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An assessment of the environmental progress and climate mitigation potential for U.S. dairy

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An Assessment of the Environmental Progress and Climate Mitigation Potential for U.S. Dairy

By

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ABSTRACT

Agricultural production, especially in the case of livestock like dairy, is a significant contributor to climate change through the production of greenhouse gases (GHG) like carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O), as well as other air pollutants like ammonia (NH_3). However, increasing productivity led the U.S. dairy industry to reduce its negative environmental impacts through intensification or dilution of maintenance. In chapter 2, the aim of the study was to update a previous environmental impact assessment comparing the carbon footprint of Cheddar cheese production by Holsteins versus Jerseys in 2009. The functional unit of this analysis was 1 million metric tons of energy-corrected milk. A deterministic model simulated the two populations, establishing 16 life groups per breed needed to meet the functional unit. Production metrics like milk yield and milk components were sourced from the Council of Dairy Cattle Breeding National Metrics database for the year 2020. The software AMTS.Cattle.Pro was used to design total mixed rations for each individual life stage and breed to meeting the energy and nutritional needs of each animal. Feeds informed the background systems used to calculate land and water use, and associated feed emissions were calculated via economic allocation with emission factors sourced from ecoinvent 3.10. AMTS.Cattle.Pro also estimated daily nutrient and manure excretion, daily enteric CH_4, and voluntary water intake. Total GHG emissions were converted to carbon dioxide equivalents (CO_2e) using AR6 global warming potential (GWP) values. The results found milk yield increased for Holsteins and Jerseys by 29% and 26%, respectively, from 2009 versus 2020. Certain key performance indicators like land use decreased for Jerseys and Holsteins due to less feed intake associated with smaller populations. However, water use increased because irrigation was included in this assessment, but not in the original 2009 study. Overall, the carbon footprint for Jersey milk production was 1.6 kg CO_2e/kg

ECM and the carbon footprint for Holstein milk production was 1.8 kg CO_2e/kg ECM, which was higher than the carbon footprints of the original assessment, likely due to the different GWP values used. Overall, both breeds made advancements in productivity, helping to offset increases in resource consumption. And the carbon footprint for Jersey production was 89% of the Holstein carbon footprint, meaning the Jersey population had an environmental impact more similar to Holsteins in 2020 compared to their environmental impacts in 2009. In chapter 3, the aim of study 2 was to investigate the effects of Eminex® on GHG and NH_3 emissions from fresh dairy slurry and dairy lagoon water. Eminex® had previously reduced total GHG emissions by 99% under anaerobic and temperature-controlled conditions, but had not tested in liquid-based systems. For experiment 1, feces and urine were collected from lactating dairy cows and mixed into a homogenous slurry, prior to being allocated into twelve individual bowls with 2.2 kg/bowl. Each bowl was randomly assigned a treatment: high, low, and a control with an n = 4/group. Upon receiving treatment, bowls were sampled beneath individual OdoFlux chambers for 7 days to measure for CO_2, CH_4, N_2O, NH_3, and ethanol (EtOH) emissions. Samples were collected to determine changes to manure quality. For experiment 2, lagoon water was collected from a commercial dairy, and distributed to 12 stainless 208-L stainless steel barrels. Two treatments (n = 4/treatment) were administered: high (1 kg/m^3 lagoon water), and low (0.5 kg/m^3 lagoon water); and control (n = 4). Four barrels at a time were sampled over two, nonconsecutive 14-day periods, using OdoFlux chambers, monitoring CO_2, CH_4, N_2O, and NH_3. Slurry total solids, total nitrogen, and total carbon was similar across all treatment groups ($P > 0.05$). Acetic acid concentration in slurry increased in Eminex® treated groups compared to control ($P < 0.05$). All slurry GHG emissions, except for N_2O, declined ($P < 0.05$). Results showed that the high Eminex® treatment compared to control reduced CO_2, CH_4, and NH_3 emissions by 49.3%, 30.4%, and 34.9%,

respectively ($P < 0.05$). In lagoon water, total nitrogen increased with treatment ($P < 0.05$), while total solids and total carbon remained similar between all three treatments ($P > 0.05$). Volatile fatty acid concentration in lagoon water also saw a trend for increasing acetic acid concentration in Eminex® treated groups compared to control ($P < 0.1$). GHG emissions from lagoon water also decreased over time ($P < 0.05$). The high Eminex® treatment emitted 12.0% less CO_2 ($P < 0.1$), 85.1% less CH_4 ($P < 0.05$), and 82.7% less N_2O ($P < 0.05$). However, both Eminex® treatments, compared to control, increased NH_3 volatilization over time ($P < 0.05$). With improvements to manure composition with increasing nitrogen content, as well as significant reductions in GHG emissions, Eminex® is a promising manure additive that could mitigate the negative environmental impacts of manure management systems. Further research is needed to continue verifying its potential in different settings and at the commercial level. The final study in Chapter 4 investigated the effects of Eminex® on the microbiome of slurry and lagoon water. Samples were collected from fresh slurry and dairy lagoon water during study 2. Samples were DNA extracted prior to being sent out to an independent laboratory for shallow shotgun metagenomic sequencing (SSMS). Results of the SSMS showed that the relative abundance of the phylum Proteobacteria decreased with Eminex® treatment in lagoon water, but increased in relative abundance with Eminex® treatment in slurry. Other phyla, like Firmicutes and Actinobacteria increased in relative abundance with Eminex® in lagoon water, but not in slurry. Pathogenic phyla, like Fusobacteria, did not increase in relative abundance with Eminex® treatment in slurry, but increased substantially in untreated slurry. A principal component analysis (PCA) was also performed and confirmed distinct microbiomes between slurry and lagoon water. The PCA also noted that the high Eminex® treatment, compared to the low Eminex® treatment, elicited a faster microbiome change. This suggested that Eminex® could be applied more effectively earlier in the

manure management chain. Lastly, a linear discriminatory analysis showed that bacterial populations were at their highest in the two Eminex® treatments at day 28, and highest in the control by day 56. Ultimately, the Eminex® treatment resulted in significant changes to the manure microbiome, helping to explain how this additive reduces GHG and NH_3 emissions. Looking at the microbiome demonstrated that the Eminex® doses can be decreased and still be effective. Future research would benefit from exploring the metabolomics associated with microbes exposed to Eminex® and exploring the effects of different treatment protocols of emissions and the manure microbiome.

Keywords: dairy, carbon footprint, Jerseys, Holsteins, manure management, greenhouse gas emissions, microbiome

Chapter 1 – Literature Review

1 INTRODUCTION

Despite its environmental impact, animal agriculture is essential to healthy human diets. Animal-source foods (ASF) provide not only essential macronutrients like protein, but micronutrients like vitamin A, vitamin B12, arachidonic, eicopentaneoic (EPA), and docosahexaenoic (DHA) fatty acids (White and Hall, 2017; Leroy et al., 2023). Research has shown that without animal agriculture, U.S. diets will be higher in calories and severely deficient in essential nutrients (White and Hall, 2017). Even so, the environmental impact of the sector cannot be ignored and therefore presents a wicked challenge for all, from consumers and producers to researchers and policymakers, as populations continue to grow and demand for ASF increases around the world.

To quantify and continue advancing the sustainability of the livestock industry and protect these vital sources of high-quality protein, the entire production system must be scrutinized. Previous U.S. life cycle assessments (LCAs) have estimated that about 73% of greenhouse gas (GHG) emissions occur before the farmgate, meaning before any product leaves the farm for additional processing (Thoma et al., 2013b; Wattiaux et al., 2019). These on-farm emissions are further split between major hotspots: enteric fermentation and manure management, which equal about 25% and 24% of the carbon footprint, respectively (Thoma et al., 2013b; Wattiaux et al., 2019).

The aim of the present literature review is to explore existing topics on milk production LCAs, with additional insight into enteric fermentation and manure management as hotspots of environmental pollution. This review will also include summarizing potential interventions to address emissions sources for the main agricultural GHG emissions, ammonia (NH_3), and volatile organic compounds (VOC).

1.1 Life Cycle Assessments of Dairy Production

Currently, the primary way of quantifying the environmental, social, and economic impacts of human-made product is through a LCA. The LCA traces the inflow of resources required to produce a certain amount of product in a specific timeframe as well as the outflow of co-products and waste/pollution streams (Guinée et al., 2011). The majority of LCAs assess midpoint indicators, like global warming potential (GWP) of GHG emissions, which are used to calculate carbon footprints. A GWP is defined as the ratio of the time-integrated radiative forcing from the instantaneous release of 1 kg of a trace substance relative to that of 1 kg of a reference gas, CO_2 (IPCC, 2021). A carbon footprint is a single value of all major GHGs required to produce a given functional unit converted into carbon dioxide equivalents (CO_2e) using GWPs to allow for comparisons between different gases. It represents an estimate of the carbon impact that any activity, sector, or product has on the environment and a proxy for climate change.

This technique provides invaluable insight into the environmental impact of production systems, like milk and beef production. Many LCAs have been performed for milk, with a wide range of scopes, goals, system boundaries, functional units, co-production allocation, and impact assessments (Capper and Cady, 2012; Thoma et al., 2013b; Thoma et al., 2013c; Üçtuğ, 2019; Capper and Cady, 2020; Rotz et al., 2021; Uddin et al., 2021). The interest seems to be spurred by multiple stakeholders within the livestock agricultural sector, including but not limited to consumers and researchers. For consumers, knowing the carbon footprint of food items can help inform contentious decisions when purchasing ASFs. For researchers, carbon footprints can help identify “hot spots” of production, where large quantities of emissions or resource depletion can be mitigated through technical and scientific interventions.

There is a wide variety of potential LCAs surrounding milk, specific to feed production, processing, and consumption, as well as those for dairy products like cheese (Adom et al., 2012; Capper and Cady, 2012; Adom et al., 2013; Nutter et al., 2013). However, the focus of the present review will be on LCAs of fluid milk and cheese production based in the United States. Scientific publications of interest are summarized in Table 1.1.

Table 1.1 Summary of the fluid milk-based life cycle assessments

Functional Unit	System Boundary	Carbon Footprint (kg CO_2e/FU)*	Source
500,000 tons of Cheddar Cheese[1]	Cradle-to-farmgate	H: 8,104 x 10^3 t J: 6,442 x 10^3 t	Capper and Cady, 2012
1,000 kg Cheddar Cheese 1 ton mozzarella cheese consumed	Cradle-to-grave	1.34x10^4 1.42x10^4	Kim et al., 2013
1 kg FPCM	Cradle-to-farmgate	1.23	Thoma et al., 2013b
1 kg milk drunk by U.S. consumers	Cradle-to-grave	2.05	Thoma et al., 2013a
1 kg ECM	Cradle-to-farmgate	1.12-1.16	Naranjo et al., 2020
1 MMT ECM	Cradle-to-farmgate	1.70 x 10^9	Capper and Cady, 2020
1 kg FPCM	Cradle-to-farmgate	0.69-1.45	Rotz et al., 2021
1 kg FPCM[2]	Cradle-to-farmgate	H: 1.47 J: 1.41	Uddin et al., 2021

*Other units will be indicated, like with Capper and Cady, 2012
[1]Capper and Cady (2012) was a breed-wide comparison of carbon footprints, each breed's respective footprint is given
[2]Uddin et al. (2021) was a breed-wide comparison of carbon footprints, each breed's respective footprint is given
MMT = million metric tons; ECM = energy corrected milk; FPCM = fat and protein corrected milk; H = Holsteins and J = Jerseys

Many LCAs focus on comparisons between specific years to quantify changes and advancements made to production efficiencies, either through productivity or management changes (Naranjo et al., 2019; Capper and Cady, 2020). Others LCAs determine baseline

estimations, creating a benchmark of environmental performance for specific years that can be updated over time (Rotz et al., 2021; Uddin et al., 2021). Some LCAs are regionally limited by focusing on state-level production (Thoma et al., 2013b; Thoma et al., 2013c; Naranjo et al., 2020; Uddin et al., 2021), or by making comparisons between different dairy breeds (Capper and Cady, 2012; Uddin et al., 2021).

As shown in Table 1.1, the dairy industry has been notably improving over time through increased milk production. These improvements are often attributed to dilution of maintenance (DOM). This phenomenon is considered one of the most effective ways of reducing the carbon footprint of milk (Capper and Cady, 2012, 2020). The DOM is achieved through increasing animal productivity without increasing herd sizes. Maintaining or even shrinking herd sizes means decreasing system inputs, like feed, and increasing system outputs, like milk.

Direct comparisons to LCAs from previous years emphasizes the growth and improvements in the dairy sector. Thoma et al. (2013c) utilized data collected via survey in 2008, whereas Rotz et al. (2021) built their LCA using the Integrated Farm Systems Models (IFSM) supplemented with survey data. Despite the discrepancies in data source and the potential of scale-up error when using survey data to represent the U.S. dairy industry on a national scale, this remains a good comparison given the similarity in scope and functional unit used within each study.

Using 2008 data, the carbon footprint of 1 kg of fat-and-protein corrected milk (FPCM) was 1.23 kg CO_2e (Thoma et al., 2013c). More recently, IFSM estimated the carbon footprint of 1 kg FPCM to range from 0.69-1.45 kg CO_2e, with a weighted national average of 1.01 kg CO_2e (Rotz et al., 2021). In the decade that passed between these two publications, the carbon footprint of 1 kg FPCM decreased by 17.8%. This is a substantial reduction in the environmental impact of

1kg FPCM was likely from improvements made to nutrition, feed efficiency, genetics, and animal husbandry.

Other time comparisons showed similar, if not greater reductions to the carbon footprint of dairy production (Capper and Cady, 2020; Naranjo et al., 2020). California is one of the top milk producing states in the U.S. and when comparing the environmental performance of the California dairy industry in 1964 to 2014, Naranjo et al. (2020) calculated a 45.9% decrease in the carbon footprint over 50 years. For the entire U.S. dairy industry, Capper and Cady (2020) compared the dairy sector in 2007 to 2017. They found a reduction of 17.3% in feedstuffs, 20.8% in land, 30.5% water, and 25.2% in cattle populations. This resulted in a 19% decrease in the total carbon footprint over a decade (Capper and Cady, 2020).

The overall degree of change over time between these two studies is likely influenced by a variety of factors, the most important of which are system boundary and timeframe. Capper and Cady (2020) performed a shorter, decade long analysis of the change in the national U.S. dairy carbon footprint, calculating a carbon footprint representative of the country, but it cannot be scaled down to represent specific regions, as the diversity of the dairy industry varies widely when it comes to feed availability, husbandry practices, and manure management techniques (Niles and Wiltshire, 2019; Capper and Cady, 2020; Rotz et al., 2021; Niles et al., 2022). Also, the carbon footprint calculated by Naranjo et al. (2020) is limited to the state of California for similar reasons.

Performing LCAs for individual states/regions, like Naranjo et al. (2020) and Uddin et al. (2021), can be especially important when it comes to providing specialized insights into specific regions. Farms in the Midwest do not operate under the same standards as those in the West, which means different access to resources, different carbon footprints and different efficacies of

interventions. Therefore, it limits how carbon footprints can be interpreted beyond the scope of a particular LCA.

Cheese is another commonly assessed dairy commodity for LCAs (Capper and Cady, 2012; Kim et al., 2013). In Capper and Cady (2012), the carbon footprint of 500,000 tons of Cheddar Cheese was compared for Holsteins versus Jerseys, and they also measured the impact that recombinant bovine somatotropin (rBST) had on cheese production. The carbon footprints calculated by Capper and Cady (2012) versus Kim et al. (2013) differed due to differences in scope and specific functional units, as well as the confounding factor of breed. While both publications used Cheddar cheese as a functional unit, Kim et al. (2013) also assessed mozzarella cheese and included post-farm processing in the carbon footprint. Given the differences in sources of data between the studies as well as differences in age of data, this makes direct comparisons between these two cheese LCAs difficult.

Another important role that LCAs have yet to play lies in a second type than has been discussed. A LCA can be categorized as attributional or consequential. All the LCAs previously discussed were attributional, meaning they calculate the carbon footprint for the status quo system. The other type, consequential LCAs show look at changes to carbon footprints (using similar guidelines for scope, goal, functional unit, system boundaries, etc.) due to specific changes to that system. Research is beginning to quantify the benefits of consistent, aggressive, and significant changes to production standards for dairy using feed additives and other interventions (Beauchemin et al., 2020; Dillon et al., 2021; Fouts et al., 2022). Consequential LCAs will become invaluable resources in the future to model the effects of greater feed efficiency, feed additives, manure additives, and other sustainability interventions that are starting to be commercially available.

However, there are several challenges yet to be overcome when performing LCAs. In the literature, the largest discrepancies in carbon footprints for the same products with identical or similar functional units are due to methodological differences (Guinée et al., 2011). This makes it difficult not only to offer up one cohesive number to represent an entire production system, but to also compare across systems in such a way that comparisons are fair and comprehensible.

The United Nations Food and Agriculture Organization (FAO) developed the Livestock Environmental Assessment and Performance (LEAP) Partnership arose from the demand for guideline harmonization, and indicators for LCAs operating at different scales and within different livestock supply chains (FAO, 2024). The LEAP partnership came from the demand for guideline standardization given the wide variety of environmental assessment methods that exist (Guinée et al., 2011; Baldini et al., 2015). Their comprehensive guidelines helps steer LCA scientists operating within the livestock sector, providing guidance regarding common aspects across livestock production chains, like feed additives, water use, soil carbon stocks, nutrient flows, and GHG flows (FAO, 2024). The overall aim of the LEAP partnership is to allow for comparisons between LCAs; using the LEAP guidelines would help control for methodological differences between studies.

There is consensus among published LCAs lies in what are the most potent sources of air quality and climate pollutants are. All these LCAs showed that enteric emissions, feed production, and manure management are the largest sources of GHG emissions from dairy production, at the national (Capper and Cady, 2020; Rotz et al., 2021) and state-level (Naranjo et al., 2020; Uddin et al., 2021), regardless of FU or scope. Now that the significant sources of GHG emissions have been identified, it is essential to understand how these gases behave in the atmosphere, and what impacts on climate and human health they have.

1.2 Greenhouse Gases and Other Gaseous Emissions

Since the onset of industrialization around the globe, human activities have produced high levels of anthropogenic emissions of air quality and climate pollutants into the atmosphere. Earth's atmosphere naturally contains GHGs like water vapor, carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and ozone (O_3) at varying concentrations (EPA, 2023c). With the consumption of fossil fuels increasing starting in 1750s, the concentration of CO_2, CH_4, and N_2O in the atmosphere has grown by 47.9, 168, and 23.3%, respectively, in the last 270 years (EPA, 2023c).

Various industries, including agriculture, expanded across the globe with the onset of industrialization. Gross U.S. GHG emissions in 2020 were estimated to be about 5,973 million metric tons of CO_2e (EPA, 2021). The agricultural sector is a significant contributor to these emissions, due to its production of CO_2, CH_4, and N_2O. The agricultural industry is responsible for about 9.4% of all U.S. GHG emissions in 2021 (EPA, 2023c).

However, of the seven GHGs of concern, only three are attributed to agriculture. The other GHGs, hydrofluorocarbons, perfluorocarbons, sulfur hexafluoride, nitrogen trifluoride, ozone (O_3), and water vapor (EPA, 2021), are not pertinent to agricultural processes; therefore, they will not be further discussed. The GHG CO_2, CH_4, and N_2O are emitted by various part of the agricultural sector. For CH_4, GWP can be measured as a biogenic or of fossil origin, with GWPs of 27.2 and 29.8, respectively, and for N_2O, the GWP is 273 over a 100-year timespan (IPCC, 2021). In the U.S., enteric CH_4 emissions—that is CH_4 eructated by ruminants as a byproduct of their natural digestive systems—were 26.9% of total U.S. anthropogenic CH_4 emissions in 2020.

Manure management, another source of CH_4 in agriculture, represented 7.2% of total U.S. anthropogenic CH_4 emissions (EPA, 2022).

1.2.1 Carbon Dioxide

Carbon dioxide represents 79% of U.S. GHG emissions, from biogenic and anthropogenic sources. Of this total, 33 and 31% come from transportation and electricity generation, respectively, due to the burning of fossil fuels (EPA, 2022). Although CO_2 has a GWP of 1, it remains in the atmosphere much longer than other gases like CH_4. There are two ways that CO_2 can enter the atmosphere. The first is via the biogenic carbon cycle, where the mammalian respiration releases CO_2 following the inhalation of oxygen (Bruhwiler et al., 2018). The biogenic carbon cycle is also how CO_2 is naturally removed from the atmosphere. Plants take up CO_2 and via photosynthesis, transform the carbon compound into carbohydrates or fixating it in soil (Johnston et al., 2004; Bruhwiler et al., 2018).

In a second, more recent way, that anthropogenic CO_2 gets released into the atmosphere is via the burning of fossil fuels. This is called anthropogenic carbon, as it is released via human activities (Bruhwiler et al., 2018). While this CO_2 can also be removed from the atmosphere by plants, the concentration at which anthropogenic CO_2 is released vastly outpaces the capabilities of plants and soils to trap carbon from the atmosphere, leading to rapid temperature rise and global warming (Bruhwiler et al., 2018). Without the aid of plants actively up taking carbon, CO_2 remains in the atmosphere for over 100 years (Moore and Braswell, 1994). This categorizes it as a long-lived air pollutant. Despite having a lower GWP compared to the other GHGs of interest in agriculture, CO_2 is still emitted due to the industry's dependency on fossil fuels for machinery, processing, and distribution of goods on a global scale.

Forest fires are another source of biogenic and anthropogenic CO_2 emissions. While forest fires can happen naturally, man-made forest fires have been used to manage local landscapes since the 1850s (Klimaszewski-Patterson et al., 2024). However, due to increasing global temperatures, the prevalent of forest fires is dramatically increasing, and from 2001 to 2022, global forest fires emitted 33.9 billion tons of CO_2 (You, 2023).

1.2.2 Methane

Another carbon-based GHG is CH_4, released from a wide variety of biogenic and anthropogenic sources. Some biogenic sources include wetlands, termite digestion, and volcanic eruptions (EPA, 2022). Anthropogenic sources of CH_4 include landfills, burning biomass, coal mining, ruminant fermentation, and manure management. In the U.S. alone, CH_4 is responsible for 11% of total GHG emissions (EPA, 2022). The main source of CH_4 of interest for agriculture and livestock production comes from ruminant fermentation, which will be discussed in further detail later (section 1.4). Of total CH_4 emissions in the U.S., livestock agriculture makes up the largest source at 36% of CH_4 emissions. The next two largest sources of anthropogenic CH_4 include natural gas/petroleum and landfills, at 32% and 17% of emissions, respectively (EPA, 2022).

CH_4 is also part of the biogenic carbon cycle, mentioned above. This is because CH_4, when eructated by cattle or other ruminants, is synthesized from the breakdown and fermentation of plant carbohydrates (Russell, 2009; Huws et al., 2018; McSweeney and Mackie, 2020). Once in the atmosphere, CH_4 can be oxidized by hydroxyl radicals back to CO_2 and other atmospheric gases with a half-life of about 12 years (Hill et al., 2016; Cain et al., 2019; Lynch et al., 2021). As the carbon in CH_4 becomes CO_2 again, it reenters the biogenic carbon cycle to either become plant matter once more or become soil carbon (Johnston et al., 2004).

1.2.3 Nitrous Oxide

Arguably the most potent GHG from agriculture and livestock production, N_2O has a GWP value of 273, making it one of the more potent gases due to its intense heat trapping potential (IPCC, 2021). And like CO_2, N_2O is a long-lived climate pollutant, capable of remaining in the atmosphere for over 100 years (Prather et al., 2015; EPA, 2023c). However, it is emitted in relatively low quantities compared to the other GHGs of interest (i.e., CH_4 and CO_2), with about 5% of total U.S. N_2O emissions coming from manure management practices and 74% coming from land management practices, due to the application of artificial and organic fertilizers to soil (EPA, 2022).

Nitrogen (N) is an essential nutrient for the growth and development of both animals and plants (Fowler et al., 2013; NASEM, 2021; Park et al., 2021). The formation of N_2O is complicated, requiring both aerobic and anaerobic environments and ammonium oxidizing and denitrifying microbes, prior to emission into the atmosphere (Sabba et al., 2017; Khairunisa et al., 2023). Currently, many interventions designed to reduce agricultural GHG emissions focus on CH_4 and CO_2, whereas only in manure management are nitrogenous emissions of greater importance. Research on acidification and urease/nitrification inhibitors have helped reduce N_2O emissions. These interventions interrupt the N cycle and prevent the formation of nitrogenous gases by stopping key compounds like nitrites and nitrates from forming (Park et al., 2021; Sokolov et al., 2021).

1.2.4 Ammonia

A key nitrogenous gas is NH_3. It forms following a chemical reaction between organic N in the form of urea in urine and the enzyme urease in feces, either in animal housing facilities or in waste management areas (Van Horn et al., 1994; Aguirre-Villegas and Larson, 2017). However, NH_3 is a vital part of the N cycle, as organic N, urea and protein are transformed in ammonium (NH_4^+), nitrates (NO_3^-), nitrites (NO_2^-), prior to becoming NH_3 or other nitrogenous gases (Fowler et al., 2013; Khairunisa et al., 2023).

While NH_3 is neither a GHG nor a VOC, it is no less problematic when it comes to its impact on the environment and animal/human health, becoming hazardous when present in high concentrations (Van Horn et al., 1994; Hristov et al., 2011). Volatilization can consume up to 70 % of excreted N and be deposited into water and land-based ecosystems, leading to eutrophication (Aguirre-Villegas and Larson, 2017). However, the majority of inorganic N present in slurry and fresh manure is in the form of NH_4^+, which exists at equilibrium with NH_3 in aqueous solution (Van Horn et al., 1994).

Aside from threats to natural ecosystems, NH_3 is also a precursor to the formation of particulate matter 2.5 ($PM_{2.5}$), a small aerosol that can survive in the atmosphere for over two weeks and can carry pathogens into deep lung tissue if inhaled (Ross et al., 2021). Furthermore, NH_3 is also a precursor to the formation of N_2O (Aguirre-Villegas and Larson, 2017; Khairunisa et al., 2023). Ammonia's ability to negatively impact a broad range of ecosystems and human health like other VOCs makes it a particularly potent pollutant that must be aggressively managed.

1.3 Volatile Organic Compounds

Volatile organic compounds (VOCs) are secondary air pollutants produced from the incomplete decomposition of organic matter (Jiang et al., 2023). They are considered the major source of malodors associated with manure management (Zhang et al., 2019). They differ from GHGs primarily in their inability to trap heat in the atmosphere, although they contribute to other environmental issues. Such VOCs include hydrogen sulfide, alkanes, sulfur containing organics and aromatic compounds (Jiang et al., 2023). These VOCs can also lead to toxicity in humans at sufficiently high concentrations, as well as other undesirable chemical reactions (Zhang et al., 2019).

One detrimental chemical reaction results in the formation of O_3 (Lu et al., 2021). Ground level or tropospheric O_3 forms from a chemical interaction between sunlight, VOCs, and nitrogen oxide compounds (Shaw et al., 2007). Tropospheric O_3 is considered a GHG, capable of trapping heat in the atmosphere, but is also an important source of hydroxyl radicals, which are involved in the removal of CH_4 from the atmosphere (Lu et al., 2021). Aside from air pollution, VOCs like aromatic hydrocarbons and halogenated hydrocarbons are also capable of polluting water and terrestrial ecosystems (David and Niculescu, 2021).

Silages are another common source of VOCs, as forages are anaerobically fermented by microbes to preserve feed. Silage constitutes a major source of VOCs like acids, alcohols, ketones, esters, and aldehydes (El-Mashad et al., 2010; Hafner et al., 2013). With a wide variety of unavoidable sources of VOCs and a plethora of negative environmental and human health side effects, VOC losses must be mitigated like GHG emissions.

1.4 The Rumen Environment

The foregut digestive system found in even-toed ungulates, like cattle, goats, sheep, and deer, gives ruminants the ability to consume a wide variety of biomass with the help of the microbes inhabiting their stomachs (Huws et al., 2018; McSweeney and Mackie, 2020; Morgavi et al., 2020). The rumen is an incredibly complex digestive system populated by an even more complex ecosystem of six groups of microbes existing symbiotically with the ruminant host. The main role of these microbes is to ferment feedstuffs while simultaneously providing substrates for the animal to use (Lopez-Garcia et al., 2022).

The byproducts of digestion support the rumen microbiome and symbiotically benefit the animal with little impact to the environment beyond, with the notable exception of CO_2 and CH_4. One of the gases naturally produced during fermentation, CH_4 poses a significant challenge to producers. Ruminants are a large portion of the agricultural sector and therefore are responsible for a significant portion of the GHGs associated with livestock production. Therefore, it becomes essential to be able to modify ruminal environments away from the pathways that form CH_4 while maintaining the productivity, health, and welfare of each animal.

About 2-12% of energy that could have potentially been used for growth or production, is lost as CH_4 (Johnson and Johnson, 1995; Grainger and Beauchemin, 2011; Clemmons et al., 2019). Digestion is a relatively inefficient process as some amount of energy is always lost as heat. During digestion, highly complex polysaccharides, proteins, and other nutrients are being broken down into base components and doing so requires energy. In ruminants, CH_4 represents an additional form of energy loss that could have potentially been used for production (Johnson and Johnson, 1995).

Feed additives are used to interrupt these energy losses and in theory, make ruminants more efficient in producing milk, meat, or wool (Bell et al., 2016; Clemmons et al., 2019). However, the rumen environment is difficult to change. The majority of rumen microbes have yet to be identified and many known species are extremely difficult to culture outside the rumen (Russell, 2002; McSweeney and Mackie, 2020). This makes identifying their roles in digestion challenging and explains why past attempts to change the rumen environment in the long term and reduce enteric CH_4 have been unsuccessful (Hristov et al., 2013; Bell et al., 2016; Clemmons et al., 2019; van Gastelen et al., 2019).

There are still plenty of microbes that have been identified and classified into niches within the rumen, and offer insight into ruminant digestion, especially when it comes to how enteric CH_4 is formed (Russell, 2002). Archaeal methanogens from the phylum Euryarcheota produce CH_4. They exist symbiotically with bacteria and protozoa, cross-feeding on reducing equivalents and hydrogen (H_2) while helping to maintain the low redox environment in the rumen necessary for proper fermentation to proceed (de la Fuente et al., 2019; Matthews et al., 2019). While other metabolic pathways are also capable of H_2-consumption, like propionate production and biohydrogenation, methanogenesis remains the most energetically efficient option to remove H_2 from the rumen (Ungerfeld, 2020).

Produced under specific conditions, CH_4 is generated via anaerobic fermentation in the rumen and reticulum of ruminants (de la Fuente et al., 2019; McSweeney and Mackie, 2020). This is because methanogens prefer carbon-rich anaerobic environments that maintain a narrow neutral pH range (5.5-6.9) (Lyu et al., 2018; de la Fuente et al., 2019; McSweeney and Mackie, 2020). Through the process of rumination, complex carbohydrate structures like polysaccharides are broken down to simple sugars (McSweeney and Mackie, 2020). Further digestion transforms those

sugars into volatile fatty acids (VFAs) that will be absorbed through the rumen wall papillae and transported to the liver to undergo gluconeogenesis (McSweeney and Mackie, 2020). Methanogens gain energy from methanogenesis and are limited by the types of substrates that can be used to form CH_4 (Lyu et al., 2018; de la Fuente et al., 2019).

There are three main biochemical pathways capable of forming CH_4: hydrogenotrophic, methylotrophic, and acetoclastic (Lyu et al., 2018; Morgavi et al., 2020; Shima et al., 2020). Hydrogenotrophic is the most prolific pathway for CH_4 formation in the rumen (Lyu et al., 2018; Berghuis et al., 2019; McSweeney and Mackie, 2020; Shima et al., 2020). Hydrogenotrophs reduce CO_2 to CH_4 in a series of steps through the reductive acetyl-CoA pathway, where H_2 is used as the electron donor (Shima et al., 2020).

Methylotrophic pathways, like hydrogenotrophic pathways, have CO_2 as the starting substrate that becomes CH_4. The main exception lies in the addition of methyl-compounds when Methyl-SCoM becomes CH_4, with compounds including methanol, monomethylamine, dimethylamine, trimethylamine, and methylthiol (McSweeney and Mackie, 2020). It is estimated that about 22% of methanogens in the rumen are capable of using methyl compounds to reduce CO_2 into CH_4, hereby making the second most common pathway through which CH_4 is produced.

The acetoclastic pathway for CH_4 formation is considered diminutive, as the microbes utilizing these pathways maintain a very small population within the rumen environment (McSweeney and Mackie, 2020). Simpler than the hydrogenotrophic and methylotrophic pathways, acetoclastic pathways use acetate as the starting substrate.

Acetate is a primary VFAs formed during digestion. It is one of the end products of glycolysis, wherein the microbes break down complex polysaccharides coming from ruminant diets until glucose to be further digested (Ungerfeld, 2020). The pyruvate generated from glucose

fermentation can then be transformed into one of two products: acetyl-CoA or lactate (Ungerfeld, 2020). Acetyl-CoA and lactate are then used to synthesize the three VFAs that are essential for gluconeogenesis and growth in ruminants, becoming acetate or butyrate in the case of acetyl-CoA or propionate in the case of lactate (Ungerfeld, 2020).

1.5 Existing Enteric Methane Interventions

Interventions aimed at reducing enteric CH_4 emissions have the potential to not only reduce gas production, but also provide more usable energy for the animal. When considering the efficacy of an intervention in reducing enteric CH_4 emissions, it is important to assess the units used to quantify reductions. There are three commonly used metrics: CH_4 production, CH_4 yield, and CH_4 intensity. Methane production refers to gross CH_4 production and is often presented in a g/animal/day basis, providing the most basic metric associated with daily CH_4 losses. It reflects the total CH_4 produced by an animal, without correcting for dry matter intake (DMI) or milk yield between animals (Fresco et al., 2023).

The other metrics are related to animal production. Daily enteric CH_4 emissions is influenced to how much an animal eats and the composition of that diet, making CH_4 yield (g CH_4/kg DMI) an important metric (van Gastelen et al., 2019). Changes to diets, for example by increasing forage digestibility and/or dietary concentrate inclusion, can decrease CH_4 yield (Olijhoek et al., 2018; van Gastelen et al., 2019). CH_4 intensity is a measure of CH_4 per unit product, like milk or meat (van Gastelen et al., 2019). This measurement relates directly to dilution of maintenance, which is a phenomenon where greater production efficiency leads to less CH_4 per unit produced (Capper and Cady, 2012; Hristov et al., 2013).

As it stands, there are many interventions designed to prevent the formation of enteric CH_4 via different modes of action (Hristov et al., 2013; van Gastelen et al., 2019; Beauchemin et al., 2020; Fouts et al., 2022). Each intervention has a different potential in its ability to reduce eructated CH_4, based on overall potency and/or efficacy. The following section will look briefly into nutritional and feed additive interventions, discussing the benefits and drawbacks of existing options.

1.5.1 Feeding Strategies

Changing diets, by adding/removing certain ingredients can impact enteric emissions. Ruminant diets are traditionally composed of a diverse collection of forages (e.g., legumes, hays and straws) and concentrates (e.g., grains). The three main feeding strategies associated with reducing enteric CH_4 include changing forage:concentrate ratios, improving forage quality, and increasing dietary lipid content.

The main benefits in changing dietary ratios are related to increased starch fermentation, which promotes propionate production, hereby acting as an alternative sink to rumen H_2 to compete with methanogenesis (Beauchemin et al., 2020; Ungerfeld, 2020). With an average 386 g/kg DM increase in concentrates, CH_4 yield decreased by 26% in beef cattle, 14% in dairy cattle, and 6% in sheep (van Gastelen et al., 2019; Fouts et al., 2022). Increasing concentrates also benefits the animal by helping to improve productivity through increasing fat deposition or greater milk production (Hristov et al., 2013; Beauchemin et al., 2020; Beauchemin et al., 2022).

However, adoption of increasing concentrates or even feeding concentrates at all is limited in extensive and/or pasture-based systems, as these animals predominantly rely on grazing. Furthermore, increasing concentrates in a diet increases dietary energy density, decreases

structural carbohydrates, increases rumen outflow rate, and lowers ruminal pH (van Gastelen et al., 2019). These factors impact availability of energy and can lead to metabolic issues like acidosis (Beauchemin et al., 2020).

Alternatively, the forage quality can be improved. Ensuring a greater ratio of non-fiber carbohydrates to neutral detergent fibers (NDF) and less lignified NDF improves forage quality and reduces CH_4 (Beauchemin et al., 2020). Forages with higher digestibility leads to greater DMI and a decreased CH_4 intensity, via dilution of maintenance, as better forage quality increases production efficiency (Hristov et al., 2013). Unlike changing rations to include more concentrates, better forage quality and digestibility is a feasible intervention for CH_4 reduction for intensive and extensive systems, by enhancing the amount of digestible energy available to animals (Fouts et al., 2022). Two meta-analyses found that a 25% increase in grass silage or herbage digestibility resulted in a 10% decrease in CH_4 yield and a 19% reduction in CH_4 intensity, but absolute CH_4 usually remained constant or increased due to greater DMI and increased organic matter fermentation in the rumen (Beauchemin et al., 2022; Fouts et al., 2022).

The inclusion of fat or lipids, in the form of oils or different seeds, is popular in ruminant diets and offers up another avenue for reducing enteric emissions. A less than 4% DM inclusion of dietary lipids can reduce CH_4 up to 20% (Grainger and Beauchemin, 2011; Beauchemin et al., 2020), although efficacy is influenced by fat type (Fouts et al., 2022). Lipids are a significant source of energy; therefore, can help balance diets that might not have sufficient energy to meet an animal's daily needs. However, inclusion of lipids beyond a certain level can negatively impact rumen performance by damaging the microbial population, depleting rumen function, and depressing milk fat production, (Beauchemin et al., 2020; Martin et al., 2023). In fact, reductions

in CH_4 associated with higher inclusion of dietary lipids was attributed to decreased diet digestibility (Beauchemin et al., 2007).

1.5.2 Anti-Methanogenic Feed Additives

Beyond direct changes to rations, alternative nutritional interventions include the use of anti-methanogenic feed additives. These compounds are derived from a wide variety of sources, including natural secondary plant compounds and synthetic enzymes (Fouts et al., 2022). Regardless of type, anti-methanogenic feed additives are designed to modify the rumen to reduce enteric emissions. This can be accomplished either by impacting the microbiome or the chemical pathway of methanogenesis directly (Fouts et al., 2022). There is a range of efficacy associated with both modes of action and the source of the additive often informs how it will affect the rumen.

