The fungal milk microbiome of dairy cattle and its implications on mastitis

Rowan Cook[1,3*], Joana Lima[1], Jolinda Pollock[2], Richard J. Dewhurst[1], Sharon Huws[3], Chris J. Creevey[3], Holly J. Ferguson[1]

1. Scotland's Rural College, Peter Wilson Building, Edinburgh, EH9 3JG
2. Scotland's Rural College Veterinary Services, Midlothian, EH26 OPZ
3. Queen's University Belfast, School of Biological Science, Belfast, BT9 5DL

*Corresponding author: rowan.cook@sruc.ac.uk

Mastitis is a prevalent disease that causes economic losses through decreased milk yield, discarded milk during treatment, and veterinary costs (Ibrahim, 2017) and negatively impacts animal welfare (Hillerton, 1998).

Milk microbiome (microbes inhabiting the milk and their interactions) profiles are influenced by age, location, feed, and health status (Zapata and Quagliarello, 2015) and disruption of these communities (dysbiosis) has been associated with mastitis (Derakhshani et al., 2018).

Accurate identification of mastitis pathogens is vital to ensure appropriate treatment and reduce antimicrobial resistance development. However, current guidelines lack consistency about best practice for mastitis sample storage prior to culture, leading to potential misdiagnosis.

The objectives of this study were to compare milk microbiome profiles between animals with differing health status, explore the role of fungi in the milk microbiome and investigate differences in microbiome profiles of single vs repeated mastitis cases. We also investigated the impact of freezing and glycerol preservation on mastitis samples.

Milk samples were collected from 40 lactating cows: clinical mastitis (C, n=10), repeated clinical mastitis (R, n=10), subclinical mastitis (S, n=10, SCC > 200,000 cells/ml), and healthy (H, n=10, no clinical mastitis history and ≥ two subsequent SCCs <100,000 cells/ml). DNA was extracted from milk samples and shotgun sequenced to obtain a microbiome profile for each sample. We also tested the culturability of clinical mastitis milk samples stored at -20°C or -80°C for up to 6 months with and without glycerol (15% or 30%).

The number of taxa (microbes) and their distribution were not significantly different between sample types; however, microbial abundances were more evenly distributed in healthy samples in comparison to unhealthy samples (where *Clostridium* and *Vibrio* were more dominant, fig. 1). Samples that cultured as fungal mastitis were less dominated by the top 10 bacteria, showing more evenly distributed microbiome profiles. We also observed similar levels of variation between animals within health groups.

