diffusion method. The antimicrobial resistance (AMR) patterns of the six bacteria obtained from 221 mastitis isolates for twelve commonly used antibiotics from nine different groups/classes. Abbreviations: AMP, Ampicillin; DOX, Doxycycline; TCN, Tetracycline; CIP, Ciprofloxacin; IMP, Imipenem; CHL, Chloramphenicol; GEN, Gentamycin; NAL, Nalidixic acid; NIT, Nitrofurantoin; CFX, Cefoxitin; VAN, Vancomycin; ERY, Erythromycin. More details about AMR profiles can be found in Table 6 (this thesis, Hoque et al., 2020a).

present study indicated that imipenem and cefoxitin are the most sensitive antibiotics against four tested Gram-negative bacteria (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Shigella* spp.) while the two Gram-positive bacteria (*S. aureus* and *Bacillus* spp.) were highly sensitive to imipenem, cefoxitin and vancomycin. Taken together, the antibiogram profile revealed that all of the selected mastitis causing pathogens are evolving as multidrug resistant (MDR, resistant to ≥ 5 antibiotics) and the highest resistance was found against tetracyclines (tetracycline and doxycycline) followed by quinolones (nalidixic acid) and penicillin (ampicillin) groups of antibiotics (**Figure 3.12**, **Table 6**).

3.13 *In vitro* metal sensitivity of the mastitis pathogens

The use of toxic metals in soluble forms as an alternative to prevent bovine CM appears as a novel promising idea supported by several earlier studies (Reyes-Jara et al., 2016; Vaidya et al., 2017). Zones of inhibition (ZOI) assays using the

individual metal solution (Cu, Zn, Cr, Co and Ni) demonstrated an increase in antimicrobial activity which correlated with increased metal ion solution concentration ($P < 0.001$). Thus, ZOI assays of metals demonstrated *S. aureus* (ZOI: 25.4 mm) as the most sensitive CM pathogens followed by *Bacillus* spp. (ZOI: 23.4 mm), *E. coli* (ZOI: 20.6 mm), *Enterobacter* spp. (ZOI:18.9 mm), *Klebsiella* spp. (ZOI:17.8 mm) and *Shigella* spp. (ZOI:15.4 mm) (**Figure 3.13.1**). The minimal inhibitory concentration (MIC) of the metal ions demonstrated a varying degree of response against all the tested CM pathogens, and these bacteria tolerated a wide range of metal concentration (3.4 to 38.1 µg/mL). When compared the highest MIC values of each metal, it was found that highest MIC values decreases in the following order: Zn (38.1 µg/mL, *S. aureus*), Cu (33.2 µg/mL, *S. aureus*), Ni (28.2 µg/mL, *E. coli*), Cr (17.2 µg/mL, *Enterobacter* spp.), and Co (15.3 µg/mL, *Bacillus* spp.) (**Figure 3.13.2**).

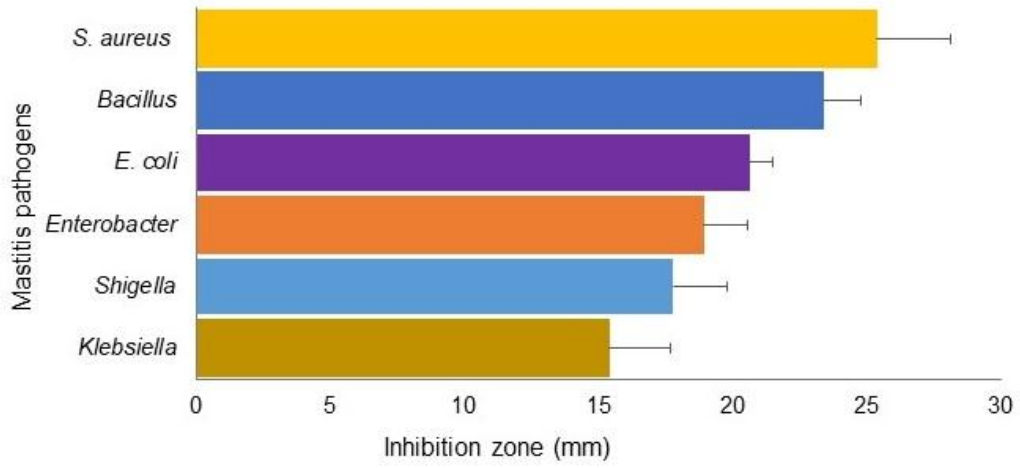


Figure 3.13.1: Antibacterial activity of heavy metals against mastitis pathogens: Cu (CuSO4), Zn (ZnO), Cr (K2Cr2O7), Co (CoCl2) and Ni (NiCl2) against bovine CM pathogens. Zone of inhibition (ZOI, mm) for six CM causing bacteria, each bar representing the mean values (values given horizontal axis of the bars, mm) and standard deviation error bar (SD error bar) for each bacterium (this thesis, Hoque et al., 2020a).

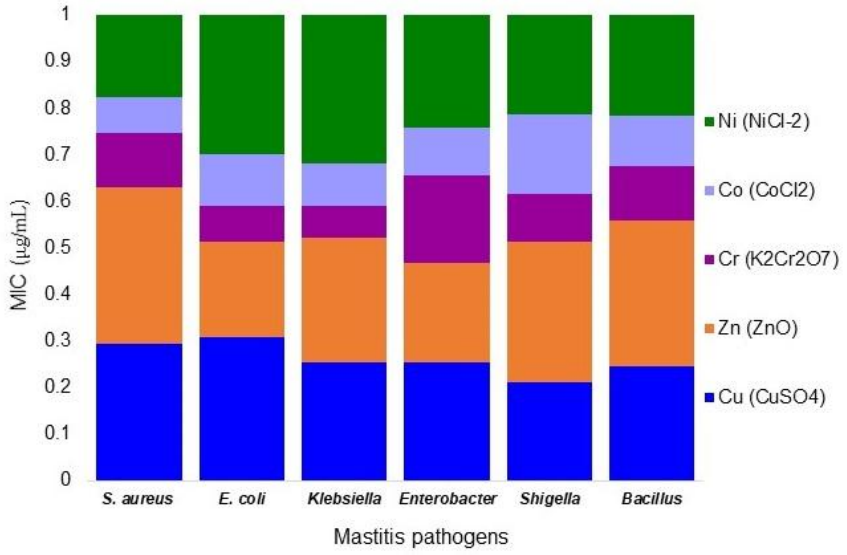


Figure 3.13.2: Antibacterial susceptibility of heavy metals against mastitis pathogens: Cu (CuSO4), Zn (ZnO), Cr (K2Cr2O7), Co (CoCl2) and Ni (NiCl2) against bovine CM pathogens. Minimal inhibitory concentration (MIC) (expressed as µg/mL) of the tested metals against representative genera/species as determined by agar well diffusion and tube dilution methods (this thesis, Hoque et al., 2020a).

For the MIC of specific bacteria, the most effective metals were found to be Cr against *Shigella* spp. (3.4 µg/mL) and *Klebsiella* spp. (5.8 µg/mL) species, Ni against *Shigella* spp. (3.5 µg/mL) species, Co against *Shigella* spp. (5 µg/mL) and *Klebsiella* spp. (7.4 µg/mL) species, and Cu and Zn against *Shigella* spp. (7.5 µg/mL, both) species. In contrast, Zn (38.1 µg/mL) and Cu (33.2 µg/mL) were the least toxic metals against *S. aureus* (**Figure 3.13.2**).

A similar phenomenon was observed for the minimal bactericidal concentration (MBC) with the greatest bactericidal activity for Cr against *S. aureus* (11.3 µg/mL) followed by Co against *E. coli* (14.3 µg/mL), Ni against *S. aureus* (23.1 µg/mL), Zn against *E. coli* (24.2 µg/mL), and Cu against *Shigella* spp. (25.1 µg/mL) species. However, Cu produced equable antimicrobial efficacy as Zn, Cr, Co and Ni against *Enterobacter* spp. (≤ 25.5 µg/mL).

3.14 *In vitro* biofilm formation (BF) assay

To assess the ability of bovine mastitis associated pathogens in producing biofilms, we randomly selected 80 isolates (15 isolates from *S. aureus*; 15 isolates from *E. coli*; 15 isolates from *Klebsiella* spp.; 15 isolates from *Enterobacter* spp.; 10 isolates from *Shigella* spp.; 10 isolates from *Bacillus* spp.) for BF assay. In this study, 76.2% (61/80) bacterial species were biofilm producers with significance differences (P = 0.028), and categorized as strong biofilm forming (SBF, 28.7%), moderate

biofilm forming (MBF, 25.2%), weak biofilm forming (WBF, 22.2%) and non-biofilm forming (NBF, 23.7%) (**Figure 3.14**).

Microscopic observation followed by 3D image analysis revealed that the intensity of green fluorescence remained higher indicating that a large number of cells were viable and attached to the surface (**Figure 3.14 a**). While investigated individually, *E. coli* (66.7%) remained as the highest biofilm producing CM pathogen followed by *Enterobacter* spp. (60.0%), *Klebsiella* spp. (46.7%), *S. aureus* (40.0%), *Shigella* spp. (30.0%) and *Bacillus* spp. (26.7%) species. Our current findings revealed that Gram-negative CM pathogens (*Enterobacter* spp., 60.0%; *E. coli*, 40.0%; *Shigella* spp., 33.3%; *Klebsiella* spp., 28.6%) had higher biofilm producing ability than Gram-positive bacteria (*S. aureus*, 16.7%). On the contrary, the majority of the *Bacillus* spp. (73.3%), *Shigella* spp. (70.0%) and *S. aureus* (60.0%) isolates remained as non-biofilm formers (NBF) (**Figure 3.14 b**). Therefore, our current findings of *in vitro* resistance analysis (antibiotics and metals resistance and biofilm assays) corroborate the resistome found in metagenome sequencing.

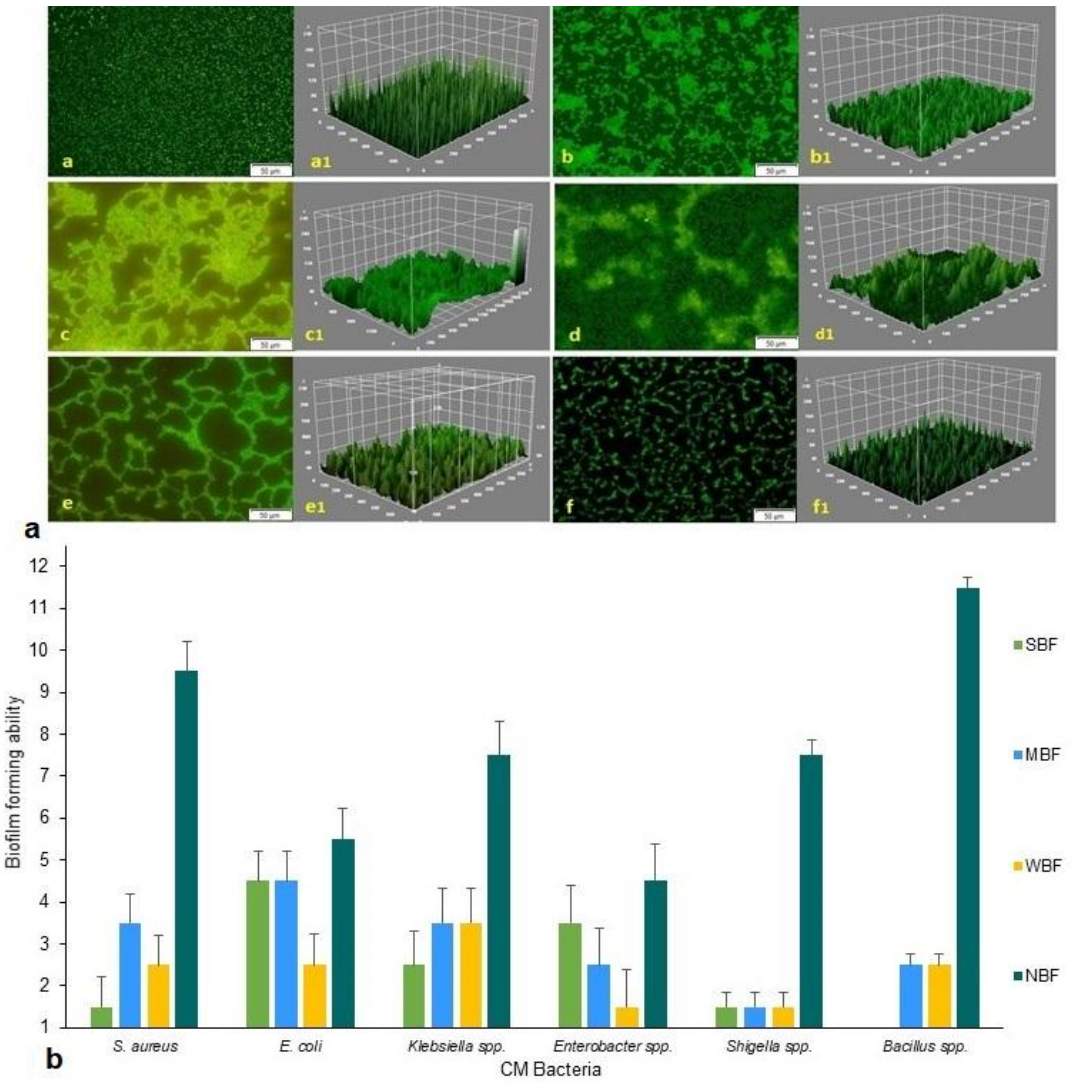


Figure 3.14: Biofilm formation (BF) ability of the six mastitis causing pathogens. BF assays was performed with solubilized crystal violet (CV) in a plate reader at 600 nm using 30% acetic acid in water as the blank and TSB as negative control. (a) Confocal fluorescence images (2D and 3D) of *S. aureus* (a, a_1), *E. coli* (b, b_1), *Klebsiella* (c, c_1), *Enterobacter* (d, d_1), *Bacillus* (e, e_1) and *Shigella* (f, f_1). Scale bars are indicated in μm. (b) Category of the biofilm formation by six bacteria. The BF ability of the tested bacteria were classified as follows: NBF, non-biofilm formers optical density (OD) ≤ optical density cut-off (ODc); WBF, weak biofilm formers (ODc < OD ≤ 2 x ODc); MBF, moderate biofilm formers (2 x ODc < OD ≤ 4 x ODc), SBF, strong biofilm formers (OD > 4 x ODc). The ODc value was set as 0.045 and the mean OD of the negative control was 0.039±0.002. Thus, bacterial biofilms were divided into breakpoint categories; OD < 0.045 non-biofilm producers; OD ≥ 0.046 but ≤ 0.090 weak biofilm producers; ≥OD 0.091–≤0.180 moderate or partial biofilm producers; > 0.181 strong biofilm producers. The results are presented as the mean ± SD, and post hoc Bonferroni test was used to compare the biofilm OD600 mean values ($P < 0.05$) (this thesis, Hoque et al., 2020a).