Additives that directly impact methanogenesis tend to be very potent and are called rumen inhibitors. One such rumen inhibitor is 3-nitrooxypropanol (3-NOP), which reduces CH_4 by inactivating the methyl-coenzyme M reductase enzyme needed to catalyze the final oxidation step to form CH_4 (Hristov et al., 2013; Duin et al., 2016; Fouts et al., 2022). Feeding 3-NOP has shown varying levels of CH_4 reduction between species, with an average 25-32% reduction in CH_4 yield measured in lactating dairy cows (Beauchemin et al., 2020). Currently, there are no long-term studies using 3-NOP and the additive has yet to receive federal approval in the U.S. to be fed commercially.

Some species of seaweed similarly interrupt methanogenesis, due to the presence of bromoform (Fouts et al., 2022). The most potent seaweed is *Asparagopsis taxiformis*, a red macroalgae that is being commercially cultivated as an anti-methane feed (Lean et al., 2021; Fouts et al., 2022). *In vivo* studies showed a 36% reduction in CH_4 yield with no effects to DMI, average

daily gain, milk yield or milk components (Lean et al., 2021). However, bromoform has negative impacts on shelf life of meat and has been detected in milk (Beauchemin et al., 2022). Moreover, bromoform is categorized as human carcinogen especially as bromine (Gao et al., 2023), so its presence in milk could potentially be harmful if found in sufficiently high concentrations.

The last group of rumen inhibitors are electron receptors, including nitrates and sulfates. These naturally occurring compounds compete for electrons in the rumen, hereby diverting the flow of metabolic H_2 away from methanogenesis (Hristov et al., 2013; Ungerfeld, 2020; Fouts et al., 2022). Animal trials found that 20 g nitrates/kg DM decreased CH_4 by 16% in dairy cattle (Beauchemin et al., 2020; Beauchemin et al., 2022). Other studies found up to 22-50% reductions in CH_4 production when feeding nitrates to cattle (Hristov et al., 2013; van Gastelen et al., 2019). Feng et al. (2020) found that different CH_4 reduction outcomes for dairy versus beef cattle when feeding nitrates were explained by breed, dose, and nitrate type (i.e., slow-release nitrates) when assessing reduction potential. Feeding nitrates has currently shown no negative effect on milk yield, milk composition, DMI, or diet digestibility, but there are risks of nitrate poisoning if animals are not properly adapted, due to the formation of methemoglobin in the blood (Fouts et al., 2022).

The second group of anti-methane feed additives are called rumen modifiers. While they do not inhibit the biochemical pathway of methanogenesis, these additives change the rumen environment to prevent CH_4 formation. The main types of rumen modifiers include ionophores and secondary plant metabolites. Ionophores are polyether compounds that increase the cell membrane permeability of gram-positive bacteria (Beauchemin et al., 2022). Rumensin/Monensin are commercially available ionophores, although traditionally used to improve productivity. Monensin has been shown to decrease CH_4 yield by 3-8%, coupled with lower DMI and greater

milk yield, but potency is lost overtime (Grainger and Beauchemin, 2011). A recent systemic review of studies feeding Monensin identified an ideal dose of 19-26 mg Monensin/kg DMI was ideal to reducing enteric CH_4, declining by about 8.12-33.31 g/day/animal (Rezaei Ahvanooei et al., 2024).Overall, the effects of ionophores are somewhat inconsistent, and highly dependent on dose and basal diets (Hristov et al., 2013; Beauchemin et al., 2022).

On the other hand, secondary plant metabolites are compounds synthesized by plants that are traditionally used as safety mechanisms to prevent herbivory, like essential oils (EO), tannins, and saponins (Carrazco et al., 2020; Beauchemin et al., 2022). Response to the feeding of secondary plant metabolites is highly variable, often dependent on dose, and the chemical structure of the compound (Beauchemin et al., 2020; Beauchemin et al., 2022; Fouts et al., 2022). Feeding the commercial EO blend Agolin significantly decreased CH_4 intensity, without negatively impacting other aspects of animal performance (Carrazco et al., 2020). However, more recent research feeding Agolin saw no significant CH_4 reductions, but did note increased milk fat content when compared to control cows (Silvestre et al., 2023).

With tannins, the mode of action involves binding of digestive enzymes, preventing DM and protein digestion in the rumen (Beauchemin et al., 2022). Inclusion rates of 14.6 g tannins/kg DM saw 10% decrease in CH_4 production and 5.9% decrease in CH_4 yield, but increased fecal N due to decreased protein digestion (Fouts et al., 2022). The anti-methanogenic effect of saponins is due to inhibition of rumen protozoa and associated methanogens, as well as increased production of propionates in the rumen (Beauchemin et al., 2022). Studies saw reductions ranging from 6-27% in CH_4 yield when feeding saponins (Hristov et al., 2013). However, high doses of saponins are considered toxic to animals and therefore must be used carefully (Beauchemin et al., 2022).

1.6 Existing Manure Management Emissions Interventions

Dairy production has steadily increased in the last few decades, leading to a greater quantity of manure and urine being managed (Barth et al., 2008; Varma et al., 2021). Because digestion is an inefficient process, there is a certain amount of organic matter still present in manure after defecation. The decomposition of this organic matter—known as volatile solids—leads to the production of GHGs and VOCs (Petersen, 2018; Jiang et al., 2023). The volatile solid content of manure is an important part of models and equations developed to estimate gas production potential from manure (Leytem et al., 2017; IPCC, 2021).

Liquid manure management systems are ideal for the formation of GHGs, due to the anaerobic environment created by storing manure and urine in solution. Volatile solids and nitrogenous compounds in feces are fermented or undergo nitrification/denitrification, leading to the release of VOCs and GHGs (Meyer et al., 2019; Wattiaux et al., 2019; Marklein et al., 2021; Yang et al., 2022; Khairunisa et al., 2023).

Given manure management's contribution to GHG emissions, it requires interventions to reduce overall environmental impact and advance the sustainability of livestock production. A manure management system (MMS) is composed of three aspects that are interconnected: 1) manure handling, defined as the means through which a farmer collects/removes manure from animal housing and/or grazing areas; 2) manure storage, which includes the infrastructure needed to handle manure and urine in large volumes until it can be used; and 3) manure application, which is how a farmer applies or utilizes stored manure (EPA, 2022; Niles et al., 2022).

All three aspects of MMS have a wide variety of options. Handling ranges from minimal effort, like little to no handling on pasture, to demanding more complicated infrastructure of daily flushing/scraping. Manure storage is similarly complex, as this part of the system, as well as

application and handling, are interdependent and are often influenced by the economic capital available to a farmer, geographical limitations, policy, farm size, land availability, and investment costs (Niles and Wiltshire, 2019; Niles et al., 2022). A popular form of manure storage is a liquid anaerobic lagoon, as these are cost effective, allow for longer storage periods, help stabilize volatile compounds, and can handle large volumes of manure and urine (Petersen, 2018).

However, lagoons create the perfect environment for gas production, especially CH_4, paired with long retention time and large surface area, which results in a high rate of emissions (Petersen, 2018; Meyer et al., 2019; Hilgert et al., 2022). The volatile solids pass through a series of chemical reactions prior to the formation of CH_4, linked by four central hydrolytic, acidogenic, acetogenic, and methanogenic microbial phases (Hanafiah et al., 2021).

Current interventions aim to change how manure is managed in the hopes of decreasing associated emissions. Such options include solid separation and dry storage, aiming to eliminate anaerobic environments that lead to CH_4 formation from the volatile solids present in manure (El Mashad et al., 2023). The main alternative manure management systems are outlined below in further detail.

1.6.1 Anaerobic Digesters

A potent option for gaseous emission mitigation is the use of anaerobic digesters, which captures biogas as a source of energy. Digesters on dairy farms can by implemented in two ways—covering a pre-existing lagoon or constructing a new standalone digester. The digester maintains the anaerobic, carbon-rich environment necessary for the production of biogas—a mixture of CH_4 and CO_2—following the fermentation of volatile solids (Kupper et al., 2020).

The fermentation process that generates CH_4 within an anaerobic digester, as well as uncovered lagoons, emulates the biochemical process in the rumen. Like rumen fermentation, organic matter undergoes hydrolysis, i.e., breaking down large molecules into their respective monomers like amino acids or monosaccharides. The next phase is called acidogenic fermentation, with the digestive enzymes of acidogens (Reis et al., 2011; Li et al., 2019). At this time, monomers from hydrolysis are fermented into VFAs (i.e. butyrate, acetate, propionate) and other compounds like alcohols, H_2, and CO_2 (Li et al., 2019; D'Silva et al., 2021). Acetogens, a small group of homo-acetic bacteria, then utilize H_2 and CO_2 resulting from previous fermentation steps, to generate more acetate. Lastly, strictly anaerobic methanogens consume the byproducts of fermentation like acetate, formate, CO_2, H_2, and use methanogenesis to synthesize CH_4 (Li et al., 2019; D'Silva et al., 2021). Similar to processes in the rumen, the hydrogenotrophic pathway dominates methanogenesis (Cárdenas et al., 2021).

Anaerobic digesters combat emissions by covering lagoons and preventing biogas, which is about 40% CO_2 and 60% CH_4, from reaching the atmosphere and have become increasingly popular in regions like California (D'Silva et al., 2021; Marklein et al., 2021). Since 2014, anaerobic digesters have offset 21.02 MMT CO_2e in California following the installation of 114 digesters (CDFA, 2023a).

Seasonal fluctuations of ambient temperature also impact CH_4 losses when manure is stored outdoors. Longer storage periods have been shown to produce more CH_4, especially in temperatures greater than 15°C (Cárdenas et al., 2021). Similarly, NH_3 volatilization is impacted by temperature, given that the equilibrium between NH_4^+ and NH_3 is temperature dependent. Volatilization increases linearly with every 1°C increase in temperature (Yang et al., 2022). Unfortunately, ambient temperature is difficult to control and therefore is not a practical solution

to reducing emissions, although some tank digesters can be temperature controlled to influence biogas formation.

The relationship between CH_4 production and temperature is linked to the efficacy of anaerobic digesters within certain climatic regions. Because the production of biogas is dependent on warmer ambient temperatures, this is a risk that anaerobic digesters built in cooler regions will not be sufficiently productive to justify construction costs (Niles and Wiltshire, 2019). However, the warm climate in California makes it the ideal region for anaerobic digesters (Marklein et al., 2021).

Additionally, the generated biogas can be used as renewable natural gas fuel in machinery or burnt for electricity, helping to reduce dependency on fossil fuels (D'Silva et al., 2021). However, anaerobic digesters are not often able to be implemented on medium-to-small sized farms (<1,000 head) due to the associated start-up and maintenance costs (Niles and Wiltshire, 2019; Niles et al., 2022; El Mashad et al., 2023). The EPA reports only 221 operational dairy anaerobic digesters as of 2023 across the entire U.S. (EPA, 2023a). While there are financial aid programs available, sponsored by local government branches like the California Department of Food and Agriculture (CDFA, 2023a), adoption remains limited as not all farms that apply will qualify for aid and in other parts of the country, no such support exists.

1.6.2 Solids Separators

Another alternative system supported for its ability to reduce GHG emissions from manure is solids separation. This technique reduces the amount of solids deposited into lagoons, hereby reducing the organic load being managed within the system. It is estimated that about 81% of dairy farms in California's San Joaquin Valley use some form of solid separation (Meyer et al., 2019).

Methane formation is directly linked to volatile solid content of manure, so when solids are separated, this reduces hydrolysis, which is considered the rate limiting step in CH_4 formation in lagoons and anaerobic digestion, by limiting the availability of organic substrates that would otherwise lead to gas formation (Li et al., 2019; Kupper et al., 2020). Additionally, the solids could be composted to be used as bedding, which would help offset some on-farm costs associated with purchased bedding (Niles and Wiltshire, 2019).

Like anaerobic digestion, solids separators are still not the perfect system when it comes to mitigating the environmental impact of MMS. First of all, it depends on the construction of a separator and additional space on which to store the solids (El Mashad et al., 2023). While solid separation remains a more cost-effective option compared to anaerobic digestion, it is not a suitable option for farms that do not utilize lagoon manure storage, as otherwise manure and urine are handled and stored as slurry.

1.6.3 Scraping versus Flushing Manure

Flushing is commonly used for manure collection, using water in flushing barns to remove manure. However, the addition of water to excreta creates an anaerobic environment (El Mashad et al., 2023). Converting manure collection from flushing to scraping is one of the alternative systems being funded by the CDFA's Alternative Manure Management Program (AMMP), as handling dry manure has been shown to reduce GHG emissions (CDFA, 2023a; El Mashad et al., 2023). However, scraping manure rather than flushing results in severe pollution swapping between CH_4 and NH_3. A study comparing flushing versus scraping manure at different daily frequencies showed that while CH_4 emissions significantly decrease with scraping, NH_3 emissions increased by 152-175% (Ross et al., 2021). Without additional interventions to mitigate the

increased NH_3 volatilization associated with scraping, any benefits from reducing CH_4 are lost by pollution swapping.

There are plenty of other alternative MMS available that are ultimately designed to reduce air, land, and water pollution. However, not all the options are equally available, effective, or feasible for farmers given the diversity in MMS that exist within the United States. Furthermore, many of these mechanical interventions do little when it comes to addressing N pollution risks. To holistically address the environmental risks associated with MMS, all potential sources of pollution need to be simultaneously addressed. Recent research has shifted to focus on changing the manure entering the systems, rather than the system itself.

Increasing popularity in liquid-based manure handling has resulted in an increase in absolute emissions and emission intensity over time, predominantly due to CH_4, while N_2O emissions remained relatively stable, at about 0.03 MMT/year (Niles and Wiltshire, 2019; Beck et al., 2023). Depending on the unit, manure-based GHG emissions are a greater contributor to global warming compared to enteric fermentation (Beck et al., 2023). When comparing the standard GWP_{100} metric to the newer GWP*, the increasing emission rate for manure CH_4 made it a significantly larger contributor of warming equivalents compared to enteric CH_4, at 90.8 MMT CO_2-warming equivalents (CO_2-we) versus 89.2 MMT CO_2-we, respectively. Conversely, GWP_{100} presented enteric CH_4 as the greater contributor, by about 206%, when compared to manure CH_4 (Beck et al., 2023). This does not mean that CH_4 from manure is more damaging than enteric CH_4, but rather that enteric emissions have remained stable while manure CH_4 emissions have increased overtime and as a result of the timeframe used in the original analysis. The overall contribution of the two different sources of CH_4 calls into question metric use and when certain calculation methodologies are appropriate over others.

It also raises additional questions as to the measures used for mitigating GHG hotspots in the livestock industry. Further insight will be needed to determine if funding is being equally and adequately distributed across all areas to truly support scientific research and development. Or could it suggest that manure emissions have been somewhat overlooked and demand more critical assessments to continue advancing the entire production system toward more sustainable practices? Currently, such speculations fall outside the scope of this review.

1.7 Manure Treatment

While there are limitations associated with changing an entire MMS, research shows that changes made directly to the excreta leads to significant reductions to GHG and VOC emissions. Much of this research has focused on altering the chemical composition of manure and urine, hereby interrupting methanogenesis, either by disrupting the natural environment needed for gas formation or directly affecting the microbial population (Chadwick et al., 2011; Kupper et al., 2020; Cárdenas et al., 2021).

1.7.1 Acidification

One excreta treatment is acidification. A review by Kupper et al. (2020) found 70% and 96% reductions in NH_3 and CH_4, respectively, during the storage period following acidification. In fact, treating slurry with sulphuric acid (H_2SO_4) saw up to 99% long term reductions of CH_4 emissions across a range of ambient temperatures (Sokolov et al., 2021). Acidification is thought to be effective because it disrupts the neutral environmental preferred by microbes that make CH_4 and NH_3 (Kupper et al., 2020; Qu and Zhang, 2021). Lowering the pH destroys the negative redox environment needed for CH_4 formation, and shifts the equilibrium between NH_4^+ and NH_3 toward

NH_4^+ formation (Hristov et al., 2011; Qu and Zhang, 2021). The only gas seemingly unaffected by acidification was N_2O.

1.7.2 Manure Additives

Like feed additives, manure additives have been developed to reduce emissions from forming at the source. The main area of concern is during manure storage, when excreta is left to sit until application, use, or disposal, and depending on the storage type, this can lead to higher emissions rates (Niles and Wiltshire, 2019; EPA, 2022; Niles et al., 2022).

SOP Lagoon is a commercially available manure additive, meant to be added to slurry or lagoon water to reduce GHG and NH_3 emissions as well as odor (Peterson et al., 2020; Chiodini et al., 2023). SOP Lagoon is a calcium sulfate dihydrate compound, which is relatively abundant in nature and traditionally used to improve soil properties, and formulated via proprietary technology (Peterson et al., 2020). Initially tested in laboratory settings, a dose of 61.6 g SOP Lagoon/m^3 lagoon water significantly reduced CO_2, CH_4, NH_3, and N_2O emissions, and also reduced odor intensity (Peterson et al., 2020). A follow up commercial on-farm trial in Italy found 80% reductions in CH_4 and 75% reductions in CO_2 after two months of treatment compared to untreated liquid manure (Chiodini et al., 2023).

Eminex®, a calcium cyanamide ($CaCN_2$) compound traditionally used in pesticides and artificial fertilizers, has recently entered the European market (Holtkamp et al., 2023). Following a 26-week incubation within a closed anaerobic system, a one-time application of Eminex® significantly reduced GHG emissions in both dairy and swine slurry (Holtkamp et al., 2023). Total CH_4 and CO_2 were reduced up to 99% at a treatment rate of 2.07 g Eminex®/kg slurry in dairy cattle slurry and fattening swine slurry, and total N_2O was decreased up to 80% and 60% for swine

and dairy cattle slurry, respectively, when compared to a control receiving no Eminex® (Holtkamp et al., 2023). However, NH_3, across both slurry types, did not significantly decrease with treatment (Holtkamp et al., 2023). Currently, Eminex® has not been tested in liquid storage systems and thus requires additional investigation to determine if it is efficacious in other forms of manure.

There are other additives that have also been investigated, although these are not traditionally used to reduce manure CH_4 emissions. Cluett et al. (2020b) investigated the usage of AgrimestMix and Penergetic-g in liquid dairy manure in reducing CH_4 emissions. Both additives were applied at two different doses, 30 mg/L and 420 mg/L, and stored at two different ambient temperatures, 20ºC and 37ºC. However, neither of these compounds were found to be effective in preventing CH_4 production, regardless of temperature or dose (Cluett et al., 2020b).

1.8 The Manure Microbiome

To fully comprehend why MMS lead to GHGs and VOCs, one must understand the manure microbiome. Microbes are directly responsible for the formation of gases as byproducts of fermentation or to get rid of specific molecules within the manure to maintain the environmental conditions needed for fermentation to proceed (Barret et al., 2013; D'Silva et al., 2021). The rumen microbiome is similar, with methanogens existing symbiotically with bacteria and protozoa to maintain the neutral pH and negative redox environment required for organic matter fermentation to occur (Ungerfeld, 2018, 2020).

1.8.1 Core Manure Microbial Phyla

Dungan and Leytem (2014) collected manure from 30 farms in Idaho to establish a clone library for the manure microbiome. They identified five key phyla present in the greatest relative

abundance: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Synergistetes. The majority of clones from Bacteroidetes belonged to the *Bacteroides* genus and the majority of the Firmicutes clones belonged to the genus *Clostridium*. Genera from the phylum Proteobacteria were much more diverse and only a single genus was identified for both Actinobacteria and Synergistetes (Dungan and Leytem, 2014). They also identified a wide variety of methanogens, split between the classes Methanomicrobia and Methanobacteria. However, of the seven archaeal genera identified, six were not of rumen origin (Dungan and Leytem, 2014).

1.8.2 Rumen Microbiome versus Manure Microbiome

Other studies compared rumen microbial populations to manure microbial populations, expanding on the work of Dungan and Leytem (2014) that methanogens in manure versus the rumen had separate origins. Rumen fluid and manure samples were collected directly from Jersey cows (Ozbayram et al., 2018), rather than from manure storage areas like in the study by Dungan and Leytem (2014). The most abundant phyla identified in the rumen of these dairy cows were Bacteroidetes (54%), Fibrobacteres (12%), Firmicutes (10%), Lentisphaerae (8%), Proteobacteria (5%) and Tenericutes (4%). There was some overlap between the microbiome of the rumen versus the manure microbiome, the latter of which was dominated by the phyla Firmicutes (46%), Bacteroidetes (36%), Lentisphaerae (6%), Proteobacteria (5%), and Verrucomicrobia (2%) (Ozbayram et al., 2018). The authors stated that the similarity in composition, especially for Bacteroidetes and Firmicutes which maintained high relative abundance levels in the rumen and manure, confirmed that these microbes originated in the gastrointestinal tract and the changes in respective relative abundance reflected their movement through an animal's digestive tract (Ozbayram et al., 2018). These phyla differed from those identified by Dungan and Leytem (2014),

but this is likely because the manure microbiomes came from two different sources: straight from the cow versus a storage pit.

Beyond phyla, the most dominant families identified in manure samples were *Ruminococcaceae* and *Bacteroidaceae*, which are both cellulose fermenters and involved in the decomposition of solids present in manure (Ozbayram et al., 2018). As for the methanogen community, there were also some stark differences. The manure methanogenic community was predominantly found to be of the genus *Methanocorpusculum*, as compared to the dominant rumen methanogenic genera, *Methanobacterium*, *Methanobrevibacter*, and *Methanomicrobium* (Ozbayram et al., 2018). The methanogens from the rumen were predominantly identified as hydrogenotrophic methanogens, consuming H_2 and CO_2 to form CH_4. Manure methanogens are more diverse, including acetoclastic and methylotrophic methanogens (Ozbayram et al., 2018; Kurth et al., 2020), implying that the methanogen population in manure has more diverse metabolic capabilities.

1.8.3 Influence of Manure Management on the Microbiome

Aside from studying microbiome communities, it is also important to explore how MMS potentially influence the microbiome, which was explored by Pandey et al. (2018), García-Lozano et al. (2019), and Khairunisa et al. (2023). Manure is managed in different ways and how it is handled often influences how it is stored. National and regional surveys of dairy farmers have found that those employing flushing systems are more likely to utilize anaerobic lagoons and those scraping manure are more likely to have dry storage pits (Meyer et al., 2011; Niles et al., 2022).

It is unsurprising that such factors also influence the microbiome. When comparing lagoon water to slurry collected in California, Pandey et al. (2018) found that there was a distinct

separation between liquid manure and solid manure microbiomes, with liquid samples being dominated by species like *Acinetobacter* (9.9%), *Psychrobacter* (2.5%), and *Enterococcus* (2.5%), whereas solid samples were dominated by *Planifilum* (6.4%), *Acinetobacter* (6.0%), and *Flavobacteriaceae* (4.4%). There were also certain genera, like *Sulfuriomonas* that were unique to liquid samples and *Thermos* that were unique to solid samples (Pandey et al., 2018). They also noted that regardless of whether solid manure was composted or not, its microbial population remained similar to other solid manures compared to liquid manure. This confirmed their hypothesis that manure handling and storage have significant impacts of microbial populations.

Given that there is population differentiation between handling and storage approaches, García-Lozano et al. (2019) explored differences in sampling spots within the same storage area, focusing on an anaerobic digester fed with dairy manure. They found 1,445 operational taxonomic units (OTUs) within this digester, which clearly showed a diverse and complex ecosystem within the lagoon. Only six of these OTUs were shared between the five different sample spots being analyzed (García-Lozano et al., 2019). Of the OTUs, the dominant phyla were Firmicutes (33.9%), Proteobacteria (21.4%), Latescibacteria (6.8%), and Thermotogae (6.8%), which are similar to those identified by Dungan and Leytem (2014) and Ozbayram et al. (2018). This implies much like in rumen microbes, there exists a core community of microbes seen in all manure, regardless of external factors. Externalities like temperature, type of manure, and substrate availability likely influence the abundance of certain taxa across a range of environments within a lagoon.

For the five sampling spots, influent, beginning, middle, final, and effluent, the individual compositions were as follows: the dominant phyla in the influent were Firmicutes (46.3%), Proteobacteria (33.2%) and Spirochaetes (9.5%). These were the same dominant phyla in the beginning sampling spot; however, overall abundances varied (García-Lozano et al., 2019).

Differences in phyla were more prominent between the middle and final spots, where middle samples were dominated by Proteobacteria (27.3%), Thermotogae (16.8%), and Latescibacteria (13.2%), and the final spot samples also included Firmicutes (12.3%) as a dominant phylum. The effluent samples had Firmicutes (33.9%), Synergistetes (15.7%), and Proteobacteria (14.2%) as the dominant phyla, and additionally had substantially higher abundances of Synergistetes and Lentisphaerae (8.13%) compared to other sampling points (García-Lozano et al., 2019).

This study even went so far as to assess metabolic activity at the different sites and determined that while a wide range of activity, like nucleotide metabolism, energy metabolism, membrane transport, carbohydrate metabolism, and more, were present across all sampling sites, the same metabolic functions were notably less abundant at the influent site (García-Lozano et al., 2019). This was likely due to the constant influx of waste entering the digester at this spot, bringing with it new microbes that fulfilled different ecological niches. Those new microbes would have to be able to metabolically survive in the new environment and/or compete with existing microbial taxa to become established (Sukhum et al., 2021).

While García-Lozano et al. (2019) explored differential microbial populations within a single anaerobic digester, Khairunisa et al. (2023) explored microbial populations within two different manure pit storage types, one earthen and one concrete, in Virginia. They found that between the two pits, there was no significant difference in species richness, but the composition of the two populations differed. This was similar to the distinct differences in sampling spots noted by García-Lozano et al. (2019). They noted that not only location, but depth of sampling influenced the microbiome. For example, *Ruminococcaceae* and *Syntrophomonas* were differentially abundant near the surface versus the middle of the earthen pit, while between the middle and

bottom samples, there were five differentially abundant amplicon sequence variants (Khairunisa et al., 2023).

There was also a wide variety in species of the Euryarchaeota phylum, with five distinct genera of *Methanophilaceae*, *Methanomassiliioccaeae*, *Methanocorpusculum*, and *Methanoculleus* identified in the earthen pit, some of which also identified by Ozbayram et al. (2018). Conversely, *Methanosarcina* was comparatively more prominent in the concrete pit (Khairunisa et al., 2023). The relative abundance of methanogens between earthen pit and concrete pit also differed, at 7.7% and 5.9%, respectively. Overall, the hydrogenotrophic family, *Methanocorpusculaceae* made up 95% of Euryarcheota identified in both storage systems. However, unlike bacteria, relative abundance of methanogens did not differ significantly between sampling depths (Khairunisa et al., 2023).

There are a wide variety of factors that influence the microbiome present in manure, starting with the diets fed to the cattle, how manure is handled/collected, how it is stored, and ambient parameters like temperature (Pandey et al., 2018; García-Lozano et al., 2019). The origins of microbes can also influence population dynamics, whether from the environment or the digestive tract (Dungan and Leytem, 2014; Ozbayram et al., 2018). These influences also apply to methanogens, helping to explaining why manure stored in different forms have different gas production potentials (Khairunisa et al., 2023).

Therefore, it is likely that a manure additive's ability to reduce GHG and VOC emissions is directly linked to the microbiome, either through negatively impacting the environment needed for normal fermentation (e.g., decreasing or increasing pH), or by directly inhibiting methanogenesis and other biochemical pathways. Such research has yet to be conducted to thoroughly explore the mode of action of many manure additives and potential implications to the

manure microbiota. Such research would lend additional insight into how manure additives impact pathogenicity, as many microbes present in manure are considered opportunistic pathogens and cause food-borne illnesses (Sukhum et al., 2021).

2 DISSERATION SUMMARY

The first experimental chapter of this dissertation explores the U.S. milk production, calculating the carbon footprints of Holsteins versus Jerseys. These are the two most popular dairy breeds used for fluid milk, cheese, and other dairy products throughout the country. Their individual breed carbon footprints were last estimated on a national scale in 2009. A new assessment is needed to better reflect current practices, productivity levels, and breed specific changes, as well as new methodologies and recently published GWP values. All these factors will update the environmental performance of the two breeds and provide a more relevant and updated snapshot into the entire production chain.

The second and third experimental chapters focus on the efficacy of a new manure additive on mitigating GHGs and NH_3 emissions. There are many hotspots of GHG emissions associated with milk production, manure management is especially potent as it is a source of all three agricultural GHGs and NH_3, although the quantity of gases is dependent on the type of system being used. Manure additives are being explored as potential interventions to target these gases to continue offsetting emissions and preventing negative environmental impacts.

The second experimental chapter explores the effects of the manure additive Eminex® on the gaseous emissions from manure. Previous research applying this additive in a closed anaerobic system found significant reductions in GHG emissions. However, as the experiment system did not accurately represent a manure management system, further research was necessary. The

present experiment was conducted in two sources, looking at emissions from fresh dairy slurry and dairy lagoon water in the hopes of emulating a wide range of standard U.S. dairy manure management systems.

The third experimental chapter investigates the impact of Eminex® on the microbiome of fresh slurry and lagoon water in the hopes of understanding its mode of action and exploring why it performs so aggressively when reducing GHG and NH_3 emissions. This was assessment was conducted via shallow shotgun metagenomic sequencing across specific testing days to determine how the treatment changed the overall population makeup and the influence those changes had on emissions.

Chapter 2 - A Comparison of the Environmental Footprint of U.S. Jersey versus Holstein Milk Production in 2020

ABSTRACT

The aim of the present study was to update a previous environmental impact assessment comparing the carbon footprint of Cheddar cheese production by Holsteins versus Jerseys in 2009. To make the update more comparable between breeds, as Jersey milk is more suited to cheese production compared to Holstein milk, the functional unit of this analysis was changed to 1 million metric tons of ECM. A deterministic model was used to model the two populations, establishing 16 life groups per breed needed to meet the functional unit. Production metrics like milk yield and milk components were sourced from the Council of Dairy Cattle Breeding National Metrics database for the year 2020. A professional dairy nutritionist used the software AMTS.Cattle.Pro to design total mixed rations for each individual life stage and breed to meeting the energy and nutritional needs of each animal. Feed ingredients informed the background systems used to calculate land and water use, and associated feed emissions through economic allocation with emission factors sourced from ecoinvent 3.10. AMTS.Cattle.Pro also estimated daily nutrient and manure excretion, daily enteric CH_4, and voluntary water intake. Total greenhouse gas (GHG) emissions were converted to carbon dioxide equivalents (CO_2e) using AR6 global warming potential (GWP) values. From 2009 to 2020, milk yield increased for Holsteins and Jerseys by 29% and 26%, respectively. Certain key performance indicators like land use decreased overtime for Jerseys and Holsteins due to less feed intake associated with smaller populations. However, water use increased from 2009 to 2020 since irrigation was included in this assessment, but not in the original 2009 comparison. Overall, the carbon footprint for Jersey milk production versus Holstein milk production was 1.6 kg CO_2e/kg ECM and 1.8 kg CO_2e/kg ECM, respectively, which was higher than the carbon footprints from 2009, likely due to the different GWP values used. The carbon footprint for Jersey production was 89% of the Holstein carbon footprint, making it more

similar to Holsteins. Overall, both breeds made substantial advancements in productivity, helping to offset increases in resource consumption.

1 INTRODUCTION

Despite milk's essential role in human diets, dairy cattle have impacts on the environment due to the release of gaseous emissions and nutrient losses (EPA, 2022). Concerns about livestock's impact on the climate, air and water quality have sparked consumer, policymaker, and industry interest in quantifying the environmental impact of food production chains in the United States and around the globe (Beauchemin et al., 2020; Smith et al., 2021; van Selm et al., 2022).

In 2020, the U.S. emitted approximately 6 million metric tons (MMT) of GHG in CO_2e, 11.2% of which came from agriculture (EPA, 2022). The GHGs of interest in animal agriculture are methane (CH_4) and nitrous oxide (N_2O). Enteric fermentation from ruminants like cattle, and manure management are responsible for 27% and 9% of U.S. CH_4 emissions, respectively (EPA, 2022). As for N_2O, 74% comes from agricultural land management practices and 5% from manure management (EPA, 2022). These gases are of special interest because of their high warming potential. Compared to CO_2, CH_4 is 27.2-29.8 times more potent, meaning it captures 27.2-29.8 times more heat compared to a single molecule of CO_2 (IPCC, 2021). Furthermore, N_2O is 273 times more potent compared to CO_2 (IPCC, 2021).

With its contribution to GHG emissions, the U.S. dairy sector has been thoroughly analyzed to measure its impact (Thoma et al., 2013b; Thoma et al., 2013c; Capper and Cady, 2020; Rotz et al., 2021). Such studies helped establish carbon footprint baselines for the sector, allowing the industry to measure how the dairy sector has reduced its impact over time. These studies estimated the carbon footprint of the U.S. dairy industry ranged from 0.65 to 1.70 kg CO_2e/kg fat

and protein corrected milk (FPCM) over the last several years (Thoma et al., 2013b; Thoma et al., 2013c; Capper and Cady, 2020; Rotz et al., 2021). However, these studies looked at the U.S. dairy industry as a whole and did not consider breed specific production differences.

The U.S. dairy industry is composed of several different dairy cattle breeds, but the two most predominant breeds are Jersey and Holstein (CDCB, 2022). Jersey and Holstein cows each occupy a unique niche within the dairy sector for various reasons. One of the most notable reasons is the size difference between cattle. Holstein cows are on average 181.4 kg heavier than Jersey cows (Prendiville et al., 2011; Capper and Cady, 2012; Uddin et al., 2021). Holstein cows also have greater average annual milk yield and dominate the fluid milk industry (Capper and Cady, 2012). Average daily milk yield is about 38.3 kg milk/d and 26.4 kg milk/d for Holstein and Jersey cows, respectively (CDCB, 2022).

On the other hand, Jerseys are known for their high concentration of fat and protein milk components, meaning their milk is better suited for dairy products such as cheese and ice cream, with an average percent milk fat and milk protein content of 4.89 and 3.70, respectively (Capper and Cady, 2012; CDCB, 2022). These breed differences result in disparities of resource use and overall environmental impact for Jerseys compared to Holsteins, as previously shown by Capper and Cady (2012) and Uddin et al. (2021).

Capper and Cady (2012) assessed the environmental impact of the Jersey versus Holstein populations' milk production using a functional unit (FU) of 500,000 tonnes of Cheddar cheese. The authors found that Jersey cows had a cheese yield of 0.125 versus 0.101 kg cheese/kg milk for Holstein cows. The difference in cheese yield per breed resulted in a difference in resource use. Across almost all characteristics of interest, the Holstein population required more land and water and had a larger carbon footprint compared to the Jersey population (Capper and Cady, 2012).

They also showed that the Jersey breed population required more animals because of lower milk production per cow to reach the 500,000 tonnes of Cheddar cheese. However, the smaller total body mass of the Jersey population resulted in lower maintenance requirements for both the entire population as well as individual animals (Capper and Cady, 2012).

A second study to investigate breed specific carbon footprints was conducted by Uddin et al. (2021), where the carbon footprints of Holstein versus Jersey cows were assessed across four different diets of varying forage levels. One major difference between Uddin et al. (2021) and Capper and Cady (2012) was the scope of the study. While the Capper and Cady (2012) study was cradle-to-farmgate in scope, meaning processes beyond the farmgate were not included in the analysis, the Uddin et al. (2021) analysis included end of life processing.

Due to the inherent differences in size, milk yield, and milk components for the two breeds, it is essential to quantify the environmental impact for Holstein and Jersey populations. The aim of the present study was to reassess the environmental footprint of the Jersey versus Holstein dairy breed from the Capper and Cady (2012) study and the effect of changes in breed characteristics over the intervening 11 years. The comparison will allow the genetic, feed quality and nutrition, production, and husbandry improvements in the last decade to be acknowledged, refined, and continued.

2 MATERIALS AND METHODS

The overall major steps in the methodology for the present study were similar to the Capper and Cady (2012) study. However, within each step there were significant changes to improve the accuracy of the estimates. These included but were not limited to a change in the FU, sources of data, and updated internal and external algorithms. It is therefore suggested that while comparisons

within years between breeds are appropriate for both studies, comparing between the two studies should be made with caution, to avoid speculation as to the cause for resulting differences.

2.1 Goal and Scope

The goal of the present study was to measure the environmental impact of milk production of the U.S. Jersey versus Holstein dairy populations on an energy corrected milk (ECM) basis: ECM = [0.25x milk kg] + [12.2x fat kg] + [7.7 x protein kg] (Tyrrell and Reid, 1965). The scope of the present study focused on the U.S. dairy herd population for the two dairy breeds. The milk production system was analyzed as the foreground system, for which the environmental impact including GHG emissions, land use, and water use was quantified. Background systems included crop production and the necessary flows supporting crop cultivation.

2.2 Functional Unit and Reference Flows

The FU for the present study was a major difference from the Capper and Cady (2012) study. Whereas the earlier study used a FU of 500,000 tonnes of Cheddar cheese, the FU for the present study was one million metric tonnes (MMT) of saleable ECM. The use of ECM is a more commonly used milk metric to assess environmental impact, compared to Cheddar cheese. Furthermore, given the inherent differences in milk composition between Jersey versus Holstein breeds due to component density, using a fluid milk basis was a more straightforward comparison (Uddin et al., 2021).

2.3 System Boundary

The system boundary for the present study was cradle-to-farm gate. It included the background systems of feed production, transportation, and associated resources (i.e., water, fertilizers, pesticides, land, etc.) needed to support the two dairy populations. Seed production and subsequent transport to crop areas were excluded because they contributed less than 1% of total emission (Landis et al., 2007; Werth et al., 2021). For on-farm processes, the analysis included feeding, milking, and milk storage and refrigeration.

2.4 Allocation

With byproducts often produced in large quantities following the processing of grains and other foodstuffs and commonly used as feedstuffs for dairy cattle, the present study used economic allocation to assess the production of those byproducts. Mass allocation would likely attribute an unfair share of emissions to the byproduct feeds. Biophysical allocation was used to account for dairy animals leaving the dairy production chain for meat. Using the equation from IDF (2015), we assumed that all mature cows removed from herds, minus an 5% of cows that died prematurely (CDCB, 2022), were sold for meat. Carcass weights for mature Jersey and Holstein cows were sourced from Berry et al. (2018) and Coyne et al. (2019), respectively. For the Holstein and Jersey breeds, the allocation factor to milk was 0.951 and 0.945, respectively. Heifers removed from dairy populations were assumed to be sold to other farms for dairy purposes, rather than raised for meat.

