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Association of Enteric Methane-Emitting Phenotype of Lactating Holstein Dairy Cows and Different Biological and Functional Parameters

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ABSTRACT

Enteric methane, as a product of microbial fermentation in ruminants, is responsible for 27.4% of anthropogenic methane emissions in the United States. Enteric methane production also theoretically reduces feed efficiency in beef and dairy cattle, as energy that would otherwise be used for growth or lactation can be directed toward methanogenesis. There is recent and ongoing research into different management practices that may reduce enteric methane emissions, as well as into the effects of selecting for phenotypically low-methane-emitting ruminants. This thesis aims to describe the associations between phenotypically high- and low-methane-emitting lactating Holstein dairy cows and biological and functional parameters, including the production, intensity, and yield of enteric carbon dioxide and hydrogen gases, body weight, body condition score, milk composition, milk yield, dietary intake, apparent total-tract digestibility, and urinary composition and output. This thesis further aims to evaluate if selection for low-methane-emitting lactating Holstein dairy cows may also select for smaller, less feed-efficient, and lower-producing cattle. From October to November 2025, fifty lactating Holstein dairy cows at the Penn State Dairy Barns were screened for enteric methane, carbon dioxide, and hydrogen gas emissions. Samples taken included feces for evaluation of apparent total-tract digestibility using neutral detergent fiber, acid detergent fiber, indigestible neutral detergent fiber, and ash analyses, milk for true protein, fat, lactose, and milk urea nitrogen analyses, and urine for urea nitrogen analysis and creatinine analysis, which was used for calculation of total urine output and nitrogen balance. Production data on body weight, body condition score, and milk yield were recorded, along with daily feed offered and daily feed refused for the calculation of dietary intake. The total mixed ration offered to the cows was analyzed for dry matter, organic

matter, and ash content. Following screening, cows were classified into high-, medium-, and low-phenotypic enteric methane emission groups based on average daily enteric methane production. Phenotypically high-enteric-methane-emitting and phenotypically low-enteric-methane-emitting cows were examined for associations between enteric methane-emitting phenotype and the above-listed biological and functional parameters.

Diurnal variation in enteric methane, carbon dioxide, and hydrogen gas production was observed in all phenotypic enteric methane emission groups, with increased gas production following feeding and decreased gas production after milking and during sleeping. Phenotypically low-enteric-methane-emitting cows were associated with having lower dry matter, organic matter, neutral detergent fiber, and acid detergent fiber intake, and reduced yields of energy corrected milk than phenotypically high-enteric-methane-emitting cows. Tendency for phenotypically low-enteric-methane-emitting cows to have lower enteric methane intensity and milk yield was observed. No significant differences in enteric methane, carbon dioxide, and hydrogen gas yield, enteric carbon dioxide and hydrogen gas intensity, milk composition, apparent total-tract digestibility of nutrients, and urine contents and total output were observed between phenotypic enteric methane-emitting groups.

Findings of this study raise concerns that genetic selection for lower enteric methane production may select for lower-producing cattle, potentially as a function of decreased dietary intake. Further, results push for future research on examination of additional factors that may be associated with the low-enteric methane-emitting phenotype, such as feed passage rate and rumen volume, so as not to select for lower-producing cattle when attempting to mitigate enteric methane emissions in the livestock industry.

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LIST OF ABBREVIATIONS

ADF	Acid Detergent Fiber
CP	Crude Protein
DIM	Days in Milk
DM	Dry Matter
DMI	Dry Matter Intake
ECM	Energy Corrected Milk
iNDF	Indigestible Neutral Detergent Fiber
MUN	Milk Urea Nitrogen
MY	Milk Yield
NDF	Neutral Detergent Fiber
NEL	Net Energy for Lactation
OM	Organic Matter
RDP	Rumen Degradable Protein
RES	Rumen Escape Starch
RFID	Radio-Frequency Identification
RMP	Residual Methane Production
RUP	Rumen Undegradable Protein
TMR	Total Mixed Ration
VFA	Volatile Fatty Acid

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Chapter 1

Literature Review

Environmental Effects of Domestic Cattle

With heightened concerns about global warming and climate change, the agricultural industry is facing increasing pressure to reduce its environmental effect; today, 9.4% of greenhouse gas emissions in the United States come from the agricultural sector alone (U.S. Environmental Protection Agency, 2024). One of the most potent greenhouse gases released from the agricultural industry is methane (CH_4), and increasing amounts of it have been released in recent years. In 2022, agriculture generated 14% more CH_4 than it had in 1990 (U.S. Environmental Protection Agency, 2024). The largest portion of CH_4 emissions comes from enteric fermentation, which accounts for 32.5% of CH_4 emissions in the agricultural sector and 27.4% of all anthropogenic CH_4 emissions in the United States (U.S. Environmental Protection Agency, 2024). Of all the domestic ruminants that produce enteric CH_4, such as sheep, goats, and cattle, cattle emit the most, contributing to 77% of total emissions (Gerber et al., 2013b). The CH_4 and nitrous oxide (N_2O) produced by cattle have warming potentials 28 and 265 times those of carbon dioxide (CO_2), respectively (Grossi et al., 2018). As a product of ruminal fermentation, enteric CH_4, which is eructated from the rumen of the animal, makes up about 91.4% of the CH_4 emitted from dairy cattle (Grossi et al., 2018). The remaining 8.6% of CH_4 produced passes through the cattle's digestive tract and is excreted in feces (Grossi et al., 2018). Livestock manure management, or the capturing, storage, and treatment of manure for different recycling purposes and usages, accounts for an additional 10.9% of CH_4 emissions in the agricultural sector and

9.23% of all anthropogenic CH_4 emissions in the United States (U.S. Environmental Protection Agency, 2024).

The number of individual cattle in the United States is the lowest it has been in 75 years, at 94.2 million head as of July 1, 2025 (USDA, 2025). Of these cattle, 9.45 million are lactating dairy cows (USDA, 2025). Despite this decrease in animal numbers, the amount of product generated, such as milk and beef, has increased due to advances in production efficiency, breeding programs, and animal nutrition. From 2004 to 2024, the United States increased milk production by 32%, from 170.8 billion pounds to 225.9 billion pounds, and the United States commercial beef production, on a carcass weight basis, increased by roughly 10.2%, from 24.5 billion pounds to 27 billion pounds (Knight, 2025; Gillespie and Njuki, 2026). Even with these changes in productivity, demand for livestock products is rising due to global population growth, urbanization, increasing incomes in many countries, and lifestyle and dietary changes (*Livestock and the environment*, n.d.). Developing sustainable agricultural practices to feed a growing world, where 600 million people are predicted to experience food insecurity by 2030, is a vital part of meeting the United Nations' second sustainable development goal, achieving zero hunger (United Nations, 2022). With countries setting further greenhouse gas emission reduction goals, and with demands for livestock products increasing, it is important to continue to investigate the reduction of the cattle industry's environmental effects for a more sustainable and productive future.

Methanogenesis in the Rumen

Enteric fermentation is a form of anaerobic metabolism that occurs in the digestive tract of ruminant animals, in which carbohydrates from the animal's diet are catabolized by microorganisms to produce fatty acids, CO_2, and dihydrogen gas (H_2) (Giesenhagen, 2023). As H_2 product, in both gaseous and dissolved forms, accumulates in the rumen, it can inhibit further carbohydrate fermentation by slowing reaction rates (Grossi et al., 2018). One metabolic pathway that microorganisms have evolved to remove excess H_2 is methanogenesis, in which certain archaea in the rumen utilize dissolved H_2 as a reducing agent to generate a proton-motive force that drives adenosine triphosphate (ATP) synthesis. Methanogenesis usually takes one of three major pathways: the methylotrophic, aceticlastic, and hydrogenotrophic pathways, although additional, less common pathways exist. The methylotrophic pathway uses methanol and methylated amine substrates to produce CH_4, while the aceticlastic pathway uses acetate as a substrate for CH_4 and CO_2 production (Kurth et al., 2020).

In the rumen, where hydrogenotrophic methanogenesis is the primary pathway, CO_2 and H_2 are the substrates, and the greenhouse gas CH_4 is the product. In this biochemical pathway, methanogenic archaea produce and use the enzyme formylmethanofuran dehydrogenase to bind CO_2 to the amino group of coenzyme methanofuran, producing formylmethanofuran (Szuhaj et al., 2023). Enzyme formylmethanofuran-tetrahydromethanopterin formyltransferase then catalyzes the transfer of a formyl group from coenzyme formylmethanofuran to coenzyme N5-Formyl-tetrahydromethanopterin, and methenyl-tetrahydromethanopterin cyclohydrolase catalyzes the dehydration synthesis of N5-Formyl-tetrahydromethanopterin to 5,10-Methenyl-tetrahydromethanopterin (Acharya et al., 2006; Szuhaj et al., 2023). In a separate reaction, an enzyme called coenzyme F_{420}-reducing hydrogenase reduces the methanogenic coenzyme F_{420} to

F_{420}-H_2 using dissolved H_2 (Szuhaj et al., 2023). Coenzyme F_{420}-H_2 then oxidizes 5,10-Methenyl-tetrahydromethanopterin with catalysis from the enzyme F_{420}-reducing methylene-tetrahydromethanopterin to produce methylene tetrahydromethanopterin (Kurth et al., 2020; Szuhaj et al., 2023). Now that the two substrates have been taken into one reaction pathway, enzyme 5,10-methylene-tetrahydromethanopterin reductase reduces methylene-tetrahydromethanopterin to 5-methyl-tetrahydromethanopterin (Szuhaj et al., 2023). Located in the plasma membrane of methanogens, tetrahydromethanopterin *S*-methyltransferase then transfers a methyl group from 5-methyl-tetrahydromethanopterin to produce methyl-coenzyme M (Szuhaj et al., 2023). In the final step of this pathway, methyl-coenzyme M reductase reduces methyl-coenzyme M to CH_4 (Szuhaj et al., 2023). The entirety of this process is beneficial to methanogenic archaea as the reduction of CO_2 substrate produces the proton gradients needed to generate ATP in an energy-harvesting pathway.

Regardless of the benefit to ruminal microorganisms, methanogenesis in the rumen poses a potential productivity loss to the animal, as anywhere from 2 to 12%, but on average 6% of gross energy from the feed is put toward CH_4 production instead of toward use by the animal (Johnson and Johnson, 1995). The percentage of energy loss has been shown to fluctuate with the ratio of produced propionate to acetate volatile fatty acid (VFA), which ranges from 0.9 to 4, where production of more propionic acid in comparison to acetic acid reduces energy loss to methanogenesis, but production of less propionic acid in comparison to acetic acid increases it (Johnson and Johnson, 1995).

Although increases in dry matter intake (DMI) are associated with increased enteric CH_4 production, in terms of animal productivity, higher intake results in a lower percentage of gross energy from the diet being lost to methanogenesis (Johnson and Johnson, 1995). It also appears

that the level of gross energy loss to methanogenesis increases when cows are fed less-digestible diets with less available carbohydrate (Johnson and Johnson, 1995).

As mentioned previously, produced CH_4 is not further metabolized by the ruminant or its hosted microorganisms, so it is primarily eructated from the animal. Enteric CH_4 production is largely dependent on the amount of H_2 gas produced, so redirection of H_2 to other metabolic pathways or reduction in the amount of H_2 produced entirely are possible ways to reduce enteric CH_4 output. Propionate-forming bacteria can utilize pyruvate from glycolysis and H_2 to produce propionate instead of CH_4, which is one of three main volatile short-chain fatty acids produced in ruminant animals (Wang et al., 2023). Other pathways on H_2 redirection include conversion of CO_2 to acetate in homoacetogenesis; of nitrate, nitrite, and ammonia to N_2O in nitrate reduction; of sulfate to hydrogen sulfide (H_2S) in sulfate reduction; of alkenes to alkanes in the saturation of fatty acids; and potentially of feeds to microbial biomass (Wang et al., 2023).

There are naturally occurring differences in enteric CH_4 emission levels, with associations between enteric-CH_4-emitting phenotype and animal genetics, as well as between enteric-CH_4-emitting phenotype and ruminal microbiome composition. The cattle genome has also been shown to be associated with the overall function of the ruminal microbiome (Martínez-Álvaro et al., 2022). Host genomic effects on the microbiome, which are the ability of an animal's genome to influence and interact with its hosted microorganisms, were seen in 29 rumen microbial genera, 22 metagenome assembled uncultured genomes, and 115 genes associated with enteric CH_4 emissions in one observational study (Martínez-Álvaro et al., 2022). These host genomic effects may influence methanogenesis directly, such as with the *cofG* gene, affect microbial interspecies communication mechanisms, such as with the *ABC.PE.P* gene, affect substrate availability for archaea, with the *bcd* and *pceB* genes, have host-microbiome

interactions, with the *TSTA3* gene, and affect genetic information processes, with the *RP-L35* gene (Martínez-Álvaro et al., 2022).