3.15 Mastitis-associated changes in metabolic functional potentials

Changes in the functional and metabolic aspects of the milk microbiota during the progression of bovine mastitis was assessed through annotation of gene functions of the identified microbial (bacteria, archaea and virus) genomes using KEGG (the

Kyoto Encyclopedia of Genes and Genomes) pathway and SEED subsystem modules of the MR pipeline. The functional annotation of the WMS reads through the KEGG pathway database, integrated with the MR pipeline, identified 8.55% and 5.98% putative genes with known and unknown protein functions, respectively. This finding suggests that many of the genes associated with different metabolic and functional potentials of the mastitis microbiome are yet unexplored (Table 2). By examining the correlation between the different gene families of the same KEGG pathway for mastitis and H milk microbiomes, we found significant differences (P = 0.002, Kruskal–Wallis test) in their relative abundances, and positive correlation with different states of bovine mastitis. Consistent with the significant shifts in microbiome diversity and composition in different states of bovine mastitis (CM, RCM, SCM) and healthy controls, KEGG orthology (KO) modules such as carbohydrate metabolism, TCA cycle, energy metabolism, oxidative phosphorylation (OP) and cellular processes were significantly (P = 0.002, Kruskal–Wallis test) overrepresented in mastitis milk microbiomes compared to healthy milk microbiota. It was also evident that genes coding for citrate metabolism (TCA cycle) had more than two-fold over expression in CM (16.55%) and SCM (12.50%) associated microbiomes than RCM (6.75%) and H (8.79%) milk microbiota.

Again, within this same pathway, the genes coding for citrate synthase (*gltA*) had higher relative abundance in RCM related microbiome (31.25%) followed by SCM (21.57%), CM (15.41%) and H milk microbes (13.86%). The H milk microbiome however were enriched with the genes of pyruvate metabolism (19.31%). The RCM and SCM milk microbiome had relatively higher abundances of genes coding for oxidative phosphorylation (74.04% and 78.86%, respectively) and carbon fixation pathways in prokaryotes (4.81% and 5.71%, respectively) while genes encoding for nitrogen (13.67%) and sulfur (16.47) metabolism remained predominantly abundant in CM-microbiomes (**Figure 3.15.1**). In addition, within the KEGG orthology module of oxidative phosphorylation pathways, the gene families of NADH dehydrogenase (*ndh*), ATPase subunits (*atpA* and *atpD*) and inorganic pyrophosphatase (*ppa*) had higher relative abundances in CM milk metagenome. Conversely, genes coding for cytochrome c oxidase subunits (*coxA*, *coxB* and *coxC*) and protoheme IX farnesyltransferase (*cyoE*) remained predominantly abundant in H milk metagenome (5.47% and 4.77%, respectively).

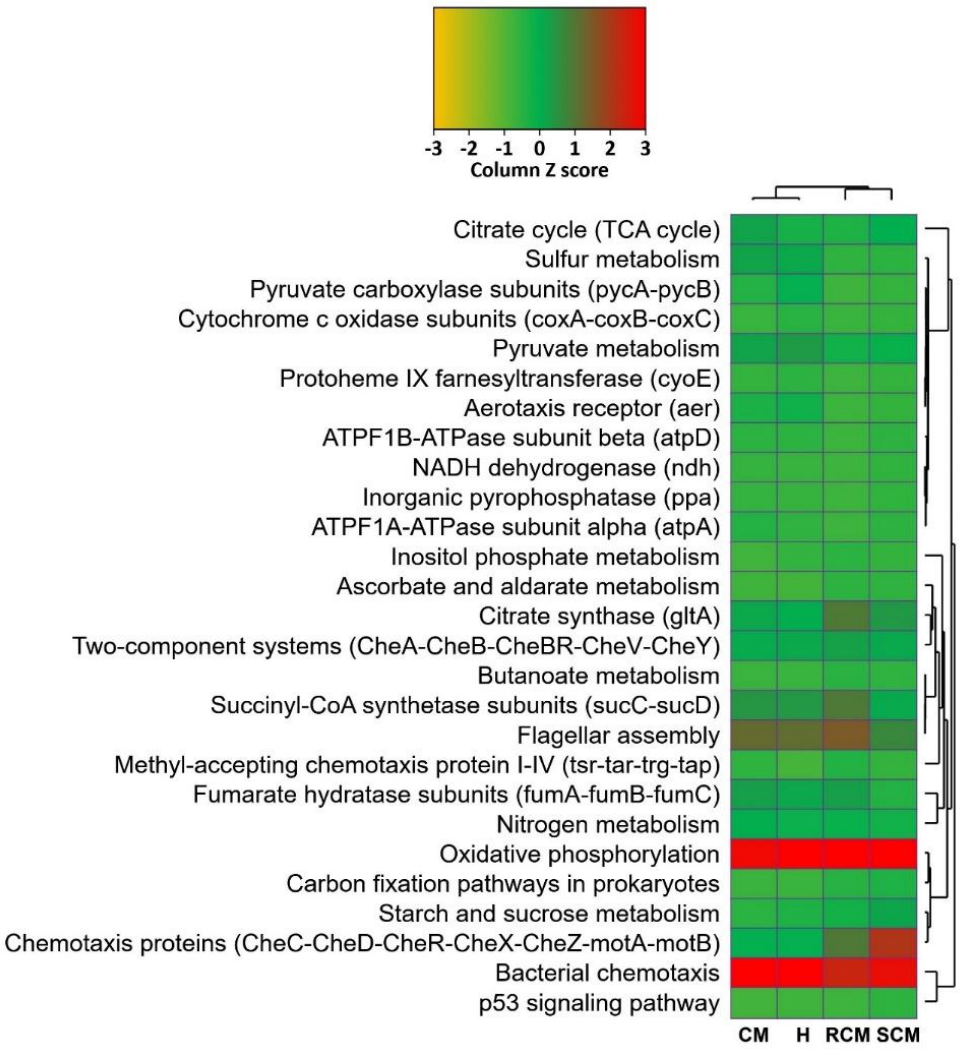


Figure 3.15.1: Projection of the mastitis and healthy milk metagenomes onto KEGG pathways. Shotgun whole metagenome sequencing (WMS) reveals differences in metabolic functional pathways in CM, RCM, SCM and H milk microbiomes. Heatmap showing the average relative abundance hierarchical clustering of the predicted KEGG Orthologs (KOs) functional pathways of the microbiota across all samples. The color code indicates the presence and completeness of each gene, expressed as a value (Z score) between -3 (low abundance), and 3 (high abundance). The red color indicates the highest abundance, whilst light green cells accounts for lower abundance of the respective genes or KOs in each metagenome.

The gene and/or protein families associated with bacterial chemotaxis (*CheC, CheD, CheR, CheX, CheZ, motA, motB*) were predominantly enriched in SCM-pathogens (57.89%), and those coding for methyl-accepting chemotaxis (*tsr, tar, trg* and *tap* genes) had higher relative abundances in RCM (5.56%) and CM (4.13%)-causing pathogens. Moreover, genes responsible for bacterial flagellar assembly (41.30%), succinyl-CoA synthetase alpha-beta subunits (*sucC, sucD*) (31.25%), and two-component systems (*CheA, CheB, CheBR, CheV, CheY*) (18.52%) remained over

expressed in RCM pathogens, and genes coding for p53 signaling pathway had relatively higher expression in SCM (1.56%) and H (1.10%) milk microbiota (**Figure 3.15.1**).

We next sought to gain further insights into the microbiome dysbiosis of four metagenome groups by performing functional profiling using the SEED protein database in MR tool, in order to identify genes and/or proteins and associated-pathways that were significantly different between the metagenome groups. The proteins annotated at deeper level (function) classification identified 25 SEED functions to be statistically different (P = 0.001, Kruskal–Wallis test) across the four metagenomes. Overall, the mastitis associated microbiomes showed higher relative abundances of these 25 SEED functions than H milk microbial community except for plasmid related functions (highest in H microbes; 6.90%) (**Figure 3.15.2)**. Of the annotated SEED functional groups, the BarA-UvrY (*SirA*)-two-component regulatory system was found as the most abundant functional pathway with highest relative abundance in RCM-pathogens (100.00%) followed by CM (85.60%), H (83.66%) and SCM (33.33%)-milk microbiomes.

Further analyses revealed that, the mastitis microbiomes were significantly enriched in genes encoding phage integration and excision (36.16%), autoinducer-2 (AI-2) transport and processing (*lsrACDBFGE* operon, 34.74%), pathogenicity islands (15.28%), *YjgK* cluster-linked to biofilm formation (15.04%), NADPH quinone oxidoreductase-2 (3.34%) and *TyrR* associated virulence (2.93%) compared to the microbes of other three metagenome groups. Elevated SEED functional pathways in RCM-causing microbiomes were dominated by genes involved in biofilm adhesin biosynthesis (80.0%), quorum sensing and biofilm formation (29.41%), glutathione non-redox reactions (20.83%), regulation of oxidative stress (ROS) response (12.50%), transposable elements (12.06%), phage DNA synthesis (2.38%), and gene transferring agent (1.89%). In contrast, the SCM microbiomes particularly showed the elevated abundance of the genes coding for protection from ROS (33.33%), oxidative stress (28.57%), virulence regulation (19.35%), and phage related functions such as *r1t*-like *Streptococcal* phages (35.59%), phage regulated gene expression (12.71%), phage packaging machinery (11.86%), phage replication (9.32%) and prophage lysogenic conversion (2.54%) modules (**Figure 3.15.2**).

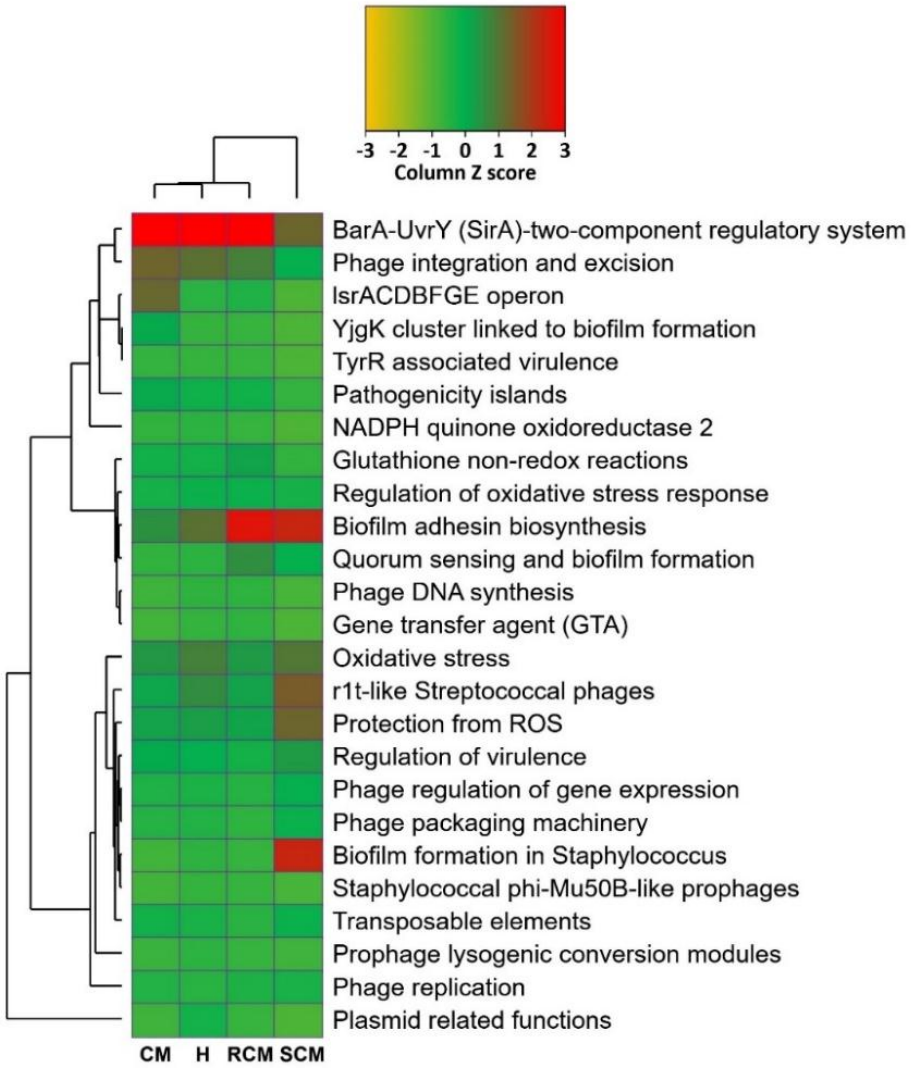


Figure 3.15.2: Functional annotation of the of the mastitis and healthy milk metagenomes using different levels of SEED subsystems. Comparison of metagenomic profiles of CM, RCM, SCM and H milk microbiotas at different levels of SEED subsystems (up to functions). The selected subsystems showing significant ($p<0.05$) differences among four sample groups is shown. The less abundant subsystems in a given metagenome are shown in green, and subsystems that are more abundant are represented in red color. The color codes indicated the presence and completeness of each subsystem module, expressed as a value between -3 (low abundance), and 3 (high abundance). The red color indicates the more abundant patterns, whilst light green cells accounts for less abundant SEED modules in that particular sample.

3.16 Cows-to-mouse fecal microbiota transplantation (FMT)

3.16.1 Gross and histopathological signs of mouse mastitis

The cows-to-mouse fecal microbiota transplantation (FMT) from mastitis cows, but not from healthy cows, to germ-free (GF) mice resulted in clinical mastitis (CM) clinical symptoms in the mammary glands, and inflammatory changes both in mammary and intestinal tissues compared to their healthy counterpart. On the mammary gland surface, gross visible signs of severe inflammation that corresponded

to mastitis was observed in each mouse of the clinical mastitis (MCM) group (**Figure 3.16.1 a-b**), however, no pathological changes were visually apparent in healthy mouse (MH) group (**Figure 3.16.1 c**). The gross physical clinical symptoms of the murine mastitis were supported by histopathologic section that evaluates mammary gland tissue damage (e.g., mammary alveolus thickening, hyperemia, and edema) and extent of inflammatory cell infiltration (i.e., stained leukocyte cells). For example, under hematoxylin-eosin (HE) staining, the MCM group had broken lobules of the mammary gland, damaged acini, and destroyed epithelial cells, with influx of inflammatory cells including macrophages, neutrophils, and blood cells detected in the mammary lobule (**Figure 3.16.1 d-e**); conversely, the MH group had no apparent pathological changes in the mammary tissues (**Figure 3.16.1 f**).

