

**Lactation-long changes in milk microbiota, milk composition,
and metabolic profiles of Holstein and Jersey cows and the
environmental microbiota of their barns**

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DECLARATION

I would like to sincerely declare that this thesis titled “**Lactation-long changes in milk microbiota, milk composition, and metabolic profiles of Holstein and Jersey cows and the environmental microbiota of their barns**” submitted in partial fulfillment of the requirements for the award of Doctor of Philosophy (Ph.D.) in Agriculture, Graduate school of environmental and Life Science, Okayama University was solely composed by myself under the tutelage of my supervisors.

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1 CHAPTER 1

GENERAL INTRODUCTION

The global demand for bovine fluid milk and dairy products is burgeoning due to population growth and the steady improvement of per capita consumption, especially in developing countries (Britt et al., 2018). Even in developed nations like the USA, where the demand for dairy products had been perceived to be stagnant, it is still growing, albeit at a relatively slower rate of 1% per annum (Jacobson, 1986). On the other hand, a report by Adesogan & Dahl, (2020) indicates that the demand for milk and milk products in developing Asian and African nations could be in the region of 35%. The improved livelihoods, urbanization, and high population growth in developing countries in Africa and Asia spur the increment in demand for dairy products as a rich source of nutrients. With the world population also anticipated to grow, especially in the two continents mentioned above, the demand for dairy products is projected to continue on the same trajectory for the next few decades (Britt et al., 2018).

In response to the above ever-growing demand for milk and dairy products, dairy production is anticipated to concomitantly increase with the demand. Different reports have different projections; for instance, Britt et al., (2018) & Food and Agriculture Organization, (2020) have projected a 1% per annum increment in dairy production and demand, particularly in developed countries. Due to the high demand for dairy products in the developing countries and relatively lower international trade level in milk-averaging out at 8%-owing to its perishability, milk production in the developing countries is predicted to soar to bridge the forecast gap in the milk supply chain (Food and Agriculture Organization, 2020). The Milk symposiums report by Adesogan & Dahl, (2020) highlighted the projected regional milk production, where the highest milk production is expected in South Asia and the lowest in Western Europe.

The abovementioned projected high milk production is expected against the backdrop of various production challenges facing the dairy sector. Heat stress is among those challenges bedeviling bovine milk production. The increased temperature-humidity index beyond the bovine thermal comfort zone induces physiological responses that lead to a decline in milk production, among other physiological disorders (Polsky & von Keyserlingk, 2017). Areas anticipated to heighten milk production are also expected to continue recording high temperature-humidity index over the same period of projected high milk production. To achieve the anticipated high milk production to meet the excessive demand for milk, the dairy sector must adopt mitigating and abatement strategies. Some of those strategies include the incorporation of foolproof ventilation and misters in cowsheds. According to Polsky & von Keyserlingk, (2017), this mitigation strategy has been proven to lower rectal body temperature, improve dry matter intake, and increase milk production. Moreover, some breeds of cows are more vulnerable to heat stress than

others. For instance, Jersey cows are more resistant to heat stress than Holstein cows. A study by Smith et al., (2013) found that Holstein milk yield declined when Holstein experimental cows were subjected to moderate and severe heat stress, while the milk yield of Jersey cows only declined when they were subjected to extreme heat stress. On the other hand, Jersey cows' milk fat was unaffected by any category of heat stress, unlike Holstein cows' milk fat, which declined in both moderate and severe heat categories. These findings indicated that Jersey cows are more resistant to heat stress than Holstein cows. Hence, it implies Jersey cows might perform better in areas with elevated ambient temperature than Holstein cows.

In addition to the milk production challenges, there is a different aspect of demand for milk that has deviated from just the demand for bovine fluid milk to bovine milk with high milk solids. Unlike the demand for high milk volumes in developing nations, milk consumers in developed countries have developed a predilection for milk with higher total solids. In some niches in the marketplace, more emphasis is put on Solids-not-fat (Smith & Carpenter, 1976). Notably, high milk protein and minerals have continually received much attention from consumers; hence, the dairy industry in such niches is steamrolled to capitulate to consumers' demand by striving to churn out dairy products with high protein content and minerals. In such niches, the rearing of Jersey cows is steadily gaining traction due to the high protein content of the milk they produce.

One of the major stumbling blocks to the projected amped milk production is the propensity of cows to contract reproductive or nutritional diseases or disorders, especially during the periparturient period. During this critical period in milk production, cows, particularly the higher producer ones, are vulnerable to manifold health challenges. Soon after parturition, cows experience the typical Negative Energy Balance (NEB) due to the heavy milk production burden coupled with lower feed intake (Churakov et al., 2021; Fenwick et al., 2008). The severity of NEB hinges on the breed of the animal, among several other factors. Holsteins have a higher propensity to suffer severe NEB than their counterparts breeds like Jerseys. This likelihood predisposes Holsteins to declined reproductive and productive performances. Brown et al., (2012) ascribed the lower reproductive performance in the Holsteins to the severe form of NEB they suffer during the early lactation period. This finding was corroborated by De Vries & Veerkamp, (2000), who found that 10 MJ of NEL/d lower nadir of energy balance corresponded to a delay of ovulation of 1.25 d. Also, this severe NEB blights the milk quality and the production ability of the Holsteins. During the early lactation period, there is a strong correlation between NEB and a decline in milk fat. Although this correlation steadily declines as Days in Milk (DIM) increase, it persists at about 180 DIM (De Vries & Veerkamp, 2000). This correlation shows the vulnerability of cows reared affects the composition of the milk produced.

The quality and safety of milk produced and marketed are most important to both milk processors and consumers. The quality of milk hinges on milk composition, while milk's safety depends highly on microbial milk quality. The milk microbes are broadly categorized into three categories: technologically beneficial microbes, pathogenic microbes, and spoilage microbes (Q. D. Nguyen et al., 2020). The former category contributes to the milk fermentation, conferring

desirable attributes to dairy products. Some strains of lactic acid bacteria have been isolated, purified, and commercially stocked. These strains impart rheological and sensorial traits to milk. The latter category spoils milk by acting on milk constituents like protein and fat. This category produces heat-tolerant extracellular enzymes, peptidases and lipases.

Thermo-stable peptidase and lipases act on the milk protein and lipids, respectively. The impact of the abovementioned enzymes includes bitter taste and aged gelation (Quigley et al., 2013). The source of milk microbiota varies widely from naturally present in milk from the cowshed environment and milking/storage equipment. Freshly drawn milk usually has an undetectable level of psychrotrophic bacteria, but this population rapidly proliferates to dominate the milk microbiota (de Oliveira et al., 2015). Milk microbiota from the cowshed environment includes the cowshed beddings and airborne dust microbiota. Nguyen et al., (2020) found that the microbiota of these two sources was clustered with milk microbiota; hence, it implied the likelihood of the two being common sources of contamination to milk. Milk equipment, on the other hand, harbors a complex population of microbiota with the potential to spoil milk. Due to the psychrotrophs' knack for forming a biofilm, which is a community of bacterial cells embedded in a self-produced protective matrix of extracellular polymeric substance, they can survive adverse environmental conditions such as detergent, acids, and heat from the clean-in-place (CIP) water (Yin et al., 2019).

The modern dairy industry is dynamic, going by the milk production expectations placed on it and the challenges it has to surmount to meet the demand for milk. The stakeholders in the dairy industry are adopting an array of strategies to mitigate the challenges, boost milk production, and churn out quality and safe milk to the market. The quality and safety of milk highly hinge on the initial microbial load of raw milk delivered to a milk processor. Murphy et al., (2016) illustrated how the initial microbial load of raw milk influences the quality of dairy products. From the processor's point of view, the quality of processed dairy products highly depends on the bacterial count just before processing. The extent of quality defects of processed milk depends on the types of microbes, the microbial load, the growth stage, and the spoilage enzymatic activities of the bacteria present in raw milk before thermo-processing. As Nguyen et al., (2020) asserted, milk composition influences milk microbiota, and milk composition changes across an entire lactation period. Hence, we conducted experiments to investigate how milk composition and microbiota change throughout a lactation period for both Jersey and Holstein cows. Below are the synopses of the three experiments we conducted.

In experiment one, we collected milk and blood samples every other month from eight lactating Holstein cows from the beginning to the end of their lactation. We also collected airborne dust and bedding samples every time we collected milk samples. We determined milk composition and milk microbiota from the milk samples collected. Blood samples, on the other hand, were used to measure metabolic profiles, while environmental microbiota was determined from both airborne dust and bedding samples collected.

In experiment two, we collected milk sample, blood, and rumen fluid samples from eight Jersey cows every two months throughout their lactation period. Airborne dust samples from the cowshed were also collected after every two months. The samples were used to meet the experimental objective as described above.

In experiment three, a pilot study, we collected samples from the Jersey farm described in experiment two. In this experiment, we collected swab samples from teat cups (TC), bulk tank outlet (BTO), and bulk tank milk. We cultured both the swab and milk samples, representatively isolated bacteria, and purified our isolates. The bacterial isolates obtained in this experiment were used to test their biofilm-forming ability and their milk spoilage enzymatic activities.

References

- Aarestrup, F. (2012). Sustainable farming: Get pigs off antibiotics. *Nature*, 486(7404), 465–466. <https://doi.org/10.1038/486465a>
- Abebe, R., Hatiya, H., Abera, M., Megersa, B., & Asmare, K. (2016). Bovine mastitis: Prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. *BMC Veterinary Research*, 12(1), 1–11. <https://doi.org/10.1186/s12917-016-0905-3>
- Adesogan, A. T., & Dahl, G. E. (2020). MILK Symposium Introduction: Dairy production in developing countries. *Journal of Dairy Science*, 103(11), 9677–9680. <https://doi.org/10.3168/jds.2020-18313>
- Aghamohammadi, M., Haine, D., Kelton, D. F., Barkema, H. W., Hogeveen, H., Keefe, G. P., & Dufour, S. (2018). Herd-level mastitis-associated costs on Canadian dairy farms. *Frontiers in Veterinary Science*, 5(MAY), 1–12. <https://doi.org/10.3389/fvets.2018.00100>
- Azooz, M. F., El-Wakeel, S. A., & Yousef, H. M. (2020). Financial and economic analyses of the impact of cattle mastitis on the profitability of Egyptian dairy farms. *Veterinary World*, 13(9), 1750–1759. <https://doi.org/10.14202/vetworld.2020.1750-1759>
- Ben, Y., Fu, C., Hu, M., Liu, L., Wong, M. H., & Zheng, C. (2019). Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review. *Environmental Research*, 169(July 2018), 483–493. <https://doi.org/10.1016/j.envres.2018.11.040>
- Bernabucci, U., Basiricò, L., Morera, P., Dipasquale, D., Vitali, A., Piccioli Cappelli, F., & Calamari, L. (2015). Effect of summer season on milk protein fractions in Holstein cows. *Journal of Dairy Science*, 98(3), 1815–1827. <https://doi.org/10.3168/jds.2014-8788>
- Bernabucci, U., Lacetera, N., Baumgard, L. H., Rhoads, R. P., Ronchi, B., & Nardone, A. (2010). Metabolic and hormonal acclimation to heat stress in domesticated ruminants. *Animal*, 4(7), 1167–1183. <https://doi.org/10.1017/S175173111000090X>
- Bowen, L., Ebrahim, S., de Stavola, B., Ness, A., Kinra, S., Bharathi, A. V., Prabhakaran, D., & Reddy, K. S. (2011). Dietary intake and rural-urban migration in India: A cross-sectional study. *PLoS ONE*, 6(6), 1–8. <https://doi.org/10.1371/journal.pone.0014822>
- Britt, J. H., Cushman, R. A., Dechow, C. D., Dobson, H., Humblot, P., Hutjens, M. F., Jones, G. A., Ruegg, P. S., Sheldon, I. M., & Stevenson, J. S. (2018). Invited review: Learning from the future—A vision for dairy farms and cows in 2067. *Journal of Dairy Science*, 101(5), 3722–3741. <https://doi.org/10.3168/jds.2017-14025>
- Brown, K. L., Cassell, B. G., McGilliard, M. L., Hanigan, M. D., & Gwazdauskas, F. C. (2012). Hormones, metabolites, and reproduction in Holsteins, Jerseys, and their crosses. *Journal of Dairy Science*, 95(2), 698–707. <https://doi.org/10.3168/jds.2011-4666>
- Brown, K., Mugoh, M., Call, D. R., & Omulo, S. (2020). Antibiotic residues and antibiotic-

- resistant bacteria detected in milk marketed for human consumption in Kibera, Nairobi. *PLoS ONE*, 15(5), 1–8. <https://doi.org/10.1371/journal.pone.0233413>
- Churakov, M., Karlsson, J., Edvardsson Rasmussen, A., & Holtenius, K. (2021). Milk fatty acids as indicators of negative energy balance of dairy cows in early lactation. *Animal*, 15(7), 100253. <https://doi.org/10.1016/j.animal.2021.100253>
- Connor, E. E. (2015). Invited review: Improving feed efficiency in dairy production: Challenges and possibilities. *Animal*, 9(3), 395–408. <https://doi.org/10.1017/S1751731114002997>
- Cowley, F. C., Barber, D. G., Houlihan, A. V., & Poppi, D. P. (2015). Immediate and residual effects of heat stress and restricted intake on milk protein and casein composition and energy metabolism. *Journal of Dairy Science*, 98(4), 2356–2368. <https://doi.org/10.3168/jds.2014-8442>
- de Oliveira, G. B., Favarin, L., Luchese, R. H., & McIntosh, D. (2015). Psychrotrophic bacteria in milk: How much do we really know? *Brazilian Journal of Microbiology*, 46(2), 313–321. <https://doi.org/10.1590/S1517-838246220130963>
- De Vries, M. J., & Veerkamp, R. F. (2000). Energy balance of dairy cattle in relation to milk production variables and fertility. *Journal of Dairy Science*, 83(1), 62–69. [https://doi.org/10.3168/jds.S0022-0302\(00\)74856-9](https://doi.org/10.3168/jds.S0022-0302(00)74856-9)
- FAO. (2017). Food outlook October 2015: Biannual report on global food markets. In *Food outlook: Biannual report on global food markets* (Issue November). www.fao.org/publications
- Fenwick, M. A., Llewellyn, S., Fitzpatrick, R., Kenny, D. A., Murphy, J. J., Patton, J., & Wathes, D. C. (2008). Negative energy balance in dairy cows is associated with specific changes in IGF-binding protein expression in the oviduct. *Reproduction*, 135(1), 63–75. <https://doi.org/10.1530/REP-07-0243>
- Food and Agriculture Organization. (2020). OECD-FAO Agricultural Outlook 2020-2029. In *OECD Publishing Paris, /Food and Agriculture Organization of the United Nations, Rome*. OECD. <https://doi.org/10.1787/1112c23b-en>
- Food and Agriculture Organization of the United Nations. (2021). Dairy market review. *Food and Agriculture Organization of the United Nations, April*, 1–13.
- Golkar, Z., Bagasra, O., & Gene Pace, D. (2014). Bacteriophage therapy: A potential solution for the antibiotic resistance crisis. *Journal of Infection in Developing Countries*, 8(2), 129–136. <https://doi.org/10.3855/jidc.3573>
- Hanrahan, L., McHugh, N., Hennessy, T., Moran, B., Kearney, R., Wallace, M., & Shalloo, L. (2018). Factors associated with profitability in pasture-based systems of milk production. *Journal of Dairy Science*, 101(6), 5474–5485. <https://doi.org/10.3168/jds.2017-13223>
- Huijps, K., Hogeveen, H., Lam, T. J. G. M., & Oude Lansink, A. G. J. M. (2010). Costs and efficacy of management measures to improve udder health on Dutch dairy farms. *Journal of Dairy Science*, 93(1), 115–124. <https://doi.org/10.3168/jds.2009-2412>

- Jacobson, R. E. (1986). Review of Current and Future Consumption Trends for Milk and Dairy Products. *Journal of Dairy Science*, 69(5), 1447–1453. [https://doi.org/10.3168/jds.S0022-0302\(86\)80554-9](https://doi.org/10.3168/jds.S0022-0302(86)80554-9)
- Janzen, J. J. (1970). Economic Losses Resulting from Mastitis. A Review. *Journal of Dairy Science*, 53(9), 1151–1160. [https://doi.org/10.3168/jds.S0022-0302\(70\)86361-5](https://doi.org/10.3168/jds.S0022-0302(70)86361-5)
- Keys, J. E., Pearson, R. E., & Weinland, B. T. (1979). Starter Culture, Temperature, and Antibiotic Residue in Fermentation of Mastitic Milk to Feed Dairy Calves. *Journal of Dairy Science*, 62(9), 1408–1414. [https://doi.org/10.3168/jds.S0022-0302\(79\)83437-2](https://doi.org/10.3168/jds.S0022-0302(79)83437-2)
- Liu, J., Li, L., Chen, X., Lu, Y., & Wang, D. (2019). Effects of heat stress on body temperature, milk production, and reproduction in dairy cows: A novel idea for monitoring and evaluation of heat stress — A review. *Asian-Australasian Journal of Animal Sciences*, 32(9), 1332–1339. <https://doi.org/10.5713/ajas.18.0743>
- Michael, C. A., Dominey-Howes, D., & Labbate, M. (2014). The antimicrobial resistance crisis: Causes, consequences, and management. *Frontiers in Public Health*, 2(SEP), 1–8. <https://doi.org/10.3389/fpubh.2014.00145>
- Mohamed, M. A., Sheikh Elmi, A. A., Dubad, A. B., Sheikh Hassan, Y. H., Osman, A. M., & Bitrus, A. A. (2020). Antibiotic residue in raw milk collected from dairy farms and markets in Benadir, Somalia. *PAMJ - One Health*, 2(19), 1–8. <https://doi.org/10.11604/pamj-oh.2020.2.19.24814>
- Murphy, S. C., Martin, N. H., Barbano, D. M., & Wiedmann, M. (2016). Influence of raw milk quality on processed dairy products: How do raw milk quality test results relate to product quality and yield? *Journal of Dairy Science*, 99(12), 10128–10149. <https://doi.org/10.3168/jds.2016-11172>
- Muturi, E. W. (2020). Effect of Mastitis on Milk Production in Dairy Cows in Kenya. *Journal of Animal Health*, 2(1), 85–91. <https://doi.org/10.47604/jah.1170>
- Nguyen, Q. D., Tsuruta, T., & Nishino, N. (2020). Examination of milk microbiota, fecal microbiota, and blood metabolites of Jersey cows in cool and hot seasons. *Animal Science Journal = Nihon Chikusan Gakkaiho*, 91(1), e13441. <https://doi.org/10.1111/asj.13441>
- Nguyen, T. T., Wu, H., & Nishino, N. (2020). An investigation of seasonal variations in the microbiota of milk, feces, bedding, and airborne dust. *Asian-Australasian Journal of Animal Sciences*, 33(11), 1858–1865. <https://doi.org/10.5713/ajas.19.0506>
- Nisha, A. R. (2008). Antibiotic residues - A global health hazard. *Veterinary World*, 1(12), 375–377. <https://doi.org/10.5455/vetworld.2008.375-377>
- Petrović, J. M., Katić, V. R., & Bugarski, D. D. (2008). Comparative Examination of the Analysis of β -Lactam Antibiotic Residues in Milk by Enzyme, Receptor–Enzyme, and Inhibition Procedures. *Food Analytical Methods*, 1(2), 119–125. <https://doi.org/10.1007/s12161-007-9007-y>
- Polsky, L., & von Keyserlingk, M. A. G. (2017). Invited review: Effects of heat stress on dairy

- cattle welfare. *Journal of Dairy Science*, 100(11), 8645–8657. <https://doi.org/10.3168/jds.2017-12651>
- Quigley, L., O’Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews*, 37(5), 664–698. <https://doi.org/10.1111/1574-6976.12030>
- Rahman, M. S., Hassan, M. M., & Chowdhury, S. (2021). Determination of antibiotic residues in milk and assessment of human health risk in Bangladesh. *Heliyon*, 7(8), e07739. <https://doi.org/10.1016/j.heliyon.2021.e07739>
- Sachi, S., Ferdous, J., Sikder, M. H., & Azizul Karim Hussani, S. M. (2019). Antibiotic residues in milk: Past, present, and future. *Journal of Advanced Veterinary and Animal Research*, 6(3), 315–332. <https://doi.org/10.5455/javar.2019.f350>
- Smith, D. L., Smith, T., Rude, B. J., & Ward, S. H. (2013). Short communication: Comparison of the effects of heat stress on milk and component yields and somatic cell score in Holstein and Jersey cows. *Journal of Dairy Science*, 96(5), 3028–3033. <https://doi.org/10.3168/jds.2012-5737>
- Smith, G. C., & Carpenter, Z. L. (1976). Eating quality of meat animal products and their fat content. In *Fat Content and Composition of Animal Products*. <http://www.nap.edu/catalog/22>
- Summer, A., Lora, I., Formaggioni, P., & Gottardo, F. (2019). Impact of heat stress on milk and meat production. *Animal Frontiers*, 9(1), 39–46. <https://doi.org/10.1093/af/vfy026>
- Thoma, G., Popp, J., Shonnard, D., Nutter, D., Matlock, M., Ulrich, R., Kellogg, W., Kim, D. S., Neiderman, Z., Kemper, N., Adom, F., & East, C. (2013). Regional analysis of greenhouse gas emissions from USA dairy farms: A cradle to farm-gate assessment of the American dairy industry circa 2008. *International Dairy Journal*, 31(1), S29–S40. <https://doi.org/10.1016/j.idairyj.2012.09.010>
- Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences of the United States of America*, 112(18), 5649–5654. <https://doi.org/10.1073/pnas.1503141112>
- World Population Prospects 2017 - Volume II: Demographic Profiles. (2021). In *World Population Prospects 2017 - Volume II: Demographic Profiles: Vol. II*. <https://doi.org/10.18356/9789210001021>
- Yin, W., Wang, Y., Liu, L., & He, J. (2019). Biofilms: The microbial “protective clothing” in extreme environments. *International Journal of Molecular Sciences*, 20(14). <https://doi.org/10.3390/ijms20143423>
- Zhang, X., & Cai, X. (2011). Climate change impacts on global agricultural land availability. *Environmental Research Letters*, 6(1). <https://doi.org/10.1088/1748-9326/6/1/014014>

2 CHAPTER 2

LITERATURE REVIEW

2.1 The current dairy status, trends, and prospects

2.1.1 The current dairy status and trends

According to the Food and Agriculture Organization of the United Nations (2021), global milk production averaged 928 million tons in 2021, up by 1.5 percent from 2020. The most significant expansions were in Asia and North America, and expansions were moderate in Europe, Africa, Oceania, Central America, and the Caribbean. On the contrary, South America registered a slight milk output decline. In Asia, the countries leading milk production are China, India, Pakistan, and Japan, while milk production shrank in S. Korea, Syria, and Saudi Arabia. The significant Asian expansion in milk production has been driven by increased productivity of large-scale dairy farms, enhanced domestic milk production capacity, and government subsidies in the dairy sector. In other parts of the world, like South America and Africa, milk production has declined slightly or stagnated in the last few years owing to prolonged drought, high cost of production, and stiff competition from imported cheap dairy products. For instance, numerically, milk production in Asia changed from 360 million tons in 2019 to 398 million tons in 2021, while milk production remained unchanged during the same period (Africa produced 48+1 million tons in 2020 and 2021 and South America produced 80+2 million tons in 2020 and 2021).

The demand for different dairy products is on the rise/decline, depending on the determinant market drivers. For instance, skim milk product exports were anticipated to increase by 5.0 percent in 2021, nudging a record high of 2.7 million tons owing to the strong demand from China, Indonesia, Mexico, and Vietnam. On the other hand, the world Cheese trade was forecasted to increase by 4 percent to 3.6 million tons in 2021. This record increment was predicted to be driven by significant import expansions in the Russian Federation, the United States, and China. Notably, the economic growth and increasing consumer demand in China spurred a 34 percent anticipated growth in cheese imports, equivalent to about 240 000 tons.

Conversely, in 2021, butter exports were expected to slump by 2.0 percent to 1 million tons. The drop in butter exports mainly reflected the anticipated import declines in the United Kingdom, the United States, and the Russian Federation. This decline in butter exports has been ascribed to the lower shipments from the leading butter exporters like the European Union and New Zealand, which prefer to allocate the limited milk produced to making cheese –the most profitable dairy product under the current prices (Food and Agriculture Organization of the United Nations, 2021)

2.1.2 The prospects

According to the Department of Economic and Social Affairs of the United Nations ("World Popul. Prospect. 2017 - Vol. II Demogr. Profiles," 2021), the world's population is estimated to reach 9.7 billion in 2050, with the majority believed to be residing in Africa or Asia. This population increment, especially in developing countries in Asia and Africa, is expected to trigger a concomitant increment in demand for milk and milk products. Globally, this population increment is expected to increase, on the fresh milk equivalent basis, the world annual milk consumption to 119 kg per person worldwide by 2067 from the current 87 kg per person (Britt et al., 2018). In addition to the population growth, rural to urban migration, especially in developing countries, is expected to drive the demand for dairy products. In most cases, the population that migrates to the urban centers has improved per capita consumption power compared to its counterpart in rural areas. Due to the mushrooming of urban centers in Asian and African developing countries, the demand for dairy products is expected to go up. For instance, a study in India by Bowen et al., (2011) found that rural-to-urban migration prompted the increase in demand for dairy products. While the rural-to-urban migration in developing nations is anticipated to occasion a rise in demand for dairy products, such migration is not expected to affect the demand for milk products in developed countries, where the demand for milk is relatively the same in both rural and urban areas. The demand for dairy products is expected to nudge 600 billion kilograms of milk in 2067, more than it is produced today. To meet this demand, the annual bovine milk production per cow must double from 2,405 to 4,531 kg (Britt et al., 2018).

In addition to the high milk volume needed by 2067, the demand for milk solids is also expected to rise in developed countries. Most market segments in countries like the US and New Zealand will continue to favor milk solids over milk yield. Milk yield and milk solids projections by Britt et al., (2018) show that milk solids and milk yields will double simultaneously by 2067. In the countries above, among other developed countries, the emphasis will be placed on increased fat and protein yields within a lower milk volume. Though it will be challenging, it is anticipated that the demand for milk with higher solids will soar in the future. Farmers are expected to leverage genomic selection and feedstuff quality and digestibility improvements to fulfill this demand for higher milk solids.

2.2 Dairy challenges and opportunities

2.2.1 Challenges the dairy sector is facing

The dairy sector is facing myriad challenges in meeting the ever-soaring demand for milk and milk products. These challenges, if not sufficiently mitigated, can impede the full realization of the dairy sector's potential. They include the following:

2.2.1.1 Climate change and the constriction of arable land

Consistently, global temperatures have been rising, and this trend is forecasted to continue in the future. The worst aspect of this global warming is that it is severe in the northern hemisphere, where over 80 percent of the worldwide milk production occurs, according to FAO, (2017). The increased global temperature will induce heat stress in dairy cows. Heat stress, a non-specific physiological response of dairy cows to soared ambient temperatures, can substantially negatively affect dairy production (Liu et al., 2019). When the environmental temperatures are elevated, it adds a load of heat cows have to dissipate. High-producing cows consume and ferment herculean feedstuff loads, emitting a lot of heat. This situation is exacerbated by the heat emanating from the environment. This heat load overwhelms cows' already limited ability of heat dissipation, especially Holstein, owing to the relatively low surface area to body weight ratio, short, dense body surface hair, and underdeveloped sweat glands. Heat stress blights the milk production ability, not only because of the reduced feed intake, but due to physiological discomfort experienced by cows. For instance, in the report by Summer et al., (2019) cows subjected to heat stress reduced their feed ingestion and produced less milk in comparison with cows reared in a thermos-comfortable environment. Contrastingly, the decrease in milk production was not significant when cows kept in a thermo-comfortable shed were fed the same quantity of feed ingested by the heat-stressed group. Additionally, when heat-stressed cows returned to the thermo-comfortable shed, milk production increased to the physiological normal level. This finding demonstrated that milk production by heat-stressed cows is not entirely due to the heat-induced feed ingestion reduction. Bernabucci et al., (2010) found a decrease of 0.27 kg of milk per temperature humidity index (THI) unit when Holstein cows were exposed to THI higher than 68. Other studies reported a decline in milk production as higher as 0.41 kg per cow per day for each THI unit increment above 69.

A hot and humid environment not only affects milk yield, but it also negatively affects milk quality as well. Reports have been inconclusive regarding the impact of heat stress on milk fat. Some reports like Bernabucci et al., (2015) showed a significant decline in milk fat during summer (3.20 g/100 g) as opposed with the values observed in winter (3.80 g/100 g) and in spring (3.61 g/100 g). On the other hand, Cowley et al., 2015) found no significant differences for milk fat content between cows in thermos-comfortable barn and those subjected to heat stress. Elevated THI influence on milk protein has been crystal-clear, and the reports by Bernabucci et al., (2015) & Cowley et al., (2015) were in agreement.

In a study by Bernabucci et al., (2015) higher milk casein content was reported in winter (2.75 g/100 g) and spring (2.48 g/100 g) than in the summer season (2.27 g/100 g). On the other hand, Cowley et al., (2015) found that cows subjected to heat stress produced milk with less protein than cows kept in a thermo-comfortable environment. Interestingly, Cowley et al., (2015) demonstrated that the decline in milk protein content is largely related to the direct effect of heat stress, contrary to what was believed to be due to a reduction in feed intake. Concerning the proportion of casein in milk, Cowley et al., (2015) found that the differences between cows kept in a temperature-comfortable shed and the group heat-stressed were 28.1 and 26.8 g/L.

Climatic changes will lead to a reduction in the acreage of arable land. For instance, Africa and South America, where about 40% of arable land is situated, according to Zhang & Cai, (2011), will lose 0.5– 18% and 1–21% of arable land, respectively. This kind of arable land loss occasioned by global warming is expected in other regions such as Europe and Asia. Although the greatest potential for agricultural expansion lies in South America and Africa, which have just put about 20% of their arable land into agriculture, the realization of the full potential might be stymied by the rapid population growth in the said regions. According to Britt et al., (2018), countries with less arable land per capita will increasingly be compelled to convert the use of the limited arable land they possess from growing animal fodder to producing human food. Some currently existing permanent grassland will likely be converted to arable land in developing countries, which will significantly reduce land for grazing. This will, in the long run, have dire sequels on dairy production; if not aptly bridled, it might hamper the anticipated increment in milk production.

2.2.1.2 Feed efficiency and farm profitability

Feed efficiency, which in dairy science refers to the quantity of milk produced per kilogram of dry matter consumed, highly influences a farm's profitability. Higher feed costs and a slump in milk prices, coupled with other existing dairy challenges, negatively affect the profit margin of dairy enterprises. The profit margin declines because the cost of feeds generally represents about 60% of the total cost of milk production (Connor, 2015). This cost is only comparable to poultry and swine production only for lactating cows. However, in dairy production, there is a stage during heifer rearing where heifers consume feeds but produce no milk and during the typical 2-month dry period. Hence, computing dairy production's feed efficiency is more intricate than the above-mentioned production systems. In addition to the importance of efficient feed conversion in farm profitability, it is also of paramount importance to the general environmental health. Currently, greater emphasis is put on curtailing the emission of greenhouse gases (GHG) from animal production. A study by Thoma et al., (2013) reported that the three most significant contributors to GHG emissions in the US dairy farms were feed production, enteric methane and manure. Further, they attributed the GHG differences between farms to feed conversion efficiency. Feed efficiency was regarded as the major contributor to the farm-to-farm differences since feed is the primary farm input. Hence, feed efficiency, combined with a dairy cow's

specific genetic potential and physiological status, directly affects both enteric emissions and the quantity and quality of manure excreted.

Since dairy farms are commercial enterprises, they must be profitable. Hence, profitability is critically essential to sustainability of dairy farms. The profitability of dairy farms highly hinges on the average fixed cost per cow or unit of milk sold, the price of milk in the marketplace, and the feed cost. Britt et al., (2018) averred that feed represents the highest production cost incurred by farmers irrespective of their scale of production, their location, or the local cost of feed. The average fixed cost per unit of milk produced by a dairy farm may favor small-scale producers who heavily rely on unpaid family labor and sink negligible outlay in dairy technology and equipment. However, it is important to note that although large-scale farmers incur colossal sums in farm machinery, equipment, land, labor, and technology, they have the advantage of economies of scale. Milk is the only commodity dairy farmers sell to keep their enterprises afloat and profitable. So, the stability of milk prices is of paramount importance. However, the volatility in milk prices is inevitable due to the imbalance of demand and supply. Dairy farmers' profits nosedive when the demand for milk slumps since it is relatively easier to increase milk output when demand increases but difficult to reduce milk output when supply exceeds demand. Britt et al., (2018) exemplified the importance and impact of volatility in milk prices. In their report, they looked at the volatility of milk prices in New Zealand in the last two decades, and they concluded that such price volatility will definitely edge out less profitable farms.

Hanrahan et al., (2018) reported that supplementary feeds usually reduce the profitability of any dairy farm, especially during peak production periods responding to high demand for milk. When the demand for milk is higher, farmers resort to the excessive use of supplementary feeds to boost milk production. This excessive use of supplemental feeds may eat into the net profit farmers earn.

2.2.1.3 Animal health and antibiotic residues in milk

The most globally widespread and costliest animal disease with a direct negative impact on milk production is mastitis (Abebe et al., 2016). Mastitis, an inflammation of the mammary gland, is caused by infectious or environmental pathogens. Since mastitis is multi-etiological, it is a complex disease in terms of its control and treatment. It is typically characterized by chemical, physical, and bacteriological changes in the milk. Also, glandular tissues undergo pathological changes, particularly in severe clinical cases. It is usually classified as either clinical or subclinical based on the visibility of inflammation of the mammary gland or its effects on milk. Subclinical mastitis (SCM) does not manifest visible symptoms on the udder or visible milk quality defects.

In most cases, elevated SCC is used as an indicator for SCM. Clinical mastitis (CM), on the other hand, manifests visible clinical signs such as udder swelling and redness. Both categories of mastitis blight the profits farmers earn by reducing milk yield, jettisoning mastitic milk, and premature culling of animals bedeviled with mastitis (Muturi, 2020). Huijps et al., (2010)

identified the reduction in milk production as the loss component with the severest economic impact on the total cost of mastitis. Janzen, (1970) reported that losses in total milk production due mastitis range from 5.0 to 25.0%. Similar findings were reported by Azooz et al., (2020), where a decrease in milk production and quality premium losses were the most critical mastitis-induced loss components. They argued that although the losses vary, reduced milk production is the most enormous indirect cost related with mastitis. They further termed losses incurred due to high SCC as hidden costs or a lost opportunity for income. The magnitude of the other two components of the losses incurred due to mastitis, milk disposal and culling, hinges on the severity of the disease. According to Aghamohammadi et al., (2018) the most considerable percentage of the costs, 48%, was attributed to SCM, 34% was attributed to CM, and 15% was incurred in implementing preventive measures. Their questionnaire survey found that for SCM cases, the two most costly components of the losses were the resultant milk yield reduction and culling at 72 and 25% of SCM costs, respectively. On the other hand, the first, second, and third most crucial cost components for CM costs were culling at 48%, milk yield reduction losses averaged out at 34%, and discarded milk at 11% of CM costs.

It is estimated that the world used over $63,151 \pm 1,560$ tons of antimicrobials over a decade ago (Van Boeckel et al., 2015). This usage is believed to have increased several folds. In fact, it is believed that the amount of antibiotics used in the livestock industry is double the amount used by humans (Aarestrup, 2012). In the dairy industry, antimicrobials are used for prophylactic and/or therapeutic purposes. However, antibiotics have recently been administered not just to treat or prevent infections but also for growth promotion and feed additives (Sachi et al., 2019). This unscrupulous act poses a serious threat to human health. Since milk is consumed globally, it has become an apt medium for transmitting antimicrobial residues. With studies reporting that significant portions, in the region of 30%–70%, of antibiotics are released unaltered into animal products like milk (Sachi et al., 2019), the challenge of antibiotic residues in the dairy sector has been a concern to both processors and consumers. When farmers administer antimicrobials to dairy animals for the abovementioned purposes, most antimicrobial drugs are metabolized for detoxification and excretion. The excretion of antimicrobial drugs is through urine and feces. However, after excretion, a significant portion of the drugs persists in milk as residues. If the residue concentration exceeds the permitted level, it is termed antibiotic residues. Antibiotic residues persist in milk if the recommended withdrawal period for residues to decline to negligible or safe limits is not observed. Once the residues get to ready-to-consume animal-derived foods like milk, they pose three major health concerns. These concerns are the potential threat to direct toxicity to humans, the alteration of human gut microflora, and the possible antibiotic therapy failure in clinical situations due to the development of resistant bacterial strains (Nisha, 2008 & Rahman et al., 2021). Additionally, hypersensitive milk consumers develop allergic responses to antibiotic residues in milk exceeding antibiotic residues limit (Petrović et al., 2008).

