Induction of mastitis by cow-to-mouse fecal and milk microbiota transplantation causes microbiome dysbiosis and genomic functional perturbation in mice

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Abstract

Background: Mastitis pathogenesis involves a wide range of opportunistic and apparently resident microorganisms including bacteria, viruses and archaea. In dairy animals, microbes reside in the host, interact with environment and evade the host immune system, providing a potential for host-tropism to favor mastitis pathogenesis. To understand the host-tropism phenomena of bovine-tropic mastitis microbiomes, we developed a cow-to-mouse mastitis model.

Methods: A cow-to-mouse mastitis model was established by fecal microbiota transplantation (FMT) and milk microbiota transplantation (MMT) to pregnant mice to assess microbiome dysbiosis and genomic functional perturbations through shotgun whole metagenome sequencing (WMS) along with histopathological changes in mice mammary gland and colon tissues.

Results: The cow-to-mouse FMT and MMT from clinical mastitis (CM) cows induced mastitis syndromes in mice as evidenced by histopathological changes in mammary gland and colon tissues. The WMS of 24 samples including six milk (CM = 3, healthy; H = 3), six fecal (CM = 4, H = 2) samples from cows, and six fecal (CM = 4, H = 2) and six mammary tissue (CM = 3, H = 3) samples from mice generating 517.14 million reads (average: 21.55 million reads/sample) mapped to 2191 bacterial, 94 viral and 54 archaeal genomes. The Kruskal–Wallis test revealed significant differences (p = 0.009) in diversity, composition, and relative abundances in microbiomes between CM- and H-metagenomes. These differences in microbiome composition were mostly represented by Pseudomonas aeruginosa, Lactobacillus crispatus, Klebsiella oxytoca, Enterococcus faecalis, Pantoea dispersa in CM-cows (feces and milk), and Muribaculum spp., Duncaniella spp., Muribaculum intestinale, Bifidobacterium animalis, Escherichia coli, Staphylococcus aureus, Massilia oculi, Ralstonia pickettii in CM-mice (feces and mammary tissues). Different species of Clostridia, Bacteroida, Actinobacteria, Flavobacteria and Betaproteobacteria had a strong co-occurrence and positive correlation as the indicator species of murine mastitis. However, both CM cows and mice shared few mastitis-associated microbial taxa (1.14%) and functional pathways regardless of conservation of mastitis syndromes, indicating the higher discrepancy in mastitis-associated microbiomes among lactating mammals.

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Introduction

Mastitis is one of the most prevalent infectious diseases in the dairy animals worldwide. It has a negative impact on the agro-economy due to reduced milk production, early culling, and high therapeutic costs [1–4]. The disease is caused by a wide range of apparently resident and opportunistic microbes including bacteria, viruses, and archaea of variable origin, where the severity and outcome of the disease depends on the cross-talk between host and pathogen [5–8]. During mastitis, an imbalance of microbiomes occurs due to the inclusion of the pathogenic and opportunistic microorganisms in susceptible mammary glands. This invading encroachment is favoured by the compromised immune status of the host and the interactions between the opportunistic pathogens and the resident microbiota of the mammary gland [5, 7–11]. Depending on the host–pathogen interactions [12–14], bovine

Conclusions: We successfully induced mastitis by FMT and MMT that resulted in microbiome dysbiosis and genomic functional perturbations in mice. This study induced mastitis in a mouse model through FMT and MMT, which might be useful for further studies focused on pathogen(s) involved in mastitis, their cross-talk among themselves and the host.

Keywords: Cows, Mice, Mastitis, Feces, Milk, Mammary gland, Microbiomes, Transplantations, Dysbiosis

Graphical Abstract
that many of these microbes can migrate to the mammary gland. Therefore, it is very likely that the milk could be deep inside the upper parts of the mammary gland. Therefore, it is very likely that many of these microbes can migrate to the mammary gland from extra-mammary sites such as the gut, via the entero-mammary axis and/or the environment.

In the last decade, the advent of shotgun metagenomics and downstream bioinformatic analyses allow for robust surveys of host-microbiome interactions in dairy populations. A large body of literature suggests that disruption of gut microbiota homeostasis is linked to metabolic, immune, gastrointestinal, and mammary gland diseases. Previous studies demonstrated that changes in the gut microbiota can result in proliferation of specific pathogenic bacteria which can thereafter enter the mammary gland through the entero-mammary pathway.

The colonization of bovine gut microbes into germ free (GF) mouse model demonstrates the influence of bovine gut microbes on the development of mastitis. The usefulness of a mouse mastitis model as a complementary tool to investigate differences between bovine associated coagulase-negative Staphylococci (CNS) has already been established. Ma et al. recently reported that cow-to-mouse fecal microbiota transplantation can modulate intestinal microbiome dysbiosis, which was one cause of mastitis. In this study, we found that in addition to fecal microbiota, CM milk microbiota transplantation is one of the important causes of mastitis. Therefore, the cown-to-mouse mastitis milk microbiota transplantations (FMT and MMT) might be useful for further molecular biological studies on mastitis using the mouse model. Previous studies reported that humanized gnotobiotic mouse models have contributed to advancements in biomedicine, bridging the gap between human and animal gut pathophysiology.

In the pathophysiology of bovine mastitis, dysbiosis of the milk microbiomes occurs with an increase in opportunistic pathogens, and a reduction in healthy milk commensal microbes. The opportunistic pathogens of the gut use very efficient strategies to evade host defenses in order to colonize and invade mammary tissues. Therefore, damage occurs to mammary epithelial cells and disruption of the cow immune system causes clinical episodes of CM. Therefore, to investigate the host-tropism of bovine mastitis microbiomes using mouse model, this study attempted to explore whether dysbiosis of fecal and milk microbiota can lead to clinical episodes of mastitis in pregnant mice. In the present study, we induced mastitis in mice by transplantation of bovine fecal or milk microbiota from CM cows into GF pregnant mice and investigated the changes in the fecal and mammary tissue microbiota using a high-throughput WMS followed by metagenomic and metabolic functional analyses along with histopathological changes in mice mammary gland and colon tissues (Graphical abstract). Our study revealed that both CM cows and mastitis induced mice shared a limited number of microbial taxa and related genomic functional potentials. We also observed a high degree of discrepancy in the microbiomes in milk, mammary tissue and feces of the mastitis affected animals. The developed mouse model would be useful for identification of the primary pathogen(s) of bovine mastitis, and their molecular interactions with hosts that are critical for sustainable management of this economically important disease in the dairy industry worldwide.

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 entero-mammary axis to confirm the dysbiosis of microbiomes and associated genomic functional features.