Further, phenotypically low-enteric-CH_4-emitting lactating Holstein dairy cows have a lower ratio of acetate to propionate in the rumen at about 2.073 than do phenotypically high-enteric-CH_4-emitting cows at a ratio of 2.875, which aligns with previous findings that increased propionate production is associated with lowered enteric-CH_4 emission (Stepanchenko et al., 2023). These phenotypically low-enteric-CH_4-emitting cows also had greater abundances of *Succinivibrionaceae* and *Veillonellaceae* in their ruminal microbiomes; the presence of these archaeal families is associated with higher molar concentrations of propionate in the rumen (Stepanchenko et al., 2023).

Methods for Enteric Methane Emission Measurement

There is ongoing research into different methods for measuring enteric CH_4 levels; although many have proven effective, they vary in feasibility and cost. Different methods of enteric CH_4 emission measurement may be used to achieve different goals; some are better for measuring emissions from individual animals, others are better for measuring CH_4 emissions from entire herds, and still others are better for measuring the total amount of greenhouse gas emissions from entire farms. Currently, effective techniques for measuring enteric CH_4 emissions in long-term studies include using respiration chambers, automated head-chamber systems like GreenFeed, and the sulfur hexafluoride (SF_6) tracer gas method (Hristov et al., 2025). Using respiration chambers, animals are housed in airtight containers where concentrations of inflowing and outflowing gases, as well as gas flow rates, are recorded (Zhao

et al., 2020). Green Feed machines record the radio-frequency identification (RFID) tags of animals who voluntarily visit the machine and dispense bait; when the animal begins feeding and releasing enteric gases, the machine's fan pulls CH_4, CO_2, and H_2 emissions toward non-dispersive infra-red analyzers for gas quantification, using propane as a tracer gas (Hristov et al., 2015). In the SF_6 tracer gas method animals are equipped with canisters of SF_6 gas, which continuously release SF_6 gas into the rumen through a permeation tube, and a capillary tube attached to the animal's halter samples eructated gases for storage into a second canister (Pfau, n.d.) Then, ratios of SF_6:CH_4 are analyzed to determine total enteric CH_4 production (Hristov et al., 2015).

Levels of enteric CH_4 emissions are not only influenced by DMI and dietary composition, but also change with many biological and production factors, including frame size, rumen capacity, feed-passage rate, body weight (BW), body condition score (BCS), and stature. To account for these factors, emissions are often reported as CH_4 yield or CH_4 intensity, rather than solely in CH_4 production measured in grams produced per day. CH_4 yield is the grams of CH_4 produced adjusted per unit of DMI, to be expressed in grams CH_4/kilogram of DMI, and CH_4 intensity is the grams of CH_4 produced adjusted per unit of product produced, such as milk or beef, expressed in grams CH_4/kilogram of product. For example, enteric CH_4 emissions increase with DMI, but when CH_4 emissions are compared with increases in milk production in dairy cattle and average daily gain in beef cattle, which are also positively correlated with increased intake, CH_4 intensity decreases (Min et al., 2022).

As we aim to decrease CH_4 intensity in dairy cattle, to reduce emissions while supporting increased productivity, different enteric CH_4 mitigation strategies are often compared by measuring enteric CH_4 reduction potential in percentages, per standard unit of energy corrected

milk (ECM) produced. Expressing enteric CH_4 emissions in terms of intensity rather than production avoids incorrect assumptions about strategy efficacy that may result from comparisons of gross greenhouse gas production. As different cow breeds as well as different individual cows within a breed have different fat and protein contents in the milk they produce, milk composition is mathematically scaled to 4 percent fat and 3.3 percent protein to standardize milk and allow for comparisons of milk yield (MY), as ECM, between groups.

Management Strategies for Enteric Methane Emission Reduction

When examining strategies to reduce enteric CH_4 emissions in a given herd, it is important to consider factors such as feed availability, treatment costs, and implementation feasibility, all of which relate to workforce availability and herd size. Further, as ruminant species, ruminant breed, animal age, and animal production level all influence rumen function and therefore the efficacy of a given management strategy, these factors should be considered during strategy selection (Hristov et al., 2025).

Two of the largest factors that influence enteric CH_4 emissions in cattle are dietary intake and feed digestibility. Higher feed digestibility is typically reflected in diets with higher grain content and lower indigestible fibers, such as lignin, which is commonly found in forages like hay and straw. However, indigestible fibers should not be eliminated from cattle diets, as the bulk they provide to the feed stimulates rumen contractions important for digestion, increases saliva production, and adds to the ruminal mat that holds feed for further fermentation.

Additionally, physically effective fibers, or those that are longer than 0.31 inches, help to prevent rumen acidosis by acting as a filter to decrease feed passage rate and increase ruminal

pH (Peña and Gartman, n.d.). Increased ruminal pH not only harms the animal directly but also reduces the ability of microorganisms to assist digestion by either halting their fermentation or killing them. It is important to balance increasing feed digestibility with maintaining sufficient physically effective fiber to support rumen and microbial health. Further, many stakeholders in the beef and dairy industries believe in feeding high-forage diets to provide cows with feed sources that cannot be utilized by monogastric animals, and to meet requirements for "grass-fed" labels, through which they can market their products.

Regardless of what enteric CH_4 emission reduction methods a given corporation, farm, or organization plans to incorporate, they should be aware of the "pollution swapping" effect, which is the unintentional or unknown increase in an additional environmental pollutant that happens because of a reduction in the targeted environmental pollutant (Gerber et al., 2013b). It should be noted that as less substrate is fermented in the rumen of the animal, more fermentable substrates, like organic matter and nitrogenous contents, may be excreted and later fermented in the manure to release CH_4 and N_2O gases (Hristov et al., 2013; Grossi et al., 2018). Further, management methods of manure itself chosen to for reduction of specific potent greenhouse gases may result in increased emissions of others; storing liquid manure in lagoons avoids the aerobic conditions needed for N_2O production and results in more CH_4 production, but using solid liquid as fertilizers removes some anaerobic conditions so that less CH_4 is produced, but so that more N_2O is produced (Grossi et al., 2018).

Potential ways to reduce CH_4 emissions resulting from enteric fermentation can be summarized into the following categories: manipulation of diet composition or quality, manipulation of feed processing, inclusion of nutritive or non-nutritive feed additives, vaccination, and genetic selection for phenotypically low-enteric-CH_4-emitting animals (Grossi

et al., 2018). With regards to feed additives, they can be divided into two general categories: those that directly inhibit methanogenesis, called direct inhibitors, and those that indirectly inhibit methanogenesis, called rumen modifiers (McFadden et al., n.d.). Direct inhibitors, such as nitrates like 3-nitrooxypropanol, directly interfere with methanogenesis and methanogenic microorganisms (McFadden et al., n.d.). Rumen modifiers indirectly affect production of CH_4 by modifying substrate levels required for methanogenesis and altering rumen environmental conditions that drive reaction rates (McFadden et al., n.d.).

Enteric CH_4 emissions decrease with increased forage quality; diets with higher grain content and greater digestibility direct H_2 toward the production of VFAs, particularly propionate, rather than toward methanogenesis (Hristov et al., 2013). One study demonstrated that steers fed diets with higher neutral detergent fiber (NDF) content showed higher enteric CH_4 intensity in terms of average daily gain; despite enteric CH_4 production not differing between high- and low-quality forage diets (Santander et al., 2023). High-quality, low-NDF diets were associated with more energy-efficient cattle, as they produced more meat without increasing CH_4 production (Santander et al., 2023). Similarly, grass-finished beef cattle on pasture emitted 30% more CH_4 and N_2O emissions than did grain-finished beef cattle in feedlots (Pelletier et al., 2010). This increase in greenhouse gas emissions is associated with both decreased quality of feed offered in pasture (e.g., forages vs. high-energy grains) and increased finishing time required for grass-finished cattle.

Differences in silage composition are associated with differences in enteric CH_4 emissions; greater inclusion of corn and legume silages and lesser inclusion of grass silage in the diet may result in lower enteric CH_4 emissions. In one study, cows fed 40% grass silage and 10%

grass silage on a DM-basis produced 11% less enteric CH_4 and 45% less N_2O emissions than did cows fed 50% grass silage on a DM-basis (van Gastelen et al., 2023).

As mentioned previously, increased DMI is associated with increased enteric CH_4 production, but is also associated with the Y_m factor of the diet, which is the percentage of the animal's gross energy intake that is lost to methanogenesis (Hristov et al., 2013). This may be a result of increased feed passage rate, which gives microorganisms less time to digest and ferment feed (Hristov et al., 2013). This may also lead to more fermentable substrate in manure, which can subsequently cause later greenhouse emissions with the pollution-swapping effect (Hristov et al., 2013). Further, increased DMI increases enteric CH_4 intensity by a greater amount when the diet is less digestible (Hristov et al., 2013).

Feeding a diet higher in starch than in fiber can also help redirect H_2 toward proportionate synthesis, as anaerobic starch fermentation by *Bacteroidia* produces VFAs such as propionate (Hatew et al., 2015; Giesenhagan, 2023; Döring and Basen, 2024). Further, inclusion of starch into the diet may reduce enteric CH_4 production as rumen escape starch (RES) bypasses rumen fermentation and proceeds to digestion in the small intestine; however, digestion levels here are typically lower at only 40% to 62% of RES being digested, posing concerns for feed efficiency (Harmon et al., 2004; Dijkstra et al., 2011; Yin et al., 2026).

Inclusion of concentrates in the diet reduces enteric CH_4 yield; this inclusion is more effective in reduction when DMI is greater than 40% concentrate composition (Hristov et al., 2013). Further, improvements to forage quality, such as through chemical treatments with alkali solutions, which make fibers more digestible, lead to reductions in enteric CH_4 emissions (Shah et al., 2025).

The inclusion of dietary lipids or oil supplementation may also reduce enteric CH_4 emissions by up to 15% per unit of ECM (Grossi et al., 2019). Caution must be taken in ensuring that dietary lipid supplementation does not bring the total fat percentage of the diet's dry matter (DM) content past 10%, at which point it can lower feed intake, resulting in lower milk production, and can change the composition of microbial populations, resulting in reduced fiber fermentation capabilities (Grossi et al., 2019). Again, inhibition of carbohydrate fermentation in the rumen may increase greenhouse gas emissions from manure, as the excreted fermentable substrate is instead fermented in the manure. Typically, between 5-8% lipid supplementation of fats such as whole cottonseed, ground canola, brewer's grains, coconut oil, maize meal, linseed oil, fish oil, and calcium palmitate has been found to be effective in reducing enteric CH_4 emissions (Grainger and Beauchemin, 2011). Another study found that supplementation of the diet with olive oil, sunflower oil, and linseed oil at 6% of DM decreased CH_4 production by 21-28% compared with unsupplemented controls (Vargas et al., 2020).

Nitrate supplementation in feed is also associated with enteric CH_4 emission reduction, particularly in low-protein diets. By acting as alternate electron acceptors for dissolved H_2, nitrates prevent methanogenic archaea from producing CH_4. Other compounds that may act as electron acceptors include methylene blue, riboflavin, nicotinamide adenine dinucleotide, sulfates, methyl-viologen, and benzyl-viologen (Knapp et al., 2014). One compound, 3-nitrooxypropanol, has been effective in many short-term studies and is currently being evaluated for long-term effects on dairy cattle health and for whether enteric CH_4 emission reductions are persistent, or whether microbes can adapt to its presence. One meta-analysis reports that supplementation of 10 g/kg DM of nitrate reduced enteric CH_4 emission intensity by 20.2% (Dicks et al., 2026).

Compounds derived from plants, or plant secondary compounds, have also been shown to reduce enteric CH_4 emissions. Tannins have the potential to reduce daily enteric CH_4 production by 4.5 g CH_4/day, as they repress fiber digestion by 6 to 12% and subsequently inhibit methanogenesis in methanogenic archaea (Martins et al., 2024; Souza et al., 2026).

Ionophores, which are antibiotic compounds isolated from *Streptomyces species*, upregulate propionate synthesis in the rumen, thereby removing the H_2 substrate that methanogens would otherwise use for enteric CH_4 production (Marques and Cooke, 2021). As they can indirectly reduce enteric CH_4 emission levels, they can be added to cattle diets to reduce enteric gas emissions and have been identified as being particularly effective in high-concentrate diets (Hristov et al., 2013).

The addition of plant matter, such as red algae species, is another option for reducing enteric CH_4 emissions. *Asparagopsis taxiformis* and additional *Asparagopsis spp.* have been identified as effective, with potential to reduce enteric CH_4 emission intensity by about 22.6% (Stefenoni et al., 2021; Martins et al., 2024). Further, supplementation of the red algae species *Laurencia snackeyi* in the diet has been shown to reduce enteric CH_4 emission intensities by 26% in Holstein cows (Wasson et al., 2026).