2.5 Model Assumptions

The deterministic model required a variety of assumptions to simplify parameterization and allow for as consistent a comparison between the two dairy breed populations as possible. First

of all, the central analysis for this model was based on an “animal-day”. An animal-day is a measure of the amount of each resource (e.g., feed, water, etc.) required to keep a single animal alive for one day. Cow-days (for mature cow stages) were calculated based on the estimated productive life of a dairy cow, sourced from the Council for Dairy Cattle Breeding (CDCB) National Performance Metrics database (CDCB, 2022).

The model also assumed no seasonality throughout the year, meaning no seasonal differences in crop availability or milk production. We also assumed thermoneutrality for all animals, defined as the metabolic state of an animal in an ambient temperature where it did not need to generate or lose heat (Vialard and Olivier, 2020). For dairy cattle, this temperature has been established to be between 7-25° Celsius (NASEM, 2021).

For animal characteristics, the model assumed a standard 60-d dry period (per communications with the CDCB) and 5% lactose content in milk for both breeds due to limited data availability. For reproduction, it was assumed that 75% of all cows and heifers were bred via artificial insemination, rather than natural cover. The model also assumed all animals were kept in confined, intensive systems, with no organic production of milk or feeds, as confined systems make up the majority of U.S. dairy farms (Rotz et al., 2021). To account for additional on-farm water usage, aside from water consumed by animals, an additional fixed 28.4 L per lactating cow used for milking equipment sanitation, the same values used by Capper and Cady (2012) and adapted from Holter and Urban (1992).

Lastly, because the FU for the present study stipulated saleable milk, it assumed 5 days of milk lost to colostrum production and 3 days of milk lost due to mastitis treatment. Mastitis incidence was estimated with CDCB data based on somatic cell counts, using 500,000 cells/mL as

the threshold to indicate an infection (CDCB, 2022). These above assumptions were identical to those made by Capper and Cady (2012).

2.6 Modeled Dairy Populations

The deterministic modeled established two simulated dairy populations, one for the Holstein breed and one for the Jersey breed to estimate the environmental impacts of each breed in the U.S. Performance parameters established the population needed to produce one MMT of saleable ECM (see Table 2.1) were sourced from National Performance Metrics data from CDCB (2022) for the year 2020. The present study data source differed from Capper and Cady (2012), where data was sourced from DRMS (Raleigh Dairy Processing Center).

Table 2.1 Jersey and Holstein cattle specific performance metrics

Performance Metrics	2009 Jersey[1]	2020 Jersey[2]	2009 Holstein[1]	2020 Holstein[2]
Total ECM, MT	1,000,000	1,000,000	1,000,000	1,000,000
Total milk, MT	892,459	884,171	1,050,089	1,038,836
Lactating cows	117,271	106,548	98,764	88,152
Total animal population	241,526	193,365	209,742	165,111
Daily milk yield, (kg)	20.9	26.4	29.1	37.6
ECM milk yield, (kg)	23.4	29.9	27.7	36.2
Milk fat, %	4.80	4.89	3.80	3.86
Milk protein, %	3.70	3.70	3.10	3.13
Calving interval, (mo)	13.7	13.2	14.1	12.9
Dry period, (d)	60	60	60	60
Cull rate, %	30.0	37.6	34.5	35.9
Number of lactations	3.00	2.42	2.54	2.44
Age at first calving, (mo)	25.3	22.3	26.1	24.0

[1]Adapted from Table 1 in Capper and Cady (2012)
[2]Data for 2020, sourced from the CDCB database, accessed May 2022.

Table 2.2 Body weight and performance data per feeding group within the model for Holstein and Jersey for 2020 assessment

Feeding groups	Jersey 2020			Holstein 2020		
	BW	Milk yield	ADG	BW	Milk yield	ADG
Heifers						
Weaned	167	--	0.589	227	--	0.861
Bred	327	--	0.453	473	--	0.680
Calving	392	--	0.408	568	--	0.544
Primiparous cows						
Colostrum	435	19.9	--	566	30.4	--
Transition	435	19.9	--	566	30.4	--
13.6 – 27.1 kg/d	435	21.1	--	566	26.3	--
27.2 – 40.7 kg/d	435	27.8	--	566	33.5	--
Multiparous cows						
Colostrum	512	28.7	--	680	40.2	--
Transition	512	28.7	--	680	40.2	--
13.6 – 27.1 kg/d	512	20.4	--	680	23.9	--
27.2 – 40.7 kg/d	512	33.3	--	680	34.2	--
40.8 – 54.3 kg/d	512	--	--	680	45.4	--
>54.3 kg/d	512	--	--	680	--	--
Dry cows						
Close up	566	--	--	752	--	--
Far off	544	--	--	725	--	--
Bulls						
Replacement	272	--	0.680	362	--	0.816
Mature	680	--	--	907	--	--

BW = body weight, kg; ADG = average daily gain, kg/d; milk yield = kg/d

Close up dry cows were defined as those with less than 21 days from calving and far off cry cows were more than 21 days from calving. Body weights for mature cows and bulls provided by breed associations and heifer body weights adapted from PSU (2017).

2.7 Feeding System Inventory

Rations were designed for the sixteen feeding groups in each dairy population. Characteristics for each feeding group are listed in Table 2. Each ration was designed to meet the energy and protein requirements for each group, based on life stage, physiological characteristics, and breed. Rations for lactating dairy cows were based on dry matter intake (DMI), days in milk (DIM), body weight (BW), and milk production levels, whereas heifer diets were based on average daily gain (ADG) and BW, similar to bull rations. It was also assumed that all dairy animals were fed dairy total mixed rations (TMR), as was standard for most U.S. commercial farms. Diets were designed to represent a standard dairy TMR, without additives like Rumensin® and other additives (e.g., Agolin®). The diets were meant to be representative of the typical dairy farm in the U.S to inventory primary feed utilization and dry matter intakes. Total feed utilization was also modified based on losses from shrink. This assessment used three different shrink values based on the type of feed at 15% for silages, 7% for concentrates, and 7.75% for forages (Greene, 2013; Hafner et al., 2013; Schroeder, 2013).

A professional dairy nutritionist formulated diets for each feeding group using AMTS Cattle Professional (AMTS.Cattle.Pro, Version 4.7, Ithaca, NY, USA) software, based on the Cornell Net Carbohydrate and Protein (CNCPS) system for the present study (Foundation, 2018). This differed from the DairyPro software originally used by Capper and Cady (2012). However, DairyPro was also a CNCPS-based software. Both represented the most updated available software for ration formulation at the time of the present study.

The current AMTS.Cattle.Pro, software also provided outputs of daily voluntary water intake (L/d), daily enteric CH_4 (L/d), manure and urine production (lbs/d), and nitrogen and phosphorus (N and P, g/d) nutrient losses (Van Amburgh et al., 2019). These outputs were used to

calculate on-farm water consumption by animals, on-farm enteric CH_4 emissions, and on-farm manure management emissions. Each feedstuff ingredient, except for micronutrient mixes, were used to quantify crop land usage. Data on average crop yields per feedstuff were sourced from the USDA NASS (2022) from 2017 to 2021 to calculate a regressed mean yield for 2020. Total feed required from the sixteen feeding groups was summed up and divided by the weighted yield by feed to estimate the acreage needed to produce that amount of feed. This methodology was repeated for all individual feed ingredients for both breed populations similar to Capper and Cady (2012).

In contrast to Capper and Cady (2012), the present study included micronutrient mixes, like minerals, vitamins, fats, and amino acids, in the overall calculations for total feed intake and feed emission intensity. Feed emissions were calculated using the life cycle inventory database, ecoinvent 3.10 (Ecoinvent, 2023). Emission factors used of economic allocation and AR6 global warming potential (GWP) values to match the scope, assumptions, and boundaries of the present study.

2.8 Environmental Impact Assessment

Emissions from the two simulated dairy populations were assessed with three impact categories: GHG emissions as kg of CO_2e/FU, land use as hectares per FU, and water use as L per FU. Land use focused on crop production associated with feed production. Water use included daily water consumption by animals, sanitation water, and irrigation water. The present report used AR6 GWP values of 1, 27.2, and 273 for CO_2, CH_4, and N_2O respectively, assuming a 100-year timeframe (IPCC, 2021), which differed from the AR5 GWP (IPCC, 2006) values used by Capper and Cady (2012).

Enteric CH_4 emissions rates were obtained from AMTS.Cattle.Pro and manure CH_4 and N_2O emissions were calculated using IPCC (2021) equations. The IPCC (2021) equations differentiate by animal life stage and manure management system, due to differences in emission potential based on management style. Therefore, the present study assumed equal distribution of Holstein and Jersey animals within their respective populations across the seven most popular manure management systems in the U.S., as per EPA (2022) definitions. This assumption was made due to lack of data available on animal distribution within specific manure management types.

Additionally, N_2O values only included direct emissions, as indirect emissions coming from volatilization and leaching were not accounted for in Capper and Cady (2012). Using percentages, it was also possible to show some relative differences between the two studies. To make the comparison, results from Capper and Cady (2012) were converted from the original FU of Cheddar cheese to match the current FU of salable ECM. Results from Capper and Cady (2012) were converted to ECM by taking the gross milk production needed for 500,000 tonnes of Cheddar cheese and determining how much of that milk was needed to meet one MMT of saleable ECM. For Jerseys, this was about 22.4% and for Holsteins, 21.2%. All outcomes were subsequently converted with the above proportions to transform the data from Cheddar cheese to ECM.

3 RESULTS AND DISCUSSION

The present study compared the environmental impact and productivity of the Jersey and Holstein milk production in 2020 as an average of the population, rather than comparing individual animal groups. Direct comparisons between 2009 and 2020 need to be made with caution regarding

speculation for the cause of some changes over time. New values for the key performance indicators (KPI) in the present study are outlined in Table 2.3.

Results for the conversion from Cheddar cheese to ECM are presented in Figure 2.1. While the four breed trait performance ratios changed somewhat, the ratios for the seven environmental KPIs changed very little, indicating that the effect of using saleable ECM instead of Cheddar cheese had little effect on the ultimate outcome of the 2009 comparison between breeds. It was also of interest to demonstrate how Jerseys performed relative to Holsteins in 2020. Figure 2.2 shows Jersey performance, as indexed to Holstein performance, the latter of which was set at 100% for 2020. Anything above or below 100% indicates a difference between the performance of the two dairy breeds. Relative differences from Capper and Cady (2012) are also provided in Figure 2.2.

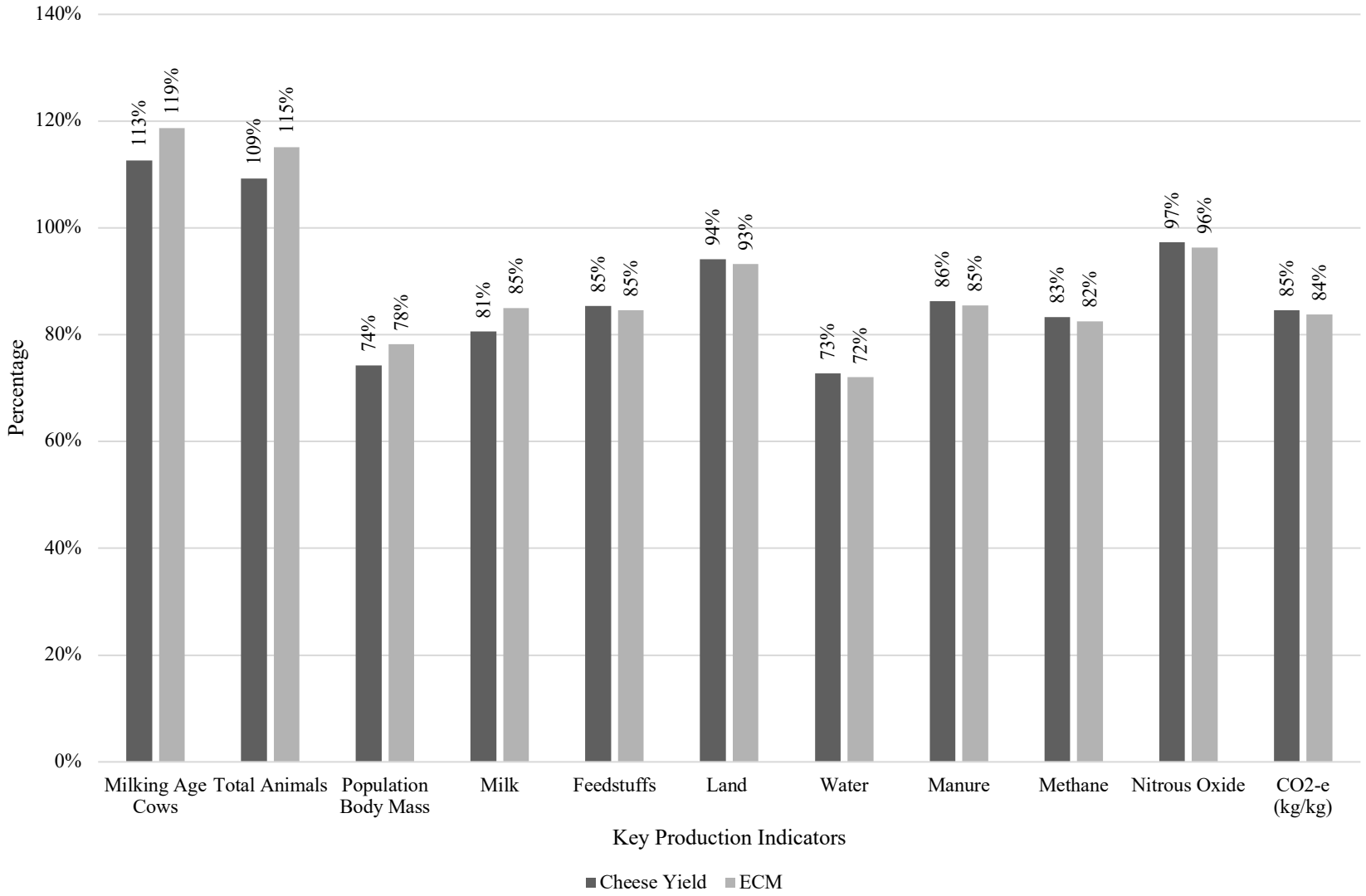

Figure 2.1 The performance of the Jersey population as a proportion of the Holstein population across key performance indicators when changing the functional unit from cheese yield to ECM.

3.1 Dairy Populations and Milk Production

Overall, there were some interesting changes over time noticed in both dairy breeds. First, Jersey animals were heavier in 2020 versus 2009. Throughout all the Jersey population life stages included in the model, reported BW increased (Table 2.2). For example, in 2020 compared to 2009, multiparous Jersey cows and dry Jersey cows were about 13% and 25% heavier, respectively. As these factors informed AMTS.Cattle.Pro when determining DMI, changes were reflected in model outputs and other results. As shown in Table 3, there were reductions in total populations and total lactating cows for both Holsteins and Jerseys. The decrease resulted from improvements to milk yield in both breeds, thereby resulting in a smaller number of cows needed to produce one MMT of saleable ECM over time. However, the Jersey breed had a smaller drop in lactating cow numbers (-9.1% compared to -10.7%, for the Holstein breed; Table 2.3). This might be explained by the Holstein cows showing a greater increased overall daily ECM yield (30% vs. 27%, respectively; Table 2.1) from 2009 to 2020.

For total populations, the current study, like in the work authored by Capper and Cady (2012), showed that in 2020, the Jersey breed compared to the Holstein breed, still required a larger support population of non-productive animals like heifers and dry cows. For the total population in the present study, Holsteins had a larger drop in total animals from 2009 to 2020, versus Jerseys (Table 2.3). However, it should also be noted that as a percent of the total population, the Jersey population had a smaller percentage of heifers primarily due to a younger age at first calving.

Both breeds saw increases in percent milk fat, but only Holstein cows were reported to have increases in milk protein (Table 2.1) over time. Because correcting raw milk production to ECM uses standardized milk protein and milk fat values (Tyrrell and Reid, 1965), the increasing amount of milk fat in both breeds would explain the drop in raw milk required to meet the FU of

the current study between 2009 and 2020 for both breeds. Selecting for higher yield would depress milk components. These results could also be due to the change in data sources from DRMS to CDCB.

Due to the higher component content of Jersey versus Holstein cow milk (Table 2.1), the former did not need to produce as much milk (Table 2.3). However, because Jersey versus Holstein average ECM per cow was lower, they needed approximately 18,000 more animals to reach the goal of one MMT of salable ECM (Table 2.3). However, when population animal body mass, milk production and component levels, were considered together, the 2020 Jersey population ate less feed overall and produced less excreta (i.e., feces and urine; Table 2.3).

Table 2.3 Annual resource consumption per dairy breed population

Parameters	2020 Jersey	2020 Holstein
Feed consumed, MT	890,668	969,891
Feed loss, MT	83,956	94,416
Feedstuffs[1], MT	974,625	1,064,307
Land, ha	363,353	472,271
On-farm water[4], x 10^9 L	4.67	4.65
Crop irrigation water x 10^9 L	1,403	1,666
Total water, x 10^9 L	1,408	1,671
Nitrogen excretion, MT	15,383	19,164
Phosphorus excretion, MT	2,039	2,426
Excreta[2], MT	2,332,506	2,646,096
Enteric methane, MT	15,383	16,020
Manure methane, MT	2,796	3,922
Total methane, MT	18,180	19,943
Nitrous oxide, MT	653	680
Carbon footprint[3], MT	1,623,632	1,823,302

All values, unless stated otherwise in the table, are in metric tonnes
[1]Feed values given on a dry matter basis
[2]Excreta refers to manure and urine together
[3]Total carbon footprint includes carbon dioxide, methane, and nitrous oxide emissions given in CO_2e
[4]On-farm water includes water intake per animals and sanitation water associated with milking procedures

3.2 Feedstuffs

Over time, there were changes to the eleven KPIs of interest in the present study, compared to Capper and Cady (2012). On an individual animal basis, Jerseys ate more in 2020 than in 2009. In 2009, Jerseys ate 15% less feed than Holsteins and in 2020, Jerseys ate only 7% less feed than Holsteins (Figure 2.2). Changes to DMI due to AMTS.Cattle.Pro improvements from software updates could potentially explain some of the differences seen between feed intake in 2009 and 2020 for both breeds. Even so, as AMTS.Cattle.Pro is confounded by year due to regular updates, it would be difficult to tell how much is due to software changes

The greater BW and milk yield seen in Jerseys in 2020 versus 2009, better explained the feed intake increase (Table 2.1 and 2.2). Milk yield and feed intake have been found to be positively correlated, meaning that when daily milk yield increased, so did feed intake and vice versa (Loker et al., 2012). Given larger animals have a greater maintenance energy requirement (Speakman, 2005), it would make sense that larger Jerseys in 2020 ate more than their 2009 counterparts. Tradeoffs like this will be important to consider in the future, as larger animals tend to have higher environmental impacts (Capper and Cady, 2012; Uddin et al., 2021).

By producing more milk, both breeds reduced the percentage of their feed intake that went to their maintenance requirement by spreading it over increased units of production. This meant that both breeds became more efficient over time. They were better at converting pounds of feed into pounds of milk or gain, thereby requiring less feed overall to be productive (Berry and Crowley, 2013). Gains in efficiency are one of the means through which milk production can continuously reduce its overall environmental impact via dilution of maintenance (DOM) (Bauman et al., 1999; Capper and Cady, 2012; Arndt et al., 2015; Capper and Cady, 2020). A lower gross

volume of feed translated into less land needed to meet the cumulative amount of feed needed to support the Jersey population in 2020.

Figure 2.2 demonstrated that overall, the Jersey breed continued to be more efficient with respect to feed required and carbon footprint. However, in two important categories, feed consumption and enteric CH_4, Holsteins closed the gap most likely for two reasons. First, Holstein daily ECM production increased by 30% from 2009 to 2020, whereas Jerseys daily ECM production increased by 27% over the same period, which was not only a percentage increase, but a real increase in ECM production as well, because Holstein started at higher production levels (Table 2.1).

The larger increase in ECM production by Holsteins was primarily due to a greater increase in daily milk yield than for Jerseys (8.44 kg vs. 6.43 kg respectively). Secondly, based on information from the respective breed associations, only Jersey animals increased in size from 2009 to 2020. This meant that the feed required for maintenance increased in the Jersey population but not in the Holstein population. The amount of CH_4 emitted is directly proportional to the amount of feed required, thus leading to the increase in eructated CH_4 emissions from Jersey over time (Figure 2.2).

3.3 Nutrient Losses and Excreta

As for nutrient losses in 2020 versus 2009, the Jersey population saw reductions in losses of N and P, while Holsteins also saw reductions in N losses, but not in P. This resulted in both breeds' N losses becoming more similar, from 86% in 2009 to 88% in 2020 (Figure 2.2). The amount of N lost during an animal's productive life is influenced by milk N use efficiency, replacement rate, and production characteristics (Fokolos and Moorby, 2018).

The improvements to milk yield and milk protein content—for Holsteins, at least—could result in less N being lost in feces and urine. Fokolos and Moorby (2018) found that feeding strategy and dietary composition were important factors influencing N loss and retention, thereby meriting additional research into acceptable crude protein feeding levels and precision nutrition practices for the dairy industry.

Differences in the digestive capacities between Jersey versus Holstein animals could also explain differences in nutrient losses. However, studies comparing the two breeds did not find significant effects of breed on nutrient digestion, (Knowlton et al., 2010; Olijhoek et al., 2018; Uddin et al., 2021), with the notable exception of neutral detergent fibers (Olijhoek et al., 2018).

3.4 Water Use and Land Use

Land use also dropped for Jersey in 2020 to 77% of Holstein land use, compared to 93% in 2009 (Figure 2.2). The change was probably attributed to the decreased feed utilization because of the smaller Jersey population in 2020 compared to 2009. However, when comparing Jersey 2020 to 2009 performance, there was an increase in total land use (Table 2.3). Such differences could be attributed to the different diets formulated for the current study versus the older Capper and Cady (2012) study. Six primary ingredients were used in Capper and Cady (2012) study, whereas in the current study, there were nine primary ingredients in the dairy TMRs.

There were also trends showing increasing average yield for most of the concentrate feed ingredients, like soybean and corn, based on estimated 2020 yields. For example, soybean yield in 2009 was 44 bushels per acre and increased to 51 bushel per acre in 2020 (USDA NASS, 2022). Increasing yield per acre would mean that fewer acres of land would be needed to produce feed. However, certain forages showed the opposite trend when calculating the estimated yields, where

alfalfa yields dropped from 3.35 to 3.27 tons per acre from 2017 to 2020 (USDA NASS, 2022). The differences in yield across the different feed ingredients would contribute to the changes in land use.

Water use, on the other hand, increased for Jerseys from 2009 to 2020, as a proportion of Holstein water use (Figure 2.2) and on a gross water use basis (Table 2.3). Data for crop irrigation rates in the present study were obtained from the 2017 USDA Agricultural Census, which is the latest data available on irrigation. As 2020 was the year being assessed in the current study, and used USDA crop data from 2020, irrigation data from 2017 could potentially impact estimates (USDA, 2019; USDA NASS, 2022). USDA trends showed irrigation decreasing in certain areas of the country, like California, but increasing in others states like Nebraska (ERS, 2022).

Given the lack of regional differentiation within the present study, performing an assessment based on specific regions might lend better insights into overall water use in the dairy sector for the future. Additionally, irrigation water was not included in water use by Capper and Cady (2012), only water consumption and sanitation water for lactating cows. The inclusion of irrigation water explains why the gross total water use in 2020 was significantly higher compared to 2009 for both breeds.

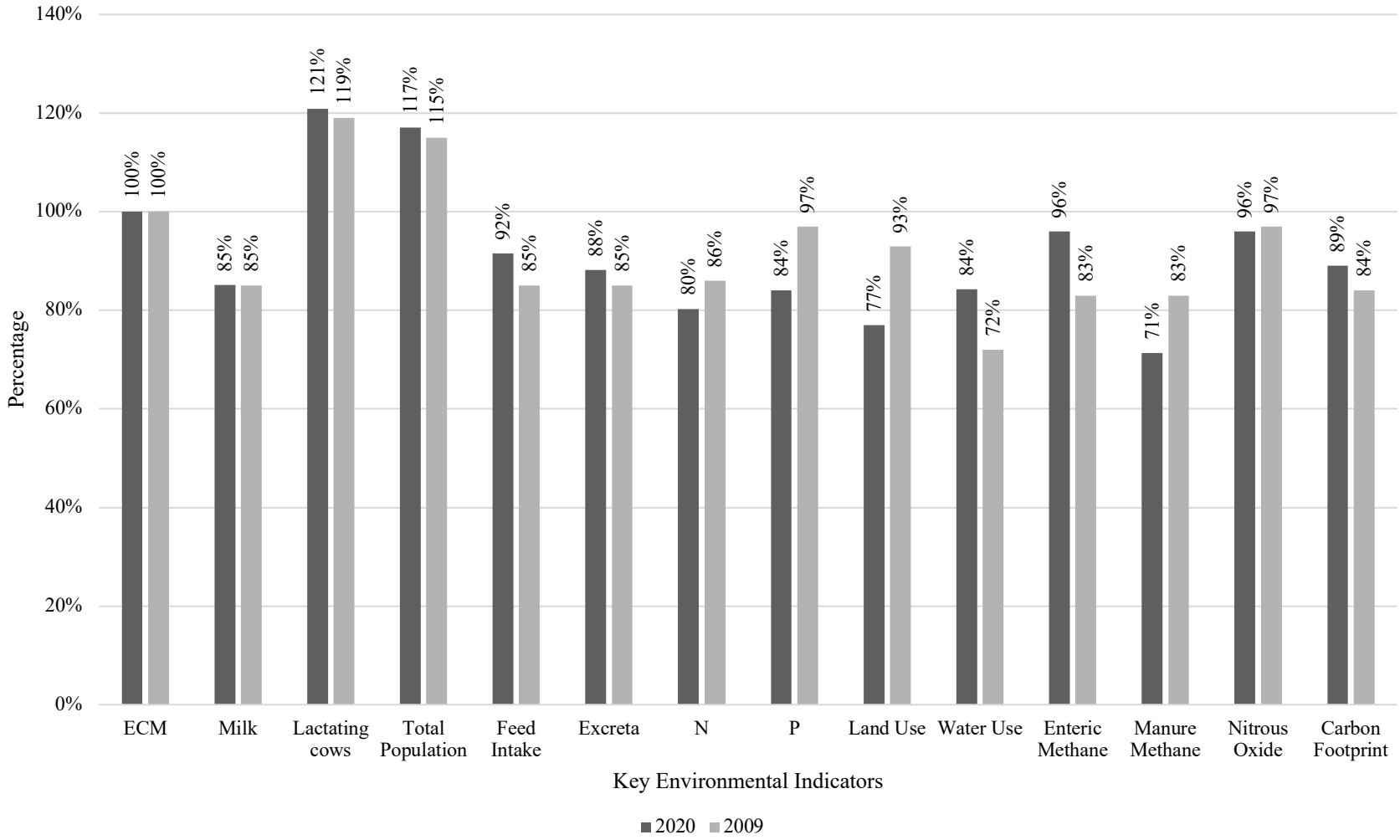

Figure 2.2 Key performance indicators for Jersey as a proportion of Holstein, comparing 2009 to 2020. Values were calculated by taking Jersey values from Table 3 and dividing them by the same Holstein values to determine the proportional performance. Any values above 100% indicate that Jerseys compared to Holsteins either require more of that resource (e.g., water) or are larger in number (e.g., total population).

3.5 Carbon Footprint of Holsteins and Jerseys

Due to lack of differentiation in sources of CH_4 and N_2O, significant updates to the model, differences in calculation methodologies, and data sources, and as stated earlier, direct comparisons between Capper and Cady (2012) results and the current study are not recommended. Even so, Table 2.3 offers some insights into how environmental KPIs have changed over time.

In the present study, the carbon footprint of the Jersey population was found to be 89% that of the Holstein population. In 2009, the carbon footprint for Jerseys was 84% that of Holsteins (Figure 2.2). With the advancements in breeding, both breeds now require fewer animals to produce the same quantity of milk. This efficiency has led to a reduction in their environmental impact compared to what it would have been without these improvements. A reduction in emission intensity is due to dilution of maintenance, a phenomenon highlighted by Capper and Cady (2012). It was defined as maintenance nutrient requirements being spread over more units of production, thereby lowering resource use and GHG emissions per unit milk (Bauman et al., 1999; Capper and Cady, 2012).

A decreased feed demand for these two populations benefited both breeds, as feed production tends to be a major source of emissions from N_2O and CO_2, due to the use of machinery, pesticides, and artificial fertilizers (Rotz et al., 2021) as well as CH_4 from enteric fermentation. It was also a major source of water consumption, as irrigation of purchased and homegrown feeds was found to account for over 60% of on-farm water use (Rotz et al., 2021).

Enteric CH_4 is highly correlated with DMI and fiber digestion (Johnson and Johnson, 1995). Highly fibrous, poorly digestible diets are often associated with higher enteric emissions, whereas high concentrate and highly digestible diets with lower enteric emissions (Johnson and Johnson, 1995; Beauchemin et al., 2020). With the higher feed intake seen across both breeds, it

could be possible to see a higher total CH_4 emissions (gross CH_4 emissions) in 2020, but lower emission intensity (units of CH_4 per unit milk), due to the increased daily milk yield. The present study did not consider any commercially available feed additives proven to reduce enteric CH_4 emissions (Carrazco et al., 2020). Performing another simulation with the inclusion of such additives merits further investigation as to the upstream and downstream impacts of these dietary interventions.

Manure was another major source of on-farm emissions. Manure emits both CH_4 and N_2O, varying greatly depending on manure handling, storage, and application (Uddin et al., 2020; EPA, 2022). In 2020, the Jersey population had fewer N_2O emissions compared to the Holstein population (Figure 2.2). This was possibly due to Jerseys, despite having a larger total population, still losing less N that could lead to nitrogenous emissions compared to the Holstein population.

The formation of N_2O is a complex process, requiring both aerobic and anaerobic conditions to allow for the nitrification-denitrification cycle to convert available forms of N like nitrites and ammonium, into gas (Broucek, 2020). However, it is notable that certain manure management systems, like solid storage and dry lots, are more prone to producing N_2O than others (IPCC, 2021). There has been a push to implement more, dry manure systems in major dairy regions of the U.S., to prevent the formation of CH_4 (CDFA, 2023a). However, preventing one type of emissions without considering them all could lead to pollution swapping, where the mitigation of one source of pollutants results in the increase of another (Stevens and Quinton, 2009; Quinton and Stevens, 2010).

Manure, depending on management type, could also generate varying quantities of CH_4 emissions due to microbes fermenting fecal organic matter (Uddin et al., 2020). Despite the increasing popularity of liquid-based manure management systems like open lagoons, such

systems can generate 4 to 20 times more CH_4 compared to dry systems (Petersen, 2018; Niles and Wiltshire, 2019; EPA, 2022). Differences in manure CH_4 between Jerseys versus Holsteins in the present study, was likely due to differences in population numbers and the total amount of manure being produced (Table 2.3).

Uddin et al. (2020) found that when comparing breed, diet, and time on CH_4 emissions, there were no significant effects of breed or diet found on CH_4 from manure. Additionally, manure CH_4 is influenced by a variety of environmental factors like precipitation and temperature (Niles and Wiltshire, 2019; Niles et al., 2022). As the model in the present study assumed thermoneutrality and no seasonality, those factors would not explain any differences in manure emissions between the two breeds in 2020.

The carbon footprint of the two breeds also differed from previous assessments of the entire U.S. milk production chain by Thoma et al. (2013b), Rotz et al. (2021), and Capper and Cady (2020). These differences were likely due to differences in scope, data sources, FU, and assumptions made in each study. A lack of standardization across life cycle assessments and modeling has led to discrepancies between studies and often makes direct comparisons difficult (Guinée et al., 2011; Baldini et al., 2015).

Thoma et al. (2013b) also assessed the influence of farm size within each region, while the present study focused on simulated herds, rather than specific farm sizes, which differentiates the assessment. Furthermore, Thoma et al. (2013b) used farm survey data from 2008, so there was the potential for scaling up error, which was avoided as the present study did not rely on survey data but rather database information for the entire U.S. population. There was also a difference in quantifying enteric CH_4, where Thoma et al. (2013b) used equations from Ellis et al. (2007) to

calculate enteric CH_4, whereas the present study used AMTS.Cattle.Pro (Van Amburgh et al., 2019).

There were certain similarities, as they also used the IDF (2015) methodology of allocating emissions/impacts from milk production to meat, based on culled cows, resulting in a ratio allocating 87% of emissions to milk, while the ratios for the current study were 94% for Jerseys and 95% for Holsteins. The differences in allocation factors were attributed to different assumptions on how many and which dairy animals would leave the herds, and these ratios would also impact results for both studies.

Another U.S. dairy sector life cycle assessment was performed by Rotz et al. (2021), using the Integrated Farm System Model (IFSM). As Rotz et al. (2021) also evaluated dairy production in 2020, it offered the closest comparable assessment of the U.S. milk production system to the current study. They found a wide variation in carbon footprints across the U.S., from 0.69 to 1.45 kg CO_2e/kg FPCM, with a final weighted mean of 1.01 CO_2e/kg FPCM (Rotz et al., 2021). When the calculated carbon footprints of the current study are converted to a per kg ECM basis, it is easier to compare to the findings of Rotz et al. (2021) to the present study given the similar FU and scope of the study.

Furthermore, like Thoma et al. (2013b), Rotz et al. (2021) performed a regional analysis, this time looking at the effects of different husbandry systems on environmental impact and used sampled data from herds. For the present study, the model worked with confined systems, so there were clear resource use differences, especially compared to smaller Amish systems, organic systems, and other grazing operations. However, Rotz et al. (2021) did confirm that confinement dairies were dominant across all regions of the U.S., supporting the assumptions made in the present study.

Another important difference was sources of data used in the present study versus Rotz et al. (2021). The latter, as mentioned, used IFSM to simulate crop production, feed use, animal production, and manure nutrients (Rotz et al., 2021). Those same inputs for the present study came from a variety of sources, like CDCB, USDA NASS, and AMTS.Cattle.Pro.

The last similar assessment was Capper and Cady (2020) which used the same deterministic model and the same FU as the present study but did not use breed-specific production and feed rations. Capper and Cady (2020) calculated a carbon footprint of 1.70 kg CO_2e/kg ECM, which fell between the carbon footprints for Jerseys and Holsteins in the present study. A direct comparison with the present study is more comparable, as it used the same deterministic model as the present study, but did not include breed specific performance parameters (Capper and Cady, 2020). Furthermore, the model has since been updated, resulting in computational differences between Capper and Cady (2020) and the present study. Therefore, like Thoma et al. (2013b) and Rotz et al. (2021), a direct comparison in outcomes remained difficult.

Ultimately, the carbon footprints from the present study are higher than Thoma et al. (2013b), Rotz et al. (2021), and Capper and Cady (2020) due to the difference in GWP values. The most recently updated GWP report differentiating between biogenic and anthropogenic CH_4 sources (IPCC, 2021). The AR6 GWP for CH_4 is higher (25 vs 27.2 and 29.8) and GWP for N_2O is lower (298 vs. 273), compared to AR4 GWPs. And AR6, GWP for CH_4 is lower (28 and 34 vs 27.2 and 29.8) and GWP for N_2O is higher (265 vs. 273), compared to AR5 over a 100-year timeframe (GHGP, 2016; IPCC, 2021). Currently, there are no known ways of converting carbon footprints to different GWP, per personal communication with Dr. Greg Thoma. Therefore, the carbon footprint of new assessment using AR6 GWP values will always have the potential to have higher carbon footprints even if gross gas production has decreased.

Moreover, the present study and Capper and Cady (2020) have substantially higher carbon footprints compared to Rotz et al. (2021) likely due to the lack of regional analysis. Rotz et al. (2021) investigated the environmental performance of different regions in the U.S., whereas the present study assumed identical dairy systems across the country. While this helped simplify the national analysis, it sacrificed insight into regional differences that could increase or decrease the carbon footprint of milk production.

3.6 Population Impacts to the Carbon Footprint

It is often difficult to highlight the individual contributions of a single lifestage to the carbon footprint of a specific population. However, as the model utilized in this study was broken down by lifestage and each group received a specific ration and individualized excreta outputs, it allowed for differentiation of impacts between groups of animals.

As shown in Figure 2.3, lactating cows (both multi- and primiparous together), contributed to 67.5% of the Jersey carbon footprint, and 68.7% of the Holstein carbon footprint. Their large contribution was because mature cows had the greatest BW and consumed the most feed across both populations (Table 2.2). When comparing only multiparous cows, the Holstein multiparous cows had a greater contribution to the breed's carbon footprint, compared to Jerseys. This was likely attributed to, again, their larger size which resulted in greater milk production, higher feed intake, and subsequently higher manure and urine losses too (Barth et al., 2008). For primiparous cows, Jerseys had a larger contribution to the carbon footprint. The difference was likely due to the higher population of primiparous cows needed in the lactating Jersey group to meet the FU of the present study.

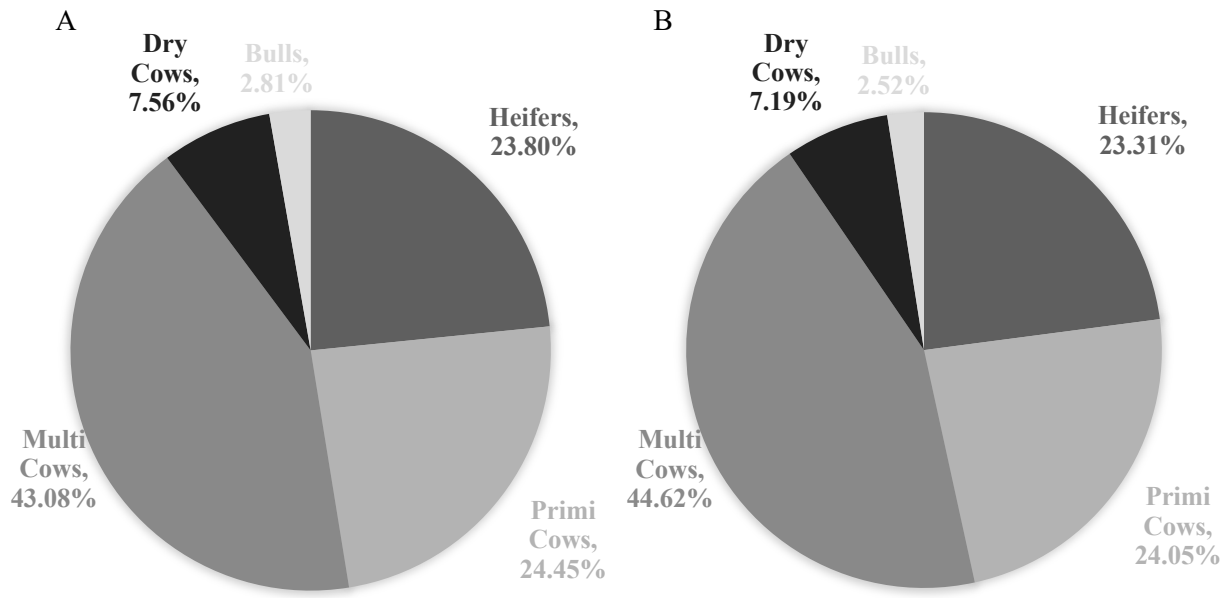

Figure 2.3 The carbon footprint of the Jersey population (A) and Holstein population (B), separated out by animal life stages.