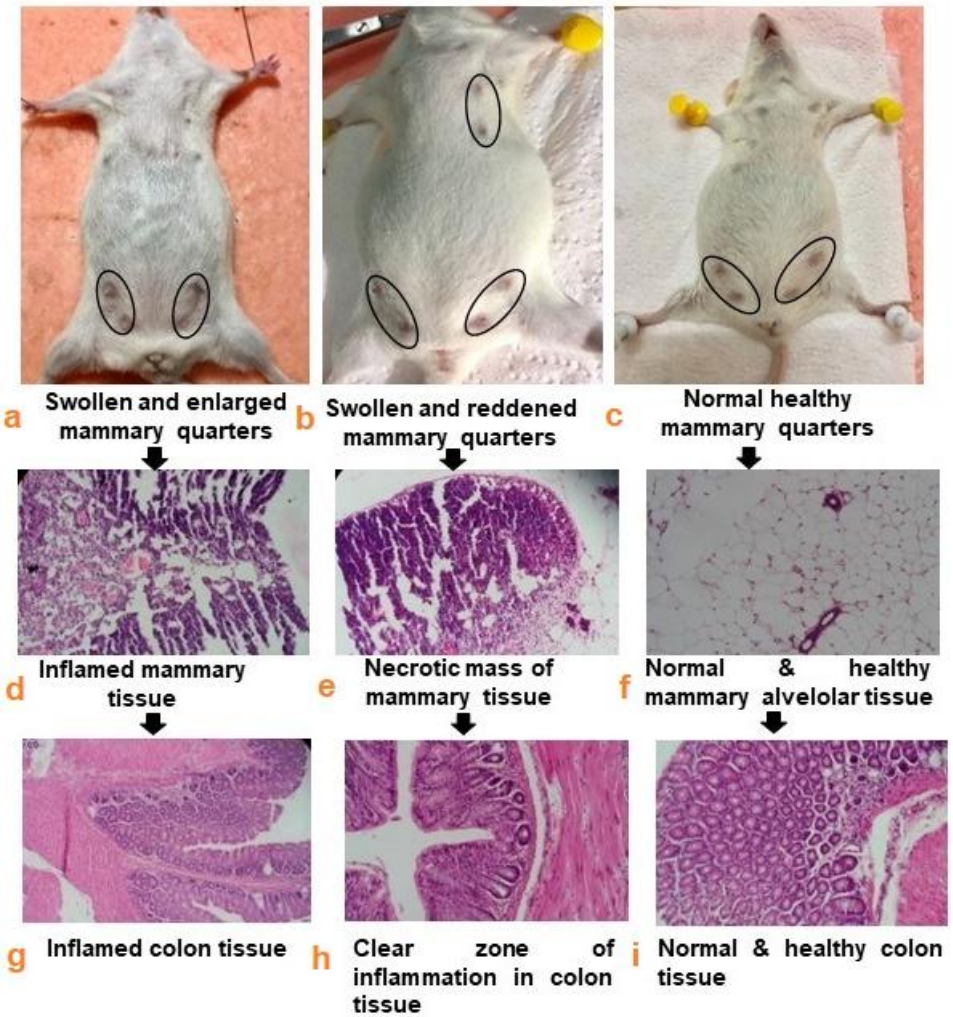


Figure 3.16.1: Histological analysis of mouse mammary and gut tissues after FMT. (a-c) Visible changes in mammary gland surface, where two abdominal mammary glands were swelling in the mastitis group of mice (MCM, a & b) on day 25 after FMT. Breast of mice are highlighted by black circles. (d-f) Representative photomicrographs of hematoxylin-eosin stained mammary gland tissue (× 200 magnification). (g-i) Representative photomicrographs of hematoxylin-eosin stained jejunum (× 100), and colon tissue (× 100).

The pathological section of murine intestinal and colon tissues also revealed severe inflammatory changes in the mucosal structure of the MCM group, and these hiotological changes were characterized by the necrosis of epithelial cells, extension of the sub-epithelial space, and structural damage of villi (**Figure 3.16.1 g-h**). However, the MH group mice exhibited normal intestinal mucosa with integral villi, and well-arranged villi structure (**Figure 3.16.1 i**). In fact, for each of the tissues tested, pathological grade of injury was significantly higher in group MCM than MH group (P = 0.037, Kruskal-Wallis test).

3.16. 2: *In vitro* cultural identification of mouse mastitis pathogens

To identify the bacteria associated with murine mastitis, the 24 fecal samples from MCM group were subjected to *in vitro* isolation and identification. A total of 140 isolates of six bacteria (46 isolates from *S. aureus*; 17 isolates from *E. coli*; 24 isolates from *Klebsiella* spp.; 15 isolates from *Enterobacter* spp.; 16 isolates from *Shigella* spp.; 22 isolates from *Bacillus* spp.) were obtained by standard selective microbiological and biochemical procedures. Mixed infections with one or more of *S. aureus*, *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Shigella* spp., and *Bacillus* spp. were common and none of the samples were negative for growth. The identified bacteria associated with murine mastitis (data represented as percent and number of isolates as well) has been illustrated in **Figure 3.16.2**. Biochemical tests revealed that *S. aureus* was the major pathogen (32.86%), followed by *Klebsiella* spp. (17.14%), *Bacillus* spp. (15.71%), *E. coli* (12.14%), *Shigella* spp. (11.43%), and *Enterobacter* spp. (10.72%). Four isolates which were otherwise characteristic of *E. coli* were negative by the indole test. The culture-based and biochemical identification of the bacteria was further validated by metagenome sequencing.

Figure 3.16.2: *In vitro* cultural identification of clinical mastitis pathogens. Using selective culture media and biochemical tests, we identified six selected pathogens (*S. aureus*, *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Shigella* spp., and *Bacillus* spp.) in mastitic mouse fecal samples.

3.16.3 Microbiome composition in mouse mastitis

The WMS of twelve mouse fecal samples after fecal transplant revealed association of diverse groups of microbes in murine mastitis as identified through MG-RAST analysis tools. We observed significant (P=0.012, Kruskal–Wallis test) changes in both microbiome composition and their relative abundances between MCM and MH metagenome groups after 25 days of FMT. The MCM mouse fecal samples had higher number of microbial taxa than health mice fecal samples. At the domain level, bacteria were the most abundant community, with an average abundance of 99.26%, followed by viruses (0.52%), and archaea (0.22%). The relative abundance of the identified microbiomes always remained higher in MCM fecal samples compared to that of MH samples, however, the abundance fluctuated more (CV = 431.22 vs 321.54).

The unique and shared distribution of microbial taxa found in mouse clinical mastitis (MCM) and mouse healthy (MH) fecal samples is shown in Venn diagrams (**Figure 3.16.3**). A total of 546 bacterial genera in MCM and 285 in MH metagenomes were detected in MG-RAST analysis, of which 52.20% genera were present in the both sample sets (**Figure 3.16.3 A**).

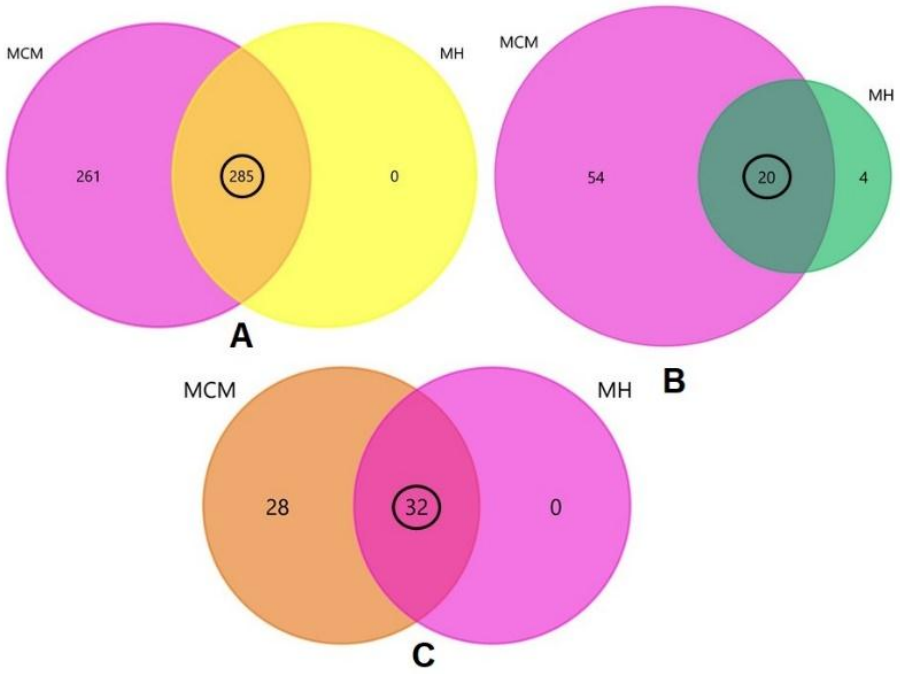


Figure 3.16.3: Taxonomic composition of mouse fecal microbiome. Venn diagrams representing the shared and distinct microbiome between mouse clinical mastitis (MCM) and mouse healthy (MH) fecal samples after fecal microbiota transplantation (FMT). (A) Venn diagram comparison of bacteria at genus level found in MG-RAST analysis, (B) Venn diagram comparison of viruses at genus level by MG RAST, and (C) Venn diagram comparison of archaea at genus level by MG RAST. Microbiomes shared between the conditions are indicated in black circles.

The MG-RAST tool simultaneously detected 78 and 60 genera of virus and archaea, respectively in theses samples. Among the detected viral genera, 74 and 24 genera were respectively found in MCM and MH samples, and of them 25.64% (20/78) genera shared between the conditions (**Figure 3.16.3 B**). In addition, 60 archaeal genera were detected in both metagenomes, of which 60 and 32 genera were found in MCM and MH samples, respectively, and among them 46.67% (28/60) archaeal genera had sole association with MCM (**Figure 3.16.3 C**).

3.16.4 Mouse mastitis-associated changes of in bacteriome composition at genus level

The metagenomic analyses successfully detected 546 bacterial genera among selected MCM samples of which 261 (47.80%) unique genera had unique association with mastitis. The most abundant bacterial genera in MCM fecal samples were *Lactobacillus* (26.37%), *Bifidobacterium* (18.19%), *Bacteroides* (16.05%), *Akkermansia* (9.89%), *Clostridium* (3.0%), *Prevotella* (2.68%), *Parabacteroides* (2.4%), *Staphylococcus* (2.2%), *Ruminococcus* (1.4%), *Desulfovibrio* (1.2%), *Porphyromonas* (1.17%), *Alistipes* (1.0%), and rest of the bacterial genera had less than 1.0% relative abundances (**Figure 3.16.4 A**). Conversely, *Bacteroides* (29.38%),

Chlorobium (14.36%), *Prevotella* (9.33%), *Clostridium* (6.91%), *Acinetobacter* (4.48%), *Xylella* (2.54%), *Helicobacter* (2.5%), *Burkholderia* (2.27%), *Haemophilus* (2.17%), *Ralstonia* (2.05%), *Histophilus* (1.34%), *Bradyrhizobium* (1.1%) were the predominantly abundant bacterial genera identified in MH fecal samples, and rest of the genera had < 1.0% relative abundances (**Figure 3.16.4 B**). However, 1.50% and 0.33% bacterial genera in MCM and MH mice fecal samples, respectively remained unclassified.

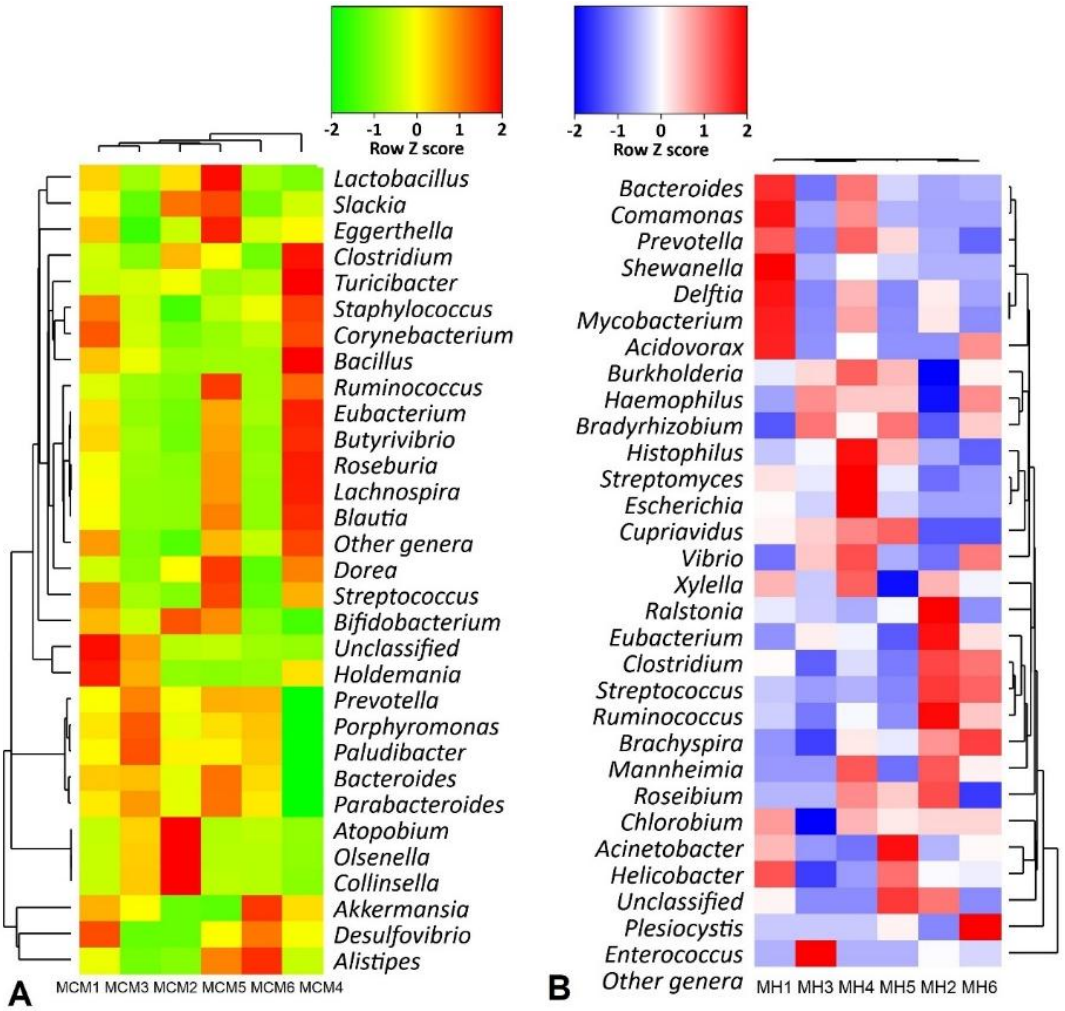


Figure 3.16.4: Difference between clinical mastitis (CM) and healthy (H) mice fecal microbiota at genus level after FMT. (A) The clustered heatmaps showing 31 top abundant bacterial genera found (A) in CM mice and (B) in H mice fecal samples. In both cases, top 30 bacterial genera having highest relative abundance were depicted in the heatmaps, and rest of the genera are denoted as "Other genera". The color codes indicate the presence and completeness of each genus, expressed as a value between -2 (lowest abundance) and 2 (highest abundance). The red color indicates the highest relative abundance, whilst white and yellow cells accounts for medium abundance and blue and green showing lowest abundance of the microbial genera.