Results

Fecal or milk microbiome transplantation from mastitis cows induced mastitis in GF mice

To elucidate whether CM-associated cow microbiota could develop mastitis syndrome in mice, fecal or milk microbiota from seven CM and five H cows were transplanted to 40 GF pregnant mice. The sample groups were- CCMF: cow clinical mastitis feces; HCF: healthy cow feces; CCMM: cow clinical mastitis milk; HCM: healthy cow milk; MCMF: mouse clinical mastitis feces; HMF: healthy mouse feces; MCMMT: mouse clinical mastitis
mammary tissue; and HMMT: healthy mouse mammary tissue (Additional file 1). Among the 40 recipient mice, 20 underwent to FMT and MMT from CM samples while the remaining 20 were treated with H cow fecal and milk microbiota (controls). By comparing the post-intervention (FMT and MMT) inflammatory responses among the four groups, we found that fecal microbiota from CM cows induced a higher incidence of mastitis (90.0%) in mice compared to milk microbiota (80.0%). However, clinical signs of CM were not apparent before 10 days-post challenges (i.e., before 27 days of gestation). Remarkably, none of the mice in the control groups (either H-cows FMT or MMT) developed mastitis syndromes. Comparison of murine post-microbiota transplantation inflammatory responses revealed that gut and milk microbiota from CM cows induced a much greater inflammatory (of mammary gland and colon) response than those from healthy cows. On mammary gland surface, severe gross pathological changes (e.g., swelling and congestion of mammary glands) that corresponded to CM were observed in the mastitis groups (CCMF and CCMM), however no pathological changes were visually apparent in healthy groups i.e., HCF or HCM (Fig. 1A). The gross syndrome of CM was further supported and confirmed by histopathological changes in the mammary and colon tissues. The CM-associated histopathological changes included damage of the mammary gland tissue (e.g., loss of architecture of alveoli, haemorrhages, involuted alveoli, degenerative changes in the epithelium of the alveoli and ducts, mammary alveolus thickening, hyperemia, and edema) and extent of inflammatory cell infiltration (i.e., infiltration of polymorphonuclear cells). For example, observable histopathological lesions under hematoxylin–eosin staining includes broken lobules of the mammary gland, damaged acinar structures, and destroyed epithelial cells, with inflammatory cells including macrophages, neutrophils, and blood cells detected in the mammary lobule, supporting connective tissue and lining of the epithelium (Fig. 1B); on the other hand, in control groups (HCF or HCM), no pathological changes were apparent. In addition, predominant changes in the colon tissues of the CM mice (Fig. 1C) included moderate to severe inflammatory cell infiltration into mucosa and submucosa, disorder in mucosal structure (epithelial necrosis, extension of the subepithelial space, and structural damage of villi). Conversely, the mice receiving H cows fecal and milk microbiota did not show such pathological changes in the colon tissues and exhibited normal intestinal mucosa with well-arranged villi structure (Fig. 1C). Moreover, the CM mice had significantly higher ($p=0.0013$, Kruskal–Wallis test) pathological grade of injury in their mammary gland and colon tissues than those of H mice (Fig. 1D). Generally, healthy mammary tissue possesses more fat vacuoles than mastitis udder [31], and thus, occurrence of mastitis was not affected by the lactation periods.

Microbiome structure and composition in gut, milk and mammary tissues differed in cows and mice

To test for differences in taxonomic diversity and structure of microbiomes, we analyzed the WMS data using an open-source cloud-based metagenomic mapping-based method of IDSeq [32]. We found significant differences in microbial α-diversity (i.e., within-sample diversity) estimated through observed species in each sample (i.e., richness), Chao1, Shannon, and Simpson indices in the CM and H samples regardless of the host. The species level α-diversity remained significantly higher in H control samples of both cows ($p=0.0079$, Kruskal–Wallis test) and mice ($p=0.0068$, Kruskal–Wallis test) compared to CM samples (Fig. 2A). The PCoA plot based on Bray–Curtis distances (Fig. 2B) and NMDS plot based on weighted UniFrac distances (Fig. 2C), showed significant microbial disparity between CM and H samples ($p=0.001$, Kruskal–Wallis test) and hosts (i.e., cows and mice) ($p=0.005$, Kruskal–Wallis test). The phylum-level microbiome composition also showed distinct differences ($p=0.002$, Kruskal–Wallis test) across the detected microbial domains (i.e., bacteria, archaea and viruses) (Fig. 2D).

At the domain level, bacteria were the most abundant (99.78%) microbial community followed by viruses (0.13%) and archaea (0.09%) (Additional file 2). In this study, we detected 1731 bacterial species including 1590 and 979 in CM and H cow fecal samples, respectively (Fig. 3A), of which 43.44% species had sole association with CM (Fig. 3A, Additional file 3). Similarly, 618 species of bacteria were identified in cows milk including 592 and 79 species in CM and H milk samples, respectively, and the CM samples had sole association of 87.22% species (Fig. 3B, Additional file 3). Conversely, 1065 species including 853 in CM and 721 in H fecal samples of mice were detected (Fig. 3C), and of them, the CM fecal samples had sole association of 32.30% species (Fig. 3C). Likewise, 115 species of bacteria were identified in mouse mammary tissue including 69 in CM and 77 in H tissues (Fig. 3D), and the CM mice mammary tissues had sole association of 33.04% species (Fig. 3D, Additional file 3). Comparing the bacterial taxa in CM and H sample in both hosts, we found that only 1.14% and 0.65% bacterial species shared across the CM (Fig. 3E) and H (Fig. 3F) samples, respectively.

In addition to bacterial fraction of the microbiomes, we detected 94 viral (Additional file 4), and 54 archaeal (Additional file 5) genera from both cows and mice (Additional file 3). By comparing these genera across the
In cow CM and H samples, we found 78 and 62 genera, respectively, and majority of these genera (75.0%) were found to be shared between CM and H samples. The milk samples of both CM and H cows harboured 29 viral genera (CM = 13 and H = 26) and, only 10.44% genera had sole association with CM. Likewise, 74 viral genera were detected in mice fecal samples (CM = 69, H = 57), of which 70.27% genera were shared.

**Fig. 1** Histopathological analysis of mouse gut and mammary tissues after microbiota transplantation. A Pathological changes in mammary gland surface, where different abdominal mammary glands were found as swollen and red in the mastitis (CM) group of mice on Day 27 after FMT (fecal microbiota transplantation) and MMT (milk microbiota transplantation). Mammary glands of mouse are highlighted by red circles. B Representative photomicrographs of mammary epithelial tissue after haematoxylin and eosin staining (×100 magnification). The lesion in the mammary epithelial cells is characterised by a central area of necrosis, broken lobules, damaged acinuses and destroyed epithelial cells, with with large numbers of inflammatory cells, predominantly neutrophils (dark bluish) and macrophages (red) (yellow arrows), in the mammary lobule, supporting connective tissue and lining of the epithelium. C Representative photomicrographs of colon (crypts, lamina propria, muscularis mucosae and submucosa) tissue after haematoxylin and eosin staining (×100 magnification). Histopathological changes in the colon tissue include moderate to severe inflammatory cell infiltration into mucosa and submucosa, disorder in mucosal structure: epithelial necrosis, extension of the subepithelial space, and structural damage of villi (yellow arrows). Scale bars: 100 μm and 50 μm. D The tissue injury scores in the mammary gland and colon. The X-axis represents the groups of samples (CM and H) while the Y-axis denotes the injury scores as measured through Chiu Scoring System (×100 magnification).
between the conditions. The mammary tissue of mice possessed only 24 viral genera (CM = 20, H = 21), and among these genera, 70.84% were shared between the conditions. In this study, we found that fecal samples from both cows and mice harboured 54 archaeal genera (CM = 54, H = 54, in each category), and of them, 100% of the genera were found to be shared between the conditions (CM and H) (Additional file 5). Moreover, 32 and 46 archaeal genera were detected in cow’s milk and mice mammary tissues, respectively. In this study, the fecal and milk samples of both CM and H cows were found to share 17 archaeal genera (Additional file 5) while the fecal and mammary tissue samples of both CM and H mice shared 16 archaeal genera (Additional file 5).