As for the other, non-productive (i.e., non-lactating) animals within both populations, heifers accounted for the largest portion of the carbon footprint, almost to the same degree as primiparous cows. Jersey versus Holstein heifers had a greater lifecycle contribution to the carbon footprint.

For both the Jersey and Holstein population, dry cows had a greater contribution considering they were much larger animals compared to heifers and released similar quantities of enteric CH_4 as primiparous cows, according to the estimates from AMTS.Cattle.Pro, for both Jerseys and Holsteins. However, the populations of heifers were much larger compared to the dry cows, hereby resulting in an overall larger contribution to the carbon footprint of both dairy breeds.

The breakdown by lifestage offered interesting insights to where sustainability interventions and technologies should be focused on the future. Currently, efforts are overwhelmingly directed at lactating cows, as the single largest contributors to the carbon footprint

of both dairy breeds. Ensuring that feed additives and other interventions can safely reduce enteric emissions and the environmental impact of lactating cows without negatively impacting milk production or health remains essential (van Gastelen et al., 2019; Beauchemin et al., 2020).

Even so, it does pose a new challenge to address emissions coming from heifers and how those impacts will be mitigated. Recent research investigated early life programing, which aimed to either temporarily or permanently alter the rumen microbiome of young dairy calves via the feeding of 3-nitrooxypropanol, was found to be successful (Meale et al., 2021). Further research will be needed to determine the long-term effects of dosing calves with feed additives and impacts to overall productivity.

4 CONCLUSIONS

Overall, the environmental impact of milk production for both Jersey versus Holstein populations decreased from 2009 to 2020. Many of the factors informing environmental impact, such as land use and water use for crop production changed for Jerseys between 2009 and 2020, with land use decreasing and water use increasing over time. Background system changes were paralleled by changes impacting dairy breed performance directly. This was seen through improvements to daily milk yield and milk fat and milk protein. Despite some increases to certain KPIs pertaining to resource use, daily milk yield improved sufficiently to prevent those changes from negatively impacting the overall carbon footprint for either breed.

Interestingly, while both breeds improved from 2009 to 2020, the Holstein population improved more than the Jersey population. Breed improvements of Holsteins over Jerseys were attributed to greater overall ECM production as measured by the differential increase from 2009 to 2020, and a somewhat greater increase to milk components. Furthermore, reportedly heavier

Jerseys negatively impacted their previous environmental performance, making the carbon footprint of Jerseys in 2020 more like Holsteins. Even so, the Jersey population still had a lower overall environmental impact, as was already described in Capper and Cady (2012). However, the gap in environmental performance between the breeds is shrinking.

Chapter 3 - Effect of Eminex® on Greenhouse Gases and Ammonia Emissions from Dairy Cattle Slurry and Lagoon Water

ABSTRACT

Dairy manure management is responsible for a considerable amount of greenhouse gas emissions (GHG) in California. Aside from redesigning existing infrastructure to adopt alternative manure management systems, there are few options available to farmers to mitigate emissions without a substantial investment. Eminex®, a manure additive, has shown potential in reducing emissions in fresh slurry, reducing total GHG emissions by 99%, but has not been tested in liquid-based manure management systems. The aim of the present study was to investigate the effects of Eminex® on GHG and NH_3 emissions of fresh dairy slurry and dairy lagoon water. For experiment 1, feces and urine were collected from lactating dairy cows and mixed into a homogenous slurry, prior to being allocated into twelve individual bowls at a rate of 2.2 kg/bowl. Each bowl was randomly assigned a treatment: high (1.0 kg/m^3; SL-HD), low (0.5 kg/m^3; SL-LD), and a control (SL-CONT) with an n = 4/group. Upon receiving treatment, bowls were individually sampled beneath an OdoFlux chamber for 7 days to measure for CO_2, CH_4, N_2O, and NH_3 emissions. Samples were also taken to determine impacts to manure quality. For experiment 2, lagoon water was collected from a commercial dairy, and distributed to twelve 208-L stainless steel barrels. Two treatments (n = 4/treatment) were administered: high (1 kg/m^3 lagoon water), and low (0.5 kg/m^3 lagoon water); and compared to an untreated control (n = 4). Each barrel was sampled over two, 14-day periods, staggered to four barrels at a time, using OdoFlux chambers, for CO_2, CH_4, N_2O, NH_3, and EtOH. Slurry total solids, total nitrogen, and total carbon was similar across all treatment groups ($P > 0.05$). Acetic acid concentration in slurry increased in Eminex® treated groups compared to control ($P < 0.05$). All slurry GHG emissions, except for N_2O, declined ($P < 0.05$). Results showed that the high Eminex® treatment compared to control reduced CO_2, CH_4, and NH_3 emissions by 49.3%, 30.4%, and 34.9%, respectively ($P < 0.05$). In lagoon water, total nitrogen

increased with treatment ($P < 0.05$), while total solids and total carbon remained similar between all three treatments ($P > 0.05$). Volatile fatty acid concentration in lagoon water also saw a trend for increasing acetic acid concentration in Eminex® treated groups compared to control ($P < 0.1$). GHG emissions from lagoon water also decreased over time ($P < 0.05$). The high Eminex® treatment emitted 12.0% less CO_2 ($P < 0.1$), 85.1% less CH_4 ($P < 0.05$), and 82.7% less N_2O ($P < 0.05$). However, both Eminex® treatments, compared to control, increased NH_3 volatilization over time ($P < 0.05$). With improvements to manure composition through increasing nitrogen content, as well as significant reductions in GHG emissions, Eminex® is a promising manure additive that could mitigate the negative environmental impacts of manure management systems. Further research is needed to continue verifying its potential in different settings and at the commercial level.

1 INTRODUCTION

Livestock agriculture, especially ruminants, is a well-recognized source of gaseous emissions greenhouse gases (GHG) and others that can lead to criteria pollutants with adverse human health impacts and climate change (Hou et al., 2014; Pratt et al., 2015). Emissions comes from a variety of sources, but the major animal related sources are enteric fermentation and manure management.

Within the United States, enteric fermentation and manure management are responsible for 26.8% and 9.1%, respectively, of anthropogenic methane (CH_4) emissions (EPA, 2023). Manure management also contributes about 4.6% of direct nitrous oxide (N_2O) emissions. These are not the only emissions, as the process of forming N_2O can also result in the release of intermediate compounds like ammonia (NH_3) and other nitrogenous gases like nitrous oxides (NO_x) as well (Fowler et al., 2013; EPA, 2023c; Khairunisa et al., 2023). While NH_3 and NO_x gases are not categorized as GHGs, they still pose a threat to air quality, waterways, terrestrial ecosystems, and human health (Hristov et al., 2011; Fowler et al., 2013; Wattiaux et al., 2019; Peterson et al., 2020).

A life cycle assessment by Thoma et al. (2013a) found that about 72% of all emissions associated with milk production occur before the farmgate. These on-farm emissions for average milk consumed in the U.S. are split at 25% and 24% for enteric and manure emissions, respectively. The remaining 23% is split between feed rations and on-farm energy use. As it stands, much research has been dedicated to reducing enteric emissions, with rumen modifiers and rumen inhibitors (Hristov et al., 2013; Beauchemin et al., 2022; Fouts et al., 2022).

Similar efforts have gone into reducing manure-associated emissions, as most excreta in the U.S. is handled as slurry, a mixture of urine and feces from housed livestock that can also be

mixed with other organic materials and/or water (Kupper et al., 2020). This becomes a potent mixture that allows for the formation of GHGs. Alternative manure management strategies like anaerobic digesters, are meant to prevent GHGs from entering the atmosphere as organic matter decomposes in these systems (Hou et al., 2014). Ultimately, the use of such interventions is not common due to economic, environmental and social factors that prevent the widespread application of alternative manure practices (McCrory and Hobbs, 2001; Niles et al., 2022). Certain practices are also limited to only targeting specific, rather than multiple GHGs (Hou et al., 2014).

Currently, California leads the U.S. in gross milk production, home to 1.72 million lactating cows (USDA, 2021; El Mashad et al., 2023). A mature lactating cow produces 58-69 kg of manure daily (Varma et al., 2021), meaning 36.4 to 43.3 million metric tons (MMT) of manure must be managed annually. In California, manure is collected in solid, liquid, or slurry form, and housing predetermines manure collection options, with free stalls either flushing or scraping (Meyer et al., 2019; Ross et al., 2021). Anaerobic manure management systems (MMS), like lagoons, are one of the most popular systems in the United States. About 54% of California dairy farms use anaerobic lagoons as their primary form of manure storage (EPA, 2023b). These systems are notorious for the formation of CH_4 due to the anaerobic decomposition of volatile solids in manure by microbes into volatile fatty acids and subsequently, GHGs like CH_4 and CO_2 (Peterson et al., 2020; Sokolov et al., 2021).

The aqueous, anaerobic environment also leads to the production of criteria pollutants like ammonia (NH_3). Gaseous emissions remove essential nutrients, like C and N, from manure that will later be used as fertilizer and represent environmental and human health threats (Qu and Zhang, 2021). Manure management is directly responsible for about 10% of U.S. CH_4 emissions (EPA, 2023c) and 57% of CH_4 emissions in California (CARB, 2022). Even so, lagoons are still

advantageous, capable of holding large quantities of excreta for long periods of time (Niles and Wiltshire, 2019). Recently, there has been additional political pressure for farmers to continue reducing on-farm emissions. Local California legislation, specifically Senate Bill 1383 mandates a 40% reduction in GHGs originating from the agricultural sector below 2013 levels (Lara et al., 2016).

Two sponsored programs by the California government developed to help reduce and offset emissions: the Alternative Manure Management Project (AMMP) and the Dairy Digester Research & Development Project (DDRDP). The AMMP offers funds to help farmers install new MMSs that prevent GHG formation, like solids separators and dry storage. Current AMMP projects are expected to offset 1.1 MMT carbon dioxide equivalents (CO_2e) over the next 5 years (CDFA, 2023a). Similarly, the DDRDP provides farmers financial assistance to install anaerobic digesters. Since launching in 2014, DDRDP has funded 117 anaerobic digesters (CDFA, 2023b). The current functioning digesters in California are estimated to have cumulatively offset 21.02 MMT CO_2e (CDFA, 2023b). However, not all dairies qualify for financial aid and moreover, similar projects are not available throughout the U.S. Therefore, additional means of reducing GHG and NH_3 emissions from MMSs are essential to reducing the environmental impact of dairy production.

Aside from alternative MMSs, which require costly equipment and on-farm infrastructural changes (Meyer et al., 2019; El Mashad et al., 2023), research has also looked at treating the manure itself (Hou et al., 2014; Peterson et al., 2020; Sokolov et al., 2021; Holtkamp et al., 2023). Manure treatments have gained special attention from researchers as cost-effective alternatives to reduce manure emissions. They change the composition and the microbiome of manure to prevent gas formation, while also mitigating NH_3 volatilization, malodor, and handling issues (McCrory and Hobbs, 2001; Peterson et al., 2020; Chiodini et al., 2023; Holtkamp et al., 2023). One option,

SOP Lagoon, shows significant reductions in gaseous emissions in both research and commercial settings (Peterson et al., 2020; Chiodini et al., 2023). It contains calcium sulfate dihydrate and when applied to dairy lagoon water from a commercial dairy in California in a controlled research setting, and significantly reduced CH_4 by 22.7%, N_2O by 45.4%, CO_2 by 14.7%, and NH_3 by 45.9% (Peterson et al., 2020). When used in the slurry tank of commercial dairy farms in Italy, there were 80% reductions in CH_4 emissions (Chiodini et al., 2023).

Another option is urease inhibitors, which reduce nitrogenous emissions. Urease inhibitors stop urea hydrolysis and NH_3 oxidation and helped enhance N use efficiency by delaying nitrification and denitrification (Park et al., 2021). The application of the urease inhibitor, hydroquinone (HQ) and nitrification inhibitor, dicyandiamide (DCD) to swine slurry saw average reductions in NH_3 volatilization, N_2O emissions and NO_3^- leaching of 30.0% and 16.3%, 40.7% and 59.8%, and 7.0% and 12.9%, respectively, across the two treatments, paired within increased N retention into plant matter when manure was applied to land (Park et al., 2021). However, HQ and DCD were designed to only targeted nitrogenous emissions.

Another potent additive for reducing manure management emissions is calcium cyanamide ($CaCN_2$). It is made by combining calcium carbonate with charcoal, and passing it through nitrogen gas under white heat conditions forms $CaCN_2$ (Dixon, 2012). A common compound used in fertilizers and pesticides, $CaCN_2$ shows promising mitigation potential when applied to dairy and swine slurry (Holtkamp et al., 2023). Researchers used Eminex® (Alzchem Group AG, Germany), a granulated commercial form of $CaCN_2$ (40% N, 18% $CaCN_2$), in controlled research settings and saw reductions of 99%, 99% and 88% for CO_2, CH_4, and N_2O, respectively (Hermann et al., 2021; Holtkamp et al., 2023). However, the experimental set up, as stated by Holtkamp et al. (2023), was not accurately reflective of a standard manure management system. Furthermore,

Eminex® has never been tested in lagoon water before, as this is not a common MMS in Europe, where it was developed and tested (Holtkamp et al., 2023).

The aim of the current research is to determine the potency of Eminex® on reducing GHGs and other gaseous emissions from fresh dairy slurry and dairy lagoon water. It is hypothesized that a one-time application of Eminex® will significantly reduce gaseous emissions within storage both forms of excreta.

2 MATERIALS AND METHODS

A complete randomized design was utilized to determine the efficacy of Eminex® as a manure additive on emissions from slurry and lagoon water. The experimental unit for the slurry trial was the bowl and for lagoon water, the experimental unit was the barrel. Eminex® was applied to fresh dairy slurry and to dairy lagoon water in two separate experiments, presented below in sections 2.1 and 2.2, respectively.

2.1 Slurry Collection and Experimental Setup

Dairy feces and urine were collected from lactating dairy cows (days in milk: 102; milk yield: 40.8 kg/d) at the UC Davis Dairy Research Facility over two days, one week apart. Cows were manually palpated to urinate and defecate, helping to eliminate the risk of cross contamination between feces and urine, which would result in premature NH_3 volatilization.

Collected manure and urine were transported to the UC Davis Feedlot where the experiment was set up (see Figure 1). Feces and urine were combined at a ratio of 1.7:1.0 feces to urine (Hristov et al., 2011), and homogenized for 60 seconds using an electric hand drill and paddle extension to ensure adequate mixing. The slurry was subsampled to establish baseline chemical

composition and pH was measured prior to experiment start. The slurry mixture was then split into six ceramic bowls, aliquoting 2.26 kg per bowl. There were two rows of six 208-L stainless steel barrels holding the ceramic bowls. Each bowl had a diameter of 25.4 cm and a depth of 5.08 cm (volume = 398.9 cm^3). Each bowl then received a randomly assigned treatment. There were two treatments (n = 4/treatment): low at a rate of 0.5 kg Eminex®/m^3 slurry (1.3 g/bowl; SL-LD), and high at a rate of 1 kg Eminex®/m^3 slurry (2.6 g/bowl; SL-HD). A control with no additive (SL-CONT; n = 4) was also tested. The 'high' treatment was based on the manufacturer recommended dose. It was also of interest to test Eminex® at a lower dosage, so 50% of the initial dose was selected.

Each treatment was applied to two plates per row, for a total of four plates per treatment and 12 bowls overall (n = 4/group). However, due to equipment limitations, it was only possible to measure emissions for six bowls at a time, resulting in two rounds of emissions measuring. Each bowl was stirred for 30 seconds to ensure proper distribution of treatment. Each plate was immediately covered by an OdoFlux flux chambers (FC; Odotech Inc., Montreal, Quebec, Canada) to begin emissions measuring. The same protocol for manure and urine collection and treatment administration for the second round of six plates was repeated a week later. All FCs and plates were spaced 1 m apart to prevent carry over and contamination between treatments.

All the bowls were additionally sampled to analyze total solids (TS), volatile fatty acids (VFAs), total N (TN), and total C (TC) on days 0 and 7 and frozen at -20ºC prior to being sent out to an independent lab for chemical analysis (Dairy One Forage Lab, Ithaca, NY, USA).

2.2 Lagoon Water Collection and Experimental Setup

Lagoon water was collected from an uncovered lagoon on a 1,000-head commercial dairy in Solano County, CA. Lagoon water was collected in a plastic tote on three separate days every two weeks, using a trash pump (Honda Trash Pump WT20X) and the lagoon water was transported back to the University of California, Davis Feedlot Research Facility.

Figure 3.1 Barrel and FC setup within the UCD Feedlot Research Facility

189L of lagoon water was dispensed into four, 208L stainless steel barrels per collection (Uline, Pleasant Prairie, WI, USA). Lagoon water was measured with 19L jugs to ensure equal distribution for each barrel. After filling the barrels, each was homogenized via an electric hand drill with a paddle extension for 60 seconds. Day 0 samples and pH were also taken at this time. Following sampling and homogenization, Eminex® was applied.

Each barrel was randomly assigned a specific treatment prior to study start. There were two treatments (n = 4/treatment): low at a rate of 0.5 kg Eminex®/m^3 lagoon water (100.6 g/barrel; LW-LD), and high at a rate of 1 kg Eminex®/m^3 lagoon water (201.1 g/barrel; LW-HD). A control with no additive (LW-CONT; n = 4) was also tested. As this product has never been tested in lagoon water before, it was decided to also use the manufacturer recommended dose and 50% dose for this second experiment within the study.

After adding Eminex®, barrels were homogenized for another 60 seconds to ensure proper distribution. The four filled barrels were covered by individual OdoFlux Flux chambers (FC; Odotech Inc., Montreal, Quebec, Canada) to start emissions sampling, shown in Figure 1. The same collection and treatment procedure above was repeated until 12 barrels were filled, for a total of three collections, with four barrels filled per collection.

The staggering of collection, filling, and treatment application was unavoidable due to equipment restrictions, as only four FC were available for this experiment. There were three rows of four barrels, with treatments randomly distributed throughout each row (12 barrels total). On each row's respective d 0, the first sampling period started and continued for 14 days. At the end of the sampling period, the FCs were moved to the next row. On each row's respective d 42, the FCs started the second 14-day sampling period. Barrels not being actively sampled were left uncovered. Samples were collected from each barrel to analyze TS, TN, TC, and VFAs on days 0, 14, 28, and 56 of the trial and frozen at -20 ºC prior to being sent out to an independent lab for analysis (Dairy One Forage Lab, Ithaca, NY, USA).

2.3.1 Emissions Sampling and Measurements

The following sections of emissions sampling, calculations, and statistical analysis apply to the experiments for slurry and lagoon water. The main differences was in sampling periods, as slurry bowls were sampled for 7 d total, and lagoon water barrels were stagger sampled for 28 d total. Air samples were samples by an INNOVA 1412 photo-acoustic multi-gas analyzer (LumaSense Technologies Inc., Ballerup, Denmark) to quantify CO_2, CH_4, N_2O, NH_3, and ethanol (EtOH) emissions from slurry and lagoon water. The INNOVA 1412 analyzer had the following detection limits: minimum of 0.4 ppm CH_4, 1.5 ppm CO_2, 0.03 ppm N_2O, 1.0 ppm NH_3, and 0.08 ppm EtOH. Each FC was sampled at 20-minute intervals in sequence over a 24-h period.

2.3.2 Emissions Calculations

Concentrations of measured gases in the FCs over the 24-h period were truncated to remove the first 12 and last 1 minute of each 20-minute sample period to avoid carry-over effects between treatments. The total flux for each gas was calculated with the following equation:

$$Total\ flux\left(\frac{mg}{hr}\right) = \frac{Cn \times\ FL\ \times\ 60}{MW} \times MV\ \times Conv$$

Cn is the net concentration of each gas that was calculated as the difference between the measured concentration from each sample minus the background concentration of the fresh inlet air in either ppm or ppb. FL is the ambient air flow rate at 8L/min and 60 is the conversion of minutes to hour. MW is the molecular weight of the gas in grams per mole. Conv is a conversion factor of 10^{-3} for the concentration of ppm and 10^{-6} for the concentration of ppb. MV is the volume of one molar gas at temperature 20ºC (24.04 L/mole). Surface area emission rate (mg/h/m^2) of each sampled bowl and barrel was calculated with the following equation:

$$Surface\ emission\ rate\ = \frac{Total\ flux}{Surface\ area}$$

Surface area is the cross-section area of the barrel directly under the flux chamber, approximately 0.05 m^2 for slurry bowls and 0.25 m^2 for lagoon water barrels.

2.4 Statistical Analysis

Gaseous data and manure composition data were analyzed using a linear mixed effects model with repeated measures over time using the 'lme4' package of R version 4.1.2 (R Core Team, 2021). Assumptions of normality and homogeneity of variances were checked, and appropriate logarithmic transformations were applied when necessary. A two-way ANOVA was used to explore the effect of treatment, day, and their interaction, on emissions and the chemical composition of the lagoon water and slurry, separately, according to the base model:

$$Y_{btd} = \mu + \beta_b + \beta_t * \beta_d + \varepsilon_{btd}$$

Where Y_{btd} = the dependent response variable; μ = overall mean of the response variable; β_b= barrel/bowl (experimental unit, random variable); β_t = treatment; β_d = day; and ε_{btd} = the error terms for the models in question. Least squares means (LSM) were determined using "emmeans". Pairwise treatment LSM comparisons were conducted using the Tukey's HSD post-hoc analysis. The significance of fixed effects was evaluated using *P*-values. Differences were declared significant at $P < 0.05$, and trends at $P < 0.1$.

3 RESULTS

Results are presented below, separated by experiment. Section 3.1 outlines the results for fresh slurry emissions and chemical composition and section 3.2 outlines results for lagoon water emissions and chemical composition. It must be noted that Holtkamp et al. (2023) determined that

Eminex® began suppressing emissions within 45 minutes of application. This likely explained why Eminex® treatments compared to the untreated control had lower emissions on d 1 GHG and NH_3 emissions from slurry and lagoon water (Figure 3.2-3.10).

3.1 Fresh Slurry Physical Characteristics

Table 3.1 Least squares means and SEM of the chemical composition of the fresh dairy slurry

Parameters	Day 0	Treatments			SEM	*P-value*		
		SL-CONT	SL-LD	SL-HD		*T*	*D*	*TxD*
Total Solids, %DM	13.20	14.0	11.5	11.7	1.61	NS	0.001	NS
Total N, %DM	0.57	0.54	0.55	0.56	0.012	0.01	0.02	0.005
Total C, %DM	44.6	45.1	44.9	44.7	0.103	NS	0.01	NS
pH	8.03	7.79	7.88	7.93	0.117	NS	NS	NS
Acetic acid, ppm	2,863	4,887[a*]	5,319[*]	5,361[b]	145	0.08	<0.001	0.02
Propionic acid, ppm	661.4	800	820	832	22.9	NS	<0.001	NS
Butyric acid, ppm	402.7	509	509	515	34	NS	0.001	<0.001
Iso-butyric acid, ppm	55.8	83.5	77.7	78.9	7.95	NS	<0.001	NS
Lactic acid, ppm	99.7	131[a]	203[b]	194[b]	15.9	0.02	<0.001	0.001

Different letters between columns indicate significant differences between those values at $P < 0.05$. Symbol '*' in columns indicates a trend at $P < 0.1$; NS = not significant ($P > 0.05$); SL-CONT = control group, no treatment; SL-LD = low dose; SL-HD = high dose. %DM = percent dry matter. T = treatment, D = day, TxD = treatment x day interaction.

To reiterate, SL-CONT represents the control group (no Eminex® added), SL-LD represents the low dose group (0.5 kg Eminex®/m^3), and SL-HD represents the high dose group (1.0 kg Eminex®/m^3). Chemical composition of slurry is presented in Table 3.1.

Slurry TS decreased over time ($P = 0.001$), but remained similar across all treatment groups. Slurry TN also decreased over time ($P = 0.02$), and all treatment groups were similar. Slurry TC increased over time ($P = 0.01$), and all treatments were similar. The pH of slurry did not vary between treatment groups.

Table 3.1 also provides results on VFA concentrations, focusing on the three main VFAs, acetic, propionic, and butyric acids, as well as iso-butyric acid and lactic acid. Acetic acid concentration increased over time ($P < 0.001$). There was a trend for the effect of treatment ($P = 0.08$), where SL-HD, compared to SL-CONT, had a 9.7% greater acetic acid concentration ($P < 0.05$), while SL-LD and SL-HD were similar. There was a trend between SL-LD and SL-CONT, as SL-LD containing 8.8% more acetic acid ($P < 0.1$). The interaction between day and treatment was also significant ($P = 0.02$).

The concentration of propionic acid in slurry also increased over time ($P < 0.001$), by 21.0%, 24.0%, and 25.8% for SL-CONT, SL-LD, and SL-HD respectively, when compared to d 0. However, all three treatment groups were similar.

The concentration of butyric acid in slurry also increased with time, by 26.6% for SL-CONT and SL-LD, and 28.1% for SL-HD ($P = 0.001$). All three treatment groups were similar. However, there was a significant interaction effect ($P < 0.001$).

The concentration of iso-butyric acid increased over time ($P < 0.001$), increasing by 49.7%, 39.3%, and 41.5%, for SL-CONT, SL-LD, and SL-HD, respectively, compared to d 0. All three treatment groups were similar. There was no significant effect of treatment alone or the interaction.

Lactic acid concentration increased over time ($P < 0.001$), increasing by 31.4%, 103.7%, and 94.6%, for SL-CONT, SL-LD, and SL-HD, respectively, compared to d 0. Both treatment groups had greater lactic acid concentration compared to SL-CONT ($P = 0.02$), but were similar to each other. There was also a significant interaction effect ($P = 0.001$).

3.1.1 Fresh Slurry Gaseous Emissions

Gaseous flux data are presented in Table 3.2. Eminex® treatments, SL-HD and SL-LD, reduced emissions of most gases compared to the SL-CONT. The first GHG, CO_2 decreased over time ($P = 0.02$). Both SL-LD and SL-HD versus SL-CONT emitted 44.4% and 49.3% less CO_2, respectively, with a trend for the effect of treatment on CO_2 ($P < 0.1$). The differences between groups were significant ($P < 0.05$), but SL-LD and SL-HD were similar. There was no significant interaction effect. CH_4 emissions decreased over time ($P = 0.01$), reduced by 30.1% and 30.4% for SL-LD and SL-HD, respectively, compared to SL-CONT with a trend for the effect treatment on CH_4 ($P <0.1$). Emissions between SL-CONT and the two treated groups differed ($P < 0.05$), but the two treated groups were similar. There was no significant interaction effect. Lastly, N_2O was similar across all three treatment groups.

Table 3.2 Least squares means and SEM of gas fluxes from fresh dairy slurry

Gas Production	Treatment			SEM	*P-values*		
	SL-CONT	SL-LD	SL-HD		*T*	*D*	*TxD*
CO_2 (mg/h/m^2)	2,733^a	1,519^b	1,387^b	345	0.06	0.02	NS
CH_4 (mg/h/m^2)	37.5^a	26.2^b	26.1^b	3.22	0.06	0.01	NS
N_2O (mg/h/m^2)	1.2	1.1	1.1	0.42	NS	NS	NS
NH_3 (mg/h/m^2)	378^a	269^b	246^b	41.3	0.04	<0.001	NS
EtOH (mg/h/m^2)	13.2^a	22.3^b	22.2^b	3.54	<0.001	<0.001	NS

Values presented are cumulative emission rates per gas. Columns with different subscript letters indicates statistically significant differences at $P < 0.05$, with '*' indicating trends at $P < 0.1$. NS = not significant ($P < 0.05$); T = treatment, D = day, TxD = treatment by day interaction

The two other gaseous emissions measured during this experiment were NH_3 and a volatile organic compound (VOC), ethanol (EtOH). NH_3 emissions decreased over time ($P < 0.05$) with significant effect of treatment ($P = 0.04$). NH_3 losses from SL-LD and SL-HD compared to SL-CONT decreased by 28.8% and 34.9%, respectively ($P < 0.05$), but SL-LD and SL-HD were similar. There was no significant interaction effect.

Conversely, EtOH emissions increased over time ($P < 0.001$). SL-LD and SL-HD, compared to SL-CONT, emitted 68.8% and 67.9% more EtOH ($P < 0.001$). Differences between groups were significant ($P < 0.05$), while SL-LD and SL-HD were similar. There was no significant interaction effect.

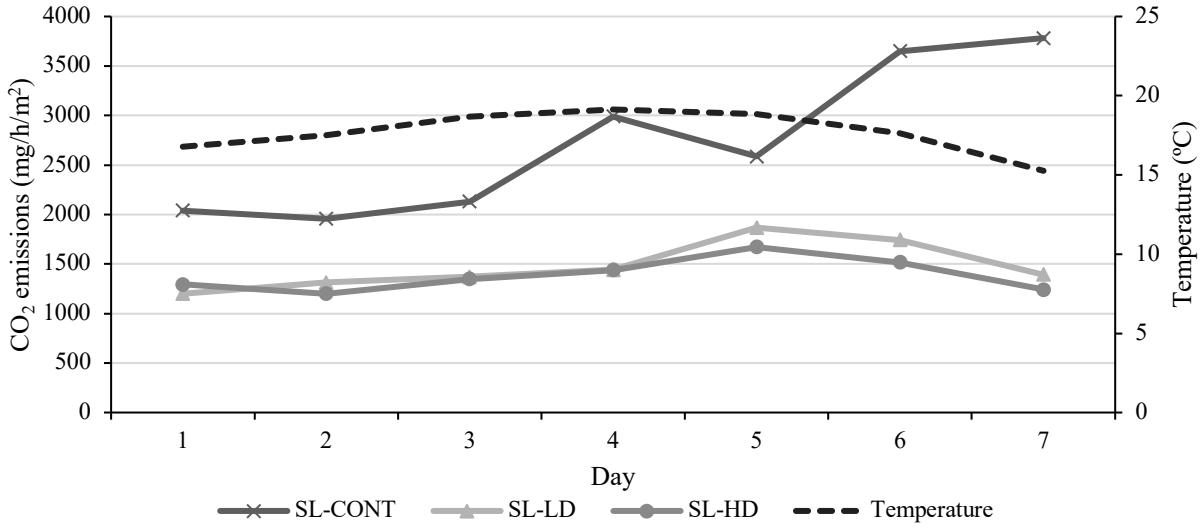

Figure 3.2 Daily CO_2 emissions throughout the one-week slurry incubation period. SL-CONT = control; SL-LD = low dose; SL-HD = high dose

Figure 3.2 shows CO_2 emission over time. The control group, SL-CONT, gradually increased in gas production throughout the sampling period, whereas SL-LD and SL-HD demonstrated consistently lower gas production over the 7 days. Interestingly, the two treatments showed similar levels of CO_2 suppression throughout the entire sampling period.

For CH_4 emissions (Figure 3.3), there were also significant reductions in emissions overtime. Like CO_2, SL-CONT maintained higher CH_4 emissions throughout the entire sampling period, while treated groups (SL-LD and SL-HD) suppressed emissions. The two treatment groups produced similar degrees of suppression throughout the sampling period.

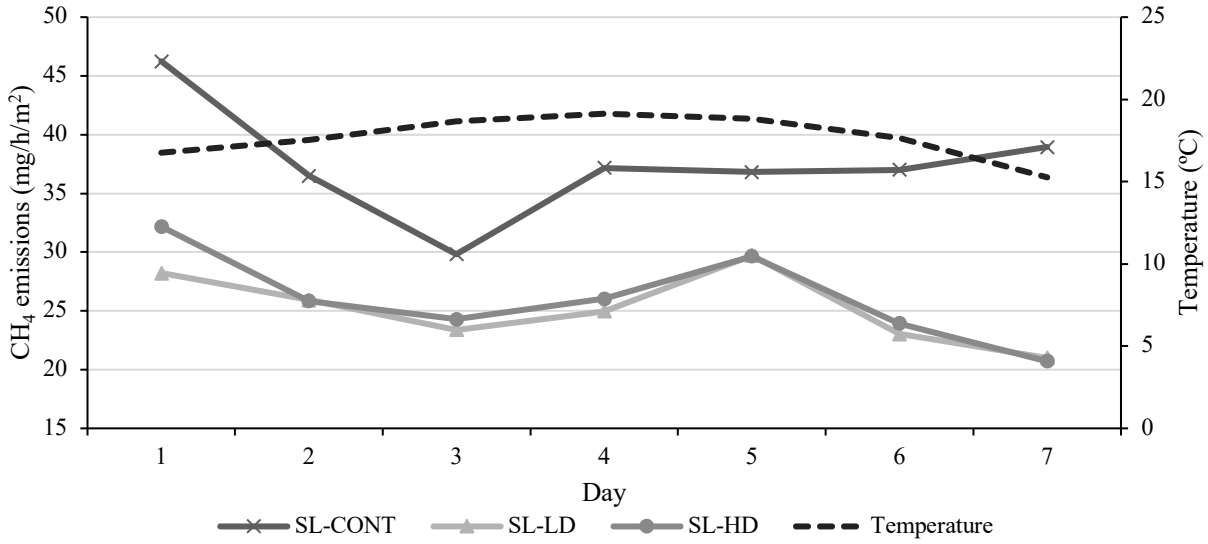

Figure 3.3 Daily CH_4 emissions throughout the one-week slurry incubation period. SL-CONT = control; SL-LD = low dose; SL-HD = high dose

Throughout the week-long incubation, N_2O emissions across the three treatments were similar. All three groups maintained relatively similar levels of emissions (Figure 3.4) throughout the sampling period. Treated slurries did maintain lower emissions levels on d 3 and 4, prior to sampling on d 4. On d 6, N_2O production for SL-HD and SL-LD started to decline more dramatically which continued into d 7. This differed from the behavior noted in the other two GHGs, where suppression of N_2O between treatments seemed to differ from day to day.

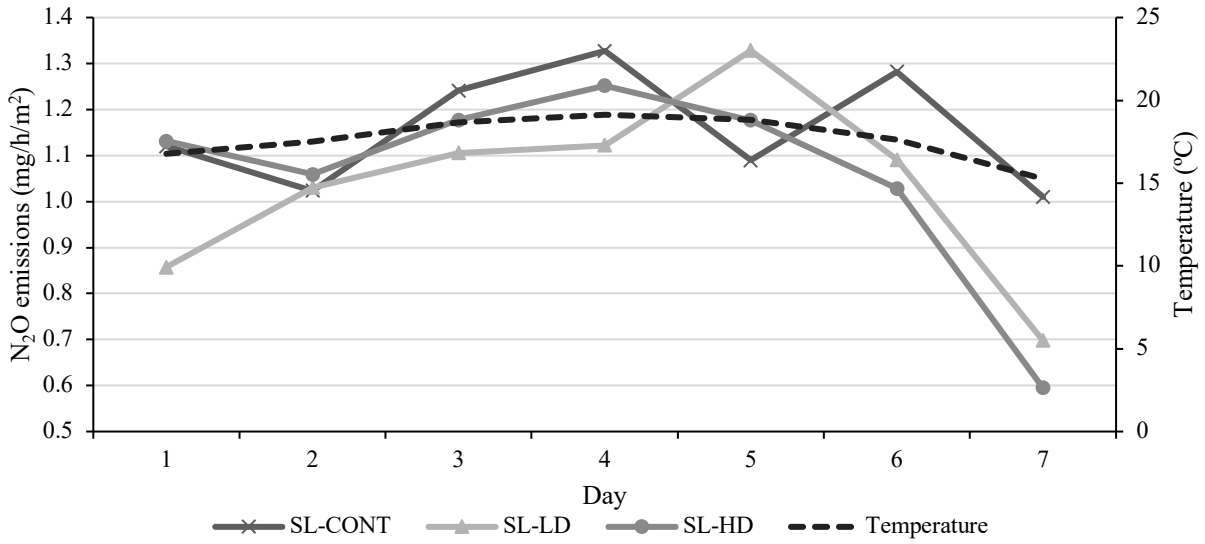

Figure 3.4 Daily N_2O emissions throughout the one-week slurry incubation period. SL-CONT = control; SL-LD = low dose; SL-HD = high dose

The second nitrogenous emission of interest, NH_3, declined overtime with Eminex® treatment (Figure 3.5). While volatilization peaked early in the sampling period, the SL-HD and SL-LD groups consistently declined in emissions overtime. Both treatment levels seemed to elicit similar degrees of NH_3 suppression throughout the sampling period. This demonstrated the same consistency seen for CH_4 and CO_2, but not for N_2O. And on d 7 when SL-CONT started increasing, the treated groups did not.