To milk processors, antibiotic residues in milk exceeding the acceptable antibiotic residues limit pose a technological problem due to their capacity to partially or totally inhibit or slow down the growth of starter cultures used in milk product processing (Keys et al., 1979). The fermentation of milk can either be slowed down, partially inhabited, or completely stopped depending on the active substance of the antibiotic residue, the residual concentration of the active antibiotic residue, and the sensitivity of bacterial cultures used to ferment the milk.

Antibiotic resistance is the other negative impact of antimicrobial residues in animal products like milk. Antibiotics are used to prevent and treat animal diseases; hence, Brown et al., (2020) termed them as insurance against livestock losses to diseases. In developed countries, the production, sale, access, prescription, and use of antibiotics used (is) to be strictly controlled. However, with the advent of online shopping, widespread and affordable expedited access to even currently restricted antimicrobial drugs such as rifampicin and ciprofloxacin is possible (Michael et al., 2014). This hassle-free access to antimicrobial drugs in developed countries may erode the gains made in such countries.

On the other hand, antibiotics are heavily used in countries with high prevalence and incidence of animal disease, especially in developing nations (Mohamed et al., 2020). In such a setting, as Brown et al., (2020) & Mohamed et al., (2020) intimated, there is little or no capacity for testing antibiotic residues in milk, regulations for the use of antimicrobial agents are not strictly complied with, and there is no enforcement of antibiotic withdrawal periods. Because of the aforementioned shortcomings in such settings, farmers end up misusing, overusing, or indiscriminately administering antibiotics with no observance of the recommended withdrawal period. Consequently, antibiotics are shed into milk and other animal products. Consumers end up ingesting milk and milk products with excessive quantities of antimicrobial residues.

What's more, the most considerable portion of antimicrobial drugs is metabolized and excreted by animals into the environment via urine and feces (Sachi et al., 2019). This long-term exposure to antibiotics through the milk and other animal-derived food leads to the development of antibiotic-resistant bacterial strains. The excretion of antibiotics into the environment leads to the growth and proliferation of antibiotic-resistant bacterial strains. This is because the occurrence of antibiotics in the environment may lead to genetic or mutational changes in environmental microbiota. Environmental microbiota that initially was sensitive to antibiotics becomes resistant; hence such bacteria survive antibiotic exposure and proliferate as antibiotic-resistant bacteria (Ben et al., 2019). The ramifications of the emergence of antibiotic-resistant bacteria are dire and include frequent hospitalization, lengthy hospitalization, and the severity of diseases that used to be easily curable (Mohamed et al., 2020). In some severe cases, humans succumb to illnesses caused by antibiotic-resistant strains. For instance, as reported by (Golkar et al., 2014), antibiotic-resistant bacterial infections take a heavy toll on human lives. In the US, methicillin-resistant *Staphylococcus aureus* kills more Americans yearly than combined emphysema, HIV/AIDS, Parkinson's disease, and homicide.

The other clinically unrelated concern in the dairy industry emanating from antimicrobial drugs is the slowdown in research, development, and production of new antibiotics. Golkar et al., (2014) intimated that the development of new antibiotics has slowed down significantly, and in some cases, the production of antibiotics stopped. This reality, according to them, is due to market failure since the production of antibiotics is no longer as profitable as the production of drugs for chronic illnesses, diabetes, and asthma. This situation is compounded by the lack of incentives, delayed publishing of workable guidelines that instruct how companies should design antimicrobial clinical trials, and slow adoption of the new antibiotics by physicians. These economic and regulatory disincentives have demoralized pharmaceutical companies, and if nothing is urgently done, the future of antibiotics might be bleak. The world might be back to the era before the advent of antibiotics.

2.2.1.4 Milk quality and safety

Milk quality depends on chemical parameters (fat and protein content and absence of inhibitory substances). In contrast, milk safety largely hinges on the amount and type of microbes present in milk (Fusco et al., 2020). From a microbiological viewpoint, microbes affect both milk quality and safety. For this reason, milk processors put emphasis on the microbial quality of raw milk at processing factories' reception (Munsch-Alatossava & Alatossava, 2019). This microbiological threshold (total bacteria count) in raw milk varies among countries; it ranges from below 100,000 cfu/ml to below 300, 000 cfu/ml. According to Murphy et al., (2016), the quality of processed dairy products depends on the microbiological quality of raw milk, so the quality of processed dairy products is not harmonious. Microbiologically poor-quality raw milk leads to quality defects in processed milk products due to the deteriorative actions of heat-resistant bacteria-produced enzymes. The quality defects are gelling and development of fruity rancid and/or bitter taste (Marchand et al., 2009 & von Neubeck et al., 2015)

Milk safety concerns stem from milk contamination. Raw bovine milk has various sources of contamination ranging from feed, the environment (air, water, barn, and pasture), to the cow's udder (Fusco et al., 2020 & Munsch-Alatossava & Alatossava, 2019). It becomes difficult to effectively prevent contamination to achieve the microbiological threshold, especially in developing nations (Kiambi et al., 2022 & Nyokabi et al., 2021). Since raw milk, without pasteurization, is still consumed both in developing and developed countries (Fusco et al., 2020, Kiambi et al., 2022 & States et al., 2017) due to the affordability and accessibility of pasteurized milk and the desire to consume the wholesome milk, respectively. If milk is contaminated with potentially pathogenic microbes, consumers are at risk of food-borne diseases (FDA, 2020 & States et al., 2017). *Salmonella spp.*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 are some of the bacteria associated with food-borne illnesses emanating from milk, especially raw milk (Fusco et al., 2020). Pasteurization is usually intended to reduce pathogenic microorganisms in milk, if present, to a level that cannot pose a significant health hazard. What's more, pasteurization used to be aimed primarily at reducing the load of *Mycobacterium tuberculosis* in milk if present; presently, however, there are many pathogens of safety concerns

as above-mentioned (Fusco et al., 2020). Also, milk contamination with quality-deteriorative and health-injurious bacteria has been reported (Tracy, 2008 & Martin et al., 2018).

2.2.2 The possible opportunities in the dairy sector that can be tapped into

As mentioned above, the dairy sector is bedeviled by a myriad of challenges that, if not mitigated or circumvented, can thwart the full realization of its potential.

2.2.2.1 Mitigation of methane emission

The dairy sector should promptly address sector-caused climate change by either accelerating its current efforts and/or adopting more innovative strategies to mitigate greenhouse gas emissions.

The current mitigation strategies that must be ramped up include the following: improving digestibility is touted as a promising strategy that can continue to suppress methane production and emission by dairy cows. According to Gerber et al., (2011), incorporation of more digestible feed ingredients into dairy cows' diet (e.g. non-fermentable carbohydrates), and/or by enhancing the efficiency with which lactating cows use the diet (e.g. through physical, chemical or enzymatic pre-feeding treatments) will lead to a marked reduction in methane emission per unit of milk output. While this strategy is promising, it has a limit that cannot be exceeded due to the potential adverse health consequences, rumen acidosis, of diets containing very high levels of fermentable carbohydrates.

On the other hand, improving animal health and reproductive efficiency is another strategy that is being used to reduce methane emissions by dairy cows and should be accelerated. Poor animal health is attributed to reduced feed intake, digestibility, and increased inefficiency at nutrient utilization, leading to an increment in methane emission. For instance, Mostert et al., (2018) & Özkan Gülzari et al., (2018) found out the prevention of different types of foot lesions and subclinical mastitis, respectively, could significantly reduce methane emissions. Poor reproductivity means that more breeding animals are needed in the herd to achieve production targets and more culling and replacements are also required to maintain the herd size (Llonch et al., 2017). Garnsworthy, (2004), through a modeling approach, predicted that simple restoration of fertility to the 1995 level could reduce methane emission by 12%, while further improvements in herd fertility could result in methane reduction by up to 24%.

Feed additives are proving to be game-changers in the bid to reduce enteric methane emission. For instance, microalgae, such as euglena, have elevated lipids (PUFA), resulting in a significant reduction in enteric methane (Abbott et al., 2020). Lean et al. (2021) reported that microalgae significantly reduced methane emissions and boosted milk production, fat, and protein. The use of rumen modifiers to create unfavorable conditions for methanogens or suppression of the activities of bacteria in a symbiotic relationship with methanogens is emerging as a promising strategy to lower enteric methane production (Fouts et al., 2022). Nitrates are becoming reliable rumen modifiers to reduce enteric methane emission by creating an alternative H₂ sink and/or

through nitrite toxicity; Zhou et al., (2012) found out that methanogens could hardly survive nitrite toxicity in the rumen.

Selective breeding has considerable potential for sustained long-term enteric methane over generations (Fouts et al., 2022). Since enteric methane emissions vary between breeds, individual animals, and across the animal's lifetime, according to De Haas et al., (2011), the direct selection of low-emitting cows for breeding based on emission measurements is still promising. Though at its nascent stage, genome selection for methane emission reduction also has a vast potential for a sustained long-term worldwide reduction in enteric methane. Manzanilla-Pech et al., (2022) found a strong association for methane production on chromosome 13 after conducting a genome-wide association study on 1,962 Danish Holstein dairy cattle and 38,253 SNPs. Though they acknowledge that there is a lot to be done, researchers in this sector agree that once methane measurement technologies improve and data on genotypes and phenotypes are acquired, the integration of genome selection into breeding programs has a huge potential to reduce methane emissions throughout the world.

2.2.2.2 Mitigating mastitis and antimicrobial resistance

Since mastitis results from a failure in the intricate interrelationship between three factors: host resistance, mastitis-causing bacteria, and the environment, conventional means to prevent mastitis may be inefficient (Pyörälä, 2002). National Mastitis Council has developed a 5-point mastitis control program. However, those procedures are less effective against environmental pathogens, especially *Streptococcus uberis*, which accounts for a significant proportion of subclinical mastitis (Kerro Dego, 2021). Later on, environmental hygiene and comfort were included in a 10-point control program. Environmental hygiene has been underscored as an essential mastitis-combating approach. Since antibiotics are proving ineffective due to the resistance of mastitis pathogens against antimicrobials in use, vaccines trigger local production of immunoglobulins which neutralize endotoxin and activate complement cell-mediated immunity. In general, vaccines do not prevent new mastitis infections but significantly lessen the clinical severity of mastitis, reduce the infection duration, and have less effect on milk production (Kerro Dego, 2021 & Pyörälä, 2002). It is worth noting that vaccines against mastitis have not had significant success; hence, much research effort is supposed to be devoted to making vaccines more efficacious. Therapeutically, antibiotic treatment has become less effective due to the development of antibiotic-resistant pathogenic strains. Plant-derived compounds are advantageous over antibiotics since they do not induce resistance against pathogens even after prolonged exposure. Also, plant-derived compounds are less toxic and can inhibit the inflammation caused by mastitis pathogens (Wn & Sg, 2020).

To circumvent the antimicrobial resistance witnessed in animal husbandry, researchers like Baker et al., (2018) have suggested various technologies that can be applied to address antimicrobial resistance. Technologies like active uptake approaches, potentiation, and reverse vaccinology are some of the promising ways to address the ever-soaring antimicrobial resistance.

References

- Aarestrup, F. (2012). Sustainable farming: Get pigs off antibiotics. *Nature*, 486(7404), 465–466. <https://doi.org/10.1038/486465a>
- Abbott, D. W., Aasen, I. M., Beauchemin, K. A., Grondahl, F., Gruninger, R., Hayes, M., Huws, S., Kenny, D. A., Krizsan, S. J., Kirwan, S. F., Lind, V., Meyer, U., Ramin, M., Theodoridou, K., Soosten, D. von, Walsh, P. J., Waters, S., & Xing, X. (2020). Seaweed and seaweed bioactives for mitigation of enteric methane: Challenges and opportunities. *Animals*, 10(12), 1–28. <https://doi.org/10.3390/ani10122432>
- Abebe, R., Hatiya, H., Abera, M., Megersa, B., & Asmare, K. (2016). Bovine mastitis: Prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. *BMC Veterinary Research*, 12(1), 1–11. <https://doi.org/10.1186/s12917-016-0905-3>
- Aghamohammadi, M., Haine, D., Kelton, D. F., Barkema, H. W., Hogeveen, H., Keefe, G. P., & Dufour, S. (2018). Herd-level mastitis-associated costs on Canadian dairy farms. *Frontiers in Veterinary Science*, 5(MAY), 1–12. <https://doi.org/10.3389/fvets.2018.00100>
- Azooz, M. F., El-Wakeel, S. A., & Yousef, H. M. (2020). Financial and economic analyses of the impact of cattle mastitis on the profitability of Egyptian dairy farms. *Veterinary World*, 13(9), 1750–1759. <https://doi.org/10.14202/vetworld.2020.1750-1759>
- Baker, S. J., Payne, D. J., Rappuoli, R., & De Gregorio, E. (2018). Technologies to address antimicrobial resistance. *Proceedings of the National Academy of Sciences of the United States of America*, 115(51), 12887–12895. <https://doi.org/10.1073/pnas.1717160115>
- Ben, Y., Fu, C., Hu, M., Liu, L., Wong, M. H., & Zheng, C. (2019). Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review. *Environmental Research*, 169(July 2018), 483–493. <https://doi.org/10.1016/j.envres.2018.11.040>
- Bernabucci, U., Basiricò, L., Morera, P., Dipasquale, D., Vitali, A., Piccioli Cappelli, F., & Calamari, L. (2015). Effect of summer season on milk protein fractions in Holstein cows. *Journal of Dairy Science*, 98(3), 1815–1827. <https://doi.org/10.3168/jds.2014-8788>
- Bernabucci, U., Lacetera, N., Baumgard, L. H., Rhoads, R. P., Ronchi, B., & Nardone, A. (2010). Metabolic and hormonal acclimation to heat stress in domesticated ruminants. *Animal*, 4(7), 1167–1183. <https://doi.org/10.1017/S175173111000090X>
- Bowen, L., Ebrahim, S., de Stavola, B., Ness, A., Kinra, S., Bharathi, A. V., Prabhakaran, D., & Reddy, K. S. (2011). Dietary intake and rural-urban migration in India: A cross-sectional study. *PLoS ONE*, 6(6), 1–8. <https://doi.org/10.1371/journal.pone.0014822>
- Britt, J. H., Cushman, R. A., Dechow, C. D., Dobson, H., Humblot, P., Hutjens, M. F., Jones, G. A., Ruegg, P. S., Sheldon, I. M., & Stevenson, J. S. (2018). Invited review: Learning from the future—A vision for dairy farms and cows in 2067. *Journal of Dairy Science*, 101(5), 3722–3741. <https://doi.org/10.3168/jds.2017-14025>

- Brown, K., Mugoh, M., Call, D. R., & Omulo, S. (2020). Antibiotic residues and antibiotic-resistant bacteria detected in milk marketed for human consumption in Kibera, Nairobi. *PLoS ONE*, 15(5), 1–8. <https://doi.org/10.1371/journal.pone.0233413>
- Connor, E. E. (2015). Invited review: Improving feed efficiency in dairy production: Challenges and possibilities. *Animal*, 9(3), 395–408. <https://doi.org/10.1017/S1751731114002997>
- Cowley, F. C., Barber, D. G., Houlihan, A. V., & Poppi, D. P. (2015). Immediate and residual effects of heat stress and restricted intake on milk protein and casein composition and energy metabolism. *Journal of Dairy Science*, 98(4), 2356–2368. <https://doi.org/10.3168/jds.2014-8442>
- De Haas, Y., Windig, J. J., Calus, M. P. L., Dijkstra, J., de Haan, M., Bannink, A., & Veerkamp, R. F. (2011). Genetic parameters for predicted methane production and potential for reducing enteric emissions through genomic selection. *Journal of Dairy Science*, 94(12), 6122–6134. <https://doi.org/10.3168/jds.2011-4439>
- FAO. (2017). Food outlook October 2015: Biannual report on global food markets. In *Food outlook: Biannual report on global food markets* (Issue November). www.fao.org/publications
- FDA. (2020). The Dangers of Raw. *Food Facts*, August, 1–4.
- Food and Agriculture Organization of the United Nations. (2021). Dairy market review. *Food and Agriculture Organization of the United Nations*, April, 1–13.
- Fouts, J. Q., Honan, M. C., Roque, B. M., Tricarico, J. M., & Kebreab, E. (2022). Enteric methane mitigation interventions. *Translational Animal Science*, 6(2), 1–16. <https://doi.org/10.1093/tas/txac041>
- Fusco, V., Chieffi, D., Fanelli, F., Logrieco, A. F., Cho, G. S., Kabisch, J., Böhnlein, C., & Franz, C. M. A. P. (2020). Microbial quality and safety of milk and milk products in the 21st century. *Comprehensive Reviews in Food Science and Food Safety*, 19(4), 2013–2049. <https://doi.org/10.1111/1541-4337.12568>
- Garnsworthy, P. C. (2004). The environmental impact of fertility in dairy cows: A modelling approach to predict methane and ammonia emissions. *Animal Feed Science and Technology*, 112(1–4), 211–223. <https://doi.org/10.1016/j.anifeedsci.2003.10.011>
- Gerber, P., Vellinga, T., Opio, C., & Steinfeld, H. (2011). Productivity gains and greenhouse gas emissions intensity in dairy systems. *Livestock Science*, 139(1–2), 100–108. <https://doi.org/10.1016/j.livsci.2011.03.012>
- Golkar, Z., Bagasra, O., & Gene Pace, D. (2014). Bacteriophage therapy: A potential solution for the antibiotic resistance crisis. *Journal of Infection in Developing Countries*, 8(2), 129–136. <https://doi.org/10.3855/jidc.3573>
- Hanrahan, L., McHugh, N., Hennessy, T., Moran, B., Kearney, R., Wallace, M., & Shalloo, L. (2018). Factors associated with profitability in pasture-based systems of milk production. *Journal of Dairy Science*, 101(6), 5474–5485. <https://doi.org/10.3168/jds.2017-13223>

- Huijps, K., Hogeveen, H., Lam, T. J. G. M., & Oude Lansink, A. G. J. M. (2010). Costs and efficacy of management measures to improve udder health on Dutch dairy farms. *Journal of Dairy Science*, 93(1), 115–124. <https://doi.org/10.3168/jds.2009-2412>
- Janzen, J. J. (1970). Economic Losses Resulting from Mastitis. A Review. *Journal of Dairy Science*, 53(9), 1151–1160. [https://doi.org/10.3168/jds.S0022-0302\(70\)86361-5](https://doi.org/10.3168/jds.S0022-0302(70)86361-5)
- Kerro Dego, O. (2021). Control and Prevention of Mastitis: Part Two. *Animal Reproduction in Veterinary Medicine*, 1–11. <https://doi.org/10.5772/intechopen.93484>
- Keys, J. E., Pearson, R. E., & Weinland, B. T. (1979). Starter Culture, Temperature, and Antibiotic Residue in Fermentation of Mastitic Milk to Feed Dairy Calves. *Journal of Dairy Science*, 62(9), 1408–1414. [https://doi.org/10.3168/jds.S0022-0302\(79\)83437-2](https://doi.org/10.3168/jds.S0022-0302(79)83437-2)
- Kiambi, S., Fèvre, E. M., Alarcon, P., Gitahi, N., Masinde, J., Kang’ethe, E., Aboje, G., Rushton, J., & Onono, J. O. (2022). Assessment of Milk Quality and Food Safety Challenges in the Complex Nairobi Dairy Value Chain. *Frontiers in Veterinary Science*, 9(June). <https://doi.org/10.3389/fvets.2022.892739>
- Lean, I. J., Golder, H. M., Grant, T. M. D., & Moate, P. J. (2021). A meta-analysis of effects of dietary seaweed on beef and dairy cattle performance and methane yield. *PLoS ONE*, 16(7 July), 1–16. <https://doi.org/10.1371/journal.pone.0249053>
- Listeria Outbreak in Massachusetts*. (n.d.).
- Liu, J., Li, L., Chen, X., Lu, Y., & Wang, D. (2019). Effects of heat stress on body temperature, milk production, and reproduction in dairy cows: A novel idea for monitoring and evaluation of heat stress — A review. *Asian-Australasian Journal of Animal Sciences*, 32(9), 1332–1339. <https://doi.org/10.5713/ajas.18.0743>
- Llonch, P., Haskell, M. J., Dewhurst, R. J., & Turner, S. P. (2017). Current available strategies to mitigate greenhouse gas emissions in livestock systems: An animal welfare perspective. *Animal*, 11(2), 274–284. <https://doi.org/10.1017/S1751731116001440>
- Manzanilla-Pech, C. I. V., Difford, G. F., Sahana, G., Romé, H., Løvendahl, P., & Lassen, J. (2022). Genome-wide association study for methane emission traits in Danish Holstein cattle. *Journal of Dairy Science*, 105(2), 1357–1368. <https://doi.org/10.3168/jds.2021-20410>
- Marchand, S., Vandriesche, G., Coorevits, A., Coudijzer, K., Jonghe, V. De, Dewettinck, K., Vos, P. De, Devreese, B., Heyndrickx, M., & Block, J. De. (2009). International Journal of Food Microbiology Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. *International Journal of Food Microbiology*, 133(1–2), 68–77. <https://doi.org/10.1016/j.ijfoodmicro.2009.04.027>
- Martin, N. H., Boor, K. J., & Wiedmann, M. (2018). Symposium review: Effect of post-pasteurization contamination on fluid milk quality. *Journal of Dairy Science*, 101(1), 861–870. <https://doi.org/10.3168/jds.2017-13339>
- Michael, C. A., Dominey-Howes, D., & Labbate, M. (2014). The antimicrobial resistance crisis:

- Causes, consequences, and management. *Frontiers in Public Health*, 2(SEP), 1–8. <https://doi.org/10.3389/fpubh.2014.00145>
- Mohamed, M. A., Sheikh Elmi, A. A., Dubad, A. B., Sheikh Hassan, Y. H., Osman, A. M., & Bitrus, A. A. (2020). Antibiotic residue in raw milk collected from dairy farms and markets in Benadir, Somalia. *PAMJ - One Health*, 2(19), 1–8. <https://doi.org/10.11604/pamj-oh.2020.2.19.24814>
- Mostert, P. F., van Middelaar, C. E., de Boer, I. J. M., & Bokkers, E. A. M. (2018). The impact of foot lesions in dairy cows on greenhouse gas emissions of milk production. *Agricultural Systems*, 167(September), 206–212. <https://doi.org/10.1016/j.agsy.2018.09.006>
- Munsch-Alatossava, P., & Alatossava, T. (2019). Quality and Safety of Bovine Raw Milk: Present Challenges and Technological Solutions. *Milk Production, Processing and Marketing*, 1–16. <https://doi.org/10.5772/intechopen.83507>
- Murphy, S. C., Martin, N. H., Barbano, D. M., & Wiedmann, M. (2016). Influence of raw milk quality on processed dairy products: How do raw milk quality test results relate to product quality and yield? *Journal of Dairy Science*, 99(12), 10128–10149. <https://doi.org/10.3168/jds.2016-11172>
- Muturi, E. W. (2020). Effect of Mastitis on Milk Production in Dairy Cows in Kenya. *Journal of Animal Health*, 2(1), 85–91. <https://doi.org/10.47604/jah.1170>
- Nisha, A. R. (2008). Antibiotic residues - A global health hazard. *Veterinary World*, 1(12), 375–377. <https://doi.org/10.5455/vetworld.2008.375-377>
- Nyokabi, S., Luning, P. A., de Boer, I. J. M., Korir, L., Muunda, E., Bebe, B. O., Lindahl, J., Bett, B., & Oosting, S. J. (2021). Milk quality and hygiene: Knowledge, attitudes and practices of smallholder dairy farmers in central Kenya. *Food Control*, 130(June), 108303. <https://doi.org/10.1016/j.foodcont.2021.108303>
- Özkan Gülzari, Ş., Vosough Ahmadi, B., & Stott, A. W. (2018). Impact of subclinical mastitis on greenhouse gas emissions intensity and profitability of dairy cows in Norway. *Preventive Veterinary Medicine*, 150(November 2017), 19–29. <https://doi.org/10.1016/j.prevetmed.2017.11.021>
- Petrović, J. M., Katić, V. R., & Bugarski, D. D. (2008). Comparative Examination of the Analysis of β -Lactam Antibiotic Residues in Milk by Enzyme, Receptor–Enzyme, and Inhibition Procedures. *Food Analytical Methods*, 1(2), 119–125. <https://doi.org/10.1007/s12161-007-9007-y>
- Pyörälä, S. (2002). New strategies to prevent mastitis. *Reproduction in Domestic Animals*, 37(4), 211–216. <https://doi.org/10.1046/j.1439-0531.2002.00378.x>
- Rahman, M. S., Hassan, M. M., & Chowdhury, S. (2021). Determination of antibiotic residues in milk and assessment of human health risk in Bangladesh. *Heliyon*, 7(8), e07739. <https://doi.org/10.1016/j.heliyon.2021.e07739>
- Sachi, S., Ferdous, J., Sikder, M. H., & Azizul Karim Hussani, S. M. (2019). Antibiotic residues

- in milk: Past, present, and future. *Journal of Advanced Veterinary and Animal Research*, 6(3), 315–332. <https://doi.org/10.5455/javar.2019.f350>
- States, U., Costard, S., Espejo, L., Groenendaal, H., & Zagmutt, F. J. (2017). 15-1603 *Unpasteurized Products Outbreaks*. 23(6), 2009–2014.
- Summer, A., Lora, I., Formaggioni, P., & Gottardo, F. (2019). Impact of heat stress on milk and meat production. *Animal Frontiers*, 9(1), 39–46. <https://doi.org/10.1093/af/vfy026>
- Thoma, G., Popp, J., Shonnard, D., Nutter, D., Matlock, M., Ulrich, R., Kellogg, W., Kim, D. S., Neiderman, Z., Kemper, N., Adom, F., & East, C. (2013). Regional analysis of greenhouse gas emissions from USA dairy farms: A cradle to farm-gate assessment of the American dairy industry circa 2008. *International Dairy Journal*, 31(1), S29–S40. <https://doi.org/10.1016/j.idairyj.2012.09.010>
- Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences of the United States of America*, 112(18), 5649–5654. <https://doi.org/10.1073/pnas.1503141112>
- von Neubeck, M., Baur, C., Krewinkel, M., Stoeckel, M., Kranz, B., Stressler, T., Fischer, L., Hinrichs, J., Scherer, S., & Wenning, M. (2015). Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *International Journal of Food Microbiology*, 211, 57–65. <https://doi.org/10.1016/j.ijfoodmicro.2015.07.001>
- Wn, C., & Sg, H. (2020). Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments - A review. *Asian-Australasian Journal of Animal Sciences*, 33(11), 1699–1713. <https://pubmed.ncbi.nlm.nih.gov/32777908/>
- World Population Prospects 2017 - Volume II: Demographic Profiles. (2021). In *World Population Prospects 2017 - Volume II: Demographic Profiles: Vol. II*. <https://doi.org/10.18356/9789210001021>
- Zhang, X., & Cai, X. (2011). Climate change impacts on global agricultural land availability. *Environmental Research Letters*, 6(1). <https://doi.org/10.1088/1748-9326/6/1/014014>
- Zhou, Z., Yu, Z., & Meng, Q. (2012). Effects of nitrate on methane production, fermentation, and microbial populations in in vitro ruminal cultures. *Bioresource Technology*, 103(1), 173–179. <https://doi.org/10.1016/j.biortech.2011.10.013>

3 CHAPTER 3

Changes in the microbiota of milk & rumen fluid, milk production, and blood metabolites of Jersey cows throughout a lactation period

3.1 Abstract

The imminent paradigm shift to the sustainable production of quality milk leads to increased adoption of Jersey cows worldwide. This budding adoption of Jersey cows is against the backdrop of a paucity of information on how milk microbiota changes as the lactation period of Jersey cows progresses in relation to metabolic profiles and ruminal microbiota. To that end, we collected rumen fluid, milk, and blood samples every other month from eight healthy lactating Jersey cows from the beginning to the end of their lactation period. Blood metabolites were determined by the use of commercial analysis kits and microbiota of milk and rumen fluid was examined by Illumina MiSeq sequencing. NEFA, GOT, and Haptoglobin were low during the early stage of lactation, while GPT, Phosphorus, and total protein were relatively higher towards the end of the lactation. Burkholderiaceae and Oxalobacteriaceae were the predominant milk microbiota, and the two taxa changed inconsistently across the lactation. The core milk taxa were statistically unchanged across the entire lactation. Prevotellaceae and Rikenellaceae predominated the microbiota of rumen, while S24-7 and Lactobacillaceae were the most abundant taxa in cowshed's airborne dust. According to PCoA, the microbiota of airborne dust was closely clustered with milk microbiota. This clustering showed that airborne dust, in part, was the source of milk contamination. Milk contamination by Burkholderiaceae and Oxalobacteriaceae was strongly linked with the liver's GOT and GPT fluctuations. Milk fat constantly increased throughout lactation, and this increment was strongly correlated with Oscillospiraceae in the rumen. This study has demonstrated that airborne dust microbiota may contaminate Jersey milk, particularly when liver function is impaired.

3.2 Introduction

Due to the sustained burgeoning global demand for milk, Holstein Friesians have been the most reared breed globally, owing to their unparalleled high milk production (Nguyen et al., 2020). This unmatched milk production usually makes Holstein Friesians experience rather intensive negative energy balance after calving down. Reproductively, Holstein Friesians reportedly have had a lower conception rate than other breeds like Jersey (Washburn et al., 2002). Moreover, as Washburn et al., (2002) also documented, Holstein Friesians are likely to have a higher incidence of mastitis than Jersey cows. Additionally, Holstein Friesians' feed intake is expectedly higher than other breeds, while their feed conversion is comparably lower (Mackle et al., 1996). The facts mentioned above, coupled with lower milk constituents, fat and protein, produced by Holstein Friesians in comparison with other breeds, have gradually galvanized dairy stakeholders into seeking alternative breeds that are more reproductive & productive-efficient, disease-resistant, and environmentally benign. Jersey cows, in contrast to Holstein Friesians, have been touted to be more efficient productively & reproductively (Mackle et al., 1996; Prendiville et al., 2011), comparatively more resistant to mastitis (Washburn et al., 2002), versatile (Huson et al., 2020), and environmentally benign (Uddin et al., 2021). These traits are endearing Jersey cows to farmers worldwide.

What's more, milk produced by Jersey cows has been gaining traction from both farmers and milk processors due to superior milk fat and protein content that fetches higher milk prices for farmers (Lim et al., 2020) and has superior processed dairy products characteristics (Yoo et al., 2019). As Nguyen et al., (2020) asserted, the difference in milk composition between milk produced by Jersey and Holstein Friesians may lead to different microbial profiles. Milk yield and constituents change across an entire lactation period due to dietary changes, metabolic status, and seasonal influence. For instance, Janovick Guretzky et al., (2006) observed lower non-esterified fatty acids in Jersey cows compared to Holstein postpartum in an experimental control group. It is still unclear how such lactation metabolite fluctuations may influence milk microbiota across the lactation. Additionally, milk microbiota is influenced by the cowshed environment, such as airborne dust and bedding (Wu et al., 2019). However, unlike the manifold research that has been sunk into the lactation-long microbial changes of milk produced by Holstein Friesians, to our knowledge, there is no single, comprehensive study that has investigated how microbiota of milk produced by Jersey cows changes in relation to metabolic profiles and ruminal microbiota across a lactation period.

Hence, we conducted this study to comprehensively investigate how the microbiota of milk produced by healthy Jersey cows change across an entire lactation period in relation to the lactation-long metabolic and ruminal microbiota changes. Moreover, we looked into how microbiota of airborne dust collected from an experimental cows' cowshed changed as the experimental cows' lactation period progressed and how the said airborne dust microbiota influenced the milk microbiota. Milk microbiota and metabolic profile correlations and the possible relationships between milk constituents and ruminal microbiota were analyzed.

3.3 Materials and Methods

3.3.1 Sample collection

Milk samples were collected from eight lactating Jersey cows randomly selected from a herd reared in a dairy college located in the Hiruzen region, Okayama, Japan. These experimental cows had delivered within the same month; hence, this was one of the inclusion criteria. The collection of milk samples began in the first month of the lactation period and continued every other month until the end of the lactation period (the first month corresponded to September and the last month was June). Each milk sample was collected after cleaning the surface of the udder and four teats. The first few streams of foremilk were discarded, and the subsequent streams from the four teats were aseptically collected into a 50 ml collection tube. The tubes were labeled and frozen in liquid nitrogen forthwith. The collected milk samples were kept in a liquid nitrogen-containing box during transportation and frozen at -31° C till further analyses.

Airborne dust samples were collected from the experimental cowshed by placing three opened Petri dishes in different locations approximately one meter above the ground for about five minutes. After the collection time elapsed, each petri dish was washed with 1 ml of sterile saline to make a composite sample. The composite sample was shipped to the lab on ice and frozen at -31° C till DNA extraction. Rumen fluid was collected as Wu et al., (2019) outlined. Briefly, rumen fluid was obtained using a flexible stainless spring tube (Lumenar stomach evacuator outfit, Fujihira Industry Co. Ltd., Tokyo, Japan) and emptied into autoclaved bottles after being sieved with sterile cheesecloth. Filtered rumen fluid was then aliquoted into 1.5 ml microfuge tubes and stored at -31° C. Blood samples, on the other hand, were taken from caudal veins and transported on ice.

3.3.2 Milk composition and blood metabolites analyses

The somatic cell count (SCC) in milk was estimated by N-acetyl- β -d-glucosaminidase (NAGase) activity assay. The content of fat, protein, solids-not-fat, and milk urea nitrogen (MUN) in milk was determined using a CombiFossFT + analyzer (Foss Allé, Hillerød, Denmark) as reported by Nguyen et al. (2020).

Plasma samples were analyzed for total cholesterol (T-Cho), albumin (Alb), non-esterified fatty acids (NEFA), total protein, calcium, phosphorus, blood urea nitrogen (BUN), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) by following the instructions of commercially available kits (Fujifilm Wako Pure Chemicals Co.). A commercial bovine Hp ELISA kit (Life Diagnostics, Inc.) was used to determine the plasma haptoglobin (Hp) content.

3.3.3 Bacterial DNA extraction

Bacterial DNA from milk and airborne dust samples were extracted from 1 ml of milk and airborne dust as described by Nguyen et al. (2020). Briefly, 1 ml of milk was centrifuged at 16,000 x g for 15 min at 4°C. The fat layer was scraped off and the supernatant was pipetted out.

1 ml of airborne dust was centrifuged at the same condition as aforementioned, and the supernatant was removed. The DNA pellets were washed and re-suspended in 1 × PBS (phosphate-buffered saline). 250 µL of the re-suspended DNA pellets of milk and airborne dust and filtered rumen fluid were transferred into 2 ml screw-cap microcentrifuge tubes containing 0.4g zirconia beads (0.1 mm 3:1 0.5 mm) and 1 ml of lysis buffer (0.5M NaCl, 0.05M Tris-HCl, 0.05M EDTA, 4% sodium dodecyl sulfate). The mixture was homogenized by a mini-bead beater, incubated at 70° C for 15 minutes, and the resultant supernatant was mixed with 200 µL ammonium acetate. After centrifugation at 16,000 × g for 10 min at 4°C, the resultant supernatant was mixed with an equal amount of 2-propanol, incubated on ice for 30 minutes, centrifuged, and the DNA pellets washed and re-suspended in 100 µL of TE. The DNA was purified from the DNA suspension by using the DNeasy Stool Mini Kit (Qiagen, Germantown, MD, USA).