**Gut and milk bacteria of mastitis cows are distinct from those of healthy cows**

To test associations between microbiomes (gut and milk) and clinical mastitis (CM), fecal and milk microbiota from seven crossbred Holstein cows diagnosed with CM were compared to five physically similar, age-matched, crossbred Holstein cows that served as the control.
(Additional file 1; Materials and method). We found significant differences ($p = 0.009$, Kruskal–Wallis test) in the relative abundance of the bacterial species in fecal and milk samples of dairy cows. Among the detected bacterial species, *Pseudomonas aeruginosa* (21.0%), *Lactobacillus crispatus* (16.10%), *Klebsiella oxytoca* (10.34%), *Enterococcus faecalis* (10.0%), *Nocardia pseudobrasiliensis* (5.0%), *Lactobacillus vaginalis* (4.85%), *Clostridioides difficile* (4.23%), *Ralstonia insidiosa* (4.0%), *Bifidobacterium pseudolongum* (3.80%), *Mucobilaculum* sp. (2.32%), and *Duncaniella dubosi* (2.0%) were the top abundant species in the CM-fecal samples of cows (Fig. 3, Additional file 6). Conversely, *P. aeruginosa* (32.7%), *E. faecalis* (30.05%), *Lachnospiraceae bacterium* (3.03%), *Clostridiales bacterium* (2.28%), and *Phocaeicola dorei* (2.15%) had higher relative abundances in H-cow fecal samples compared to those of CM cows (Fig. 3, Additional file 6). Similarly, the CM-cow milk samples were dominated by *Pantoea dispersa* (24.29%), *K. oxytoca* (20.68%), *Actinoalloteichus* sp. (11.63%), *N. pseudobrasiliensis* (8.87%), *Staphylococcus aureus* (4.7%), *C. botulinum* (4.19%), *Acinetobacter baumannii* (4.0%), *Acinetobacter johnsonii* (3.5%), *K. pneumoniae* (3.44%) and *Escherichia coli* (3.02%). On the contrary, *P. aeruginosa* had the highest relative abundance (40.35%) in H-cow milk samples followed by *P. dispersa* (13.86%), *A. baumannii* (11.0%), *Prevotella melaninogenica* (10.0%), *Actinoalloteichus* sp. (5.25%), *K. oxytoca* (4.0%) and *N.
Pseudobrasiliensis (3.05%). The remaining bacterial species detected from the metagenomic data had relatively lower (<3.0%) abundances and remained mostly abundant in CM-associated fecal and milk samples of cows (Fig. 3, Additional file 6).

Cow-to-mouse FMT and MMT reveal distinct gut and mammary gland bacteria between mastitis and healthy mice

We further investigated whether cow-to-mouse FMT and MMT treatment could produce distinct disease outcomes among the challenged mice. The WMS of both fecal and mammary tissues obtained from seven-CM and five-H mice at Day 27 of gestation (Methods) showed distinct changes in both composition and relative abundances of bacteria at the species level. The CM-mice fecal samples had a higher number of bacterial species (n = 853) than H-mice fecal samples (n = 561). The CM-related fecal metagenome of mice (MCMF) was dominated by Muribaculum sp. (38.30%), Duncaniella sp. (10.17%), Muribaculum intestinale (9.61%), Bifidobacterium animalis (8.36%), D. dubosii (7.14%), E. faecalis (5.0%), Akkermansia muciniphila (4.42%), L. crispatus (2.87%), B. pseudolongum (2.15%) and Lactobacillus murinus (2.10%) (Fig. 3, Additional file 6). In contrast, the H-mice fecal metagenome (HMF) was dominated by Muribaculum sp. (26.74%), A. muciniphila (20.61%), Duncaniella sp. (7.89%), M. intestinale (6.82%), D. dubosii (4.71%), K. pneumoniae (3.5%), P. aeruginosa (3.2%), and E. faecalis (2.0%), and rest of the species detected in both groups had comparatively lower relative abundances than that of CM-mice (Fig. 4, Additional file 6). The mammary tissues of the challenged mice had a significantly lower number of bacterial species compared to fecal samples (115 vs. 1065). In the mammary tissue of CM mice (MCMMT), E. coli (42.48%) was identified as the single most prevalent species followed by S. aureus (9.7%), Massilia oculi (5.90%), Ralstonia pickettii (4.13%), Curtobacterium flaccumfaciens (3.84%), P. aeruginosa (2.9%), A. johnsonii (2.37%), A. junii (2.36%), and Cutibacterium acnes (2.06%) (Fig. 4). Conversely, the H mice mammary tissue metagenome (HMMT) was mostly dominated by Ralstonia pickettii (9.84%), S. aureus (9.53%), A. johnsonii (8.85%), P. aeruginosa (8.74%), Helicobacter cinaedi (7.04%), M. oculi (5.74%), A. junii (4.51), and E. coli (3.28%). The remaining species detected in both MCMMT and HMMT metagenomes had relatively lower (<3.0%) abundances (Fig. 4, Additional file 6).

Indicator and shared bacterial taxa in the mastitis and healthy mice metagenomes

The indicator species analysis identified 46 differentially abundant (IndVal values ≥0.6, p < 0.01) bacterial species in mouse CM (MCMF and MCMMT) and H (HMF and HMMT) metagenomes (Fig. 5). In this study, mice CM samples showed the highest number of differentially enriched species (including 24 species in MCMF samples and eight species in MCMMT samples). Among the indicator species identified, Paenibacillus durus, Stenotrophomonas maltophilia, Pontibacter russatus, Caproiciproducens sp. NJN-50, Pseudobutyrovibrio xylanivorans, Treponema brennaborensis, Capnocytophaga sputigena, Roseimicrobium sp. ORNL1, Christensenella massiliensis, and Blautia obeum were the top scoring (IndVal ≥0.8, p = 0.002) species in MCMF samples. Similarly, P. polymyxa, Niabella ginsenosidivorans, Hymenobacter sedentarius and several species that were classified at higher taxonomic ranking (IndVal ≥0.7, p = 0.03) were differentially abundant in the MCMMT samples. Remarkably, indicator species analysis confirmed that P. durus, S. maltophilia, P. russatus and Paenibacillus polymyxa were all good indicators of the murine CM (Fig. 5, Additional file 2). On the contrary, the H-mice samples had only seven differentially enriched bacterial species. The most abundant species (IndVal ≥0.78, p = 0.01) in the HMF samples were Bifidobacterium choerinum, Desulfovibrio sp. IOR2, and M. intestinale while Azorarcus olearius, Bacteroides sp. A1C1, B. uniformis and B. animalis were enriched (IndVal ≥0.79, p = 0.01) in HMMT samples (Fig. 5).