It is important to note that all methods of reducing enteric CH_4 emissions that involve dietary supplements can have dose-dependent effects on emission intensity, reduction potential, animal production, feed intake, and animal health. When evaluating the efficacy of feed additives in reducing enteric CH_4 emissions, it is vital to consider any associated health effects, as animal health should be the number one priority. Further, regarding feed additives, the effectiveness of supplementation can vary with changes in basal diet composition. Last, there is also potential for

ruminal microbial communities to adapt to the presence of anti-methanogenic feed additives, so evaluation for long-term efficacy is needed.

Vaccinations against methanogenic archaea may reduce enteric CH_4 production, but the long-term efficacy of this strategy remains uncertain, given potential microbial adaptations in response to vaccination (Hristov et al., 2013). Vaccination against *Methanobrevibacter* has been shown to reduce enteric CH_4 intensity in sheep by 7.7%, but the vaccine was unable to target a wide range of methanogens in the rumen (Wright et al., 2004).

The use of multiple enteric CH_4 reduction strategies together may have compounding effects on gross production, yield, and intensity. For example, a combination of feeding a diet higher in concentrate feed components and dietary starch and supplementing the feed with multiple additives, like canola oil and 3-nitrooxypropanol together, may have synergistic enteric CH_4 reduction effects (Ku-Vera et al., 2020; Martins et al., 2024).

As there are natural phenotypic differences in enteric CH_4 emission levels among individual ruminants due to differences in host genetics and ruminal microbiome composition, genetic selection for low-enteric-CH_4-emitting cattle is an appealing strategy for enteric CH_4 mitigation, given the potential for cumulative CH_4 reduction over generations and the long-term persistence of this reduction. Although there has been increased effort to examine the relationship between the enteric CH_4-emitting phenotype and ruminant performance in recent years, additional research is needed to identify differences in productive and functional characteristics between phenotypically high- and low-enteric-CH_4-emitting lactating dairy cattle.

Chapter 2

Introduction

This thesis aims to describe the associations between phenotypically high- and low-CH_4-emitting lactating Holstein dairy cows and biological and functional parameters, including the production, intensity, and yield of enteric CO_2 and H_2 gases, BW, BCS, milk composition, MY, dietary intake, apparent total-tract digestibility, and urinary composition and output. This thesis further aims to evaluate if selection for low-CH_4-emitting lactating Holstein dairy cows may also select for smaller, less feed-efficient, and lower-producing cattle. Previously conducted studies have identified concerns that phenotypically low-CH_4-emitting cattle may have decreased ability to digest organic matter and fiber (Stepanchenko et al., 2023; Kjedsen et al., 2024). Another study found that growing Hereford steers with lower residual feed intake were associated with lower enteric CH_4 emissions (26.8% decrease), where feed efficiency was higher for lower- CH_4-emitting cattle, as average daily gain remained the same for both groups (Dini et al., 2018). Further, DMI is strongly correlated, up to 86%, with differences in levels of enteric CH_4 production, but rumination time has not been proven to be correlated with DMI nor enteric CH_4 production in dairy cattle (Zetouni et al., 2018). Lower enteric CH_4 production has also been associated with increased feed passage rate in RUSITEC rumen simulations and, similarly, with lower feed retention time in sheep, as there is less time for methanogenesis to occur when feed fermentation time is reduced (Goopy et al., 2014; Arowolo et al., 2022). Smaller rumen size, as determined by x-ray computer tomography, has also been observed in low-enteric- CH_4-emitting sheep (Goopy et al., 2014). However, few studies directly examine differences in feed intake, feed digestibility, feed efficiency, and production between phenotypically high- and phenotypically low-CH_4-emitting ruminants, specifically high-producing lactating dairy cattle

(Denninger et al., 2020). There is increasing demand for this research with growing interest in selection for lower-CH_4-emitting cattle.

Different compositions of the ruminal microbiome, as well as different levels of methanogenic gene expression, have also been shown to correlate with enteric CH_4 emissions in ruminants (Shi et al., 2014; Stepanchenko et al., 2023). Higher transcription levels of methyl-coenzyme M reductase have been associated with high-enteric-CH_4-emitting sheep (Shi et al., 2014). Greater relative abundances of *Methanobrevibacter gottschalkii* clade species have also been observed in high-enteric-CH_4-emitting sheep, and greater relative abundances of *Succinivibrionaceae* and *Veillonellaceae*, which favor propionate production, have been observed in low-enteric-CH_4-emitting cattle (Shi et al., 2014; Stepanchenko et al., 2023).

As enteric CH_4-emitting traits, in terms of production, yield, and intensity, have been shown to be moderately heritable, there is a need to evaluate for potential genetic linkages to avoid unintentional selection for less feed-efficient, lower-producing, and less healthy animals. Further, identified associations between the animal's enteric CH_4-emitting phenotype and any production and biological factors should be investigated to explain how certain heritable CH_4 traits act on the animal to alter methanogenic conditions and the resulting biological or productive processes. Current practices for genetic selection of low-enteric-CH_4-emitting cattle are largely based on evaluation of enteric CH_4 yield, and more recently, evaluation of residual CH_4 production (RMP), which examines the level of expected CH_4 production for a given DMI, BW, or MY compared to the cows' observed CH_4 emission level (Negussie et al., 2017; Kolathingal-Thodika et al., 2026). This method acknowledges established associations between enteric CH_4 production and DMI and BW. Although selecting cows with lower RMP would not directly select for lower DMI and BW, it should be examined whether lower DMI and BW

reappear down the generational line (Kolathingal-Thodika et al., 2026). Last, the selection of low-enteric-CH_4-emitting cattle should be evaluated alongside the output of CH_4 and N_2O in manure, to look for potential pollution-swapping effects.

Chapter 3

Materials and Methods

All animals used in this experiment were cared for according to the guidelines of the IACUC protocol ID PROTO202502990, which was reviewed and approved by The Pennsylvania State University's Animal Care and Use Committee.

Selection of Cows for the Trial

Fifty lactating Holstein dairy cows from The Pennsylvania State University Dairy Complex were randomly selected for this trial, but preferentially selected to be near 30-100 days in milk (DIM) by the time their respective phase began, to reduce variation in reported CH_4 yields due to differences in DMI and energy balance that are associated with variations in lactation stage (Hristov et al., 2025). Selected cows were split into 3 groups for screening, with Phases 1 and 2 each evaluating 16 cows and Phase 3 evaluating 18 cows. Selected cows were screened for enteric CH_4 emissions, DMI, MY, BW, and BCS, and were sampled for feces, urine, and milk.

Housing, Diet, & Animal Care

Animals were housed in the tie-stall barn at the Pennsylvania State Dairy Complex, which can accommodate 60 cattle at a time and is equipped with tunnel ventilation. One ad-libitum automatic water bowl was available for every 2 cows. Cows were tied in individual stalls, bedded with sand, overnight and during the day when they were not in the milking parlor

or exercise yard. An individual feed cart was held in front of each individual cow stall. In the tie stall barn, the 16 cows in the Phase 1 group were housed from 10/01/2025 to 10/18/2025, the 16 cows in the Phase 2 group were housed from 10/19/2025 to 11/05/2026, and the 18 cows in the Phase 3 group were housed from 11/06/2025 to 11/29/2025. Each phase of cows underwent one week of adaptation, where they became familiar with the tie-stall barn environment, followed by two weeks of sampling.

Cows were fed the same diet (Table 1) throughout the experiment, which was formulated to meet National Academies of Sciences, Engineering, and Medicine (NASEM) requirements for net energy of lactation and metabolizable protein for a herd of mixed-parity early-lactation cows weighing 650kg, producing 42.7kg/d milk with 4.00% milk fat, 3.28% true protein, and 4.30% lactose, and having 24.75 kg/d DMI.

The amount of feed offered and refused each day was recorded and was used to calculate intake. Each morning, cows were offered 110% of their intake from the previous day. Amount of feed offered was not dropped more than 10lbs from one day to the next, and cattle were not offered less than 90lbs/day.

Table 1. Ingredients and Composition of Diet Fed During this Experiment

Item	Inclusion, %
Ingredient, % of DM	
Corn silage	47.8
Alfalfa haylage	11.5
Canola meal	8.90
Ground corn	8.50
SoyPLUS	8.09
Soybean, whole roasted	5.36
Molasses	4.68
Cottonseed, whole	2.30
Mineral and vitamin premix[1]	1.95
Grass-Hay mix	0.90
Composition, % if DM unless noted otherwise	
CP[3]	17.0
Ash[2]	5.69
RDP[3]	10.5
RUP[3]	6.50
NDF[2]	29.3
ADF[2]	16.6
Ether extract[3]	3.33
NEL[3], Mcal/kg	1.78
Ca[3]	0.72
P[3]	0.43

[1]The mineral (PSU Lactating Mineral) and vitamin premix contained, on a predicted DMI-basis, 179 g/d Ca, 106 g/d P, 69 g/d Mg, 152 g/d Cl, 335 g/d K, 117 g/d Na, 60 g/d S, 3 mg/d Co, 430 g/d Cu, 39 mg/d I, 3484 mg/d Fe, 2823 mg/d Mn, 11 mg/d Se, 1453 mg/d Zn, 94553 IU/d Vitamin A, 15156 IU/d Vitamin D, and 456 IU/d Vitamin E.
[2]Values calculated using nutrient analysis of individual feed ingredients (Cumberland Valley Analytical Services Inc., Waynesboro, PA) and their inclusion rate in the diet.
[3]Estimated based on NRC (2001) by Cumberland Valley Analytical Services Inc.

Total mixed ration (TMR) offered, refused feeds, and feed components of the diet were sampled twice weekly. TMR, refusals, and feed components sampling dates that occurred are listed in Table 6 of Appendix A. Upon collection, approximately 200 grams of each sample were weighed into aluminum pans and dried in a forced-air oven for 72 hours at 55ºC. After drying, samples were ground through a 1 mm sieve in a Thomas Scientific Wiley Mill and composited

across timepoints to create a representative TMR sample for the experiment. Composited TMR sample was prepared for analysis of DM, organic matter (OM), ash, NDF, acid detergent fiber (ADF), and indigestible neutral detergent fiber (iNDF) for apparent total tract digestibility.

Production Data Collection and Analysis

Cows were milked twice daily, in the a.m. and p.m., and individual milk production was automatically recorded (DeLaval milk meter, MM27BC) for each cow. For each cow, representative milk samples were collected twice per day, at the a.m. and p.m. milking sessions, for two days, to yield a total of 4 milk samples per cow. Milk sampling dates that occurred are listed in Table 7 of Appendix A. Milk samples were analyzed for composition of true protein, fat, lactose, and milk urea nitrogen (MUN) contents using infrared spectroscopy (MilkoScan 4000, Foss). Average milk yield, in kg/d, from combined a.m. and p.m. milkings, from the dates that each cow spent in the tie-stall barn, and average milk composition values were used to calculate total yields of milk fat, true protein, lactose, and MUN in kg/d. ECM was calculated using the following equation (Sjaunja et al., 1990):

$$ECM, \frac{kg}{d} = MY, \frac{kg}{d}$$

$$\times \{\frac{[(38.3 \times \%\ milk\ fat \times 10) + (24.2 \times \%\ milk\ true\ protein \times\ 10) + (16.54 \times\ \%\ lactose \times 10) + 20.7]}{3140}\}$$

Cow body weight was automatically recorded twice daily as cows exited the milking parlor using the AfiFarm 3.04 scale system (SAE Afikim). Cow body condition score was automatically recorded twice daily using cameras installed at the platform scales (DeLaval BCS), which photographed cows' lower backs for online scoring calculations.

Enteric Gas Emissions Collection and Analysis

During each sample and data collection period, enteric CH_4, CO_2, and H_2 emissions were collected using two Green Feed units (C-Lock Inc.), which were calibrated according to the manufacturer's instructions. For each cow, gas samples were collected over a representative 24-hour period twice. In each of these periods, gas samples were collected 8 times, over 3 consecutive days, at 4:00 AM, 10:00 AM, 4:00 PM, and 10:00 PM on Day 1, 2:00 PM and 8:00 PM on Day 2, and 2:00 AM and 8:00 AM on Day 3. Each individual gas sample was collected by removing the cows' feed cart and replacing it with the Green Feed machine for 5 minutes, following a 3-minute baseline recovery period of background air between animals. Cows lowered their heads to the Green Feed machine, which scanned their unique radio-frequency identification ear tags, signaling the machine to dispense bait feed (Stocker Grower 14, Purina Animal Nutrition LLC). Bait feed intake was recorded for every animal at every gas sampling period. Enteric gas emissions samplings that occurred are listed in Table 8 of Appendix A. Individual enteric CH_4, CO_2, and H_2 productions, in g/day, and DMI in kg/d were used to calculate CH_4, CO_2, and H_2 yields, in g/kg of DMI. Individual enteric CH_4, CO_2, and H_2 productions, in g/day, and MY and ECM in kg/d were used to calculate CH_4, CO_2, and H_2 intensities, in g/kg of MY or ECM.