3.3.4 16S rRNA gene amplicon MiSeq sequencing

The primers reported previously by Nguyen et al. (2020) targeting the hypervariable region V4 of the 16S ribosomal gene were used to generate the first-round PCR amplicons. The PCR protocol was as reported in the aforementioned work, with the exception of cycle numbers, where 30 cycles were used in the present work for DNA derived from milk and airborne dust and 25 cycles for DNA obtained from rumen fluid. The Resultant first-round PCR products were electrophoresed on a 1% agarose gel, bands were excised, and purified Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., LTD., Tokyo, Japan). With adapter-attached primers, the purified first-round PCR amplicons were used as DNA templates for the second-round PCR, and the thermocycling program described in work cited above was used. The second-round PCR amplicons were purified as described above and sequencing was done at FASMAC Co., Ltd.

3.3.5 Bioinformatics and statistical analyses

The Quantitative Insights Into Microbial Ecology (QIIME2) version 2022.2 was used to analyze the pair-end raw sequences. Raw sequences, which were about 250bp, were truncated at the site whose quality score was below 25(forward reads; 177 reverse reads 134). Quality-truncated reads with a 60 bp overlap were pair-end joined, and the quality assessed, chimera-free, pair-end sequences were grouped into operational taxonomic units with a similarity threshold of above 97%.

The JMP software version 14.2.0 was used to statistically analyze all microbial, metabolites, and milk composition data. Tukey test was used to determine if there were statistical differences between means. Principal coordinate analysis (PCoA) was performed by using the primer7 version 7.0.14 (Primer-E, Plymouth Marine Laboratory, Plymouth, UK) to determine how milk and airborne dust microbiota was clustered.

3.4 Results

3.4.1 Jersey's milk production, milk composition, and blood metabolic profiles

The average daily production for the experimental cows was 22.61 kg per day; the milk production ranged from 14.77 to 27.30 kg, peaking in the second month postpartum. Milk fat and protein steadily increased as the lactation period advanced; fat and protein contents ranged between 4.49 to 5.85% and 3.39 to 4.54%, respectively. Solids-not-fat and milk urea nitrogen were higher at the advanced stages of the lactation period (9.01-9.83% SNF and 8.71-12.29 mg/dl MUN). Milk somatic cell counts remained relatively stable across the lactation period and below the 2.0×10^5 cells per ml threshold (table 1).

Metabolically, the late lactation's blood total protein was significantly higher than the early lactation period level, and the albumin content was numerically higher towards the end of the lactation period. On the contrary, the blood urea nitrogen was higher in the first four months of the lactation period, sharply increased in the fifth lactation month, and plummeted in the subsequent months till the end of the lactation period. Energy-wise, non-esterified fatty acids (NEFA) were the highest within the first month after parturition, 0.39mEq/L, and decreased progressively towards the penultimate month of the lactation up to 0.085mEq/L. There was an uptick in NEFA in the last month of lactation (0.14mEq/L). The total cholesterol content in blood was the lowest in the first month of lactation; it peaked the second month after parturition and progressively declined to 179.88 mg/dl. The calcium and phosphorus levels irregularly fluctuated throughout the lactation period, with an average of 8.88 and 4.80 mg/dl, respectively. The liver functions biomarkers, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), also fluctuated across the lactation period, with both enzymes peaking in the fourth month after delivery (115.59 IU/L GOT and 33.62 GPT). Haptoglobin levels decreased gradually as the lactation period advanced, from 62.56 to 10.60µg/L (tabl

	SEPT	OCT	DEC	FEB	APR	JUN	SE
Days in milk	26.88	55.88	118.88	179.88	238.88	302.14	1.07
Milk yield (kg)	26.99 ^a	27.30 ^a	25.93 ^{ab}	20.43 ^{bc}	20.29 ^{bc}	14.77 ^c	0.67
Milk Composition							
Fat (%)	4.49 ^c	4.69 ^{bc}	5.31 ^{ab}	5.31 ^{ab}	5.49 ^{ab}	5.85 ^a	0.08
Protein (%)	3.39 ^b	3.47 ^b	4.23 ^a	4.54 ^a	4.54 ^a	4.46 ^a	0.04
Solids-not-fat (%)	9.01 ^b	9.20 ^b	9.83 ^a	10.02 ^a	9.97 ^a	9.83 ^a	0.04
SCC ($\times 10^3$ cells/ml)	103.71	70.14	87.57	121.57	202.43	169.43	5.94
Milk Urea Nitrogen (mg/dl)	8.96 ^c	3.86 ^d	8.71 ^c	12.29 ^a	10.26 ^{bc}	11.63 ^{ab}	0.20
Blood Metabolites							
Albumin (g/dl)	3.85 ^c	4.03 ^{bc}	4.23 ^{bc}	7.04 ^a	4.33 ^b	4.35 ^b	0.04
Blood Urea Nitrogen (mg/dl)	9.87 ^b	7.11 ^b	8.78 ^b	22.90 ^a	2.37 ^c	3.02 ^c	0.27
Total cholesterol (mg/dl)	160.35 ^b	229.30 ^a	214.39 ^{ab}	208.53 ^{ab}	196.19 ^{ab}	179.88 ^{ab}	5.65
NEFA (mEq/L)	0.39 ^a	0.29 ^b	0.11 ^c	0.11 ^c	0.085 ^c	0.14 ^c	13.59
Total Protein (g/dl)	4.98 ^b	5.08 ^b	8.10 ^a	8.18 ^a	8.37 ^a	9.19 ^a	0.13
Calcium (mg/dl)	8.28 ^{bc}	9.25 ^{ab}	8.15 ^c	10.04 ^a	8.94 ^{abc}	8.61 ^{bc}	0.10
Phosphorus (mg/dl)	3.43 ^b	4.37 ^{ab}	5.27 ^a	4.52 ^{ab}	5.45 ^a	5.77 ^a	0.15
GOT (IU/L)	112.29 ^a	75.98 ^c	115.59 ^a	76.25 ^{bc}	87.57 ^{bc}	98.11 ^{ab}	2.02
GPT (IU/L)	16.49 ^c	16.32 ^c	33.62 ^a	27.95 ^{ab}	22.71 ^{bc}	19.55 ^c	0.63
Haptoglobin ((μ g/L))	62.56 ^a	51.86 ^{ab}	47.46 ^{ab}	13.27 ^b	15.31 ^b	10.60 ^b	3.68

Rows whose means have different letter superscripts depict statistical differences among means

Abbreviations: SCC; somatic cell count, NEFA; non-esterified fatty acid, GOT; glutamic oxaloacetic transaminase, and GPT; glutamic pyruvic transaminase, SE; standard error

Table 3. 1 Jersey milk yield, composition, and blood metabolites across the lactation

Indices/Taxa	SEPT	OCT	DEC	FEB	APR	JUN	SE
Diversity indices							
chao1	404.94 ^a	312.81 ^{ab}	270.00 ^{ab}	387.51 ^a	373.73 ^a	216.89 ^b	13.09
Simpson	0.95 ^a	0.87 ^a	0.63 ^b	0.92 ^a	0.87 ^a	0.73 ^b	0.01
Shannon	6.01 ^a	4.87 ^a	3.56 ^b	5.75 ^a	5.17 ^a	3.37 ^b	0.12
Observed OTUs	393.88 ^a	303.00 ^{abc}	261.75 ^{bc}	376.25 ^{ab}	359.75 ^{ab}	210.43 ^c	12.57
Phyla/Families							
Proteobacteria	48.07c	59.08bc	74.32ab	47.23c	61.42bc	80.46a	1.44
Burkholderiaceae	22.44 ^{cd}	12.19 ^d	62.28 ^a	12.15 ^d	36.73 ^b	29.20 ^{bc}	0.97
Oxalobacteraceae	8.75 ^b	0.87 ^b	2.21 ^b	1.71 ^b	9.81 ^b	41.38 ^a	1.00
Bradyrhizobiaceae	3.31 ^a	0.64 ^c	2.09 ^{ab}	0.53 ^c	3.20 ^a	0.96 ^{bc}	0.10
Enterobacteriaceae	0.32 ^c	36.75 ^a	0.29 ^c	25.80 ^b	0.26 ^c	4.88 ^c	0.99
Moraxellaceae	1.95	3.84	1.14	2.80	1.67	0.76	0.26
Caulobacteraceae	3.84 ^{ab}	0.16 ^d	2.54 ^{bc}	0.41 ^d	4.05 ^a	1.13 ^{cd}	0.11
Firmicutes	33.60a	26.63ab	15.80bc	36.40a	23.40abc	12.57c	1.10
Erysipelotrichaceae	6.70 ^a	4.66 ^{ab}	2.72 ^{ab}	5.14 ^{ab}	4.26 ^{ab}	1.90 ^b	0.38
Ruminococcaceae	5.51	2.65	3.27	7.09	5.05	2.55	0.42
Lactobacillaceae	2.40	2.59	1.53	2.30	2.00	1.96	0.18
Streptococcaceae	1.26 ^b	2.81 ^a	0.75 ^b	1.85 ^{ab}	0.80 ^b	0.51 ^b	0.12
o__Clostridiales	2.35 ^a	2.04 ^{ab}	0.96 ^b	2.54 ^a	1.45 ^{ab}	0.67 ^b	0.12
Bacillaceae	0.81 ^b	3.94 ^a	0.06 ^b	2.70 ^a	0.13 ^b	0.65 ^b	0.13
Lachnospiraceae	2.45	1.04	1.61	4.25	3.26	0.80	0.34
Staphylococcaceae	3.27	2.32	1.49	3.10	1.40	0.52	0.34
Actinobacteria	5.16a	3.77ab	1.68b	3.25ab	2.64ab	1.38b	0.23
Bifidobacteriaceae	3.14	2.35	0.88	2.38	1.39	0.75	0.22
Bacteroidetes	9.19a	7.80ab	4.45ab	8.39a	5.91ab	3.09b	0.41
Bacteroidaceae	1.81 ^{ab}	0.92 ^b	0.96 ^b	2.29 ^a	1.26 ^{ab}	0.66 ^b	0.13
Others	0.59	0.83	1.50	2.46	3.56	1.30	0.17

Bacterial diversity indices and Phyla/Families having a relative abundance of >2% in at least one sample

Means with different superscripts show statistical differences

Abbreviations: SE standard error

Table 3. 2 Jersey milk bacterial diversity indices and the most abundant bacterial phyla and families

3.4.2 Milk microbiota and diversity

Alpha diversity and evenness indices, Chao1, Simpson, Shannon, and observed OUT number varied inconsistently throughout the lactation period; the lowest bacterial diversity and evenness were observed mid-lactation and the last month of the lactation period (table2). Proteobacteria and Firmicutes were the predominant phyla averaging out at 48.07-80.46% and 12.57-36.40%, respectively. Averagely, the most abundant families across the lactation period were Burkholderiaceae (29.2%), Enterobacteriaceae (11.4%), Oxalobacteriaceae (10.8%), Ruminococcaceae (4.4%), and Erysipelotrichaceae (4.2%). Notably, the three most abundant families changed intermittently across the lactation period. For instance, Oxalobacteriaceae changed from 0.87 to 41.38%, while Enterobacteriaceae abundance varied from 0.29 to 36.75%. Typical milk microbiota like lactobacillus, Lachnospiraceae, and Moraxellaceae remained statistically unchanged across the lactation period (table 2). Only Burkholderiaceae and Oxalobacteriaceae had seasonal variations: the former was highest in winter (37.06%), while the latter was highest in summer (15.94%), as shown in Table 3.3.

Table 3. 3 seasonal impact on the most abundant milk bacterial taxa

Family	Summer	winter	SE
Burkholderiaceae	20.93 ^b	37.06 ^a	0.76
Oxalobacteriaceae	15.94 ^a	4.58 ^b	0.65
Enterobacteriaceae	14.38	8.78	0.72
Erysipelotrichaceae	4.53	4.04	0.14
Lactobacillaceae	2.33	1.95	0.06
Bifidobacteriaceae	2.14	1.55	0.08
Bradyrhizobiaceae	1.67	1.94	0.06
Ruminococcaceae	3.61	5.13	0.16
Caulobacteraceae	1.73	2.33	0.08
Streptococcaceae	1.57	1.14	0.05
o__Clostridiales	1.73	1.65	0.05
Others	0.89	2.51	0.06

Rows having different letter superscripts indicate means are different statistically

3.4.3 Airborne dust microbiota

The most prevalent families in the airborne dust collected from the experimental cows' cowshed were S24-7(17.5%), Lactobacillaceae (14.3%), Ruminococcaceae (12.5%), and Lachnospiraceae (9.4%). Burkholderiaceae and Oxalobacteriaceae abundance in the airborne dust averaged out at 4.9% and 4.0%, respectively. In April, the penultimate month of the lactation period of the experimental cows, the two families mentioned above were undetected in the airborne dust.

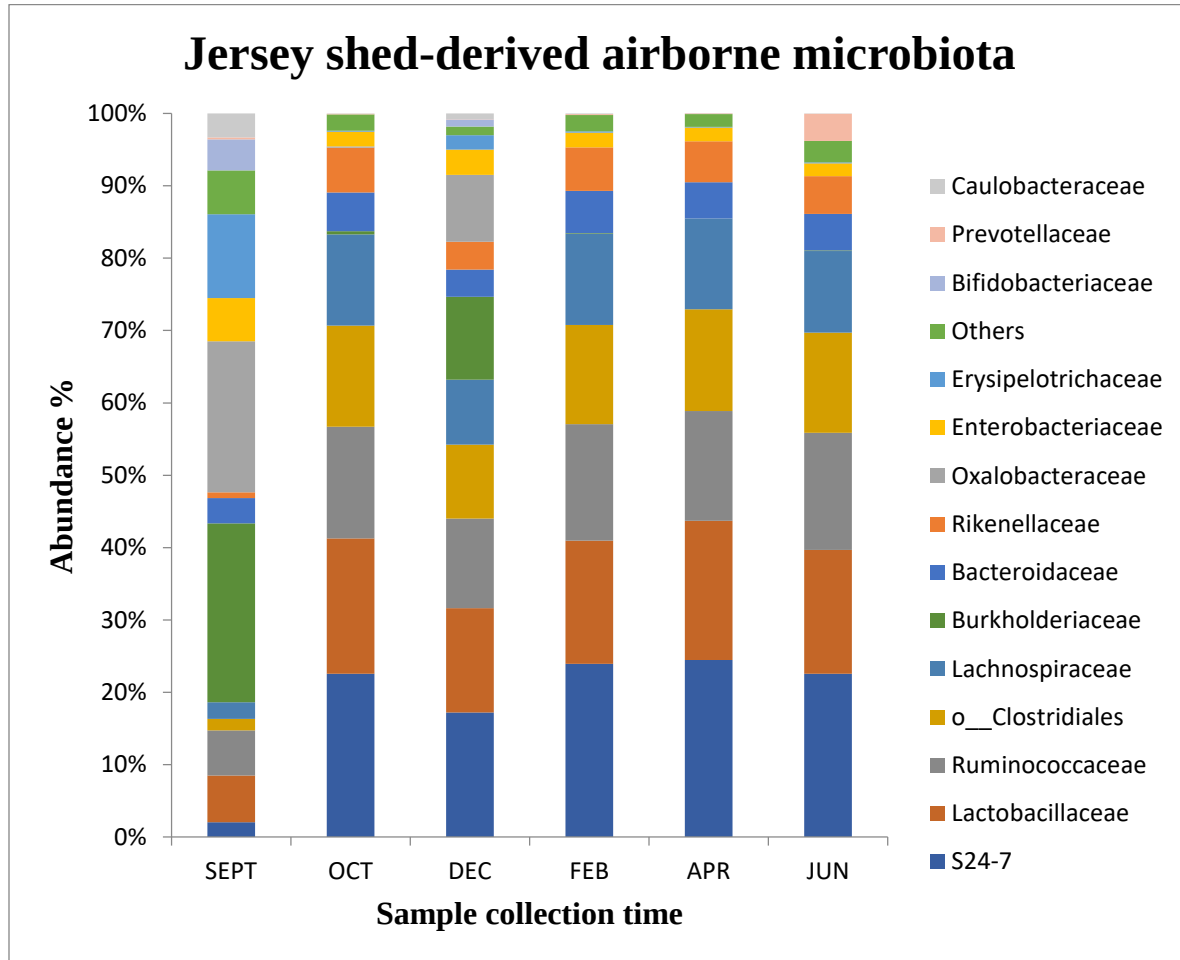


Figure 3. 1 The most abundant families of Jersey cows' cowshed-collected airborne dust

3.4.4 The relationship between milk and airborne dust microbiota

Airborne dust microbiota, as analyzed by PCoA, was closely grouped with milk microbiota, with the September airborne dust microbiota being grouped with milk microbiota. In September and December, equivalently during the first and fourth months of the lactation period, Burkholderiaceae and Oxalobacteriaceae were higher in milk and airborne dust (table 2; figure 1).

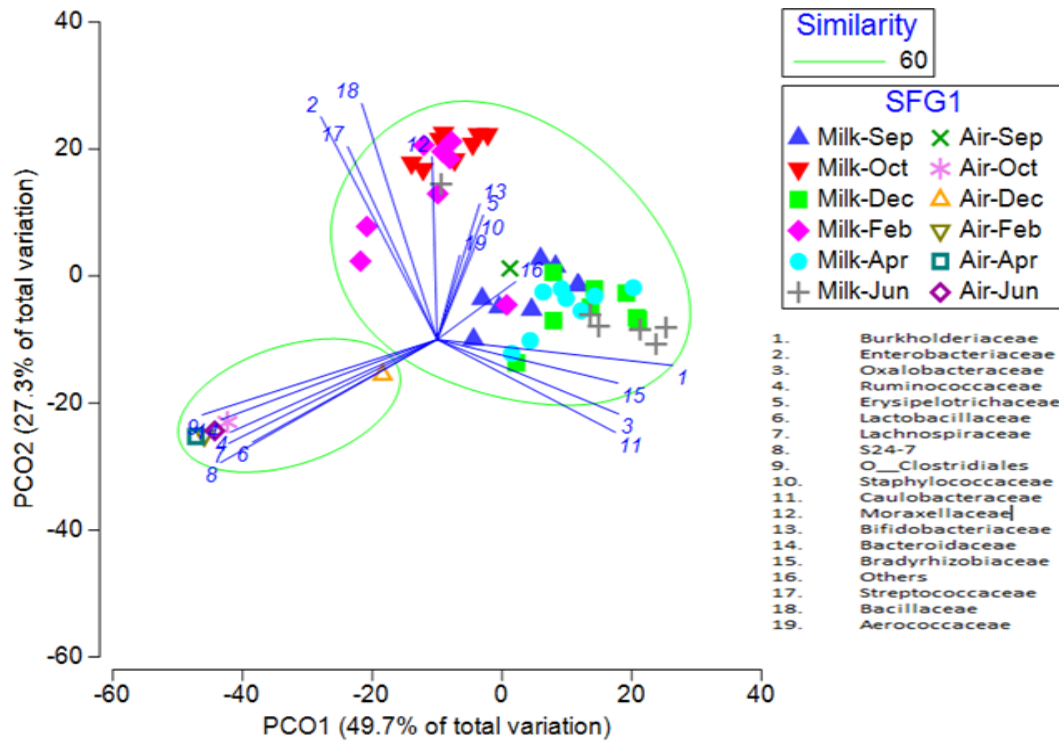


Figure 3. 2 Principal coordinates plot characterizing the microbiota of airborne dust and milk.

3.4.5 The notable relationships between milk microbiota and blood metabolites

According to network analysis, as shown in figure 3.3, the invasion of Burkholderiaceae and Oxalobacteriaceae in milk was strongly and positively correlated with GOT and GPT (Spearman $r=0.5$ $p<0.001$ and $r=0.42$ $p<0.001$, respectively). GOT was also negatively correlated with Enterobacteriaceae (Spearman $r=-0.33$ $p<0.05$). Bradyrhizobiaceae was positively associated with GOT (Spearman $r=0.37$ $p<0.05$) and negatively related to the calcium level in the blood (Spearman $r=-0.42$ $p<0.05$). The abundance of Bacillus was negatively and positively influenced by GOT (Spearman $r=-0.35$ $p<0.05$) and the level of calcium (Spearman $r=0.47$ $p<0.05$) in blood, respectively.

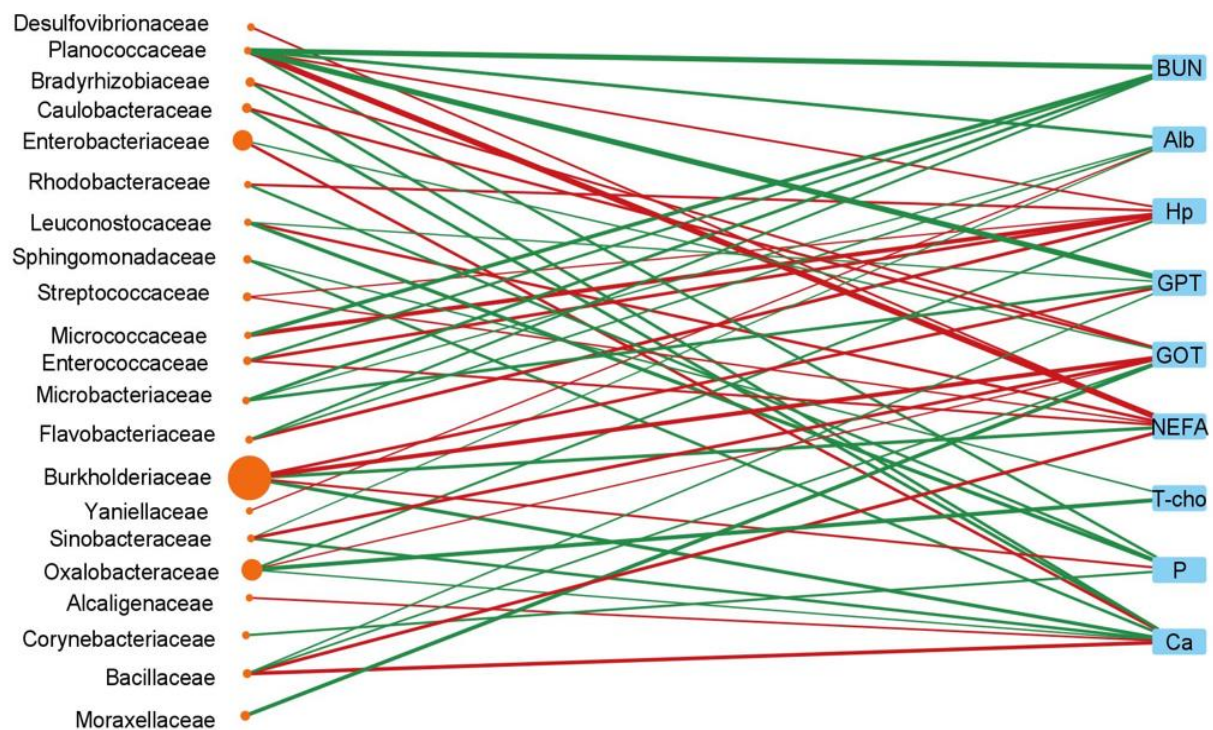


Figure 3. 3 network analysis showing the relationship between milk microbiota and blood metabolites. Only strong (correlation coefficient $R > 0.3$ or <-0.3) and significant ($P < 0.01$) correlations were chosen to be displayed in the network. The edge width and color (red, positive; green, negative) are proportional to the correlation strength. The orange circle (nodes) sizes are proportional to the relative abundance of milk bacterial families. The light blue rectangles indicate blood metabolites.

3.4.6 The relationship between milk composition and blood metabolites

MUN and BUN were 8.96 and 6.13 mg/dl at the onset of the lactation, but they both declined to the lactation-lowest values of 3.86 and 5.57 mg/dl in the subsequent lactation month, respectively. From the lactation-lowest values, both BUN and MUN sharply increased over the next four months to the lactation-peak values of 12.29 and 14.19 mg/dl, respectively. After peaking, the two indicators of protein metabolism declined over the next two months and then increased towards the end of the lactation to 11.63 mg/dl and 10.64 mg/dl for MUN and BUN, respectively.

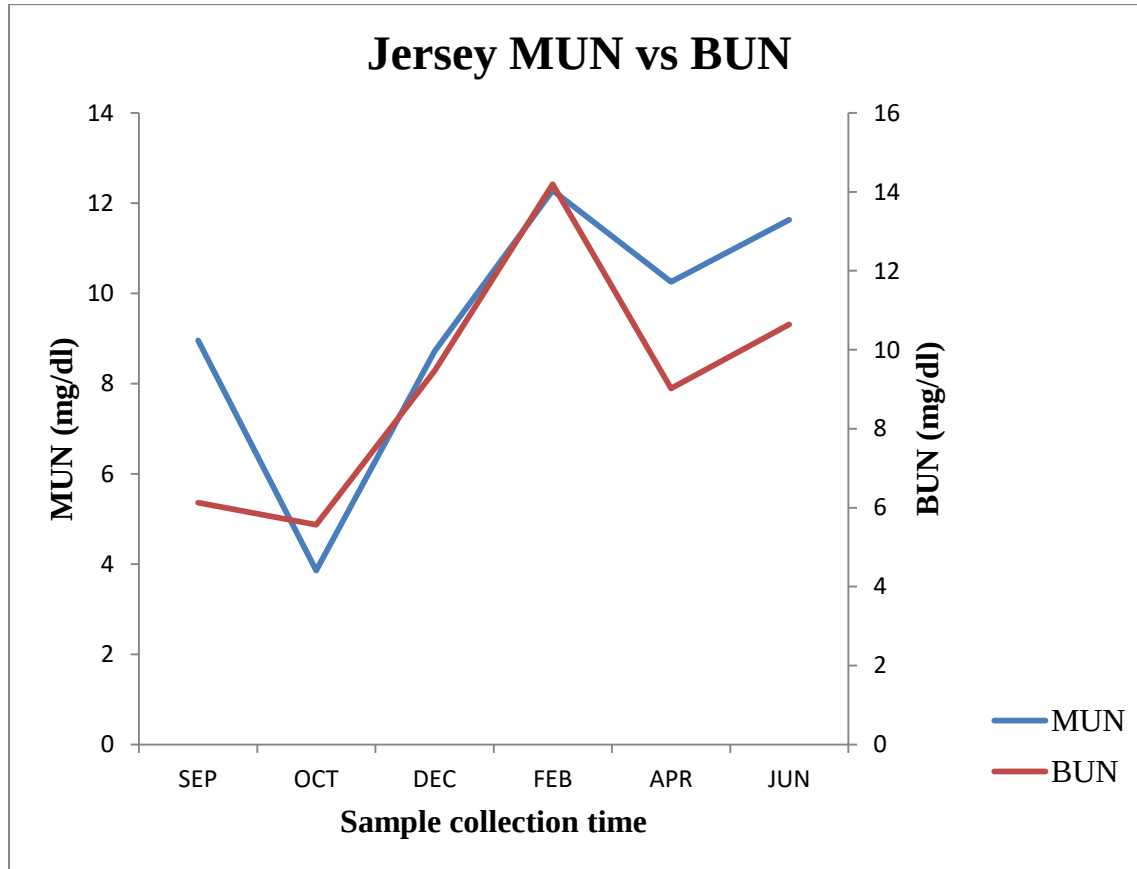


Figure 3. 4 the equilibrium relationship between MUN and BUN across the lactation

3.4.7 Rumen microbiota

Prevotellaceae predominated the rumen microbiota at 20%, followed by Rikenellaceae (11%), Lachnospiraceae (9%), and Ruminococcaceae (8%). Prevotellaceae and Ruminococcaceae remain statistically unchanged throughout the lactation period, while Rikenellaceae lowest and Lachnospiraceae highest mid-lactation (Table 4).

Table 3. 4 the most abundant Jersey cows' ruminal bacterial families across a lactation period

Month	Sept	Oct	Dec	Feb	April	SE
Families						
Prevotellaceae	19.40	17.82	20.72	20.83	20.24	0.49
Rikenellaceae	11.48 ^{abc}	10.21 ^{bc}	8.95 ^c	13.93 ^a	12.62 ^{ab}	0.25
Lachnospiraceae	6.26 ^c	9.99 ^b	13.80 ^a	7.18 ^{bc}	8.59 ^a	0.28
Ruminococcaceae	7.54	7.90	7.68	8.81	7.73	0.33
Oscillospiraceae	4.58 ^c	6.12 ^{abc}	7.22 ^{ab}	5.59 ^{bc}	7.45 ^a	0.16
F082	4.23 ^{bc}	3.70 ^c	5.06 ^{abc}	6.01 ^a	5.57 ^{ab}	0.14
RF39	6.81 ^a	4.86 ^b	3.20 ^c	3.80 ^{bc}	4.33 ^{bc}	0.14
Christensenellaceae	3.13 ^b	5.08 ^a	5.07 ^a	4.03 ^{ab}	3.92 ^{ab}	0.14
Clostridiaceae	4.19 ^a	4.35 ^a	4.02 ^a	2.27 ^b	2.16 ^b	0.15
Methanobacteriaceae	6.14 ^a	3.02 ^b	0.97 ^b	3.52 ^{ab}	2.41 ^b	0.25
Leuconostocaceae	5.89	7.20	0.01	0.08	0.00	0.92
RF16	2.23	2.73	3.22	2.06	2.63	0.16
Muribaculaceae	1.55 ^b	2.41 ^{ab}	2.70 ^a	1.73 ^{ab}	2.59 ^{ab}	0.10
Hungateiclostridiaceae	1.32 ^{bc}	1.12 ^c	2.23 ^a	1.08 ^c	1.89 ^{ab}	0.06
Acidaminococcaceae	1.40 ^{ab}	1.09 ^b	1.58 ^{ab}	1.70 ^a	1.81 ^a	0.05
Spirochaetaceae	0.94 ^{ab}	0.76 ^b	1.50 ^a	0.86 ^{ab}	1.24 ^{ab}	0.07
Erysipelotrichaceae	1.47	0.99	0.84	1.21	0.78	0.04

Rumen bacterial families with a relative abundance >1% in at least one sample.

Rows having means with different letter superscripts indicate statistical differences among means. Abbreviation: SE; Standard Error

3.5 Discussion

Milk production gradually decreased as the lactation progressed from approximately 27 kg per day one month after parturition to 15 kg per day in the tenth month of the lactation period. Milk production peaked at approximately 56 days in milk (27.3 kg of milk per day), contrary to what was previously reported (Hickson et al., 2006), where Jersey cows milked twice a day peaked at 36 days in milk producing about 16 kg of milk per day. This difference could be ascribed to either the dietary management differences between the two experimental herds and/or the average parity of the two herds. O'Sullivan et al. (2019) reported that a cow's milk production is influenced by its parity. As O'Sullivan et al. (2019) previously reported, this is a usual milk production trend, and the days in milk were comparable to other Jersey cows (Beneberu et al., 2022). Milk composition: fat, protein, and SNF were higher in the late lactation than in the early lactation stage. Milk fat progressively increased across the entire lactation, which was comparable to what Bainbridge et al. (2016) observed. However, milk protein and SNF slumped towards the end of the lactation period. Though the milk protein and SNF trends are not congruent with what Bainbridge et al. (2016) & Lim et al. (2020) reported, they also observed an inconsistent decline in both protein and SNF in milk, unlike the milk fat, which concomitantly increased as the lactation progressed in their respective studies. Although milk fat depicts a stable increment as DIM increases, it is confounding to note that Macciotta et al. (2002) reported lower prediction accuracy for milk fat in Simmental cows as opposed to milk yield and protein. Somatic cell count, which is a conventionally accepted indicator of a cow's susceptibility or resistance to intra-mammary infection according to Sharma, Singh, and Bhadwal (2011), remained insignificantly unchanged across the entire lactation period, except in the eighth month of the lactation period. In the eighth month, when the average SCC was elevated beyond the accepted $1.0\text{--}2.0 \times 10^5$ cells/mL threshold (Bytyqi et al., 2010), two of the eight experimental cows had a separate average of 5.96×10^5 cells/mL. Although this level is way above the threshold mentioned above, the two cows did not depict mastitis-related symptoms. Intriguingly, one of the two cows had an elevated SCC averaging out at 3.88×10^5 cells/mL throughout the entire lactation period.

Milk and blood urea nitrogen, which are the final product of protein metabolism, are arguably helpful as a practical indicator for rapid monitoring of the dietary crude protein and energy intake if other major sources of variations are controlled (Hammond, 1998; Rajala-Schultz & Saville, 2003). Rajala-Schultz and Saville, (2003) observed lower MUN concentration in the first month after parturition and an increment in MUN as DIM increased. This observation is congruous with this study's results. Gustafsson and Palmquist (1993) found out that MUN equilibrated with BUN rapidly, and a somewhat similar observation was made in this study with a few exceptions. The few exceptions can only be attributed to various factors influencing these two parameters (Rajala-Schultz & Saville, 2003). NEFA, which are the estimator of energy balance in lactating cows, were expectedly higher at the beginning of the lactation and decreased as the lactation advanced (Grum et al., 2002).

Contrary to NEFA, total cholesterol was lowest at the beginning of the lactation, increased mid-lactation, and slightly declined at the end of the lactation period. This trend of the energy estimator implied that the experimental cows had sufficient energy, especially during mid-lactation (Kaneene et al., 1997). During the periparturient period, lactating cows are susceptible to reproductive and metabolic disorders. For instance, when the conversion of NEFA to triglycerides exceeds the metabolic capacity of hepatocytes, triglycerides accumulate in hepatocytes, resulting in impairment in liver function (Ospina et al., 2010). Impaired liver function induced by metabolic disorders and other infections is indicated by elevated GOT, GPT, and Haptoglobin levels. GOT and haptoglobin were expectedly higher during the early lactation period. This finding agrees with a study by Stojević et al. (2005) that previously reported the highest activities of liver enzymes within the first 45 days after parturition and a subsequent decline as the lactation advanced. This might be indicative of rather subtle hepatic stress the experimental cows were undergoing during the early lactation period. As Chan et al. (2004) & Cui et al. (2019) intimated, haptoglobin and GOT are blood biochemical indicators of an acute infection, not non-infective disorders. As such, there is a dearth of information linking hepatic function enzyme changes in blood of lactating cows with ingress of microbial opportunists. However, this study's network analysis strongly linked the trend of GOT in blood with changes in the most predominant family observed in this study, Burkholderiaceae (Spearman $r = 0.5$ $p < 0.001$).

Burkholderiaceae, Enterobacteriaceae, and Oxalobacteriaceae predominated milk microbiota and depicted variable fluctuations throughout the lactation period. Seasonally, only Burkholderiaceae and Oxalobacteriaceae changed: the former was higher in winter, while the latter was higher in summer. Nguyen et al., (2020) reported seasonal effects on just five bacterial families; they observed several monthly changes at the family level. This observation is comparable to this study's findings since several families changed monthly. Dahlberg et al., (2019) initially reported Burkholderiaceae and Oxalobacteriaceae among the fifteen most abundant families in milk before filtering milk contaminants. After filtering and utilizing a recently published decontam R-package, the families mentioned above were filtered and regarded as milk contaminants. Salter et al. (2014) also reported the abovementioned families as potential contaminants in milk emanating from the environment; the probable source of the bacteria from these families reportedly being soil and water. The two families were also detected in airborne dust and changed comparably to the fluctuations in milk microbiota. Nguyen et al., (2020) found out that airborne dust microbiota was clustered together with milk microbiota. This study's PCoA clustered the microbiota of the first airborne dust sampling, which corresponded to the first month of lactation. In contrast, the microbiota of the third airborne dust sampling was closely grouped with milk microbiota. The Persistence of these bacterial families in milk, even after their detection in the airborne dust diminished, could be attributed to the diversity of sources of these contaminants (Salter et al. 2014 & Nguyen et al., (2020). Hence, it can be conjectured that airborne dust was the most probable source of milk contamination at the beginning of the lactation, but other sources also contributed to milk contamination towards the end of the

lactation. It is worth noting that other widely known milk contaminants that deteriorate milk quality, such as Pseudomonadaceae, were detected at a minute level. This observation is opposite to the findings by Nguyen et al., (2020), where Pseudomonadaceae predominated milk microbiota. This stark contrast in milk microbiota abundance between the two studies can be ascribed to the method of collecting milk samples, where the cited research collected milk from the sampling device attached to the milking parlor.