A microbial co-occurrence network was built based on correlations of relative abundance of the indicator species between mice-CM and -H samples. The network analysis (Fig. 6) presented 46 nodes and 1449 edges (significant positive correlations, r > 0.6 and Spearman’s corrected p = 0.001), all connected into one cluster with a clustering coefficient of 0.503. In the co-occurrence network, Clostridia had the highest number of edges (19.57%) followed by Bacteroidera (13.04%), Actinobacteria (10.87%), Flavobacteria (8.7%), Betaproteobacteria (8.7%), Gammaproteobacteria (6.52%), and Spirochaetia (6.52%), indicating strong co-occurrence among the species of these classes (Fig. 6). Pseudoclostridium thermosuccino genes and Pseudobutyrovibrio xylanivorans (phylum: Firmicutes, class: Clostridia) had the highest co-occurrence (Correlation coefficient: 0.55, p = 0.005, in each) showing positive correlation with 45 indicator species and had only a negative correlation with Azorarcus species. Likewise, Labilbaculum antarcticum (phylum: Bacteroidetes, class: Bacteroidia) was correlated with 25 bacterial species including P. russatus, Polynucleobacter necessaries, Treponema succinifaciens etc. and had no negative correlation with other indicator species (Fig. 6). In contrast, Azorarcus sp. and Lacrimispora sphenoides were mutually exclusive in the mouse habitats, and their abundance in
Fig. 4 Species-level taxonomic clustering of bacteria. The heatmap shows the hierarchical clustering of sample groups based on the relative abundance of the top ranked 70 bacterial species identified in cow and mouse mastitis (CCMM, CCMF, MCMF, MCMMT) and healthy (HCM, HCF, HMF, HMMT) metagenomes. The relative values in the heatmap (after normalization), depicted by colors, indicate the aggregation degree or content of bacterial species among samples at the phylum and order level. The color bar (light blue to red) displays the row Z-scores (0–40): red color indicates high abundance, light blue color low abundance. The color of the squares on the left indicates the relative abundance of the bacterial species in each group. The distribution and relative abundance of the bacterial species in the study metagenomes are also available in Additional file 2.
mouse gut and mammary tissues was negatively correlated (Fig. 6).

**CM-associated changes in viral and archaeal fraction (genus-level) of microbiomes in mice**

Consistent with the variation in the bacterial component of the microbiomes, we concurrently found notable differences in the relative abundances of the viral (Fig. 7, Additional file 4) and archaeal (Fig. 8, Additional file 5) components in CM and H samples of both cows and mice (Additional file 2). For instance, the CM mice fecal samples (MCMF) were enriched with higher relative abundances of Siphovirus (61.98%), Mastadenovirus (14.17%) and Myovirus (12.21%), and Gammaparetovirus (37.77%), whereas the CM mice mammary tissues (MCMMT) were predominated by Ichnovirus (21.90%), Betaretrovirus (15.45%), and Macavirus (12.69%) (Fig. 7, Additional file 2). In contrast, Siphovirus (61.98%), Mastadenovirus (14.17%) and Myovirus (12.21%) in H mice fecal samples (HMF), and Gammaparetovirus (36.12%), Ichnovirus (23.0%), Betaretrovirus (14.43%), Macavirus (13.11%) and Rhadinovirus (5.04%) in H mice mammary tissues (HMMMT) had higher relative abundances compared to the CM samples. The remaining viral genera in the CM and H samples of mice had relatively lower abundances (<5.0%) (Fig. 7, Additional file 2).

The present microbiome study demonstrated significant differences \((p=0.012, \text{Kruskal–Wallis test})\) among the archaeal community in the sample categories of both hosts (cow and mice) with mastitis at the genus level. The CM cow fecal metagenome (CCMF) was dominated by Aeropyrum (11.12%), Ferroplasma (3.81%), Cenarchaeum (3.72%), Desulfurococcus (3.31%), Halococcus (2.90%), Halobacterium (2.77%), Halogemericum (2.33%) and Methanobrevibacter (1.06%) genera whereas Methanosphaerula (33.05%), Methanoculleus (5.08%), Methanoplanus (2.54%) and Pyrococcus (2.54%) were identified as the top abundant archaeal genera in CM cows milk samples (CCMM) (Fig. 8, Additional file 7). Among the identified archaeal genera in the mastitis induced mice metagenomes, Acidilobus (23.74%), Aciduliprofundum (14.47%), Archaeoglobus (8.34%), Aeropyrum (6.93%), Caldivirga (5.12%), Candidatus (3.04%), Cenarchaeum (2.65%), Desulfurococcus (2.54%), Ferroglobus (2.51%), Ferroplasma (2.26%), Halalakalicoccus (2.26%), Haloarcula (2.01%), Halofexax (1.98%), Halobacterium (1.79%), Halogemericum (1.67%), Halomicrobium (1.63%) in MCMF metagenome, and Methanosaeta (46.23%), Methanoplanus (22.64%), Methanospaerula (61.32%), Methanosarcina (3.77%), Acidilobus (1.89%), Aciduliprofundum (1.89%), Methanobrevibacter (1.89%), Methanoculleus (1.89%), and Pyrococcus (1.89%) in MCMMT metagenome were the most dominant genera (Fig. 8, Additional files 2, 7). Conversely in healthy mice, Aciduliprofundum (15.02%) in HMF, and Archaeoglobus (11.03%), Halobacterium (4.26%), Halalkalicoccus (3.01%), Halogemericum (2.76%), Haloquadratum (2.51%), Methanoculleus (2.51%), Halomicrobium (2.01%) and Ignicoccus (2.01%) in HMMT were the most abundant archaeal genera. Although, the remaining archaeal genera detected in this study had a relatively lower abundance (<1.0%), their abundance always remained higher in CM associated murine samples (Fig. 8, Additional files 2, 7).

**CM-associated genomic functional perturbations of microbiomes in murine mastitis**

The WMS data were further analyzed using an assembly-based hybrid method of MG-RAST 4.0 (MR) [33] to compare the genomic functional potentials of the microbiomes. In MR analysis, 90.29 million reads (43.15% of cleaned reads) mapped to putative genes with known protein functions after filtering against host associated reads (Data S1). We identified 154 differentially abundant KEGG orthologues; KOs (including MCMF = 149, HMF = 144, MCMMT = 55, HMMT = 109, CCMF = 142, HCF = 131, CCMM = 110 and HCM = 129) and 61 SEED functions (including MCMF = 59, HMF = 59, MCMMT = 44, HMMT = 56, CCMM = 59, HCF = 61, CCMM = 56 and HCM = 60) at different subsystem levels across the bovine and murine metagenomes (CM and H). By comparing the composition and relative abundances of the different Kos or SEED functions in the same KEGG pathway or SEED subsystem between CM- and H-metagenomes, we found significant differences \((p=0.003, \text{Kruskal Wallis test})\) in their relative abundances indicating positive correlations with CM in both hosts (Fig. 9, Additional file 2). Moreover, by measuring the relative abundances of these functional pathways between CM- and H-mice, our analysis revealed

(See figure on next page.)