3.16.5 Mouse mastitis-associated changes of viruses at genus level

Of the identified viral genera, 54 (69.23%) and 4 (5.12%) genera had sole association with mastitis (MCM) and healthy (MH) conditions of mice, respectively (**Figure 3.16.5**). In MCM metagenome, *Siphovirus* (53.30%) was identified as the single most abundant genera, and other abundant genera in this metagenome were *Myovirus* (11.90%), *Mastadenovirus* (10.79%), *SPO1-like viruses* (2.88%), *Caudovirus* (2.37%), *T4-like viruses* (1.72%), *Lambda-like viruses* (1.05%), *Podovirus* (0.72%), *Phi29-like viruses* (0.61%), and rest of the viral genera had less than 0.5% relative abundances. *Gammaretrovirus* (36.94%), *Ichnovirus* (22.46%), *Betaretrovirus* (14.94%), *Macavirus* (12.91%), *Rhadinovirus* (4.88%), *Simplexvirus* (1.58%), *Varicellovirus* (1.42%), *Whispovirus* (1.32%), *Chlorovirus* (0.86%), and *Mononegavirus* (0.61%) were the predominating viral genera in MH metagenome (**Figure 3.16.5**). The MCM metagenome however had 11.1% unclassified viral genera which were absent in MH metagenome. The rest of the viral genera in both metagenomes had < 0.5% relative abundances (**Figure 3.16.5**).

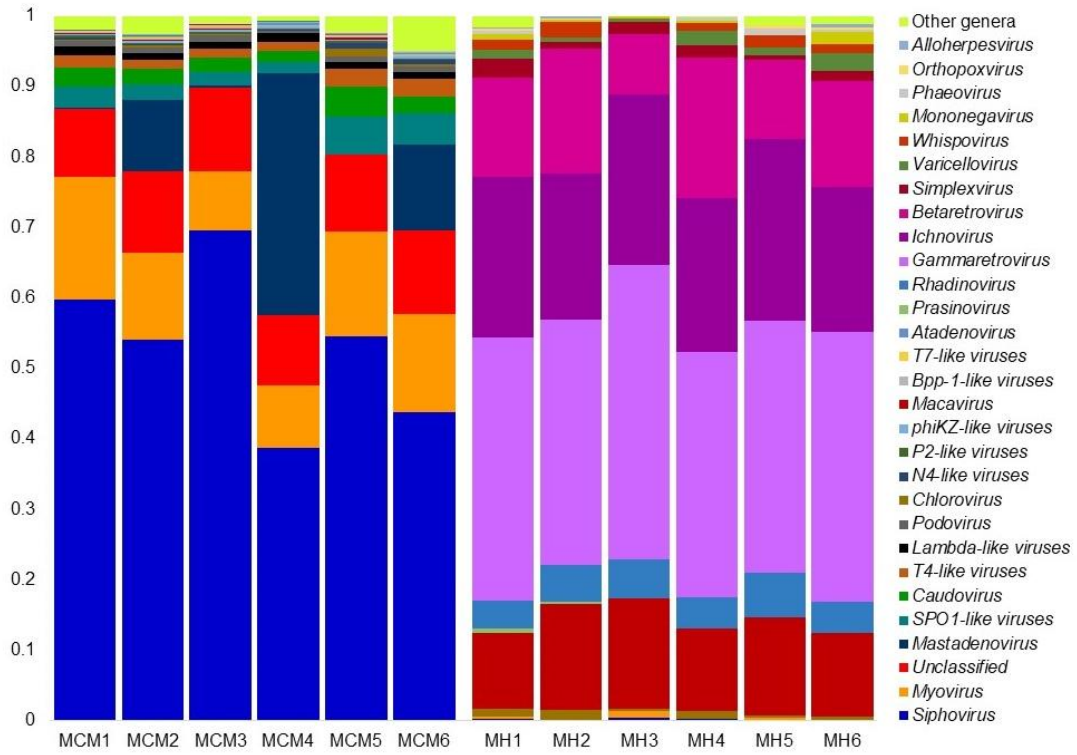


Figure 3.16.5: Taxonomic profile of 30 top abundant viral genera in mouse clinical mastitis (MCM) and mouse healthy (MH) fecal samples obtained by MG RAST analysis. The 29 most abundant viral genera are sorted by ascending order of the relative abundance in 12 samples, with the remaining genera grouped into the 'Other genera'. Each stacked bar plot represents the abundance of viruses in each sample of the corresponding category. Notable differences between the viral populations are those where the taxon is abundant in MCM samples and effectively undetected in the MH milk.

3.16.6 Mouse mastitis-associated changes of archaea at genus level

The relative abundance of the detected archaea genera also varied significantly (P = 0.002, Kruskal–Wallis test) in both MCM and MH metagenomes. Of the detected archaeal genera 53.34% (32/60) genera were found to be shared in both metagenomes. The predominating archaeal genera in MCM samples were *Methanosarcina* (13.71%), *Methanobrevibacter* (13.64%), *Methanococcus* (10.97%), *Methanocorpusculum* (6.35%), *Methanococcoides* (3.61%), *Pyrococcus* (3.55%), *Methanothermobacter* (3.42%), *Methanosphaera* (3.24%), *Methanocaldococcus* (3.21%), *Methanospirillum* (2.76%), *Thermococcus* (2.76%), *Methanoculleus* (2.57%), *Euryarchaeota* (2.45%, *Methanoregula* (2.30%), and *Archaeoglobus* (2.10%) (**Figure 3.16.6 A**).

Again, *Natrialba* (38.1%) was found as the most abundant archaeal genera in MH samples, and other top abundant genera in these samples were *Halorhabdus* (15.48%), *Methanosarcina* (8.34%), *Haloterrigena* (4.46%), *Methanobrevibacter*, *Halogeometricum* and *Haloquadratum* (2.78%, each), *Halorubrum* and *Halomicrobium* (2.39%, each) (**Figure 3.16.6 B**). The remaining of the archaeal genera in both metagenomes had less than 2.0% relative abundances (**Figure 3.16.6**).

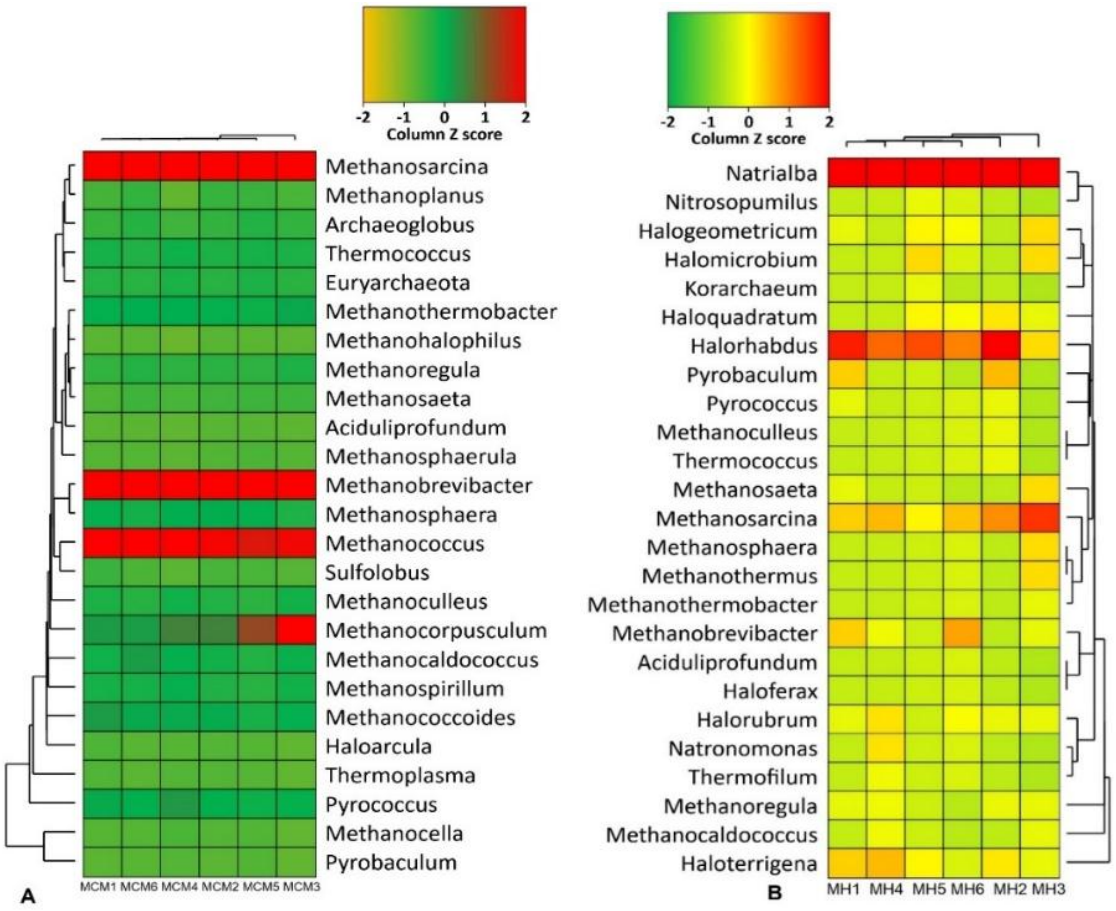
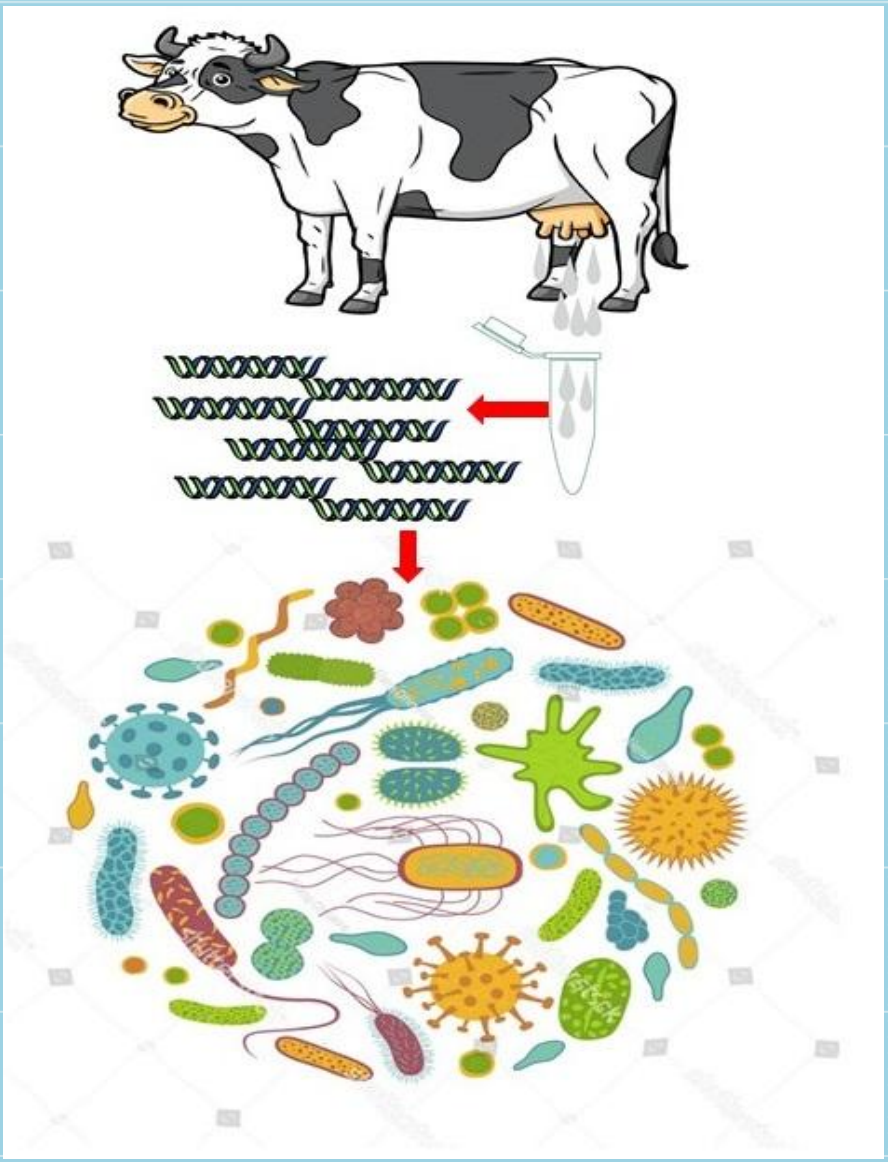


Figure 3.16.6: Taxonomic abundance of top 25 archaeal genera in mouse clinical mastitis (MCM) and mouse healthy (MH) fecal samples at genus level after FMT through MG-RAST analysis. (A). The clustered heatmap showing 25 top abundant archaea genera found in MCM fecal samples. (B). The clustered heatmap of the 25 top abundant archaeal genera found in MH mice feces. The color codes indicate the presence and completeness of each genus, expressed as a value (Z score) between -2 (lowest abundance) and 2 (highest abundance). The red color indicates the highest relative abundance, whereas green and yellow cells represent lowest abundance of the microbial genera.



Chapter 04:

Discussion

4. Discussion

The pathophysiology of bovine mastitis (CM, RCM, SCM) involves multiorganism (bacteria, archaea and viruses), and is considered as a host-pathogen interaction driven by host and microbial determinants (bacterial population mainly) (**Figure 1:1)**. This investigation demonstrated that the interaction between mastitis pathogens and the host immune system (host-pathogen interaction) is complex, and controlled by both the inherent genetic make-up of the host (e.g. innate and/or adaptive immune responses), and genomic make-up of the microbiomes involves with the disease (e.g. compositions, abundances, related VFGs, ARGs and metabolic functions) (Hoque et al., 2019; Hoque et al., 2020a, this thesis) (**Figure 1:1**). The mastitis associated microbiomes have developed various strategies to alter and evade host defenses in order to survive in the mammary glands or teats. The possible dynamic shift of microbiome compositions with the progress of different pathophysiological states of mastitis reported in this thesis/ dissertation, are determined by its favoring genomic potentials including virulence, antimicrobial resistance, and metabolic functions. Furthermore, Cows-to-mouse fecal microbiota transplantation (FMT) from mastitis (not from healthy) cows, to germ-free (GF) mice revealed that microbiome 'dysbiosis' could be one of the causes of mastitis, and might shed new light on rational selection of animal models to study and interpret host-tropism of bovine mastitis pathogens.

4.1 Mastitis-associated changes in microbiomes diversity and composition

The present study represents the first ever proof-of-concept to decipher dynamics changes in microbiome composition and abundances in CM, RCM, SCM and H-milk samples through the cutting-edge WMS technology. The findings generated from the WMS data are much higher in taxonomic resolution, and predicted protein functions, and are consistent with previous 16S rRNA partial gene-based studies (Oikonomou et al., 2014; Falentin et al., 2016; Patel et al., 2017; Cremonesi et al., 2018). The microbiome diversity (alpha and beta diversity) measures provided that microbial dysbiosis is closely linked to different stages of mastitis. We observed increased microbial diversity and species richness in CM, RCM, and SCM-microbiomes relative/compare to healthy-controls. Beta diversity also revealed a substantial microbial disparity between healthy-controls and mastitis-milk samples keeping the closest relationship of the CM-microbiomes to RCM followed by SCM,

and H-microbiomes (**Figure 3.2**). Regardless of higher taxonomic abundances, the bovine mastitis-associated microbiome remained inconsistent and fluctuates more within CM, RCM, and SCM-samples than those of H-milk (**Figure 3.3.2)**, agreeing with several recent studies (Oikonomou et al., 2014; Cremonesi et al., 2018; Hoque et al., 2019; Hoque et al., 2020a, this thesis). These variations could be related to host factors (genotype, immune status, parity, stage of lactation, position and shape of udder and teats), farm management practices (housing condition, milking hygiene, stress factors, nutritional management, antimicrobials use), and invading microorganisms (microbe-microbe response, virulence factors, antibiotic resistance, triggering of pro-and anti-inflammatory responses) (Derakhshani et al., 2018; Oikonomou et al., 2020).