Milk microbial taxa believed to be the typical core bovine milk microbiota (Kable et al., 2016; Oikonomou et al., 2020), such as Lactobacillaceae, Lachnospiraceae, and Moraxellaceae, depicted stability throughout the lactation period. This observation is congruent with research findings by Nguyen et al., (2020), who found no monthly or seasonal changes in the putative core milk microbiota. Cremonesi et al., (2018) also found out Rendena cows' milk microbiota was largely stable, unlike Holstein Friesian cows' milk microbiota over four sampling periods. Their observation may point to the fact that core milk microbiota might be stable depending on the breed and health of a herd.

In harmony with Wu et al., (2019), Prevotellaceae was the predominant taxon. This family has been widely associated with the degradation of concentrate soluble carbohydrates in rumen (Petri et al., 2012). As Petri et al., (2012) reported, Ruminococcaceae abundance in the rumen is likely to be influenced by forage intake; hence, this might explain the difference in abundance between the study by Wu et al., (2019) and this study. Diet is the main factor shaping the rumen microbiome as reported by Khafipour et al., (2016) and Petri et al., (2012). They argued that core members of rumen microbiota are stable in adult cows; hence, this might explain why Prevotellaceae and Ruminococcaceae were stable across the lactation period as the diet proffered to the cows enrolled in this study remained unchanged. In addition to the rumen microbiome contributing to ruminal fermentation, manifold bacterial families have been associated with different milk production traits. However, the associations of the ruminal microbiome to milk production have not been conclusive. For instance, Lopes et al., (2021) reported that inoculation of Prevotella led to increased milk fat content, while Jami et al., (2014) observed a significant negative correlation between Prevotellaceae and milk fat content. This study, on the other hand, did not observe any significant correlation between Prevotellaceae and milk fat. However, a strong positive correlation (Spearman $r=0.57$ $p<0.001$) between Oscillospiraceae and milk fat content was observed. Research reports reporting correlations between milk protein and ruminal microbiota are scanty; Xue et al., (2019) reported a positive correlation between Succinivibrionaceae and milk protein, while the current study observed that Oscillospiraceae and Acidaminococcaceae were positively correlated with milk protein (Spearman $r=0.46$ $p<0.001$ for both). While manifold correlations between ruminal microbiota and milk components have been reported, Jami et al., (2014), Lopes et al., (2021) & Xue et al., (2019) have unanimously agreed on the need for in-depth, expansive research to practically corroborate these findings due to inconsistencies reported in various reports.

3.6 Conclusion

Burkholderiaceae and Oxalobacteriaceae, which were predominant milk microbial families and putative milk contaminants, changed inconsistently across the lactation period. The probable source of the bacterial families was airborne dust in the cowshed, but it was apparent it was not the only source of the two bacterial families. The ingress of the two bacterial families into milk was strongly associated with the liver function impairment depicted by the GOT fluctuations. On the other hand, the core milk microbiota did not significantly change across the lactation period despite the irregular fluctuations of the predominant milk microbiota. Milk fat content increased as the DIM increased, and this milk fat increment was associated with Oscillospiraceae in the rumen. The ruminal microbiota was predominated by Prevotellaceae, which was stable across the lactation period, but other predominant ruminal microbiota changed variably across the lactation period. This study agrees with the others that recommended the need for further research into the association of ruminal microbiota with milk constituents.

References

- Bainbridge, M. L., Cersosimo, L. M., Wright, A. D. G., & Kraft, J. (2016). Content and composition of branched-chain fatty acids in bovine milk are affected by lactation stage and breed of dairy cow. *PLoS ONE*, 11(3), 1–17. <https://doi.org/10.1371/journal.pone.0150386>
- Beneberu, N., Shibabaw, W., Getahun, K., & Alemayehu, K. (2022). *Milk Productive Performances of Pure Jersey Dairy Cattle at Adea-Berga Dairy Research Center , Central Highland of Ethiopia*. 10(1), 1–6. <https://doi.org/10.11648/j.ijgg.20221001.11>
- Bytyqi, H., Zaugg, U., Sherififi, K., Hamidi, A., Gjonbalaj, M., Muji, S., & Mehmeti, H. (2010). Influence of management and physiological factors on somatic cell count in raw cow milk in Kosova. *Veterinarski Arhiv*, 80(2), 173–183.
- Chan, J. P. W., Chu, C. C., Fung, H. P., Chuang, S. Te, Lin, Y. C., Chu, R. M., & Lee, S. L. (2004). Serum haptoglobin concentration in cattle. *Journal of Veterinary Medical Science*, 66(1), 43–46. <https://doi.org/10.1292/jvms.66.43>
- Cremonesi, P., Ceccarani, C., Curone, G., Severgnini, M., Pollera, C., Bronzo, V., Riva, F., Addis, M. F., Filipe, J., Amadori, M., Trevisi, E., Vigo, D., Moroni, P., & Castiglioni, B. (2018). Milk microbiome diversity and bacterial group prevalence in a comparison between healthy holstein friesian and rendena cows. *PLoS ONE*, 13(10), 1–17. <https://doi.org/10.1371/journal.pone.0205054>
- Cui, L., Wang, H., Ding, Y., Li, J., & Li, J. (2019). Changes in the blood routine, biochemical indexes and the pro-inflammatory cytokine expressions of peripheral leukocytes in postpartum dairy cows with metritis. *BMC Veterinary Research*, 15(1), 1–10. <https://doi.org/10.1186/s12917-019-1912-y>
- Dahlberg, J., Sun, L., Waller, K. P., Ostensson, K., Mcguire, M., Agenas, S., & Dicksved, J. (2019). Microbiota data from low biomass milk samples is markedly affected by laboratory and reagent contamination. *PLoS ONE*, 14(6), 1–17. <https://doi.org/10.1371/journal.pone.0218257>
- Grum, D. E., Drackley, J. K., & Clark, J. H. (2002). Fatty acid metabolism in liver of dairy cows fed supplemental fat and nicotinic acid during an entire lactation. *Journal of Dairy Science*, 85(11), 3026–3034. [https://doi.org/10.3168/jds.S0022-0302\(02\)74388-9](https://doi.org/10.3168/jds.S0022-0302(02)74388-9)
- Gustafsson, A. H., & Palmquist, D. L. (1993). Diurnal Variation of Rumen Ammonia, Serum Urea, and Milk Urea in Dairy Cows at High and Low Yields. *Journal of Dairy Science*, 76(2), 475–484. [https://doi.org/10.3168/jds.S0022-0302\(93\)77368-3](https://doi.org/10.3168/jds.S0022-0302(93)77368-3)
- Hammond, C. (1998). Use of BUN and MUN as Guides for Protein and Energy in Cattle. *Revista Corpoica*, 2(2), 44–48.
- Hickson, R. E., Lopez-Villalobos, N., Dalley, D. E., Clark, D. A., & Holmes, C. W. (2006). Yields and persistency of lactation in Friesian and Jersey cows milked once daily. *Journal of Dairy Science*, 89(6), 2017–2024. [https://doi.org/10.3168/jds.S0022-0302\(06\)72269-X](https://doi.org/10.3168/jds.S0022-0302(06)72269-X)

- Huson, H. J., Sonstegard, T. S., Godfrey, J., Hambrook, D., Wolfe, C., Wiggans, G., Blackburn, H., & VanTassell, C. P. (2020). A Genetic Investigation of Island Jersey Cattle, the Foundation of the Jersey Breed: Comparing Population Structure and Selection to Guernsey, Holstein, and United States Jersey Cattle. *Frontiers in Genetics*, 11(April). <https://doi.org/10.3389/fgene.2020.00366>
- Jami, E., White, B. A., & Mizrahi, I. (2014). Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS ONE*, 9(1). <https://doi.org/10.1371/journal.pone.0085423>
- Janovick Guretzky, N. A., Carlson, D. B., Garrett, J. E., & Drackley, J. K. (2006). Lipid metabolite profiles and milk production for Holstein and Jersey cows fed rumen-protected choline during the periparturient period. *Journal of Dairy Science*, 89(1), 188–200. [https://doi.org/10.3168/jds.s0022-0302\(06\)72083-5](https://doi.org/10.3168/jds.s0022-0302(06)72083-5)
- Kable, M. E., Srisengfa, Y., Laird, M., Zaragoza, J., McLeod, J., Heidenreich, J., & Marco, M. L. (2016). The core and seasonal microbiota of raw bovine milk in tanker trucks and the impact of transfer to a milk processing facility. *MBio*, 7(4). <https://doi.org/10.1128/mBio.00836-16>
- Kaneene, J. B., Miller, R. A., Herdt, T. H., & Gardiner, J. C. (1997). The association of serum nonesterified fatty acids and cholesterol, management and feeding practices with peripartum disease in dairy cows. *Preventive Veterinary Medicine*, 31(1–2), 59–72. [https://doi.org/10.1016/S0167-5877\(96\)01141-5](https://doi.org/10.1016/S0167-5877(96)01141-5)
- Khafipour, E., Li, S., Tun, H. M., Derakhshani, H., Moossavi, S., & Plaizier, K. J. C. (2016). Effects of grain feeding on microbiota in the digestive tract of cattle. *Animal Frontiers*, 6(2), 13–19. <https://doi.org/10.2527/af.2016-0018>
- Lim, D. H., Mayakrishnan, V., Lee, H. J., Ki, K. S., Kim, T. Il, & Kim, Y. (2020). A comparative study on milk composition of Jersey and Holstein dairy cows during the early lactation. *Journal of Animal Science and Technology*, 62(4), 565–576. <https://doi.org/10.5187/JAST.2020.62.4.565>
- Lopes, D. R. G., de Souza Duarte, M., La Reau, A. J., Chaves, I. Z., de Oliveira Mendes, T. A., Detmann, E., Bento, C. B. P., Mercadante, M. E. Z., Bonilha, S. F. M., Suen, G., & Mantovani, H. C. (2021). Assessing the relationship between the rumen microbiota and feed efficiency in Nellore steers. *Journal of Animal Science and Biotechnology*, 12(1), 1–17. <https://doi.org/10.1186/s40104-021-00599-7>
- Macciotta, N. P. P., Vicario, D., Pulina, G., & Cappio-Borlino, A. (2002). Test Day and lactation yield predictions in Italian Simmental cows by ARMA methods. *Journal of Dairy Science*, 85(11), 3107–3114. [https://doi.org/10.3168/jds.S0022-0302\(02\)74398-1](https://doi.org/10.3168/jds.S0022-0302(02)74398-1)
- Mackle, T. R., Parr, C. R., Stakelum, G. K., Bryant, A. M., & MacMillan, K. L. (1996). Feed conversion efficiency, daily pasture intake, and milk production of primiparous Friesian and Jersey cows calved at two different liveweights. *New Zealand Journal of Agricultural Research*, 39(3), 357–370. <https://doi.org/10.1080/00288233.1996.9513195>

- Nguyen, Q. D., Tsuruta, T., & Nishino, N. (2020). Examination of milk microbiota, fecal microbiota, and blood metabolites of Jersey cows in cool and hot seasons. *Animal Science Journal = Nihon Chikusan Gakkaiho*, 91(1), e13441. <https://doi.org/10.1111/asj.13441>
- Nguyen, T. T., Wu, H., & Nishino, N. (2020). An investigation of seasonal variations in the microbiota of milk, feces, bedding, and airborne dust. *Asian-Australasian Journal of Animal Sciences*, 33(11), 1858–1865. <https://doi.org/10.5713/ajas.19.0506>
- O’Sullivan, M., Horan, B., Pierce, K. M., McParland, S., O’Sullivan, K., & Buckley, F. (2019). Milk production of Holstein-Friesian cows of divergent Economic Breeding Index evaluated under seasonal pasture-based management. *Journal of Dairy Science*, 102(3), 2560–2577. <https://doi.org/10.3168/jds.2018-15559>
- Oikonomou, G., Addis, M. F., Chassard, C., Nader-Macias, M. E. F., Grant, I., Delbès, C., Bogni, C. I., Le Loir, Y., & Even, S. (2020). Milk Microbiota: What Are We Exactly Talking About? *Frontiers in Microbiology*, 11(February), 1–15. <https://doi.org/10.3389/fmicb.2020.00060>
- Ospina, P. A., Nydam, D. V., Stokol, T., & Overton, T. R. (2010). Evaluation of nonesterified fatty acids and β -hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. *Journal of Dairy Science*, 93(2), 546–554. <https://doi.org/10.3168/jds.2009-2277>
- Petri, R. M., Forster, R. J., Yang, W., McKinnon, J. J., & McAllister, T. A. (2012). Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *Journal of Applied Microbiology*, 112(6), 1152–1162. <https://doi.org/10.1111/j.1365-2672.2012.05295.x>
- Prendiville, R., Pierce, K. M., Delaby, L., & Buckley, F. (2011). Animal performance and production efficiencies of Holstein-Friesian, Jersey and Jersey $\times$ Holstein-Friesian cows throughout lactation. *Livestock Science*, 138(1–3), 25–33. <https://doi.org/10.1016/j.livsci.2010.11.023>
- Rajala-Schultz, P. J., & Saville, W. J. A. (2003). Sources of variation in milk urea nitrogen in Ohio dairy herds. *Journal of Dairy Science*, 86(5), 1653–1661. [https://doi.org/10.3168/jds.S0022-0302\(03\)73751-5](https://doi.org/10.3168/jds.S0022-0302(03)73751-5)
- Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., Turner, P., Parkhill, J., Loman, N. J., & Walker, A. W. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*, 12(1), 1–12. <https://doi.org/10.1186/s12915-014-0087-z>
- Sharma, N., Singh, N. K., & Bhadwal, M. S. (2011). Relationship of somatic cell count and mastitis: An overview. *Asian-Australasian Journal of Animal Sciences*, 24(3), 429–438. <https://doi.org/10.5713/ajas.2011.10233>
- Stojević, Z., Piršljin, J., Milinković-Tur, S., Zdelar-Tuk, M., & Ljubić, B. B. (2005). Activities of AST, ALT and GGT in clinically healthy dairy cows during lactation and in the dry period. *Veterinarski Arhiv*, 75(1), 67–73.

- Uddin, M. E., Aguirre-Villegas, H. A., Larson, R. A., & Wattiaux, M. A. (2021). Carbon footprint of milk from Holstein and Jersey cows fed low or high forage diet with alfalfa silage or corn silage as the main forage source. *Journal of Cleaner Production*, 298, 126720. <https://doi.org/10.1016/j.jclepro.2021.126720>
- Washburn, S. P., White, S. L., Green, J. T., & Benson, G. A. (2002). Reproduction, mastitis, and body condition of seasonally calved holstein and jersey cows in confinement or pasture systems. *Journal of Dairy Science*, 85(1), 105–111. [https://doi.org/10.3168/jds.S0022-0302\(02\)74058-7](https://doi.org/10.3168/jds.S0022-0302(02)74058-7)
- Wu, H., Nguyen, Q. D., Tran, T. T. M., Tang, M. T., Tsuruta, T., & Nishino, N. (2019). Rumen fluid, feces, milk, water, feed, airborne dust, and bedding microbiota in dairy farms managed by automatic milking systems. *Animal Science Journal*, 90(3), 445–452. <https://doi.org/10.1111/asj.13175>
- Xue, M. Y., Sun, H. Z., Wu, X. H., Guan, L. L., & Liu, J. X. (2019). Assessment of rumen bacteria in dairy cows with varied milk protein yield. *Journal of Dairy Science*, 102(6), 5031–5041. <https://doi.org/10.3168/jds.2018-15974>
- Yoo, J., Song, M., Park, W., Oh, S., Ham, J. S., Jeong, S. G., & Kim, Y. (2019). A comparison of quality characteristics in dairy products made from Jersey and Holstein milk. *Food Science of Animal Resources*, 39(2), 255–265. <https://doi.org/10.5851/kosfa.2019.e20>

4 CHAPTER 4

Milk microbiota of Holstein cows is stable despite changes in milk composition, blood metabolites, and environmental microbiota.

4.1 Abstract

Holsteins are renowned for high milk production, which predisposes them to nutritional disorders, particularly during the periparturient period. Although farmers mount dietary intervention strategies to stave off such disorders, periodic monitoring of metabolites is necessary since such interventions do not yield the expected outcome always. Similarly, monitoring milk microbiota occasionally is equally essential due to the influence of the initial milk microbiota composition and diversity on processed milk quality. Since raw bovine milk is highly susceptible to microbial contamination, especially from the ambient environment like airborne dust and beddings, regular hygiene monitoring of the ambient environment is vital. In light of the reasons mentioned above, we performed this study to monitor how lactating Holsteins fare throughout the lactation metabolically and how the microbiota of their milk and airborne dust of where they are housed was. After every other month, from the beginning of the lactation to the end, we collected blood and milk samples from 8 Holstein cows reared at Okayama Prefecture Livestock Research Institute (Okayama, Japan). The experimental cows were housed in a free stall barn and fed total mixed ration silage throughout the lactation. We also collected one airborne dust and bedding sample every time we collected other samples. Commercially available kits were used to analyze blood metabolites, while a CombiFoss™ analyzer was used to analyze milk composition. The microbiota of Milk, bedding, and airborne dust was sequenced on a MiSeq platform. Milk fat and protein increased as DIM increased. MUN, total cholesterol, and NEFA were unchanged across the lactation. Hepatic enzymes, GOT and GPT, were unusually low at the beginning of the lactation and increased towards the end. Milk and airborne dust microbiota were surprisingly stable across the lactation and the collection months, respectively. Milk microbiota was dominated by Lactobacillaceae, Lachnospiraceae, Muribaculaceae, and Micrococcaceae at 15.4%, 15.4%, 14.4%, and 10.2%, respectively. Airborne dust, on the other hand, was dominated by S24-7 (21.2%), Lactobacillaceae (17.3%), Ruminococcaceae (14.7%), o__Clostridiales (13.4%), and Lachnospiraceae (11.3%). Aerococcaceae, Corynebacteriaceae, and Ruminococcaceae were the most abundant bedding bacterial families at 26%, 14%, and 11%, respectively. Milk and airborne dust microbiota was clustered together and separately from bedding microbiota. This indicated bedding did not influence milk microbiota.

4.2 Introduction

High quality and quantity of milk production is the prime goal of farmers globally. Milk quality, from the processors' perspective, is indicated by the level of protein and fat in milk. Although Holstein cows are known for the highest milk production throughout the lactation period among other dairy breeds, the proportion of fat in milk is usually the lowest among other breeds (Dobson et al., 2007; Mogollón-García et al., 2020). Due to this exceptionally high milk production across the entire lactation period, Holstein cows are susceptible to physiological and nutritional disorders (González et al., 2011). These disorders are experienced mainly during the early lactation period when cows have a jaded appetite. Holstein cows have markedly reduced dietary intake during the early lactation period if proper nutritional management practices are not instituted. This leads to a negative energy balance, where cows are compelled to mobilize energy reserves. For instance, González et al., (2011) argue that due to milk production and limited feed intake, high-yielding dairy cows experience lipomobilization. This lipomobilization, as reported by Bradford et al., (2015), may lead to fatty infiltration of up to 25% of liver tissue. If this fat accumulation in hepatic tissue exceeds its oxidation ability, Holsteins develop fatty liver disease. Lactating Holsteins continue to experience such physiological changes due to high milk production, body maintenance, and reproductive changes as the lactation period advances. These inevitable physiological changes trigger metabolic changes, which, if not controlled, might blight milk production (Dubuc et al., 2011; Kim et al., 2016). Mezzetti et al., (2021) elucidated how the transition period should be handled holistically, and particularly, they underscored the welfare condition of the lactating high-yielding cows, superior nutritional management, and proper modulation of metabolic processes that would more likely overwhelm the control of homeostatic mechanisms. Farmers manage to maintain metabolic profiles of lactating Holstein cows within the physiologically normal levels through dietary manipulations. The said dietary interventions, though aimed at maintaining lactating cows physiologically healthy, do not invariably occasion the expected outcomes (Moyes et al., 2010). In their research, Moyes et al., (2010) found that manipulating dietary protein to starch was not a potential strategy to solve physiological imbalance experienced by high-yielding cows during early lactation. Hence, blood metabolic profiles are conventionally conducted to monitor the metabolic and physiological statuses of lactating cows. Caixeta & Omontese, (2021) emphasized the necessity of regularly monitoring dairy cows during the early lactation period since it helps in detecting early metabolic anomalies, diagnose clinical and subclinical diseases, and initiate restorative health management to avoid health and milk production impairment. Different metabolic indicators such as NEFA and total cholesterol are usually monitored to indicate energy status (González et al., 2011), albumin and BUN to indicate protein status, and GPT & GOT to indicate the liver health of lactating cows (Stojević et al., 2005).

Also, the safety and shelf-life of milk and milk products highly hinge on raw milk microbiota (Desmasures & Gueguen, 1997; Schmidt et al., 2012). Milk, by virtue of being nutritionally rich, harbors manifold bacteria (Quigley et al., 2013). A while ago, aseptically freshly drawn milk was considered sterile (Evans, 1916), but this assumption was controverted

when aseptically freshly drawn milk was proven to be inhabited by microbiota (McGuire & McGuire, 2017). These bacteria that inhabit freshly drawn milk can be pathogenic, or spoilage, and/or technologically beneficial (Nguyen et al., 2020). Although milk is generally exposed to microbial annihilative thermal processing before consumption, dairy researchers have been unanimous that the microbial composition of milk before thermal processing affects the quality of the resultant milk products (Barbano et al., 2006). On the other hand, some milk products, such as cheese, are produced from raw milk, so the knowledge of raw milk microbiota remains crucial.

Several studies have investigated different aspects of milk composition, metabolic profiles, and microbiota. These preceding studies have found out seminal fundamental results, which have informed dairy stakeholders differently. However, there is still a paucity of information about how milk production, metabolic profiles, and milk microbiota change throughout the lactation periods of clinically healthy Holstein Friesian cows. So, this study was designed to gain insights into how clinically healthy lactating cows metabolically fare throughout their lactation periods, milk yield and components, and how aseptically freshly drawn milk microbiota are bi-monthly across the lactation cycles of clinically healthy Friesian lactating cows. Also, this study looked into the microbiota of airborne dust and beddings collected in the barn housing the experimental cows. This was necessary since environmental microbiota had been reported to influence milk microbiota (Nguyen et al., 2020 & Wu et al., 2019)

4.3 Materials and Methods

4.3.1 Sample collection

Milk samples were collected from eight lactating Holstein cows randomly selected from a herd reared in a research institute in Okayama, Japan. These experimental cows were randomly selected from a subset of the herd that calved down within the same month. The collection of milk samples began in the first month of the lactation period and continued every other month until the end of the lactation period (the first month corresponded to August and the last month was April). Each milk sample was collected after cleaning the surface of the udder and four teats with clean lukewarm water and wiping the teats with clean towels. The first few streams of foremilk were discarded, and the subsequent streams from the four teats were aseptically collected into a 50 ml collection tube. The tubes were labeled and frozen in liquid nitrogen forthwith. The collected milk samples were kept in a liquid nitrogen-containing box during transportation and frozen at -31°C till further analyses.

Airborne dust samples were collected from the experimental cowshed by placing three opened Petri dishes in different locations approximately one meter above the ground for about five minutes. After the collection time was over, each petri dish was immediately covered with a lid and sello-taped. In the laboratory, each petri dish was rinsed with 1 ml of sterile saline to make a composite liquid sample (T. T. Nguyen et al., 2020). The composite samples were frozen at -31°C till DNA extraction. Rumen fluid was collected as Wu et al., (2019) outlined. Blood samples, on the other hand, were taken from caudal veins into vials containing anticoagulant, heparin, and transported on ice.

4.3.2 Milk composition and blood metabolites analyses

The somatic cell count (SCC) in milk was estimated by N-acetyl- β -d-glucosaminidase (NAGase) activity assay. The content of fat, protein, solids-not-fat, and milk urea nitrogen (MUN) in milk was determined using a CombiFossFT + analyzer (Foss Allé, Hillerød, Denmark) as documented by Nguyen et al. (2020).

Plasma samples were analyzed for total cholesterol (T-Cho), albumin (Alb), non-esterified fatty acids (NEFA), total protein, calcium, phosphorus, blood urea nitrogen (BUN), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) by following the instructions of commercially available kits (Fujifilm Wako Pure Chemicals Co.). A commercial bovine Hp ELISA kit (Life Diagnostics, Inc.) was used to determine the plasma haptoglobin (Hp) content.

4.3.3 Bacterial DNA extraction

Bacterial DNA from milk and airborne dust samples were extracted from 1 ml of milk and airborne dust as described by Nguyen et al. (2020). Briefly, 1 ml of milk was centrifuged at $16,000 \times g$ for 15 min at 4°C . The fat layer was scraped off and the supernatant was pipetted out. 1 ml of airborne dust was centrifuged at the same condition as aforementioned, and the

supernatant was removed. The DNA pellets were washed and re-suspended in 1 × PBS (phosphate-buffered saline). 250 µL of the re-suspended DNA pellets of milk and airborne dust and 0.2g of beddings were transferred into 2 ml screw cap microcentrifuge tubes containing 0.4g zirconia beads (0.1 mm 3:1 0.5 mm) and 1 ml of lysis buffer (0.5M NaCl, 0.05M Tris-HCl, 0.05M EDTA, 4% sodium dodecyl sulfate). The mixture was homogenized by a mini-bead beater, incubated at 70° C for 15 minutes, and the resultant supernatant was mixed with 200 µL ammonium acetate. After centrifugation at 16,000 x g for 10 min at 4°C, the resultant supernatant was mixed with an equal amount of 2-propanol, incubated on ice for 30 minutes, centrifuged, and the DNA pellets washed and re-suspended in 100 µL of TE. The DNA was purified from the DNA suspension using the DNeasy Stool Mini Kit (Qiagen, Germantown, MD, USA).

4.3.4 16S rRNA gene amplicon MiSeq sequencing

To generate an amplicon library for MiSeq 16S rRNA gene sequencing, the primers and the PCR protocol were as reported previously. Briefly, the primers were the forward primer 5'-ACACTCTTCCCTACACGACGCTCTTCCGATCTGTGCCAG

CMGCCGCGGTAA-3 & the reverse primer 5-GTGACTGGAGTTCAGACGTGTGCTCTTCC

GATCTGGACTACHVGGGTWTCTAAT-3 targeting the hypervariable region V4 of the 16S ribosomal gene. The PCR protocol used to generate the first-round PCR products was as follows: The initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s, and the final elongation at 72 °C for 5 min. The amplicons were run on 1% agarose gel and the targeted bands were excised. The excised bands were purified using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan). The purified first-round PCR amplicons were used as template DNA samples for the second-round PCR.

With adapter-attached primers, the purified first-round PCR amplicons were used as DNA templates for the second-round PCR, and the second-round PCR was performed by following this thermo-cycling program: the initial denaturation was at 94°C for 2 mins followed by 10 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, elongation at 72°C for 30 sec, and the final elongation of 72°C for 5 min. The resultant second-round PCR amplicons were purified as described above and sequencing was done at FASMAC Co., Ltd.

4.3.5 Bioinformatics and statistical analyses

The Quantitative Insights Into Microbial Ecology (QIIME2) version 2022.2 was used to analyze the pair-end raw sequences. Raw sequences, which were about 250bp, were truncated at the site whose quality score was below 25(forward reads; 177 reverse reads 134). Quality-truncated reads with a 60 bp overlap were pair-end joined, and the quality assessed, chimera-free, pair-end sequences were grouped into operational taxonomic units with a similarity threshold of above 97%.

The JMP software version 14.2.0 was used to statistically analyze all microbial, metabolites, and milk composition data. Tukey test was used to determine if there were statistical differences between means. Principal coordinate analysis (PCoA) was performed by using the primer7 version 7.0.14 (Primer-E, Plymouth Marine Laboratory, Plymouth, UK) to determine how milk and airborne dust microbiota was clustered.

4.4 Results

4.4.1 Holsteins' milk production and milk composition

Milk yield during the second month post parturition, when the collection of milk samples began, was about 32Kg per day, as shown in table 4.1. Milk production continued to increase steadily till the six month post-parturition (peak production 35Kg per day). After the peak milk production, milk production declined to about 31Kg per day at about 300 days in milk (DIM). Milk fat, on the other hand, increased progressively throughout the lactation period from 3.2% two months after calving down to 4.1% just before drying off. Protein content in milk also increased throughout the lactation period from about 2.9% two months after giving birth to 3.6% after approximately 9 months of milk production. Solids-not-fat were higher during the late lactation, but milk urea nitrogen (MUN). Somatic cells remained unchanged and below the generally accepted threshold of 2.0×10^5 cells/ml.

Table 4. 1 days in milk, milk yield, and milk composition of Holsteins across a lactation period

Months	AUG	OCT	DEC	FEB	APR	SE
DIM	67.75	123.75	185.75	241.75	298.75	
YIELD (kg/day)	31.79	33.13	35.47	31.40	30.59	4.74
Milk composition						
FAT (%)	3.17 ^b	3.50 ^{ab}	3.66 ^{ab}	3.99 ^a	4.07 ^a	0.18
PROTEIN (%)	2.86 ^b	3.13 ^{ab}	3.42 ^{ab}	3.46 ^a	3.56 ^a	0.13
SNF (%)	8.50 ^c	8.72 ^{bc}	9.18 ^{ab}	9.02 ^{abc}	9.27 ^a	0.13
SCC($\times 10^3$ cells/ml)	177.00	219.00	59.71	94.75	140.25	65.14
MUN (mg/ml)	14.30	14.10	13.21	13.18	14.94	0.8

Rows whose means have different letter superscripts depict statistical differences among means

Abbreviations: SNF; solids-not-fat, SCC; somatic cells count, and MUN; milk urea nitrogen

4.4.2 Holsteins' metabolic profiles

Total cholesterol and non-esterified fatty acids (NEFA) were unchanged statistically across the lactation period. Albumin increased from the beginning of the lactation to the penultimate month of the lactation. The last month, just before drying off, albumin content in the blood slightly declined. On the other hand, blood urea nitrogen changed significantly across the lactation period. Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), liver function indicators, were lower in the early lactation period and increased in the late lactation period, as graphically shown in figure 4.1. Calcium content was lower towards the end of the lactation, as depicted in table 4.2.

Table 4. 2 Holsteins' blood metabolites from the beginning to the end of a lactation period

Months	AUG	OCT	DEC	FEB	APR	SE
Metabolites						
Total cholesterol(mg/dl)	141.14	195.50	201.75	164.25	189.63	8.93
NEFA (uEq/L)	241.14	199.50	229.75	184.88	182.88	9.37
Albumin (g/dl)	3.69 ^b	4.10 ^{ab}	4.21 ^a	4.37 ^a	4.24 ^a	0.09
BUN (mg/dl)	9.99 ^{ab}	7.83 ^b	10.66 ^{ab}	11.58 ^a	11.10 ^a	0.52
GPT(IU/L)	3.89 ^b	4.31 ^b	4.06 ^b	5.52 ^a	5.70 ^a	0.30
GOT(IU/L)	19.61 ^b	22.33 ^{ab}	19.16 ^b	24.99 ^{ab}	28.75 ^a	1.41
Calcium (mg/dl)	10.09 ^{ab}	10.14 ^a	9.11 ^{bc}	9.69 ^{abc}	9.03 ^c	0.19

Rows whose means have different letter superscripts depict statistical differences among means

Abbreviations: BUN; blood urea nitrogen, NEFA; non-esterified fatty acid, GOT; glutamic oxaloacetic transaminase, and GPT; glutamic pyruvic transaminase, SE; standard error

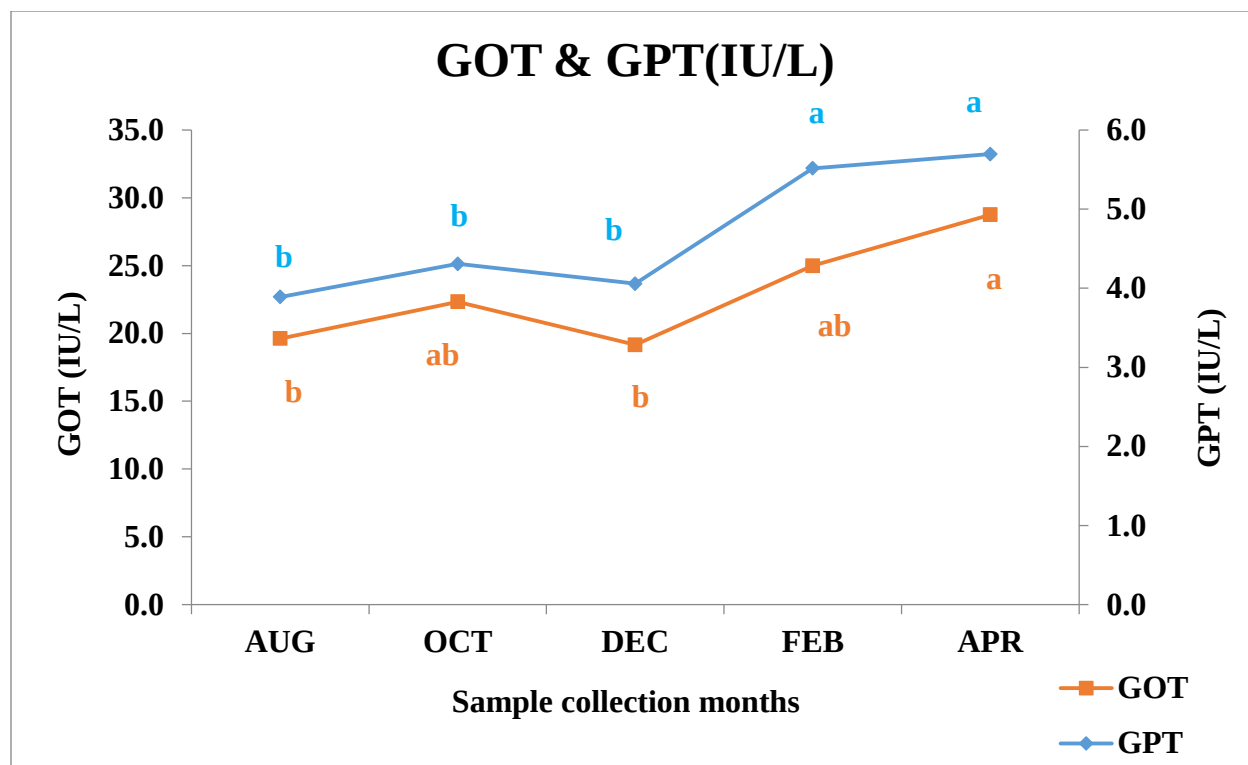


Figure 4. 1the trend of liver function indicators across the lactation

4.4.3 The relationship between milk composition and blood metabolites

Notably, Milk protein and albumin content in the blood had somewhat a similar trend across the lactation, as shown in figure 4.2. MUN and BUN, which expectedly are supposed to equilibrate rapidly, were unrelated across the lactation period. BUN concentration seemed not to have any proportionate relation with MUN, as shown in table 4.1 and 4.2

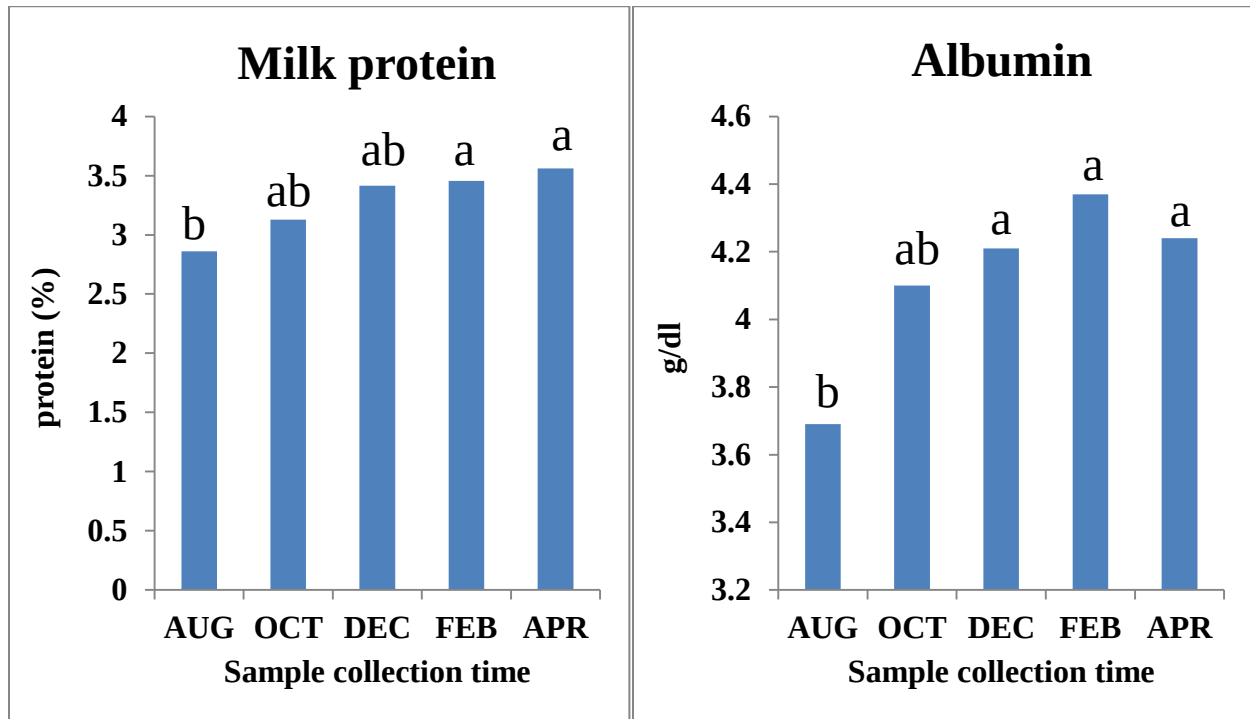


Figure 4. 2 the trend of both milk protein and albumin in the blood

4.4.4 Milk microbiota

Firmicutes, Bacteroidota, and Actinobacteriota were the three most abundant phyla, averaging at 56.7%, 22%, and 12.2%, respectively. At the family level, Lactobacillaceae, Lachnospiraceae, Muribaculaceae, and Micrococcaceae were the most predominant families at 15.4%, 15.4%, 14.4%, and 10.2%, respectively. Milk microbiota remained relatively stable across the lactation and unaffected by season as well. Putative contaminants were not detected in this survey.