Feces and Urine Collection and Analysis for Apparent Total Tract Digestibility

For each cow, approximately 300 g of feces were collected from the rectum over a representative 24-hour period. Fecal samples were collected from each cow 4 times, 6 hours apart, at 4:00 AM, 10:00 AM, 4:00 PM, and 10:00 PM. Fecal samplings that occurred are listed

in Table 9 of Appendix A. Upon collection, fecal samples were placed into aluminum pans and dried in a forced-air oven for 72 h at 55°C. After drying, samples were ground through a 1 mm sieve with a Thomas Scientific Wiley Mill and composited by cow to create representative samples for each animal across all sampling times. Composited samples were used to analyze apparent total-tract digestibility of nutrients, including NDF, ADF, DM, OM, and ash.

Composited TMR and composited fecal samples for each cow were weighed in 0.49-0.51g duplicate aliquots into F57 filter bags (ANKOM Technology) for analysis of NDF and ADF concentrations in the Ankom 200 fiber analyzer (Ankom Technology Corp.).

Composited TMR and composited fecal samples were weighed in 1-2g duplicate aliquots into overnight forced-air oven dried crucibles, dried in a forced-air oven for 48 hours at 120ºC, and reweighed for determination of DM content. Samples were then ashed for 6 hours at 600ºC in a natural-convection oven (LAB1ST NCO-30A), cooled to 120ºC in a desiccator, and reweighed to determine organic matter and ash content.

To calculate apparent total-tract digestibility, iNDF was used as a marker in a 12-day in-situ incubation procedure (Huhtanen et al., 1994; Lee et al., 2012). Composited TMR and composited fecal samples for each cow were weighed in 0.49-0.51g 4-replicate aliquots into F57 filter bags (ANKOM Technology). Each incubation of 22 bags included a lab alfalfa standard and a blank standard. Samples were placed in the rumen of a lactating Holstein dairy cow and incubated for 12 days. Upon removal, bags were washed in cold water and dried in a forced-air oven at 55ºC overnight. Subsequently, samples were analyzed using the ANKOM fiber analyzer system.

Urine samples were scheduled to be collected at the same time points as fecal samples; urine samplings that occurred are listed in Table 9 of Appendix A. For each cow at each

sampling, approximately 300 mL of urine was collected by perineal stimulation and filtered through 2 layers of cheesecloth to prevent fecal contamination. Upon collection, 10 mL of a whole urine sample was diluted into 0.6 mL of 2 M sulfuric acid (H_2SO_4) and 90 mL of distilled water solution to achieve a ratio of 60 mL of acid per 1000 mL of urine, reaching a pH less than 3.0. From each sampling time point of each experimental period, 10 mL of acidified, diluted urine will be composited and stored at -20°C. Also, upon collection at each sampling timepoint of each experimental period, 10mL of a whole urine sample will be directly composited to prepare a "fresh" urine composite sample for each cow and stored at -20ºC. Fresh composite samples will be analyzed for creatinine content, and acidified and diluted composite samples will be analyzed for urea nitrogen content.

For creatinine content analysis, fresh composited urine samples from each cow were sonicated to break up creatinine crystals, then diluted with distilled water (dH_2O) to a 1:2 dilution factor. Picric acid was weighed out to 6.33g and diluted with dH_2O to yield a total volume of 500mL; 10% sodium hydroxide (NaOH) solution was measured out to 20mL and diluted with dH_2O to yield a total volume of 100mL. Creatinine stock standard (Millipore Sigma PHR1462-1G) and dH_2O were used to create a set of standards (1 mL dH_2O, 1 mL 0.25 mg/mL creatinine, 1 mL 0.50 mg/mL creatinine, 1 mL 0.75 mg/mL creatinine, and 1 mL 1 mg/mL creatinine). In duplicate aliquots, 100 µL of each standard and urine sample was vortexed, then pipetted to a borosilicate glass tube. To each tube, 2 mL of prepared picric acid reagent, then 150µL of prepared NaOH reagent, was added, and solutions were vortexed and then allowed to incubate for 15 minutes. Each standard and sample was pipetted in a 300 µL aliquot to a well of a 96-well microplate for spectrophotometric analysis at 520 nm (SPECTROstar Nano, BMD Labtech).

Urine output, in L/d, was calculated from reported creatinine concentrations using the following equation:

$$\text{Urine Output } \left(\frac{\text{L}}{\text{d}}\right) = \frac{BW\ (kg) \times 29}{Urine\ Creatinine\ Concentration\ (\frac{mg}{L})}$$

Total daily output of urine urea nitrogen, in mg/dL, was then calculated by multiplying total urine output by the average observed urine urea nitrogen concentration.

For urine urea nitrogen analysis, acidified, composited urine samples from each cow were defrosted from storage at -20ºC and centrifuged at 10,000g and 4ºC for 15 minutes. Collected supernatant was diluted with double-distilled water (ddH_2O) to a dilution factor of 20. Samples were prepared according to the protocol in Novus Biologicals Urea Assay Kit (Colorimetric) NBP3-24543, and 300 µL aliquots of standards and samples were pipetted to individual wells of a 96-well microplate for spectrophotometric analysis at 520 nm (SPECTROstar Nano, BMD Labtech).

Division of Cows into Enteric Methane Emitting Phenotype Groups

Levels of emitted enteric gases, CH_4, CO_2, and H_2, measured in grams per day, for each of the fifty cows screened in this study, were averaged for each sampling timepoint as well as across all sampling timepoints using Program R. In Program R, cows were classified by enteric-CH_4-emitting phenotype as “High”, “Low”, or “Medium” enteric CH_4 emitters by dividing the 50 averaged CH_4 values into tertiles. Cows were then assigned to the “High” enteric-CH_4-emitting phenotype group (HM) if their average enteric CH_4 production (g/day) was above the upper tertile cutoff, or 66.7th percentile, to the “Low” enteric-CH_4-emitting group phenotype

(LM) if production was below the lower tertile cutoff, or 33.3rd percentile, and to the "Medium" enteric-CH_4-emitting phenotype group (MM) if production was between these values.

Statistical Analyses

All CH_4 data were analyzed for outliers using Program R based on absolute studentized residual values ≥ 3. Identified outliers were verified as true technical outliers and removed from the dataset. The linear mixed-effects model, created in Program R with the lmer function of the lmer package, for enteric CO_2 and H_2 gas production variables included the fixed effects of enteric-CH_4-emitting phenotype, sampling timepoint, and enteric-CH_4-emitting phenotype and sampling timepoint interaction, and random effects of cows. CH_4, CO_2, and H_2 production data from each cow were analyzed as repeated measures, using a first-order autoregressive (AR(1)) model covariance structure.

The generalized linear model, created in Program R with the lm function, for response variables including CH_4, CO_2, and H_2 intensity in g/kg of DMI, and CH_4, CO_2, and H_2 yield in both g/kg of MY and g/kg of ECM, included the fixed effect of enteric-CH_4-emitting phenotype to analyze for differences in variables between phenotypic groups.

The generalized linear model for production and biological response variables, including DMI (kg/day), MY (kg/day), ECM (kg/day), BW (kg), BCS, feed efficiency (kg/kg), ECM feed efficiency (kg/kg), milk fat percentage, milk fat production (kg/d), milk true protein percentage, milk true protein production (kg/d), milk lactose percentage, milk lactose production (kg/d), milk urea nitrogen percentage, milk urea nitrogen production (kg/d), DM intake (kg/d), OM intake (kg/d), NDF intake (kg/d), ADF intake (kg/d), apparent DM digestibility, apparent OM

digestibility, apparent NDF digestibility, apparent ADF digestibility, urine urea nitrogen (mmol/L), urine creatinine (mg/L), total urine output (L/d), and total urea nitrogen output (mg/dL), also included the fixed effect of enteric-CH_4-emitting phenotype to analyze for differences in variables between phenotypic groups.

All data are presented as least squares means, which were compared with the Tukey-Kramer test, where differences are considered significant at $P \leq 0.05$ and a non-significant tendency was declared at $0.05 < P \leq 0.10$.

Chapter 4

Results

Enteric Gas Emissions

Enteric CH_4 production (g/day) data for HM, MM, and LM cows are shown in Figure 1. Cows were considered to be HM if they, on average, produced more than the 66.7th percentile of 403.3215 g CH_4/day, were considered LM if they, on average, produced less than 33.3rd percentile of 360.7231 g CH_4/day, and were considered MM if they, on average, produced more than 360.7231 g CH_4/day but less than 403.3215 g CH_4/day. Sample sizes for different enteric CH_4-emitting cow groups were n=17 for HM cows, n=16 for MM cows, and n=17 for LM cows. Median enteric CH_4 production was 433.2040 g CH_4/day for HM cows, 331.3437 g CH_4/day for LM cows, and 381.0728 g CH_4/day for MM cows. The interquartile range for HM cows of 49.20594 g CH_4/day was larger than that of LM cows at 45.31807 g CH_4/day, and both were larger than the interquartile range of MM cows, which was 14.78297 g CH_4/day. The difference between maximum and minimum enteric CH_4 production, excluding the HM outlier of 575.2969 g CH_4/day, was lower in HM cows at 93.8055 g CH_4/day than in LM cows at 106.2516 g CH_4/day, but was greater in both HM cows and LM cows than in MM cows at 35.5351 g CH_4/day. Enteric CH_4 production values that were identified as true technical outliers were previously removed and are not shown in Figure 1.

Figure 1: Mean Enteric CH_4 Emitted for Different Enteric CH_4-Emitting Cow Groups

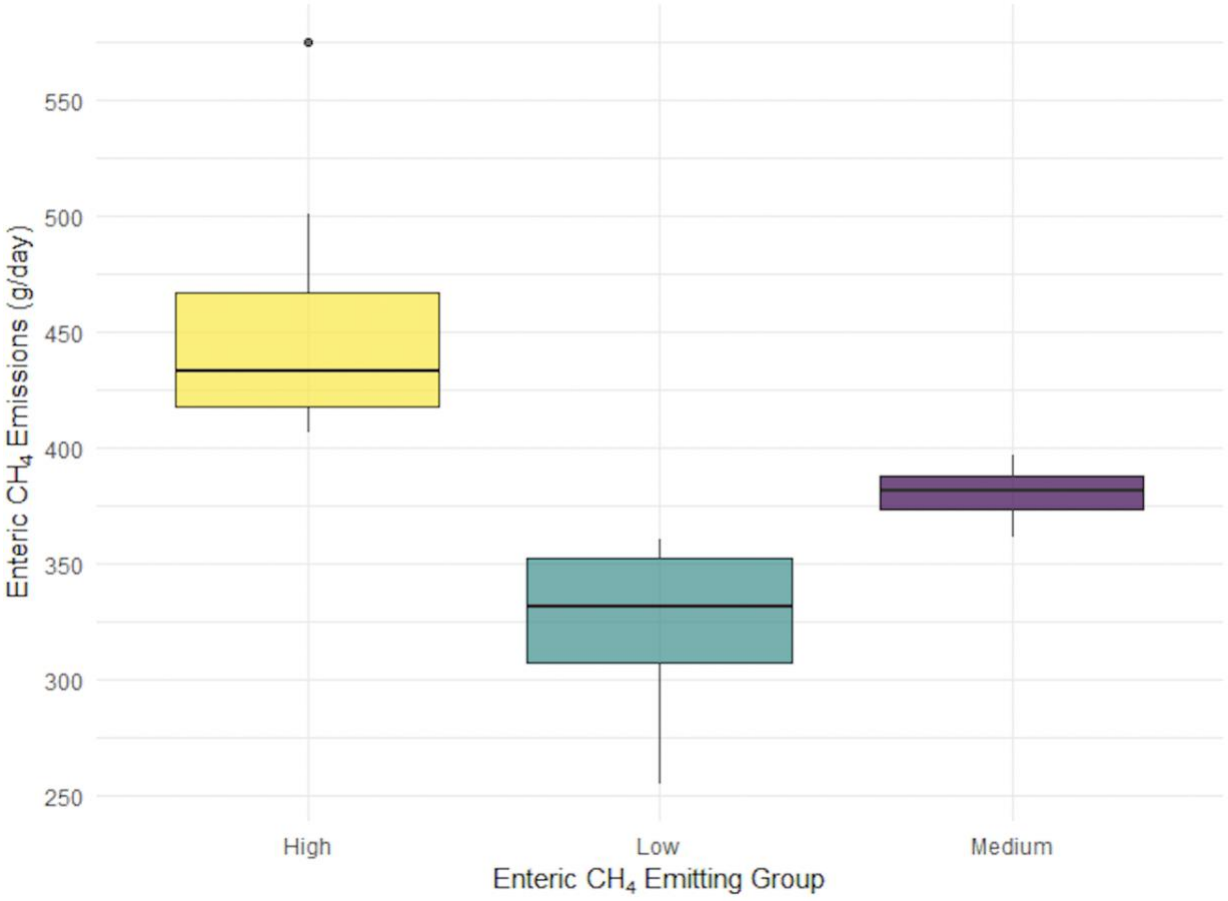

Figure 1: Mean enteric CH_4 emissions in g/day from each enteric CH_4-emitting cow group. Mean enteric CH_4 emissions in g/day for "High" enteric-CH_4-emitting cows are shown in yellow, mean enteric CH_4 emissions in g/day for "Low" enteric-CH_4-emitting cows are shown in cyan, and mean enteric CH_4 emissions in g/day for "Medium" enteric-CH_4-emitting cows are shown in purple.