4.1.1 Mastitis-associated shifts in bacteriome composition

In bovine mastitis, predominantly identified pathogens are bacteria (Oikonomou et al., 2014; Falentin et al., 2016; Cremonesi et al., 2018; Derakhshani et al., 2018), however, other concomitant microbial players like archaea and viruses (Bhatt et al., 2012; Hoque et al., 2019; Oikonomou et al., 2020; Hoque et al., 2020a, this thesis) could also be detected, highlighting the novel insights of microbiome characterizations through WMS compared to amplicon sequencing. Bovine mastitis milk microbiome was dominated by *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, and their relative abundance also varied across the samples of three mastitis categories. These results are in line with bovine (Bhatt et al., 2012; Hoque et al., 2019; Oikonomou et al., 2020; Hoque et al., 2020a, this thesis), bubaline (Catozzi et al., 2017), murine (Ma et al., 2018), and human (Patel et al., 2017) mastitis microbiome results reported earlier. Previously, *Proteobacteria* and *Firmicutes* were reported as the major phyla in bovine SCM (Bhatt et al., 2012) supporting our current findings. During the pathophysiology of mastitis, mammary epithelial cells reduce their capacity to beta-oxidation which alters the composition of indigenous microbiome, reducing the number of anaerobic *Firmicutes*, and allowing the proliferation of different genera, species and/or strains belonging to *Proteobacteria* blooms (Oikonomou et al., 2014; Boix-Amorós et al., 2016; Rizzatti et al., 2017; Derakhshani et al., 2018). To date, around 50 bacterial genera have been reported in bovine mastitis milk through 16S rRNA-based metagenomics (Falentin et al., 2016; Cremonesi et al., 2018), while our current WMS study detected 314, 187, 66 and 272

bacterial genera in CM, RCM, SCM and H milk samples, respectively (**Figure 3.3.2 a-b**). This indicates that WMS cutting-edge technology has extraordinary discriminatory power of in identifying microbiomes over other technology such as 16S rRNA sequencing metagenome identification (Oniciuc et al., 2018; Hoque et al., 2019). The core bacterial genera associated with bovine CM such as *Acinetobacter*, *Pseudomonas*, *Shigella*, *Klebsiella*, *Escherichia*, *Enterobacter*, *Staphylococcus*, *Streptococcus*, *Bacillus*, *Pantoea*, *Shewanella*, *Ralstonia* etc. remained consistent with the metagenomic data regardless of the analytic tool (**Figure 3.4.2**). Though mastitis milk samples had relatively higher taxonomic composition and abundances, their abundance remained more inconsistent in different categories of mastitis corroborating several earlier findings (Bhatt et al., 2012; Oikonomou et al., 2014; Boix-Amorós et al., 2016; Hoque et al., 2019, this thesis).

Metagenomics (Oikonomou et al., 2014; Falentin et al., 2016; Catozzi et al., 2017; Derakhshani et al., 2018; Hoque et al., 2019), and sequence (Hoque et al., 2018; Yang et al., 2019) of mastitis pathogens provided overwhelming evidence that microbial traits, for instance, virulence, antibiotic resistance, biofilm formations and metabolic potentials which are linked with pathogenicity of the pathogens/ or microbiome involves in pathogenesis are linked with strain-specific genomic characteristics (Egilmez et al., 2018; Segata, 2018; Nayfach et al., 2019). We demonstrated that microbial dysbiosis in different types of mastitis resulted in the depletion of beneficial microbes and enrichment of the opportunistic pathogens. The presence of few predominating bacterial species in the four metagenomes (particularly in RCM and SCM) suggests that the crucial differences might also be occurring at the strain-level. Furthermore, most of the species identified in each sample of the corresponding metagenome were represented by a single strain (**Figure 3.4.3)**. In this study, different strains of *Acinetobacter*, *Psedomonas*, *Escherichia*, *Aeromonas*, *Klebsiella*, *Shigella*, *Enterobacter*, *Salmonella*, *Pantoea*, *Citrobacter*, *Shewanella*, *Lactococcus*, and *Cronobacter* were predominantly abundant in CM and RCM milk samples **(Figure 3.4.4**), and routinely colonize the mammary tissue or quarters, and manifest different episodes of mastitis (Oikonomou et al., 2014; Gomes et al., 2016; Catozzi et al., 2017; Hoque et al., 2019, this thesis). On the other hand, the SCM samples were predominated by different strains of *Lactococcus*, *Chryseobacterium*, *Ralstonia*, *Serratia*, *Riemerella*, *Streptococcus*, and *Pedobacter* corroborating the findings of several earlier reports (Bhatt et al., 2012; Oikonomou et al., 2014).

Remarkably, 67.19% previously unreported bacterial strains were found in mastitis microbiomes, most of which are facultative and/or opportunistic in nature (**Figure 3.3.1 b, Table 3**). Moreover, mastitis (CM, RCM and SCM) metagenomes were enriched with different strains of soil or environmental bacteria (*Micromonospora*, *Escherichia*, *Klebsiella*, *Pantoea* and *Kluyvera*), animal skin, teat apices or oral cavity bacteria (*Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Corynebacterium*, *Enterococcus*, *Lactobacillus*, *Micrococcus* and *Aerococcus*), and gut or rumen bacteria (*Campylobacter*, *Catenibacterium*, *Aeromonas* and *Anaerobutyricum*) (**Figure 3.4.4**). These opportunistic microbiomes interfere with metabolism, host defense, and immune development to producing acute (CM), subacute (SCM), and long-standing (RCM) udder infections (Maga et al., 2013; Gomes et al., 2016; Hoque et al., 2019, this thesis).

Our present findings could support the potential existence of endogenous entero-mammary pathway through this axis the gut or rumen microbiomes migrate to the mammary gland to manifests different episodes of mastitis (Gomes et al., 2016; Hoque et al., 2019, this thesis). The healthy-milk microbiome was also dominated by different strains of environmental, gut, and animal skin originating microbes. Though the pathogenic mechanisms of these commensal microbes are largely unknown, they can cause opportunistic infections in the mammary glands and/or quarters with or without varying degrees of clinical episodes by producing different virulence factors particularly in immunocompromised hosts (Thompson-Crispi et al., 2014; Klaas and Zadoks, 2018; Hoque et al., 2019, this thesis).

We found significant differences in taxonomic diversity and abundances among the CM microbiomes of four dairy breeds. The crossbred Holstein Friesian (XHF) cows suffering from CM had higher microbial diversity at strain-level, and a significant proportion of the microbiota found to be shared with that of the other three breeds (local zebu, LZ; Sahiwal, SW; red Chattogram cattle, RCC) (**Figure 3.5 a-b**). Consistent with the results of earlier studies (Gonzalez-Recio et al., 2018; Li et al., 2019), the taxonomic profile of the CM microbiomes found in four cattle breeds were dominated by phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Fusobacteria*. This breed specific variation in taxonomic richness and diversity of the microbiome, especially in XHF and LZ cows, could associate with their increased disease resistance or immune response (Curone et al., 2018; Gonzalez-Recio et al., 2018), and rumen microbial features (e.g., taxa, diversity indices, functional

categories, and genes) (Li et al., 2019).

4.1.2 Mastitis-associated changes of archaeal and viral fraction of microbiomes

The present state of the art WMS approach also provided an opportunity for investigating the integrated cross-kingdom interactions of 'multi-microbiomes'– traditionally neglected components of the milk microbiomes; archaea and viruses, in modulating mastitis susceptibility. Unlike the bacteria, the diversity, composition, and the relative abundances of archaea and viruses remained much lower in the study metagenomes. Even though the role of these accompanying microbes in the pathophysiology of bovine mastitis has not been understood within the frames of a typical host-pathogen interaction, these taxa could act as opportunistic pathogens during the pathological changes occur in the mammary glands created by bacterial pathogens.

The archaeal fraction of the microbiome is mostly represented by the methanogenic and thermophilic genera of *Euryarchaeota* in CM and H-milk, and halophilic archaeal genera in SCM and RCM-metagenomes (**Figure 3.3.2 c, e** and **Figure 3.6.1**). Furthermore, a higher abundance of *Haloquadratum* and *Natronomonas* in SCM and RCM-milk may possess some accessory capsular switching genes for their adaptation to environmental fluctuations like bacterial pathogens to causing different magnitudes of disease (Lurie-Weinberger and Gophna, 2015). The virome analyses revealed that most of the detected viral genera belonged to the orders Caudovirales, Ortervirales and Herpesvirales, which consists of the three families of tailed bacterial viruses (**Figure 3.3.2 d, e** and **Figure 3.6.2**). The Ortervirales order, which includes five major families such as *Belpaoviridae* (new), *Caulimoviridae*, *Metaviridae*, *Pseudoviridae*, and *Retroviridae* comprises disease-causing viruses in humans and animals, and therefore, necessitate to their correct diagnostic identification and epidemiologic study (Loeffelholz and Fenwick, 2019). The host range of Caudovirales is very broad, and includes all major bacterial phyla found in bovine milk: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. We also found the association of different genera (*Macavirus* and *Rhadinovirus*) of Herpesvirales with relatively higher abundances in mastitis metagenomes inferring their association with pathogenesis of mammary gland (Shkoporov and Hill, 2019).

4.2 The *in vitro* identification of mastitis-associated pathogens

The present WMS-based metagenome study revealed different species/strains

of *Acinetobacter*, *Pseudomonas*, *Escherichia*, *Shigella*, *Klebsiella*, *Salmonella*, *Enterobacter*, *Shewanella*, *Pantoea*, *Staphylococcus*, *Streptococcus*, *Lactococcus*, *Cronobacter*, *Citrobacter*, *Chryseobacterium*, *Ralstonia*, *Serratia*, *Aeromonas*, *Riemerella*, and *Pedobacter* (**Figures 3.4.2-3.4.3**). Several earlier studies reported that *Staphylococcus*, *Escherichia*, *Enterococcus*, *Pseudomonas*, *Micromonospora*, *Klebsiella*, *Enterobacter*, *Shigella*, *Bacillus*, *Streptococcus*, *Acinetobacter*, *Corynebacterium*, *Lactobacillus*, and *Lactococcus* genera are the predominating etiology of bovine mastitis (especially CM and SCM) (Bhatt et al., 2012; Oikonomou et al., 2014; Abebe et al., 2016; Falentin et al., 2016; Catozzi et al., 2017; Curone et al., 2018; Cheng et al., 2019). Based on our WMS results, and previous reports of bovine mastitis, we selected *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Bacillus* spp., and *Shigella* spp. (**Figure 3.7** and **Figure 3.8)** for *in vitro* study. These *in vitro* findings are supported by the cutting-edge WMS-based mastitis microbiome identification of the present study, and also corroborate with the results of many of the earlier studies (Abebe et al., 2016; Reyes-Jara et al., 2016; Hoque et al., 2018; Cheng et al., 2019).

We also found that the prevalence of CM pathogens varies in different geographic regions keeping *S. aureus*, *Klebsiella* spp., and *Bacillus* spp. as the most frequently identified CM pathogens in the coastal-based intensive farms of Chattogram division. However, the distribution of CM pathogens also varied in different districts of the central region (CR) of the country where *Staphylococcus aureus* identified as the chief etiology, and *Shigella* spp. as the least detected CM pathogen. Therefore, these results showed that the prevalence of CM-pathogens could vary according to geographical locations and farming (semi-intensive, intensive and free-range grazing) systems (Reyes-Jara et al., 2016). These differences may imply that the etiology of bovine mastitis in Bangladesh could be related to types of feeding, farm locations and types, and antimicrobial uses for treatment or other factors as have been described in other countries (Preethirani et al., 2015; Reyes-Jara et al., 2016; Cheng et al., 2019).

4.3 Functional genomics of mastitis-associated microbiomes

4.3.1 Virulence factors-associated genes (VFGs) in mastitis metagenomes

In the present study, the mastitis-causing pathogens harbored a wide range of VFGs that enable them to overcome host defense, immune-mediated colonization of

the udder (Gomes et al., 2016; Derakhshani et al., 2018), and subsequent mammary gland pathogenesis by sensing metabolites derived from the microbiota (Fleitas et al., 2019). Notable that, the CM and RCM- microbiomes possessed VFGs associated with innate immune responses (*ABZJ*), nutrient uptake and/or regulation (*pvd*L), motility/chemotaxis (*chp*A), adhesion and invasion (*mot*B/*omp*A), biofilm formation (BF) and adherence (*pilJ* and *pvd*A), flagellum assembly (*flh*A), two-component regulatory system (*gac*S, *gac*A, *crc*), intracellular multiplication (*icmF1*), master regulator of BF (*bfmR*), and quorum sensing/antibiotics susceptibility altering (*pvd*Q and *pvd*H) (**Figure 3.9 b**). In addition, the SCM microbiomes had higher abundance of VFGs coding for bacteriocins production and cytotoxic activity (*EcSMS35*), capsular and fimbrial proteins (*cps*B, *stg*C), lipid biosynthesis (*rfb*), adhesin proteins (*upa*G/*eha*G), flagellum biosynthesis, assembly, motility and invasion (**Figure 3.9 b**). Conversely, the H milk microbiomes harbored higher number of capsular genes (*ABZJ*), genes required for survival in competition (*hcp.2*), pili and flagella expression/adherence (*rpo*N, *fle*Q, *fle*N), bacterial chemotaxis (*omp*A/*mot*A), alginate biosynthesis and BF. The underlying mechanisms for microbial colonization in the udder modulated by these VFGs is not well established. However, these VFGs can enable the mastitis-associated microbiomes to overcome immune-mediated colonization of the udder ecosystem, suppress regrowth of the resident commensal microbiota, and inclusion of a diverse range of opportunistic pathogens (Gomes et al., 2016; Derakhshani et al., 2018) with the progression and recurrence of the disease. Commensals can resist colonization of exogenous pathogens and inhibit overgrowth of indigenous opportunists via several mechanisms, including metabolic competition, and thus, partial recovery of commensal bacteria following CM and antimicrobial therapy may facilitate both development of new CM and recurrence of persistent infections, the RCM (Thomason et al., 2017; Derakhshani et al., 2018; Fleitas et al., 2019).