Table 4. 3 bacterial diversity indices, phyla, and families of milk produced by Holstein cows across a lactation period

Month	AUG	OCT	DEC	FEB	APR
Diversity indices					
Chao1	205	201	258	205	215
Shannon	5.2	5.14	5.37	5.2	5.24
Simpson	0.94	0.94	0.95	0.94	0.94
Phyla/family					
Firmicutes	55.71	55.76	57.33	56.96	57.91
Lactobacillaceae	15.27	15.3	15.08	15.56	15.73
Clostridia_UCG-014	6.96	7.04	6.8	7.03	7.05
Lachnospiraceae	15.12	15.33	15.03	15.67	15.61
Erysipelotrichaceae	1.27	1.31	1.41	1.32	1.38
Oscillospiraceae	2.94	3.02	3.42	3.06	3.3
Aerococcaceae	0.25	0.24	0.58	0.32	0.57
Streptococcaceae	0.45	0.46	0.7	0.52	0.49
Carnobacteriaceae	0.45	0.31	0.62	0.2	0.21
Ruminococcaceae	0.61	0.61	0.81	0.71	0.77
Clostridiaceae	0.54	0.5	0.61	0.52	0.55

Anaerovoracaceae	0.36	0.4	0.45	0.4	0.43
Staphylococcaceae	0.14	0.11	0.17	0.16	0.11
Bacilli__RF39	1.24	1.24	1.24	1.25	1.23
Bacteriodota	21.87	21.35	21.86	22.29	22.39
Prevotellaceae	0.41	0.37	0.4	0.38	0.4
Rikenellaceae	3.61	3.51	3.53	3.68	3.7
Bacteroidaceae	3.52	3.35	3.5	3.71	3.36
Muribaculaceae	14.24	14.05	14.24	14.43	14.77
Actinobacteriota	12.73	21.35	21.86	22.29	22.39
Corynebacteriaceae	1.58	1.57	1.45	1.43	1.32
Micrococcaceae	10.67	10.83	9.99	10.03	9.41
Proteobacteria					
Yersiniaceae	8.19	8.25	7.32	7.42	7
Succinivibrionaceae	0.03	0.01	0.17	0.02	0.06
Enterobacteriaceae	1.05	1.35	1.04	1.09	1.1
Phyla and families with a relative abundance of >0.5% in at least one sample					

4.4.5 Airborne dust microbiota

S24-7 (21.2%), Lactobacillaceae (17.3%), Ruminococcaceae (14.7%), o__Clostridiales (13.4%), and Lachnospiraceae (11.3%) were the most dominant five families in the airborne dust of the experimental herd's shed. The microbial composition of airborne dust across the lactation remained unchanged and stable.

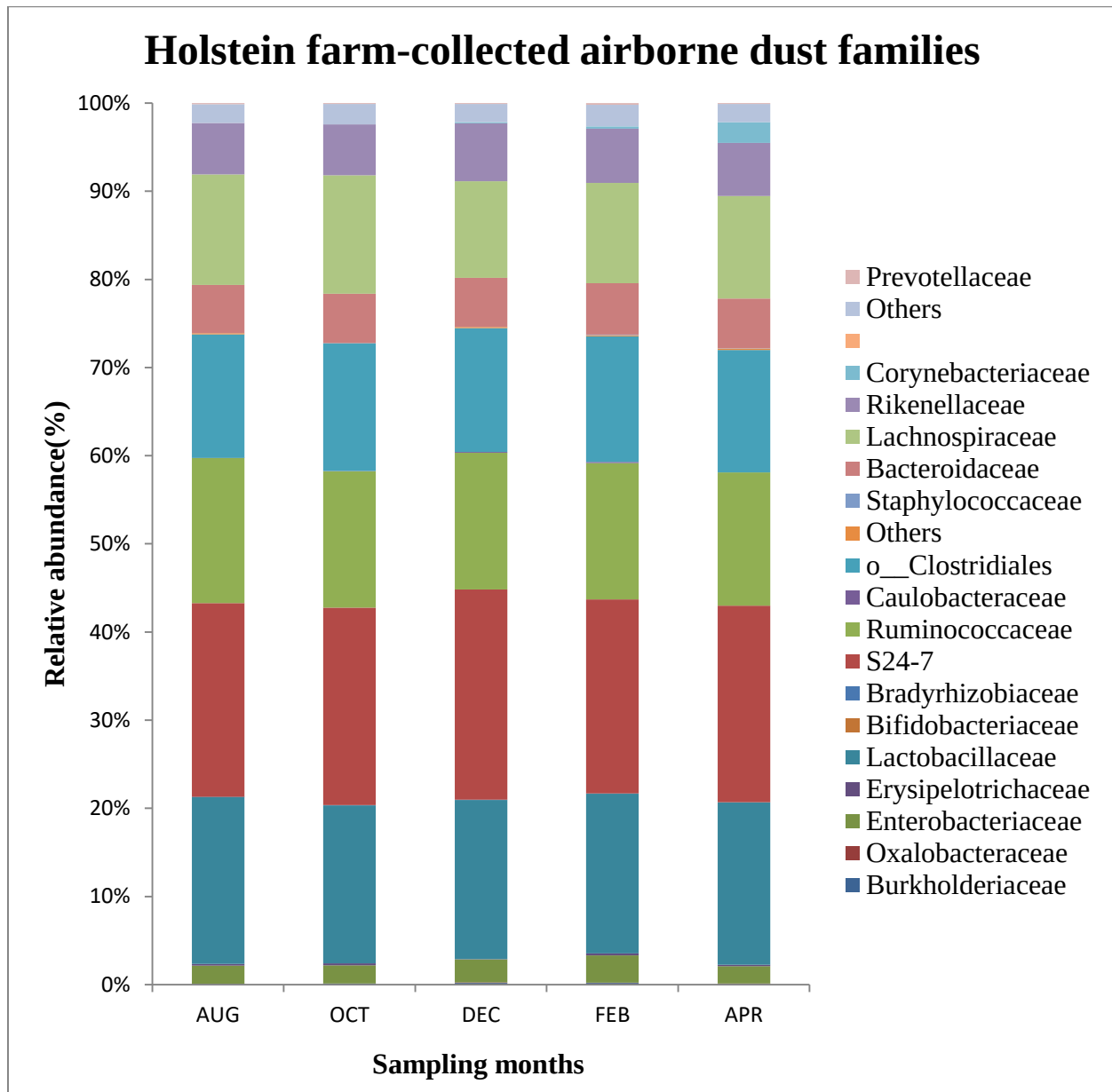


Figure 4. 3 microbiota of Holstein collected airborne dust across the lactation of Holstein cows

4.4.6 Bedding microbiota

Aerococcaceae, Corynebacteriaceae, and Ruminococcaceae were the most abundant families at 26%, 14%, and 11%, respectively. The relative abundance of Aerococcaceae was highest in August and December at 39% and 30%, respectively.

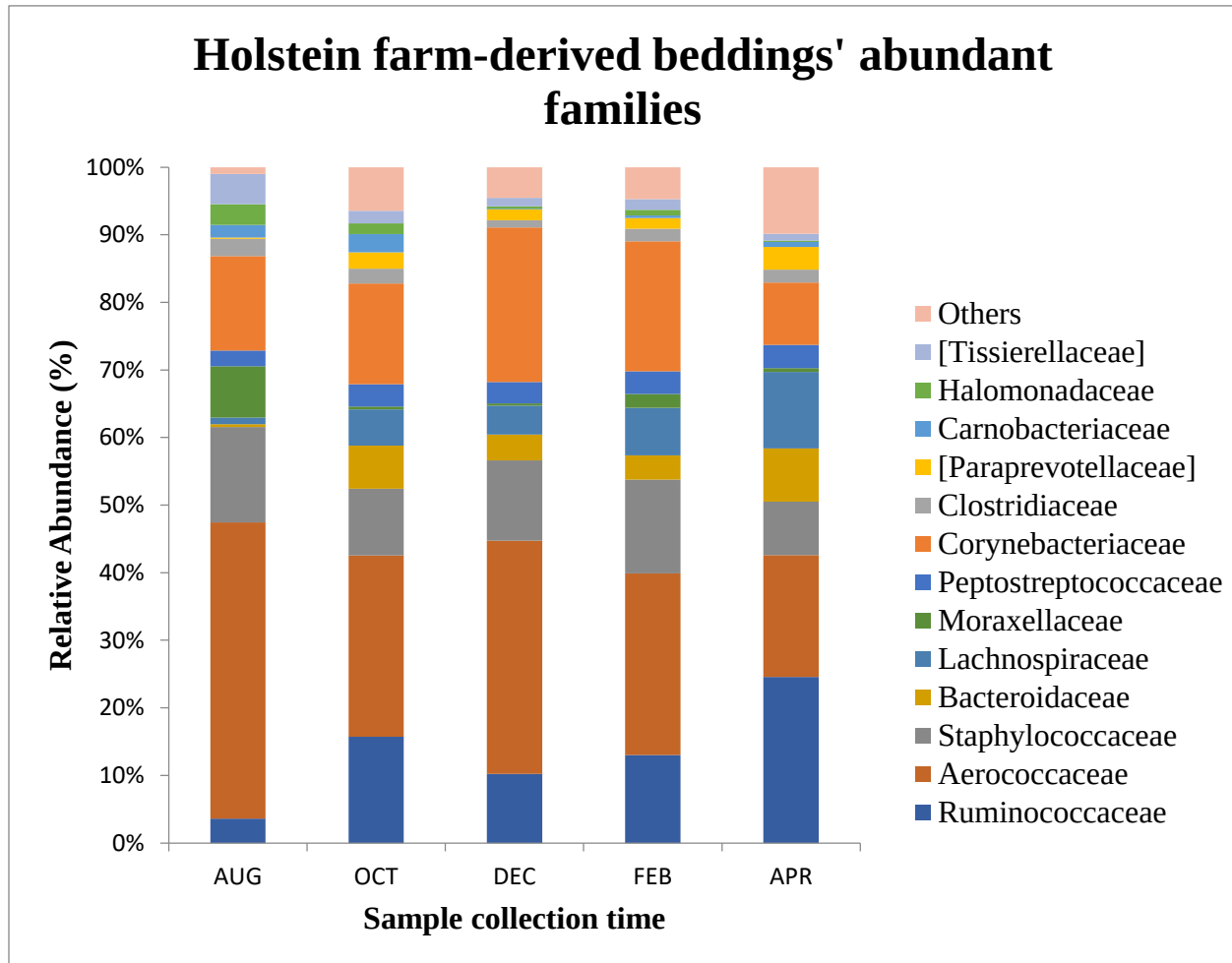


Figure 4. 4 microbiota of beddings collected from the Holstein cowshed

4.4.7 The relationship between airborne dust, bedding, and milk microbiota

According to PCoA clustering, milk and airborne microbiota were clustered together. However, bedding microbiota was clearly separated from both airborne and milk microbiota.

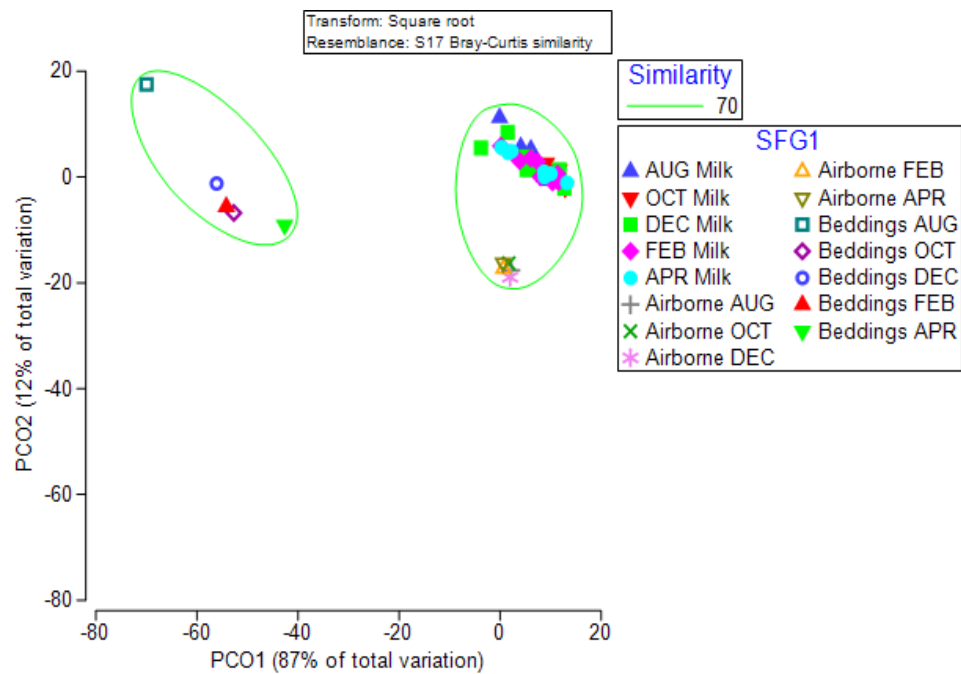


Figure 4. 5 PCoA showing clustering of bedding, milk and airborne dust microbiota

4.5 Comparison between the two breeds' milk composition, metabolic profiles and core microbiota

4.5.1 Milk composition

As expected, Jersey cows, across the entire lactation period, produced milk with superior milk fat and protein to their counterpart Holstein cows. Jersey milk fat ranged from 4.5% at the beginning of the lactation to 5.9% at the end of lactation, while Holstein milk fat was 3.2% to 4.1% at the onset and the end of the lactation, respectively. Milk protein, on the other hand, was 3.4% to 3.5% from the start to the end of the Jersey lactation. On the contrary, Holstein cows produced milk with 2.9% and 3.6% protein at the beginning and the end of the lactation, respectively. Jerseys produced about 27Kg/day in the first month of the lactation and peaked in the second month with a daily yield of 27.3 Kg/day. The milk yield decreased gradually as DIM increased to 14.8 Kg/day. On the other hand, Holsteins produced 31.8 Kg/day in the first month of our sample collection and peaked at 35.5 Kg/day in the fifth month. Holsteins milk production decreased to 30.6 Kg/day before drying off.

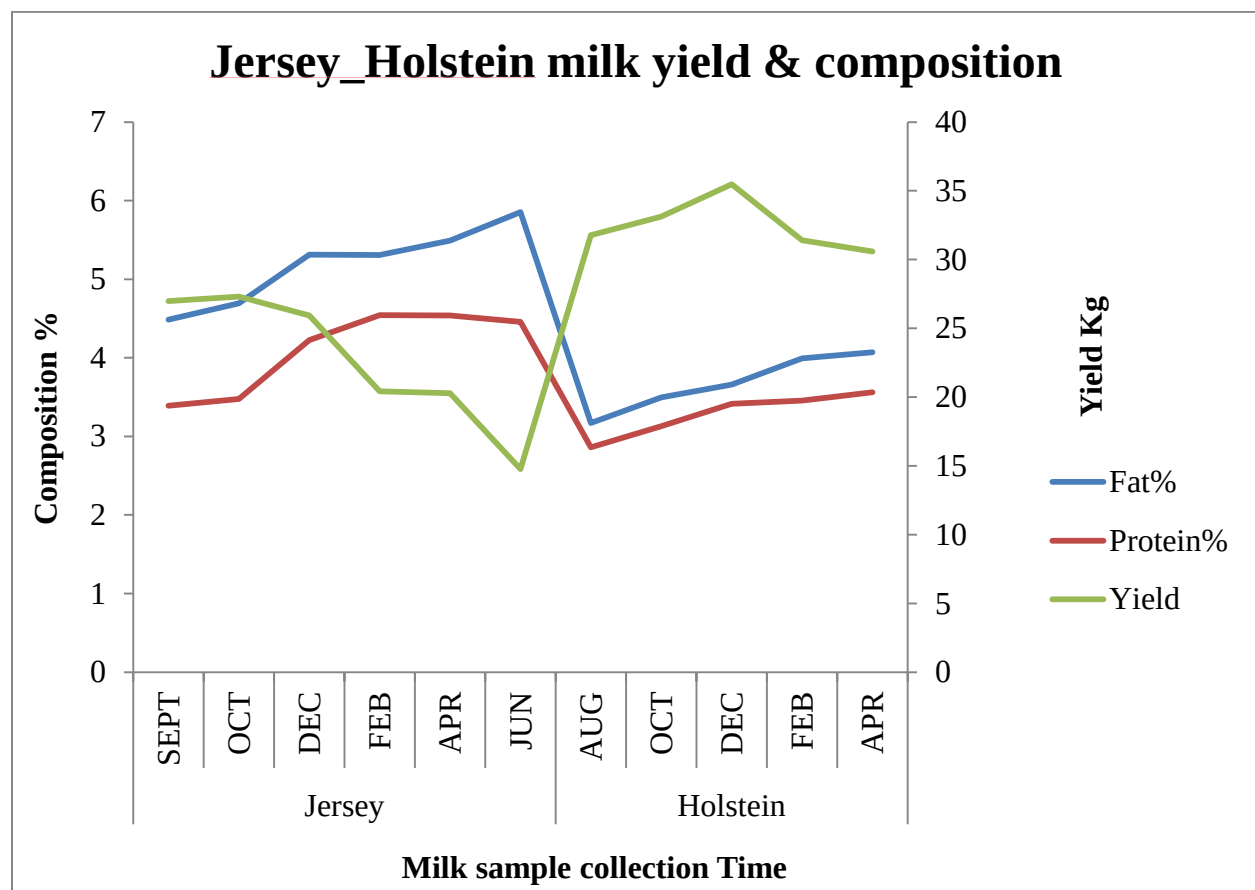


Figure 4. 6 a comparison between Holsteins and Jerseys' milk yield and composition

4.5.2 Metabolic profiles

4.5.2.1 Energy status

The two breeds experienced different energy patterns throughout the lactation. Jersey cows had the highest NEFA values at the beginning of the lactation, 0.39 mEq/L, as opposed to Holstein cows which had 0.24 mEq/L. Jersey cows' NEFA plummeted to 0.09 mEq/L in the penultimate lactation month, while the lowest Holstein cows' NEFA was 0.18 mEq/L in the same period. The total cholesterol for both breeds, on the other hand, was the lowest at the beginning of their respective lactations. Jersey cows' T-cho was 160.35 mg/dl at the beginning of the lactation, while Holstein cows' T-cho was 141.14 mg/dl. For both breeds, T-cho increased as their respective lactations advanced and peaked at 229.3 and 201.85 mg/dl for Jersey and Holstein cows, respectively.

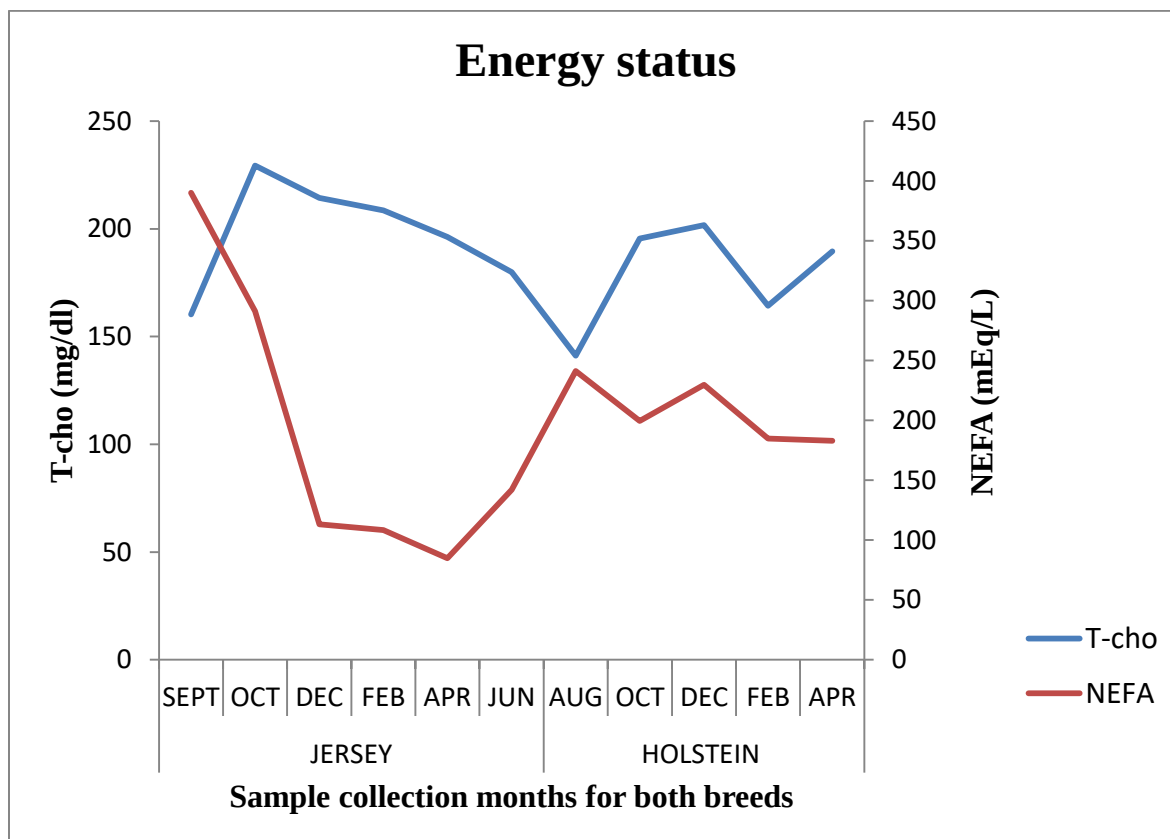


Figure 4. 7 a comparison of energy status indicators between Holsteins and Jerseys

4.5.2.2 Protein status

The two breeds had different lactation-long patterns for protein status. Jerseys' protein status and protein metabolic products fluctuated in tandem across the lactation. BUN, MUN, and Alb were 8.96 mg/dl, 6.13 mg/dl, and 3.85 g/dl, respectively, at the beginning of the Jersey lactation. While BUN and MUN dipped in the subsequent month to 3.86 and 5.57 mg/dl, there was an uptick in Alb content to 4.03 g/dl. BUN, MUN, and Alb peaked at 14.19 mg/dl, 12.29 mg/dl, and 7.04 g/dl, respectively. Holsteins' BUN, MUN, and Alb fluctuated irregularly and no unique changing pattern was observed. At the beginning of the lactation, Alb was at the lowest level of 3.69 g/dl, while MUN was at 14.30 mg/dl - the lactation-long second highest. MUN remained unchanged across the lactation, while BUN and Alb were statistically higher as the lactation progressed. BUN and Alb peaked at 11.58 mg/dl and 4.37 g/dl, respectively.

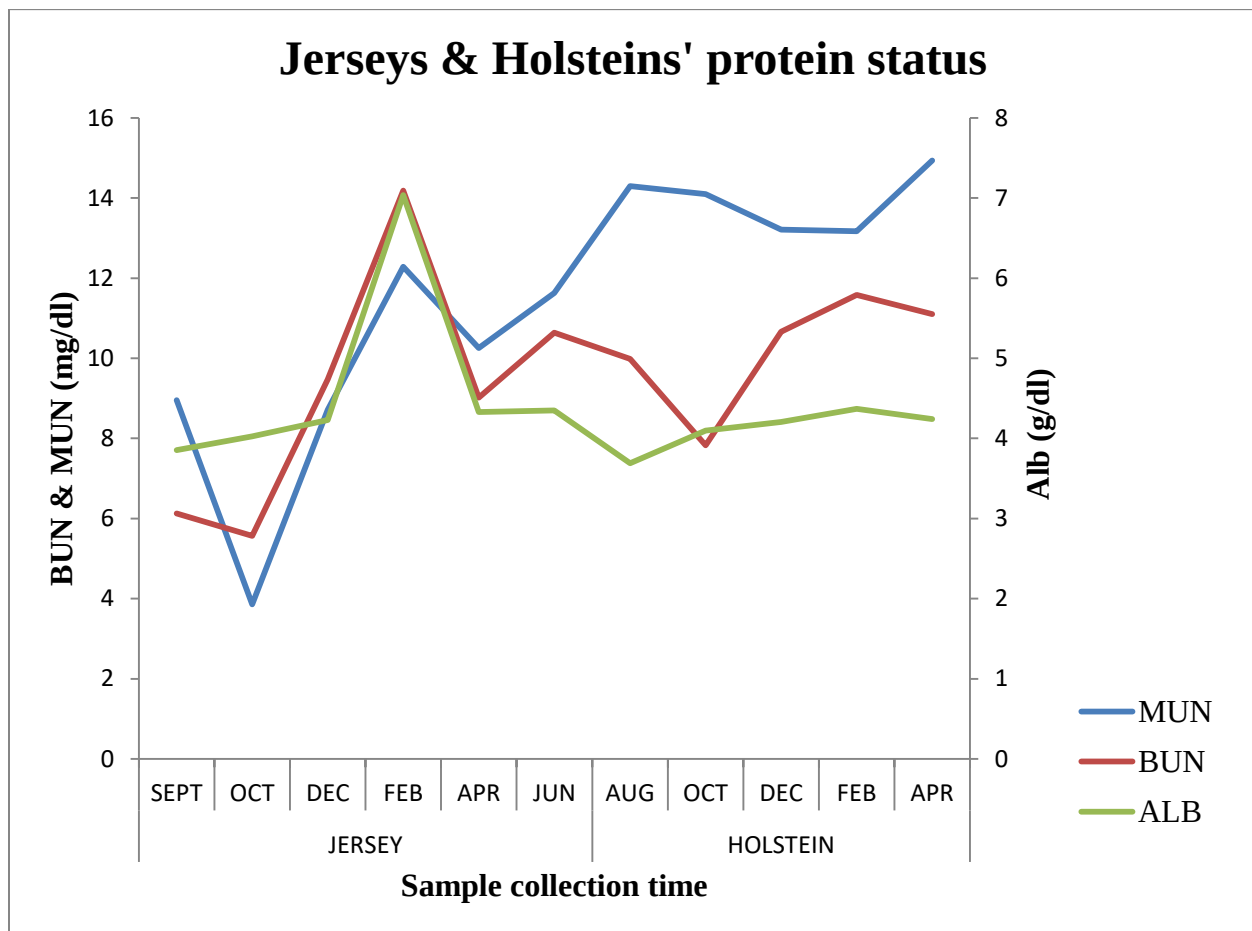


Figure 4. 8 a comparison between Jerseys and Holsteins' BUN, MUN, and Albumin

4.5.2.3 Liver function

Hepatically, the two breeds fared differently across their respective lactation periods. After parturition, Jersey cows' liver function indicators were 16.49 IU/L and 112.29 IU/L for GPT and GOT, respectively. Holstein cows, on the other hand, had 3.89 IU/L and 19.61 IU/L for GPT and GOT, respectively, at the beginning of their lactation. Jerseys' liver function indicators, GPT and GOT, fluctuated irregularly across the lactation, while Holsteins' liver function indicators steadily increased as the lactation advanced. The Jerseys' peak values for GPT and GOT were 5.70 and 28.75 IU/L, respectively, while Holsteins' peak values were 33.62 IU/L for GPT and 112.29 IU/L for GOT.

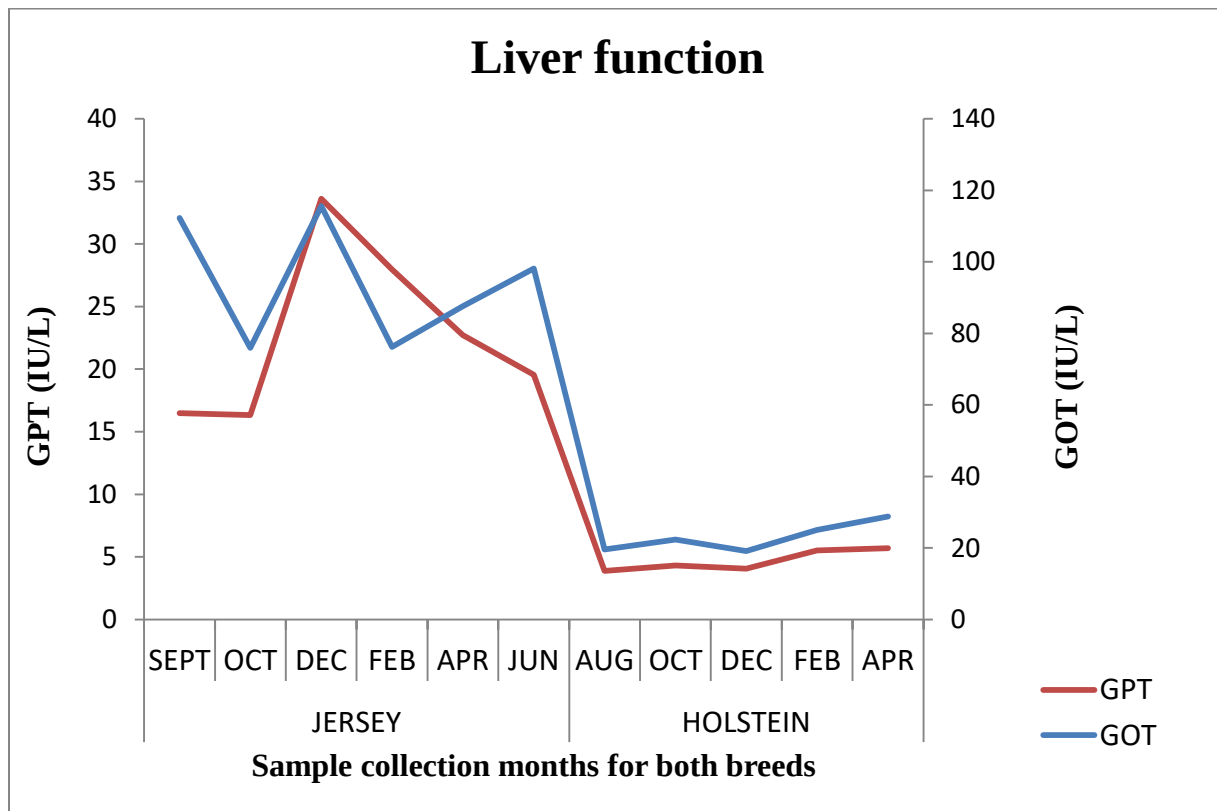


Figure 4. 9 how Holsteins and Jerseys liver function indicators fared across the lactation

4.5.2.4 Mineral content

Calcium content in blood for both breeds had somewhat a similar changing pattern. At the beginning of the lactation, Jerseys' had 8.28 mg/dl of Calcium circulating in their blood, while Holsteins had 10.09 mg/dl circulating. When their respective lactations came to an end, Holsteins had 9.03 mg/dl of Calcium circulating in their blood, while Jerseys' blood-circulating Calcium was 8.61 mg/dl.

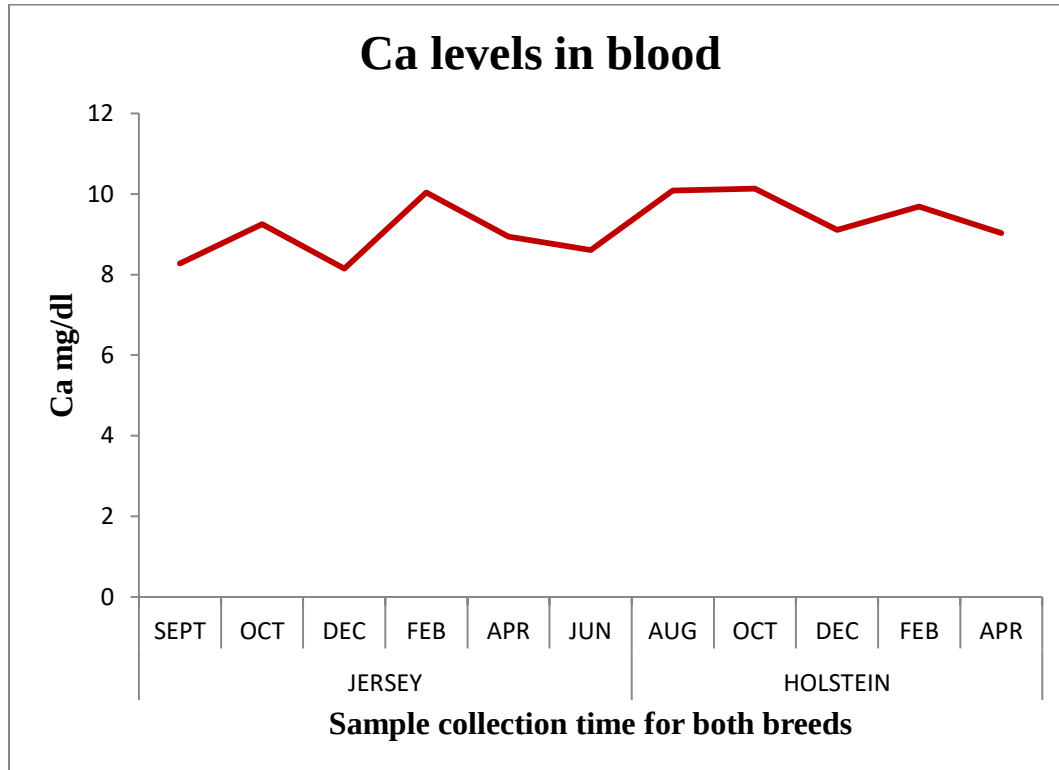


Figure 4. 10 lactation-long calcium fluctuations across the lactation for Holsteins and Jerseys

4.5.3 The core Holstein-Jersey milk microbiota

The major core milk families shared between the two breeds in this study were 18 families. Among the 18 major families shared, Lactobacillaceae, Lachnospiraceae, and Micrococccaceae were the most predominant core bacterial families for Holsteins at 15.4, 15.4, and 10.2%, respectively. As for Jersey milk, the prevalent shared core families were Ruminococcaceae, Erysipelotrichaceae, Lachnospiraceae, and Lactobacillaceae at 4.35, 4.23, 2.23, and 2.13%, respectively. The above-shared core microbiota was statistically unchanged across the lactation period for the two breeds.