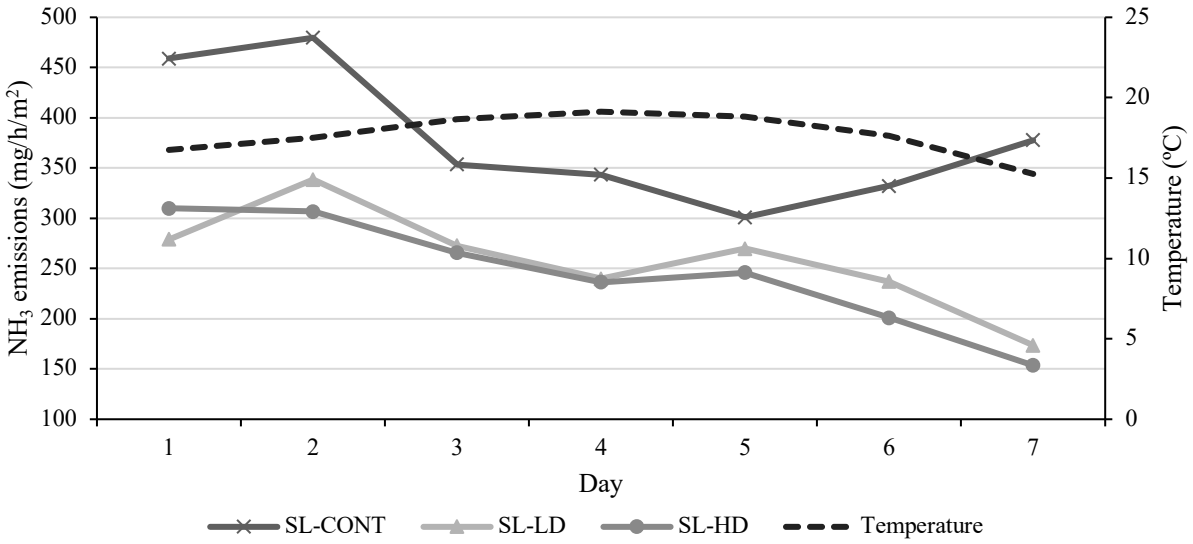

Figure 3.5 Daily NH_3 emissions throughout the one-week slurry incubation period. SL-CONT = control; SL-LD = low dose; SL-HD = high dose

Losses of EtOH also significantly varied overtime (Figure 3.6). Untreated SL-CONT consistently maintained lower levels of EtOH after diverting from the other treatments on d 2. However, SL-LD and SL-HD produced significantly more EtOH, although the production patterned mimicked the mitigation behaviors seen for CH_4, CO_2, and NH_3—with the two Eminex® levels resulting in similar levels of emissions throughout the sampling periods.

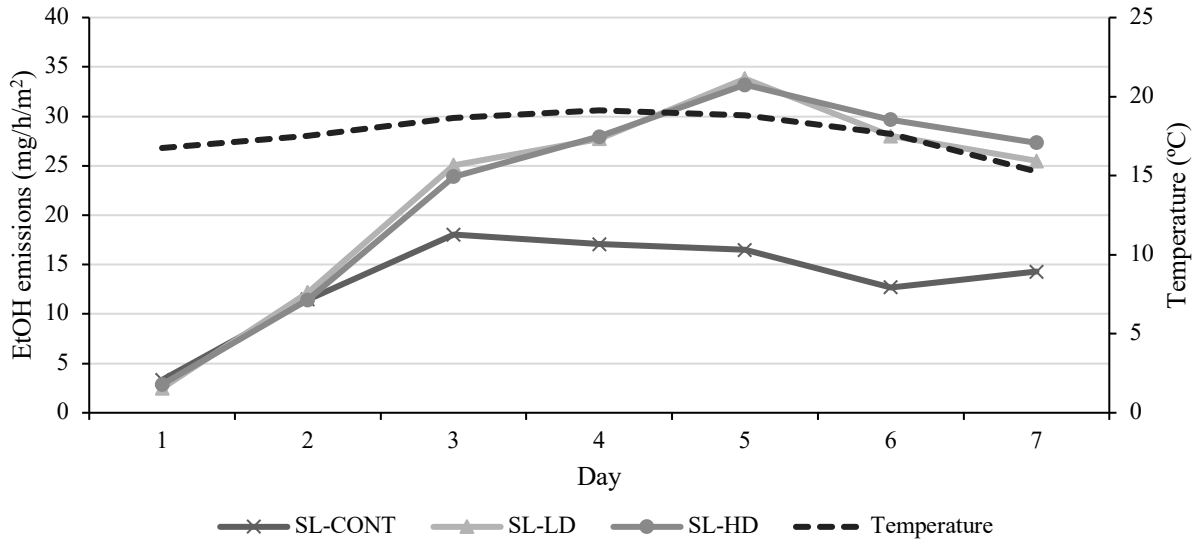

Figure 3.6 Daily EtOH emissions throughout the one-week slurry incubation period. SL-CONT = control; SL-LD = low dose; SL-HD = high dose

3.2 Lagoon Water Physical Characteristics

Chemical composition of the dairy lagoon water is presented in Table 3.3. Lagoon water TS increased over time ($P = 0.01$). TS content of treatments LW-LD and LW-HD compared to d 0 increased by 23.8% and 33.3%, respectively ($P < 0.05$). The TS content for all three treatment groups were similar.

Lagoon water TN increased over time ($P < 0.001$), with significant effect of treatment ($P = 0.02$). LW-LD and LW-HD, compared to LW-CONT, contained 33.3% more TN ($P < 0.05$). However, LW-LD and LW-HD were found to be similar.

Lagoon water TC increased with time ($P < 0.001$). TC content increased by 13.6%, 18.1%, and 10.0% for LW-LD, LW-HD, and LW-CONT, respectively, compared to d 0. However, all three treatment groups were similar.

In lagoon water, pH became more alkaline over time ($P < 0.001$). However, all three treatments were similar. The concentration of VFAs for lagoon water was also quantified.

However, only acetic acid and lactic acid are included in Table 3.3, as the rest were below the detectable limits of the Dairy One Forage Lab equipment (>1 ppm).

Table 3.3 Least squares means and SEM of the chemical composition of the dairy lagoon water

Parameters	Day 0	Treatment			SEM	*P-values*		
		LW-CONT	LW-LD	LW-HD		*T*	*D*	*TxD*
Total Solids, %DM	0.42	0.42	0.52	0.56	0.067	NS	0.01	NS
Total N, %DM	0.03	0.03[a]	0.04[b]	0.04[b]	0.004	0.02	<0.001	NS
Total C, %DM	0.20	0.22	0.25	0.26	0.015	NS	<0.001	NS
pH	7.38	7.66	7.77	7.79	0.068	NS	<0.001	NS
Acetic acid, ppm	0.0	2.9[a]	21.1[b]	18.6[b]	5.27	0.07	<0.001	0.03
Lactic acid, ppm	2.7	1.1[a]	29.5[b]	35.4[b]	8.17	0.03	<0.001	0.005

Columns with different subscript letters indicates statistically significant differences at $P < 0.05$, with '*' indicating trends at $P < 0.1$; NS = not significant ($P > 0.05$). T = treatment, D = day, TxD = treatment by day interaction.

Acetic acid concentration increased over time ($P < 0.001$). There was a trend for the effect of treatment ($P < 0.1$), with LW-LD and LW-HD acetic acid concentrations versus LW-CONT increasing by 608.7% and 526.2%. Differences between groups were significant ($P < 0.05$), but LW-LD and LW-HD were similar. Acetic acid concentrations also showed significant effects the interaction ($P = 0.03$).

The concentration of lactic acid also changed increased over time ($P <0.001$). Lactic acid concentrations in LW-LD and LW-HD treatments compared to LW-CONT, were 2,581.8% and 3,118.1% greater, respectively ($P < 0.05$). Eminex® treated groups were similar.

3.2.1 Lagoon Water Gaseous Emissions

The results for gaseous emissions are presented in Table 3.4. While EtOH emissions were included for slurry, there was no EtOH detected during the lagoon water experiment. For all GHGs,

there were reductions following treatment application, when compared to LW-CONT. Emissions of CO_2 decreased over time (P <0.001). CO_2 emissions from LW-LD and LW-HD, compared to LW-CONT, were by 2.9% and 12.0% lower, respectively (P < 0.1). Emissions from all three treatment groups were similar. For CH_4, emissions decreased over time (P < 0.001). Compared to LW-CONT, CH_4 emissions decreased by 80.9% and 85.1% for LW-LD and LW-HD, respectively, (P < 0.05), while LW-LD and LW-HD were found to be similar. There was also an interaction effect (P < 0.001). For N_2O emissions also declined over time (P < 0.001). LW-LD and LW-HD compared to LW-CONT, emitted 81.08% and 82.66% less N_2O, respectively. There was also a significant interaction effect (P < 0.001).

Unlike NH_3 emissions from slurry (section 3.1.1), NH_3 emissions increased over time (P < 0.001). Compared to LW-CONT, emissions increased by 65.26% and 65.73%, for LW-LD and LW-HD, respectively. All three treatment groups were similar.

Table 3.4 Least squares means and SEM of gas fluxes from lagoon water

Gas Production	Treatment			SEM	*P-values*		
	LW-CONT	LW-LD	LW-HD		*T*	*D*	*TxD*
CO_2 (mg/h/m^2)	507.0	492.0	446.0	82.2	NS	<0.001	NS
CH_4 (mg/h/m^2)	13.7^a	2.6^b	2.0^b	3.32	0.03	<0.001	<0.001
N_2O (mg/h/m^2)	4.4	0.84	0.77	1.08	NS	<0.001	<0.001
NH_3 (mg/h/m^2)	21.3	35.2	35.3	6.98	NS	<0.001	NS

Values presented are cumulative emission rates per gas. Columns with different subscript letters indicates statistically significant differences at P < 0.05, with '*' indicating trends at P < 0.1. NS = not significant (P < 0.05); T = treatment, D = day, TxD = treatment by day interaction

Gaseous emissions over the two sampling periods are presented in Figures 3.7-3.10. No barrels were sampled on their respective d 14 through 42, due to the staggered sampling protocol described above (section 2.3.1).

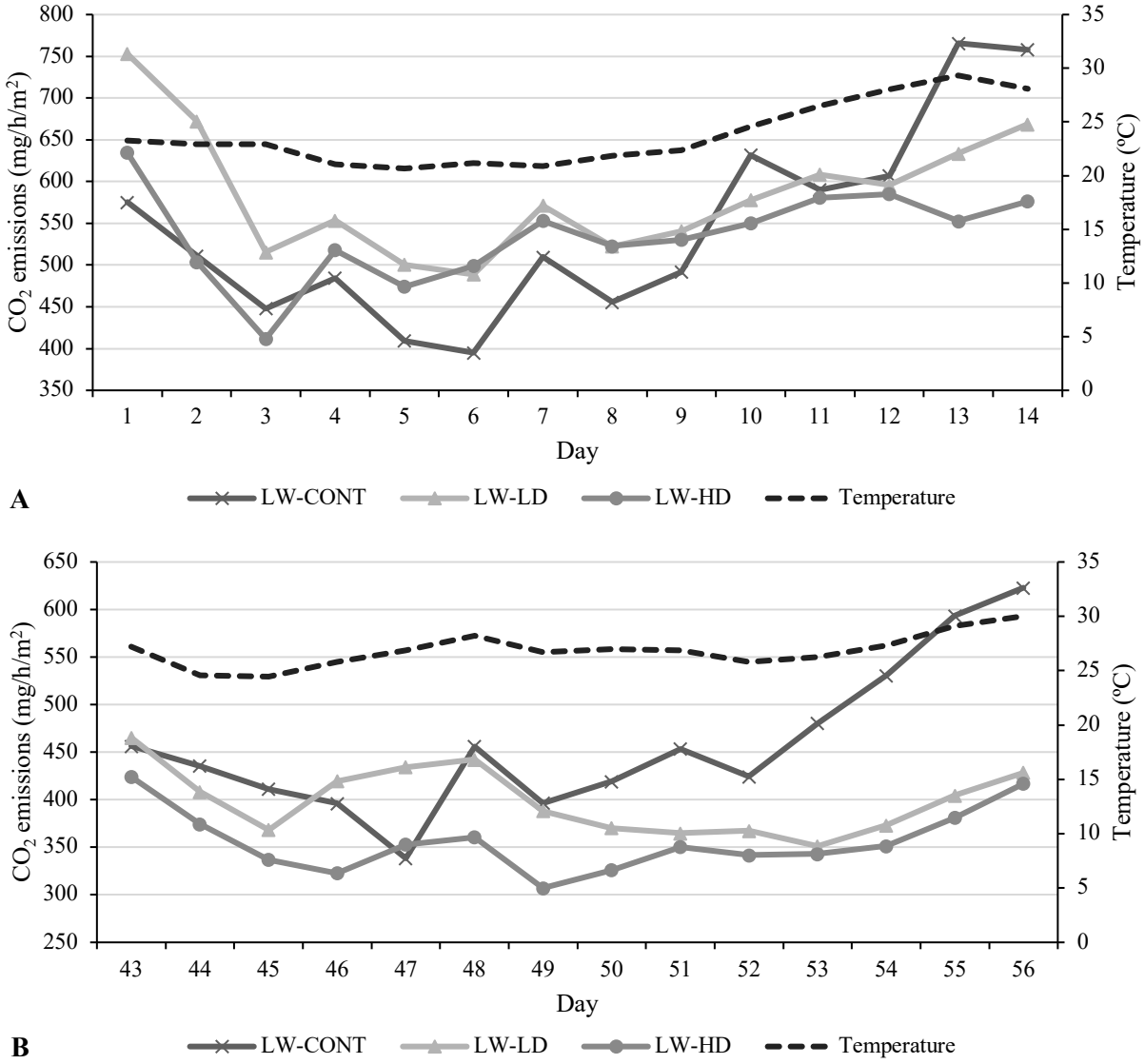

Figure 3.7a-b Daily CO_2 emissions over time following the application of treatment. A) Daily CO_2 emission rate over the first two-week incubation period, also plotted with mean ambient temperature. B) Daily CO_2 emission rate over the second two-week incubation period, also plotted with mean ambient temperature.

Overall, CO_2 emissions fluctuated across both sampling periods, regardless of treatment (Figure 3.7a). However, starting d 49, LW-CONT increased while LW-LD and LW-HD were suppressed. This behavior continued for the rest of the second sampling period, with LW-CONT reaching similar emissions levels to the first period, while LW-LD and LW-HD continued

declining (Figure 3.7b). As seen in the slurry emissions, the two treatments showed similar patterns in emission suppression throughout the sampling periods.

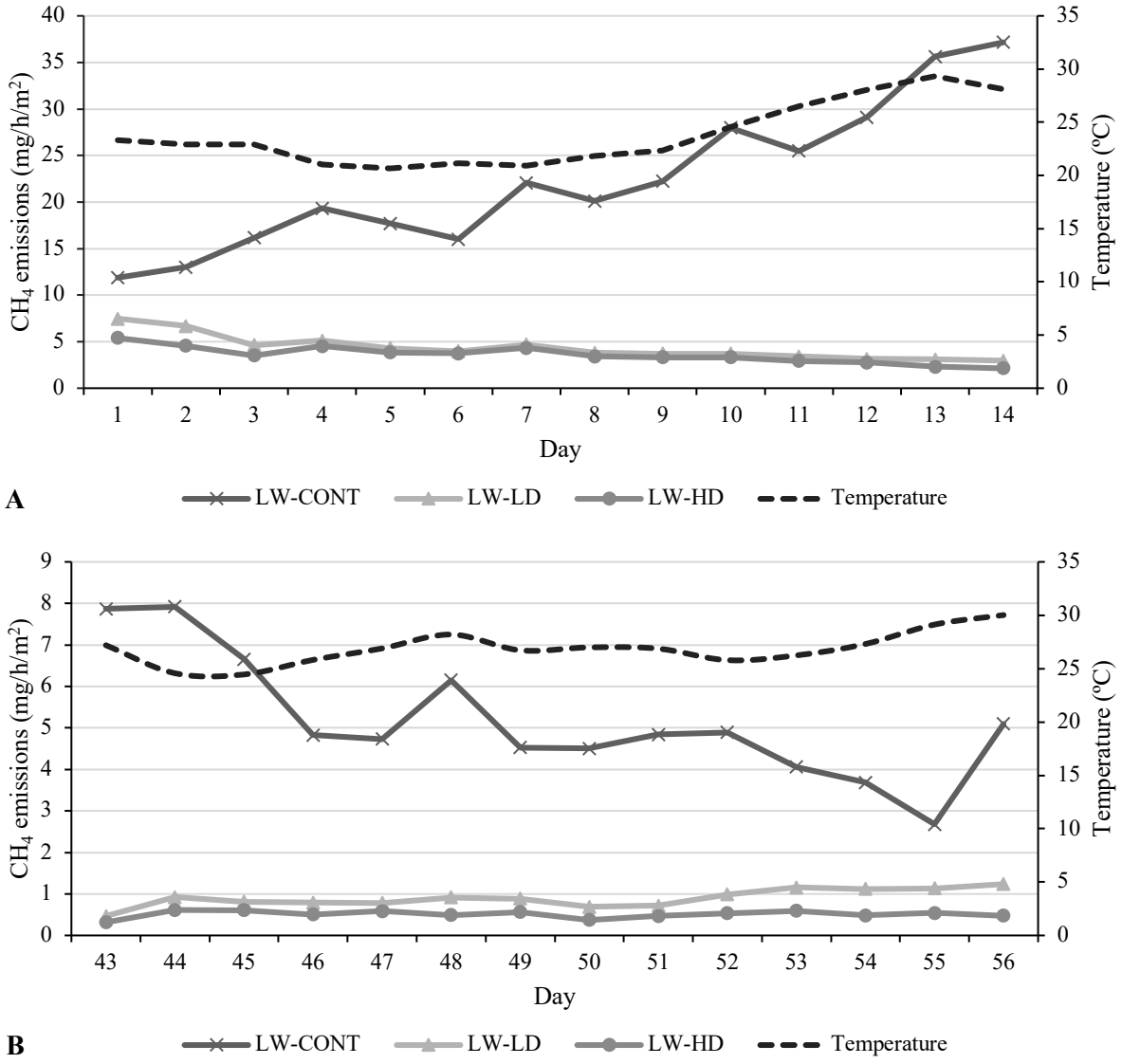

Figure 3.8a-b Daily CH_4 emissions over time following the application of treatment. A) Daily CH_4 emission rate over the first two-week measurement period, also plotted with mean ambient temperature. B) Daily CH_4 emission rate over the second two-week measurement period, also plotted with mean ambient temperature.

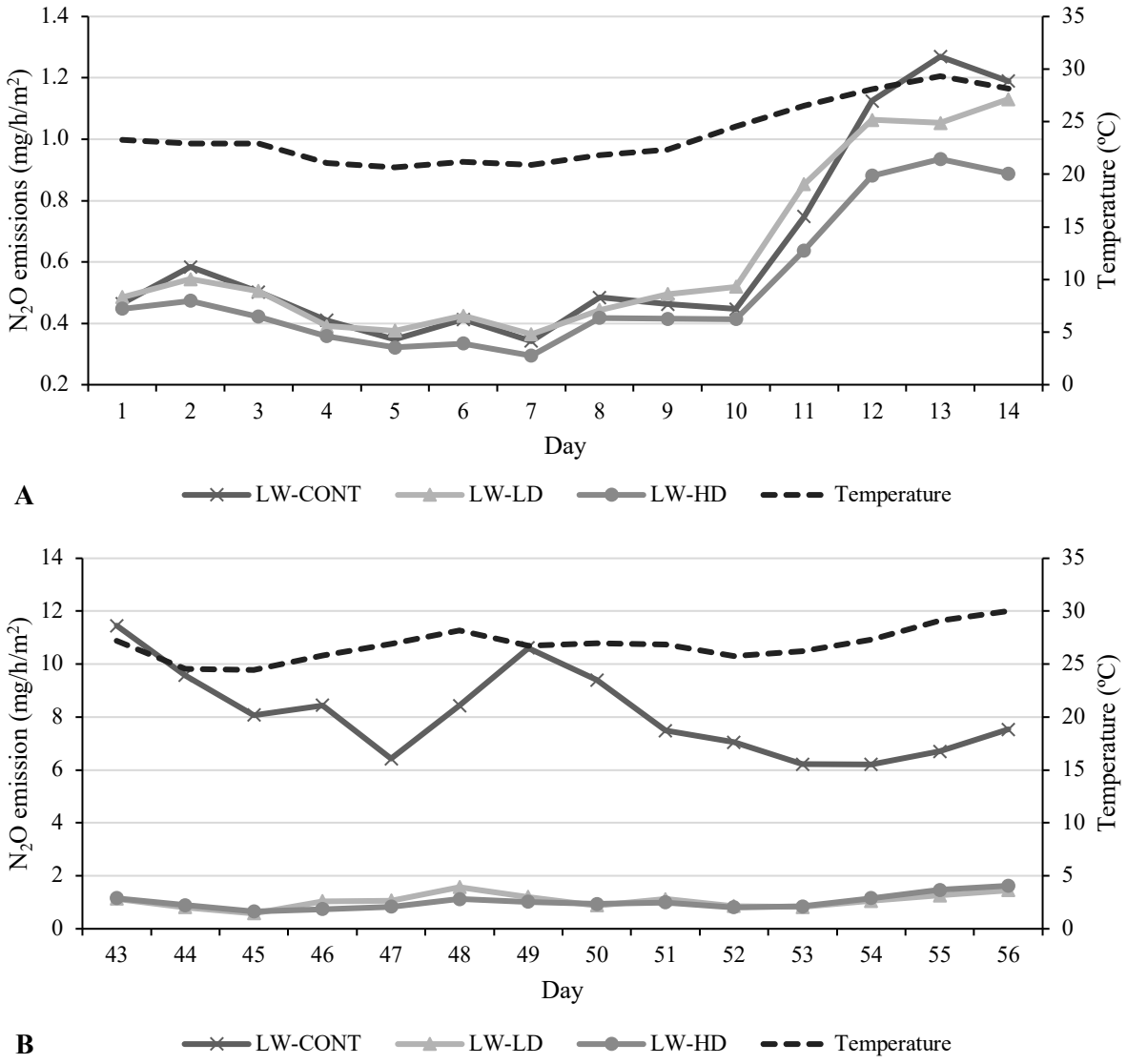

Figure 3.9a-b Daily N_2O emissions over time following the application of treatment. A) Daily N_2O emission rate over the first two-week incubation period, also plotted with mean ambient temperature. B) Daily N_2O emission rate over the second two-week incubation period, also plotted with mean ambient temperature.

Figure 3.8a shows the emissions of CH_4 overtime in the first sampling period, with LW-CONT steadily increasing, whereas LW-LD and LW-HD decreased. In Figure 3.8b, CH_4 from LW-CONT declined, but maintained greater gas levels compared to LW-LD and LW-HD treatments. Overall LW-CONT closely followed temperature fluctuations, with emissions

increasing as average daily temperature also increased. LW-LD and LW-HD treatments also continued demonstrating similar suppression patterns throughout the experiment.

Like CO_2, N_2O (Figure 3.9a-b) initially did not demonstrate obvious differences in emissions with treatment in the first sampling period. In fact, as seen in Figure 3.9a, N_2O emissions increased starting d 10 for all treatments and continued increasing the remainder of that period. However, by the second sampling period, there were clear differences in emission rates between LW-CONT and the treated groups. Interestingly, emissions from LW-LD and LW-HD seemed to stabilize in the second sampling period, while LW-CONT increased further (Figure 3.9b).

Emissions of NH_3 over the two sampling periods are presented in Figure 3.10a-b. Eminex® was not effective at reducing NH_3. Unlike the three GHGs, NH_3 losses, for LW-LD and LW-HD, were much higher than LW-CONT (Figure 3.10a). High NH_3 losses for the treated groups continued into the second sampling period, with LW-HD emitting more than LW-LD starting d 50 (Figure 3.10b).

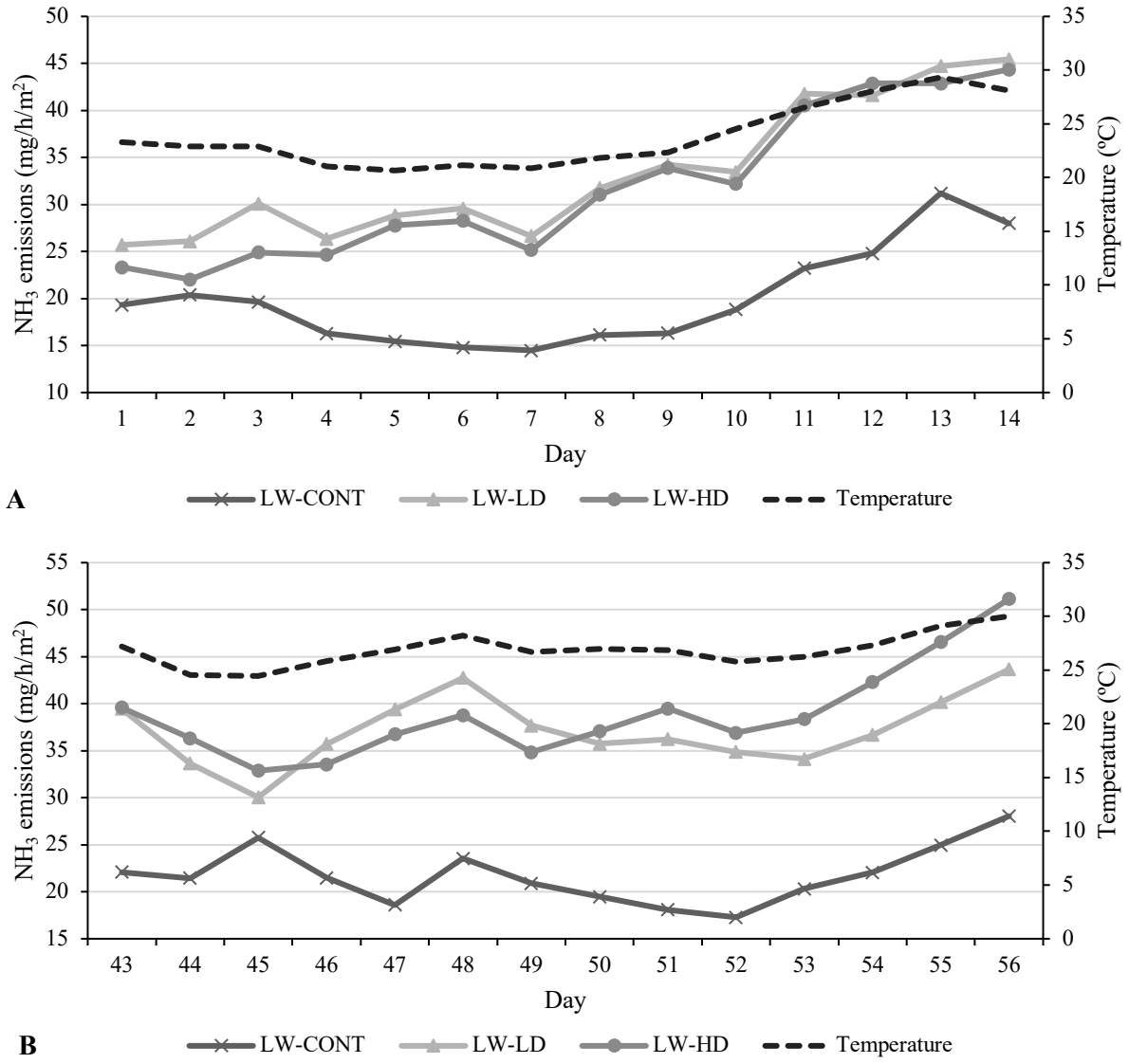

Figure 3.10a-b Daily NH_3 emissions over time following the application of treatment. A) Daily NH_3 emission rate over the first two-week incubation period, also plotted with mean ambient temperature. B) Daily NH_3 emission rate over the second two-week incubation period, also plotted with mean ambient temperature.

4 DISCUSSION

This completely randomized design trial investigated the effects of two treatments and a control (low: LD, 0.5 kg Eminex®/m^3; and high: HD, 1 kg Eminex®/m^3; control: CONT, no Eminex®) on gaseous emissions from dairy slurry and dairy lagoon water. Varying levels of

Eminex® treatment were found to significant change the chemical composition of slurry and lagoon water, as well as significantly reducing most gas production.

4.1 Dairy Slurry

Despite slurry being a mixture of manure and urine, in the absence of excessive water, and not strictly anaerobic, it still generates emissions through the breakdown of volatile solids. Volatile solids make up 70% to 80% of TS, made up of in large part by the labile fractions of waste like VFAs, degradable carbohydrates, protein and more (Petersen, 2018). These volatile fractions, including both carbon- and nitrogen-based compounds, are broken down and become gases like CH_4 or NH_3 overtime (Petersen, 2018). The aim of the present experiment was to determine the efficacy of the $CaCN_2$-based manure additive Eminex® on reducing emissions while emulating dry and liquid manure storage.

Previous research using Eminex® employed a closed, anaerobic, temperature-controlled system to measured effects on GHG and NH_3 emissions (Holtkamp et al., 2023). Bottles of dairy cattle slurry collected from a dairy farm in Germany underwent a pre-treatment of gas stripping and kept at 20.2°C and stored for up to 26 weeks, the duration of which mimicked the maximum storage length for slurry in Germany (Holtkamp et al., 2023). Even so, controlling ambient temperature and gas stripping are not part of a normal manure management system. Therefore, this project better represented U.S. manure storage conditions within a research setting.

4.1.1 Dairy Slurry Volatile Fatty Acids and Physical Slurry Characteristics

Eminex® contained high quantities of calcium hydroxide, calcium carbonate, and magnesium carbonate which has an alkalizing effect (Holtkamp et al., 2023), resulting in higher

pH, followed by a decline in pH, which was not truly seen during this project. An acidic pH changed the NH_3-ammonium (NH_4^+) equilibrium (Hristov et al., 2011; Holtkamp et al., 2023), but would not negatively impact the quality of the manure, maintaining its role as fertilizer. However, as the reductions in slurry pH of the present study were not significant and the pH never reached an acidic range, any potential mitigation benefits from acidification were not confirmed.

Alkalinization has also been shown to affect gas production from animal manure. Research found that while alkalinization improved biomass hydrolysis by increasing available soluble nutrients, the accumulation of cations has been shown to disrupt microbial metabolic pathways associated with anaerobic fermentation (Lin et al., 2015; Chen et al., 2023). Furthermore, a study that treated swine slurry with sodium hydroxide (NaOH) found significant accumulation of acetic acid and propionic acid (Lin et al., 2015).

The acetic acid, propionic acid, and butyric acid formed in slurry were end products of microbial fermentation (Hilgert et al., 2022). These short, carbon-chain molecules, within ruminant digestive systems, were vital to proper fermentation and played key roles in hydrogen consumption, glucose formation, and milk fat production (Ungerfeld, 2015; Beauchemin et al., 2020). However, VFAs like acetic acid, were also substrates for methanogenesis (Holtkamp et al., 2023).

Previous work with Eminex® by Holtkamp et al. (2023) noted a significant increase in acetic acid found in treated dairy slurry compared to untreated control slurry, which after 26 weeks of incubation, had less than 1 g acetic acid/kg dairy cow slurry. For the treated slurry groups, when compared to d 0 acetic acid levels, the quantity nearly doubled, indicating an accumulation of acetic acid overtime (Holtkamp et al., 2023). Accumulation of VFAs was also seen in the present study in the Eminex® treated groups ($P < 0.05$; Table 3.1). Due to the initial alkalinization seen

in Holtkamp et al. (2023) and in the present study, microbial hydrolysis was likely enhanced by the alkaline pH, allowing for the production of more VFAs (Lin et al., 2015).

The outcomes from the present student differed from the Holtkamp et al. (2023) VFA results, where all VFAs significantly increased in concentration. In the present study, this was not seen for propionic or butyric acid (Table 3.1). Although concentrations increased, there were no significant differences between the three treatment groups in the present study. It was possible that the differences in VFA concentrations seen in the present study versus that by Holtkamp et al. (2023) related to differences in experimental set up. As previously mentioned, the present study did not employ an anaerobic system or controlled ambient temperature. This allowed for a more accurate representation of a dry manure storage in the U.S. Therefore, it was possible that the experimental setup in Holtkamp et al. (2023) prevented additional degradation of VFAs due to factors like temperature (Hilgert et al., 2022).

The Eminex® treatment also impacted the composition of the manure. Traditionally found in artificial fertilizers, the $CaCN_2$ component of Eminex® was meant to prevent nitrification and inhibit leaching of nutrients into soils following land application (Dixon, 2012; Holtkamp et al., 2023). Following application, the $CaCN_2$ in Eminex® was converted dicyandiamide (DCD) and urea, which was converted to NH_3 and then nitrate (Dixon, 2012). As a nitrification inhibitor, DCD was designed to prevent nitrates from leaching out of the soils and into waterways (Dixon, 2012; Park et al., 2021).

In the present study, there were no treatment effects for TC or TN (Table 3.1). It was possible that some N was still lost via volatilization. The pH for the present study was alkaline (7.79-7.93; Table 3.1) compared to the pH of Holtkamp et al. (2023), which ranged from 6.57-6.61, depending on dose. The N equilibrium between NH_3-NH_4^+, when in alkaline environments,

favored NH_3 formation. However, as TN did not decrease in SL-LD and SL-HD, the N content of the slurry was likely augmented by the urea in Eminex®. Manure composition was also influenced by animal diet from which the manure and urine was collected for the two studies, which could have impacted to slurry composition (Hristov et al., 2011).

4.2 Dairy Slurry Gaseous Emissions

Despite the notable deviations between the effects of Eminex® to the chemical composition of slurry from Holtkamp et al. (2023) versus the present study, the gaseous emissions were still declined with the application of Eminex®.

4.2.1 Carbon Dioxide

Reductions in CO_2 in the present study were lower than the reductions noted by Holtkamp et al. (2023), the latter which ranged from 81% to 99%, depending on dose. The substantial difference in gaseous suppression were likely due to the differences in experimental setup, as previously mentioned. Influence of temperature, wind, and other environmental factors could have inhibited Eminex® from reducing emissions as aggressively as in Holtkamp et al. (2023). However, the present study showed that Eminex® still offered significant reductions in CO_2 in slurry stored outdoors.

However, as CO_2 has a low global warming potential (GWP) compared to CH_4 and N_2O, it is not often a gas targeted for reduction in agriculture. Even so, the reductions in CO_2 seen in the present study showed Eminex® had potential in preventing emissions in dry manure storage systems. Additional research would be needed to confirm on-farm outcomes.

4.2.2 Methane

The reductions in CH_4 following a one-time application of Eminex® to dairy slurry in the present study were less dramatic than the 99% reductions reported by Holtkamp et al. (2023). The lack of temperature control in the present study likely explained the discrepancy in CH_4 reductions compared to Holtkamp et al. (2023). Cárdenas et al. (2021) found that temperature was the most important factors when it came to predicting manure CH_4 emissions, as there was a positive correlation between CH_4 and increasing temperature. Greater ambient temperatures had been shown to accelerate the degradation of VS in manure and enhance gas diffusion potential (Kupper et al., 2020; Qu and Zhang, 2021). The ambient temperature in the present study ranged from 13.1°C to 23.5°C throughout the sampling periods, but the low and high dose Eminex® treatments consistently suppressed CH_4 emissions ($P < 0.05$).

The reduction in CH_4 explained the accumulation of VFAs seen in the present study, as acetic acid could be used for methanogenesis (Barret et al., 2013; Shima et al., 2020; Ungerfeld, 2020). CH_4 acted as a byproduct of microbial fermentation, meant to remove hydrogen (H_2) and allow fermentation to continue (Wattiaux et al., 2019). With the accumulation of acetic acid and decline in CH_4 emissions, it was clear that Eminex® interrupted methanogenesis, as the accumulation of acetic acid likely meant accumulated H_2 and reducing equivalents. What became of these byproducts and end products of required further insight into microbial populations, which is explored in Chapter 4.

Sokolov et al. (2021) reported CH_4 reductions of 52% to 59% when acidifying swine slurry, and a meta-analysis by Kupper et al. (2020) found acidification reduced CH_4 by 61% to 96% across various types of animal slurry. Acidification worked by disrupting the neutral environment required for methanogenesis (Kupper et al., 2020). After alkalization, Eminex® was meant to

lower slurry pH, like acidification to disrupt methanogenesis (Holtkamp et al., 2023), but this was not seen in the present study.

As a short-lived climate pollutant with a half-life of about 10 to 12 years and a GWP_{100} of 27.2, CH_4 remained an important GHG for mitigation (Cain et al., 2019; IPCC, 2021). Its presence in the atmosphere at high concentrations accelerated climate change, whereas preventing its entry into the atmosphere could induce global cooling (Cain et al., 2019; Lynch et al., 2021). While the abatement of CH_4 measured by this study was not as prominent as in Holtkamp et al. (2023), it demonstrated that Eminex® still effectively reduces slurry CH_4 emissions in less controlled settings.

4.2.3 Nitrous Oxide

Holtkamp et al. (2023) reported a 60% reduction in N_2O. However, the present study did not find significant reductions in N_2O with treatment. Additionally, there was more overall N_2O produced in the current study compared to Holtkamp et al. (2023), likely due to the open system which supports the formation of N_2O, as it required both aerobic and anaerobic conditions, and was shaped by environmental and chemical factors like C:N, temperature, and moisture (Pratt et al., 2015).

Most N present in feces were in organic form, whereas in urine, it was 60% to 90% urea (Park et al., 2021). As mentioned, unintentional N losses would lead pollution in soil via acidification and degradation, in atmosphere via volatilization of NH_3 and N_2O emissions, and in groundwater, causing eutrophication from nutrient leaching (Anas et al., 2020; Park et al., 2021). Nitrification inhibitors, which stop urea hydrolysis and NH_3 oxidation, have been shown to be effective at enhancing N use efficiency by delaying nitrification/denitrification (Park et al., 2021).

However, the issue of targeting only on aspect of the environmental pollution associated with manure remained, as these types of inhibitors did not affect carbon emissions.

Park et al. (2021) tested the efficacy of three different urease and nitrification inhibitors, including DCD, on nitrogenous emissions from pig slurry. They found 59.8% reductions in N_2O following a 56 day sampling period (Park et al., 2021), which was significantly longer than the 7 day sampling period of the present study. However, as DCD was also present in Eminex®, it was possible that with a longer sampling time, there might have been a more substantial reduction in N_2O emissions. Holtkamp et al. (2023) also employed a much longer 26-week sampling period. Eminex® could simply require more contact time with slurry to reduce N_2O emissions.

Of the three agricultural GHGs, N_2O had the highest GWP of 273 and an atmospheric lifetime of over 100 years (Fowler et al., 2013; Prather et al., 2015; IPCC, 2021). However, its formation was more complicated, requiring aerobic- anaerobic conditions with nitrification-denitrification taking place (Anas et al., 2020; Khairunisa et al., 2023). The gas formation would take place at the manure crust, where there existed a liquid-air interface in slurry, which would ensure the necessary conditions for N_2O formation (Kupper et al., 2020).

4.2.4 Ammonia

Holtkamp et al. (2023) noted an initial increase in NH_3 losses. As a gas, NH_3 contributed to the formation of particulate matter 2.5 ($PM_{2.5}$), capable of carrying pathogens deep into lung tissue and causing serious human health issues (Wattiaux et al., 2019). The use of Eminex® in the present study significantly reduced NH_3 losses ($P < 0.05$). The majority of NH_3 emissions occurred in the first two days of storage, as expected given the mixing of manure and urine to combine urease and urea, that was hydrolyzed to NH_3 (Wattiaux et al., 2019).