4.3.2 Resistomes composition in mastitis-associated microbiomes

Bovine milk microbiomes are a wide source of resistance to antibiotic and toxic compounds (RATC) genes. This study revealed that all of the samples in both mastitis (CM, RCM, SCM) and healthy milk metagenomes harbored RATC genes (**Figure 3.10.1**) indicating their wide and indiscriminate use in Bangladeshi dairy farms. However, most of the resistant genes in RATC functional groups remained

predominantly higher in RCM and CM milk microbes. The RATC genes detected in this study are of particular interest because there is concern that the use of this class of antibiotics or metals in veterinary medicine, particularly for food animals, may contribute to the development of resistance to this class of antimicrobial options in human (Hu et al., 2014).

The WMS data from this study provides important insights into the resistance potentials in mastitis-associated microbiomes. The results of the present study are concordant with multidrug resistant (MDR) bacteria reported elsewhere from the milk of clinically infected cows (Curone et al., 2018; Cheng et al., 2019), buffalo cows (Preethirani et al., 2015) and humans (Patel et al., 2017; Baron et al., 2018). The findings of the present study showed that multidrug resistance to efflux pumps (MREP), *Cme*ABC operon, *mdt*ABCD cluster, *Bla*R1 family, methicillin resistance in *Staphylococcus* (MRS), resistance to fluoroquinolones (RFL), and multiple metals resistance to CZCR and AR are the predominantly abundant RATC functional groups (**Figure 3.10.1**) in CM microbiomes suggesting that bovine CM milk microbiome constitutes a good reservoir ARGs (Baron et al., 2018; Hoque et al., 2019). Therefore, MDR to efflux pumps and multiple heavy metals resistance represented ubiquitous resistance mechanisms among CM microbiomes, which might be associated with unethical overuse of antibiotics in dairy animals and extensive application of toxic chemicals and metals in agricultural use (Reyes-Jara et al., 2016; Tiwari et al., 2017; Vaidya et al., 2017). The study also demonstrated that resistome is not significantly correlated with different dairy breeds (**Figure 3.10.2)**, and further investigations should be carried-out to evaluate the actual influence of breed specific bacteria on cow mammary gland diseases. The resistomes profile of the six selected CM bacteria also varied significantly both in RATC composition and relative abundances (**Figure 3.10.3**).

We found different homologues for antibiotic resistant genes (ARGs) product belonged to different protein families among the microbiome of the four metagenomes. Within the mastitis-microbiomes, the composition and diversity of ARGs varied greatly which might be associated with the differences in microbiome signature in the corresponding metagenomes, their genetic diversity, and selective pressure for the maintenance of ARGs (**Figure 3.11** and **Table 5)** in different states of mastitis (Escudeiro et al., 2019). β-Lactamases, the major resistance determinant for

β-lactam antibiotics in Gram-negative bacteria, remained with extended-spectrum both in mastitis and healthy milk microbiomes (**Figure 3.11 b**). They are sometimes augmented in mastitis causing microbiomes by additional resistance mechanisms, such as impermeability or efflux. The tetracyclines resistant genes (*tet*, *tetA*) were found only in mastitis-pathogens with apparently higher abundance in SCM-microbiomes, and this finding is in line with Nobrega et al. (2018). Aminoglycosides (*aph*), trimethoprim (*dfr*A), florfenicol (*flo*R) and sulfonamide (*sul*)-resistant genes were predominantly abundant in CM microbiomes (**Figure 3.11 c**). Higher number and classes of ARGs including macrolides, fosfomycin, and quinolones resistance genes in the RCM samples might be resulted from their infrequent use in CM, and subsequent treatment failure. The possible mechanisms for the detected ARGs include enzymatic inactivation, prevention of antibiotic-bindings to the targets, catalytic activity, folate pathway antagonist-attributed resistances, and efflux pump and/or system (**Figure 3.11 d)** conferring resistance to a wide range of antibiotics (Card et al., 2013; Fitzpatrick and Walsh, 2016; Escudeiro et al., 2019). These ARGs can easily spread by their harboring bacteria throughout different dairy environments because of wind and runoff waters, climate change, human activities, and contact with wild animals, in particular, migratory ones (Escudeiro et al., 2019).

The *in vitro* antibiogram assays revealed that the selected CM pathogens were resistant to multiple antibiotics (MDR) corroborating the high prevalence of multidrug resistant genes in the metagenomics sequences. This finding of high MDR patterns for CM pathogens are in line with many of previous studies on bovine mastitis (Preethirani et al., 2015; Cheng et al., 2019). Despite having higher prevalence of multiple metal and toxic compounds resistant genes in the metagenomics sequences, the *in vitro* resistance assay of heavy metals (Cr, Co, Cu, Zn and Ni) showed sensitivity against the tested CM pathogens.

Biofilm formation is an important virulence factor that may result in recurrent or persistent udder infections, and treatment failure through increased resistance to antibiotics and protection against host defenses. A large number of biofilm forming food spoilage and/or pathogenic bacteria including *Enterococcus faecalis*, *Enterobacter* spp., *Pseudomonas* spp., *Klebsiella* spp., *S. aureus*, *E. coli*, *B. cereus* and so on have already been isolated and identified (Singh et al., 2017; Vaidya et al., 2017) from from dairy niches which supports our current findings.

4.3.3 Metabolic functional changes in mastitis-associated microbiomes

The metabolomics analysis of the present study revealed several important predicted metabolic functions that altered in their abundances among CM, RCM, SCM and healthy-milk microbiomes as also reported previously in lactating cows (Cremonesi et al., 2018; Hoque et al., 2019, this thesis), women (Patel et al., 2017), and mice (Ma et al., 2018). We found that similar metabolic features identified in the same KEGG pathway or SEED subsystem varied in different categories of mastitis (CM, RCM and SCM) (**Figure 3.15.1** and **Figure 3.15.2)** suggesting their possible association in the early colonization and disease progression (Derakhshani et al., 2018; Jamali et al., 2018). Several recent studies have shown that bacterial metabolites are involved in modulating immune functions of their host (Round and Mazmanian, 2009; Zeng et al., 2017). The mastitis-associated microbiomes harbored a higher number of genes for nutrient transport, and carbohydrate metabolism through TCA-cycle (Turnbaugh et al., 2009; Li et al., 2018). Moreover, increased benzoate degradation by different strains of *Acinetobacter* and *Klebsiella* through TCA-cycle might promote bacterial growth and virulence factors expression during mammary gland pathogenesis (Li et al., 2014). The methyl-accepting chemotaxis proteins (MCP) or genes (*tar*, *tsr* and *tap*) act through transmembrane receptors that mediate chemotactic response, and cell-to-cell communication in certain bacteria, such as *Salmonella* and *E. coli* (Grebe and Stock, 1998). The methyl-accepting transmembrane chemotaxis genes or MCPs were upregulated in RCM and CM-associated pathogens suggesting their role in cell-to-cell communication, early phase attachment to or entry into the udder tissues, and virulence regulation (Li et al., 2014). The aerotaxis receptor gene was overexpressed in H milk microbiomes, which is thought to sense internal redox states, and mediate bacterial movement towards optimal oxygen conditions or mediate maximal growth of bacteria (Li et al., 2014; Gomes et al., 2016). The global regulating unit BarA-UvrY (*Sir*A)-two-component regulatory system is well conserved in many bacterial species (γ-Proteobacteria in particular), and regulate the production of extracellular factors (exoenzymes or toxins), quorum-sensing, motility, and diverse metabolic functions (Binnenkade and Lassak, 2011; Argov et al., 2019). In many mastitis-causing microbes, BarA/UvrY is important for regulation and coordination of pathogenicity and group behaviors (Binnenkade and Lassak, 2011; Argov et al.,

2019). The CM microbiome however were enriched in abundance of genes coding for autoinducer-2 (AI-2) transport and processing, pathogenicity islands, *Yjg*K cluster-linked to biofilm formation (BF), and *Tyr*R associated virulence (**Figure 3.15.2**). These genes are widely found among Gram-positive and Gram-negative bacteria, and associated with a diverse array of virulence mechanisms for mammary gland pathogenesis, ranging from secretion systems to BF. The altered metabolic pathways such as transport and processing, pathogenicity islands, and BF (**Figure 3.15.2**) in mastitis-pathogens are associated with a diverse array of virulence mechanisms for mammary gland pathogenesis (Gomes et al., 2016). Bacterial BF is a strain-specific or genetically-linked trait that can trigger mammary gland pathogenesis by promoting the phagocyte release, proliferation of reactive oxygen and nitrogen species, and transfer of antibiotic resistance (Escudeiro et al., 2019; Hoque et al., 2020a, this thesis). Moreover, biofilms adhesins, glutathione non-redox reactions, regulation of oxidative stress and transposable elements play an important role in many chronic bacterial infections (Gomes et al., 2016; Fleitas et al., 2019; Kelly et al., 2019; Saleh et al., 2019), and our current findings are in line with this statement since the RCM microbiomes had relatively overexpression of these genes.

4.4. Proposed mechanisms of mastitis progression

Bovine mastitis, a multifaceted devastating disease, which is initiated primarily with the bacterial infections under immunosuppression or stress conditions (when the cow suffers from a severe negative energy balance at the onset, in-and-around lactation, and other environmental stress e.g. heat stress), and progress to different magnitudes of pathogenesis (Thompson-Crispi et al., 2014; Derakhshani et al., 2018). During this progression of bacterial mastitis, bacterial viruses (phages or bacteriophages) jump into, and reach the site of inflammation in the mammary tissues (due to increased intestinal permeability and transcytosis), and triggers pathogenic pathways by the lysis of macrophages (Tetz and Tetz, 2018; Argov et al., 2019). This phagocytic macrophage storming further aggravates the pathogenesis, and creates a micro-aerobic/anaerobic condition which ultimately favors the archaeal growth (Lurie-Weinberger and Gophna, 2015; Ruiz et al., 2019). These secondary pathogens (phages and archaea) follow the similar virulence mechanisms of bacterial pathogens, they replicate in the immune and epithelial cells of the udder and/or milk ducts, and make a way for opportunistic pathogens (pathobionts), already present in milk or gain

secondary access to the mammary gland to produce more severe and prolonged mastitis (Egilmez et al., 2018). Furthermore, co-occurrence different virulence factors (VFGs), resistomes (ARGs, RATC, biofilm-forming and quorum-sensing genes), and altered metabolic pathways might boost-up the microbiomes to manifest different magnitude of pathogenesis in the mammary glands or teats producing different forms of mastitis. Though our data favors this mechanism of bovine mastitis with altered genomic features of microbiomes, still it needs further verifications of identifying microbiomes dynamics with the manifestation pathogenic episodes in mastitis progressions (**Figure 4.2**).

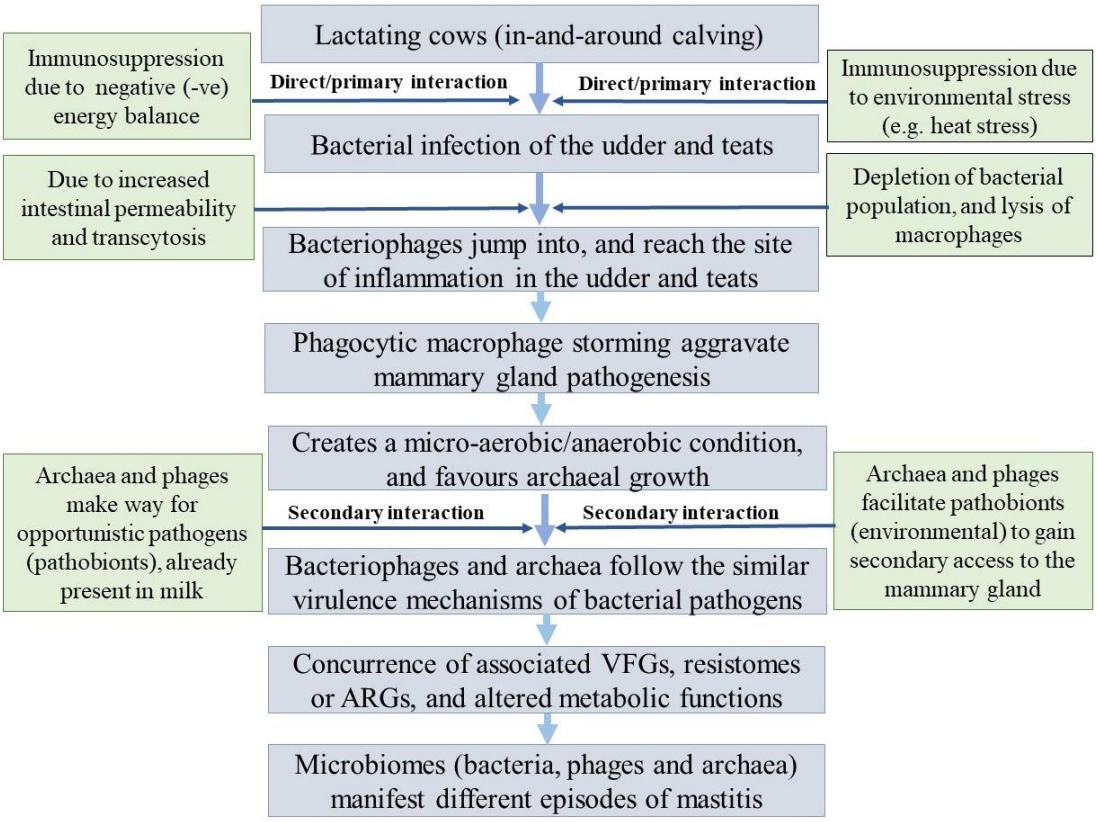


Figure 4.4 Microbiomes concept of bovine mastitis pathogenesis. Direct or primary interaction (host-pathogen) of bacteria under immunosuppression or stress conditions (when the cow suffers from a severe negative energy balance at the onset, in-and-around lactation, and other environmental stress e.g. heat stress). Indirect or secondary host-pathogen interaction with bacteriophages and archaea causing alteration of harmful microorganisms (secondary access of the opportunistic pathogens either already present in the milk or from environment of the cows). This combined association of microbiomes (bacteria, phages and archaea) triggers different magnitude of mammary gland pathogenesis, and manifests different categories of mastitis (e.g. CM, RCM or SCM).