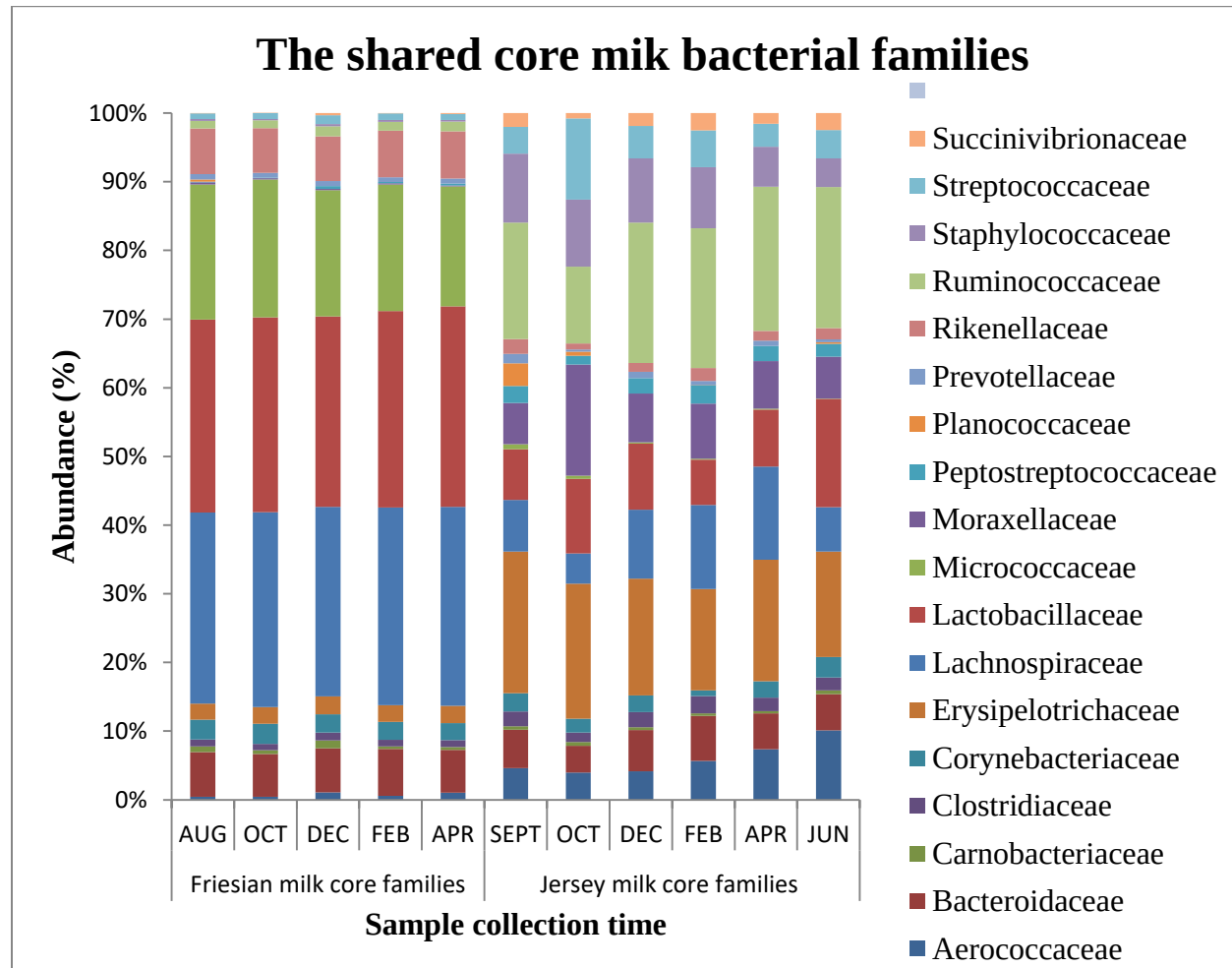


Figure 4. 11shared lactation-long core milk microbiota for Holsteins and Jerseys

4.5.4 The shared airborne dust microbiota of the two breeds' barns

The dominant shared airborne dust bacterial families for both breeds' sheds were Lactobacillaceae, Ruminococcaceae, and Lachnospiraceae (Jerseys' shed; 14.30, 12.50, & 9.35%, and 17.26, 14.73, & 11.32% at the Holsteins' shed, respectively).

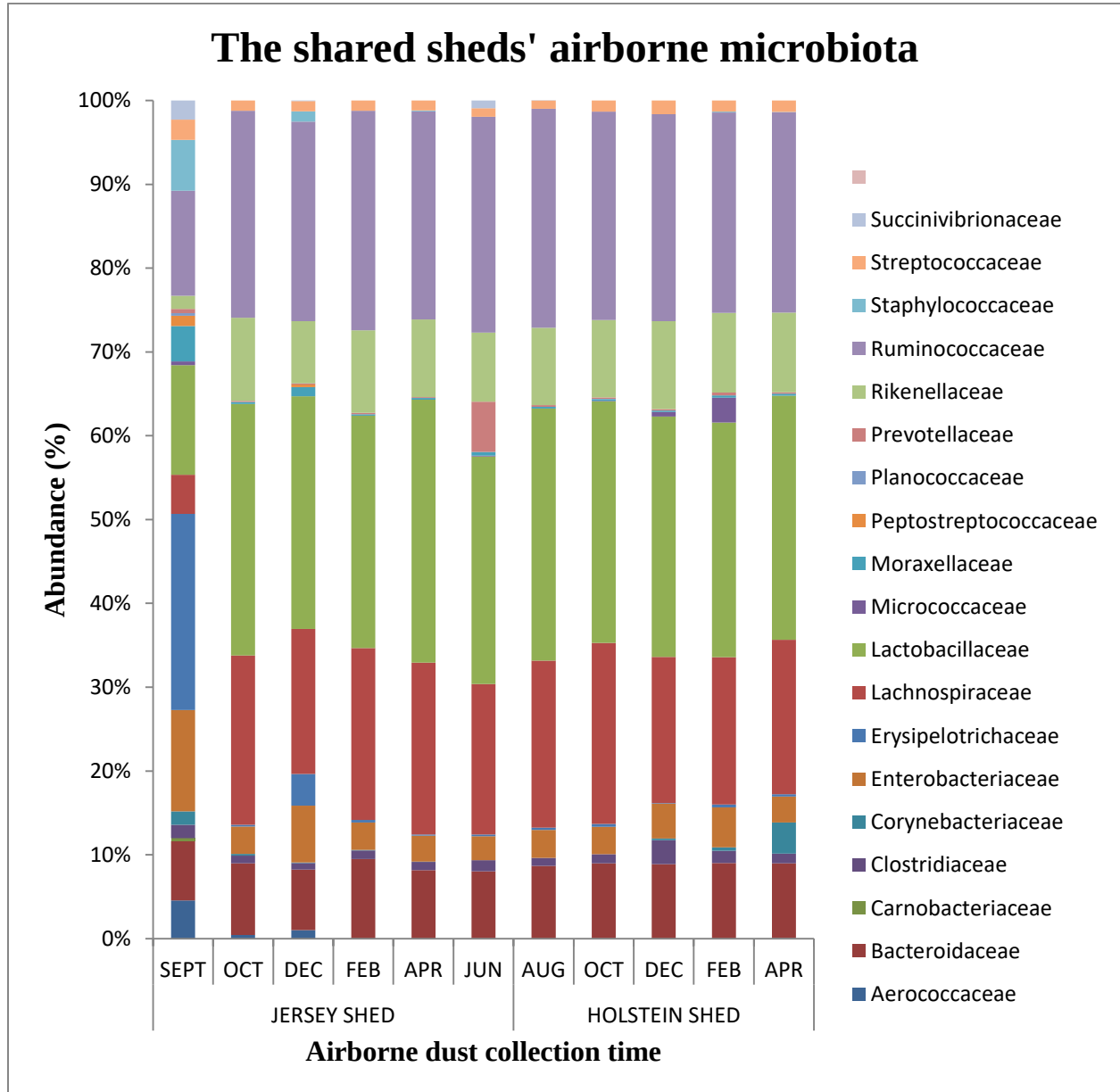


Figure 4. 12 shared airborne dust microbiota of the two breeds' barns

4.5.5 The influence of airborne dust on milk microbiota

Milk microbiota for both breeds was grouped separately. On the one hand, Holstein milk microbiota was clustered with the microbiota of airborne dust collected from Holstein shed and Jersey shed, except the Jerseys' shed September-collected airborne dust. On the other hand, Jersey milk microbiota was fragmented into four clusters and associated with only the microbiota of airborne dust collected in September.

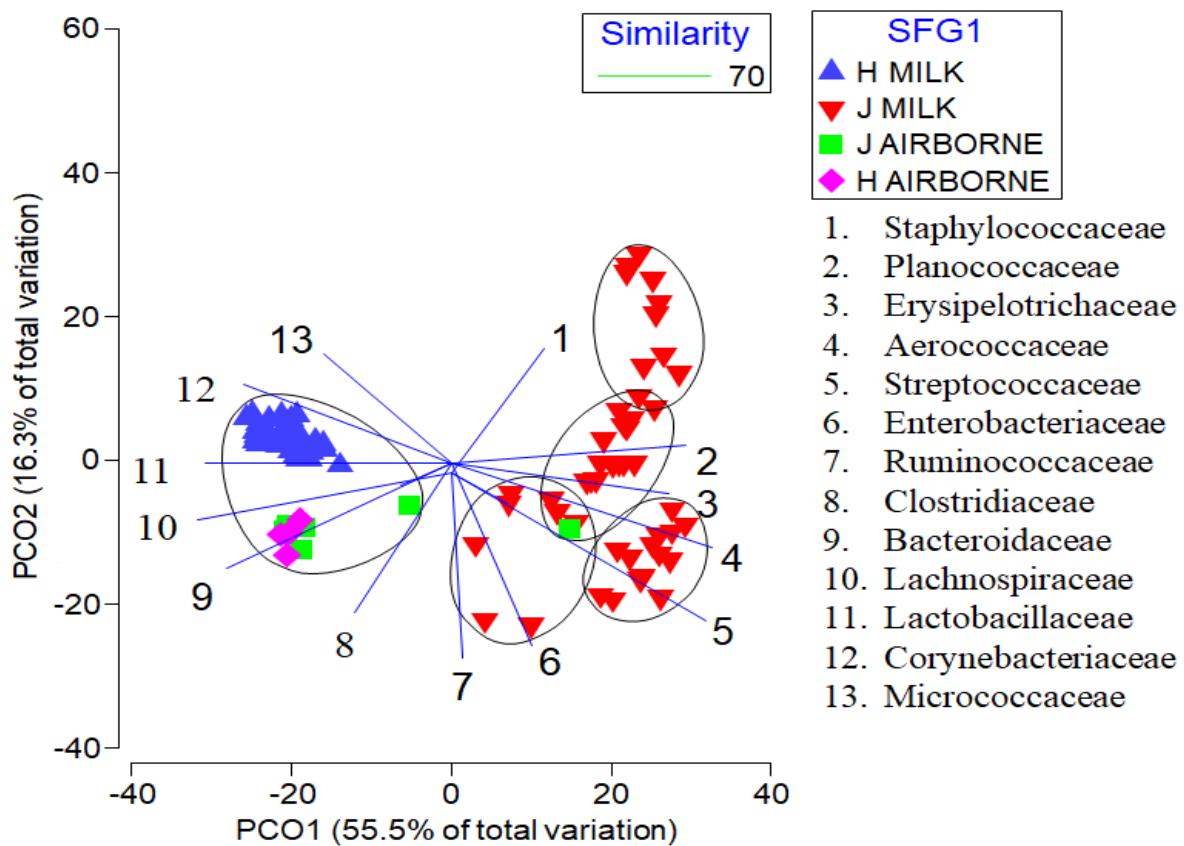


Figure 4. 13 clustering of Jersey and Holstein milk microbiota and their shed-collected airborne dust microbiota

4.6 Discussion

Holsteins maintained a steady increment in milk production till about the sixth month (186 DIM) into the lactation period, when milk production peaked at 35.5 kg/day. Milk production subsequently declined to the lactation lowest milk yield, 30.6 kg/day, just before drying off (299 DIM). This milk yield is in the same region as the one previously reported by Bernabucci et al., (2015). However, Hickson et al., (2006) reported that Holstein milked twice daily had milk peak production at 26.2 kg/day on 37 DIM. This peak production and time are incongruous with our findings since even our experimental herd's lowest production after ten months in milk production was higher than their study's peak yield. This difference in peak milk yield and time between the two studies can be ascribed to different factors, among them being the genetic makeup and dietary management differences between the two herds (Kumar, 2017 & Min et al., 2005). According to the study by Min et al., (2005), providing a high-quality diet increased Alpine goats' milk yield by up to 22%. Additionally, they also reported that peak time concomitantly increased with the increase in dietary supplementation; this improvement was from 29 DIM for the control group to 43 DIM for the group that was fed the highest quality diet.

Similarly, milk composition, milk protein and fat, increased as lactation advanced. This finding is in agreement with Sharma et al., (1990) & Shibru et al., (2019); in their study, milk fat and protein increased as the lactation progressed. Somatic cell count (SCC) remained unchanged across the lactation and below the threshold, 200,000 cells/mL, which generally serves as a marker for subclinical mastitis (Zecconi et al., 2019). This means our experimental herd was healthy and might not have had subclinical mastitis throughout the lactation. MUN was also statistically stable across the lactation and averaged out at 14 mg/dl. MUN below this value in high-producing Holstein cows is regarded as an indicator of protein insufficiency (Webb & de Bruyn, 2021). In the study by Rajala-Schultz et al., (2001), which had a high and low-producing Holstein group, found that the high-yielding Holstein group had 13.6 mg/dl as compared to 11.1 mg/dl for the low-producing herd. This might mean our high-producing herd was partaking of sufficient dietary protein across the lactation. BUN, on the other hand, significantly increased as lactation advanced. This could have been occasioned by the improvement of dietary protein provided to the herd as DIM increased. Though we did not determine the dietary protein content, we could surmise it premised on the findings of Hoffman et al., (2001) that Albumin is positively correlated with dietary protein. So, BUN being one of the end products of protein metabolism, according to Hammond, (1998), might have increased due to dietary protein improvement. However, the two end-products of protein metabolism, BUN and MUN, did not equilibrate across the season as it had been intimated by Gustafsson & Palmquist, (1993). It is crystal clear that Holstein had a predilection for apportioning urea in circulation to milk over plasma. Averagely, Holstein milk had 14 mg/dl as compared to 10.2 mg/dl every month across the lactation period. The same observation was made by Rodriguez et al., (1997), where MUN for 12 Holstein cows was higher than BUN in their experiment. This observation points to the necessity to investigate whether this observation is repeatable and whether it has a significant physiological meaning. Albumin and milk protein had a notable upward trend as lactation

advanced. While albumin has been shown to be directly influenced by dietary protein status (Hoffman et al., 2001; Raggio et al., 2007), no study, according to our knowledge, has directly linked the influence of albumin on milk protein. Nonetheless, it is worth noting that as Linn et al., (1988) & Larson, (1979) intimated, plasma proteins, albumin, might transudate into mammary lacteal secretions through leaky junctional complexes. Due to the lack of incontrovertible evidence that shows the influence of albumin on milk protein, we can only conjecture that the trend observed in this study is a concomitant result induced by an increment in dietary protein. For instance, in the study by Raggio et al., (2007), an increase in metabolizable protein in the diet resulted in a 15% increment in milk protein and a paltry 4% in albumin concentration. However, Bobbo et al., (2017) & Rowlands et al., (1977) reported that high-producing cows had higher plasma albumin concentrations. The authors of the two reports did not weigh in on this positive correlation between plasma concentrations and milk productivity. So, it remain unclear whether plasma albumin has a direct influence on milk productivity and milk protein.

Glutamic Oxaloacetic Transaminase (GOT)/ Aspartate aminotransferase (AST) and Glutamic Pyruvic Transaminase (GPT)/ Alanine aminotransferase (ALT) are liver enzymes which are sensitive to hepatic disturbance emanating from various factors like metabolic disorders or the stage of lactation (Aguirre et al., 2018). We found that the two enzymes were lower at the beginning of the lactation period and rose gradually towards the end of lactation. Our findings were in contrast with the results reported by Andjeli et al., (2022) & Boots & Ludwick, (1970). Andjeli et al., (2022) attributed the higher activities of AST and ALT in the early lactation period to fat infiltration into hepatic cells triggered by lipomobilization. The said lipomobilization was induced by negative energy balance (NEB). In their study, the lipomobilization biomarker, NEFA, was strongly positively correlated liver function indicator enzymes. In our study, NEFA were statistically unchanged across the lactation. This suggested that during the early lactation stage our experimental herd did not experience NEB, which could explain why the level of liver enzymes was relatively low compared to the late lactation period.

Milk microbiota was surprisingly stable across the lactation and dominated by Firmicutes (56.7%) at the phylum level. At the family level, Lactobacillaceae, Lachnospiraceae, Muribaculaceae, and Micrococcaceae had over 55% dominance. Cremonesi et al., (2018) also found out that Holstein milk was dominated by Firmicutes at 66%. Cremonesi et al., (2018) & Oikonomou et al., (2014) also reported that Lachnospiraceae was one of Holstein milk's dominant families. However, the level of dominance reported in their reports was lower than in the present study. Other reports, like Nguyen et al., (2020), Nguyen et al., (2020) & Wu et al., (2019), found other families dominating raw bovine milk microbiota. In their reports, Pseudomonadaceae (16.1%–59.7%), Staphylococcaceae (10.3%), and Aerococcaceae (24.3%) were the most dominant families, respectively. However, in their reports, families that constituted the dominant milk microbiota in the present study were also part of the dominant microbiota in each report, though at relatively lower abundance. The variation in the dominant raw bovine microbiota and the relative abundance of the dominant families has been attributed to

the lack of the standard sampling procedure, the influence of the dwelling environment of experimental herds, postpartum stages of herds, and nutritional management (Parente et al., 2020 & Steinberg et al., 2022). This surprising stability in milk microbiota across the lactation is inexplicable and has not been reported, yet.

Airborne dust microbiota was also surprisingly stable and dominated by S24-7, Lactobacillaceae, Ruminococcaceae, o__Clostridiales, and Lachnospiraceae, with a combined relative abundance of about 80%. Reports by Nguyen et al., (2020) & Wu et al., (2019) had Staphylococcaceae (13.4%) and Aerococcaceae(25.2%), respectively. In the two reports, S24-7 was not among the five most abundant families. However, in the two reports, Ruminococcaceae and Lachnospiraceae were among the five most abundant families. The surprising stability in airborne microbiota observed in the present study was not observed by Nguyen et al., (2020). Their study observed seasonal differences among many taxa detected, especially the five most abundant families. Hence, the stability observed in the present study remains inexplicable.

Unlike the surprising stability observed in the microbiota of milk and airborne dust, bedding microbiota was not stable and was dominated by Aerococcaceae, Corynebacteriaceae, and Ruminococcaceae were the most abundant families. The three families represented over 50% relative abundance of the bedding microbiota. Aerococcaceae and Ruminococcaceae varied widely; the former was highest in August and lowest in April and the latter was the highest in April and lowest in August. The dominance of Aerococcaceae and Ruminococcaceae in beddings in the present study is in agreement with the reports by Nguyen et al., (2020) & Wu et al., (2019). The higher dominance of Ruminococcaceae in April in our study was observed in winter by Nguyen et al., (2020).

Bedding microbiota, according to PCoA clustering, did not have any influence on milk microbiota. However, airborne dust microbiota closely resembled milk microbiota; hence, they were clustered together. While the influence of the cowshed microbiota, airborne and beddings, on milk microbiota has been appreciated, very few reports have reported it (Nguyen et al., 2020). In contrast to our study, the microbiota of beddings in the studies by Nguyen et al., (2020) & Wu et al., (2019) were clustered together with milk microbiota.

Throughout the lactation, Jerseys produced milk superior in protein and fat to the one produced by Holsteins. Conversely, Holsteins sustained higher milk production in comparison to Jerseys across the lactation. The findings of this present study are in agreement with reports by Hickson et al., (2006), Lembeye et al., (2016) & Prendiville et al., (2011). The differences in milk production and milk composition between the two breeds have largely been attributed to genetic differences and environment to a small extent. For instance, Ahlborn-Breier & Hohenboken, (1991) argued that Holstein and Jersey breeds have been separated for about 40 generations. Hence, the two breeds are expected to have a greater genetic distance and exhibit a more significant heterosis effect.

Both breeds had different NEFA changing patterns across the lactation; Jersey had the greatest variations of NEFA compared to Holsteins. Jersey had higher NEFA in the first month than Holsteins. This suggests Jerseys had higher lipomobilization than Holsteins at the beginning of the lactation due to a greater NEB. Janovick Guretzky et al., (2006) also found out that Jersey cows were in greater negative energy balance postpartum than Holsteins. While nutrition management during the periparturient period might influence the extent of NEB lactating cows undergo, Parker Gaddis et al., (2018) indicated that there is a genetic component related to ketosis (accumulation of ketone bodies) susceptibility in Jersey cattle.

Generally, the two breeds had a somewhat similar amount of albumin in circulation, except for an upshot observed in Jersey cows in February (the 6th month in lactation). This upshot in albumin was reflected in the end-products of protein metabolism (MUN and BUN). The two end-products fared differently across the lactation for the two breeds. MUN was higher in Holsteins than BUN, suggesting that Holsteins apportion higher urea in circulation to milk in comparison to blood. Rodriguez et al., (1997) also reported this kind of observation. Webb & de Bruyn, (2021) concluded that Holsteins have a higher MUN threshold than Jerseys. Unlike in Holsteins, BUN and MUN in Jerseys equilibrated across the lactation as it was suggested by Gustafsson & Palmquist, (1993).

Jersey cows seemed to have undergone hepatic stress across the lactation, unlike their counterparts, Holsteins, based on the amount and irregular fluctuations of hepatic enzymes, GOT and GPT, in circulation. This observation could be attributable to the nutritional management and genetic makeup of the two breeds (Andjeli, 2022; Parker Gaddis et al., 2018). The elevation of the two enzymes at the onset of lactation can be associated with NEB, marked by a sharp rise in NEFA. However, it is still unclear why the two enzymes remained higher in Jerseys' circulation despite the decline in NEFA as the DIM increased. Although ketosis, an elevation of ketone bodies in the circulation system, has been reported to be higher in Jersey and its crosses unlike Holstein (Anderson et al., 2007), it's still not clear whether it is only because of their genetic predisposition suggested by Parker Gaddis et al., (2018).

Calcium levels in the plasma of the two breeds were quite comparable, and only slight changes were observed as the DIM increased for each breed. Calcium is vital to lactating cows due to its importance in bone metabolism, muscle contraction, and milk synthesis. Hypocalcemia (hypocalcemia), insufficiency of Calcium in circulation, affects lactating cows, especially at the onset of lactation. While other studies have reported that Jersey cows are at higher risk of suffering from milk fever (Chiwome et al., 2017, Lean et al., 2006, & Roche & Berry, 2006), there was a notable difference in the level of plasma Calcium between the two breeds.

According to Kable et al., (2016) & Oikonomou et al., (2020), Lachnospiraceae, Ruminococcaceae, Lactobacillaceae, and Micrococcaceae, among others, constitute the core

milk microbiota. The shared core bovine milk microbiota of milk produced by the two breeds was relatively stable across their respective lactations in the present study. In agreement with our study, Nguyen et al., (2020) also observed monthly stability in most core bovine milk microbiota. The microbiota of airborne dust samples collected from barns housing both Jerseys and Holsteins was somewhat similar for all months, except the first month of Jersey-collected airborne dust. This could explain why the microbiota of airborne dust for all the other months, irrespective of the shed of origin, was clustered with the microbiota of Holstein milk. The Microbiota of the remaining Jersey shed-collected airborne dust, the September sample, which was characterized by putative contaminants, was clustered with the microbiota of Jersey-produced milk. This observation pointed to the importance of cowshed hygiene since the contamination of airborne dust might end up in milk.

4.7 Conclusion

Holsteins sustained high milk production that peaked over five months post-parturition, and milk fat and protein of Holstein-produced milk increased gradually throughout the lactation. On the other hand, Holsteins seemed to prefer to apportion more urea in circulation to milk over blood. The energy status of the Holstein herd was unchanged across the lactation, and the experimental herd did not suffer severe NEB. It remains inexplicable why hepatic enzymes rose towards the end of lactation. Milk microbiota was unusually stable across the lactation and was dominated by technologically important microbiota. This lactation-long stability was similarly observed in airborne dust microbiota of the barn housing the experimental herd.

Comparatively, Holsteins produced more milk than Jerseys, while Jerseys' milk contained higher milk fat and protein than Holsteins. However, the two breeds' plasma calcium contents were within the same range as the lactation progressed. The end products of protein metabolism equilibrated for Jerseys but did not for Holsteins across the lactation. Notably, Jerseys seemed to suffer to a greater extent from hepatic stress compared to Holsteins, as depicted by the irregular fluctuations of hepatic enzymes in Jerseys. The microbiota of milk produced by Holsteins was unusually stable, while that of milk produced by Jerseys fluctuated significantly across the lactation. However, the shared core milk microbiota was unchanged as DIM increased. The microbiota of airborne dust of the barns that housed the two experimental herds was largely similar, except for the September-collected airborne dust sampled from Jerseys' barn. Hence, cowshed hygiene was demonstrated to be vital in a bid to produce microbiologically hygienic milk, as contamination in airborne dust might end up in milk.

References

- Aguirre, E. L., Quezada, M., Uchuari, M., & Mamani, G. (2018). ALP-AST/GOT-ALT/GPT-Bilirubin in Serum from Bos Taurus Cows in the Postpartum Period and Maintained by Grazing in the Humid Tropic Region. *ARC Journal of Animal and Veterinary Sciences*, 4(January 2019), 2455–2518. <https://doi.org/10.20431/2455-2518.0404004>
- Ahlborn-Breier, G., & Hohenboken, W. D. (1991). Additive and Nonadditive Genetic Effects on Milk Production in Dairy Cattle: Evidence for Major Individual Heterosis. *Journal of Dairy Science*, 74(2), 592–602. [https://doi.org/10.3168/jds.S0022-0302\(91\)78206-4](https://doi.org/10.3168/jds.S0022-0302(91)78206-4)
- Anderson, T., Shaver, R., Bosma, P., & De Boer, V. (2007). Case Study: Performance of Lactating Jersey and Jersey-Holstein Crossbred Versus Holstein Cows in a Wisconsin Confinement Dairy Herd. *Professional Animal Scientist*, 23(5), 541–545. [https://doi.org/10.1532/S1080-7446\(15\)31017-2](https://doi.org/10.1532/S1080-7446(15)31017-2)
- Andjeli, B. (2022). *Relationships between Milk and Blood Biochemical Parameters and Metabolic Status in Dairy Cows during Lactation*.
- Barbano, D. M., Ma, Y., & Santos, M. V. (2006). Influence of raw milk quality on fluid milk shelf life. *Journal of Dairy Science*, 89 Suppl 1, E15–E19. [https://doi.org/10.3168/jds.s0022-0302\(06\)72360-8](https://doi.org/10.3168/jds.s0022-0302(06)72360-8)
- Bernabucci, U., Basiricò, L., Morera, P., Dipasquale, D., Vitali, A., Piccioli Cappelli, F., & Calamari, L. (2015). Effect of summer season on milk protein fractions in Holstein cows. *Journal of Dairy Science*, 98(3), 1815–1827. <https://doi.org/10.3168/jds.2014-8788>
- Bobbo, T., Fiore, E., Giancesella, M., Morgante, M., Gallo, L., Ruegg, P. L., Bittante, G., & Cecchinato, A. (2017). Variation in blood serum proteins and association with somatic cell count in dairy cattle from multi-breed herds. *Animal*, 11(12), 2309–2319. <https://doi.org/10.1017/S1751731117001227>
- Boots, L. R., & Ludwick, T. M. (1970). Plasma Glutamic-Oxaloacetic and Glutamic-Pyruvic Transaminase Activities in Holstein Cattle. I. Effects of Stage of Lactation, Gestation, and Level of Milk Production. *Journal of Dairy Science*, 53(4), 449–452. [https://doi.org/10.3168/jds.S0022-0302\(70\)86229-4](https://doi.org/10.3168/jds.S0022-0302(70)86229-4)
- Bradford, B. J., Yuan, K., Farney, J. K., Mamedova, L. K., & Carpenter, A. J. (2015). Invited review: Inflammation during the transition to lactation: New adventures with an old flame. *Journal of Dairy Science*, 98(10), 6631–6650. <https://doi.org/10.3168/jds.2015-9683>
- Caixeta, L. S., & Omontese, B. O. (2021). Monitoring and improving the metabolic health of dairy cows during the transition period. *Animals*, 11(2), 1–17. <https://doi.org/10.3390/ani11020352>
- Chiwome, B., Kandiwa, E., Mushonga, B., Sajeni, S., & Habarugira, G. (2017). A study of the incidence of milk fever in Jersey and Holstein cows at a dairy farm in Beatrice, Zimbabwe. *Journal of the South African Veterinary Association*, 88(1), 6–11. <https://doi.org/10.4102/jsava.v88i0.1457>

- Cremonesi, P., Ceccarani, C., Curone, G., Severgnini, M., Pollera, C., Bronzo, V., Riva, F., Addis, M. F., Filipe, J., Amadori, M., Trevisi, E., Vigo, D., Moroni, P., & Castiglioni, B. (2018). Milk microbiome diversity and bacterial group prevalence in a comparison between healthy holstein friesian and rendena cows. *PLoS ONE*, 13(10), 1–17. <https://doi.org/10.1371/journal.pone.0205054>
- Desmasures, N., & Gueguen, M. (1997). Monitoring the microbiology of high quality milk by monthly sampling over 2 years. *Journal of Dairy Research*, 64(2), 271–280. <https://doi.org/10.1017/S0022029996002130>
- Dobson, H., Smith, R. F., Knight, C. H., & Sheldon, I. M. (2007). The High-producing Dairy Cow and its Reproductive Performance Defining the Problem with respect to Milk Yield. *Reproduction in Domestic Animals*, 42(Suppl.2), 17–23.
- Dubuc, J., Duffield, T. F., Leslie, K. E., Walton, J. S., & LeBlanc, S. J. (2011). Effects of postpartum uterine diseases on milk production and culling in dairy cows. *Journal of Dairy Science*, 94(3), 1339–1346. <https://doi.org/10.3168/jds.2010-3758>
- Evans, A. C. (1916). The bacteria of milk freshly drawn from normal udders. *Journal of Infectious Diseases*, 18(5), 437–476. <https://doi.org/10.1093/infdis/18.5.437>
- Foods, D., Options, A. P., Committee, M., Options, T., Attributes, N., Products, A., Isbn, C., Pdf, T., Press, N. A., Press, N. A., Academy, N., Academy, N., & Press, N. A. (1988). Designing Foods. In *Designing Foods*. <https://doi.org/10.17226/1036>
- González, F. D., Muiño, R., Pereira, V., Campos, R., & Benedito, J. L. (2011). Relationship among blood indicators of lipomobilization and hepatic function during early lactation in high-yielding dairy cows. *Journal of Veterinary Science*, 12(3), 251–255. <https://doi.org/10.4142/jvs.2011.12.3.251>
- Gustafsson, A. H., & Palmquist, D. L. (1993). Diurnal Variation of Rumen Ammonia, Serum Urea, and Milk Urea in Dairy Cows at High and Low Yields. *Journal of Dairy Science*, 76(2), 475–484. [https://doi.org/10.3168/jds.S0022-0302\(93\)77368-3](https://doi.org/10.3168/jds.S0022-0302(93)77368-3)
- Hammond, C. (1998). Use of BUN and MUN as Guides for Protein and Energy in Cattle. *Revista Corpoica*, 2(2), 44–48.
- Hickson, R. E., Lopez-Villalobos, N., Dalley, D. E., Clark, D. A., & Holmes, C. W. (2006). Yields and persistency of lactation in Friesian and Jersey cows milked once daily. *Journal of Dairy Science*, 89(6), 2017–2024. [https://doi.org/10.3168/jds.S0022-0302\(06\)72269-X](https://doi.org/10.3168/jds.S0022-0302(06)72269-X)
- Hoffman, P. C., Esser, N. M., Bauman, L. M., Denzine, S. L., Engstrom, M., & Chester-Jones, H. (2001). Short communication: Effect of dietary protein on growth and nitrogen balance of holstein heifers. *Journal of Dairy Science*, 84(4), 843–847. [https://doi.org/10.3168/jds.S0022-0302\(01\)74542-0](https://doi.org/10.3168/jds.S0022-0302(01)74542-0)
- Janovick Guretzky, N. A., Carlson, D. B., Garrett, J. E., & Drackley, J. K. (2006). Lipid metabolite profiles and milk production for Holstein and Jersey cows fed rumen-protected choline during the periparturient period. *Journal of Dairy Science*, 89(1), 188–200.