Higher temperatures and wind/air velocity also influenced NH_3 losses (Grant and Boehm, 2015). Because the bowls containing slurry were partially protected by the FCs from wind, air velocity did not contribute to significant changes in levels of NH_3 emissions. Crusting was another physical factor that reduced gaseous emissions from manure, and was also related to lower NH_3 emissions depending on what proportion of the surface was covered by a crust (Grant and Boehm, 2015). By the end of the sampling period, the slurry had formed thin crusts. This could have artificially decreased NH_3, as the gas was unable to escape. However, this was likely not the case, as shown in Figure 3.5. The crust of all bowls was broken on d 4 to allow for sampling to occur. NH_3 emissions from SL-LD and SL-HD did not significantly increase in the days that followed, as SL-CONT maintained higher emissions for the rest of the sampling period.

Other options for reducing NH_3 included acidification and certain inhibitors. Acidification reduced NH_3 by 83% (Hou et al., 2014) while urease inhibitors reduced NH_3 by 16.3% (Park et al., 2021). It was possible that had the slurry pH decline as expected, NH_3 reductions could have been higher, due to the expected acidification and nitrification inhibitor potential of Eminex®. Further research is needed to confirm this.

4.2.5 Ethanol

The last gas monitored in the present study was ethanol (EtOH). These emissions significantly increased with treatment ($P < 0.05$). As a carbon-based molecule (CH_3CH_2OH), the increased EtOH losses explained why, despite reductions in both CO_2 and CH_4 emissions, the TS and TC of SL-LD and SL-HD decreased over time ($P < 0.05$).

While EtOH is not a GHG, it still has negative environmental impacts. To start, EtOH is part of a larger group of compounds called volatile organic compounds (VOC), which included

other alcohols, aldehydes, ketones, ester, ethers, aromatic hydrocarbons, halogenated hydrocarbons, terpenes, amines, and a variety of other carbon and nitrogen containing compounds (Filipy et al., 2006). Such compounds were often associated with malodor from manure as well as tropospheric ozone (O_3) formation (Filipy et al., 2006; Shaw et al., 2007; El-Mashad et al., 2010; Yuan et al., 2017). O_3 formed when VOCs react with nitrous oxides and sunlight in the atmosphere and EtOH was a VOC capable of forming O_3 precursors (Shaw et al., 2007; Willey et al., 2019; Lu et al., 2021).

Furthermore, it was previously mentioned that suppression of CH_4 resulted in an accumulation of H_2 in slurry. Buildup of H_2 and reducing equivalents would negatively impact microbial fermentation (Ungerfeld, 2015, 2018, 2020; D'Silva et al., 2021). Eminex® could've forced microbes to find alternative hydrogen sinks to continue fermentation and inadvertently augmented EtOH emissions. Unfortunately, this increase in EtOH represented pollution swapping, which was defined as the implementation of environmental mitigation measures meant to reduce one pollutant resulting in the increase of another pollutant (Stevens and Quinton, 2009). The equipment used in the present study did not have the capacity to measure other such VOCs, so it would be prudent for future research to assess how other Eminex® affected other VOCs.

4.3 Dairy Lagoon Water

Prior to this experiment, Eminex® had never been tested in a liquid manure storage, which was one of the most popular forms of manure storage in the U.S. and in the state of California. Therefore, the aim of the second experiment was to determine the efficacy of Eminex® in mitigating GHG and NH_3 emissions. Unlike in slurry, EtOH was not detected in sufficient quantity and will not be further discussed.

4.3.1 Dairy Lagoon Water Volatile Fatty Acids and Physical Parameters

In the present study, a one-time application of Eminex® improved the composition of lagoon water, resulting in more TC compared to untreated lagoon water ($P < 0.05$; Table 3.3). As lagoon water would be used as fertilizer (Meyer et al., 2011; Niles and Wiltshire, 2019; Niles et al., 2022), increasing available nutrients for plants would benefit farmers. Similar results were noted by Holtkamp et al. (2023) after treating dairy slurry with Eminex®.

Our study showed an effect of treatment on TN in lagoon water ($P < 0.05$; Table 3.3), likely due to the urea in Eminex® (Dixon, 2012; Park et al., 2021; Holtkamp et al., 2023). However, the NH_3 losses over time seen in Table 3.4, should have decreased the lagoon water TN. However, between treatments, LW-HD and LW-LD still contained higher amounts of TN compared to LW-CONT ($P < 0.05$).

Like the slurry results of the present study, acidification of lagoon water was also not seen, unlike in previous research with Eminex® (Holtkamp et al., 2023). It was possible that the open experimental set-up, temperature, and diluted wastewater prevented the additive from changing the lagoon water pH as previously seen. However, Holtkamp et al. (2023) incubated their slurry for 26 weeks with continuous pH monitoring. While continuous measurements were not possible in the current study due to equipment limitations, it was possible that the lagoon water and slurry could have acidified over time. But Eminex® was still effective following alkalinization and significantly suppressed GHGs. Increased NH_3 emissions will be further discussed below (section 4.4.4)

The concentration of VFAs was also of interest. Holtkamp et al. (2023) noted an accumulation of the three primary VFAs in dairy slurry. The present study saw increased acetic

acid concentration. Previous research noted the acetic acid was often the most abundant VFA in manure (Page et al., 2015), which was also noted in the present study for slurry and lagoon water. Reducing CH_4 should have caused an accumulation of VFAs, as acetoclastic methanogens used acetate as a substrate for CH_4 formation (Berghuis et al., 2019; Ungerfeld, 2020). The other VFAs, like propionic acid and butyric acid, were not present in detectable concentrations. This could have contributed to the alkaline pH, as higher concentrations of VFAs tend to decrease pH (Atasoy and Cetecioglu, 2022; Holtkamp et al., 2023). Additionally, this study took place during summer in California, and higher ambient temperatures could have caused VFAs to degrade faster in the lagoon water and therefore be undetectable (Page et al., 2015).

4.4 Gaseous Emissions

Uncovered lagoons had a greater potential for high emissions due to a greater ambient air turbulence over a larger surface area than other manure storage systems (Kupper et al., 2020). In the present study, all three GHGs decreased with treatment.

4.4.1 Carbon Dioxide

As shown in Table 3.4 and Figures 3.7a-b, Eminex® decreased CO_2 emissions from lagoon water over time ($P < 0.05$). The suppression of carbon-based GHGs likely resulted in a greater TC content seen in LW-LD and LW-HD compared to LW-CONT ($P < 0.05$). Compared to other manure additives like SOP Lagoon, Eminex® performed similarly. There was a 12.0% reduction for LW-HD compared to LW-CONT ($P < 0.1$). This reduction was similar to the 14.7% reduction in CO_2 seen when the manure additive SOP Lagoon was applied to lagoon water (Peterson et al.,

2020). SOP Lagoon efficacy was even better on-farm, showing CO_2 reductions up to 75% (Chiodini et al., 2023).

The fluctuations in CO_2 (Figure 3.7a-b) likely contributed to the more alkaline pH, as CO_2 has been shown to increase surface pH in waste (Hristov et al., 2011). Raising the pH at the manure air interface has also been shown to enhance NH_3 losses (Petersen, 2018). The fluctuations in CO_2 emissions were likely caused by the experimental setup. The FCs covering the barrels provided minimal cover. In uncovered liquid manure, heat and wind turbulence affected mass transfer between dissolved organic carbon, leading to steep concentration gradients and greater O_2 consumption (Petersen, 2018). With a sufficient O_2 present at the surface levels of manure, aerobic microorganisms were found to be capable of transforming CH_4 into CO_2 via oxidation (Møller et al., 2004; Peterson et al., 2020).

4.4.2 Methane

High ambient temperatures did not negatively impact Eminex®'s ability to reduce emissions. At higher temperatures, volatile solids in manure degraded faster, leading to greater CH_4 emissions (Meyer et al., 2019; Cárdenas et al., 2021; Hilgert et al., 2022). Eminex® had previously been tested in temperature-controlled settings at 20.2ºC (Holtkamp et al., 2023). The present study occurred at ambient temperature from June through August in California with temperatures spiking to 35ºC.

Previous research with Eminex® measured 99% CH_4 reductions following a 26-week incubation (Holtkamp et al., 2023). This was greater than the 85.1% CH_4 reduction in the present study noted in LW-HD ($P < 0.05$). However, it must be kept in mind that Holtkamp et al. (2023) severely controlled slurry environment, providing ideal conditions for CH_4 mitigation. The present

study did not control environmental factors and collected lagoon water a few hours before treatment application. The significant CH_4 reductions of the present study for the two Eminex® treatments proved it still effectively prevents gas production at high ambient temperatures and in aqueous conditions.

Other manure treatments to reduce CH_4 include acidification and SOP Lagoon. Acidification disrupted the neutral environment ideal for methanogenesis (Sokolov et al., 2021). Sokolov et al. (2021) treated liquid manure with 0.03 mL H_2SO_4 and CH_4 emissions decreased by about 52% to 59%. In the present study, CH_4 decreased by 85.1% for LW-HD, making it comparatively more potent ($P < 0.05$). SOP Lagoon measured 80% CH_4 reductions when applied to liquid manure on a commercial farm (Chiodini et al., 2023). Eminex® has yet to be tested in a normal lagoon, which receives constant inflow of fresh excreta from barns throughout the day. Therefore, it is important to assess whether Eminex® can maintain its potent gas reductions in a commercial lagoon setting.

The AMMP program recommended swapping from flush to scrape manure systems to reduce water consumption and reduce CH_4 by storing manure under aerobic conditions or sun dried (Ross et al., 2021; El Mashad et al., 2023). However, research measuring GHG and NH_3 emissions from dairy manure collection via scraping versus flushing showed that while scraping reduced CH_4, NH_3 flux increased by 152% to 175% (Ross et al., 2021).

4.4.3 Nitrous Oxide

The LW-HD treatment decreased N_2O emissions by 82.6% ($P < 0.05$). Holtkamp et al. (2023) measured reductions of 60% in dairy slurry, but reported minimal overall emissions of N_2O. Since N_2O requires oxygen to form (Wattiaux et al., 2019; Park et al., 2021), the anaerobic systems

used by Holtkamp et al. (2023) likely prevented N_2O formation and potentially prevented Eminex® from suppressing N_2O more substantially.

Other manure treatments helped in reducing nitrogenous emissions. Nitrification inhibitors, like DCD, prevented urea hydrolysis and NH_3 oxidation. This effectively enhanced nitrogen use efficiency by delating nitrification/denitrification, resulting in N_2O reductions up to 59% (Park et al., 2021; Yang et al., 2022). However, inhibitors were designed only to target nitrogenous emissions, which would result in continued losses of CO_2 and CH_4. In addition to more aggressive N_2O reductions compared to nitrification/urease inhibitors, Eminex® also targeted carbon-based GHGs, making it a better intervention for farmers.

4.4.4 Ammonia

In the present study, NH_3 emissions increased by 65.7% with Eminex® application ($P < 0.05$). NH_3 was also an indirect source of N_2O emissions, after being oxidized to nitrites (Fowler et al., 2013; Petersen, 2018; Anas et al., 2020). Holtkamp et al. (2023) reported increased NH_3 emissions (+50% to 200%), followed by 60% reductions at the end of the 26-week incubation.

Other studies showed that wind speed and temperature explained almost 60% of variation in NH_3 losses, with up to 35% more NH_3-N loss in warmer climates (Petersen, 2018). In fact, Yang et al. (2022) calculated a strong positive correlation between NH_3 and temperature. Increasing temperature and wind speed enhanced emissions since this directly affected diffusion and convection of gases near emitting surfaces (Kupper et al., 2020). Because the present study housed the lagoon water at ambient temperature and exposed to wind, it was possible that climactic factors resulted in increased volatilization.

Like temperature, the pH of the lagoon water was an important factor affecting NH_4^+-NH_3 equilibrium in aqueous environments (Hristov et al., 2011). Research showed that acidic pH favored NH_4^+ formation over NH_3, while alkaline pH shifted the equilibrium toward NH_3 volatilization, with the greatest release occurring between pH 7-10 (Hristov et al., 2011). The pH for Eminex® treated groups were 7.77-7.79 (Table 3.3), favoring NH_3 volatilization. Additionally, increased NH_3 losses contribute to N_2O reductions, as the reactive N capable of becoming N_2O was prematurely emitted as NH_3 (Chadwick et al., 2011; Wattiaux et al., 2019).

Using additives were but one of many options when it came to reducing NH_3 emissions from manure. Animal diets were said to have equally important contributions to nitrogenous content in feces and urine (Hristov et al., 2011). A meta-analysis from Hou et al. (2014) found that lowering crude protein in animal diets decreased NH_3 emissions by 24% to 65%. Dietary manipulation would therefore open another route of preemptive nutrient management to prevent future N pollution.

Given that increased NH_3 volatilization was not seen in the present study when applying Eminex® to slurry, it is possible that treating manure prior to flushing into a lagoon could potentially circumvent this issue.

4.5 Future of Eminex®

Eminex®'s ability to target all GHGs and NH_3 make it ideal to manure-based emissions. Furthermore, its efficacy across different types of manure management would open it up for use by almost all dairy farmers. Eminex® is easy to apply, existing as a granulated powder than can easily poured into a lagoon or settling basin. Its weakness lies in the high manufacturer

recommended dose of 1 kg Eminex®/m^3 manure and cost. Eminex costs about €3/kg or $3.29/kg (personal communication).

At the manufacturer recommended dose, a 1,000 head dairy with a 95,000 m^3 lagoon would require 95 tons of Eminex® and cost the farmer $312.55/animal. As shown in slurry and lagoon water, there were no significant differences between the high and low dose, meaning that substantial reductions in emissions can still be achieved with lower, more practical doses. Research is needed to determine how low the dose can be while maintaining aggressive emissions reductions.

5 CONCLUSION

Eminex® demonstrated consistent, strong mitigative effects for GHGs in slurry and lagoon water. CO_2, CH_4, and NH_3 were significantly reduced when fresh dairy slurry was treated with Eminex® at two different doses. However, N_2O was unchanged and EtOH significantly increased. In lagoon water, CO_2, CH_4, and N_2O all significantly decreased in emission rate with Eminex® treatment. However, NH_3 significantly increased. For both slurry and lagoon water, the quality of waste also improved following the application of treatment, through increased TN, TC, and TS. It is possible that changing when Eminex® is applied, like during manure collection or prior to manure entering a lagoon, could potentially offset the high NH_3 emissions seen in lagoon water and the high EtOH seen in slurry.

Furthermore, Eminex® showed gas reductions at 50% of the manufacturer recommended dose, with no significant differences between the treatment levels. It would be prudent to test its efficacy again on a commercial farm manure management system to quantify emissions reductions. Even so, Eminex® remains a potent option for farmers to continue reducing GHG

emissions associated with manure management in the dairy sector. Further research should establish lower doses to minimize labor and financial costs. The next chapter will elucidate how Eminex® affects the microbial populations present in fresh dairy slurry and lagoon water.

Chapter 4 – Comparing Microbiomes of Fresh Dairy Slurry and Dairy Lagoon Water under the Effects of the Manure Additive, Eminex®

ABSTRACT

Manure additives like Eminex® have been shown to effectively reduce greenhouse gas (GHG) and ammonia (NH_3) emissions from slurry and lagoon water. However, the impacts of manure additives on the microbiome of manure is not often assessed. The aim of the present study was to quantify how Eminex® changed the microbiome of dairy slurry and dairy lagoon water once receiving Eminex®, dosed at low (0.5 kg Eminex®/m^3 manure) and high (1.0 kg Eminex®/m^3 manure) levels, for both forms of manure. Samples were collected from fresh slurry and dairy lagoon water and underwent DNA extraction prior to being sent out to an independent laboratory for shallow shotgun metagenomic sequencing (SSMS). Results of the SSMS show that the relative abundance of the phylum of Proteobacteria decreased with Eminex® treatment in lagoon water, but Proteobacteria increased in relative abundance with Eminex® treatment in slurry. Other phyla, like Firmicutes and Actinobacteria increased in relative abundance with Eminex® in lagoon water, but not in slurry. Pathogenic phyla, like Fusobacteria, did not increase in relative abundance with Eminex® treatment in slurry, but increased substantially in untreated slurry. A principal component analysis (PCA) was also performed and confirmed distinct microbiomes between slurry and lagoon water. Additionally, the PCA also noted that the high dose Eminex® treatment elicited a faster microbiome change as compared to the low Eminex® dose. This suggested that Eminex® could be applied more effectively earlier on in the manure management chain. Lastly, a linear discriminatory analysis showed that bacterial populations were at their highest in the two Eminex® treatments at d 28, but only highest in the control by d 56, indicating that the untreated populations continued to grow throughout the experiment. Ultimately, the Eminex® treatment resulted in changes to the manure microbiome, helping to explain how this additive reduces GHG and NH_3 emissions. However, delving into the microbiome also elucidated

that the Eminex® doses can be reduced and still be effective when applied at earlier stages of manure management. Future research would benefit from exploring the metabolomics associated with microbes exposed to Eminex® and exploring the effects of different treatment protocols of emissions and the manure microbiome.

1 INTRODUCTION

The natural decomposition of the labile fractions of manure releases greenhouse gases (GHG) like carbon dioxide (CO_2) and methane (CH_4) (Petersen, 2018). The breakdown of organic matter within manure is mediated by the presence of bacteria. Some of these microbes are natural inhabitants of an animal's gastrointestinal tract while others exist more regularly in the soil or air. Regardless, they all play a part in breakdown of organic matter and the generation of GHGs. Manure and urine also contain large amounts of N. The N cycle, through which reactive N transforms into various molecular states, can result in ammonia (NH_3) and nitrous oxide (N_2O) (Chadwick et al., 2011; Fowler et al., 2013).

Manure management is a hotspot of GHG emissions in U.S. dairy production, responsible for about 10% of total U.S. CH_4 and 75% of total U.S. N_2O emissions (EPA, 2023c). To reduce these deleterious emissions and their significant environmental impact, there must be a fundamental understanding of the microbiome of manure in its major storage forms (i.e., dry and liquid).

As it stands, researchers have recently started exploring the microbiome of fresh dairy slurry and lagoon water. Research has primarily used 16s rRNA amplification to identify present bacterial and archaeal populations (Dungan and Leytem, 2014; Pandey et al., 2018). While it remains an important tool to quantifying microbial populations, there are issues associated with amplification bias and the cost associated with having to amplify more than one domain (Hillmann et al., 2018).

Beyond that, research has yet to investigate the impacts of manure additives on manure microbial populations. While several studies have been published identifying potent additives capable of decreasing GHGs and NH_3 emissions, none have yet determined the impact of such

additives on the microbiome (Cluett et al., 2020a; Park et al., 2021; Chiodini et al., 2023; Holtkamp et al., 2023). By ignoring the impacts these additives have on the manure microbiome, valuable knowledge and insight into pathogenicity, future emission potential, and soil interactions are lost.

The aim of the present study was to establish a baseline microbiome population in fresh dairy slurry and dairy lagoon water using shallow shotgun metagenomic sequencing as well as to determine what the effects of Eminex® are on the microbial populations. It is hypothesized that while the core populations of these two forms of excreta will be similar, there will be significant differences that may explain different gas forming potentials and that the population structure will be fundamentally changed by Eminex® by selecting against microbes involved in gas production.

2 MATERIALS AND METHODS

2.1 Slurry Collection and Experimental Set Up – Experiment 1

Dairy feces and urine were collected at the UC Davis Dairy Research and Teaching Facility over two days from 15 lactating dairy cows (days in milk: 102 d; avg. milk yield: 40.8 kg/d). Collected urine and feces was pooled, combined at a ratio of 1.7:1.0 feces:urine (Hristov et al., 2011), and homogenized for 60 seconds to make a slurry. Day 0 samples were collected from the stock slurry mix and frozen at -80 ºC prior to further analysis. The two treatments (low, SL-LD; high, SL-HD) and a control (SL-CONT; no Eminex®) were used, with each group containing four bowls (n = 4). Samples were collected again on d 7, and frozen and stored at -80ºC prior to further analysis.

2.2 Lagoon Water Collection and Experimental Set Up – Experiment 2

Lagoon water was collected from an uncovered lagoon on a commercial dairy farm. Lagoon water was pumped from the lagoon via a trash pump into a plastic tote and transported to the UC Davis Feedlot Research Facility. Lagoon water samples were collected on d 0 and frozen at -80 ºC prior to further analysis. 208-L stainless steel barrels were filled with lagoon water and randomly assigned a specific treatment. There were two experimental treatments and one control (n = 4/group): control (LW-CONT; no additive), low (LW-LD; 0.5 kg Eminex®/m^3 lagoon water), and high (LW-HD; 1 kg Eminex®/m^3 lagoon water). The high dose was the manufacturer recommended dose for Eminex®. Barrels were sampled on each barrel's respective d 14, 28, and 56. These samples were frozen and stored at -80ºC prior to further analysis. Additional details on the experimental set up are provided in section 2, chapter 3.

2.3 Shallow Shotgun Metagenomic Sample Preparation

Microbial samples were DNA extracted using a Quick-DNA Fecal/Soil Microbe Mini Prep Kit (Zymo Research Co.), following kit instructions. Extracted DNA was subsequently suspended in TE buffer and frozen prior to being sent out in dry ice to Novogene for shallow shotgun metagenomic sequencing (SSMS; Novogene Corporation Inc., China). This project opted for SSMS over 16s rRNA genome amplicon sequencing to avoid amplification bias and as it is limited in the information 16s rRNA sequencing can provide across different taxonomic levels (Hillmann et al., 2018). Quality of DNA samples were assessed via Quibit Fluorometer and standard agarose gel electrophoresis by Novogene. The sections below were provided by Novogene as an overview of sample processing, sequencing, and statistical analysis.

2.4 Library Construction

A total of 1 μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were developed using NEBNext® Ultra™ DNA Library Prep kit for Illumina (NEB, USA), following manufacturer recommendations, with index codes added to attribute sequences to each sample. Qualified DNA were randomly fragmented to 350 bp size using a Covaris ultrasonicator.

DNA fragments subsequently underwent end repair, dA tailing, adaptor ligation, and purification/PCR purification. Resulting libraries were again assessed by Quibit Fluorometer, then diluted to 2 ng/μl and further analyzed for size distribution by Agilent 2100 Bioanalyzer. Libraries were quantified again using real-time PCR, aiming for a final concentration >3nM.

2.5 Sequencing and Statistical Analysis

Qualified libraries were pooled by library concentration and desired read depth. The clustering of the index-coded samples was performed on a cBot Cluster Generation System, according to manufacturer instructions. Pooled libraries were then sequenced using the Illumina NovaSeq 6000 with paired end 150 bp (PE 150) strategy. Kraken 2 was used to characterize the taxonomic composition of the SSMS samples. Kraken is the metagenomic sequence classification software launched in 2013, with Kraken 2 (v2.0.7-beta) launched in August 2018. The procedure went as follows: the double-end clean reads were combined for each metagenomics sample and converted into fasta sequences. The Kraken2 software was used to compare and annotate the fasta sequence obtained in the previous step. The comparison results for each sample was integrated and species abundance was performed. Statistical analysis included MetaStats and was performed with the 'pheatmap' package, principal component analysis (PCA) was performed with the

'FactoMineR' package; and linear discriminatory analysis (LDA) was performed with the 'MASS' package in the statistical software R across all taxonomic levels (R Core Team, 2021), and differences were determined to be significance at $P < 0.05$ and trends at $P < 0.1$.

3 RESULTS

Bioinformatics results are presented below. The core of the analysis focused on intergroup differences, PCA, and LDA. The aim was to explain and explore the differences between the microbiome on d 0 and d 7 for slurry and d 0 and d 56 lagoon water. Furthermore, the experiments needed to explain the impact of Eminex® on the microbiome in these two forms of stored excreta. All results are exclusively presented at two taxonomic levels: genus and phylum. This offers insight at a high (phylum) and low (genus) level of taxonomic organization.

3.1 Fresh Dairy Slurry versus Dairy Lagoon Water

The chemical composition of fresh dairy slurry and dairy lagoon water are presented in Table 4.1. Overall, fresh dairy slurry had higher total solids (TS), total carbon (TC) and total nitrogen (TN) compared to the dairy lagoon water. Conversely, the dairy lagoon water had a lower, more neutral pH compared to fresh dairy slurry. And the fresh dairy slurry had higher, detectable levels of volatile fatty acids (VFA) compared to dairy lagoon water, for which only lactic acid (not considered a VFA) were present in detectable levels on d 0.

Table 4.1 Chemical composition of the fresh dairy slurry and dairy lagoon water

Parameters	Fresh dairy slurry	Dairy lagoon water
Total Solids, %DM	13.2	0.42
Total Carbon, %DM	44.6	0.03
Total Nitrogen, %DM	0.57	0.2
pH	8.03	7.38
Lactic Acid, ppm	99.6	2.67
Acetic Acid, ppm	2,863	--
Propionic Acid, ppm	661.4	--
Butyric Acid, ppm	55.8	--
Isobutyric Acid, ppm	402.7	--

Values presented are least squared means of each parameter. Rows with '--' indicate lack of data given those volatile fatty acids were not found to be present in sufficient levels to be detectable.

The SSMS identified 3,600 species across 2 domains, 3 kingdoms, 39 phyla, 78 classes, 173 orders, 380 families, and 1,182 genera. The two main domains were Archaea and Bacteria, with the kingdoms divided into Archaea, Bacteria, and Viruses.

The relative abundance of the top phyla in both forms of manure are presented in Table 4.2. These means represent d 0 values based on samples taken after slurry mixing and lagoon water collection and prior to treatment start. There was a greater relative abundance in the phyla Bacteroidetes ($P = 0.04$) and Fusobacteria ($P = 0.001$) in fresh dairy slurry compared to dairy lagoon water. Comparatively, Proteobacteria ($P = 0.0001$), Tenericutes ($P = 0.005$), Spirochaetes ($P = 0.0005$), Verrucomicrobia ($P < 0.0001$), and Deinococcus-Thermus ($P = 0.0023$) had higher relative abundance in lagoon water compared to slurry. Only three phyla showed significant differences in slurry versus lagoon water, including Euryarcheota, the only prominent archaeal phylum.

Table 4.2 Mean relative abundance at the phylum level for fresh dairy slurry and dairy lagoon water on day 0

Phylum	Fresh dairy slurry	Dairy lagoon water	*P*-value
Proteobacteria	12.64	61.91	<0.0001
Firmicutes	51.32	0.17	0.0027
Actinobacteria	23.69	19.38	NS
Euryarchaeota	5.08	2.91	NS
Bacteroidetes	4.19	2.77	0.0469
Tenericutes	0.55	0.83	0.0050
Spirochaetes	0.33	1.65	0.0005
Cyanobacteria	0.59	0.59	NS
Verrucomicrobia	0.12	0.39	<0.0001
Fusobacteria	0.36	0.18	0.0010
Deinococcus-Thermus	0.14	0.38	0.0023

Values are mean relative abundance as percentages of the top phyla, for both dairy slurry and lagoon water. NS = not significant ($P > 0.05$).

3.2 Relative Abundance with Eminex® Treatment

Eminex® was applied at two different treatment levels, used in slurry and lagoon water. Results for the average relative abundance of the top ten phyla and top ten genera for lagoon water are presented in Table 4.3 and Table 4.4, respectively. These values compared relative abundane for each group on d 0 versus d 56. The top ten phyla and top ten genera for slurry are presented in Table 4.5 and Table 4.6, respectively. These values compared d 0 to d 7 relative abundance for each group.

3.2.1 Lagoon Water Relative Abundance

Of the most abundant phyla isolated from lagoon water, there were significant differences for LW-HD across all the phyla when comparing relative abundances on d 0 to d 56. The phyla Proteobacteria, Euryarchaeota, Bacteroidetes, Spirochaetes, Cyanobacteria, and Verrucomicrobia significantly decreased in relative abundance for LW-HD on d 56 ($P < 0.05$). For LW-LD on d 56,

Proteobacteria, Euryarchaeota, Bacteroidetes, Cyanobacteria, Verrucomicrobia, and Deinococcus-Thermus decreased in relative abundance ($P < 0.05$). For LW-CONT on d 56, the phyla differed, with significant decreases seen instead in Actinobacteria and Tenericutes, in addition to other previously mentioned phyla (Table 4.3).

Table 4.3 Mean relative abundance of the top ten phyla in lagoon water

Phyla	Day 0	Day 56			P-values		
		LW-CONT	LW-LD	LW-HD	LW-CONT	LW-LD	LW-HD
Proteobacteria	61.91	69.90	57.60	53.68	0.002	0.001	0.003
Firmicutes	0.17	6.43	7.88	11.58	NS	NS	0.001
Actinobacteria	19.38	15.75	25.94	25.58	0.003	0.012	0.005
Euryarchaeota	2.91	1.21	2.31	1.95	0.0002	0.037	0.003
Bacteroidetes	2.77	3.63	2.05	2.18	0.003	0.001	0.004
Tenericutes	0.83	0.40	0.72	1.10	0.001	NS	0.003
Spirochaetes	1.65	0.63	1.28	0.35	0.0001	NS	0.002
Cyanobacteria	0.59	0.36	0.38	0.15	0.0006	0.0005	0.001
Verrucomicrobia	0.39	0.20	0.20	0.27	0.0003	0.0001	0.0001
Deinococcus-Thermus	0.38	0.26	0.31	1.26	0.001	0.003	0.003

Table presents the mean relative abundance of the top ten phyla identified from lagoon water samples. Significant differences are indicated by $P < 0.05$ and trends at $P < 0.1$. NS = not significant ($P > 0.05$).

There were also significant increases in relative abundances noted across the three different treatment groups (Table 4.3; $P<0.05$). For LW-HD, Firmicutes, Actinobacteria, Tenericutes, and Deinococcus-Thermus all increased in relative abundance ($P < 0.05$). For LW-LD, only Actinobacteria significantly increased in relative abundance ($P = 0.012$). And for LW-CONT, Proteobacteria ($P = 0.002$) and Bacteroidetes ($P = 0.003$) increased in relative abundance.

Table 4.4 Mean relative abundance of the top ten genera from lagoon water

Genera	Day 0	Day 56			P-values		
		LW-CONT	LW-LD	LW-HD	LW-CONT	LW-LD	LW-HD
Bifidobacterium	0.81	0.18	0.28	0.32	0.0001	<0.0001	0.0006
Escherichia	0.25	0.93	0.84	0.20	NS	NS	NS
Jeotgalicoccus	0.03	0.009	0.007	0.01	0.0007	0.0008	0.001
Nitrosomonas	0.04	11.1	0.04	0.04	0.016	NS	NS
Pseudomonas	4.77	4.28	4.19	3.75	0.043	NS	0.001
Fusobacterium	0.11	0.04	0.04	0.05	0.0003	<0.0001	0.0001
Corynebacterium	1.49	0.52	1.06	1.04	0.0002	<0.0001	0.002
Jeotgalibaca	0.03	0.008	0.009	0.01	0.0005	0.0001	0.006
Sporosarcina	0.02	0.04	0.07	0.23	0.009	NS	0.0002
Thauera	7.90	1.73	1.12	0.85	0.0005	0.001	0.0008

Table presents the mean relative abundance of the top ten genera identified from lagoon water samples. Significant differences are indicated by $P < 0.05$ and trends at $P < 0.1$. NS = not significant ($P > 0.05$).

At the lower level of taxonomic organization, the top ten genera identified by SSMS in lagoon water are presented in Table 4.4. There were significant changes in relative abundance of the genera with treatment. When comparing LW-HD d 56 to d 0, *Bifidobacterium*, *Jeotgalicoccus*, *Pseudomonas*, *Fusobacterium*, *Corynebacterium*, *Jeotgalibaca*, and *Thauera* all significantly decreased in relative abundance ($P < 0.05$).

Comparing LW-LD d 56 to d 0, *Bifidobacterium*, *Jeotgalicoccus*, *Fusobacterium*, *Corynebacterium*, *Jeotgalibaca*, and *Thauera* are decreased in relative abundance ($P < 0.05$). For LW-CONT d 56 versus d 0, *Bifidobacterium*, *Jeotgalicoccus*, *Pseudomonas*, *Fusobacterium*, *Corynebacterium*, *Jeotgalibaca*, and *Thauera* significantly decreased in relative abundance ($P < 0.05$).

As for increases in relative abundance, the Eminex® treatment groups LW-HD and LW-LD on d 56 only had significant increases in the genus *Sporosarcina* ($P < 0.05$). On the other hand,

the relative abundance of *Nitrosomonas* ($P = 0.016$) and *Sporosarcina* ($P = 0.009$) increased in LW-CONT on d 56.

3.2.2 Slurry Relative Abundance

The dairy slurry samples were also assessed via SSMS, with the top ten phyla presented in Table 4.5. The mean relative abundance of the top phyla identified in dairy slurry, when comparing SL-HD on d 7 to d 0 saw significant decreases in the relative abundance of Bacteroidetes ($P = 0.013$). There were no other significant changes to relative abundance with the high dose Eminex® treatment.

Table 4.5 Mean relative abundance of the top ten phyla from fresh slurry

Phyla	Day 0	Day 7			P-values		
		SL-CONT	SL-LD	SL-HD	SL-CONT	SL-LD	SL-HD
Proteobacteria	12.64	30.24	27.38	20.36	0.001	<0.0001	NS
Firmicutes	51.32	29.06	41.25	45.69	NS	NS	NS
Actinobacteria	23.69	23.09	20.09	24.13	NS	NS	NS
Euryarchaeota	5.08	3.05	4.67	3.78	NS	NS	NS
Bacteroidetes	4.19	2.37	2.13	2.05	NS	0.026	0.013
Tenericutes	0.55	0.37	0.44	0.61	NS	NS	NS
Spirochaetes	0.33	0.21	0.26	0.27	NS	NS	NS
Cyanobacteria	0.59	0.38	0.42	0.51	0.004	0.010	NS
Verrucomicrobia	0.12	0.07	0.09	0.09	NS	NS	NS
Fusobacteria	0.36	10.52	2.41	1.63	0.026	NS	NS

Table presents the mean relative abundance of the top ten phyla identified from fresh slurry samples. Significant differences are indicated by $P < 0.05$ and trends at $P < 0.1$. NS = not significant ($P > 0.05$).

For SL-LD on d 7 compared to d 0, there were significant decreases in Bacteroidetes ($P = 0.026$) and Cyanobacteria ($P = 0.01$). As for SL-CONT on d 7 versus d 0, there were significant decreases in only Cyanobacteria ($P = 0.004$).

The relative abundance also increased across the treatment groups. For Eminex® treatment, SL-LD on d 7 compared to d 0, Proteobacteria significantly increased in mean relative abundance ($P < 0.001$). As for SL-CONT on d 7 compared to d 0, Proteobacteria ($P = 0.001$) and Fusobacteria ($P = 0.026$) increased significantly in mean relative abundance.

Table 4.6 Mean relative abundance of the top ten genera from fresh slurry

Genera	Day 0	Day 7			P-values		
		SL-CONT	SL-LD	SL-HD	SL-CONT	SL-LD	SL-HD
Bifidobacterium	15.56	10.57	10.61	12.03	0.02	NS	NS
Jeotgalicoccus	7.04	0.32	0.07	0.45	0.06	NS	NS
Methanobrevibacter	4.19	2.65	4.07	3.12	0.09	NS	NS
Sporosarcina	4.13	0.08	0.11	0.16	NS	NS	NS
Bacillus	3.02	1.31	1.67	2.26	NS	0.07	NS
Clostridium	3.01	2.84	3.25	3.40	NS	NS	NS
Olsenella	2.92	1.88	2.59	3.51	NS	NS	NS
Staphylococcus	2.73	0.95	1.57	3.57	NS	NS	NS
Carnobacterium	2.65	0.35	0.60	0.86	NS	NS	NS
Lactobacillus	2.15	1.17	1.57	2.41	NS	NS	NS

Table presents the mean relative abundance of the top ten phyla identified from fresh slurry samples. Significant differences are indicated by $P < 0.05$ and trends at $P < 0.1$. NS = not significant ($P > 0.05$).

The mean relative abundance of the top ten genera from fresh dairy slurry are presented in Table 4.6. For SL-HD, there were no significance changes in relative abundance of any of the identified genera. For SL-LD, the relative abundance of *Bacillus* tended to decrease from d 0 to d 7 ($P = 0.07$). For the untreated control, there was a decrease in relative abundance of *Bifidobacterium* ($P = 0.02$). There were also trends noted for decreasing relative abundance in *Jeotgalicoccus* ($P = 0.06$) and *Methanobrevibacter* ($P = 0.09$) between d 0 and d 7 for SL-CONT.

3.3 Principal Component Analysis

PCA explored how well specific components explained the variation seen within a data set. It also allowed for complicated, dynamic datasets to be presented in two dimensions while preserving as much statistical information as possible (Jolliffe and Cadima, 2016). Two PCA plots are presented below, combining sample groupings for fresh dairy slurry and dairy lagoon water.