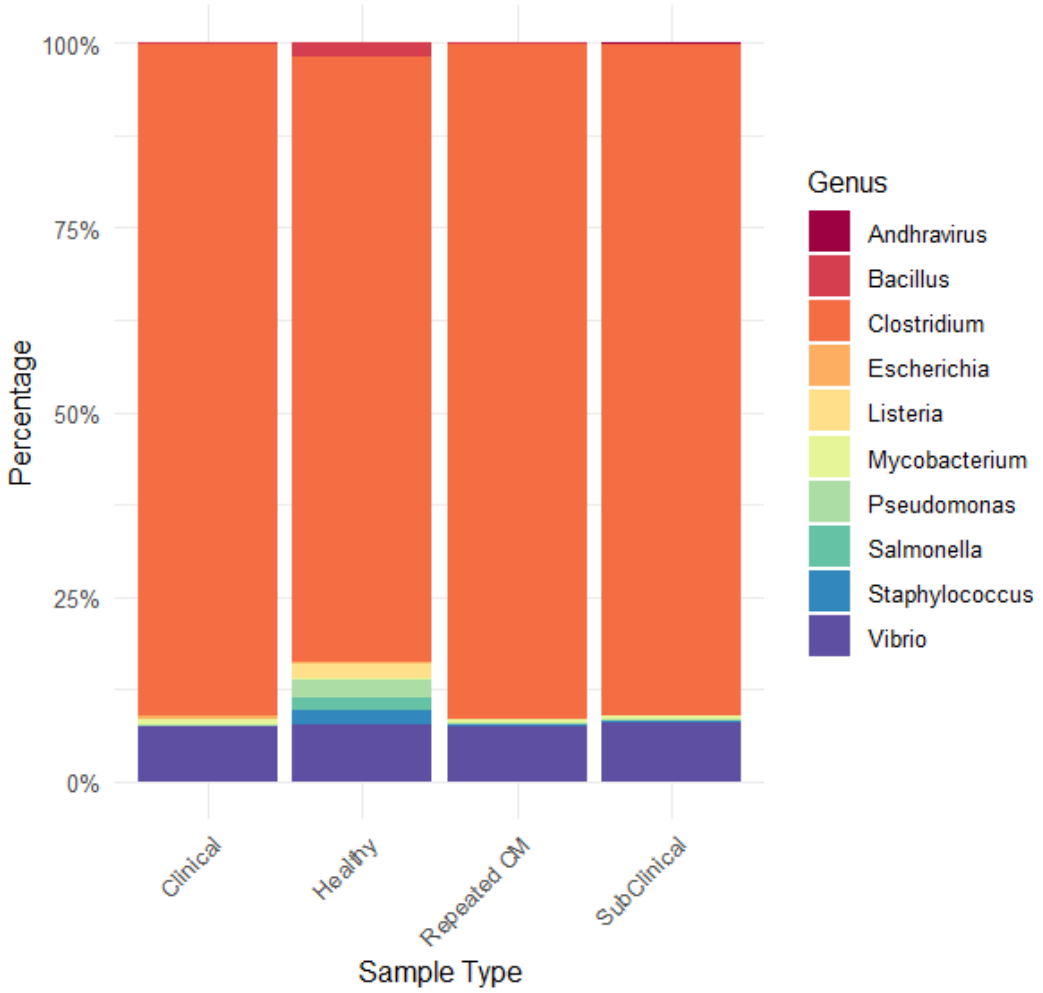

Figure 1 Relative abundance of the top 10 most abundant genera from metagenomic shotgun sequencing data. Data is grouped by cow mastitis health status, with 10 samples in each group.

Partial least squares discriminant analyses were used to compare C, S and R microbiome profile to H, and C to R, and to identify the most important microbes in differentiating between the samples (table 1). For example, *Companilactobacillus, which* increased in C compared to H, has been observed on dairy farms, and spreads through vectors such as flies (Neupane et al., 2024). Both *Sphingobacterium* and *Chryseobacterium,* increased in C compared to H, have been identified in milk samples with *Sphingobacterium* being associated with increased SCC and *Chryseobacterium* thought to be an opportunistic bacterium (Hagi et al., 2013; Kuang et al., 2009; Oikonomou et al., 2014).

Comparing microbiome profiles of C with R showed that R had decreased relative abundance of pathogens traditionally associated with mastitis, including *Enterococcus* and *Streptococcus*, potentially due to previous antibiotic administration. *Limosilactobacillus,* increased in R, is a lactic acid bacterium which inhibits the growth of common mastitis pathogens including *E. coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus agalactiae*

(Chauhan et al., 2024). *Limosilactobacillus* has also been shown to be effective in reducing mastitis in humans when administered as a probiotic (Ozen et al., 2023).

The microbiome of the clinical mastitis cases diagnosed as of fungal origin during culture (*Candida parasilopsis*, yeast) did not include fungal taxa. Whilst shotgun sequencing methods rely on DNA, fungi were identified during culture using MALDI-TOV which relies on protein molecules. After DNA extraction, DNA of these specific fungi may have been present at too low concentrations to be identified or have degraded.

Table 1 Most important microbes to differentiate sample types. H, C, S, R, refer to healthy, clinical, subclinical, and repeated clinical mastitis cases, respectively. Shaded cells represent increase in relative abundance in the C, S, and R in comparison to H, and R in comparison to C.

H vs C	H vs S	H vs R	C vs R
Cutibacterium	*Geothrix*	*Catellatospora*	*Sulfuriferula*
Companilactobacillus	*Catellatospora*	*Proteiniclasticum*	*Limosilactobacillus*
Sphingobacterium	*Neisseria*	*Tissierella*	*Enterococcus*
Chryseobacterium	*Gluconobacter*	*Cutibacterium*	*Streptococcus*
Herbinix	*Dyadobacter*	*Tepiditoga*	*Citrobacter*

Our investigation into freezing temperature and duration showed that culture results were impacted by both, leading to inaccurate pathogen identification. The impact of freezing on clinical mastitis samples varied by pathogen. *Serratia liquefaciens* was identified in a fresh sample. After one month, this sample resulted in sterile growth, *Escherichia coli* was identified in milk samples even after six-month storage at -20°C or -80°C, unlike other Gram-negative pathogens. Previous work has shown after one year at -20°C, *E. coli* was no longer culturable (Leclair et al., 2019). These results highlight the potential risk of inaccurate pathogen identification after freezing samples.