[https://doi.org/10.3168/jds.s0022-0302\(06\)72083-5](https://doi.org/10.3168/jds.s0022-0302(06)72083-5)

- Kable, M. E., Srisengfa, Y., Laird, M., Zaragoza, J., McLeod, J., Heidenreich, J., & Marco, M. L. (2016). The core and seasonal microbiota of raw bovine milk in tanker trucks and the impact of transfer to a milk processing facility. *MBio*, 7(4). <https://doi.org/10.1128/mBio.00836-16>
- Kim, D. U., Lee, S. C., Jeong, J. K., Choi, I. S., Moon, S. H., Kang, H. G., & Kim, I. H. (2016). Effects of dystocia on the postpartum complications, milk production and reproductive performance in dairy cows. *Journal of Veterinary Clinics*, 33(2), 87–92. <https://doi.org/10.17555/jvc.2016.04.33.2.87>
- Kumar, V. (2017). Genetic and Breeding Aspects of Lactation. *Trends and Advances in Veterinary Genetics*. <https://doi.org/10.5772/66179>
- Larson, B. L. (1979). Biosynthesis and secretion of milk proteins: A review. *Journal of Dairy Research*, 46(2), 161–174. <https://doi.org/10.1017/S002202990001699X>
- Lean, I. J., DeGaris, P. J., McNeil, D. M., & Block, E. (2006). Hypocalcemia in dairy cows: Meta-analysis and dietary cation anion difference theory revisited. *Journal of Dairy Science*, 89(2), 669–684. [https://doi.org/10.3168/jds.S0022-0302\(06\)72130-0](https://doi.org/10.3168/jds.S0022-0302(06)72130-0)
- Lembeye, F., Lopez-Villalobos, N., Burke, J. L., & Davis, S. R. (2016). Milk production of Holstein-Friesian, Jersey and crossbred cows milked once-a-day or twice-a-day in New Zealand. *New Zealand Journal of Agricultural Research*, 59(1), 50–64. <https://doi.org/10.1080/00288233.2015.1130724>
- McGuire, M. K., & McGuire, M. A. (2017). Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk. *Current Opinion in Biotechnology*, 44, 63–68. <https://doi.org/10.1016/j.copbio.2016.11.013>
- Mezzetti, M., Cattaneo, L., Passamonti, M. M., Lopreiato, V., Minuti, A., & Trevisi, E. (2021). The Transition Period Updated: A Review of the New Insights into the Adaptation of Dairy Cows to the New Lactation. *Dairy*, 2(4), 617–636. <https://doi.org/10.3390/dairy2040048>
- Min, B. R., Hart, S. P., Sahlu, T., & Satter, L. D. (2005). The effect of diets on milk production and composition, and on lactation curves in pastured dairy goats. *Journal of Dairy Science*, 88(7), 2604–2615. [https://doi.org/10.3168/jds.S0022-0302\(05\)72937-4](https://doi.org/10.3168/jds.S0022-0302(05)72937-4)
- Mogollón-García, H. D., Nieto-Sierra, D. F., & Castro-Rincón, E. (2020). Productive performance of Holstein and the crossbreeding Kiwi Cross x Holstein cattle. *Agronomy Mesoamerican*, 31(2), 329–339. <https://doi.org/10.15517/am.v31i2.38541>
- Moyes, K. M., Friggens, N. C., & Ingvarsen, K. L. (2010). Nutritional strategies to combat physiological imbalance of dairy cows during early lactation: The effect of changes in dietary protein to starch ratios. *Acta Agriculturae Scandinavica A: Animal Sciences*, 60(3), 166–174. <https://doi.org/10.1080/09064702.2010.515242>
- Nguyen, Q. D., Tsuruta, T., & Nishino, N. (2020). Examination of milk microbiota, fecal microbiota, and blood metabolites of Jersey cows in cool and hot seasons. *Animal Science*

- Journal = Nihon Chikusan Gakkaiho*, 91(1), e13441. <https://doi.org/10.1111/asj.13441>
- Nguyen, T. T., Wu, H., & Nishino, N. (2020). An investigation of seasonal variations in the microbiota of milk, feces, bedding, and airborne dust. *Asian-Australasian Journal of Animal Sciences*, 33(11), 1858–1865. <https://doi.org/10.5713/ajas.19.0506>
- Oikonomou, G., Addis, M. F., Chassard, C., Nader-Macias, M. E. F., Grant, I., Delbès, C., Bogni, C. I., Le Loir, Y., & Even, S. (2020). Milk Microbiota: What Are We Exactly Talking About? *Frontiers in Microbiology*, 11(February), 1–15. <https://doi.org/10.3389/fmicb.2020.00060>
- Oikonomou, G., Bicalho, M. L., Meira, E., Rossi, R. E., Foditsch, C., Machado, V. S., Teixeira, A. G. V., 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). <https://doi.org/10.1371/journal.pone.0085904>
- Parente, E., Ricciardi, A., & Zotta, T. (2020). The microbiota of dairy milk: A review. *International Dairy Journal*, 107, 104714. <https://doi.org/10.1016/j.idairyj.2020.104714>
- Parker Gaddis, K. L., Megonigal, J. H., Clay, J. S., & Wolfe, C. W. (2018). Genome-wide association study for ketosis in US Jerseys using producer-recorded data. *Journal of Dairy Science*, 101(1), 413–424. <https://doi.org/10.3168/jds.2017-13383>
- Prendiville, R., Pierce, K. M., Delaby, L., & Buckley, F. (2011). Animal performance and production efficiencies of Holstein-Friesian, Jersey and Jersey × Holstein-Friesian cows throughout lactation. *Livestock Science*, 138(1–3), 25–33. <https://doi.org/10.1016/j.livsci.2010.11.023>
- Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews*, 37(5), 664–698. <https://doi.org/10.1111/1574-6976.12030>
- Raggio, G., Lobley, G. E., Berthiaume, R., Pellerin, D., Allard, G., Dubreuil, P., & Lapierre, H. (2007). Effect of protein supply on hepatic synthesis of plasma and constitutive proteins in lactating dairy cows. *Journal of Dairy Science*, 90(1), 352–359. [https://doi.org/10.3168/jds.S0022-0302\(07\)72636-X](https://doi.org/10.3168/jds.S0022-0302(07)72636-X)
- Rajala-Schultz, P. J., Saville, W. J. A., Frazer, G. S., & Wittum, T. E. (2001). Association between milk urea nitrogen and fertility in Ohio dairy cows. *Journal of Dairy Science*, 84(2), 482–489. [https://doi.org/10.3168/jds.S0022-0302\(01\)74498-0](https://doi.org/10.3168/jds.S0022-0302(01)74498-0)
- Roche, J. R., & Berry, D. P. (2006). Periparturient climatic, animal, and management factors influencing the incidence of milk fever in grazing systems. *Journal of Dairy Science*, 89(7), 2775–2783. [https://doi.org/10.3168/jds.S0022-0302\(06\)72354-2](https://doi.org/10.3168/jds.S0022-0302(06)72354-2)
- Rodriguez, L. A., Stallings, C. C., Herbein, J. H., & McGilliard, M. L. (1997). Effect of Degradability of Dietary Protein and Fat on Ruminal, Blood, and Milk Components of Jersey and Holstein Cows. *Journal of Dairy Science*, 80(2), 353–363. [https://doi.org/10.3168/jds.S0022-0302\(97\)75945-9](https://doi.org/10.3168/jds.S0022-0302(97)75945-9)

- Rowlands, G. J., Little, W., & Kitchenham, B. A. (1977). Relationships between blood composition and fertility in dairy cows - a field study. *Journal of Dairy Research*, 44(1), 1–7. <https://doi.org/10.1017/S0022029900019889>
- Schmidt, V. S. J., Kaufmann, V., Kulozik, U., Scherer, S., & Wenning, M. (2012). Microbial biodiversity, quality and shelf life of microfiltered and pasteurized extended shelf life (ESL) milk from Germany, Austria and Switzerland. *International Journal of Food Microbiology*, 154(1–2), 1–9. <https://doi.org/10.1016/j.ijfoodmicro.2011.12.002>
- Sharma, A. K., Wilcox, C. J., Martin, F. G., & Thatcher, W. W. (1990). Effects of Stage of Lactation and Pregnancy and Their Interactions on Milk Yield and Constituents. *Journal of Dairy Science*, 73(6), 1586–1592. [https://doi.org/10.3168/jds.S0022-0302\(90\)78829-7](https://doi.org/10.3168/jds.S0022-0302(90)78829-7)
- Shibru, D., Tamir, B., & Goshu, G. (2019). *iMedPub Journals Effect of Season , Parity , Exotic Gene Level and Lactation Stage on Milk Yield and Composition of Holstein Friesian Crosses in Central Highlands of Ethiopia*. 1–5.
- Steinberg, R. S., Silva, L. C., Souza, M. R. De, Reis, R. B., Patrícia, C. L., Lacorte, G. A., Nicoli, J. R., Neumann, E., & Nunes, Á. C. (2022). Changes in bovine milk bacterial microbiome from healthy and subclinical mastitis affected animals of the Girolando , Gyr , Guzera , and Holstein breeds. *International Microbiology*, 0123456789. <https://doi.org/10.1007/s10123-022-00267-4>
- Stojević, Z., Piršljín, J., Milinković-Tur, S., Zdelar-Tuk, M., & Ljubić, B. B. (2005). Activities of AST, ALT and GGT in clinically healthy dairy cows during lactation and in the dry period. *Veterinarski Arhiv*, 75(1), 67–73.
- Webb, E. C., & de Bruyn, E. (2021). Effects of milk urea nitrogen (Mun) and climatological factors on reproduction efficiency of holstein friesian and jersey cows in the subtropics. *Animals*, 11(11). <https://doi.org/10.3390/ani11113068>
- Wu, H., Nguyen, Q. D., Tran, T. T. M., Tang, M. T., Tsuruta, T., & Nishino, N. (2019). Rumen fluid, feces, milk, water, feed, airborne dust, and bedding microbiota in dairy farms managed by automatic milking systems. *Animal Science Journal*, 90(3), 445–452. <https://doi.org/10.1111/asj.13175>
- Zecconi, A., Vairani, D., Cipolla, M., Rizzi, N., & Zanini, L. (2019). Assessment of subclinical mastitis diagnostic accuracy by differential cell count in individual cow milk. *Italian Journal of Animal Science*, 18(1), 460–465. <https://doi.org/10.1080/1828051X.2018.1533391>

5 CHAPTER 5

Evaluation of milk spoilage potential of bacterial isolates obtained from Jersey farm's milking equipment and milk; a pilot study

5.1 Abstract

Raw milk and milking systems & storage tanks are inhabited by a diverse microbiota. This microbiota can blight milk quality and/or shorten the shelf-life of processed dairy products due production of heat-resistant extracellular enzymes. The risk of milk spoilage is exacerbated by the formation of biofilms in cold storage facilities since biofilms enhance the heat resistance and activities of the said enzymes. A proportion of bacteria in biofilms survive sanitizers used at farms. To effectively gain insights into this complex and diverse microbial community, there is a need to use both the culture-dependent and –independent method and incorporate a viable PCR into the latter. As a pilot study, we cultured and isolated putative sanitizer-tolerant bacteria with milk spoilage potential. We also assessed their biofilm-forming abilities. The effectiveness of propidium monoazide to inhibit the amplification of DNA from dead bacterial cells was also evaluated. *Clostridium butyricum* and *Pseudarthrobacter equi* had the highest and second-highest proteolytic abilities, $16 \text{ h}^{-1} \cdot \text{ml}^{-1}$ and $6.9 \text{ h}^{-1} \cdot \text{ml}^{-1}$, respectively. Two strains of *Clostridium butyricum* liberated the highest μmol of p-nitrophenol (27.01 and 13.81 μmol (min.ml)) per minute per milliliter, *Leifsonia shinshuensis*, had the third-highest lipolytic activity 12.04 μmol (min.ml). Swab-derived isolates had a higher propensity for biofilm formation in comparison with milk-derived isolates. Out of the 58 isolates obtained from swabs, 57 could form biofilm of varied strength. On the contrary, only 22 out of the 55 isolates obtained from milk could form biofilm of different strength. Propidium monoazide was found to be effective at discriminating and inhibiting the amplification of membrane-compromised DNA. Its effectiveness largely hinges on the length of the target DNA fragment; its efficiency improves as the length of the targeted DNA fragment increases. It did not affect the sequence signal quality and homology in Sanger and MiSeq sequencing. Hence, it can effectively be incorporated into high-throughput sequencing in a bid to gain valuable insights into dairy biofilms.

5.2 Introduction

Milk, being nutritionally rich, harbors a complex microbial community, which can either be technologically important or pose safety/quality concerns to the dairy industry. Although it was initially believed that milk was sterile in a healthy cow's udder, it has been undoubtedly demonstrated that it has autochthonous microbiota inhabiting the lumen of the mammary gland (Rainard, 2017). However, milk freshly drawn from a healthy udder might not have detectable, culturable microbiota of quality concerns. Nonetheless, this microbiota, especially psychrotolerant and thermophilic bacteria, increase over time during storage in silos and transportation to the dairy factories (Hantsis-Zacharov and Halpern, 2007). Spoilage microbiota, especially psychrotrophs and thermophilic bacteria, significantly deteriorates the quality of both fluid pasteurized milk and milk products. Psychrotrophs load after milk collection depends on storage hygiene, temperature, and time; under utmost sanitary conditions, the psychrotrophs load in milk is less than ten percent of the total microbiota (Hantsis-Zacharov et al., 2007). There are microbial milk tests that are routinely conducted to evaluate the herd health and ascertain milk's microbial quality at the farm and milk factory levels for both raw and pasteurized milk, respectively; however, they might not be comprehensive, collative, and comparative to furnish in-depth information about milk microbiota in a given area. The tests conducted can either be culture-dependent or culture-independent, with the latter being more sensitive and revealing the exact microbial composition of milk. The combination of the two microbiota-analytical approaches has been recommended by Wember et al, (2019) as the best way to exhaustively study a diverse microbiota community due to the limitations and edge of each approach over the other.

Raw milk might also contain microbes emanating from unhygienic milking equipment, udder skin, airborne dust, impure water, feed, and/or fecal materials. A study previously conducted by Wu et al., (2018) underscored the influence of airborne dust on milk microbiota; however, the study intimated the need to confirm whether the influence of airborne dust on milk microbiota would exist in other dairy farms managed differently. Milking systems and Bulk Tanks (BT) are implicated in impinging on the milk microbiological quality due to either inefficient sanitation or formation of biofilms or both (Flach et al., 2014). The resultant biofilm becomes a perennial source of milk contamination, either with microbiota or with thermo-stable milk-quality degrading enzymes. It is, therefore, imperative to evaluate the efficiency of Clean In Place (CIP) at dislodging biofilms, or the possibilities of recontamination in case of an efficient sanitation process. The spoilage ability, the aptitude of producing deteriorative heat-stable enzymes, of biofilm inhabitants will also be evaluated. Bacterial isolates from raw milk will also be assessed for their ability to form biofilms and produce spoilage enzymes as described by Stepanović et al., (2000).

Milk microbial analysis can be conducted for individual cow's milk and/or Bulk Tank Milk (BTM). The latter has been generally regarded as a useful tool for the comprehensive assessment of the herd's udder health and milk microbial quality (Rodrigues et al., 2017). Although

considerable milk pathogenic microbes are exterminated by thermal treatments, HTST pasteurization and ultra-heat treatment, not all microbes and their thermo-tolerant enzymes are extirpated (Teh et al., 2011). According to Hantsis-Zacharov and Halpern, (2007), psychrotrophic bacteria and their associated heat-stable enzymes significantly contribute to the processed fluid milk and milk products' organoleptic quality deterioration, particularly during cold storage. Psychrophile-derived extracellular enzymes, protease and lipase, act on protein and lipids respectively to produce off-flavors and milk bitterness. Consequently, the resultant pasteurized milk quality highly hinges on the microbial load, composition, and diversity of raw milk prior to heat treatment. It's, therefore, necessary to monitor extensively and control the microbiological quality of raw milk before pasteurization (Skeie et al., 2019). To that end, BTM provides apt samples for the prior-pasteurization microbiological quality analyses of raw milk. Also, a study by Weber et al.,(2019) found out that the milking equipment retainers, teat cups (TC), and the outlet of the bulk milk tanks(BMTO) were the most colonized spots by biofilm-forming bacteria on both farms. Hence, in our pilot study, we swabbed both teat cups and bulk tanks' outlets in order to culture and isolate putative biofilm formers and bacteria with the highest milk spoilage potential since Teh et al., (2011) reported that biofilm dwellers are likely to produce the highest amount of proteolytic enzymes in comparison with their counterparts planktonic cells. Hence, we conducted this pilot study to isolate culturable psychrotrophic bacteria from a Jersey farm's raw milk, TC, and BMTO and assess their *in vitro* biofilm-forming, lipolytic, & proteolytic potential.

5.3 Materials and Methods

5.3.1 Sample collection

The samples for this pilot study were collected from a Jersey farm where experiment two was also conducted. The experimental farm is in Hiruzen, Okayama, Japan, and the Hiruzen area is the home to the second-highest number of Jersey cows in Japan. The area has about 26 Jersey farms. Swab samples from the teat cups and BMTO were taken using cotton swabs pre-wet with sterile saline just before milking, processed, and shipped to Okayama University on ice. The swab samples were taken immediately before the milking. BTM, on the other hand, was obtained aseptically from the BT and aliquoted into 50 ml centrifuge tubes. The tubes were shipped the same way as swab samples on ice immediately after collection.

5.3.2 Bacterial culturing and isolation

Milk and swab samples were serially diluted to the appropriate diluents depending on the type of samples. Different diluents for each sample were seeded on different agar media (*Pseudomonas* agar, *Streptococcus* & *Staphylococcus* agar, milk plate count agar, M17, and MRS) for cultivation of bacteria. The plates were incubated at 7° C and 37° C for 7 days and 2 days, respectively. After the cultivation period elapsed, distinct and representative colonies were isolated with applicator sticks and streaked on respective medium for purification. After three rounds of successful streaking, bacterial isolates were regarded as pure. Pure isolates were sub-cultured in respective broths of the above agar media. Isolates were maintained in the respective broths during downstream experiments. To preserve pure isolates, they were put in respective broths supplemented with 15% of pure cryopreservative, glycerol.

5.3.3 Biofilm assessment

All the isolates from both swabs and milk were subjected to a preliminary assessment of the biofilm-forming potential. The assessment was conducted as described by Stepanović et al., (2000). Briefly, 500 µl of all purified isolates in their respective culture suspensions were put into wells of a sterile 24-well flat-bottomed plastic tissue-culture plate with a lid in triplicate. For negative controls, each respective broth was used. Plates with cultured medium were incubated at 37° C for 48 hours. After the incubation time elapsed, the culture of each well was aspirated, and wells washed thrice sterile phosphate-buffered saline (pH=7.4). The plates were vigorously shaken during washing to dislodge bacterial cells that were non-adherent. After sufficient rinsing and emptying of the plates, the adherent cells in each well were fixed with 500 µl of 99% methanol for 20 minutes. After the fixation period was over, the content of the wells were emptied, and the adherent cells in each well were stained with 500 µl of 2% Hucker crystal violet. The excess stain was rinsed off with distilled water, and the plates were let to dry adequately. After sufficiently drying up of wells, the adherent cells were resolubilized with 200 µl of glacial acetic acid, and the optical density (OD) of each well was measured at 570 nm by use of a microplate reader. All the isolates, depending on their (or lack of) abilities to form a biofilm, were broadly categorized into four major groups (Zain et al., 2016). The categories were: non-

biofilm formers, weak biofilm formers, moderate biofilm formers, and strong biofilm formers in accordance with the criterion spelled out by Stepanović et al., (2000) & Zain et al., (2016).

5.3.4 The screening for the ability to produce extracellular enzymes

5.3.4.1 Proteolytic enzymes

All the isolates were preliminarily subjected to screening for the ability to produce protease on skim milk agar (nutrient agar supplemented with 1% w/v of locally purchased skim milk). Overnight grown cultures were streaked on skim milk agar plates, and the plates were incubated at 37 °C for 48 hours and 7 °C for 7 days. Isolates were regarded as positive for proteolysis if, after incubation, there was a transparent halo around the colony (Hahne et al., 2019).

Isolates that were positive protease activity on the screening medium above were selected for the quantification of protease production. The isolates were grown overnight in the respective broth supplemented with skim milk and used for the quantification of proteolysis in accordance with the guidelines reported by Yuan et al., (2018) after slight customization. Briefly, the overnight grown cultures were centrifuged at 12 000× g for 10 min at 4 °C, and 100 µl cell-free supernatant of each isolate was used for the quantification of the respective isolate's proteolytic potential. The quantification was conducted on azocasein substrate prepared according to Hoong et al., (2012). The proteolysis reaction was initiated by adding 100 µl cell-free supernatant will to microfuge tubes containing 300 µl phosphate-buffered saline (PBS; 50 mmol/L, pH=7.4) and 100 µl azocasein(1.5% (15 g/L). The microfuge tubes were incubated at 37° C for one hour. This reaction between extracellular protease and substrate azocasein was stopped at the top of the hour with the addition of 500 µl of 20% (0.2 g/ml) trichloroacetic acid. The reaction mixtures were then centrifuged at 12 000 × g and 4° C for 5 minutes. 100 µl of the resultant supernatants were put in wells of a microtiter plate in triplicate. A control, which was sterilized PBS instead of cell-free supernatant, was included in the microtiter plate. Absorbance was measured at 366 nm using a spectrometer-based microplate reader. Protease was expressed as the increment of absorption at 366 nm per hour and milliliter ($\Delta A \text{ h}^{-1} \cdot \text{ml}^{-1}$). This assay was repeated thrice.

5.3.4.2 Lipolytic enzymes

As with the screening for proteolytic activities, all isolates were also screened for lipolytic activities. Tributyrin agar supplemented with phenol red to improve the visualization of clearing halo. The agar medium was prepared in accordance with Ramnath et al., (2017). The isolates that produced wide halos were considered positive for lipolytic activities and were selected for the quantification of lipolysis.

P-Nitrophenol palmitate (pnpp) was used as a substrate for lipolytic enzymes produced by bacterial isolates. The substrate was formulated as follows: 5 mg of pnpp were dissolved in 1 ml of isopropanol and 9ml of 50mM Tris-HCL solution maintained at pH 8 and containing 40 mg of Triton X-100 (Bussamara et al., 2010). The bacterial isolates that evinced lipolytic potential on the Tributyrin Agar were grown overnight. The overnight-grown cultures were centrifuged at 12

000 × g and 4° C for 10 minutes. After centrifugation, the resultant supernatant was filtered by 0.45µm cellulose acetate filter to obviate the interference of dairy protein. To initiate a lipolytic reaction, 100 µl of cell-free supernatant was mixed with 0.9 ml of pnpp in microfuge tubes. The tubes were incubated at 37 °C for 30 minutes. After the incubation period elapsed, 0.5 ml of 95 % ethanol was added to each microfuge tube to stop the lipolytic reaction. The tubes were then centrifuged at 1500 × g and 4 °C for five minutes. 150 µl of the supernatant for each isolate were transferred in triplicate to wells of a microtiter plate, and the absorbance was taken at 410 nm. A negative control, which was sterilized PBS instead of cell-free supernatant, was included in the microtiter plate in triplicate, also. The lipolytic activity will be expressed as the amount of p-nitrophenol (µmol) liberated per minute per milliliter (Bussamara et al., 2010; Yuan et al., 2018). Three replicates were performed for the above lipolytic assay.

5.3.5 Assessment for the effectiveness of propidium monoaxide (PMA)

To effectively study the bacterial dwellers of a biofilm, especially at a dairy farm where sanitation measures are conducted every day, there is need for discrimination between live and dead bacterial cells. The effectiveness of PMA at discriminating between live and dead bacterial cells and the impact of sample treatment with PMA on bacterial identification were investigated as follows: *Enterococcus durans* and *Leifsonia shinshuensis* were used 11 formulations for this investigation. Overnight grown cultures of both species were aliquoted into two portions, and one portion was exposed to heat denaturation at 100° C for 15 minutes. The loss of viability was confirmed by failure of colonies to grow on respective agar plates after incubating at the optimal growth temperature (Nocker et al., 2006).

The eleven formulations were as follows:

1. Live *Enterococcus durans*
2. Live *Leifsonia shinshuensis*
3. Dead *Leifsonia shinshuensis*
4. Dead *Enterococcus durans*
5. Dead *Leifsonia shinshuensis* with PMA
6. Dead *Enterococcus durans* with PMA
7. Live *Leifsonia shinshuensis* and Live *Enterococcus durans*
8. Live *Leifsonia shinshuensis* and Live *Enterococcus durans* with PMA
9. Dead *Leifsonia shinshuensis* and Dead *Enterococcus durans* with PMA
10. Live *Leifsonia shinshuensis* Dead *Enterococcus durans* with PMA
11. Dead *Leifsonia shinshuensis* and Live *Enterococcus durans* with PMA

A 20 mM PMA stock solution was prepared by dissolving the PMA powder into dimethyl sulfoxide (DMSO), and the tube containing the stock solution was stored at -20° C after it was wrapped in aluminum foil (García-Cayuela et al., 2009).

For single strain formulations like formulation 1, 1 ml of the overnight grown cultures were used, while 500 µl of each overnight grown cultures were mixed for two-strain formulations like

formulation 7. Cultures were centrifuged at 10,000×g for 20 min to collect cell pellets. Cell pellets were re-suspended in 500 µl of PBS (pH=7.4), and 2.5 µl of PMA stock solution was added (final concentration of 100µM). For maximal PMA contact and interaction with bacterial DNA, tubes containing bacterial suspension and PMA were incubated in dark for 15 minutes and occasionally shaken. After incubation, the tubes were horizontally placed 20 cm from a 500W halogen lamp and on ice to prevent overheating during photo-induction. Samples were exposed to light under aforementioned conditions for 15 minutes. Then, PMA-treated samples were centrifuged at 10,000×g for 15 min to re-collect cell pellets (Desfossés-Foucault et al., 2012). PMA-treated cell pellets were then re-suspended in 250 µl of PBS and used for the repeated bead-beating DNA extraction as described by (Nguyen et al., 2020). Primers 27 F and 1492 R were used to amplify 16 S rRNA gene of extracted DNA. Purification of the resultant amplicons and sequencing was as described below.

5.3.6 Bacterial identification

After the purification of colonies and evaluating the potential of the colonies to form biofilm and produce extracellular milk spoilage enzymes, DNA was extracted as described above. All those bacterial isolates with the potential to form biofilm and produce lipolytic and proteolytic enzymes were subjected to denaturing gradient gel electrophoresis (DGGE) to characterize the isolates according to their genetic similarities as described by Wu & Nishino, (2016). Briefly, the extracted DNA for the targeted isolates was amplified using a primer set GC357f (5' – CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGA GGCAGCAG–3') and the reverse primer 517r (5' –ATTAC CGCGGCTGCTGG–3'). The thermocycling program, the touchdown PCR, was as follows: an initial denaturation for ten minutes at 95° C. The initial denaturation was followed by the first round of 9 cycles of denaturation at 93° C for 30 seconds, annealing at 65° C for 30 seconds, and extension at 72° C for 1 minute. In the second round of 9 cycles, only the annealing temperature was reduced to 60° C, while the rest remained as the first round of cycles. In the third round of cycles, denaturation was still at 93° C for 30 seconds, annealing at 55° C for 30 seconds, and extension was also at 72° C. The resultant amplicons were separated via DGGE by using the DCode Universal Mutation Detection System (Bio-Rad Ltd., Tokyo, Japan). The concentration of polyacrylamide, the denaturation gradient, and the denaturants used was as those outlined by Wu & Nishino, (2016). Depending on the banding patterns, the representative isolates were selected for sequencing and identification.

The original DNA of the representative isolates was used as the template for the 16S rRNA gene amplification. Primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') 1492R (5'-GGTTACCTTGTTACGACTT-3') were used, and the thermocycling program was as follows: the initial denaturation at 96° C for 1 minute was followed by 30 thermocycling cycles of denaturation at 96° C for 10 seconds, annealing at 55 C for 10 seconds, extension at 72° C for 1 minute, and a final extension at 72° C for 5 minutes. The resultant amplicons were purified

using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and used as DNA templates for Sanger sequencing using BrilliantDye™ Terminator v3.1 Cycle Sequencing Kit(NimaGen BV Lagelandseweg 56 6545 CG Nijmegen The Netherlands) by ABI PRISM 3130 sequencer (Applied Biosystems Inc., Foster City, CA). For sequencing, only the forward, 27F, primer was used. This PCR program was used for BrilliantDye™ Terminator v3.1 was an initial denaturation at 96° C for 45 seconds and 27° cycles of denaturation at 96° C for 10 seconds, annealing at 50° C, and elongation at 60° C for 4 minutes. The Basic Local Alignment Search Tool (BLAST) was used to search for the closest relatives of high-quality raw sequences(Zain et al., 2016). The sequences that had over 98% homology on BLAST GeneBank were considered identified.

5.3.7 Bioinformatics and statistical analysis

The data obtained from this study, depending on its nature, will be analyzed using relevant analytical tools. For instance, analysis of variance will be used to analyze the bacterial load and composition among farms, while heatmap will be used to represent the relative abundance of major taxa. For the sources of milk bacterial contaminants, the SourceTracker algorithm will be used as previously applied by Wu et al., (2018).

5.4 Results

5.4.1 Milk isolates

After sequencing and identifying the representatives of the clusters of isolates obtained, 49 isolates were identified to the species level. The majority of isolates were cultured on staphylococcus agar (18), pseudomonas agar base (14), and streptococcus agar (8). *Staphylococcus aureus* (10), *Streptococcus uberis* (6), and *Enterococcus durans* (4) were the most species isolated from milk.

Table 5. 1 representative bacteria isolated from milk samples

No.	Bacterial identity	Culturing medium	No. of isolates
1.	<i>Aerococcus viridans</i>	Pseudomonas agar	2
2.	<i>Bacillus licheniformis</i>	Pseudomonas agar	4
3.	<i>Staphylococcus aureus</i>	Staphylococcus agar	10
4.	<i>Pseudoclavibater faecalis</i>	Staphylococcus agar	2
5.	<i>Pseudomonas indoloxydans</i>	Pseudomonas agar	3
6.	<i>Streptococcus uberis</i>	Streptococcus agar	6
7.	<i>Corynebacterium variabile</i>	Staphylococcus agar	1
8.	<i>Corynebacterium glyciniphilum</i>	Staphylococcus agar	2
9.	<i>Enterococcus durans</i>	Milk plate count agar	4
10.	<i>Streptococcus dysgalactiae</i>	Streptococcus agar	2
11.	<i>Staphylococcus chromogenes</i>	Staphylococcus agar	3
12.	<i>Leifsonia shinshuensis</i>	Milk plate count agar	2
13.	<i>Psudomonas aeruginosa</i>	Pseudomonas agar	5
14.	<i>Leifsonia soli</i>	Milk plate count agar	1
15.	<i>Lactobacillus paracasei</i>	MRS	2

5.4.2 Swab isolates

74 swab-derived bacterial isolates were selected to represent clusters obtained from DGGE bacterial fingerprints. The 74 representative isolates were sequenced and identified at the species level, and the 74 isolates belonged to 24 different species. *Lactiplantibacillus paraplantarum* had the highest selected representative isolates, 11 different representative isolates. *Latilactobacillus sakei* had 6 representative isolates, representing the second-highest diverse bacterial fingerprints on DGGE. According to the DGGE banding patterns, the third-most diverse species were *Clostridium butyricum* and *Latilactobacillus curvatus* with 5 representative isolates each. 24 out of 74 of isolates were cultured on MRS. The second-highest (16) and third-highest (14) isolates were cultured on Staphylococcus agar and milk plate count agar, respectively.

Table 5. 2 representative bacteria isolated from swab samples

No.	Bacterial identity	Culturing medium	No. of isolates
1.	<i>Rahnella inusitata</i>	Staphylococcus agar	4
2.	<i>Clostridium butyricum</i>	Pseudomonas agar	5
3.	<i>Rouxiella chamberiensis</i>	Streptococcus agar	4
4.	<i>Alkalihalobacillus clausii</i>	Staphylococcus agar	1
5.	<i>Leifsonia shinshuensis</i>	Milk plate count agar	2
6.	<i>Pseudarthrobacter equi</i>	Staphylococcus agar	2
7.	<i>Microvirga calopogonii</i>	Milk plate count agar	2
8.	<i>Staphylococcus warneri</i>	Staphylococcus agar	3
9.	<i>Latilactobacillus sakei</i>	MRS	6
10.	<i>Lactiplantibacillus paraplantarum</i>	MRS	11
11.	<i>Enterococcus gallinarum</i>	Milk plate count agar	1
12.	<i>Leuconostoc gelidum</i>	Milk plate count agar	2
13.	<i>Leuconostoc carnosum</i>	Milk plate count agar	1
14.	<i>Lactococcus lactis</i>	M17	2
15.	<i>Latilactobacillus curvatus</i>	MRS	5
16.	<i>Enterobacter wuhouensis</i>	Milk plate count agar	2
17.	<i>Pseudomonas oryzihabitans</i>	Pseudomonas agar	3
18.	<i>Microvirga calopogonii</i>	Milk plate count agar	4
19.	<i>Pantoea allii</i>	Staphylococcus agar	2
20.	<i>Cronobacter dublinensis</i>	Streptococcus agar	3
21.	<i>Pantoea eucalypti</i>	Staphylococcus agar	4
22.	<i>Trabulsiella odontotermitis</i>	M17	1
23.	<i>Enterobacter quasiroggenkampii</i>	M17	1
24.	<i>Enterobacter cloacae</i>	M17	3

5.4.3 Quantitative assessment of proteolysis (azocasein assay)

Out of bacterial isolates that were positive for proteolysis at the screening stage, five isolates with the strongest proteolytic ability (based on the diameter of halos) were selected for preliminary quantification of their hourly proteolytic abilities. *Clostridium butyricum* had the highest proteolytic ability both at the screening and quantification stage; its proteolytic activity averaged out at $16 \text{ h}^{-1} \cdot \text{ml}^{-1}$. *Pseudarthrobacter equi* had the second-highest proteolytic activity, $6.9 \text{ h}^{-1} \cdot \text{ml}^{-1}$. The two species of *Rouxiella chamberiensis* species produced the third-highest protease per hour (4 and $5.3 \text{ h}^{-1} \cdot \text{ml}^{-1}$). However, there was a difference in the proteolytic activities of the two species. *Rahnella inusitata* had the least proteolytic activity after a one-hour reaction.

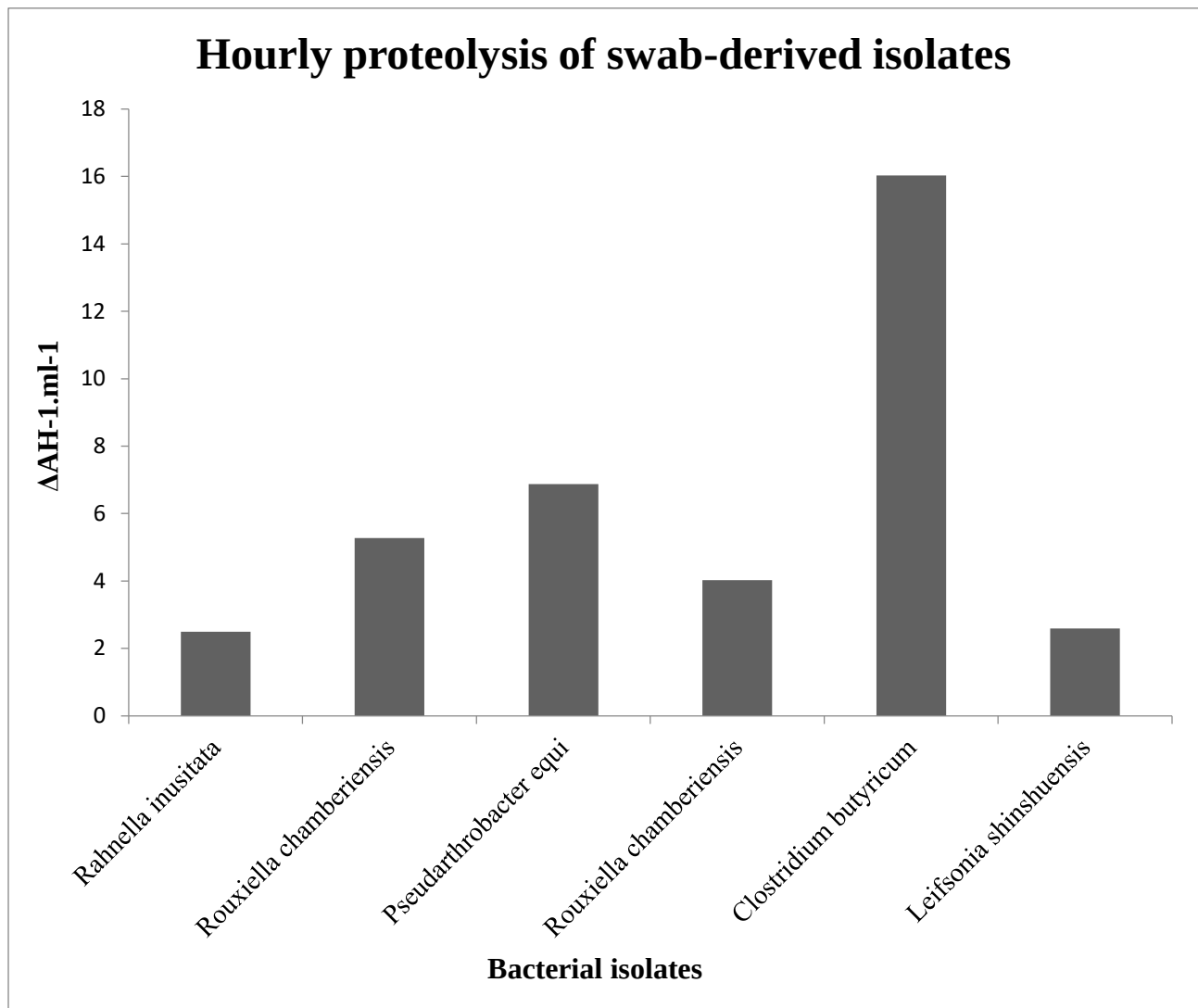


Figure 5. 1the proteolytic potential of some teat cup- and BMTO-derived isolates

5.4.4 Quantification of lipolytic activities (p-Nitrophenol palmitate assay)

Just like with the proteolytic activity, *Clostridium butyricum* had the highest lipolytic activity among the isolates that were subjected to the quantitative assessment of lipolytic activities. The two species (strains) liberated the highest μmol of p-nitrophenol (27.01 and 13.81 μmol (min.ml)) per minute per milliliter. *Leifsonia shinshuensis*, had the third-highest lipolytic activity 12.04 μmol (min.ml), unlike the low proteolytic activities it produced in the quantitative assessment of proteolysis. *Alkalihalobacillus clausii* liberated 7.38 μmol (min.ml), while *Rahnella inusitata* had the lowest lipolytic activity (2.72 and 3.19 μmol (min.ml)), just like it had the lowest proteolytic activity.