**Fig. 5** Indicator species in both mastitis (MCMF and MCMMT) and healthy (HMF and HMMT) mice metagenomes. Differentially enriched indicator species in different sample groups of mouse mastitis model. Indicator values are shown next to the taxonomic information for the indicator taxa as indicated by Indicator. Only highly significant indicator values (IndVal > 0.6, \(p<0.01\)) are displayed. Size of bubble is proportional to the mean relative abundance of each species in the corresponding sample group, and the color scale bar shows indicator value for each taxon. Red color indicate the level of significance for which group the taxon is an indicator. Grey symbols indicate group that contain a taxon, but for which that taxon is not an indicator taxa.
Fig. 5 (See legend on previous page.)
that bacterial chemotaxis (67.18%), primary immuno-
deficiency; ADA (32.11%), methanogenesis (29.16%),
phosphotransferase system (17.88%) in CM-mice feces
(MCMF), and glycolysis and gluconeogenesis (61.90%),
reactive oxygen species; ROS (43.90%), cell-to-cell com-
munication (43.15%), ABC transporters (40.25%), recom-
Bacteroides thetaiotaomicron, D. dubosii, Dunsaniella
sp. B8, Faecalibaculum rodentium, L. bacterium, L. murinus
and Muribaculum sp. TLL-A4 had strongest positive correlations (Spearman’s correlation; r > 0.5, p < 0.01) with different SEED
functions including membrane transport, quorum sens-
ing and biofilm formation, oxidative stress, rubrerythrin
mediated oxidative stress, regulation of oxidative stress
response, protein YjgK cluster linked to biofilm formation,
virulence, diseases and defenses, proteolytic pathways,
GTP or GMP pathways, methanogenesis, glycolysis and
and one-carbon metabolism (18.75%) in CM-mice mammary
tissues (MCMMT) were the predominantly enriched metabolic functions compared
to their H counterparts (Fig. 9, Additional file 8). On the contrary, genes coding for quorum sensing: autoinducer-2 synthesis (46.81%) and HIF-1 signalling pathway (21.16%) in H-mice feces (HMF), and flagellar assembly (46.08%), bacterial secretion system (39.25%), and citrate synthase; gltA (15.61%) in H-mice mammary
tissues (HMMT) were the enriched metabolic functions
compared to the CM associated microbiomes. Moreover,
remaining KOs and SEED modules identified in this
study also varied in their relative abundances and had
relatively lower abundance in H metagenomes (Fig. 9,
Additional file 8).

Relationships between predominant microbial species
and their genomic functional potentials in murine mastitis
In this study, B. pseudolongum, Bacteroides thetaiotaomi-
cron, D. dubosii, Duncaniella sp. B8, Faecalibaculum
rodentium, L. bacterium, L. murinus and Muribaculum sp.
TLL-A4 had strongest positive correlations (Spearman’s correlation; r > 0.5, p < 0.01, Fig. 10) with different SEED
functions including membrane transport, quorum sens-
ing and biofilm formation, oxidative stress, rubrerythrin
mediated oxidative stress, regulation of oxidative stress
response, protein YjgK cluster linked to biofilm formation,
virulence, diseases and defenses, proteolytic pathways,
GTP or GMP pathways, methanogenesis, glycolysis and
gluconeogenesis, and one-carbon metabolism (Fig. 10).
Conversely, C. botulinum, A. johnsonii and Actinoal-
loteichus sp. AHMU revealed significant negative correla-
tions (Spearman’s correlation; r ≥ −0.4, p < 0.05) with most
of the SEED modules (Fig. 10). Simultaneously, A. mucin-
iphila, B. pseudolongum, B. thetaiotaomicron, B. animalis,
D. dubosii, Duncaniella sp. B8, F. rodentium, L. bacterium, L. murinus and Muribaculum sp. TLL-A4 showed significantly higher positive correlations (Spearman's correlation; $r \geq 0.6$, $p < 0.01$) with KOs like cheV, motB, ABC transporters, phosphotransferase and two-component systems, ADA, RAG1/RAG2 and mcp (Additional file 9). Consistent with SEED functions, C. botulinum, M. oculi, A. johnsonii, A. junii, Actinoalloteichus sp. AHMU and H. cinaedi displayed negative correlations (Spearman's correlation; $r \geq -0.4$, $p < 0.05$) with most of the KOs identified in this study (Additional file 9).

**Discussion**

Metagenomics is a powerful tool for shedding light on microbiome signature and concurrent genomic features associated with the process of animal disease. Our cow-to-mice induced mastitis model developed in this study clearly indicated that microbiome dysbiosis and concurrent genomic functional perturbations are associated with mammalian mastitis. In this study, more than 8.5 million metagenomic reads were assigned to taxonomic composition which is thought to be sufficient enough to capture maximum microbial richness and corroborated with our previous studies [7, 8, 12]. Several previous studies [34, 35] reported that in shotgun metagenome (WMS) study, more than 90% sequence reads may come from host DNA supporting our present findings. One of the hallmark findings of this study is the identification of several new bacterial species associated with both bovine (e.g., L. crispatus, N. pseudobrasiliensis, L. vaginalis, C. difficile, R. insidiosa, B. pseudolongum, Muribaculum sp., Duncaniella sp., D. dubosii, and Actinoalloteichus...
sp.) (Fig. 4) and murine (e.g., *Muribaculum* sp., *Duncanella* sp., *M. intestinale*, *B. animalis*, *D. dubosi*, *A. muciniphila*, *L. crispatus*, *B. pseudolongum*, *L. murinus*, *M. oculi*, *R. pickettii*, *C. flaccumfaciens*, and *C. acnes*) mastitis (Fig. 5). So far, none of these species have been reported to be associated with bovine and murine CM and other lactating mammals.

**Microbial dysbiosis and host-tropism occurs in mastitis pathogenesis**

In the present study, the fecal microbiota of cows and mice differed significantly at the species level. The prevalence of these microbiota significantly varied across the CM and H metagenomes of both the hosts. The association of *P. aeruginosa*, *K. oxytoca*, *P. dispersa*, *E. faecalis*, *S. aureus*, *A. baumannii*, *A. johnsonii*, *K. pneumoniae* and *E. coli* in bovine CM corroborated with our previous studies [7, 8, 12]. However, the relative abundance of these species remained substantially higher in this study than what we reported in earlier studies [7, 8, 12]. Consistent with these findings of taxonomic discrepancies in microbiome signature and abundances between two respective sample categories (CM vs. H). We also observed the distinct changes in the genomic functional features of the microbiomes in the two hosts (cow and mice) and sample categories between groups (CM and H) (Fig. 9).

The findings of the present study revealed that majority of the species of *Lactobacillus* were identified with higher abundances in the CM fecal samples of both cows and mice, which support their positive association with CM as also reported previously in a number of studies [24, 36, 37]. Lactic-acid bacteria of the genera *Lactobacillus*, *Bifidobacterium*, *Bacillus* and *Enterococcus* are commonly found in the human [38], avian [39] and animals [37, 40] gut. Different species and/or strains of these genera are good candidates to compete with pathogens for mammary gland colonization [41]. However, no direct scientific evidence is currently available regarding the

![Fig. 8](image_url)