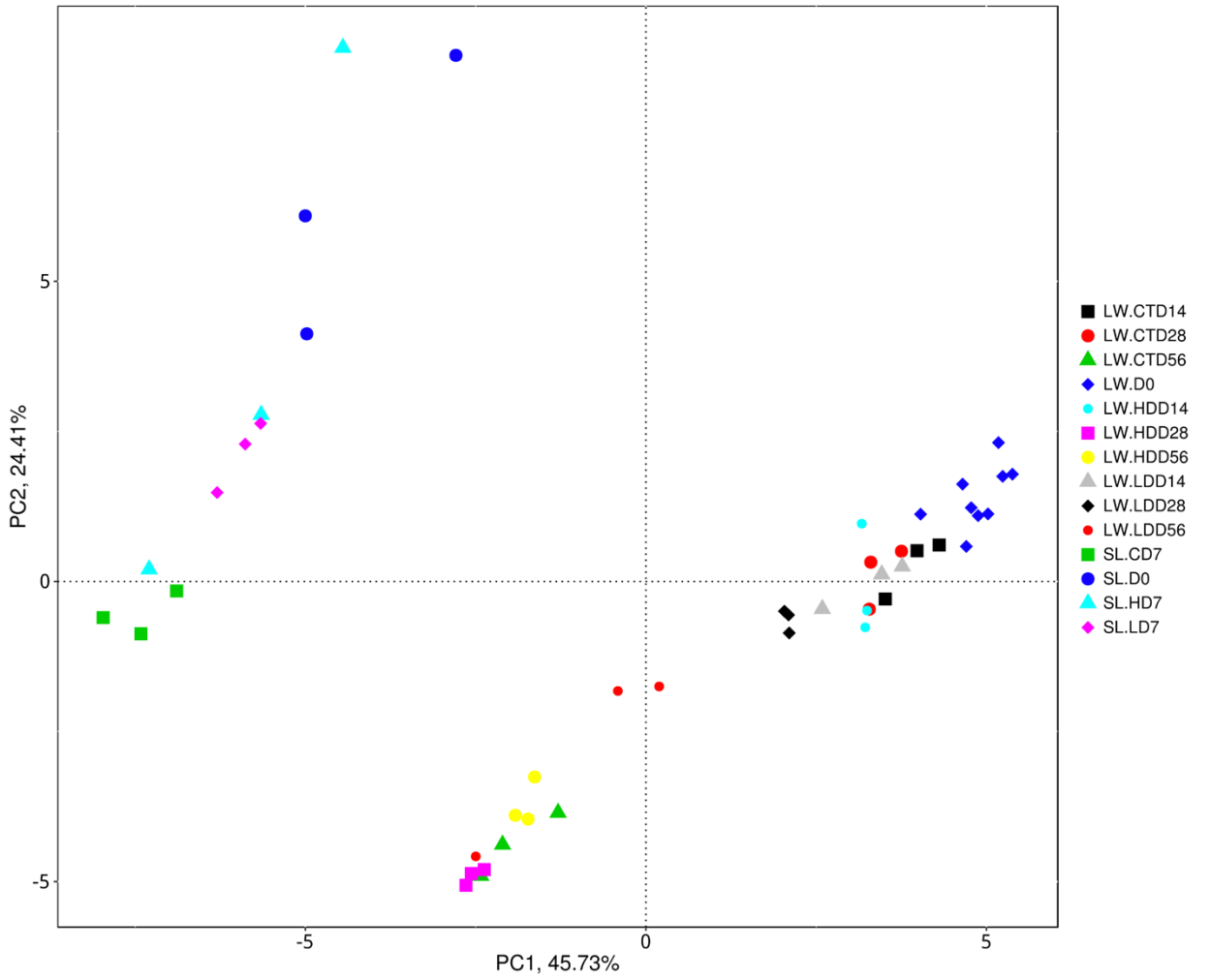

Figure 4.1 Principal component analysis (PCA) plot at the phylum level, of experiment 1 and experiment 2 together. Clustering indicates similarity between groups. Each axis represents a principle component, with the x-axis explaining 45.73% and the y-axis explaining 24.41% of variation between groups.

The axes of Figure 4.1 explained small amounts of variation at the phylum level in the dataset, at 45.73% and 24.41% for PC1 and PC2, respectively. There was clustering at the phylum level within most of the groups, which was so be expected given that each dot represents a replicate per group (Figure 4.1). Clustering was indicative of similarity between samples.

Furthermore, the lagoon water samples and slurry samples also clustered with their respective manure type (i.e., lagoon water clustered with lagoon water and slurry clustered with slurry). The slurry samples also had a wider distribution within their clusters compared to the lagoon water samples. Eminex® treated slurry groups clustered away from SL-CONT on d 7, seemingly to resemble d 0 samples more closely, which had a wide distribution of samples with no apparent clustering pattern.

Tight clustering occurred for d 56 of the lagoon samples for all three treatment groups. The d 28 samples clustered closer to the d 14 and d 0 samples, with the notable exception of LW-HD on d 28. Unlike in the slurry samples, there was comparatively tighter clustering for all the lagoon water groups.

Figure 4.2 shows the PCA at the genus level for experiment 1 and experiment 2. The PCA analysis at this taxonomic level did not explain much variation. The axes PC1 and PC2 only explained 42.85% and 13.23% of the variation in the dataset, respectively, which was comparatively lower than in Figure 4.1.

The major patterns of clustering of slurry versus lagoon water was the same in Figure 4.2 as in Figure 4.1, with the slurry samples clustered together, and the lagoon water samples clustered together. The slurry samples had tighter clustering at the genus level, with the d 0 and SL-HD d 7 having the widest distribution of points on the graph. However, the same phenomenon was noted,

with the SL-CONT d 7 samples having the tightest cluster patterns, for the genus and phylum level (Figure 4.1 and 4.2).

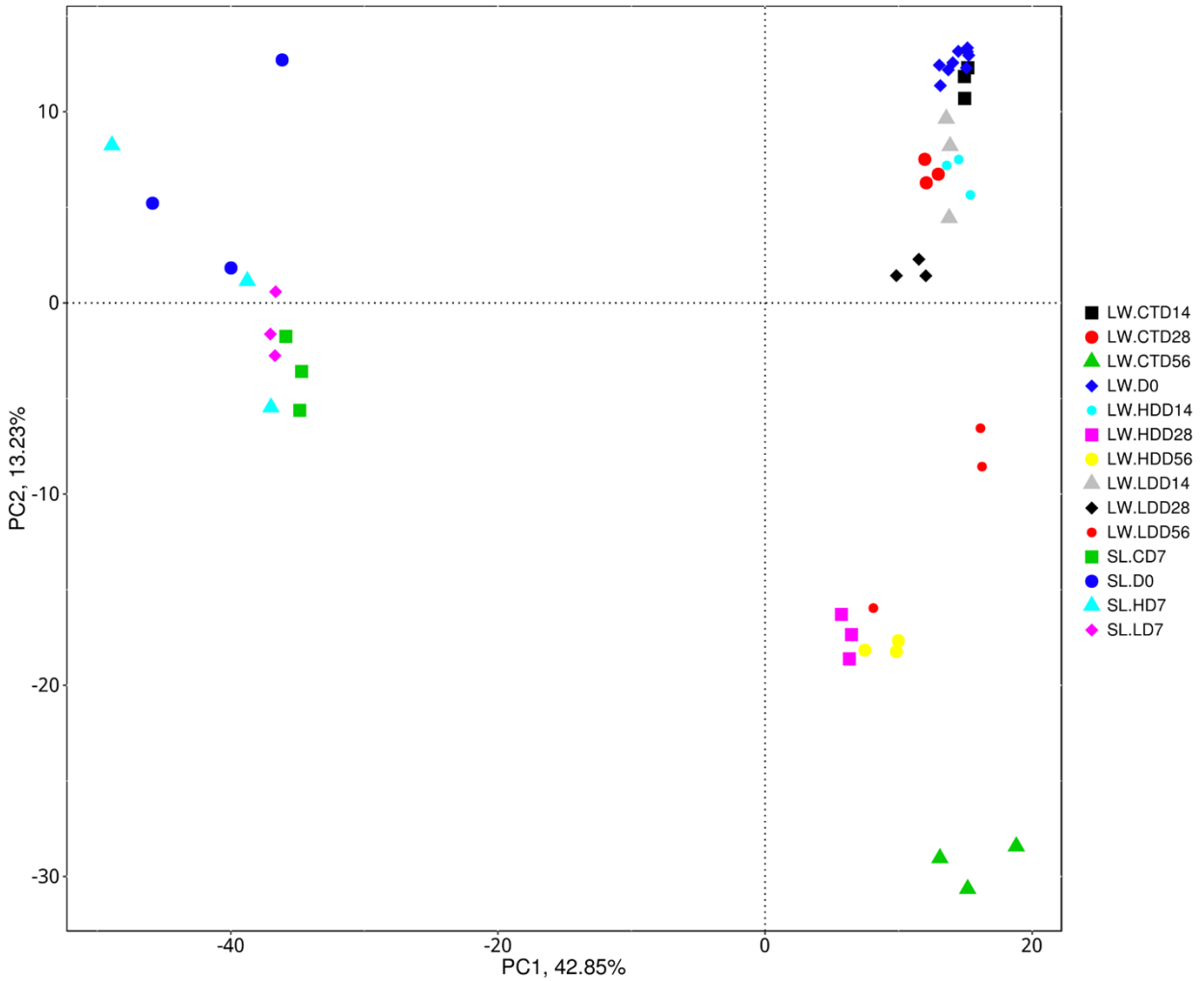

Figure 4.2 Principal component analysis (PCA) plot at the genus level, of experiment 1 and experiment 2 together. Clustering indicates similarity between groups. Each axis represents a principle component, with the x-axis explaining 42.85% and the y-axis explaining 13.23% of variation between groups.

For the lagoon water groups, there was a wide distribution between the groupings. All samples, regardless of treatment, clustered close to d 0 at d 14. However, by d 28, LW-HD and LW-LD already differentiated and began to cluster closer to the d 56 samples, whereas LW-CONT d 28 stayed closer to d 14 samples. All three samples became remarkably different by d 56

compared to previous sample days, with LW-LD and LW-HD grouping together while LW-CONT clustered away from both Eminex® treatment groups.

3.4 Linear Discriminatory Analysis Effect Size

Linear discriminatory analysis effect size (LEfSe) measures the degree of difference between the relative abundance of specific taxonomic levels when comparing two or more groups. A positive value indicates that relative abundance of that taxonomy increased compared to the other group(s), whereas a negative value indicates a decrease in relative abundance in comparison (Chang et al., 2022). The LDA score was based on differences between all the presented sampling days, meaning positive/negative differences for any taxonomic levels were based on scores between all the presented taxonomic levels.

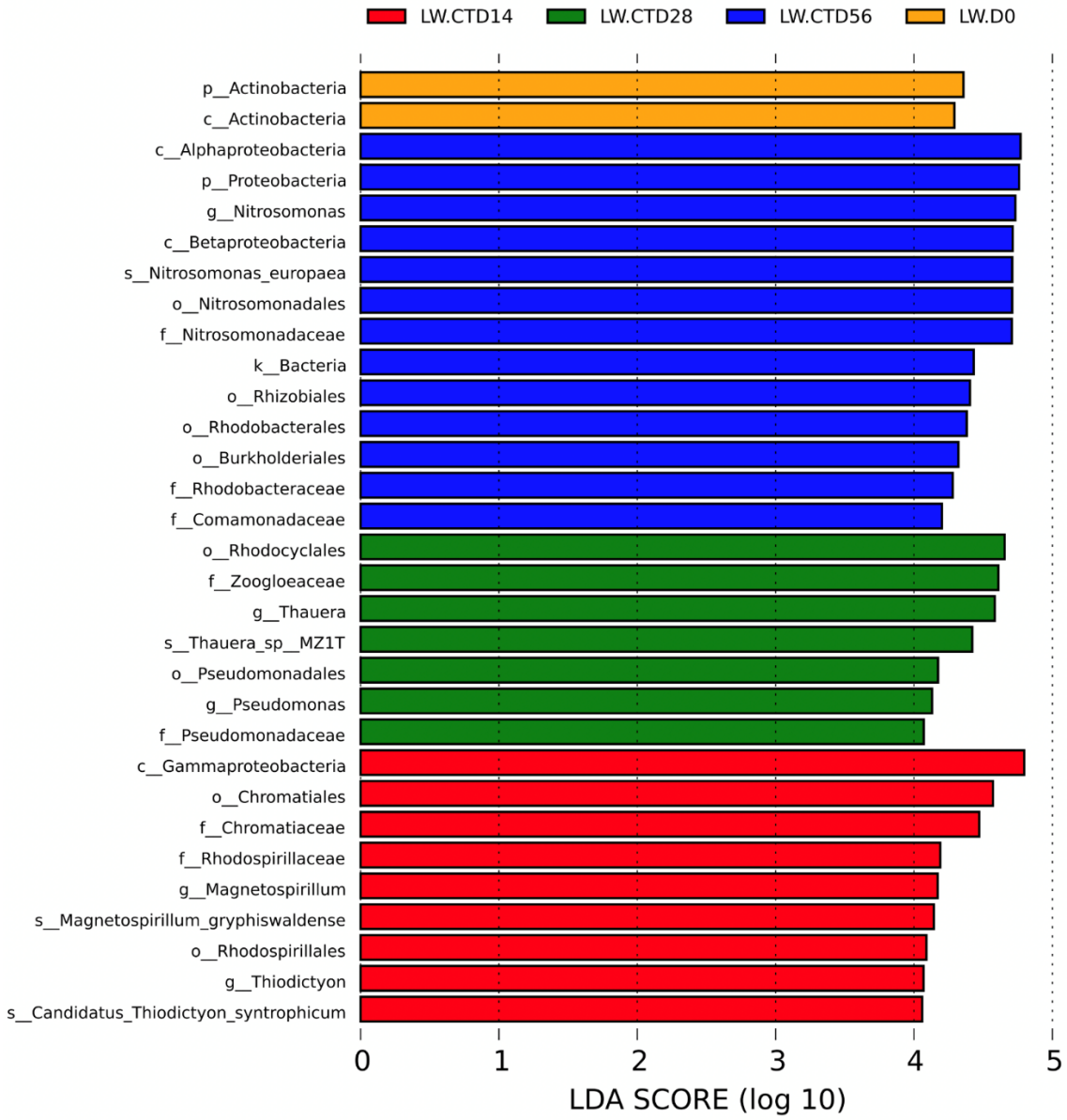

Figure 4.3 The linear discriminatory analysis compares the LW-CONT group across all the samples days. Any missing days indicates that there were no significant contributors at any taxonomic level to differences between those test days. Letters represent different taxonomic rankings of k = kingdom, p = phylum, c = class, g = genus, s = specie, o = order, and f = family.

Figure 4.3 shows the LEfSe outcomes for LW-CONT across the four sampling days. On d 0, the greatest differences belong to the phylum Actinobacteria and the class Actinobacteria. For d 14, the greatest differences belonged to the class Gammaproteobacteria, and the order

Chromatiaceae. For d 28, the greatest differences belonged to the order Rhodocyclales and the family Zoogloeaceae. And for d 56, the greatest differences were attributed to the class Alphaproteobacteria and the phylum Proteobacteria. Additionally, d 56 showed the greatest number of taxonomic differences in relative abundance compared to the three other days.

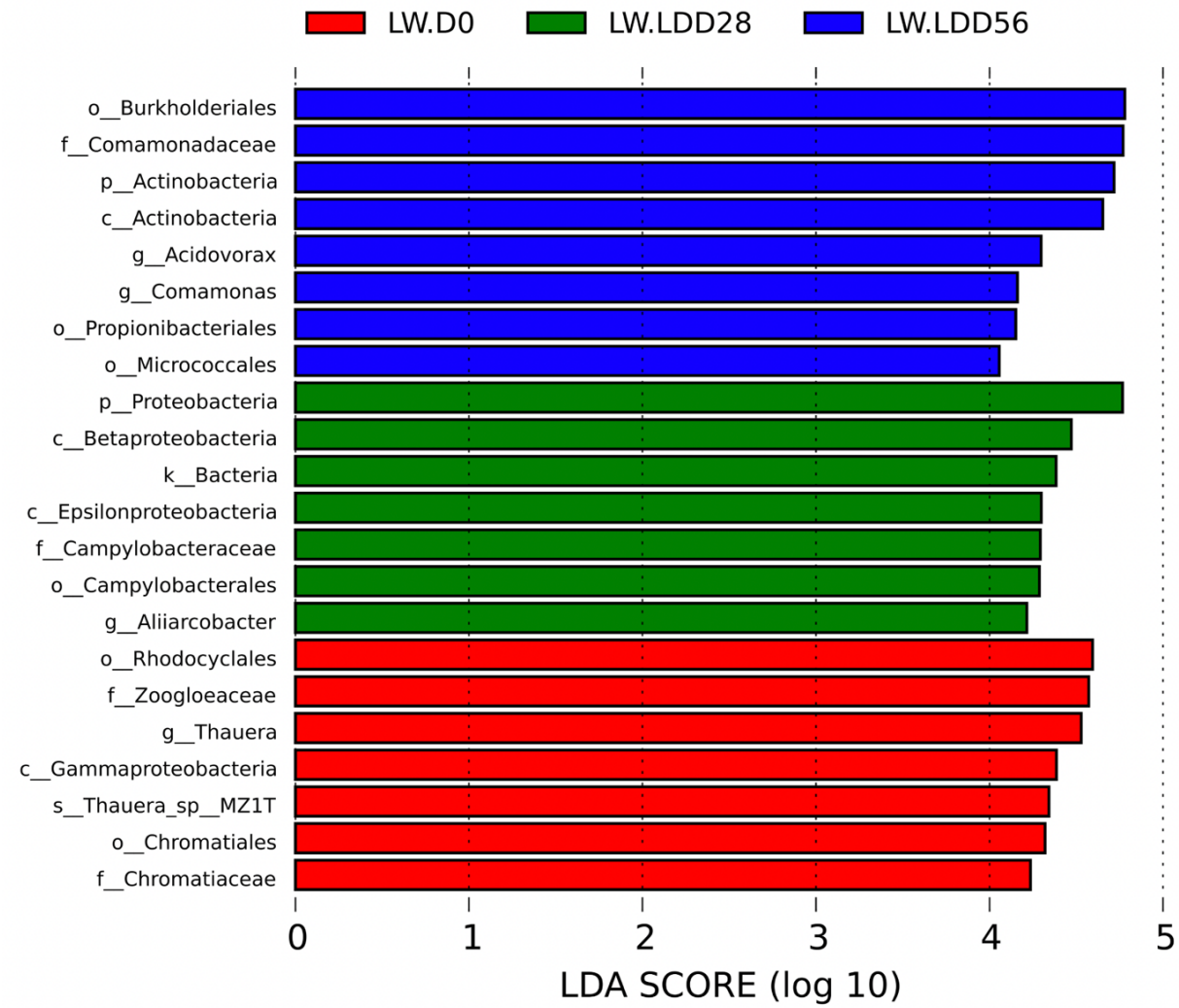

Figure 4.4 The linear discriminatory analysis compares the LW-LD group across all the samples days. Any missing days indicates that there were no significant contributors at any taxonomic level to differences between those test days. Letters represent different taxonomic rankings of k = kingdom, p = phylum, c = class, g = genus, s = specie, o = order, and f = family.

Figure 4.4 shows the LEfSe outcomes for LW-LD samples across all sampling days. There were only differences in relative abundance of specific taxonomic levels on d 0, 28, and 56 of the experiment. LW-LD d 14 did not have any sufficiently discriminatory differences in relative abundance compared to the other sample days and was not included in this figure. The greatest difference on d 0 was attributed to the order Rhodocyclales and the family Zoogloeaceae. For d 28, the greatest differences were attributed to the phylum Proteobacteria and the class Betaproteobacteria. For d 56, the greatest differences were attributed to the class Comamonadaceae and the order Burkholderiales. LW-LD d 56 also had the greatest number of taxonomic differences with increasing relative abundance.

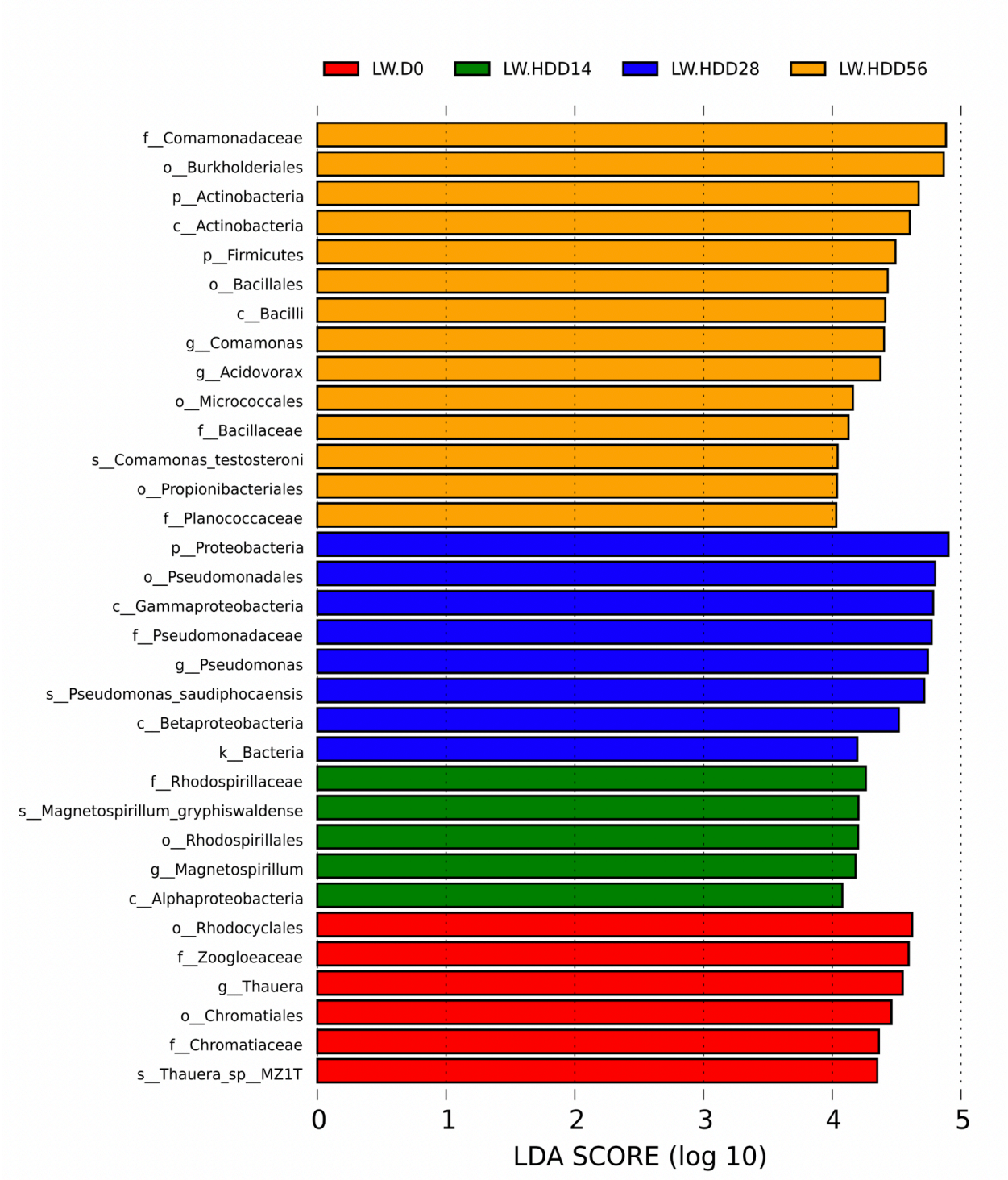

Figure 4.5 The linear discriminatory analysis compares the LW-HD group across all the samples days. Any missing days indicates that there were no significant contributors at any taxonomic level to differences between those test days. Letters represent different taxonomic rankings of k = kingdom, p = phylum, c = class, g = genus, s = specie, o = order, and f = family.

Similarly, Figure 4.5 shows the LEfSe outcomes for LW-HD samples across all sampling days. Within this treatment group, all sampling days had positive differences in relative abundance. For d 0, the greatest differences were attributed to the order Rhodocyclales and the family Zoogloeaceae. For d 14, the greatest differences were attributed to the family Rhodospirillaceae and the species *Magnetospirillum gryphiswaldense*. For d 28, the greatest differences were attributed to the phylum Proteobacteria and the order Pseudomonadales. Compared to the other sampling days, d 56 showed the most increases across taxonomic levels, the greatest of which belonged to the class Comamonadaceae and the order Burkholderiales, same as d 56 for LW-LD.

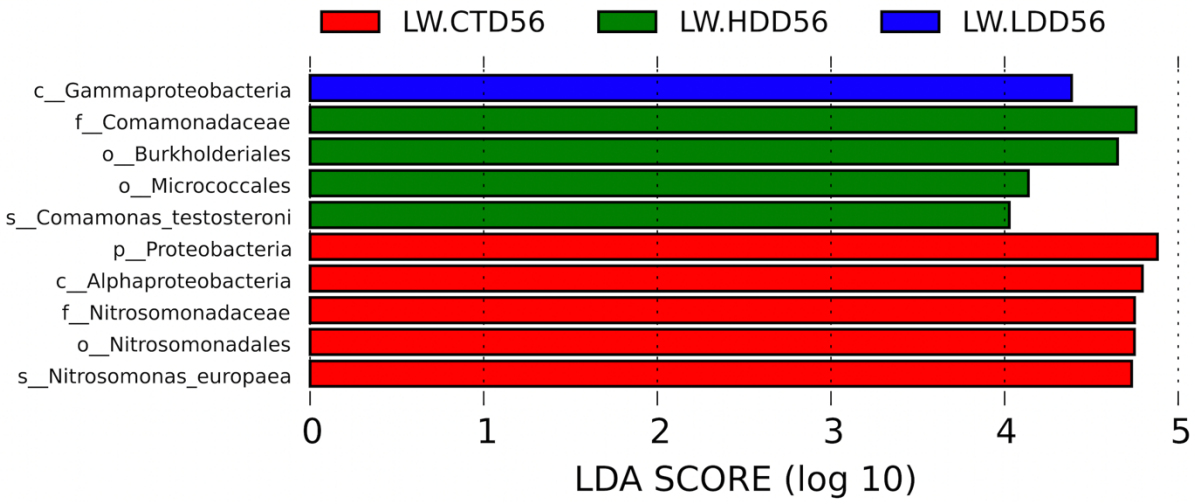

Figure 4.6 Linear discriminatory analysis between the three lagoon water groups on day 56 samples. Identifies which taxonomic levels contribute the most to differences between the samples. Red = LW-CONT; Green = LW-HD; Blue = LW-LD. Letters represent different taxonomic rankings of k = kingdom, p = phylum, c = class, g = genus, s = specie, o = order, and f = family.

Figure 4.6 shows the LEfSe outcomes of the three treatments (HD = high, LD = low, CONT = control) on the last day of the experiment, day 56. There were prominent, positive differences between the three groups. For LW-CONT, there were five taxonomic levels that differed from

LW-LD and LW-HD, with the greatest differences attributed to the phylum Proteobacteria and the class Alphaproteobacteria. For LW-LD, only one taxonomic level was different between the groups, which was the class Gammaproteobacteria. For LW-HD, there were four taxonomic levels that were different in relative abundance compared to the other groups. The greatest difference was attributed to the family Comamonadaceae and the order Burkholderiales.

As for the slurry experiment, the LEfSe found no differences across different taxa to explain significant amounts of variation between the samples.

4 DISCUSSION

4.1 Fresh dairy slurry versus dairy lagoon water

The physical characteristics of fresh dairy slurry and dairy lagoon water may explain why certain microbes were able to thrive compared to others. Table 4.1 shows parameters for measurements taken on d 0 of the experiment. To start, slurry contained a higher TS content, unsurprisingly, as it had not been diluted by water compared to lagoon water. This would provide microbes with more organic matter to ferment and breakdown. The pH of the two forms of manure was also different, with fresh slurry being more alkaline (pH 8.03) compared to lagoon water (pH 7.38).

Furthermore, VFAs act as substrates and end products of microbial fermentation (Ungerfeld, 2015; Bengelsdorf et al., 2018). Especially acetic acid can either be transformed by methanogens into CH_4 or compete with methanogens for CO_2 and H_2 during formation by acetogenic bacteria. As seen in Table 4.1, there was a high concentrate of acetic acid in the fresh slurry, while there was no detectable acetic acid in the lagoon water.

4.1.1 Baseline Microbiota for Slurry and Lagoon Water

The relative abundance at the phylum level for fresh dairy slurry versus dairy lagoon water (Table 4.2) showed distinct differences in microbial populations. Over the 11 phyla presented in Table 4.2, eight had significant differences in relative abundance in slurry versus lagoon water. Manure microbiome experiences taxonomic shifts when transitioning between fresh manure and stored manure (Sukhum et al., 2021). The slurry in this experiment was collected fresh as feces and urine then mixed into slurry; it represented fresh manure that would later be stored in a lagoon if this were part of a standard manure management system. Anaerobic lagoons, as a form of manure storage, have a different microbiome environment, compared to fresh slurry, especially when it comes to substrate availability, lack of oxygen, and pH levels (Dungan and Leytem, 2014).

One of the phyla that had a higher relative abundance in lagoon water compared to slurry was Proteobacteria ($P < 0.0001$). This phylum was diverse, primarily involved in protein and amino acid fermentation, as well as key facilitators in the N cycle (Hatzenpichler, 2012; Prosser et al., 2020). They were dominant in the gastrointestinal tract (GIT) and contained several pathogenic species like *Pseudomonas* (Rizzatti et al., 2017; Peng et al., 2021). Some Proteobacteria were also colloquially referred to as 'purple bacteria' and caused the purplish-pink color seen in anaerobic lagoons, sustained by the organic and mineral N present in lagoons (Adessi and De Philippis, 2013; Pratt et al., 2015). Many species from this phylum were involved in NH_3 oxidation into nitrate/nitrite, and eventually N_2O formation (Hatzenpichler, 2012; Anas et al., 2020; Khairunisa et al., 2023). They had also been shown to participate in VFA degradation and hydrogen (H_2) consumption (García-Lozano et al., 2019).

Firmicutes was another major phyla that exhibited differences in relative abundance in slurry versus lagoon water, often found in the GIT with additional important agroecological roles

(Rizzatti et al., 2017; Hasmi et al., 2020). This phyla contained genera involved in plant growth promoting bacteria (i.e., N-fixation), biocontrol and bioremediation (Hasmi et al., 2020). The relative abundance in fresh slurry was 51.3%, which was significantly greater compared to relative abundance of 0.17% in dairy lagoon water ($P < 0.05$). The high relative abundance of Firmicutes was likely attributed to their natural presence in the GIT, as this slurry came directly from cows. However, Firmicutes, as a phylum, do not grow well in manure, compared to Proteobacteria, probably because Firmicutes do not have the same capability to utilize manure-derived carbohydrates for growth when in soil and therefore do not thrive with manure application (Li et al., 2020). Proteobacteria, on the other hand, secreted enzymes capable of liposaccharide biosynthesis and carbohydrate metabolism in manure. This could explain why, regardless of waste type, the relative abundance of Firmicutes decreased when comparing slurry versus lagoon water, especially compared to Proteobacteria.

Conversely, certain orders in the phylum Firmicutes, specifically anaerobic *Clostridiales*, played a major role in the hydrolytic stage of anaerobic digestion (García-Lozano et al., 2019). For lagoon water, the microbial population dynamics and abundance changed depending on where samples were collected. Khairunisa et al. (2023) quantified microbial populations in an earthen storage pit and a concrete storage pit for slurry and found that locations such as inlet, middle of the pit, outlet, and even sampling depth all had significant differences in beta diversity, which was a measure of differences in population dynamics between different sampling locations.

Actinobacteria was another abundant phylum, with relative abundance of 23.7% and 19.4% for slurry and lagoon water, respectively (Table 4.2). This phylum contained Gram-positive, aerobic bacteria, commonly found in alkaline soils and aquatic ecosystems (Ul-Hassan and Wellington, 2009; De Simeis and Serra, 2021; Mitra et al., 2022). Actinobacteria had also been

isolated from animal fecal matter, with species capable of fermenting lignocellulolytic carbohydrates (Jiang et al., 2013; Mitra et al., 2022). They also code for enzymes involved in sugar degradation (Chen et al., 2022). This phylum also has positive impacts on plant growth, through the colonization of root systems and production of various plant growth factors. They also produce other anti-microbial compounds that protect plants from pathogenic microbes and improve plant resistance to variations in ambient temperature, soil pH, and moisture (Mitra et al., 2022). Given the prominent presence of this phylum within both fecal matter and soils, it was unsurprising that it had high relative abundance across both slurry and lagoon water. Furthermore, the relative abundance of Actinobacteria has a positive correlation with more alkaline pH, and a negative correlation with higher concentrations of lactic acid (Chen et al., 2022). As noted in Table 4.1, slurry had a pH 8.03 on d 0, making it more alkaline compared to lagoon water, at pH 7.38. Therefore, this would explain why Actinobacteria were present in a higher relative abundance in slurry compared to lagoon water at the beginning of the experiment. Conversely, fresh slurry had a higher concentration of lactic acid present compared to lagoon water (Table 4.1). Despite the plethora of benefits the bacteria of this phylum offered to plants, it contained a variety of pathogenic strains as well (Jiang et al., 2013).

Bacteroidetes was another prominent phylum that maintained low levels of relative abundance across both slurry and lagoon water (Table 4.1), with fresh dairy slurry maintaining a relative abundance nearly double that of lagoon water ($P < 0.05$). Previous research inoculating anaerobic digesters with cream, starch, and other forms of carbohydrates found Bacteroidetes existed in a relative abundance of about 30%, which was much higher than in the present study (Kampmann et al., 2012). However, this phylum was also capable of fermenting cellulose and existed at much higher relative abundance (54%) within the rumen environment, and produced H_2

and acetate as end products of fermentation (Ozbayram et al., 2018). As the values presented in Table 4.2 came from samples taken prior to treatment start, the low relative abundance cannot be attributed to the effects of Eminex®. Therefore, it was assumed that the stark differences in relative abundance of the present study were due to the substrates (i.e., non-structural carbohydrates) being fed into the digester in the study of Kampmann et al. (2012), implying that Bacteroidetes bacteria thrived on carbohydrate-based substrates available in a rumen, which were limiting in lagoon water and slurry.

Furthermore, Bacteroidetes and Firmicutes were predominantly found to colonize and inhabit GITs, in humans and ruminants alike (Rizzatti et al., 2017; Ozbayram et al., 2018; Cholewinska et al., 2021). Studies on sheep showed that diet had significant influence on the Firmicutes and Bacteroidetes abundance in feces (Cholewinska et al., 2021). As the slurry and lagoon water were collected from two different dairy farms, it was possible that dietary influence changed the relative abundance outcomes for both phyla. Additionally, as these phyla are normally found in the rumen and other parts of the GIT, the bacteria identified in manure were likely those that traveled through the tract attached to pieces of feed that were also passed. It is not uncommon to see pieces of feed in manure or have microbes traveling throughout the GIT, it also explained why the relative abundance of these phyla were so low in manure. Existing outside their ideal environment could mean that Bacteroidetes and Firmicutes struggled to compete for resources with other microbes that had better metabolic capacity to survive outside the GIT (Cholewinska et al., 2021; Sukhum et al., 2021).

Tenericutes was another phylum identified in the slurry and lagoon water samples. Recently, Tenericutes was reclassified as the phylum Mycoplasmatota (Munson et al., 2023). However, since Novogene and its results maintain the old nomenclature, it will be referred to as

Tenericutes throughout this report. Like Firmicutes and Proteobacteria, the relative abundance in lagoon water was significantly greater than the relative abundance in fresh dairy slurry ($P < 0.05$; Table 4.2). This phylum has been found to inhabit a wide variety of habitats, include marine and terrestrial ecosystems, and the GIT, exhibiting a strong adaptability and diverse metabolic versatility (Wang et al., 2020). However, many of the genera within this phylum, specifically *Mycoplasma*, *Ureaplasma*, and *Acholeplasma* were known to be commensal or obligate pathogens of humans and domesticated animals (Wang et al., 2020). Recent research has identified several clades within Tenericutes as H_2-producers and possess genes known to be involved in amino acid fermentation, carbohydrate storage, as well as carbon fixation (Wang et al., 2020). Much remains to be elucidated about Tenericutes, as the recent reclassification opened more questions about its role in GIT and other environments (Wang et al., 2020; Munson et al., 2023).

Spirochaetes were a phylum of anaerobic bacteria, which explained why the relative abundance in lagoon water was significantly higher compared to slurry. This phylum was also part of the natural GIT microbiota (Pandey et al., 2018). They had been shown to be involved in the decomposition and degradation of organic matter, like rice straw (Tanahashi et al., 2005). This phylum had been identified by other studies as a common phylum present in manure/waste water (de la Guardia-Hidrogo and Paz, 2021), despite seemingly being present in low relative abundance, about 3% in the middle of a lagoon, but increased in relative abundance (+6%) near influent areas (García-Lozano et al., 2019). Due to their anaerobic classification, their limited presence in the collected samples could be attributed to collection. García-Lozano et al. (2019) noted that sampling different spots affect which microbes were present and their relative abundance within a dairy lagoon. This was determined by variability in substrate availability, pH, redox potential, VFA concentration and more, all influencing where within a lagoon certain species thrived. Samples for

the present study were taken using a 3.05 m hose with a mesh cage on the end to prevent the collection of any sludge or solids, about 1.52 m from the edge of the lagoon. It could be that the environmental conditions within that area of the lagoon were not supportive of Spirochaetes and had the present study had the capability to sample from elsewhere in the lagoon, the results might have been different. Furthermore, the presence of oxygen near the surface of the lagoon as well as the introduction of oxygen into the lagoon water during pumping (which was unavoidable) could've led to decreased relative abundance of Spirochaetes (Table 4.2).

de la Guardia-Hidrogo and Paz (2021) found Verrucomicrobia at a relative abundance of 11.6% in fresh manure, although other studies found it at lower relative abundance in slurry and lagoon water, about 1-2% (Ozbayram et al., 2018; García-Lozano et al., 2019), compared to 0.12% and 0.39% for fresh slurry and lagoon water, respectively, on d 0 in the present study ($P < 0.0001$). This phylum was widely abundant across diverse habitats, especially in soils where they were considered metabolically essential and active members of the microbiome, capable of existing in aerobic and anaerobic conditions but optimal growth occurred at about 2% to 8% oxygen concentration (Wertz et al., 2012). Organisms within this phylum utilized plant matter as energy sources, primarily various oligo- and polysaccharides, which were subsequently transformed into acetate as an end product of fermentation (Wertz et al., 2012).

Fusobacteria was the more prominent pathogenic phyla identified in the slurry and lagoon water samples. The bacteria of this phylum have been shown to cause foot rot, ulcers, hepatic abscesses, and stomatitis (Booth, 2014; Brennan and Garrett, 2019). Unlike most other phyla previously discussed, this one's relative abundance was lower in lagoon water compared to slurry, at 0.18% and 0.33%, respectively ($P < 0.05$; Table 4.2). Fusobacteria were anaerobic, and found to be more abundant in manure samples versus soil in other studies (Sukhum et al., 2021). Species

within the phylum were known cross-feeders, relying on amino acid products from other microbes to survive, while producing butyrate and NH_3 as end products of fermentation (Sakanaka et al., 2022). Like the other anaerobic phyla already discussed, the low relative abundance of d 0 was likely due to the collection protocol of the present study. Alternatively, low abundance could have been influenced by the pH of slurry and lagoon water, as ideal growth environment for Fusobacteria was about pH 7.4 (Rogers et al., 1991), which was neutral compared to the relatively alkaline pH of the fresh slurry on d 0 and very close to the pH of lagoon water (Table 4.1).

Deinococcus-Thermus were classified as an extremophilic phylum of obligately aerobic bacteria, previously isolated from hot arid deserts and geothermal springs, but also the GIT (Theodorakopoulos et al., 2013; Ott et al., 2019; Wang and Osborn, 2024). On d 0, the phylum was present in fresh slurry and lagoon water at relative abundances of 0.14% and 0.38%, respectively ($P < 0.05$; Table 4.2). They have been shown to be metabolically linked to lactic acid and acetate oxidation (Fredrickson et al., 2000), although little was otherwise known about this phylum and research is ongoing (Ott et al., 2019; Wang and Osborn, 2024).