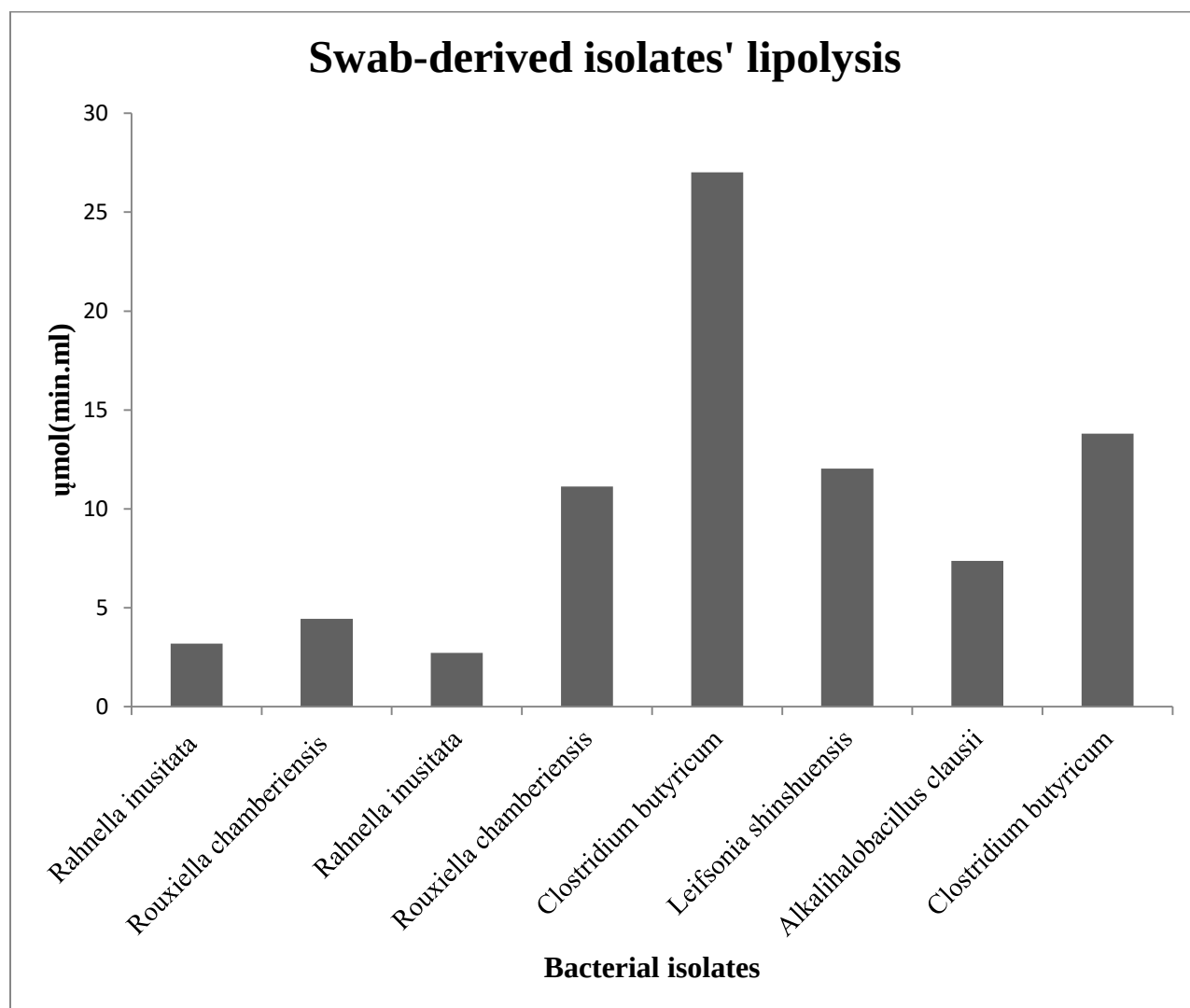


Figure 5. 2 the ability of teat cup and BMTO-derived isolates to produce lipolytic enzymes

5.4.5 A preliminary assessment of the biofilm-forming potential of isolates

5.4.5.1 Swab-derived isolates

The ability of 58 isolates obtained from a teat cups' and BMTO's swabs to form biofilm was preliminarily assessed. Out of the 58 isolates, 46 were strong biofilm formers. 9 isolates produced biofilm of moderate strength, 2 were weak biofilm producer, and 1 did not form biofilm.

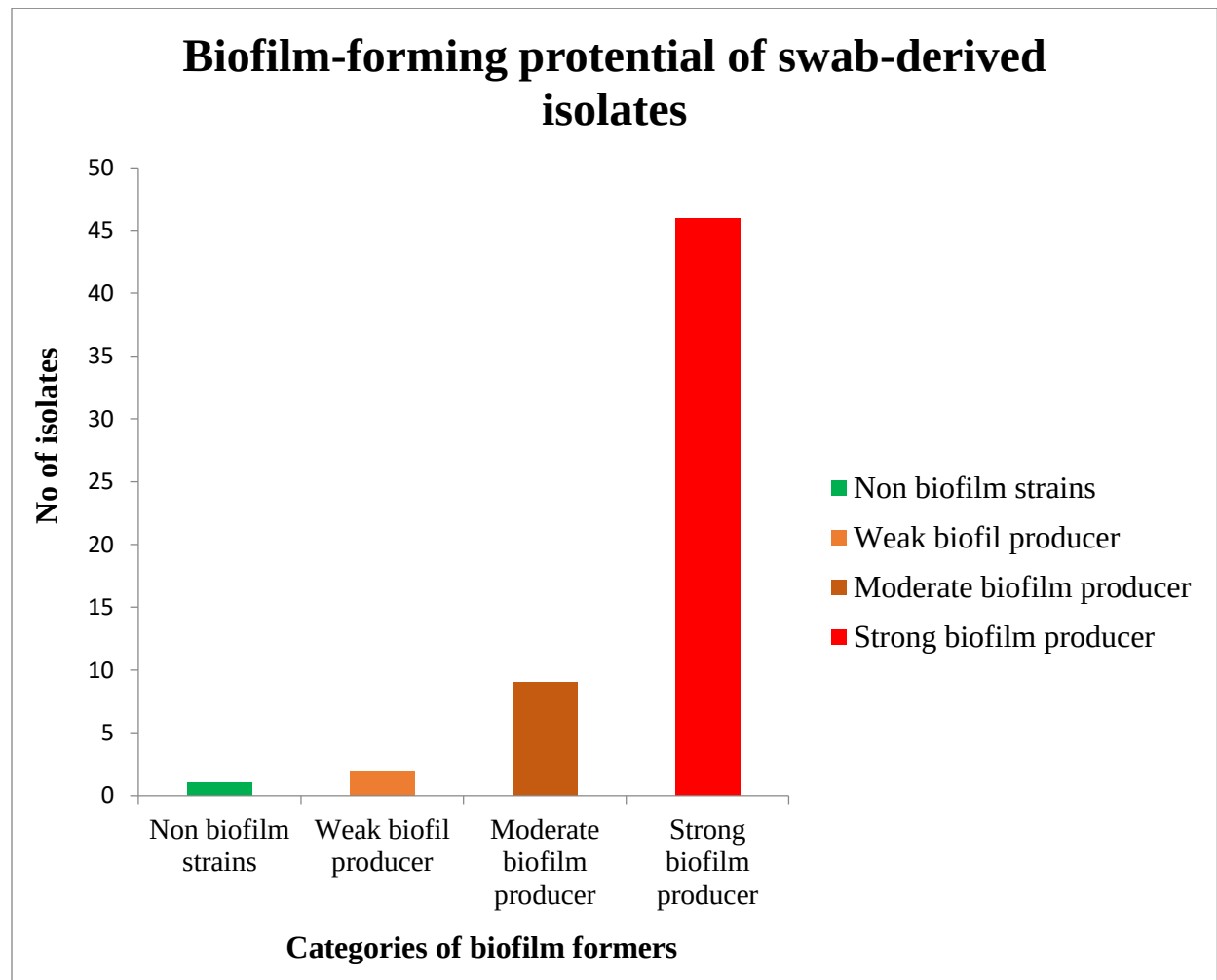


Figure 5. 3 the biofilm forming potential of teat cups' and BMTO's swab-derived strains

5.4.5.2 Milk-derived isolates

In this pilot experiment, the ability of 55 milk-derived isolates to form biofilm was assessed. 33 out of 55 did not form biofilm. 6, 5, 11 isolates produced weak biofilm, biofilm of moderate strength, and strong biofilm, respectively.

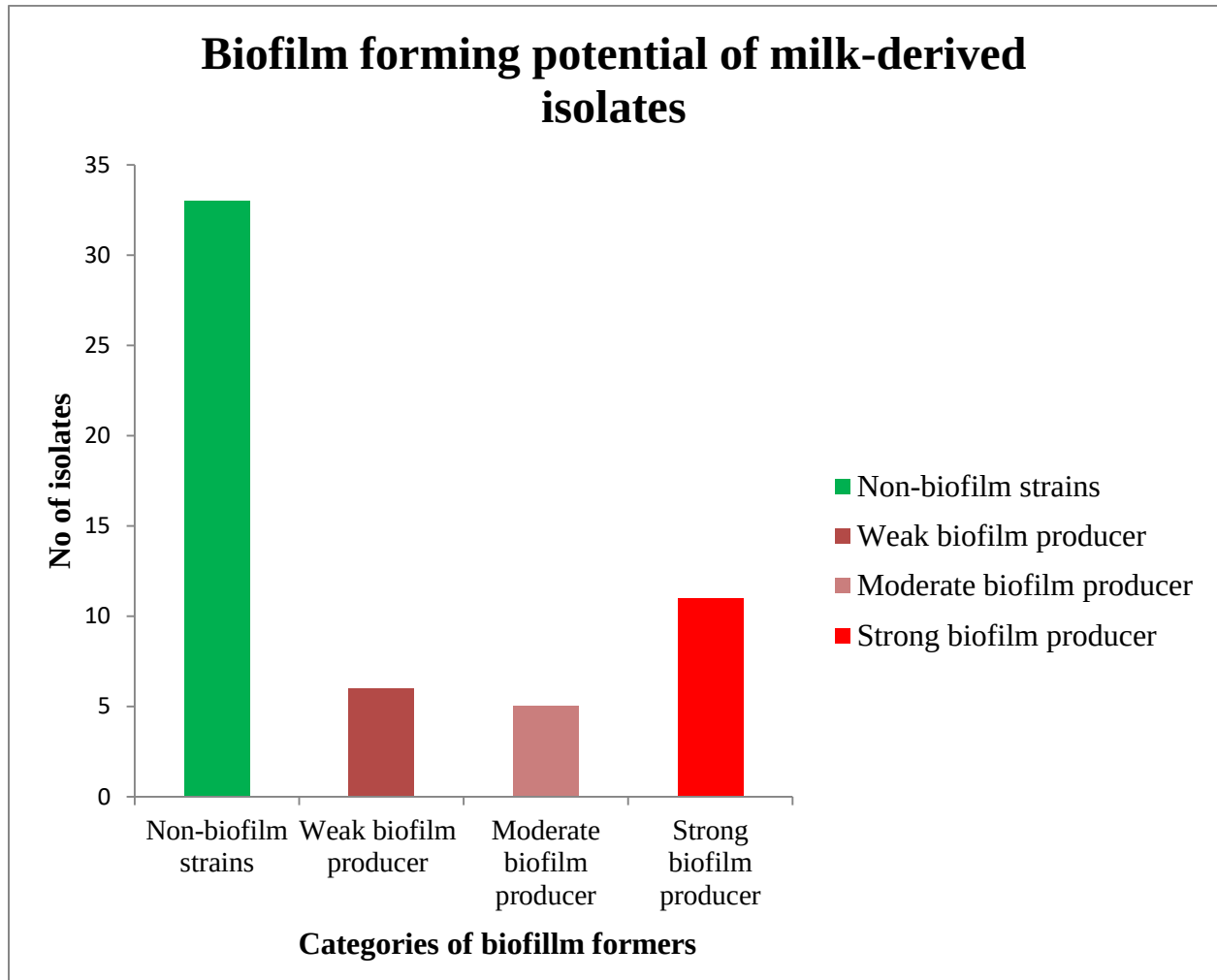


Figure 5. 4 the potential of BTM-derived isolates to form biofilm

5.4.6 Discrimination between live and dead cells

The order of the samples

1. **Live** *Enterococcus durans*
2. **Live** *Leifsonia shinshuensis*
3. **Dead** *Leifsonia shinshuensis*
4. **Dead** *Enterococcus durans*
5. **Dead** *Leifsonia shinshuensis* **with PMA**
6. **Dead** *Enterococcus durans* **with PMA**
7. **Live** *Leifsonia shinshuensis* and **live** *Enterococcus durans*
8. **Live** *Leifsonia shinshuensis* and **live** *Enterococcus durans* **with PMA**
9. **Dead** *Leifsonia shinshuensis* and **dead** *Enterococcus durans* **with PMA**
10. **Live** *Leifsonia shinshuensis* and **dead** *Enterococcus durans* **with PMA**
11. **Dead** *Leifsonia shinshuensis* and **live** *Enterococcus durans* **with PMA**

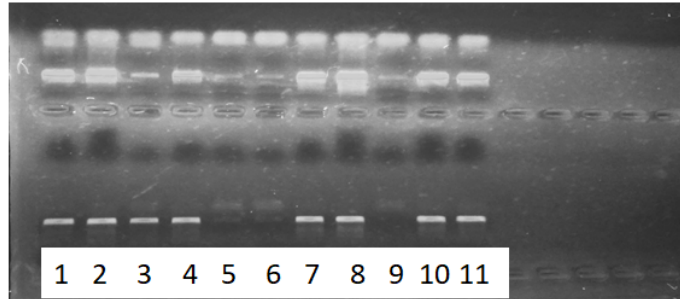


Figure 5. 5 the schematic arrangement and composition of samples used for the assessment of effectiveness of PMA in dead-and-live cells discrimination

The effectiveness of PMA in discriminating between live and dead cells

From the inset gel image above, PMA prevented the amplification of dead *Enterococcus durans* and *Leifsonia shinshuensis* in the well number 5 and 6. Also, when the two species were mixed, PMA also successfully thwarted the amplification of their targeted gene as shown in the well number 9. The effectiveness of PMA at preventing the amplification of dead cells' DNA treated with PMA seemed to reduce with the size of the gene to be amplified. The lower lane of bands in the inset above shows bands of 1500kb amplicons, while the upper lane of bands shows bands of 200kb. While PMA successfully prevented the amplification of larger fragments in the lower lane's well number 5, 6, and 9, there were faint bands observed in the upper lane's larger DNA fragments (well number 5, 6, and 9 of the inset's upper lane).

5.4.7 The impact of PMA treatment on bacterial sequencing and identification

PMA treatment did not affect the sequencing and identification of bacterial isolates. PMA successfully enabled the prevention of amplification of dead *Enterococcus durans* and *Leifsonia shinshuensis* in both formulation number 10 and 11, respectively. Similarly PMA treatment excluded the dead bacteria DNA signals in the identification of *Enterococcus durans* and *Leifsonia shinshuensis* in the sample number 10 and 11, respectively.

Table 5. 3 the identity of bacteria used for the assessment of PMA effectiveness in discriminating live cells from dead cells as described in fig 5.5 above

The order of sample	Bacteria identity	Homology (%)	Accession number
1	<i>Enterococcus durans</i>	100	MT604840.1
2	<i>Leifsonia shinshuensis</i>	100	MN709196.1
7	<i>Enterococcus sp.</i>	79.21	KF048929.1
8	<i>Uncultured bacterium</i>	82.89	HM316872.1
10	<i>Leifsonia shinshuensis</i>	100	MN709196.1
11	<i>Enterococcus durans</i>	100	HE646418.1

5.5 Discussion

Milk, due to its high nutritional value, harbor a diverse microbiota and is susceptible to bacterial contamination from manifold sources and bacteria-induced spoilage (Garedew et al., 2012 & Reta et al., 2016). The main sources of contamination are cow's udder & health (Garedew et al., 2012 & Velázquez-Ordoñez et al., 2019), barn's environment like airborne dust (Wu et al., 2019), milking equipment and storage tanks (Velázquez-Ordoñez et al., 2019 & Vidal et al., 2017). Weber et al., (2019) found out that there are spots in milking and storage systems on dairy farms that are more colonized than others. Milk retainers, teat cups, and BMTO were found to be highly colonized by psychrotrophic bacteria than other sampling spots. We swabbed these spots for our pilot study and isolated putative biofilm-forming bacteria. In addition to swabs, we also collected BTM and isolated bacteria from it. After genetic fingerprinting using DGGE, 49 and 74 isolates from milk and swabs, respectively, were selected to represent unique genetic fingerprint clusters. The isolates were found to represent 38 different species and 19 genera.

Even if a significant proportion of microbiota inhabiting milk is extirpated during thermo processing, pasteurization, their heat-stable extracellular enzymes survive pasteurization (Baglinière et al., 2017). After surviving thermo processing, extracellular enzymes, lipase and peptidase, lead to spoilage of thermally processed milk. Lipases are said to impart rancid taste (Deeth, 2006 & von Neubeck et al., 2015) on milk due to hydrolysis of milk fat globules. Peptidases, on the other hand, impart a bitter taste on milk and/or gelling of UHT milk (Marchand et al., 2009 & Nörnberg et al., 2010) due to the casein micelle destabilization. According to Adams et al., (1975) & Nörnberg et al., (2010), the ultimate ramifications of the heat-resistant enzymes are the shortening of the shelf-life of processed dairy products and unacceptable off-flavors.

From our pilot study, *Clostridium butyricum* had the highest proteolytic ($16 \text{ h}^{-1} \cdot \text{ml}^{-1}$) and lipolytic (27.01 and $13.81 \mu\text{mol} (\text{min} \cdot \text{ml})$) activities, higher than the one reported by Yuan et al., (2018) where *Yersinia intermedia* had the highest proteolytic activity at $14 \text{ h}^{-1} \cdot \text{ml}^{-1}$ and *Aconetobacter guillouiae* had the highest lipolysis liberating $14 \mu\text{mol} (\text{min} \cdot \text{ml})$. Nörnberg et al., (2010), on the other hand, found out that *Burkholderia cepacia* 1A4 had the highest proteolysis at $80 \pm 11 \text{ U/ml}$. Isolates identified as the same species like *Clostridium butyricum* and *Rouxiella chamberiensis* had different lipolytic and proteolytic activities, respectively. For instance, the two species of *Clostridium butyricum* liberated 27.01 and $13.81 \mu\text{mol} (\text{min} \cdot \text{ml})$, while the two species of *Rouxiella chamberiensis* had proteolytic activities at 4 and $5.3 \text{ h}^{-1} \cdot \text{ml}^{-1}$. This phenomenon, where different species in the same genus and different strains in the same species having different enzymatic activities, has been reported previously by Baur et al., (2015) & Yuan et al., (2018). For instance, in the report by Yuan et al., (2018), *Pseudomonas fragi* strains a & b and *Acinetobacter johnsonii* strains a & b had different lipolytic and proteolytic activities at the same condition. This difference in enzymatic activities between strains of the same species was ascribed to the genetic diversity differences between strains (Baur et al., 2015). Also, the tight regulation of the aprX gene coding for the protease and lipA gene that encodes the lipase may

explain the difference in enzymatic activities between strains (Yuan et al., 2018). Furthermore, Marchand et al., (2009) found out that there existed a broad spectrum of aprX gene heterogeneity (similarities ranged between 63 to 99%) among strains of *Pseudomonas*. This heterogeneity would underscore the differences in proteolytic activities among strains of the same species.

Biofilms are communities of microbial cells adhered to a substratum and encased in extracellular polysaccharides and non-cellular materials (Marchand et al., 2012 & Teh et al., 2014), have become a great concern in the dairy industry. According to the reports by Latorre et al., (2010) & Marchand et al., (2012), biofilms have become perennial sources of microbial contamination in the dairy industry due to continual sloughing off the biofilm dwellers into milk. What's more, Teh et al., (2014) asserted that biofilms are an unrecognized source of extracellular enzymes like protease and lipase. According to them, biofilms were previously an unrecognized reservoir of bacterially produced enzymes with the potential to spoil milk. They further postulated that the heat-resistance of biofilm-emanating bacterially produced enzymes might be enhanced by the protective nature of exopolysaccharides in a biofilm matrix. Additionally, Teh et al., (2013) found that sessile bacterial cells inhabiting a biofilm produced higher enzymatic activities than their counterpart planktonic cells. The aforementioned facts underscored the importance of biofilms in the dairy industry.

In light of the aforementioned reasons, we performed a pilot study to assess the potential of bacterial isolates from BTM and swabs. We found out that the majority of milk-derived isolates were not biofilm formers, while 22 out of the 55 milk-derived isolates could form biofilm of varied strength. On the other hand, in stark contrast, the majority (46 out of 58) of the swab-derived isolates formed strong biofilms and only 1 isolate could not form a biofilm. The rest, 11, isolates formed biofilms of different strengths: 9 isolates formed biofilms of moderate strength and 2 isolates formed weak biofilms. Rollet et al., (2009) found out that sessile and detached cells had enhanced affinities for substratum as opposed to counterpart planktonic cells. This implied that biofilm-derived bacterial cells could retain physiological biofilm characters after they returned to a planktonic growth mode. This can explain why the majority of swab-derived isolates had a higher potential to form biofilm, especially a strong biofilm, unlike milk-derived isolates.

Unlike the culture-dependent approach that has been the usual method used to study biofilm, the culture-independent has been proposed to be a superior technique to study biofilm since it offers the overall view of the biofilm microbial community composition (Varjani et al., 2018). However, the application of culture-independent method in studying dairy biofilms is also limited by the fact that bacterial dwellers of biofilms have been reported to depict variable resistance to sanitizers utilized in clean in place (CIP) due to the protective nature of the polymeric matrix encasing bacterial cells (Lee et al., 2015). Hence, a proportion of a dairy biofilm community might lose viability after CIP while others might retain it. Thus, a direct application of culture-independent in studying dairy biofilms after CIP might either under- or

overestimate the biofilm cells that are sensitive to sanitizers in use (Weber et al., 2019). Therefore, to comparatively use both approaches and gain overall insights into the bacterial community of dairy biofilms, the use of viability dyes has been suggested to a way to surmount this challenge (Erkus et al., 2016).

Propidium monoazide (PMA) is fluorescent dye that is usually applied for staining membrane the DNA of compromised cells; it is a modified, superior version of the previously used fluorescent dyes like propidium iodide (PI) and ethidium monoazide (EMA) (Erkus et al., 2016 & Nocker et al., 2006). Unlike previously used fluorescent dyes, PMA circumvented the drawbacks of other dyes. It improved overall selectivity for only membrane compromised cells unlike EMA due to its membrane-impermeant nature. It is identical to PI, albeit it has an azide group which enables it to readily cross-link to DNA of a membrane-compromised cell upon light exposure. The aforementioned qualities make it more promising than the other previously used fluorescent dyes. Although quite promising, it has not been sufficiently used in the dairy sector, especially in the study of complex microbial communities like biofilms using high-throughput sequencing methods. For this reason, we decided to apply it at a pilot level to assess its applicability in studying dairy biofilms using a culture-independent method.

From our pilot study, we found out that PMA successfully inhibited the amplification of DNA of membrane-compromised cells (heat-exposed bacterial cells). This inhibition was not bacteria-dependent since it prevented the amplification of DNA of heat-denatured *Enterococcus durans* and *Leifsonia shinshuensis*. What's more, PMA was able to selectively inhibit the amplification of DNA of membrane-compromised cells in case of a mixture of both live and dead cells of different species. For instance, in the figure 5.5 above, PMA inhibited the amplification of DNA of dead *Enterococcus durans* and *Leifsonia shinshuensis* in samples no. 10 and 11, respectively. Just like Banihashemi et al., (2012) found out that the targeted DNA length affects the effectiveness of PMA, our pilot study had similar findings. In their study, they found out that 174-, 899- or 1512-bp fragments of the cpn60 gene of heat-treated *Campylobacter jejuni* yielded faint, fainter, and no bands, respectively. In our pilot experiment as shown in the figure above, PMA treatment completely inhibited the amplification of 1500 kb of 16S rRNA gene of heat-killed *Enterococcus durans* and *Leifsonia shinshuensi*, while faint bands were observed for 200 kb of 16S rRNA gene of the aforementioned bacteria.

We found out that PMA treatment of samples did not affect Sanger sequencing. When PMA was used in samples no. 10 and 11, as expected, we could successfully identify *Leifsonia shinshuensis* and *Enterococcus durans*, respectively. PMA treatment of samples did not affect the signal quality or the homology of a bacterium as shown in the table 5.3 above. Though the data is not shown in this manuscript, PMA treatment of samples also did not affect MiSeq sequencing.

5.6 Conclusion

Despite CIP, key spots in the milking and storage systems were still inhabited by bacteria with the milk spoilage potential. Milk in this system was contaminated with bacteria with the ability to produce milk-quality deteriorative enzymes. Bacteria isolated from swabs after CIP showed they could readily form strong biofilms, and their isolation after CIP meant they either survived sanitizers used or re-contaminated the swabbed surfaces after CIP. PMA can be incorporated in the culture-independent study of dairy biofilms to accurately gain an overall view of bacteria that resist sanitizers in use in the dairy industry.

References

- Adams, D. M., Barach, J. T., & Speck, M. L. (1975). Heat Resistant Proteases Produced in Milk by Psychrotrophic Bacteria of Dairy Origin. *Journal of Dairy Science*, 58(6), 828–834. [https://doi.org/10.3168/jds.S0022-0302\(75\)84645-5](https://doi.org/10.3168/jds.S0022-0302(75)84645-5)
- Baglinière, F., Tanguy, G., Salgado, R. L., Jardin, J., Rousseau, F., Robert, B., Harel-Oger, M., Vanetti, M. C. D., & Gaucheron, F. (2017). Ser2 from *Serratia liquefaciens* L53: A new heat stable protease able to destabilize UHT milk during its storage. *Food Chemistry*, 229, 104–110. <https://doi.org/10.1016/j.foodchem.2017.02.054>
- Banihashemi, A., Van Dyke, M. I., & Huck, P. M. (2012). Long-amplicon propidium monoazide-PCR enumeration assay to detect viable *Campylobacter* and *Salmonella*. *Journal of Applied Microbiology*, 113(4), 863–873. <https://doi.org/10.1111/j.1365-2672.2012.05382.x>
- Baur, C., Krewinkel, M., Kranz, B., von Neubeck, M., Wenning, M., Scherer, S., Stoeckel, M., Hinrichs, J., Stressler, T., & Fischer, L. (2015). Quantification of the proteolytic and lipolytic activity of microorganisms isolated from raw milk. *International Dairy Journal*, 49, 23–29. <https://doi.org/10.1016/j.idairyj.2015.04.005>
- Bussamara, R., Fuentesfria, A. M., Oliveira, E. S. de, Broetto, L., Simcikova, M., Valente, P., Schrank, A., & Vainstein, M. H. (2010). Isolation of a lipase-secreting yeast for enzyme production in a pilot-plant scale batch fermentation. *Bioresource Technology*, 101(1), 268–275. <https://doi.org/10.1016/j.biortech.2008.10.063>
- Deeth, H. C. (2006). Lipoprotein lipase and lipolysis in milk. *International Dairy Journal*, 16(6), 555–562. <https://doi.org/10.1016/j.idairyj.2005.08.011>
- Desfossés-Foucault, É., Dussault-Lepage, V., Le Boucher, C., Savard, P., LaPointe, G., & Roy, D. (2012). Assessment of probiotic viability during Cheddar cheese manufacture and ripening using propidium monoazide-PCR quantification. *Frontiers in Microbiology*, 3(OCT), 1–11. <https://doi.org/10.3389/fmicb.2012.00350>
- Erkus, O., de Jager, V. C. L., Geene, R. T. C. M., van Alen-Boerrigter, I., Hazelwood, L., van Hijum, S. A. F. T., Kleerebezem, M., & Smid, E. J. (2016). Use of propidium monoazide for selective profiling of viable microbial cells during Gouda cheese ripening. *International Journal of Food Microbiology*, 228, 1–9. <https://doi.org/10.1016/j.ijfoodmicro.2016.03.027>
- García-Cayuela, T., Tabasco, R., Peláez, C., & Requena, T. (2009). Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR. *International Dairy Journal*, 19(6–7), 405–409. <https://doi.org/10.1016/j.idairyj.2009.02.001>
- Garedew, L., Berhanu, A., Mengesha, D., & Tsegay, G. (2012). Identification of gram-negative bacteria from critical control points of raw and pasteurized cow milk consumed at Gondar town and its suburbs, Ethiopia. *BMC Public Health*, 12(1), 1. <https://doi.org/10.1186/1471->

- Hahne, J., Isele, D., Berning, J., & Lipski, A. (2019). The contribution of fast growing, psychrotrophic microorganisms on biodiversity of refrigerated raw cow's milk with high bacterial counts and their food spoilage potential. *Food Microbiology*, 79, 11–19. <https://doi.org/10.1016/j.fm.2018.10.019>
- Hoong, K., Flint, S., Palmer, J., Andrewes, P., Bremer, P., & Lindsay, D. (2012). International Journal of Food Microbiology Proteolysis produced within bio films of bacterial isolates from raw milk tankers. *International Journal of Food Microbiology*, 157(1), 28–34. <https://doi.org/10.1016/j.ijfoodmicro.2012.04.008>
- Latorre, A. A., Van Kessel, J. S., Karns, J. S., Zurakowski, M. J., Pradhan, A. K., Boor, K. J., Jayarao, B. M., Houser, B. A., Daugherty, C. S., & Schukken, Y. H. (2010). Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes*. *Journal of Dairy Science*, 93(6), 2792–2802. <https://doi.org/10.3168/jds.2009-2717>
- Lee, J. S., Bae, Y. M., Lee, S. Y., & Lee, S. Y. (2015). Biofilm Formation of *Staphylococcus aureus* on Various Surfaces and Their Resistance to Chlorine Sanitizer. *Journal of Food Science*, 80(10), M2279–M2286. <https://doi.org/10.1111/1750-3841.13017>
- Marchand, S., De Block, J., De Jonghe, V., Coorevits, A., Heyndrickx, M., & Herman, L. (2012). Biofilm Formation in Milk Production and Processing Environments; Influence on Milk Quality and Safety. *Comprehensive Reviews in Food Science and Food Safety*, 11(2), 133–147. <https://doi.org/10.1111/j.1541-4337.2011.00183.x>
- Marchand, S., Vandriesche, G., Coorevits, A., Coudijzer, K., Jonghe, V. De, Dewettinck, K., Vos, P. De, Devreese, B., Heyndrickx, M., & Block, J. De. (2009). International Journal of Food Microbiology Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. *International Journal of Food Microbiology*, 133(1–2), 68–77. <https://doi.org/10.1016/j.ijfoodmicro.2009.04.027>
- Nguyen, Q. D., Tsuruta, T., & Nishino, N. (2020). Examination of milk microbiota, fecal microbiota, and blood metabolites of Jersey cows in cool and hot seasons. *Animal Science Journal = Nihon Chikusan Gakkaiho*, 91(1), e13441. <https://doi.org/10.1111/asj.13441>
- Nocker, A., Cheung, C. Y., & Camper, A. K. (2006). Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods*, 67(2), 310–320. <https://doi.org/10.1016/j.mimet.2006.04.015>
- Nörnberg, M. F. B. L., Friedrich, R. S. C., Weiss, R. D. N., Tondo, E. C., & Brandelli, A. (2010). Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. *International Journal of Dairy Technology*, 63(1), 41–46. <https://doi.org/10.1111/j.1471-0307.2009.00542.x>
- Ramnath, L., Sithole, B., & Govinden, R. (2017). Identification of lipolytic enzymes isolated from bacteria indigenous to Eucalyptus wood species for application in the pulping industry.

- Biotechnology Reports*, 15(July), 114–124. <https://doi.org/10.1016/j.btre.2017.07.004>
- Reta, M. A., Bereda, T. W., & Alemu, A. N. (2016). Bacterial contaminations of raw cow's milk consumed at Jigjiga city of Somali regional state, Eastern Ethiopia. *International Journal of Food Contamination*, 3(1). <https://doi.org/10.1186/s40550-016-0027-5>
- Rollet, C., Gal, L., & Guzzo, J. (2009). Biofilm-detached cells, a transition from a sessile to a planktonic phenotype: A comparative study of adhesion and physiological characteristics in *Pseudomonas aeruginosa*. *FEMS Microbiology Letters*, 290(2), 135–142. <https://doi.org/10.1111/j.1574-6968.2008.01415.x>
- Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, 40(2), 175–179. [https://doi.org/10.1016/S0167-7012\(00\)00122-6](https://doi.org/10.1016/S0167-7012(00)00122-6)
- Teh, K. H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., & Lindsay, D. (2014). Biofilm- An unrecognised source of spoilage enzymes in dairy products? *International Dairy Journal*, 34(1), 32–40. <https://doi.org/10.1016/j.idairyj.2013.07.002>
- Teh, K. H., Flint, S., Palmer, J., Lindsay, D., Andrewes, P., & Bremer, P. (2011). Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. *International Dairy Journal*, 21(10), 742–747. <https://doi.org/10.1016/j.idairyj.2011.04.013>
- Teh, K. H., Lindsay, D., Palmer, J., Andrewes, P., Bremer, P., & Flint, S. (2013). Lipolysis within single culture and co-culture biofilms of dairy origin. *International Journal of Food Microbiology*, 163(2–3), 129–135. <https://doi.org/10.1016/j.ijfoodmicro.2013.02.009>
- Varjani, S., Srivastava, V. K., Sindhu, R., Thakur, I. S., & Gnansounou, E. (2018). Culture based approaches, dependent and independent, for microbial community fractions in petroleum oil reservoirs. *Indian Journal of Experimental Biology*, 56(7), 444–450.
- Velázquez-Ordoñez, V., Valladares-Carranza, B., Tenorio-Borroto, E., Talavera-Rojas, M., Antonio Varela-Guerrero, J., Acosta-Dibarrat, J., Puigvert, F., Grille, L., González Revello, Á., & Pareja, L. (2019). Microbial Contamination in Milk Quality and Health Risk of the Consumers of Raw Milk and Dairy Products. *Nutrition in Health and Disease - Our Challenges Now and Forthcoming Time*. <https://doi.org/10.5772/intechopen.86182>
- Vidal, A. M. C., Netto, A. S., Vaz, A. C. N., Capodifoglio, E., Gonçalves, A. C. S., Rossi, G. A. M., Figueiredo, A. S., & Ruiz, V. L. A. (2017). *Pseudomonas* spp.: Contamination sources in bulk tanks of dairy farms. *Pesquisa Veterinaria Brasileira*, 37(9), 941–948. <https://doi.org/10.1590/s0100-736x2017000900008>
- von Neubeck, M., Baur, C., Krewinkel, M., Stoeckel, M., Kranz, B., Stressler, T., Fischer, L., Hinrichs, J., Scherer, S., & Wenning, M. (2015). Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *International Journal of Food Microbiology*, 211, 57–65. <https://doi.org/10.1016/j.ijfoodmicro.2015.07.001>
- Weber, M., Liedtke, J., Plattes, S., & Lipski, A. (2019). Bacterial community composition of biofilms in milking machines of two dairy farms assessed by a combination of

- culture-dependent and -independent methods. *PLoS ONE*, 14(9), 1–14. <https://doi.org/10.1371/journal.pone.0222238>
- Wu, B., & Nishino, N. (2016). Identification and isolation of *Lactobacillus fructivorans* from wilted alfalfa silage with and without molasses. *Journal of Applied Microbiology*, 120(3), 543–551. <https://doi.org/10.1111/jam.13031>
- Wu, H., Nguyen, Q. D., Tran, T. T. M., Tang, M. T., Tsuruta, T., & Nishino, N. (2019). Rumen fluid, feces, milk, water, feed, airborne dust, and bedding microbiota in dairy farms managed by automatic milking systems. *Animal Science Journal*, 90(3), 445–452. <https://doi.org/10.1111/asj.13175>
- Yuan, L., Sadiq, F. A., Liu, T. jie, Li, Y., Gu, J. si, Yang, H. yi, & He, G. qing. (2018). Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes. *Journal of Zhejiang University: Science B*, 19(8), 630–642. <https://doi.org/10.1631/jzus.B1700352>
- Zain, S. N. M., Flint, S. H., Bennett, R., & Tay, H. S. (2016). Characterisation and biofilm screening of the predominant bacteria isolated from whey protein concentrate 80. *Dairy Science and Technology*, 96(3), 285–295. <https://doi.org/10.1007/s13594-015-0264-z>