*Fig. 8* The genus-level taxonomic profile of archaea identified in cow and mouse mastitis (CCMF, CCMM, MCMF, MCMMT) and healthy (HCF, HCM, HMF, HMMT) samples. Stacked bar plots showing the relative abundance and distribution of the 20 top abundant archaeal genera, with ranks ordered from bottom to top by their decreasing proportion of relative abundances, with the remaining genera keeping as ‘Other genera’. Each stacked bar plot represents the abundance of archaeal genera in each sample of the corresponding category. Notable differences in archaeal population are those where the taxon is abundant in CM (cow and mouse) samples, and effectively undetected in the H- (cow and mouse) controls. The distribution and relative abundance of the archaeal genera in the study metagenomes are also available in Additional file 2.
The typical role of these microbes in the pathophysiology of mastitis. *K. pneumoniae* and *N. pseudobrasiliensis* are the emerging opportunistic environmental pathogens, and transmission of these pathogens to the mammary glands might occur from feces and bedding materials [7, 8, 42]. *E. faecalis*, one of the major pathogens of bovine mastitis, generally present in organic bedding materials and feces being opportunistic invaders of mammary glands [17, 30]. Additionally, a novel species of *Actinobacteria* phylum, *Actinoalloteichus* sp. was predominantly found only in CM cows milk samples (not in mouse samples), which argues against *Actinoalloteichus* sp. as the causative agent of murine mastitis. However, *Actinoalloteichus* has recently been detected as the most abundant genus in milk [43] supporting our present findings. *P. dispersa* can cause a variety of infections in immunocompromised dairy cows [8, 10], however, *P. melaninogenica*, a member of the normal microbiota of the human breast tissue [44], has never been described in cow feces and milk until now. Notably in this study, microbial communities originating from H-control samples have differed from those of CM samples. For instance, the detection of *P.*
Fig. 10 The correlation between top abundant 30 bacterial species and their genomic functional potentials at different levels of SEED subsystem in CM and H control metagenomes of both cows and mice. The scale bar at the right side of the heatmap represent the Spearman's correlation coefficient ($r$). Blue and red indicate positive and negative correlation, respectively. The color density, circle size, and numbers reflect the scale of correlation. *Significant level (*$p$ < 0.05; **$p$ < 0.01; ***$p$ < 0.001)
aeruginosa, E. faecalis, P. dispersa, A. baumannii and P. melaninogenica at higher levels within normal H fecal and milk is intriguing (Fig. 4). *Pseudomonas* is an animal skin microbe, which can colonize the udders and/or quarters from contaminated water and fecal sources [12, 45] and thus, potentially act as opportunistic pathogen by causing intramammary infections [8, 10]. This study therefore revealed a close association between the gut microbiome and milk microbes in the pathogenesis of bovine CM as also reported previously [7, 8, 46]. Additionally, *M. oculi* which was predominantly identified in the mammary tissue of both CM and H mice had significantly lower abundance in fecal and milk samples of cows. This soil bacterium has recently been detected in human clinical specimen [47], however, this species has not been reported in either cows or mice.

Notably, we detected some organisms that are indicative of the mastitis in GF mice (Fig. 5). Our results showed that CM mice sample groups had the highest number of indicator bacterial species. For example, *P. durus, S. malthophilia, P. russatus* and *P. polymyxa* were identified as good indicators of the murine CM (IndVal ≥ 0.8, p = 0.002). This high indicator value suggested that these species were found in most of the samples from CM mice and were comparatively less abundant in H mice samples (Fig. 5). Moreover, different species of *Paenibacillus* have potential contributions to maintaining mammary gland homeostasis [17], and we therefore suggest that association of *Paenibacillus* and *Pontibacter* in mammalian mastitis could be as opportunistic pathogens. In addition, the multidrug resistant bovine CM pathogen, *S. malthophilia* [17, 48] was found with higher relative abundances in CM mice fecal samples indicating its potential role in the induction of mastitis in GF mice. We further found that different species of *Clostridia, Bacteroidia, Actinobacteria, Flavobacteriia* and *Betaproteobacteria* had strong co-occurrence and positive correlation as indicator species of murine mastitis. In this study, *P. thermosuccinogenes, P. xylanivorans, M. intestinale, B. uniformis, P. polymyxa, and B. animalis* showed more connections and overlap suggesting their cooperative or syntrophic interactions during the pathogenesis of murine mastitis (Fig. 6). This is further supported by the higher relative abundance of these microbial taxa in CM mice samples.

**Multi-microbial pathogenesis favors mastitis through metabolomic cross-talk**

Mastitis is a polymicrobial (including bacteria, viruses, archaea) disease where both archaea and viruses are considered as traditionally neglected microbes. Unlike the bacteria, the diversity, composition, and the relative abundances of viruses (Fig. 7) and archaea (Fig. 8) remained much lower in this metagenome study. Our previous studies [7, 8, 10, 12] also reported the concurrent occurrence of archaea and viruses in bovine mastitis highlighting the notion that mastitis is a multietiological disease [8, 10, 29, 49]. Archaea are major colonisers of rumen or the intestinal tracts of animals or humans [50], and thus, their abundance in milk always remains much lower. Recently, majority of the archaeal genera were detected, have been identified from a diverse group of samples including the fecal sample of cattle [51], canine [52] and swine [53], bovine milk [7, 8] and saliva of human [54] supporting our present findings. Mastitis is the result of direct interactions of bacteria with hosts (host–pathogen interactions) under immunosuppression or stressed 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). During the progression of bacterial mastitis, viruses jump into, and reach the site of inflammation in the mammary tissues, and triggers pathogenic pathways by the lysis of macrophages [55]. This phagocytic macrophage storming further aggravates the pathogenesis and creates a micro-aerobic/anaerobic condition which ultimately favors the archaeal growth [56, 57]. Therefore, both viruses and archaea may act as a predisposing factor as well as a primary etiological agent for more severe and prolonged mastitis [7, 8, 10, 12].

In this study, we also found alteration in relative abundances of some important predicted genomic functions among different sample groups of CM and H microbiomes (Fig. 9). The metabolic features identified in the same KEGG pathway or SEED subsystem varied across mastitis samples in both cows and mice, suggesting their possible association in the early colonization and disease progression [8, 17]. Higher abundance of genes associated with bacterial chemotaxis, two-component system, GTP or GMP, NF-kappa-B and Jak-STAT signalling pathways in CM microbiomes of both hosts suggests their potential roles in mastitis through several complex biologic processes including immune disorders, cell differentiation, migration, proliferation, expression of many cytokines, quorum-sensing, microbial group behaviours and oxidative stress mediators, which likely accounts for the high systemic pathogen burden [7, 58–60]. Moreover, the higher abundance of ROS in CM-samples may contribute to the development of oxidative stress and inflammatory response [61] to further aggravate the pathogenesis of mastitis. Besides, quorum sensing, biofilm formation, glutathione non-redox reactions, and methyl-accepting chemotaxis genes that were predominantly identified in CM-pathogens play an important role in many opportunistic bacterial infections [8, 30]. Conversely, the PI3K-Akt pathway related genes remained highly expressed in H-microbes, and this pathway plays
a critical role in the regulation of cell growth, proliferation, survival, motility, differentiation, angiogenesis, and metabolism [62].

The observed differences in microbiome composition and their corresponding genomic functional properties are considered to be the co-selection factor for mammary gland pathogenesis. Notably, the enriched consortia of *B. pseudolongum*, *B. thetaiotaomicron*, *D. dubosis*, *Duncanella* sp. B8, *F. rodentium*, *L. bacterium*, *L. murinus*, *M. baculum* sp. TLL-A4, *A. muciniphila* and *B. animalis* species had the strongest positive correlations with most of the KEGG and SEED functional pathways, indicating these microbial members could play potential roles in the pathophysiology of mastitis (Fig. 10).