Identified enteric CH_4 production outliers are shown in Figure 2 and were considered to be true technical outliers. The high outliers of 777.1424 g CH_4/day and 797.1996 g CH_4/day for cow number 3285 were likely due to neighboring cows attempting to reach the bait in the Green Feed machine during sampling, resulting in enteric CH_4 from multiple animals being recorded. Enteric CH_4 increase at these timepoints is accompanied by increases in both CO_2 and H_2, at 20213.7077 g CO_2/day and 2.7920 g H_2/day, for the 777.1424 g CH_4/day outlier, and at 20638.0304 g CO_2/day and 1.948970718 g H_2/day for the 797.1996 g CH_4/day outlier. Similarly, the low outliers of 29.3953 g CH_4/day for cow 3324 and 3.9003 g CH_4/day for cow 3620 were

likely due to the cows being uninterested in bait from the Green Feed machine, which resulted in them spending less time with their heads lowered into the machine to emit recordable gas. Accompanied by low CH_4 production at these instances are low emissions of CO_2 and H_2, at 872.8805 g CO_2/day and 0.0926 g H_2/day for the outlier of 29.3953 g CH_4/day, and 473.1814 g CO_2/day and 0.002893 g H_2/day for the outlier of 3.9003 g CH_4/day.

Although the high identified outlier for cow number 3324 was not accompanied by elevated CO_2 and H_2 emissions, it was still removed from the dataset, as other technical factors, such as cow restlessness during a sampling timepoint, may lead to inaccurate gas readings in the Green Feed system.

Figure 2: Enteric CH_4 Emissions from Individual Cows with Outliers Highlighted

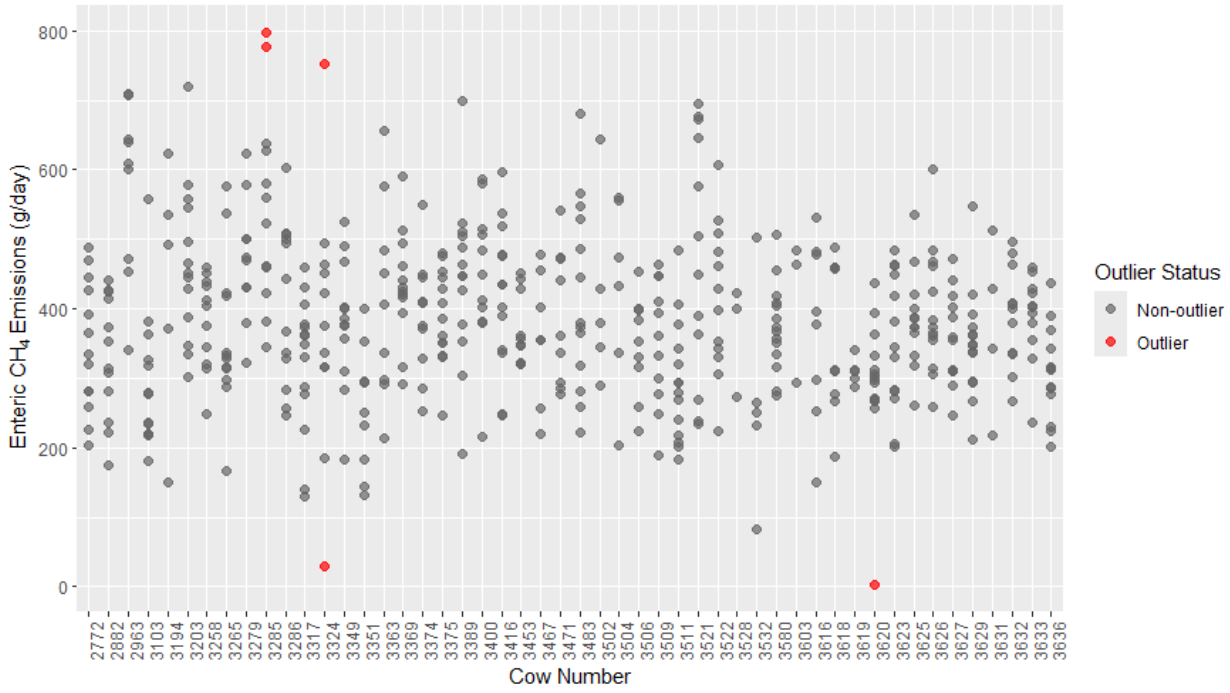

Figure 2: Enteric CH_4 emissions in grams from each cow. Each individual dot represents the level of CH_4 emitted at a given sampling timepoint. Outliers, identified by being larger than 3 studentized residuals away from the mean, are highlighted in red, while non-outliers are highlighted in grey.

Average production of CH_4 (g/day) for cows in each enteric CH_4 emitting group at each of the 8 sampling timepoints is shown in Figure 3. At all sampling timepoints, HM cows emitted

more CH_4 (g/day) on average than did MM cows, which emitted more CH_4 (g/day) on average than did LM cows. For HM cows, production of CH_4 (g/day) followed diurnal patterns, with significantly more (57.906 g CH_4/day) ($P = 0.04$) CH_4 being produced in the afternoon (4:00PM) and significantly less (-65.578 g CH_4/day and -109.343 g CH_4/day) ($P = 0.03$ and $P < 0.001$) CH_4 being produced in the late evening (10:00PM) and early morning (4:00AM), respectively, than in the morning (10:00AM). LM and MM cows followed the same diurnal pattern as HM cows, aside from one interaction effect, between LM cows and the 4:00AM sampling timepoint. At this timepoint, LM cows did not show as great a decline in enteric CH_4 production as HM cows did; the difference in enteric CH_4 production from 10:00AM to 4:00AM was 98.115 g/day less in LM cows than it was in HM cows ($P = 0.02$).

Figure 3: Average Enteric CH_4 Emissions for Different Enteric CH_4-Emitting Cow Groups Over Time

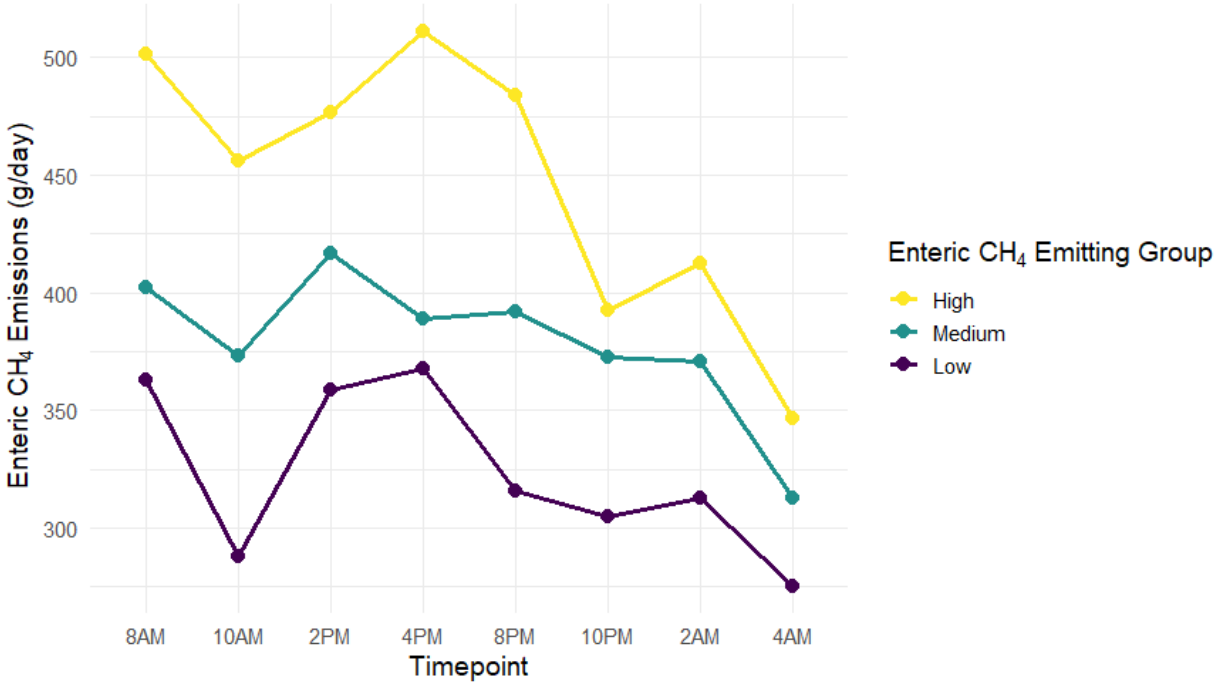

Figure 3: Mean enteric CH_4 emissions in g/day from each enteric CH_4-emitting cow group across a period of sixteen hours. Mean enteric CH_4 emissions in g/day for "High" enteric-CH_4-emitting cows are shown in yellow, mean enteric CH_4 emissions in g/day for "Medium" enteric-CH_4-emitting cows are shown in cyan, and mean enteric CH_4 emissions in g/day for "Low" enteric-CH_4-emitting cows are shown in purple.

The studentized residual outliers identified for cows 3324 and 3620, shown in Figure 4, were determined to be true technical outliers. As described above, the CO_2 outputs of 872.8805 g CO_2/day for cow 3374 and 473.1814 g CO_2/day for cow 3620 were accompanied by lower emissions of H_2 and CH_4 gases. These overall reductions in gas emissions at these timepoints can be explained by cows' disinterest in bait where they did not keep their heads in the Green Feed machine.

Figure 4: Enteric CO_2 Emissions from Individual Cows with Outliers Highlighted

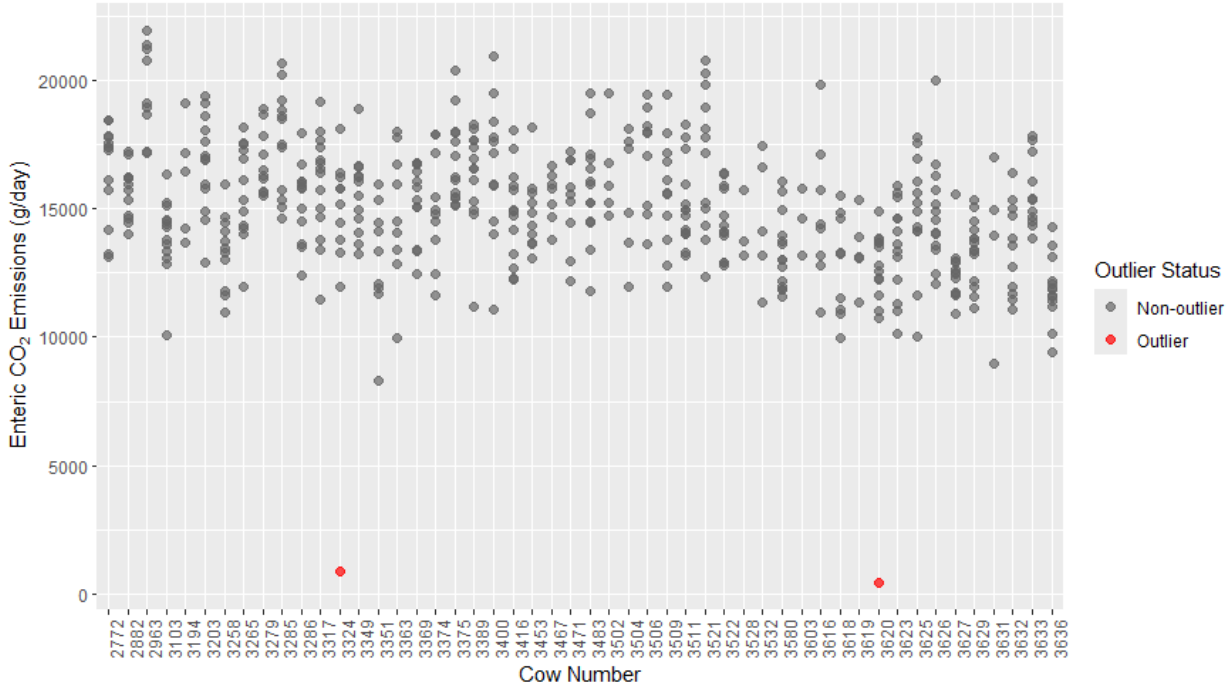

Figure 4: Enteric CO_2 emissions in g/day from each cow. Each individual dot represents the level of CO_2 emitted in g/day at a given sampling timepoint. Outliers, identified by being larger than 3 studentized residuals away from the mean, are highlighted in red, while non-outliers are highlighted in grey.