The investigation into the efficacy of glycerol as a preservative was inconclusive. Cases to which glycerol was added produced Gram-positive bacteria or *E. coli* on culture, which were unaffected by freezing.

Our work shows that milk microbiome profiles are affected by mastitis incidence. Further investigation into microbial function, biochemical pathways, and interactions is ongoing. Future guidance should discourage freezing of milk samples prior to mastitis culture and should include the addition of fungi specific culture, to ensure the most accurate results.

References

Chauhan, N., Kumar, S., Chauhan, T. & Samanta, A. K. 2024. Screening of lactic acid bacteria from the milk of Sahiwal cows and characterization of their probiotic potential for preventing bovine mastitis. *International Microbiology*, 1-26.

Derakhshani, H., Fehr, K. B., Sepehri, S., Francoz, D., De Buck, J., Barkema, H. W., Plaizier, J. C. & Khafipour, E. 2018. Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. *J Dairy Sci,* 101(12), 10605-10625.

Hagi, T., Sasaki, K., Aso, H. & Nomura, M. 2013. Adhesive properties of predominant bacteria in raw cow's milk to bovine mammary gland epithelial cells. *Folia microbiologica,* 58(515-522.

Hillerton, E. Mastitis treatment–a welfare issue. Proceedings. British Mastitis Conference, Stoneleigh, UK, 1998.

Ibrahim, N. 2017. Review on mastitis and its economic effect. *Canadian Journal of Research,* 6(1), 13-22.

Kuang, Y., Tani, K., Synnott, A. J., Ohshima, K., Higuchi, H., Nagahata, H. & Tanji, Y. 2009. Characterization of bacterial population of raw milk from bovine mastitis by culture-independent PCR–DGGE method. *Biochemical engineering journal,* 45(1), 76-81.

Leclair, R. M., Mclean, S. K., Dunn, L. A., Meyer, D. & Palombo, E. A. 2019. Investigating the effects of time and temperature on the growth of Escherichia coli O157: H7 and Listeria monocytogenes in raw cow's milk based on simulated consumer food handling practices. *International Journal of Environmental Research and Public Health,* 16(15), 2691.

Neupane, S., Park, Y., Watson, D. W., Trout Fryxell, R. T., Burgess Iv, E. R. & Nayduch, D. 2024. Bacterial Communities of House Flies from Dairy Farms Highlight Their Role as Reservoirs, Disseminators, and Sentinels of Microbial Threats to Human and Animal Health. *Insects,* 15(9), 730.

Oikonomou, G., Bicalho, M. L., Meira, E., Rossi, R. E., Foditsch, C., Machado, V. S., Teixeira, A. G., Santisteban, C., Schukken, Y. H. & Bicalho, R. C. 2014. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. *PLoS One,* 9(1), e85904.

Ozen, M., Piloquet, H. & Schaubeck, M. 2023. Limosilactobacillus fermentum CECT5716: clinical potential of a probiotic strain isolated from human milk. *Nutrients,* 15(9), 2207.

Zapata, H. J. & Quagliarello, V. J. 2015. The microbiota and microbiome in aging: potential implications in health and age-related diseases. *Journal of the American Geriatrics Society,* 63(4), 776-781.

Research Outputs

Publications:

Cook R., Lima J., Pollock J., Dewhurst R.J., Huws S., Creevey C.J., Ferguson H.J. *Freezing Milk Reduces Accuracy of Mastitis Pathogen Identification in Cattle*. In preparation

Conference presentations and proceedings:

Cook R., Lima J., Pollock J., Dewhurst R.J., Huws S., Creevey C.J., Ferguson H.J. (2023) *The impact of freezer temperature, storage duration, and glycerol cryoprotectant usage on clinical mastitis culture results*. Hannah Dairy Research Foundation Conference 2023: Next Generation Quality Dairying.

Cook R., Lima J., Pollock J., Dewhurst R.J., Huws S., Creevey C.J., Ferguson H.J. (2024) *Freezer storage impact on clinical mastitis culture results*. Scotland's Rural College Post-Graduate Research Conference 2024.

Cook R., Lima J., Pollock J., Dewhurst R.J., Huws S., Creevey C.J., Ferguson H.J. (2024) *Freezer storage impact on clinical mastitis culture results*. British Mastitis Conference 2024.

Acknowledgements

The authors wish to thank the SRUC technical and farm staff for their assistance with sample collection. This study was made possible through funding gratefully received through the Hannah Dairy Research small grants competition and the European Union's HoloRuminant project. HoloRuminant has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N° 101000213.