**Cow-to-mouse model may be a useful protocol for mastitis diagnosis, curative and preventive studies**

Laboratory animals like mice provide an effective experimental model for understanding the underlying mechanisms of host–microbe interactions [5, 63]. The mouse mastitis model seems to be a good model to study bovine mastitis compared to other laboratory animals for ease of handling, controlled environments, and low maintenance cost [64]. Despite restoring the common mastitis syndromes in both cows and mice, we observed marked differences in microbiome composition and relative abundances irrespective of the sample categories. Therefore, the findings of this study imply that mastitis is not solely caused by the resident microbiota of the mammary gland and/or its secretory product milk but can also be impacted by the alterations of gut microbiota and their genomic functional potentials. Our results highlight the paramount importance of existing entero-mammary pathways through which gut and milk microbiota (from CM host only) could transfer to induce mastitis. The results of the present study corroborated with the previous findings of Ma et al. [5] who reported that bovine mastitis is not necessarily a local infection of mammary glands, rather can be caused by a dysfunctional intestinal microbiota. Taken together, core microbiota identified in the present study distinguished gut and mammary microbiota not only with different sample sources, but also with the health and/or disease state of mice (CM vs. H). Therefore, restoration of gut and mammary gland ecosystem function, for example the mastitis-associated pathways identified in this study, could possibly serve as an effective therapeutic target for bovine mastitis, which may deserve further validation in a larger representative cohort of dairy cows. However, further research is needed to understand the mechanism that allows gut and environmental microorganisms to invade the mammary glands and/or quarters and their relationships with mammalian hosts immune system.

**Conclusions**

The omics approach employed to study mastitis pathogenesis clearly showed microbiome dysbiosis. Species bias was dependent on the host and the predicted genomic functional features in CM hosts were significantly different from the H control counterparts. Our results show that few mastitis-associated microbial taxa and/or genomic functions were shared between diseased (CM) cows and mice regardless of conservation of mastitis syndromes. Cow-to-mouse microbiota transplantation protocols for induction of mastitis might be useful for further molecular studies of mastitis, which will ultimately improve the prevention and treatment strategies in both human and animal species. Taken together, a high-level abundance of the dominant and indicator microbial communities, associated genomic functional potentials, and their simultaneous correlations with the pathogenesis of mammary glands are considered to be driving factors for the mammalian mastitis.

**Methods**

**Dairy cow selection and sampling**

Twelve (n = 12) Holstein crossbred cows (including CM = 7 and H = 5) from seven dairy farms in Dhaka district (23.81 N, 90.41 E) of Bangladesh were used as the donors of fecal and milk samples. The age, parity and lactation of the cows ranged from 2.5 to 6 years, 1 to 5, and 7 to 45 days, respectively (Additional file 1). The cows gave birth randomly throughout the year (no particular control breeding), were milked once daily with their calves used for stimulating milk letdown. The cows were fed on rice straw, cut-and-carry grasses and milling by-products as concentrate (crushed rice and/or sometimes mustard oil cake) with limited grazing [65]. California mastitis test (CMT) was employed initially to diagnose CM following a previously published protocol [2], and 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 s in positive samples and was categorized into 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) [2]. The cows having a CMT score of ≥2 along with gross visible signs of mastitis were designated as CM cows [2]. A total of 12 milk samples (including CM = 7 and H = 5) were collected from lactating cows. Approximately, 15–20 mL of milk from each cow was collected in a sterile falcon tube during the morning milking (9.0–11.0 am) with an emphasis on pre-sampling disinfection of teat-ends and hygiene during milk sampling.
Simultaneously, we collected 12 fresh fecal samples (including CM = 7 and H = 5) from the selected cows under hygienic condition. Approximately, 5–10 g of fecal sample was collected through the rectum of each cow wearing a hygienic disposable plastic glove. No lubricants were used during sample collection. Collected fecal samples were then mixed-well and placed in a sterile falcon tube after proper labelling (15 mL) [5]. For either the CM or H group, both milk and fecal samples were freshly collected, transported to the laboratory keeping in an ice box (at 4 °C temp), the content was thereafter processed and divided into aliquots. A portion of the processed samples proceeded to FMT and MMT immediately after collection, and rest of the aliquoted samples were stored at −80 °C for metagenomic DNA extraction.

**Microbiota transfer experiments**

For the FMT and MMT procedures, forty (n = 40) timed-pregnant (at Day 17 of breeding) Swiss albino mice were procured from the ICDDR’B, Dhaka, Bangladesh. The mice were randomly divided into four groups: Group-I (FMT from CM cows, n = 10) Group-II (FMT from H cows, n = 10), Group-III (MMT from CM cows, n = 10) and Group-IV (MMT from H cows, n = 10). The mice were challenged on the same day of sampling. At the day of challenge (Day 17 after mating), 0.5 g fecal sample obtained from each of the CM and H cow was resuspended with twice the fecal volume of sterile physiological saline. After thorough mixing and resting (to minimize the number 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 Group-I and Group-II [66]. Like wise, 10 mL whole milk tubes (from both CM and H cows) were centrifuged at room temperature for 10 min at 5000 × g [67]. After centrifugation, both the cream layer and supernatant liquid were removed. The pellet was resuspended to the initial sample volume of sterile physiological saline, and MMT was performed by a single oral administration of 0.5 mL of milk suspension to each mouse of Group-III and Group-IV [68]. The mice were housed in GF environment on a 12 h light/dark cycle with unlimited access to food and water. In order to prevent cross-contamination of gut microbiota, the four groups of mice were physically separated into different GF isolators after challenge. 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 [66, 69]. At the end of Day 27 of mating (10 days of challenge), the mice were sacrificed, and fresh fecal samples, mammary and gut (colon) tissues were collected. The collected fecal samples were then mixed with freshly prepared phosphate buffered saline (PBS), and finally stored at −80 °C until further processing and DNA extraction.

**Histopathological analysis**

Collection of milk and subsequent milk somatic cell count (SCC) was not feasible in challenged mice, therefore histopathological examination was performed to assess the alterations and inflammatory changes of mammary gland and colon tissues during mastitis. The mammary gland and colon tissues were dissected from mice, kept in glass tubes, and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 24 h at 4 °C. Paraffin-embedded tissues were cut on a Leica Rotary Microtome (Leica Microsystems), placed onto SuperFrost Plus slides, and dried overnight at 37 °C. Sections were deparaffinized with xylene, and gradually rehydrated through graded alcohols for staining with standard hematoxylin and eosin (H & E staining) sectioned [70]. To assess the degree of tissue injury to the mammary gland and colon, we used the Chiu Scoring System [71] in a blinded manner, where the number of inflammatory cells was counted in 12 randomly selected fields from each slide at a magnification of 400×. The slides were observed for severe, diffuse interstitial and/or alveolar infiltrate of inflammatory cells, focal to multifocal areas of tissue damage, epithelial abnormalities and extensive necrotic areas in mastitic mice [70]. The degree of necrosis in mammary gland tissues was scored on a scale of 0–3 (normal 0, mild 1, moderate 2, severe 3). The degree of colon injury was scored as grades 0 (normal mucosa), 1 (development of subepithelial spaces at villus tips), 2 (extension of the subepithelial space with moderate lifting of the epithelial layer), 3 (massive epithelial lifting with a few denuded villi), 4 (denuded villi with exposed capillaries), and 5 (disintegration of the lamina propria, ulceration, and hemorrhage) [66, 70]. The slides were assessed under an Olympus BX51 upright microscope (40× objective), and finally images were collected using an Olympus DP73 camera through cellSens entry software (Olympus Corporation, Japan), and visualized using image J software (https://image.nih.gov/ij/).