Average production of CO_2 (g/day) for cows in each enteric CH_4-emitting group at each of the 8 sampling timepoints is shown in Figure 5. At all sampling timepoints, HM cows emitted more CO_2 (g/day) on average than did MM cows, which emitted more CO_2 (g/day) on average than did LM cows. For HM cows, production of CO_2 (g/day) also followed a slightly diurnal pattern, with significantly less (-1561.1 g CO_2/day) ($P = 0.008$) CO_2 being produced in the early

morning (4:00AM), than in the morning (10:00AM). LM and MM cows followed the same diurnal pattern as HM cows, aside from one interaction effect, between MM cows and the 10:00PM sampling timepoint. At this timepoint, MM cows showed a greater increase in enteric CO_2 production than HM cows did; the difference in enteric CO_2 production from 10:00AM to 10:00PM was 1679.2 g CO_2/day greater in MM cows than it was in HM cows (P = 0.05).

Figure 5: Average Enteric CO_2 Emissions for Different Enteric CH_4-Emitting Cow Groups Over Time

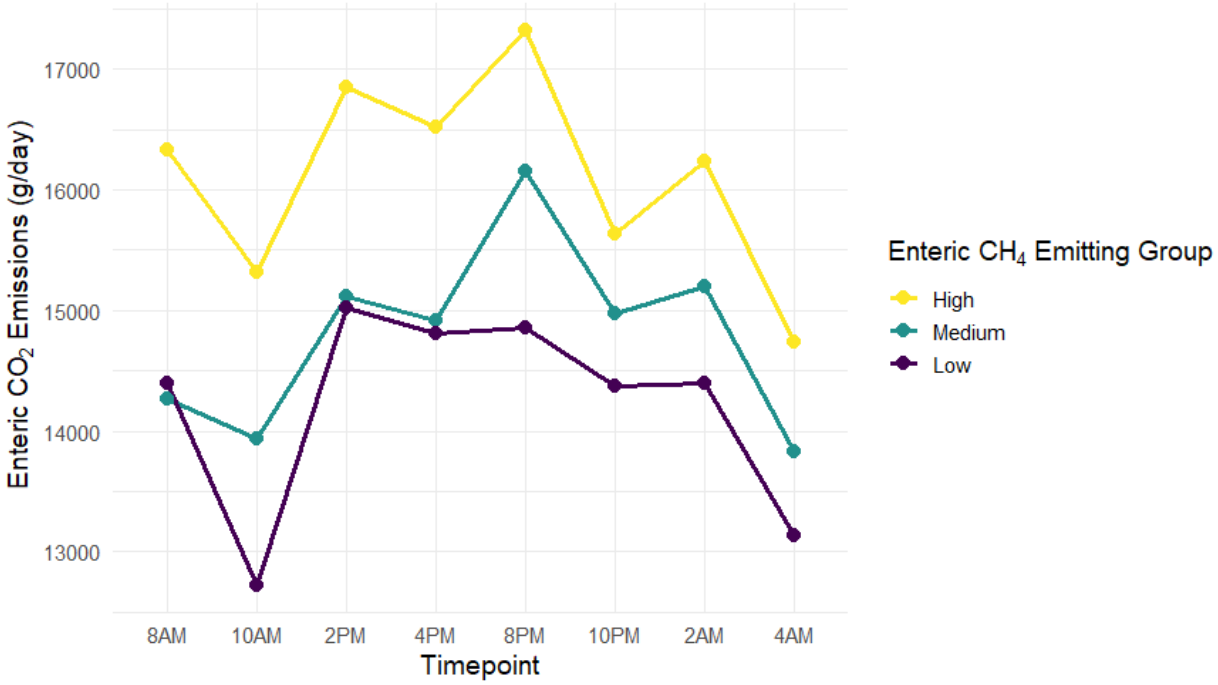

Figure 5: Mean enteric CO_2 emissions in g/day from each enteric CH_4-emitting cow group across a period of sixteen hours. Mean enteric CO_2 emissions in g/day for "High" enteric-CH_4-emitting cows are shown in yellow, mean enteric CO_2 emissions in g/day for "Medium" enteric-CH_4-emitting cows are shown in cyan, and mean enteric CO_2 emissions in g/day for "Low" enteric-CH_4-emitting cows are shown in purple.

The high outliers identified for cows 3317, 3375, 3400, 3416, 3506, 3521, and 3522, all of which were greater than 4.5 g H_2/day, as shown in Figure 6, were determined to be true technical outliers. As they did not occur at consistent time points of the day, these spikes in H_2 gas readings are unlikely to be explained by increased feed consumption rates following the morning feed drop, an association previously identified (Rooke et al., 2014). They were removed

from the dataset, as other technical factors, such as cow restlessness during a sampling time point, may lead to inaccurate gas readings in the Green Feed system.

Figure 6: Enteric H_2 Emissions from Individual Cows with Outliers Highlighted

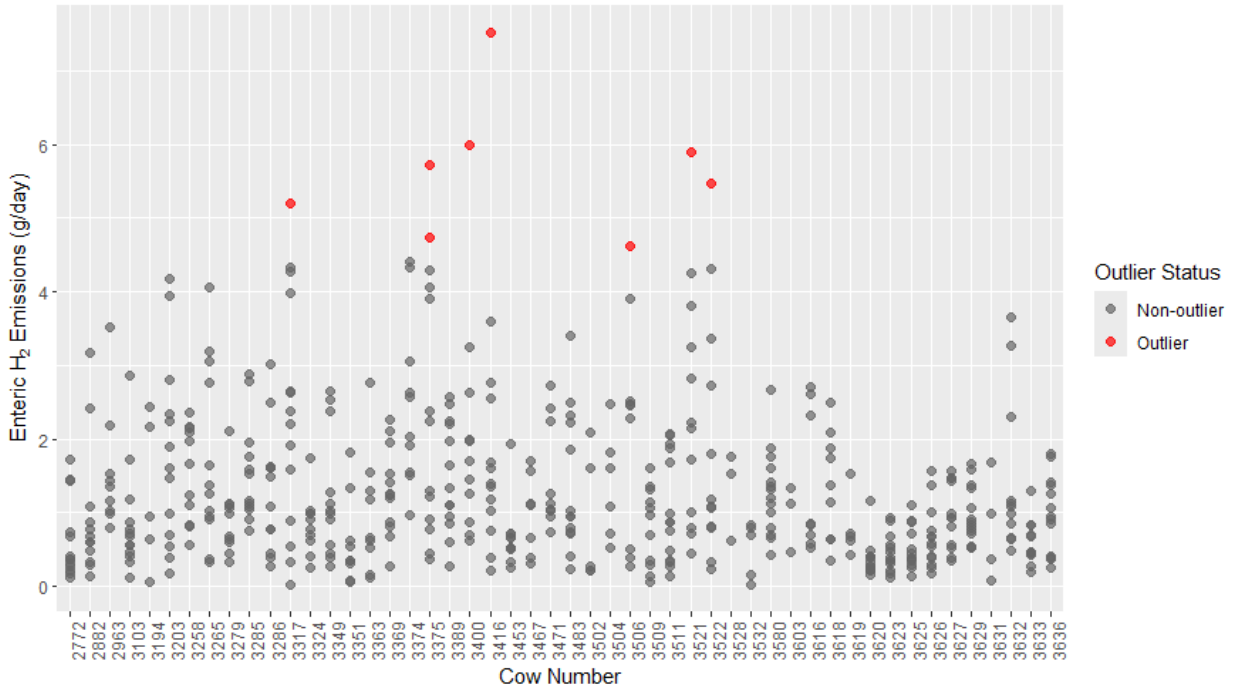

Figure 6: Enteric CH_4 emissions in g/day from each cow. Each individual dot represents the level of CH_4 emitted at a given sampling timepoint. Outliers, identified by being larger than 3 studentized residuals away from the mean, are highlighted in red, while non-outliers are highlighted in grey.

Average production of H_2 (g/day) for cows in each enteric CH_4 emitting group at each of the 8 sampling timepoints is shown in Figure 7. At all sampling timepoints, HM cows emitted more H_2 (g/day) on average than did MM cows, which emitted more H_2 (g/day) on average than did LM cows. For HM cows, production of H_2 (g/day) also followed a slightly diurnal pattern, with significantly less (-0.992732 g H_2/day) ($P < 0.001$) H_2 being produced in the early morning (4:00AM), than in the morning (10:00AM). LM and MM cows followed the same diurnal pattern as HM cows.

Figure 7: Average Enteric H_2 Emissions for Different Enteric CH_4-Emitting Cow Groups Over Time.

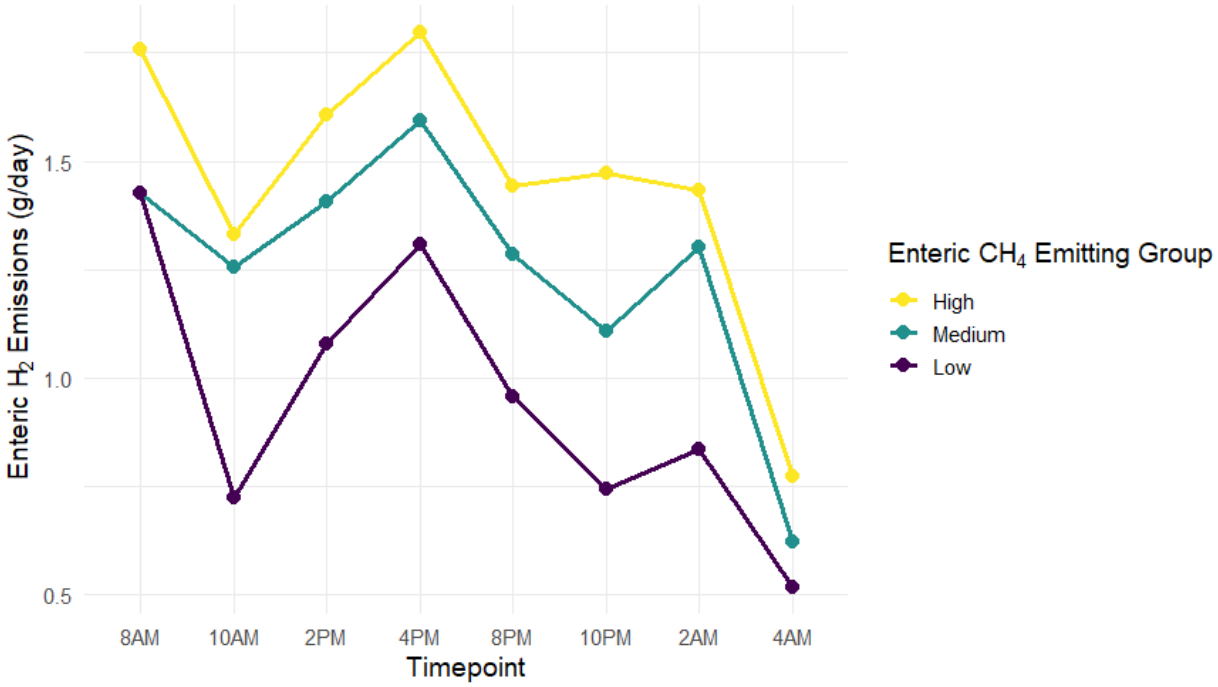

Figure 7: Mean enteric H_2 emissions in g/day from each enteric CH_4-emitting cow group across a period of sixteen hours. Mean enteric H_2 emissions in g/day for "High" enteric-CH_4-emitting cows are shown in yellow, mean enteric H_2 emissions in g/day for "Medium" enteric-CH_4-emitting cows are shown in cyan, and mean enteric H_2 emissions in g/day for "Low" enteric-CH_4-emitting cows are shown in purple.