4.5 The *in vivo* cows-to-mouse fecal microbiota transplantation (FMT)

The post-FMT inflammatory responses between two mouse groups revealed

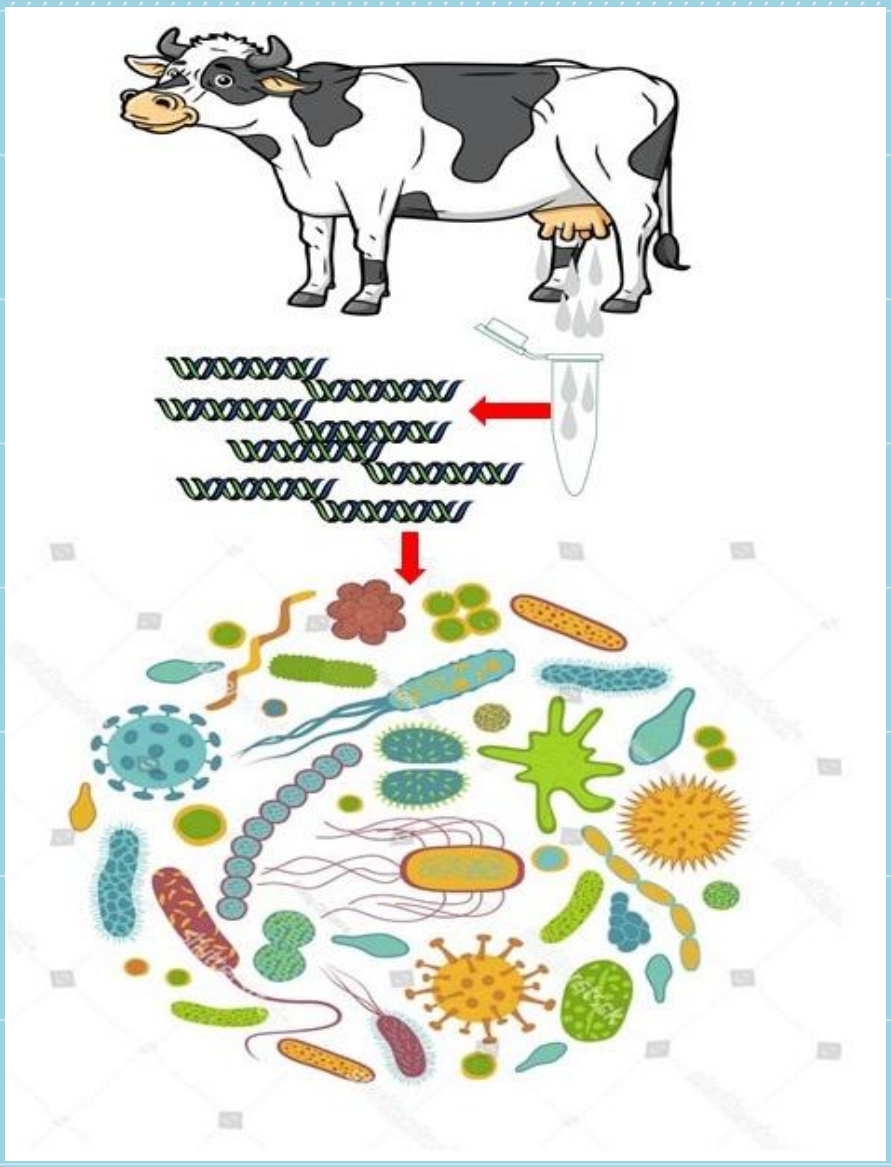
that fecal microbiota from CM cows produced typical inflammatory (in mammary gland, jejunum and colon) responses in mouse clinical mastitis (MCM) group, but not in mouse healthy (MH) control group (**Figure 3.16.1)**.The observed histopathological changes included the mammary gland tissue damage with thickened, hyperemic and edematous mammary alveoli, clear zone of inflammation, and extent of inflammatory cell infiltration with stained leukocytes as also reported earlier (Li et al., 2013; Ma et al., 2018).

The FMT also revealed microbiome diversity differences between MCM and MH groups. The composition of gut microbiota changes rapidly in mice prior to weaning. Microbiota in post-parturient MCM mice were found to be less diverse than that of MH mice, and this finding is supported by several previous studies on gut microbiome shifts different conditions (Lundberg et al., 2017). After initial colonization by FMT, the gut microbiomes of pregnant mice shift toward a high-diversity composition (**Figure 3.16.3**). While some differences in the microbiota were associated with particular FMT group, individual differences on mouse also influenced the fecal bacterial community. This is reflected by the higher relative abundances of *Lactobacillus*, *Bifidobacterium*, *Akkermansia*, *Parabacteroides*, *Staphylococcus*, *Ruminococcus*, *Desulfovibrio*, *Porphyromonas*, and *Alistipes* genera in MCM metagenome compared to the MH group (**Figure 3.16.4 A**). These fundings were further supported by the inclusion of 47.80% unique or opportunistic bacterial genera in murine mastitis corroborating with several earlier studies (Ma et al., 2018; Leystra and Clapper, 2019). The experimented mice were caged individually, and the two groups of mice were physically separated into different germ-free isolators. Therefore, the possibility for an island effect among the microbiomes of each isolator was quite impossible, and could not contributed to the differences in the fecal microbiota between the groups of mice (Zhang et al., 2017; Ma et al., 2018). Therefore, restoration of microbial community in intestinal ecosystem such as the mastitis-associated pathogens identified in this study can potentially serve as an effective strategy for therapeutics development, and devising treatment regimens for bovine mastitis, which may deserve validation in additional bovine cohorts.

Although, bacteria were the predominant fecal microbiota both in MCM and MH metagenomes, however, this *in vivo* study of cows-to mouse FMT marks an additional step towards identifying the co-occurrence of viruses and archaea as

symbiotic network. These secondary pathogens (archaea and viruses) had positive correlation with murine mastitis. The MCM metagenome had sole association of 69.23% and 46.66% viral and archaeal genera, respectively suggesting that these microbes can cease the opportunity during the pathophysiological changes in the mammary glands created by bacteria, as also reported in bovine mastitis (Hoque et al., 2019). Though, the full spectrum of outcomes from these virus-archaea-mice interactions, whether it altered host mammary gland physiology remained as a mystery, it is hypothesized that virus and archaea might follow the exact mechanisms of bacterial pathogens producing murine mastitis.

Finally, the ability to reiterate key physiological and microbiome features of bovine mastitis in germ-free mice via FMT which can shift microbiome diversity and composition nearly all lactating mammals, may promote mastitis study as a new research model to study the co-evolution of fecal and/or gut microbiome, mammalian genomes, and inflammatory diseases. The key advantageous features of the mouse mastitis model include, e.g., relative ease of disease symptom measurement, short time span of disease onset and progression, and ability to intervene disease development.

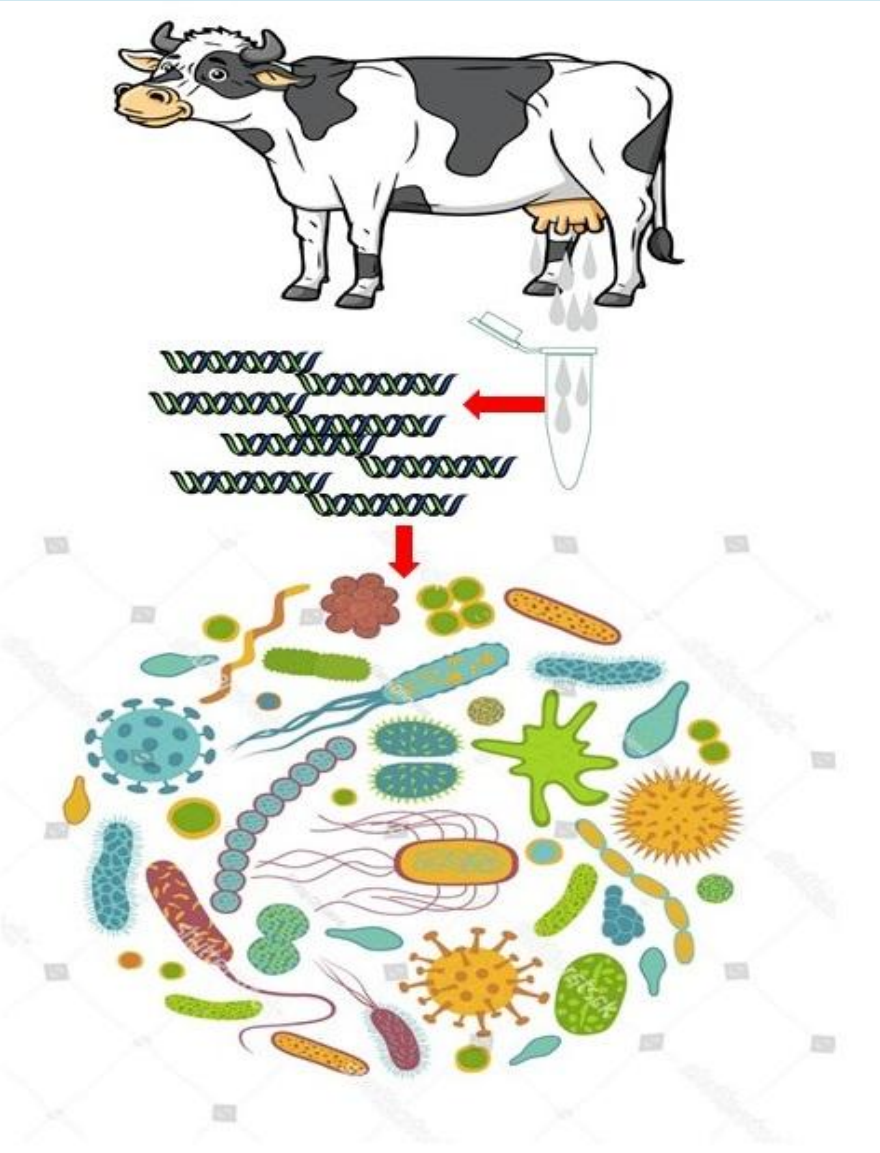


Chapter 05:

Conclusion

5. Conclusions

The shotgun metagenome sequencing of bovine mastitis (CM, RCM, SCM) and healthy milk samples identified distinct shifts in microbial diversity with significantly altered microbial communities, and predicted genomic features in mastitis and healthy cows. We revealed that microbial dysbiosis associated with different states of mastitis resulted in depletion of beneficial microbes, and enrichment of opportunistic pathogens. Our results suggested that concomitant presence of archaeal and viral fraction of microbiomes along with predominating bacteriomes in bovine milk, and co-occurrence of associated VFGs, resistomes and/or ARGs might be associated with the magnitude of pathogenesis in different types of mastitis. The ubiquitous presence of multidrug (efflux pumps mainly) and multiple metal (e.g., cobalt, zinc, cadmium, arsenic, chromium) resistance genes in mastitis pathogens may protect bacteria from the antibacterial effects of antimicrobials extruding them out of cells, and therefore persistence of the disease. Several predicted metabolic functional pathways differed between mastitis and healthy-controls, possibly reflecting metabolic changes associated with mastitis pathogenesis. Genes coding for bacterial flagellar movement and chemotaxis, regulation and cell signaling, oxidative stress, immune diseases, biofilm-formation, and quorum-sensing can be a great benefit to bacteria against the host's immune system. Therefore, an accurate and timely identification of mastitis-associated pathogens, and analyses of their VFGs, resistomes, ARGs and metabolic functional potentials through cutting-edge WMS technology will reach a point to develop microbiome-based diagnostics for this economically important disease. Thereby, the development of effective, safe and economical treatment regimens for bovine mastitis and sustainable dairying. Furthermore, our demonstration on microbiomes dysbiosis in mouse model after cow-to-mouse FMT might shed new light on rational selection of animal models to explore the role of microbiomes in clinical, subclinical and recurrent disease (mastitis) biology. Although, the baseline data presented here are promising, further studies are recommended using a larger sample size, and with the inclusion of gut/rumen microbiome sampling in addition to the milk samples for direct testing of microbial transfer across this axis to confirm the dysbiosis of microbiomes and associated genomic features.



Chapter 06:

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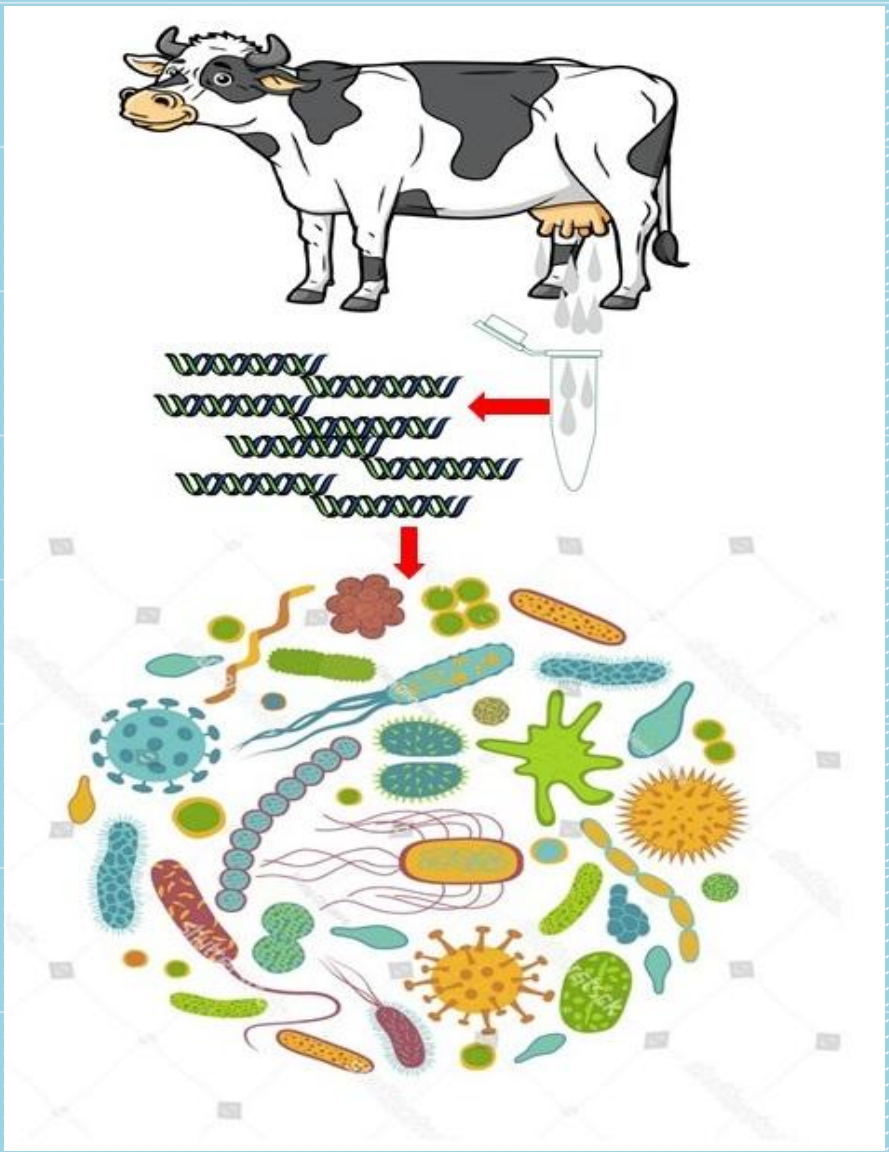
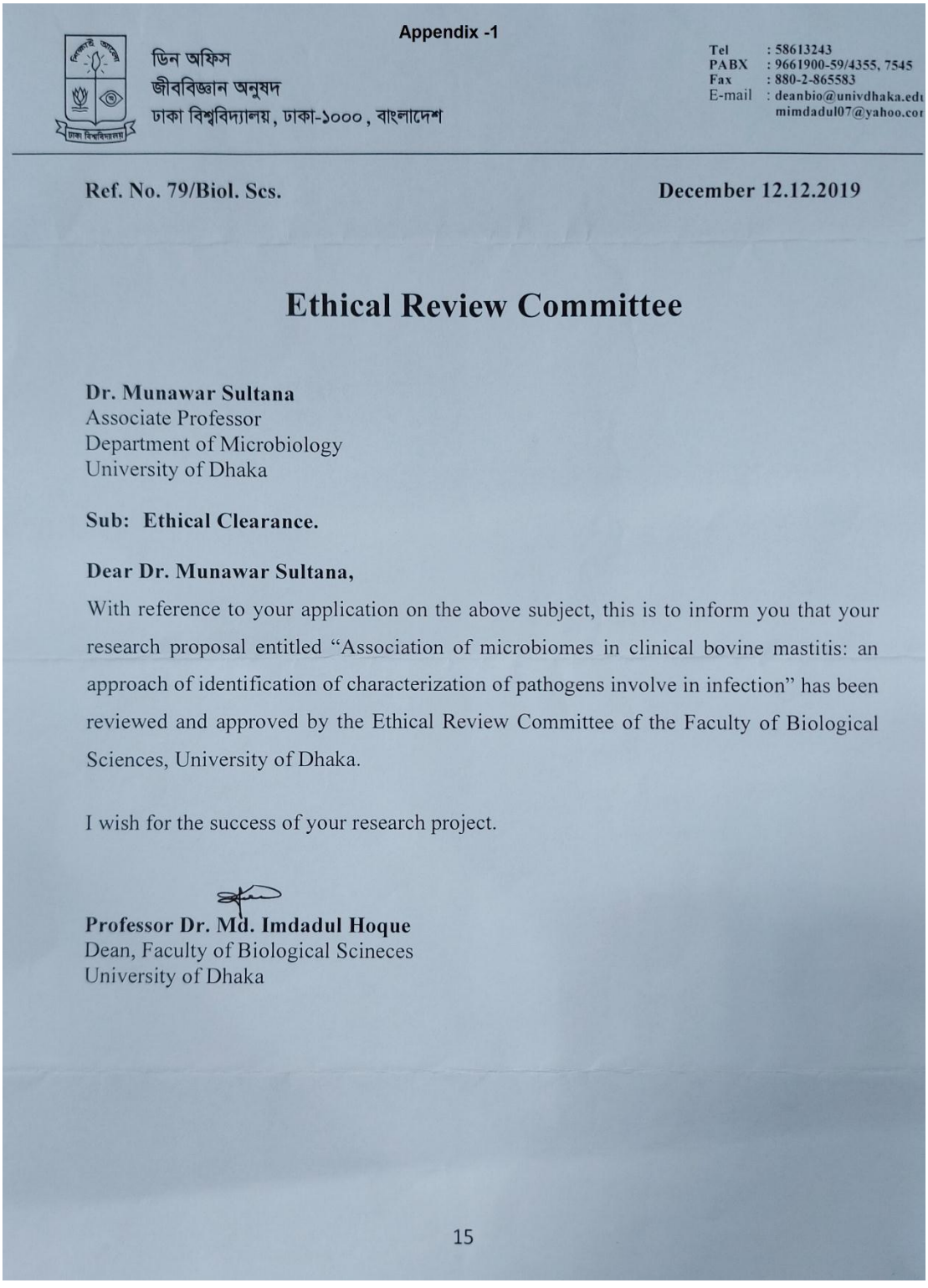