**Genomic DNA extraction and whole metagenome sequencing**

Total genomic DNA from six milk (CM = 3, H = 3), six mammary tissue (CM = 3, H = 3), and 12 fecal (CM = 8, H = 4, from both cow and mice) samples (Data S1) was extracted using Maxwell 16 automated DNA extraction platform (Promega Corporation, Madison, WI 53711-5399, USA). In brief, DNA from fecal (400 µL fecal suspension) and milk (400 µL of whole milk) samples was extracted using Maxwell® 16 FFPS Nucleic Acid Extraction Kit [7] and Maxwell® 16 FFPE Plus LEV DNA Purification Kit.
using the very sensitive Bowtie 2 algorithm [76], and fil-
WMS reads were then aligned against the target libraries
'target' genome library was constructed containing all
human genomes (Additional file 2).
and SEED subsystems after filtering the cow, mouse and
in different known protein functions in KEGG pathways
43.15% reads mapped (of total cleaned reads) mapped to the target reference
tems in the MR pipeline. In IDSeq pipeline, 4.06% reads
used minimum identity of
finally screening for metabolic functional assignment. We
reference genome of both cattle, mouse and human), and
dereplication, host DNA removal (filtering against the set
uously uploaded to the MR server with properly embed-
(with cattle (bosTau8), mouse (GRCm39) and human
DNA extraction from mammary tissue (50 mg) samples
tered with Trimmomatic [77] to remove the reads aligned
with the cattle (bosTau8), mouse (GRCm39) and human
(available from https://sourceforge.
open-source cloud-based pipeline for taxonomic assign-
and MG-RAST 4.0 (MR) [33], respectively. IDseq is an
based and assembly-based hybrid methods of IDSeq [32]
computing bacterial species and their genomic functional
ships between the relative abundances of the predomi-
nating bacterial species and their genomic functional
potentials in the pathogenesis of murine mastitis after

**WMS data processing and microbiome analysis**

The generated FASTQ files were concatenated and fil-
tered through BBduk (available from https://sourceforge.
et/projects/bbmap/) with options k=21, mink=6, ktrim=r, ftm=5, qtrim=r, trimq=20, minlen=30,
overwrite=true [75] to remove Illumina adapters,
known Illumina artifacts, and phiX. Any sequence below
these thresholds or reads containing more than one ‘N’
were discarded. The WMS data were analyzed using
both open-source cloud-based metagenomic mapping-
based and assembly-based hybrid methods of IDSeq [32]
and MG-RAST 4.0 (MR) [33], respectively. IDseq is an
open-source cloud-based pipeline for taxonomic assign-
ments with NTL (NTL; nucleotide alignment length
in bp) ≥ 50 and NT % identity ≥ 90. In IDSeq analysis, a
‘target’ genome library was constructed containing all
prokaryotic sequences from the NCBI Database. The
WMS reads were then aligned against the target libraries
using the very sensitive Bowtie 2 algorithm [76], and fil-
tered with Trimmomatic [77] to remove the reads aligned
with the cattle (bosTau8), mouse (GRCm39) and human
(hg38) genomes. The raw sequences were simultane-
ously uploaded to the MR server with properly embed-
ded metadata, and were subjected to quality filtering with
dereplication, host DNA removal (filtering against the set
reference genome of both cattle, mouse and human), and
finally screening for metabolic functional assignment. We
used minimum identity of ≥ 90% for metabolic functional
analysis through KEGG pathways and SEED subsystems
in the MR pipeline. In IDSeq pipeline, 4.06% reads
(of total cleaned reads) mapped to the target reference
genomes whereas in MR pipeline 43.15% reads mapped
to different known protein functions in KEGG pathways
and SEED subsystems after filtering the cow, mouse and
human genomes (Additional file 2).

**Metabolic functional potential analysis**

The genomic functional profile of the microbiomes
was annotated according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology [84], and
SEED subsystem [85] databases in MR pipeline using a
“Best Hit Classification” method. The functional map-
ing was performed with the partially modified set param-
ters (e-value cutoff: 1 × 10⁻30, min. % identity
cutoff: ≥ 90%, and min. alignment length cutoff: 20) of
the MR server [86]. We also investigated the relation-
ships between the relative abundances of the predomin-
ing bacterial species and their genomic functional
potentials in the pathogenesis of murine mastitis after
renormalization and permutations.
Statistical analyses
The pair-wise non-parametric Kruskal–Wallis rank sum test was used to evaluate differences in the relative percent abundance of the microbial taxa and differentially abundant SEED or KEGG functions (at different levels) in CM and H animal (cow and mice) groups. Indicator species analysis calculated an IndVal which was the product of the relative frequency and relative abundance of a species in a cluster. To test the significance of the IndVal, p values were calculated with 100 iterations, where in each iteration, the sample groupings were randomly assigned and an IndVal determined. The p values for the IndVal calculation were corrected for multiple comparisons using the false discovery rate correction [87]. To explore the relationship among the relative abundance of indicator species, we calculated Spearman’s rank correlation coefficients using the ‘mulitest’ package in R [88], and the correction was made using Benjamini–Hochberg FDR. In the co-occurrence network, each node represents one species and each edge stands for correlation between the species abundances [87]. Spearman’s correlation among the top abundant 30 bacterial species, and their genomic metabolic functions (SEED and KEGG functional pathways) were performed in Hmisc and corrplot R packages as described above (Methods).

Abbreviations
FMT: Fecal microbiota transplantation; MMT: Milk microbiota transplantation; GF: Germ free; CM: Clinical mastitis; H: Healthy; WMS: Whole shotgun metagenomic sequencing; CCMF: Cow clinical mastitis feces; HCF: Healthy cow feces; MCMF: Mouse clinical mastitis feces; HMF: Healthy mouse feces; MCMMT: Mouse clinical mastitis mammary tissue; HMMT: Healthy mouse mammary tissue.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s42523-022-00193-w.

Additional file 1. Study information (animal information, location, breeds, lactation, and parity).
Additional file 2. Information on metagenomic data, microbial structure, composition and relative abundances in each metagenome.
Additional file 3. Taxonomic structure of microbiomes identified in different sample groups.
Additional file 4. Taxonomic composition of viruses.
Additional file 5. Taxonomic composition of archaea.
Additional file 6. Taxonomic information of top seventy bacterial species identified in different metagenomic groups.
Additional file 7. Archaeal genera detected (with their relative abundances) in different metagenomes.
Additional file 8. Changes in metabolic functional profile in the study metagenomes.
Additional file 9. The correlation between predominantly abundant bacterial species and their genomic functional potentials in different KEGG orthologues (KO) in CM and H control metagenomes of both cows and mice.

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Author contributions
M.N.H. and M.A.H conceived and designed the study. M.N.H. collected field samples. M.N.H. and M.S.R carried out laboratory works including mouse model experiment, genomic DNA extraction and executed the bioinformatics analysis. M.N.H interpreted the results and drafted the manuscript. M.S., T.I., K.A.C and M.A.H contributed intellectually to the interpretation and presentation of the results. Finally, all authors have approved the manuscript for submission.

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Availability of data and materials
The sequence data and related metadata reported in this paper are available in the SRA repository of the NCBI database under BioProject Accession ID of PRJNA753332. Supplementary information supporting the findings of the study are available in this article as Additional files 1, 2, 3, 4, 5, 6, 7, 8 and 9.

Declarations
Ethics approval and consent to participate
The protocol for milk sample collection from dairy cows and in vivo mouse model challenge was approved by the Animal Experimentation Ethical Review Committee (AEERC), Faculty of Biological Sciences, University of Dhaka (under reference number: 79/Biol.ScS), and International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Permission was also obtained from the owners of sampled dairy farms. 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. Every effort was made to minimize animal suffering.

Consent for publication
Consent was obtained from participating farms prior to the start of the study.

Competing interests
The authors declare no competing interests.

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