Production of CH_4, CO_2, and H_2 across all time points was averaged for each enteric-CH_4-emitting group to obtain average daily gas production (g/day), which was then used to calculate gas intensity (g/kg DMI) and gas yield (g/kg MY) (Table 2). On average, LM cows produced 125 g/day less enteric CH_4 than did HM cows, at 323 g CH_4/day as compared to 448 g CH_4/day, respectively ($P < 0.001$). Enteric CH_4 intensity for LM cows was lower at 12.8 g CH_4/kg DMI than it was for HM cows at 15.0 g CH_4/kg DMI, and although this result was not statistically significant ($P = 0.08$), there was a tendency for enteric CH_4 intensity to be lower in LM cows. There were no significant differences in enteric CH_4 yield for both measured MY and ECM between enteric-CH_4-emitting cow groups. There was a significant difference in enteric CO_2 production between LM and HM cows, with LM cows producing a mean of 14223 g

CO_2/day and HM cows producing a mean of 16129 g CO_2/day ($P < 0.001$). However, there were no significant differences or observed tendencies in association between enteric CO_2 intensity or yield, in both measured MY and ECM, between LM and HM cow groups. Similarly, there was a significant difference in enteric H_2 production between LM and HM cows, with LM cows producing less H_2 at a mean of 0.963 g H_2/day and HM cows producing more H_2 at a mean of 1.427 g H_2/day ($P = 0.02$). There were no significant differences or observed tendencies in association between enteric H_2 intensity or yield, in both measured MY and ECM, between LM and HM cow groups.

Table 2: Enteric Gas Emissions of Phenotypically High- and Low-CH_4-Emitting Cattle

	Phenotype			
Item	LM	HM	SEM	P-value[1]
CH_4, g/day	323	448	12.7	<0.001
CH_4, g/kg DMI	12.8	15.0	0.99	0.08
CH_4, g/kg MY	7.52	8.56	0.714	0.32
CH_4, g/kg ECM	8.16	8.79	0.771	0.69
CO_2, g/day	14,223	16,129	452.0	<0.001
CO_2, g/kg DMI	558	541	34.1	0.87
CO_2, g/kg MY	325	309	23.3	0.78
CO_2, g/kg ECM	353	317	26.0	0.36
H_2, g/day	0.96	1.43	0.163	0.02
H_2, g/kg DMI	0.04	0.05	0.007	0.16
H_2, g/kg MY	0.02	0.03	0.007	0.16
H_2, g/kg ECM	0.02	0.03	0.005	0.42

[1]Main effect of enteric CH_4-emitting phenotype.

Production Variables

Production variables of HM and LM cows are summarized in Table 3. On average, LM cows had lower DMI at 26.4 kg/day than did HM cows at 30.2 kg/day (P = 0.04). No significant difference between LM and HM cows was observed for reported milk yield, although there was a tendency for LM cows to produce less milk than HM cows, at 45.5 kg milk/d compared to 52.8 kg milk/day (P = 0.08), respectively. There was a significant difference in ECM, where LM cows produced less ECM at 42.1 kg ECM/day while HM cows produced more ECM at 51.5 kg ECM/day (P = 0.02). There was no significant difference between LM and HM cows regarding milk composition, which included milk fat, milk true protein, lactose, and MUN percentages. However, due to the tendency of LM cows to produce less milk than HM cows, total production of milk fat in kg/day was observed to be significantly different; LM cows produced 1.65 kg milk fat/day as compared to HM cows producing 2.12 kg milk fat/day (P = 0.01). Similarly, total production of lactose in kg/day tended to be lower in LM cows than in HM cows, at 2.16 kg lactose/day as compared to 2.55 kg lactose/day (P = 0.06). No significant differences were observed between LM and HM cows for total production of milk protein or MUN in kg/day.

Feed efficiency, as a measure of how effectively DMI was converted to milk yield, was not significantly different between LM and HM cows. Furthermore, feed efficiency, adjusted for ECM, was not significantly different between LM and HM cows.

Body weight data could not be collected for each cow at each milking time point due to limitations regarding how long the cow remained on the platform scale before proceeding to exit the milking parlor. The amount of available data varied for each cow, and in some cases, no body weight was recorded, as with cows 3504 and 3580. As cow 3504 was classified as an HM cow, she was excluded from the BW analysis, resulting in n=16 rather than n=17 for the HM sample

size in the BW analysis. Based on this data, no significant difference in BW was observed between LM and HM cows. Further, no significant difference in BCS between LM and HM cows was observed.

Table 3: Production Variables of Phenotypically High- and Low-CH_4-Emitting Cattle

	Phenotype			
Item	LM	HM	SEM	P-value[1]
DMI, kg/d	26.4	30.2	1.54	0.04
Milk yield, kg/d	45.4	52.8	3.36	0.08
ECM, kg/d	42.1	51.5	3.23	0.02
BW, kg	645	676	18.5	0.23
BCS	2.96	3.13	0.107	0.30
Feed efficiency, kg/kg	1.76	1.76	0.115	1.00
ECM Feed efficiency, kg/kg	1.64	1.72	0.122	0.78
Milk fat, %	3.69	4.05	0.289	0.44
Milk fat, kg/d	1.65	2.12	0.112	0.01
Milk true protein, %	3.09	3.07	0.083	0.96
Milk true protein, kg/d	1.41	1.62	0.114	0.15
Lactose, %	4.77	4.82	0.048	0.48
Lactose, kg/d	2.16	2.55	0.162	0.06
MUN, %	3.30	3.17	0.993	0.99
MUN, kg/d	1.50	1.67	0.495	0.94

[1]Main effect of enteric CH_4-emitting phenotype.

Intake, Apparent Total-Tract Digestibility, and Urine Composition

Differences in enteric-CH_4-emitting phenotype were associated with some differences in dietary intake and apparent total-tract digestibility; reported intake and digestibility variables are summarized in Table 4.

As reported above, DMI was significantly lower in LM cows than in HM cows (P = 0.04). Similarly, as cows were all fed the same diet throughout the experiment, intake of OM,

NDF, and ADF were all lower in LM cows than in HM cows, at 24.9 kg OM/day compared to 28.5 kg OM/day, 7.73 kg NDF/day compared to 8.86 kg NDF/day, and 4.39 kg ADF/day compared to 5.03 kg ADF/day, respectively (P = 0.04, P = 0.04, P = 0.04).

Apparent total-tract digestibility of DM, OM, NDF, and ADF was not observed to be significantly different between LM and HM cows.

Table 4: Apparent Total-Tract Digestibility of Phenotypically High- and Low-CH_4-Emitting Cattle

	Phenotype			
Item	LM	HM	SEM	P-value[1]
Intake, kg/d				
DM	26.4	30.2	1.54	0.04
OM	24.9	28.5	1.45	0.04
NDF	7.73	8.86	0.451	0.04
ADF	4.39	5.03	0.256	0.04
Apparent total-tract digestibility, %				
DM	73.7	72.6	0.61	0.17
OM	75.4	74.5	0.62	0.32
NDF	48.9	48.3	1.34	0.89
ADF	42.7	43.1	0.48	0.93

[1]Main effect of enteric CH_4-emitting phenotype.

Reported variables regarding urine composition and production are summarized in Table 5. In calculating the average urine creatinine concentration for HM cows, the value for cow 3416 was omitted because the reported value was negative, indicating an error in the determination of creatinine concentration. As cow 3416 was classified as an HM cow, this removal caused n=16 rather than n=17 for the HM sample size in the creatinine analysis. No significant difference between LM and HM cows was observed for reported urine creatinine concentration.

Urine output was calculated for all LM cows and all HM cows, aside from 3416, for which creatinine data were not available, and 3504, for which body weight was not available. As cow 3416 and cow 3504 were both classified as HM cows, these removals resulted in n=15 rather than n=17 for the HM sample size in the urine output analysis. There was no significant difference in urine output (L/d) between LM and HM cows.

No significant difference was observed in the concentration of urine urea nitrogen between LM and HM cows. Total output of urea nitrogen in urine, for examination of nitrogen balance, was calculated by multiplying the urine urea nitrogen concentration by the urine output volume. As HM cows 3416 and 3504 had been removed from the urine output analysis, they were also removed from the urine output analysis, resulting in an HM sample size of n=15 instead of n=17. There was no significant difference in the total output of urine urea nitrogen between LM and HM cows.

Table 5: Urine Composition and Output of Phenotypically High- and Low-CH_4-Emitting Cattle

	Phenotype			
Item	LM	HM	SEM	P-value[1]
Urea Nitrogen, mmol/L	107.8	97.7	23.10	0.90
Creatinine, mg/L	825	832	74.3	0.99
Urine Output, L/d	24.1	24.9	2.36	0.94
Urea Nitrogen, mg/dL	302	274	64.8	0.90

[1]Main effect of enteric CH_4-emitting phenotype.

Chapter 5

Discussion

There are clear genetic factors that influence enteric CH_4 production in ruminants, and lactating Holstein dairy cattle have previously been shown to exhibit distinct enteric-CH_4-emitting phenotypes. These phenotypes have been associated with a variety of biological and functional parameters, including, but not limited to, DMI, BW, rumen capacity, feed retention time, feed efficiency, apparent total-tract digestibility, ruminal VFA concentrations and ratios, microbial gene expression, relative methanogen abundance, and MY. The literature has described how diurnal patterns of enteric CH_4 production, where enteric CH_4 production spikes following feeding times on dairy farms, are a result of daily fluctuations in feeding rate and energy demands for lactation (Gao et al., 2011). The observation of diurnal patterns in enteric CH_4 production in the present study support previous findings. The rate of methanogenesis decreases at night, when the cows are sleeping and not fermenting feed at the daytime rate. Increases in enteric CH_4 production from 4:00AM to 8:00AM for all cow groups also agree with previous findings, as during this period, cows waking up are beginning to consume and ferment the morning's dropped feed, providing increased substrate for methanogenesis. Further, apparent drops in enteric CH_4 production follow milking times, between 8:00AM and 10:00AM, and between 4:00PM and 10:00PM. The highest yield period for milk production occurs directly after milking, when intermammary gland pressure is reduced, lowering inhibition of milk production (Sullivan, 2005). These changes may indicate that dietary energy is directed toward lactation rather than methanogenesis when lactation energy demand is higher, but it cannot be

said that lactation directly competes with methanogenesis. There have been some associations between decreased enteric CH_4 intensity and increased milk production, as enteric CH_4 production does not increase as the same rate as MY increases (Fresco et al., 2023). Although enteric CH_4 intensity and increased milk production are correlated, resulting in a "dilution" effect, selection for cows of lower CH_4 intensity is not recommended as it does not show real reduction in total CH_4 production, just tapering of CH_4 emission per unit of milk produced as more milk is produced (Fresco et al., 2023). Although selection for increased milk is important, the correlation between increased MY and increased enteric CH_4 production does not select for more feed-efficient animals, a concept aligning with concerns that increased methanogenesis poses potential productivity losses to the animal (Johnson and Johnson, 1995). In the present study, midday spikes in enteric CH_4 production are likely due to a combination of cows actively fermenting feed and providing substrate for methanogenesis, and of a larger proportion of dietary energy not being used for lactation as milk production rates slow as udders fill.

The interaction effect between LM cows and the 4:00AM timepoint could indicate that LM cows have more consistent enteric CH_4 production throughout the day than do HM cows in the present study. Similarly, the interaction effect between MM cows and the 10:00PM timepoint could indicate that MM cows have less consistent enteric CO_2 production throughout the day than do HM cows. Both results may indicate that greater variability in the rate of methanogenesis throughout the day exists in high-enteric-CH_4-emitting cattle, which could be explained if these cows also displayed daily variation in feeding rate. As the high-enteric-CH_4-emitting cattle had greater DMI than low-enteric-CH_4-emitting cattle, they may have also had increased rates of feeding during specific times of the day, which may have caused greater increases in enteric CH_4 production. This concept has been shown to have some association in previous studies, where

increased feeding rate, or the amount of metabolizable energy intake for the duration of a feeding period, and increased intake per feeding event, are both correlated with greater enteric CH_4 production (Crowley et al., 2024).

The significant difference observed in the present study between LM and HM cows in gross enteric CH_4 production is expected, as the cows were divided into enteric-CH_4-emitting groups based on average daily enteric CH_4 production. The significant differences between LM and HM cows for gross enteric CO_2 and H_2 production, as well as the coordination of diurnal CH_4 production pattern with CO_2 and H_2 production patterns, where LM cows showed significantly less gross enteric CO_2 and H_2 production than did HM cows, is also reasonable. When less CO_2 and H_2 substrate are available for methanogenesis, less CH_4 should be expected to be produced. However, the lack of a significant difference in enteric CH_4 yield between LM and HM cow groups does not align with findings from previous studies. The lack of significant difference for enteric CH_4 yield between enteric-CH_4 emitting groups in the present study as compared to results from (Stepanchenko et al., 2023) may be a result of overall increased milk yield for cows in the present study; 45.4 kg milk/day for LM and 52.8 kg milk/day for HM cows was observed in the present study, as compared to 33.3 kg milk/day for LM cows and 32.3 kg milk/day for cows in the previous study. It is known that enteric CH_4 yield decreases with increased milk production, so cattle in the present study may have had enough increases in milk production to mask any differences in enteric CH_4 yield. It should also be noted that the difference in milk production values between cattle in these studies is likely due to the fact that cattle examined in the present study were preferentially selected to be in early lactation, between 30 and 100 DIM, while cows in the previous study were in later lactation near 250 DIM; earlier lactation cows produce significantly more milk than do late lactation cows (Arshad and Erickson,

2025). Although there was a tendency for LM cows to have lower CH_4 yield than HM cows, meaning LM cows tended to produce less CH_4 per unit of feed intake, which was in agreement with previous studies that found a significant difference in enteric CH_4 intensity between phenotypically high- and phenotypically low-CH_4-emitting cattle, the difference observed in this study was not as great as previously reported (Stepanchenko et al., 2023).