Appendices

Appendix-I



Appendix -1

ডিন অফিস
জীববিজ্ঞান অনুষদ
ঢাকা বিশ্ববিদ্যালয়, ঢাকা-১০০০, বাংলাদেশ

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Ref. No. 79/Biol. Scs. December 12.12.2019

Ethical Review Committee

Dr. Munawar Sultana
Associate Professor
Department of Microbiology
University of Dhaka

Sub: Ethical Clearance.

Dear Dr. Munawar Sultana,

With reference to your application on the above subject, this is to inform you that your research proposal entitled "Association of microbiomes in clinical bovine mastitis: an approach of identification of characterization of pathogens involve in infection" has been reviewed and approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka.

I wish for the success of your research project.

Professor Dr. Md. Imdadul Hoque
Dean, Faculty of Biological Scineces
University of Dhaka

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Appendix-II

Questionnaire for Epidemiological analysis and characterization of microbiome associated with bovine mastitis.

Farm related data		
Farm ID:	Farmer's name:	
Village:	Upazila:	District:
Farmer's Training: Yes/No	If yes- specify:	
Herd size:	i. Lactating cow: ii. Dry cow: iii. Pregnant heifer: iv. Non pregnant heifer: v. Calves (≤ 1yr):	
Housing information:	No. of shed: 1/2/3/4 Age of shed (yrs): 1= 2= 3= 4= Floor area (ft): Length: Width:	
Ventilation:	1= Natural (Shed 1/2/3/4) 2= Fan system (Shed 1/2/3/4) 3= Both (Shed 1/2/3/4)	
Shed gets sunlight:	Sufficient/ Insufficient/No	
Floor Cleaning:	Daily/Weekly	
Floor component:	i. Concrete (Shed 1/2/3/4) ii. Brick block without cementing (Shed 1/2/3/4) iii. Brick block with cementing (Shed 1/2/3/4) iv. Soil only (Shed 1/2/3/4)	
Floor condition:	i. Dry and clean (Shed 1/2/3/4) ii. Wet and clean (Shed 1/2/3/4) iii. Dry and soiled with dung (Shed 1/2/3/4) iv. Wet and soiled with dung (Shed 1/2/3/4)	
Distance between the shed and the manure pit (ft):	1= 2= 3= 4=	
Manure cleaning:	Once/ twice/ thrice daily	
Floor cleaning by:	Water/ antiseptic solution	
Cow's bath practice:	Bathed: yes/no, if yes…..times daily/weekly/monthly Bath water: Tube well/tap/pond/river water	
Milking practice		
Frequency of milking/day:	…….. times	
Milking by:	Hand/Machine	
Milking by whom:	Self/Labor/Milking man (Gosh/Goala)	
Same person milking daily:	Yes/No	
Complete milking:	Yes/No	
Whether calves allowed to suck after milking:	Yes/No	
Cows are milked sequentially:	Yes/No	
Milking hygiene practice		
Wash or wipes udder and teat before milking:	Yes/No, If yes- antiseptic solution/water	

Milking men's hand wash before milking:	Yes/No		
Teat dipping during milking with antiseptic :	Yes/No, If yes-Before/after/both, Names of dipping agent-		
Udder spray:	Yes/No, If yes- antiseptic solution/water		
Fore milk discarded:	Yes/No		
Mastitis related information			
Total number of mastitis affected cows last year:			
Total number of lactating cows last year:			
Lactating cows' information			
Cow's ID:	Name/Identification:		Breed:
Age (yr):	Body wt (Kg):	BCS:	Lactation stage:
Calving date:		Lactation No:	Milk production: ……L/day
Last puerperium			
1= Normal	2= Vaginal prolapsed	3= Uterine prolapse	
4=Milk fever	5= Retained placenta	6= Dystocia	
7= Other abnormalities (specify):			
Udder cleanliness			
1= Clean udder and teat	2= Clean udder and dirty teat		
3= Dry udder and clean teat	4= Dirty udder and teat		
History of previous mastitis:	Yes/No		
Udder and milk examination			
Udder position:	Pre-inguinal / inguinal / post-inguinal		
Udder shape:	Pendulous /Round (bag) / Bowl / Bottle		
Teat shape:	Conical / Funnel / Cylindrical /Platform		
Udder lesions:	Yes/No If yes-please specify:		
Teat lesions:	Yes/No If yes- please specify:		
Supernumerary teat:	Present/absent, If present- please specify:		
California Mastitis Test (CMT) score			
Right front: Left front: Right rear: Left rear:	- = Negative: Mixture remains fluid, no sediment or slime T= Trace: Mixture becomes slimy 1= Weak: Mixture form distinct sediment 2= Distinct: Mixture thickens immediately; tends to form a jelly 3= Strong: Mixture forms jelly; pick in center, even after stop of swirling		
Udder palpation score			
Right front: Left front: Right rear: Left rear:	1= No swelling and pain 2= Ventral quarter swollen 3= Generalized swollen quarter 4= Swollen and painful quarter and udder		
Milk score			
Right front: Left front: Right rear: Left rear:	1=Normal 2=Flakes/clots otherwise normal milk 3=Little/ no milk, moderately abnormal color 4= No normal milk ;watery, serum or blood		

Appendix-III

Sequence data information: A total of 20 whole metagenome sequences (including 5 clinical mastitis, 6 recurrent clinical mastitis, 4 subclinical mastitis and 5 healthy) were used in this study. The WMS data have been submitted in the National Center for Biotechnology Information (NCBI) under the **BioProject PRJNA529353**. The accessions numbers of sequences are given append below the Table.

The sequence data

Cow ID	Sample ID	Farm Location	Breeds	Accession Number
Cow 1	CM1	Gazipur	SW	SRR8881032
Cow 2	CM2	Gazipur	RCC	SRR8881035
Cow 3	CM3	Gazipur	LZ	SRR8881034
Cow 4	CM4	Gazipur	XHF	SRR8881037
Cow 5	CM5	Manikgonj	XHF	SRR8881029
Cow 6	RCM1	Gazipur	SW	SRR8881019
Cow 7	RCM2	Gazipur	RCC	SRR8881020
Cow 8	RCM3	Chattogram	RCC	SRR8881021
Cow 9	RCM4	Dhaka	XHF	SRR8881016
Cow 10	RCM5	Manikgonj	SW	SRR8881017
Cow 11	RCM6	Sirajgonj	XHF	SRR8881041
Cow 12	SCM1	Chattogram	SW	SRR8881040
Cow 13	SCM2	Dhaka	XHF	SRR8881039
Cow 14	SCM3	Gazipur	SW	SRR8881038
Cow 15	SCM4	Manikgonj	RCC	SRR8881045
Cow 16	H1	Chattogram	XHF	SRR8881042
Cow 17	H2	Chattogram	RCC	SRR8881047
Cow 18	H3	Sirajgonj	XHF	SRR8881046
Cow 19	H4	Dhaka	SW	SRR8881026
Cow 20	H5	Gazipur	XHF	SRR8881027

of the ribosomal gene (16S rRNA) have also been deposited in the NCBI database under accession numbers: **MN 620423–MN 620430**.

Appendix-IV

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SCIENTIFIC REPORTS
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OPEN

Metagenomic deep sequencing reveals association of microbiome signature with functional biases in bovine mastitis

Received: 3 May 2019
Accepted: 14 August 2019
Published online: 19 September 2019

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Milk microbiomes significantly influence the pathophysiology of bovine mastitis. To assess the association between microbiome diversity and bovine mastitis, we compared the microbiome of clinical mastitis (CM, n = 14) and healthy (H, n = 7) milk samples through deep whole metagenome sequencing (WMS). A total of 483.38 million reads generated from both metagenomes were analyzed through PathoScope (PS) and MG-RAST (MR), and mapped to 380 bacterial, 56 archaeal, and 39 viral genomes. We observed distinct shifts and differences in abundance between the microbiome of CM and H milk in phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria* with an inclusion of 68.04% previously unreported and/or opportunistic strains in CM milk. PS identified 363 and 146 bacterial strains in CM and H milk samples respectively, and MR detected 356 and 251 bacterial genera respectively. Of the identified taxa, 29.51% of strains and 63.80% of genera were shared between both metagenomes. Additionally, 14 archaeal and 14 viral genera were found to be solely associated with CM. Functional annotation of metagenomic sequences identified several metabolic pathways related to bacterial colonization, proliferation, chemotaxis and invasion, immune-diseases, oxidative stress, regulation and cell signaling, phage and prophases, antibiotic and heavy metal resistance that might be associated with CM. Our WMS study provides conclusive data on milk microbiome diversity associated with bovine CM and its role in udder health.

Mastitis is one of the most prevalent diseases in the dairy industry with the highest clinical and economic significance worldwide[1]. The condition usually happens when pathogenic microbes enter the mammary gland, mostly by the disruption of the physical barriers of the mammary quarters, requiring prompt and appropriate host defenses to prevent colonization and subsequent disease pathology[2]. Diverse groups of microbes are known to colonize the mammary quarters of cows and have evolved novel mechanisms that facilitate their proliferation, leading to clinical mastitis (CM). Despite knowledge of a few of these invading microbial groups, the etiology of bovine mastitis is continuously changing, with new microbial species identified as causing disease frequently. Additionally, although bacteria are the main cause of mastitis[3], other microbes like archaea, viruses, and fungi might be associated with the disease process[4] and should therefore be investigated as well. During the progression of the mastitis, dysbiosis of the milk microbiome can occur with the increase of opportunistic pathogenic bacteria and reduction of healthy commensal bacteria[5]. Until recently, investigations of the microbiome associated with bovine mastitis have been mostly restricted to individual pathogen isolation and characterization. The disease is caused by epidemiologically diverse groups of microorganisms and categorized into

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Appendix-V

frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 June 2020
doi: 10.3389/fmicb.2020.00860

Insights Into the Resistome of Bovine Clinical Mastitis Microbiome, a Key Factor in Disease Complication

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 15 December 2019
Accepted: 09 April 2020
Published: 03 June 2020

Citation:
Hoque MN, Istiaq A, Clement RA, Gibson KM, Saha O, Islam OK, Abir RA, Sultana M, Siddiki AZ, Crandall KA and Hossain MA (2020) Insights Into the Resistome of Bovine Clinical Mastitis Microbiome, a Key Factor in Disease Complication. Front. Microbiol. 11:860. doi: 10.3389/fmicb.2020.00860

Bovine clinical mastitis (CM) is one of the most prevalent diseases caused by a wide range of resident microbes. The emergence of antimicrobial resistance in CM bacteria is well-known, however, the genomic resistance composition (the resistome) at the microbiome-level is not well characterized. In this study, we applied whole metagenome sequencing (WMS) to characterize the resistome of the CM microbiome, focusing on antibiotics and metals resistance, biofilm formation (BF), and quorum sensing (QS) along with *in vitro* resistance assays of six selected pathogens isolated from the same CM samples. The WMS generated an average of 21.13 million reads (post-processing) from 25 CM samples that mapped to 519 bacterial strains, of which 30.06% were previously unreported. We found a significant (P = 0.001) association between the resistomes and microbiome composition with no association with cattle breed, despite significant differences in microbiome diversity among breeds. The *in vitro* investigation determined that 76.2% of six selected pathogens considered "biofilm formers" actually formed biofilms and were also highly resistant to tetracycline, doxycycline, nalidixic acid, ampicillin, and chloramphenicol and remained sensitive to metals (Cr, Co, Ni, Cu, Zn) at varying concentrations. We also found bacterial flagellar movement and chemotaxis, regulation and cell signaling, and oxidative stress to be significantly associated with the pathophysiology of CM. Thus, identifying CM microbiomes, and analyzing their resistomes and genomic potentials will help improve the optimization of therapeutic schemes involving antibiotics and/or metals usage in the prevention and control of bovine CM.

Keywords: whole metagenome sequencing, clinical mastitis, microbiome, resistome, *in vitro* resistance assays