Chapter 5: Final discussion
The work presented in this thesis characterize human milk microbiome associated with lactational mastitis using culture dependent and independent approach. It examines how bacterial dysbiosis alters microbiota composition and functional gene content during the course of subacute and acute mastitis. Moreover, it also examines antibacterial activity of three commonly available herbal plant extracts against bacteria isolated from mastitis milk. The key finding of the work along with future prospective and limitations are discussed below.

5.1 Overview and Conclusion:
Human milk provides important benefit to the mother-infant dyad and should be encouraged. However, infectious condition such as mastitis constitutes one of the major factor leading to undesirable weaning and thus depriving mother-infant pair from advantages of breastfeeding. Therefore lactational mastitis should be considered as public health issue.

Role of microbial dysbiosis in pathogenesis of mastitis has already been described; however, a definitive link is yet to be established. Till date only one study has profiled breast milk microbiota from mastitis suffering women using NGS techniques and that was using shotgun sequencing. So, we sought to determine culturable and unculturable bacterial diversity from breast milk of mastitis suffering women and compared them with healthy-controls. In addition, we checked antibacterial efficacy of three commonly available herbal plants against mastitis causing pathogens and used GC-MS analysis for identification of bioactive compound.

In overall, we collected a total of 50 human milk samples including, 16 subacute and 16 acute mastitis and 18 healthy-controls. We explored culturable bacterial diversity from subacute and acute mastitis samples and identified them using 16S Sanger sequencing. By culturing approach, we identified 30 bacterial species belonging to phyla Proteobacteria, Firmicutes and Actinobacteria from mastitis samples. While at genus level, Staphylococcus and Pseudomonas were the most prevalent genera, Brevundimonas,
Enterococcus, Micrococcus, Bacillus infrequently detected. Certain bacteria species such as Staphylococcus sp. (87.5% of mastitis samples), Staphylococcus aureus (75%), Pseudomonas aeruginosa (53.12%), Klebsiella pneumoniae (43.75%), and Brevundimonas diminuta (37.5%) were more often recovered from mastitis samples. Antibiogram study of 37 bacterial isolates against 19 different antibiotics proved chloramphenicol, gentamicin, ofloxacin and ciprofloxacin as most efficient antibiotics against mastitis pathogens.

Antibiotic susceptibility test indicated antimicrobial resistance acquired by different strains of bacteria isolated from mastitis samples. With this in mind, we evaluated antibacterial efficacy of three commonly available herbal plants (D. stramonium, F. racemosa and P. juliflora) extract as an alternative treatment to antibiotics against mastitis causing pathogens. Ethanolic and ethyl acetate extract of P. juliflora showed significant higher antimicrobial activity compared to other plant extracts. These were further confirmed by GC-MS analysis, in which we could identify different bioactive compound with reported antimicrobial activity.

We next determined comprehensive microbiome associated with subacute and acute mastitis cases and compared them with healthy-controls using amplicon sequencing approach. Subacute and acute mastitis women had significantly lower microbial diversity as determined by observed OTUs and Phylogenetic Diversity index. Unweighted and weighted UniFrac PCoA analysis of species level microbiota indicated distinct separation of healthy-controls from that of subacute and acute mastitis cases. Phylogenetic identification of human milk microbiota revealed that Proteobacteria and Firmicutes accounted for majority of bacterial sequences. At genus level, genera including, Aeromonas, Staphylococcus, Ralstonia, Klebsiella, Serratia, Enterococcus and Pseudomonas were significantly enriched in subacute and acute mastitis samples, while Acinetobacter, Ruminococcus, Clostridium, Faecalibacterium and Eubacterium were consistently depleted. Moreover, we observed that a group of 13 bacterial genera (Acinetobacter, Bacillus, Clostridia, Staphylococcus, Lactobacillus, Propionibacterium,
Corynebacterium, Pseudomonas, Paenibacillus, Prevotella, Ralstonia, Eubacterium and Ruminococcus), frequently identified in previous milk metagenomics study, constituted core microbiome and accounted for majority of bacterial sequences. Further analysis of our samples revealed positive aerotolerant odds ratio, indicating dramatic depletion of obligate anaerobes and enrichment of aerotolerant bacteria during the course of mastitis. Obligate anaerobes such as Ruminococcus, Clostridium, Eubacterium, Faecalibacterium, Veillonella, Pyramidobacter, Selenomonas, Hespellia, Ethanoligenens, Roseburia and Butyrivibrio drastically depleted in mastitis cases. Analysis of human milk microbiota at species level also indicated depletion of commensal prokaryotes and increased abundance of opportunistic pathogens during mastitis. In addition, predicted functional metagenomics identified several gene pathways related to bacterial proliferation and colonization (e.g. two-component system, bacterial secretion system and motility proteins) in subacute and acute mastitis samples.

This is a pilot scale study, with only 50 human milk samples collected and sequenced for identifying distinct microbial signature during subacute and acute mastitis. Nevertheless, the results present in this thesis are consistent with previous hypothesis that mastitis women have lower microbial diversity, increased abundance of opportunistic pathogens and depletion of commensal obligate anaerobes.

5.2 Future prospective:
Despite of recent advancement in molecular techniques, role of bacteria in lactational mastitis is still a debatable question. Several studies have pointed out crucial role of bacterial dysbiosis of mammary gland in aetiopathogenesis during mastitis, but the exact mechanism behind it is still unclear. To further investigate the hypothesis that bacterial dysbiosis during mastitis is the result of depletion of commensal obligate anaerobes, it will require more participants with hundreds of patients and controls. Little is known about how inflammatory environment during mastitis resulted in increased aerotolerant bacteria and depletion of obligate anaerobes. Previous study on child malnutrition has linked depletion of obligate anaerobes to increase gut redox potential in feces. Future studies targeting
mastitis microbiome should measure human milk redox potential and antioxidants present in it. Unravelling this linked between depletion of obligate anaerobes and increase redox potential will help us to understand role of milk microbiota in mastitis pathogenesis. Composition of human milk microbial community dramatically changes over time and influenced by age, geographical location and maternal diet. In such condition, longitudinal analysis of samples from individuals will be advantageous to better understand disease perturbation during mastitis.

Although 16S community profiling does not inform us what microbes are doing and what they are expressing, it is relatively affordable and widely used. Nevertheless, shotgun metagenomic sequencing provides clear and more accurate picture of functional gene content of organism and accurate species level classification. But large number of studies performed till date were only focused on describing microbial composition in healthy or disease state because shotgun sequencing technology inevitably requires higher cost. In addition to the functional gene content of microbiota, it is important to know what the active microbes are and what they are expressing during disease condition using metatranscriptomics approach. In overall, future studies dealing with human mastitis microbiome using high throughput sequencing should include combination of amplicon, shotgun and metatranscriptomics sequencing for better understanding of disease causing bacteria and their strategy.

At last, with bacteria contributing more genes than our own human genome, we need to better understand our second genome or virtual organ. The pursuit to comprehend pathogenesis of human mastitis requires more research on how underlying host genetics influences human milk microbiome during healthy and disease condition. Microbial profiling of human milk samples may distinguish individuals at high risk of developing mastitis, enabling either precaution mediations or early diagnosis. It is with high expectations that research presented in this thesis and future studies along these track improves better understanding of mastitis pathogenesis, which thusly prompt early diagnosis and treatment.