Interestingly, the differences in relative abundance of Euryarchaeota, the archaeal phylum of methanogens, between slurry and lagoon water were not statistically significant, even though slurry had a higher relative abundance (Table 4.2). This could potentially be attributed to high variation between samples that would otherwise prevent a significant difference from being identified. It was also possible that the overall population of methanogens within this region was low due to the higher concentration of oxygen, and this was reflected in the samples due to the sampling, which occurred with 1.52 m of the lagoon edge. Oxygen could penetrate as deep as 7-10 cm beneath the liquid manure surface (Khairunisa et al., 2023), eliminating the strictly anaerobic environment that obligate anaerobic methanogens need to survive.

4.2 Relative Abundance

Relative abundance acted as a measure of the abundance of a specific taxa within a sample, so a sample with 10% relative abundance of bacteria meant that 10% of that sample were bacteria (Lin and Das Peddada, 2020). It was hypothesized that Eminex® would dramatically change the microbiome of fresh slurry and lagoon water in order to reduce gaseous emissions. It was therefore essential to thoroughly assess how the relative abundance and by proxy, population was affected by the treatment. It was noted that most of the phyla that increased in relative abundance in LW-CONT seemed to decrease in relative abundance in the treatments receiving Eminex®, LW-LD and LW-HD, and vice versa as well. The microbiome of fresh slurry and lagoon water following the application of Eminex® are discussed below, at the genus and phylum level.

4.2.1 Impact of Eminex® on Lagoon Water Phyla

Overall, the relative abundance of the top phyla presented in Table 4.3 differed significantly between treatment groups. Additional discussion in this section with regards to the effects of Eminex® on the lagoon water microbiome will focus on the bacterial phyla with relative abundance >1.5%, as well as the archaeal phylum.

The phylum Proteobacteria declined in relative abundance, when comparing LW-CONT to LW-LD and LW-HD. This phylum was known to be dominated by nitrifying bacteria, called ammonia-oxidizing bacteria (AOB) and participated in the N cycle to transform NH_3 into nitrite then nitrate (Gieseke et al., 2006; Fowler et al., 2013; Samocha and Prangnell, 2019; Park et al., 2021). Eminex® contained nitrification inhibitor, dicyandiamide (DCD) that was designed to prevent nitrification as a means of preventing leaching of nitrates into soils and waterways (Dixon,

2012; Park et al., 2021). If AOB, along with the other bacteria involved in heterotrophic and homotrophic nitrification pathways (Khairunisa et al., 2023), were unable to engage in normal metabolic activities in the lagoon water due to the presence of DCD, then that could explain why the relative abundance of Proteobacteria decreased by over 16% in LW-HD compared to LW-CONT ($P < 0.05$) and why NH_3 emissions from lagoon water increased so dramatically (Table 3.4; Chapter 3). Whether there were more discrete interruptions to specific nitrification metabolic pathways would require additional metatranscriptomic research beyond the scope of the present study.

There was a significant increase in relative abundance of Firmicutes with the application of Eminex® for LW-HD by d 56 compared to d 0 ($P < 0.05$). For the other two groups, compared to d 0, there were no significant differences, likely attributed to high variation between samples. The Firmicutes phylum contained acetogenic bacteria, which produce acetate or acetic acid as end products of fermentation (Barret et al., 2013; Beauchemin et al., 2020). Holtkamp et al. (2023) reported a significant accumulation in acetic acid in dairy slurry following the application of Eminex®. The higher relative abundance of Firmicutes, noted in Table 4.3, would help explain the increased concentration of acetic acid in LW-HD. Furthermore, acetogenic bacteria consume CO_2 and H_2 to synthesize acetic acid. They potentially competed with hydrogenotrophic methanogens for those substrates (Bengelsdorf et al., 2018; Enzmann et al., 2018; Shima et al., 2020). This could have contributed to reductions in CH_4 and CO_2 emissions. Additionally, Bacteroidetes and Firmicutes both exhibited symbiotic cross-feeding relationships with methanogens, as they produce CO_2, H_2, and formate, which hydrogenotrophic methanogens consumed for methanogenesis (Bengelsdorf et al., 2018; Wirth et al., 2023).

Actinobacteria was another phylum that demonstrated significant increases in relative abundance across different treatments compared to d 0. As seen in Table 4.3, the relative abundance of Actinobacteria was about 4% lower for LW-CONT (P = 0.003), whereas for LW-LD and LW-HD, relative abundance was about 6.5% (P = 0.012) and 6.2% (P = 0.005) higher. The increase in relative abundance by d 56 could be attributed to the alkaline environment (Table 3.3; Chapter 3). While other bacteria would potentially struggle to survive within an alkaline pH, Actinobacteria would thrive, likely also existing near the surface of the lagoon water to have access to oxygen (Chen et al., 2022). The greater abundance of this bacteria would offer benefits to farmers utilizing this wastewater as crop fertilizer, as Actinobacteria produce many enzymes and compounds shown to be plant growth promoters, as well as help offer resistance to plant pathogens, and variations in salinity, pH, and temperature (Mitra et al., 2022). As other bacterial phyla like Bacteroidetes, Verrucomicrobia, and Spirochaetes decreased in relative abundance following treatment over time, it was possible that Actinobacteria increased in relative abundance to fill these ecological niches. With a diverse metabolic capacity to ferment structural and non-structural carbohydrates (Ul-Hassan and Wellington, 2009; Chen et al., 2022), leftover organic matter in the lagoon water could be broken down by Actinobacteria.

The relative abundance of Euryarcheota, the only dominant archaeal phylum identified in these samples, significantly decreased in relative abundance by d 56 as compared to d 0 samples, by 58.4%, 20.6%, and 32.9% for LW-CONT, LW-LD, and LW-HD, respectively ($P < 0.05$; Table 4.3). As mentioned in Chapter 3, CH_4 emissions from lagoon water significantly decreased. The suppression of the methanogen population, dominated by hydrogenotrophic species, contributed to the reductions in CH_4 and accumulation of end products like acetic acid. However, interestingly,

the decline in archaeal populations and CH_4 emissions did not seem to influence the pH, as the lack of CH_4 formation would imply H_2 accumulation as well.

Hydrogenotrophic methanogens used H_2 to reduce CO_2 into CH_4, hereby consuming reducing equivalents, and maintaining the redox environment required for fermentation to take place (Lyu et al., 2018; Kurth et al., 2020; Shima et al., 2020). Without an alternative H_2 sink available, pH should have become acidic due to a buildup of H_2 that would otherwise become CH_4. Propionic acid was an alternative hydrogen sink within the rumen, and given the increase relative abundance of Actinobacteria, which contained the genus *Propionibacteria*, this could be where the H_2 was shunted (Mitra et al., 2022). However, as discussed in Chapter 3, there was no propionic acid detected in lagoon water, which made this unlikely. Another explanation was linked to the increase in NH_3 emissions from lagoon water also noted in Chapter 3. Like CH_4 formation, NH_3 consumed H_2 via anaerobic reduction of nitrite (Chatterjee et al., 2021; Khairunisa et al., 2023). Despite the decline in relative abundance of the Proteobacteria phylum after treatment, which contained the genera *Nitrosomonas* and *Comomonas*, which were involved in this process (Khairunisa et al., 2023), the phylum still made up more than half the bacteria in samples. Therefore, it would not be unreasonable to suggest that rather that removing H_2 via CH_4, Proteobacteria took over for methanogens in eliminating H_2 from the environment. Future research into the biochemical activity of methanogenesis versus NH_3 volatilization under the effects of Eminex® is needed to confirm this.

The phylum Bacteroidetes significantly increased by 41.8% in relative abundance from d 0 to d 56 in LW-CONT ($P = 0.003$). Conversely, both LW-LD and LW-HD significantly declined in relative abundance, by 25.9% and 21.2%, respectively, from d 0 to d 56 ($P < 0.05$; Table 4.3). Bacteroidetes were considered a dominant rumen phylum, known to ferment structural and non-

structural carbohydrates, and producing acetic acid and H_2 as end products (Ozbayram et al., 2018). Given that the relative abundance of Bacteroidetes increased for LW-CONT, this meant that at least some amount of the bacteria coming from the GIT were capable of colonizing lagoon water, utilizing the available organic matter to survive (Cholewinska et al., 2021). The decline in relative abundance for LW-LD and LW-HD showed the Eminex® negatively impacted the Bacteroidetes phylum, although it would be difficult to determine if the effects were as a direct consequence of Eminex® itself, or indirectly, because of other bacteria and archaea being impacted by Eminex®.

4.2.2 Impacts of Eminex® on Lagoon Water Genera

Assessing microbial populations at a lower level of taxonomic organization provided additional insight into the ecological niches occupied by specific genera within the major phyla present in samples. Within lagoon water samples, the major genera are presented in Table 4.4. Of these genera, two belonged to the phylum Actinobacteria (*Bifidobacterium* and *Corynebacterium*), four belonged to the phylum Proteobacteria (*Escherichia*, *Nitrosomonas*, *Pseudomonas*, and *Thauera*), three belonged to the phylum Firmicutes (*Sporosarcina*, *Jeotgalibaca* and *Jeotgalicoccus*), and one belonged to the phylum Fusobacteria (*Fusobacterium*).

The top genera were dominated by the phylum, Proteobacteria. Of the four, only three had significant differences in relative abundance on d 56 compared to d 0 ($P < 0.05$; Table 4.4). The first was *Nitrosomonas*, which increased in relative abundance of LW-CONT compared to d 0 ($P < 0.05$), but relative abundance in LW-LD and LW-HD did not change, remaining at d 0 levels. This genus of nitrifying bacteria were involved in the natural N cycle, participating in the autotrophic nitrification of ammonium into nitrate (Khairunisa et al., 2023). For LW-CONT, there was a significant increase in relative abundance, whereas there no differences in relative

abundance between d 0 and d 56 for groups receiving Eminex®. This showed that the treatment did not allow for the proliferation of *Nitrosomonas*, likely due to the presence of DCD inhibiting nitrification (Park et al., 2021).

Other Proteobacteria genera were similarly impacted, as the genera *Pseudomonas* and *Thauera* also significantly declining in relative abundance with treatment (Table 4.4), with the only difference being that only LW-HD elicited a significant reduction in *Pseudomonas*, whereas LW-LD did not. The genus *Pseudomonas* was highly pathogenic, with special attention paid to the virulence of opportunistic *Pseudomonas aeruginosa*, as it was capable of infecting plants, livestock, and humans and was highly adaptive across a wide range of environments (de Sousa et al., 2021). The reductions in the relative abundance of *Pseudomonas* with Eminex® treatment offered insight into how this manure additive could affect pathogenicity. While additional research is warranted to confirm how Eminex® could potentially impact virulence factors, this initial study proved that the manure additive did negatively influence their survival in manure, thereby helping to reduce the risk of contamination from manure fertilizers and food-borne pathogens in the food production system.

The last Proteobacteria genus, *Thauera*, also significantly decreased in relative abundance by d 56 as compared to relative abundance on d 0 ($P < 0.05$; Table 4.4). This genus had previously been isolated from wastewater plants, and were known for having a versatile metabolism, capable of degrading aromatic compounds under anaerobic conditions (Liu et al., 2013). They were categorized as denitrifying bacteria, involved in the eventual formation of N_2O (Petersen, 2018; Khairunisa et al., 2023). As mentioned in Chapter 3, there were significant reductions in N_2O emissions from lagoon water. Therefore, the reduction in N_2O emissions could be attributed to the

reductions in the genus *Thauera* as well as other bacteria with similar nitrifying-denitrifying capabilities needed for the formation of the potent GHG (Khairunisa et al., 2023).

The relative abundance of the genus, *Bifidobacterium* declined in relative abundance across all the three treatment groups ($P < 0.05$; Table 4.4). The genus *Bifidobacterium* were beneficial bacteria inhabiting the GIT, capable of producing acetic acid, propionic acid, butyric acid, and various vitamins (Hou et al., 2020). These were aerobic, Gram-positive bacteria predominantly involved in carbohydrate fermentation within the GIT (O'Callaghan and van Sinderen, 2016). The other Actinobacteria genus, *Corynebacterium*, also significantly decreased in relative abundance with treatment ($P < 0.05$). This genus contained many pathogenic species, including the bacteria responsible for diphtheria (Oliveira et al., 2017). Despite these genera decreasing in relative abundance with the Eminex® treatment, the decrease was more aggressive for LW-CONT on d 56 compared to LW-LD and LW-HD. The alkaline pH of the lagoon water likely aided in the survival of these genera in the Eminex® treated groups, compared to LW-CONT (Table 3.1; Chapter 3), as they had been shown to prefer alkaline environments (Chen et al., 2022). What likely contributed to the decline in relative abundance, likely more than Eminex®, was the aquatic anaerobic environment of lagoon water, as these bacteria required oxygen in order to survive (Mitra et al., 2022).

The genera *Jeotgalibaca* and *Jeotgalicoccus* belonged to the phylum Firmicutes. However, these genera were relatively new, having been recently identified and isolated from fermented foods, air samples, and bodies of water (Kämpfer et al., 2021). Jeotgalicoccus was first isolated in 2003 and remained substantially unexplored compared to other members of the Firmicutes phylum (Yoon et al., 2003). Recent research within the genus struggled to isolate different species within the genus, as it remained uncharacterized when it came to DNA sequencing (Kämpfer et al., 2021).

The same issue existed for the genus *Jeotgalibaca*, which was initially described and put forth as a novel genus in 2014 (Lee et al., 2014; Zamora et al., 2017). This aerobic Gram-positive genus had only one recognized specie, but little else was known to date (Zamora et al., 2017). Therefore, without more in-depth knowledge available on not only the ecological niche that these genera fill as well as their roles in fermentation/ecological niche, but it would also be difficult to know how they were impacted by Eminex® aside from the significant decrease in relative abundance for all three treatments ($P < 0.05$).

The last genus from the Firmicutes phylum was *Sporosarcina* and its relative abundance significantly increased following the application of Eminex® for LW-HD ($P < 0.05$; Table 4.4), but not for LW-LD. Research suggested the *Sporosarcina* played a role in NH_3 volatilization, as its relative abundance in soil significantly increased following the application of urea (Coelho et al., 2022). Under the right conditions, which included an alkaline environment, species of *Sporosarcina* produced urease, capable of forming NH_3 from urea (Ma et al., 2020). As Eminex® contained urea as one of the compounds that it broke down into, this contributed to the increasing relative abundance of *Sporosarcina* in lagoon water, as well as the increased NH_3 emissions noted in Chapter 3. Given that other bacteria involved in the N cycle in the phylum Proteobacteria—like *Nitrosomonas*—were negatively impacted by Eminex®, it could be that *Sporosarcina* filled the vacant ecological niche. Further research would be essential to explore why DCD seemed to negatively impact other bacteria involved in the N cycle, but not *Sporosarcina*.

The genus *Fusobacterium* belonged to the phylum Fusobacteria, which contained a wide collection of pathogenic species that were linked to foot rot, ulcers, liver abscesses, and gastrointestinal issues in calves (Brennan and Garrett, 2019). Compared to the relative abundance

at d 0, the relative abundance of this genus significantly declined by d 56 across all three groups in lagoon water ($P < 0.05$; Table 4.4).

4.2.3 Impacts of Eminex® on Dairy Slurry Phyla

The major phyla identified in fresh dairy slurry differed from the major phyla identified in lagoon water, as shown in Table 4.5 versus Table 4.3. While some differences were specifically related to relative abundance, where starting at d 0 for both slurry and lagoon water, the certain phyla relative abundances already differed between the two experiments. Additionally, there was one phylum with lagoon water that was not a dominant phylum for slurry—Deinococcus-Thermus, which was replaced by Fusobacteria (Table 4.5). Like the lagoon water phyla, this discussion will focus on phyla that had relative abundances greater than 1.5%, helping to refine and focus the analysis on the most popular bacteria and archaea present in the slurry samples.

To start, the relative abundance of the phylum Proteobacteria increased when comparing d 0 to d 7. For SL-CONT, relative abundance significant increased by over 139% ($P = 0.001$) and for SL-LD, relative abundance increased by over 113% ($P < 0.0001$). The relative abundance of LW-HD also increased by over 61%, but this was not considered significant. As previously mentioned, Proteobacteria was a diverse phylum that predominantly engaged in protein and amino acid fermentation, as well as VFA degradation and H_2 consumption (Hatzenpichler, 2012; García-Lozano et al., 2019; Prosser et al., 2020). As these bacteria were also commonly found to inhabit the GIT and better able to grow on manure nutrients compared to other phyla, it was unsurprising that they were able to successfully colonize fresh slurry (Rizzatti et al., 2017; Li et al., 2020). While treated groups increased in relative abundance compared to d 0, it was clear that Eminex® still suppressed the growth of these bacteria, even within the shorter sampling period of 7 days.

The slurry experiment used shallow bowls meant to mimic a dry manure system, resulting in a more aerobic, oxic environment in the slurry. Therefore, the Proteobacteria likely engaged in heterotrophic nitrification and aerobic denitrification, which has been shown to produce N_2O (Khairunisa et al., 2023). This could explain why N_2O emissions from slurry were seemingly unaffected by the Eminex® treatment (Table 3.2; Chapter 3). While there were significant reductions in N_2O noted by Holtkamp et al. (2023), it was possible that more time was needed in order to see more substantial reductions in N_2O, given that the slurry was only monitored for emissions over 7 days, which was assumed as adequate time to emulate a dry manure management system in California. This was confirmed, as N_2O emissions from lagoon water also only started declining after the first 2-week sampling period (Figure 3.9a-b; Chapter 3). However, extending the sampling period for slurry beyond the typical dry storage period would not make sense, especially if Eminex® required longer to become wholly effective. Even so, Eminex® had a demonstrated effect on Proteobacteria, shrinking the population in dairy slurry and dairy lagoon water.

The next dominant phylum identified in slurry was Firmicutes (Table 4.5). Compared to d 0, the relative abundance of Firmicutes did not significantly decrease for all three treatments ($P > 0.05$). Firmicutes had been identified as a prominent phylum commonly found in fresh manure, due to its role in rumen fermentation and in order portions of the GIT (Sukhum et al., 2021). They were well studied fibrolytic bacteria and H_2 producers within the rumen, containing classes of anaerobes and facultative aerobes alike, and likely occupied similar metabolic roles within manure (Tapio et al., 2017; de la Guardia-Hidrogo and Paz, 2021). Firmicutes also produced acetic acid as end products of their fermentation In fact, the relative abundance of Firmicutes identified in the slurry samples after the sampling period were close to relative abundance levels measured by

Ozbayram et al. (2018) and greater than the relative abundance levels measured by García-Lozano et al. (2019). Given that the relative abundance of Firmicutes measured in lagoon water significantly increased and remained high in fresh slurry, it was likely that this phylum was not impacted by Eminex®, allowing for fermentation and acetic acid production to continue, while other phyla were more affected.

The relative abundance of Bacteroidetes decreased in the two Eminex® treatment groups, when comparing d 0 to d 7 by 55.3% and 57.0% for LW-LD and LW-HD, respectively ($P < 0.05$; Table 4.5), but not LW-CONT. As previously mentioned, the anaerobic and aerobic Bacteroidetes phylum occupied a similar niche as Firmicutes, prominent in the early stages of carbohydrate fermentation and producing acetic acid, and were often identified as dominant phyla across a wide range of research (Dungan and Leytem, 2014; Ozbayram et al., 2018; Larsbrink and McKee, 2020; Sukhum et al., 2021). Bacteroidetes were also net H_2 consumers to produce acetic acid, while maintain diverse metabolic capabilities, their growth inhibited by acidic pH (Flint and Duncan, 2014; Larsbrink and McKee, 2020), which was not seen in this experiment. Even so, the relative abundance declined after treatment, so it was possible that Eminex® was directly able to suppress members of this phylum.

The same effect was not seen in lagoon water where Bacteroidetes significantly decreased in relative abundance and Firmicutes significantly increased. Both phyla contain species of aerobes and anaerobes, so it would be expected that these would thrive in either environment, which had been confirmed by previous research quantifying normal manure microbiomes in liquid and solid manure (Dungan and Leytem, 2014; Ozbayram et al., 2018; Pandey et al., 2018; García-Lozano et al., 2019). Given the differences in sampling period length and the innate differences in

composition of slurry versus lagoon water (Table 4.1), it could be that longer sampling periods for slurry would see relative abundances for these phyla reflect the outcomes seen in lagoon water.

Fusobacteria was a dominant phylum in fresh slurry, but not in lagoon water. Across the three treatments, relative abundance increased by d 7 by over 2,000% in LW-CONT, over 560% in LW-LD, and over 352% in LW-HD (Table 4.5). These increases in relative abundance was only statistically significant for LW-CONT (P = 0.026). This highly pathogenic, strictly anaerobic phylum thrived in slurry (Brennan and Garrett, 2019; Sukhum et al., 2021). And it was clear at the phylum and genus level (Table 4.5 and Table 4.6), as well as in lagoon water (Table 4.4), that Eminex® negatively impacted the relative abundance of Fusobacteria. Species within the phylum had demonstrated symbiotic and mutualistic relationships with other bacteria, like *Streptococcus spp.* of the Firmicutes phylum (Brennan and Garrett, 2019). As the Firmicutes phyla maintained a high relative abundance in slurry, it could be inferred that Eminex® interrupted the symbiotic and mutualist relationships that allowed Fusobacteria to thrive in manure and other environments. Further research is recommended to isolate how Eminex® interrupted these biochemical pathways, not only for Fusobacteria, but other pathobionts present in manure.

The last dominant phylum of interest was archaeal. Euryarcheota, containing the archaeal methanogens responsible for CH_4 formation, needed to be assessed despite the lack of significant differences in relative abundance between d 0 and d 7 across all treatment groups (Table 4.5). Like with lagoon water, there was also a reduction in CH_4 emissions measured from slurry (Table 3.2; Chapter 3). As seen in Table 4.5, all three groups ranged from 3 to 4.67% relative abundance by d 7, which was greater than the relative abundance for lagoon water (Table 4.3). The higher concentration of VS available in slurry was able to sustain a larger population of bacteria and therefore, a higher population of methanogens, given that these domains exist symbiotically

(Beauchemin et al., 2020). In fact, the relative abundance of Euryacheota increased, even though CH_4 emissions decreased. This again supported the hypothesis that Eminex® could potentially have some unexplored impacted directly to the biochemical pathway of methanogenesis.

Of the ten major phyla, six did not have statistically significant changes to relative abundance (Table 4.5). It was possible that large variation between samples could have contributed to the lack of significant differences. Future research should include additional samples to help correct for such variation; the present study was limited by available funds and time.

4.2.4 Impacts of Eminex® on Dairy Slurry Genera

The top ten genera of fresh slurry saw only one significant change, noted between d 0 and d 7 in SL-CONT. The relative abundance of *Bifidobacterium* declined by over 32% between d 0 and d 7 for SL-CONT ($P = 0.02$**)**. Two other genera, *Jeotgalicoccus* and *Methanobrevibacter* was a trend between d 0 and d 7 for SL-CONT, decreasing by 95.4% and 36.8%, respectively ($P < 0.1$; Table 4.6).

The genus, *Bifidobacterium* belonged to the phylum Actinobacteria and declined relative abundance across all the three treatment groups, but only significantly for SL-CONT ($P = 0.02$). As previously mentioned, the genus *Bifidobacterium* were symbiotic gut bacteria that produced VFAs and vitamins (Hou et al., 2020). Given the higher concentration of TS present in slurry compared to lagoon water, it was not surprising that slurry had a greater relative abundance of *Bifidobacterium*, especially given their role in carbohydrate digestion (O'Callaghan and van Sinderen, 2016). Their higher relative abundance could also contribute to why a greater concentration of VFAs was detected in slurry compared to lagoon water (Table 4.1).

The decreasing relative abundance of the archaeal genus, *Methanobrevibacter*, showed that while methanogens typically existed in low relative abundance, as seen in the present study (Table 4.6), overall metabolic activity could still be high (Wirth et al., 2023). Since there were significant decreases in CH_4 emissions (Table 3.2; Chapter 3), it was possible that Eminex® negatively impacted the biochemical pathway of methanogenesis, rather than the methanogens. Further research is needed to confirm the effects of Eminex® on the metatranscriptomic activity of methanogens.

Only *Bacillus* had a trend for a treatment group, specifically for SL-LD, and decreased in relative abundance by 44.70% ($P < 0.1$). Not considered a top genus in lagoon water, *Bacillus* was part of the Firmicutes phylum. This genus was composed of aerobic, gram-positive bacteria often found in soils, water, and food products of plant origin and several pathogenic species (Schultz et al., 2017). Research inoculating composted manure with *Bacillus* species at low concentrations (0.5%) found that they enhanced carbon content by preventing mineralization and CO_2 losses (Duan et al., 2020). Inoculating swine slurry with *Bacillus* was also found to reduce volatile organic compounds, odor, and NH_3 emissions (Hwang et al., 2023). A greater relative abundance could've been part of combating ethanol emissions from slurry, which significantly increased, as discussed in Chapter 3. However, this would require additional research to confirm.

It was very likely the lack of significance noted at the genus level could be attributed to high variation between samples, given the small number of samples analyzed, due to financial limitations of the experiment. However, this does not eliminate differences at other levels of taxonomic organization, as many genera contain hundreds of species that could be individually affected by Eminex®.

4.2 Principle Component Analysis

As seen in Figure 4.1, there were a variety of patterns noted with the PCA plots. Principle component 1 (PC1) explained 45.7% of the variation seen between samples, whereas principle component 2 (PC2) explained 24.4% of the variation. These values acted as explanations of the connections between components of the two axes (Jolliffe and Cadima, 2016). Therefore, less than half of the variation at the phylum level was explained in the given plot.

To start, all the samples representing slurry were clustered together, on the left side of the graph, whereas all the lagoon water samples were clustered together on the right side of the graph. The samples from the two different types of manure being investigated also clustered by sampling day, apart from slurry on d 0 and SL-HD on d 7. These samples, while still all within the same quadrant, did not demonstrate the same tight clustering as the other samples, which was likely representative of a core population of microbes across the types of manure.

All d 56 samples, regardless of treatment, clustered away from the earlier sample days. Interestingly, the d 28 samples for LW-HD were closer to d 56 samples versus the other d 28 or even earlier samples. This could be that LW-HD elicited a more aggressive change to the population sooner, compared to LW-CONT and LW-LD. In fact, the clustering of the lagoon water samples seemed to read almost like a map, with samples becoming more dissimilar to d 0 as time progressed. The same was not seen for slurry, as the samples were more spread out and mixed regardless of sampling day.

At the genus level, the PC1 and PC2 axes also explained less than half of the variation. However, there was a unique clustering pattern. Overall, the same distinct separation between slurry and lagoon remained. However, at the genus level, LW-CONT on d 56 isolated itself from the other d 56 samples, which were otherwise somewhat clustered together. Some of the LW-LD

d 56 samples were close to the LW-HD d 56 samples, but also positioned themselves closer to the earlier sampling days. Like at the phylum level, LW-HD d 28 samples were closely clustered to d 56. Again, this was likely due to the higher concentration of Eminex® affecting the microbiome faster than at the 50% dose. In fact, it seemed that the LW-HD samples to differentiate themselves from the other groups around d 14.

The separate clustering of lagoon water and slurry samples was not unexcepted. It confirmed that each type of excreta had a unique population of core microbes. This had been noted in previous research, when comparing flushed manure to dry manure (Pandey et al., 2018), across sampling locations (García-Lozano et al., 2019), and between the rumen and manure microbiome (Ozbayram et al., 2018).

Given the distinct clustering in populations and the differences in mitigation potential across slurry and lagoon water, these PCA plots lend insight into where Eminex® might most effectively be applied on a commercial farm. As the current recommended dose of 1 kg Eminex®/m^3 slurry was high, it would be possible that a lower dose could provide a similar impact to the microbiome and still reduce gaseous emissions if applied earlier in the manure management chain, like during handling/collection, rather than storage. This would provide additional time needed for Eminex® to become effective if added to a settling basin or pit prior to manure being pumped into a lagoon or moved for drying. Future research should investigate applying Eminex® at different points of a manure management chain to establish the best point along the management chain for application to minimize dose and maximize emission mitigation potential. Research like this has yet to be done, with most research focused on treatments at the storage stage of manure management (Peterson et al., 2020; Sokolov et al., 2021; Chiodini et al., 2023).

4.3 Linear Discriminatory Analysis

When assessing the efficacy of Eminex®, it was vital to determine more discreetly how the populations changed on each sampling day across all taxonomic levels of organization. Figure 4.3 showed LW-CONT across all sampling days. As mentioned, the presence of all four sampling days indicated significant differences—positive or negative—between each day for specific taxonomic levels. Previous sections of the discussion have focused on mean relative abundance at the genus and phylum level only, whereas the LEfSe lent insight into the other taxonomic levels. In fact, the histogram plots identified which clades among all those presented were statistically and biologically different in order to explain the most variation between groups (Segata et al., 2011).

For LW-CONT, as there was no treatment added, nor addition of fresh lagoon water (in any barrel regardless of treatment), the differences in taxa throughout the experimental period reflected the natural changes in populations overtime, assuming limited available fermentable organic matter. The lagoon water seemed to be initially colonized, as compared to later sample days, by the phylum and class, Actinobacteria. As a Gram-positive aerobic bacteria involved in the fermentation of lignocellulolytic fibers and carbohydrates (Chen et al., 2022; Mitra et al., 2022), its presence in newly collected lagoon water was unsurprising, as lagoon were inundated with fresh excreta every day, multiple times a day. These bacteria would be able to thrive in the nutrient rich, mildly alkaline environment (Chen et al., 2022), given that it had a comparatively lower concentration of lactic acid compared to fresh dairy slurry (Table 4.1).

By the end of the first sampling period, when samples were collected on d 14, the Proteobacteria phylum, specifically the orders, families, and genera within the classes Alphaproteobacteria and Gammaproteobacteria, became more prominent. However, by the end of

the experiment, the lagoon water was completely dominated by Proteobacteria and various taxa within that phylum, like the class Betaproteobacteria, order Rhodobacterales, and genus *Nitrosomonas*, to name a few. This was confirmed by the increasing relative abundance of Proteobacteria for LW-CONT from d 0 to d 56 (Table 4.3). Proteobacteria have been shown to have a greater potential for deriving nutrients from manure-derived carbohydrates, compared to other phyla (Li et al., 2020), its prominence in lagoon water at such high relative abundance would be considered normal. It was also notable that the kingdom Bacteria had also increased in relative abundance by d 56 for LW-CONT. This showed that the entire microbial population was negatively affected by Eminex®, reducing the overall bacterial population in the treated groups.

The two Eminex® treatments had different outcomes regarding the specific taxa responsible for variation between the sampling days. For LW-LD, only days 0, 28, and 56 had significant effects of treatment on relative abundance for across various taxonomic levels. While there was also a similar domination by Proteobacteria (Table 4.3), the phylum Actinobacteria distinguished itself from other taxa in Figure 4.4. It significantly increased in relative abundance overtime, peaking by d 56. This confirmed, in addition to mean relative abundance (Section 4.2), that Proteobacteria and Actinobacteria were significantly impacted by Eminex®, influencing the lagoon water composition and gaseous emission potential. Interestingly, there were fewer taxa in the LEfSe of LW-LD compared to LW-CONT and LW-HD. It was therefore possible that the microbiome of LW-LD samples did not change as significantly as LW-HD compared to LW-CONT. This further supported the hypothesis that while the lower LW-LD treatment still significantly reduced GHG emissions reported in Chapter 3, it needed more time to make significant changes to the microbiome.

For LW-HD, there were significant differences across many taxonomic levels across all four sampling days (Figure 4.5). It was notable that on d 28, there was significantly more bacteria, receiving a positive LDA score. This indicated that bacterial populations grew until peaking around d 28. The same was seen for LW-LD (Figure 4.4). However, bacterial populations in LW-CONT peaked on d 56. This demonstrated the clear suppressive effect Eminex® had on bacterial populations. By d 56, there were positive LDA scores for Firmicutes and Actinobacteria, both of which had significantly increased in relative abundance (Table 4.3), but not Proteobacteria, which decreased in relative abundance. Research into the metabolic pathways of Firmicutes bacteria showed that these microbes were involved in amino acid and carbohydrate metabolism, whereas Proteobacteria was negatively correlated with the genes associated with those pathways (Li et al., 2020). The authors postulated that these phyla existed to complement to one another, with relative abundance increasing for one as the other decreased and vice versa (Li et al., 2020). That same pattern was also seen in the present study, in lagoon water and slurry. It is important to note that Proteobacteria also metabolize amino acids, but target separate types of amino acids. Proteobacteria metabolize valine, leucine, and isoleucine, whereas Firmicutes metabolize alanine and glutamate (Hatzenpichler, 2012; Li et al., 2020; Prosser et al., 2020). Actinobacteria were also an important phylum for LW-HD in LEfSe analysis. These bacteria, involved in mineral cycling of elements like C, N, P, and K could have responded to the higher levels of C and N present in the lagoon water with the application of Eminex® (Peng et al., 2021). Total N and C significantly increased in LW-HD, compared to LW-CONT (Table 3.3; Chapter 3).

When looking at the LDA plot for d 56 between the treatments, LW-CONT had more taxa belonging to the phylum Proteobacteria, compared to LW-LD and LW-HD (Figure 4.6). Many taxa identified for LW-LD also belonged to Proteobacteria, apart from the order Micrococcales of

the phylum Actinobacteria. The major classes of Proteobacteria differed between groups. While LW-CONT had more Alphaproteobacteria, LW-LD had more Gammaproteobacteria, and LW-HD had more Betaproteobacteria, which contained the specie *Comomonas testosteroni* and order Burkholderiales.

These Proteobacteria classes have some significant biological differences of interest. Alphaproteobacteria were gram-negative bacteria of a wide variety of aerobicity, including obligate aerobes and anaerobes, and facultative aerobes and anaerobes (Hördt et al., 2020). This class contained the genus *Rhizobium* of N-fixing bacteria, as well as genera *Rhodobacteraceae* and *Rhodospirallaceae*, colloquially known as purple non-sulfur bacteria and commonly found in anaerobic lagoons (Okubo et al., 2006; Khairunisa et al., 2023). Furthermore, Alphaproteobacteria included species of methylotrophs, capable to reducing CH_4 for energy and growth (Hördt et al., 2020). The presence of this class of bacteria within the LW-CONT was indicative of a normal microbiome.

For LW-LD, the class Gammaproteobacteria was characterized by bacteria with a broad range of oxygen aerobicity, like Alphaproteobacteria, temperature adaptation, sulfur oxidizers, and microbes capable of carbon fixation through chemoautotrophs and photoautotrophs metabolism (Williams et al., 2010; Dyksma et al., 2016). Given that there were significant reductions in CH_4 emissions paired with increasing total carbon (Table 3.3 and Table 3.4; Chapter 3), it was probably that this genus played some role in those compositional changes. Since the LDA plot showed that their relative abundance was higher in LW-LD compared to the other groups, indicating a taxon of significant difference to explain variation between the groups, the Gammaproteobacteria class could have proliferated to replace the Alphaproteobacteria seen in LW-CONT.

The class Betaproteobacteria seen more prominently in LW-HD contained anaerobic strains of denitrifying bacteria well adapted to alkaline conditions, that consumed fatty acids and non-structural carbohydrates (Strijkstra et al., 2014; Suzuki et al., 2014). Some bacteria within this class also grew autotrophically via the consumption of hydrogen, oxygen, and other compounds (Suzuki et al., 2014). Betaproteobacteria were also shown to hydroxylate methyl-containing molecules, because of species like *Methylophilus methylotrophus* (Muffler et al., 2011). As methylated compounds were known substrates for methylotrophic methanogens of Euryarcheotic orders Methanosarcinales, Methanobacteriales, and Methanomassiliicoccales (Vanwonterghem et al., 2016; McSweeney and Mackie, 2020). These three archaeal orders were present in lagoon water samples. With the positive LDA score attributed to Betaproteobacteria in LW-HD, it could be hypothesized that these methylotrophic bacteria competed with methylotrophic methanogens for methylated compounds, hereby helping to reduce the amount of CH_4 produced in lagoon water.

There were no LDA differences to explain variation between slurry samples. This could've been attributed to high variation between samples. However, further research would be prudent to explore what taxa most contributed to the differences otherwise noted between the samples, through relative abundance and PCA plots.

5 CONCLUSIONS

Eminex® elicited significant changes to the microbiome of dairy slurry and lagoon water. Relative abundance showed that the phylum Proteobacteria was most impacted, its population declining significantly in both forms of excreta. These bacteria were vital to the N cycle and likely caused the increase in NH_3 emissions in lagoon water noted in Chapter 3, as Eminex® included a nitrification inhibitor. Furthermore, the decrease in Proteobacteria resulted in a significant increase

of the Firmicutes and Actinobacteria phyla, which fermented organic matter and generated VFAs, like acetic acid that was found to increase in concentration with treatment (Chapter 3). Despite this increase concentration of end products that could become GHGs, the relative abundance of archaeal methanogens of the phylum Euryarcheota significantly increased in LW-LD and LW-HD, but not in SL-LD and SL-HD. However, CH_4 emissions significantly decreased with Eminex® treatment (Chapter 3). Ultimately, one of two things probably caused this: first, the substrates were used by other microbes. Because other bacterial phyla increased in relative abundance throughout the experiment, this implied that normal fermentation was proceeding and therefore, there was no accumulation of H_2 or other compounds that could stop fermentation. Firmicutes and Actinobacteria contained bacteria capable of taking up these end products. Secondly, Eminex® could have interrupted methanogenesis directly. Certain feed additives, like 3-nitrooxypropanol, were designed to stop methanogenesis by preventing the catalysis of last chemical step from creating CH_4, by inactivating the methyl-coenzyme M reductase enzyme (Duin et al., 2016). Additional research is needed to determine if Eminex® acts similarly.

While Eminex® has shown itself to be a potent additive, capable of not only suppressing GHG emissions and other air pollutants under the right conditions, but as previously mentioned, it is expensive. However, as shown in Chapter 3 and in the present chapter, Eminex® is equally effective at 50% of its recommended dose. Future research must focus on reducing the dose, because as it stands, Eminex® is not a cost-effective option for U.S. dairy farmers, unless carbon credits or subsidies could be used. However, the present study proves that applying Eminex® earlier on in the production chain, has the potential to extend the contact time needed to elicit microbiome changes, and prevent increased NH_3 emissions in lagoon water and ethanol from slurry, but also reduces the amount of Eminex® needed for treatment. If applied when manure is

collected, prior to flushing into a lagoon, like in a settling basin, the additive has the means of being more cost effective while reducing the environmental impact of manure management.

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