The lack of significant differences in enteric CO_2 and H_2 intensities, yields in MY, and yields in ECM between LM and HM cows is also consistent with findings from previous studies (Stepanchenko et al., 2023). Increased enteric CH_4 production is not necessarily accompanied by increased CO_2 and H_2 production, as these gaseous reagents may also be used in other energy-producing pathways in the rumen rather than being directly eructated, such as VFA production, including propionate synthesis. These pathways are often upregulated with increases in DMI, which is also associated with increased enteric CH_4 production; while both CH_4 and CO_2 intensities may decrease with increased DMI, there is no association between gas intensities themselves (Jones, 2025). The same concepts apply to H_2 intensity, as it is also a reagent in VFA synthesis and has been shown to increase with increasing DMI. Similarly, as increased DMI increases MY, CO_2 and H_2 yields correlate with DMI but do not directly correlate with enteric CH4 production (Hristov et al., 2005).

The lower enteric gas production levels for LM cows appear to be directly associated with decreased DMI intake, a finding that has been repeatedly reported in the literature (Johnson and Johnson, 1995; Hristov et al., 2013). Existing literature has described significant differences ($P = 0.001$) in CH_4 intensity per unit of ECM, and of CH_4 yield ($P < 0.001$) per kg DMI, digestible OM, and digestible NDF between low-Y_m and high-Y_m cows (Denninger et al., 2020).

Previous studies have identified associations between high CH_4-emiting lactating Holstein cattle and genomic predicted transmitting ability for taller stature, greater body depth, greater body weight, and overall greater frame size, but have not shown associations between examined animal body weight and enteric CH_4-emitting phenotype, consistent with the results of the present study (Stepanchenko et al., 2023). However, it should be acknowledged that both the present study and the previous study were conducted on related cows within the herd at the Pennsylvania State University Dairy Complex, where body weight was recorded in the same manner and may have been faulty. Also, it may be that observed changes in BW and CH_4 emissions are both driven by DMI changes, as DMI changes are known to affect CH_4 emission levels. A previous study on body composition traits and associated enteric CH_4 production levels in beef cattle have identified only a weak correlation between cattle birth weight and CH_4 emission levels at 1-2 years old (MPR) ($r = 0.19 \pm 0.05$), and moderate correlation between MPR and weaning weight ($r = 0.50 \pm 0.04$), yearling weight ($r = 0.57 \pm 0.03$), and weight at maturity ($r = 0.49 \pm 0.05$) (Herd et al., 2014). The authors of this paper also discuss how strong associations between CH_4 production and DMI as well as between DMI and BW may be responsible for any observed associations between CH_4 production and BW. As feed intake is so strongly correlated with various production and biological factors, any associations observed between differences in enteric CH_4 emissions and production and biological factors may reflect both parameters responding to changes in dietary intake.

Although the present study did not find a significant association of BCS with enteric-CH_4-emitting phenotype, increasing BCS, like BW, tends to increase with enteric CH_4 production (Salisbury et al., 2026). However, interactions between BW and BCS have also been observed as significant in changing CH_4 production; increases in BCS have less effect on enteric

CH_4 production in higher BW cows than in lower BW cows (P = 0.05) (Salisbury et al., 2026). Regardless, it should again be noted that observed associations between BCS and CH_4 production may be a result of changes in DMI.

In the current study, an observed tendency for low-enteric-CH_4-emitting cattle to produce less milk than high-enteric-CH_4-emitting cattle, and the significant differences in production of ECM between enteric-CH_4-emitting groups, where lower enteric-CH_4-emitting cattle produce fewer kg of ECM per day, align with previous findings in the literature. A recent study examining 4,019 Danish lactating Holstein dairy cattle found that cattle with lower enteric CH_4 emissions were associated with lower milk production (Schneider et al., 2025). The authors of that study recommend evaluation of the potential of genetic traits related to methanogenesis that are independent of milk production (Schneider et al., 2025). It has also been observed that early-lactation cattle have lower enteric CH_4 emissions than late-lactation cattle, likely because of decreased DMI in early lactation, as cows draw more energy from body reserves at that time (Schneider et al., 2025).

No significant differences in milk composition between LM and HM cows in the present study indicate that differences in enteric-CH_4-emitting phenotype do not affect the cow's ability to produce adequate amounts of milk fats, proteins, and lactose for the amount of milk produced. Enteric-CH_4-emitting phenotype only seems to be associated with the gross amount of milk production. This results in significantly greater milk fat production (kg/day) in high-enteric-CH_4-emitting cows and a tendency for high-enteric-CH_4-emitting cows to produce more lactose (kg/day), as a direct result of differences in total milk produced between enteric- CH_4-emitting groups.

The lack of a significant difference in the apparent total-tract digestibility of DM, OM, NDF, and ADF in the present study is inconsistent with observations from previous studies. A meta-analysis of five digestibility studies, both *in vivo* and and cows and sheep, revealed that digestibility was lower by a mean of 10 g/kg change in CH_4 between high-enteric-CH_4-emitting and low-enteric-CH_4-emitting models or ruminants (Løvendahl et al., 2018). Low-enteric-CH_4-emitting animals showed decreased ability to digest cell wall components of plants, such as cellulose, as indicated by lower NDF digestibility (Løvendahl et al., 2018). Additionally, previous studies have detailed significant differences in OM, NDF, and ADF digestibility between high-enteric-CH_4-emitting (HM) and low-enteric-CH_4-emitting (LM) cows, where for apparent total-tract digestibility of OM, NDF, and ADF, HM cows had values of 69.3%, 45.3%, 40.2%, and LM cows had values of 67%, 40.8%, and 33.9%, ($P = 0.03$, $P = 0.01$, $P = 0.02$), respectively (Stepanchenko et al., 2023). Similarly, in another study, high-Y_m cows had greater OM and NDF digestibility ($P < 0.001$, $P = 0.003$) than did low-Y_m cows (Denninger et al., 2020). It should be acknowledged that the lack of differences may be due to the low sample sizes used in the present study, with n=17 for LM cows and n=17 for HM cows. Although not reported in the present study, phenotypic differences in CH_4 emission levels have been shown to be associated with changes in rumen function and dietary energy availability, as a function of dietary digestibility.

Additional digestibility studies have raised concerns that decreased feed passage rate, which is associated with limited DMI, may result in more of the diet's digestible energy, up to 30%, being lost to methanogenesis, as methanogens have increased contact time with substrates (Løvendahl et al., 2018) So, although decreased DMI is associated with decreased gross enteric

CH_4 production, it may also be associated with increased CH_4 yield due to differences in digestive processes and feed passage rates (Løvendahl et al., 2018; Johnson and Johnson, 1995).

As urine nitrogen balance is an indirect way to evaluate how much dietary protein is being utilized by the animal; high levels may indicate the animal is not using all the protein it is consuming in its diet, while lower levels may indicate protein deficiency. With no significant difference in urine urea nitrogen levels between HM and LM dairy cow groups in the present there is no indication of a difference in dietary protein efficiency between enteric- CH_4-emitting groups. Differences in animals' nitrogen balance when the diet is the same can indicate differences in rumen function and in how ruminal microorganisms make protein available to cows. This result is supported by published literature, where differences between high-Y_m and low-Y_m cow groups did not show differences in cows' nitrogen balance (Denninger et al., 2020).

Chapter 6

Conclusion

Results from the present study included support for diurnal variation in enteric CH_4, CO_2, and H_2 production that follows feeding and milking patterns. Further, cows with the low CH_4-emitting phenotype were associated with having lower DMI, OM NDF, and ADF intake, and reduced yields of ECM. Results may indicate that lower gross enteric CH_4 production and lower ECM production are both associated with lower DMI, as rumen microbes receive less fermentable material to generate energy for both methanogenesis and lactation. The current study did not find any differences in apparent total-tract digestibility of nutrients, body weight, body condition score, or urinary composition and output between different enteric-CH_4-emitting groups of cows. Findings from the present study indicate that selection for low-methane-emitting lactating Holstein dairy cows may select for lower-producing cattle, but not for smaller or less feed-efficient cattle. However, differences in enteric-CH_4-emitting phenotype and associated differences in ECM production may both be a result of differences in DMI. Further research into the associations between enteric-CH_4-emitting phenotype and ECM production, where DMI is a fixed variable, is recommended. A further recommendation for future studies is to examine additional factors that may be associated with the enteric-CH_4-emitting phenotype, such as feed passage rate and rumen volume, to identify additional characteristics of low-enteric-CH_4-emitting cattle. This way, genetic selection of low-enteric-CH_4-emitting cattle as a strategy to reduce the environmental effect of the livestock industry can be a more informed process.

REFERENCES

Acharya, P., Warkentin, E., Ermler, U., Thauer, R. K., & Shima, S. (2006). The structure of formylmethanofuran: Tetrahydromethanopterin formyltransferase in complex with its coenzymes. *Journal of Molecular Biology*, *357*(3), 870–879. https://doi.org/10.1016/j.jmb.2006.01.015

Adebayo Arowolo, M., Zhang, X. M., Wang, M., Wang, R., Wen, J. N., Hao, L. Z., He, J. H., Shen, W. J., Ma, Z. Y., & Tan, Z. L. (2022). Proper motility enhances rumen fermentation and microbial protein synthesis with decreased saturation of dissolved gases in rumen simulation technique. *Journal of Dairy Science*, *105*(1), 231–241. https://doi.org/10.3168/jds.2021-20165

Arshad, M. A., & Erickson, P. (2025, September 8). *Understanding the Lactation Curve in Dairy Cows*. University of New Hampshire. https://extension.unh.edu/blog/2025/09/understanding-lactation-curve-dairy-cows

Beauchemin, K. A., McAllister, T. A., & McGinn, S. M. (2009). Dietary mitigation of enteric methane from cattle. *CABI Reviews*, 1–18. https://doi.org/10.1079/pavsnnr20094035

Crowley, S. B., Purfield, D. C., Conroy, S. B., Kelly, D. N., Evans, R. D., Ryan, C. V., & Berry, D. P. (2024). Associations between a range of enteric methane emission traits and performance traits in indoor-fed growing cattle. *Journal of Animal Science*, *102*. https://doi.org/10.1093/jas/skae346

Denninger, T. M., Schwarm, A., Dohme-Meier, F., Münger, A., Bapst, B., Wegmann, S., Grandl, F., Vanlierde, A., Sorg, D., Ortmann, S., Clauss, M., & Kreuzer, M. (2020). Accuracy of methane emissions predicted from milk mid-infrared spectra and measured by laser methane detectors in Brown Swiss Dairy Cows. *Journal of Dairy Science*, *103*(2), 2024–2039. https://doi.org/10.3168/jds.2019-17101

Dicks, L., Roman-Garcia, Y., van Gastelen, S., Maigaard, M., Ingram, P., & Schroeder, G. F. (2026). A meta-analysis of the effects of nitrate supplementation on enteric methane emission, production performance, and blood methemoglobin in dairy cattle. *Journal of Dairy Science*, *109*(3), 2444–2457. https://doi.org/10.3168/jds.2025-27514

Dijkstra, J., Oenema, O., & Bannink, A. (2011). Dietary strategies to reducing N excretion from cattle: Implications for methane emissions. *Current Opinion in Environmental Sustainability*, *3*(5), 414–422. https://doi.org/10.1016/j.cosust.2011.07.008

Dini, Y., Cajarville, C., Gere, J. I., Fernandez, S., Fraga, M., Pravia, M. I., Navajas, E. A., & Ciganda, V. S. (2018). Association between residual feed intake and enteric methane emissions in Hereford Steers. *Translational Animal Science*, *3*(1), 239–246. https://doi.org/10.1093/tas/txy111

Döring, C., & Basen, M. (2024). Propionate production by Bacteroidia gut bacteria and its dependence on substrate concentrations differs among species. *Biotechnology for Biofuels and Bioproducts*, *17*(1). https://doi.org/10.1186/s13068-024-02539-9

Fresco, S., Boichard, D., Fritz, S., Lefebvre, R., Barbey, S., Gaborit, M., & Martin, P. (2023). Comparison of methane production, intensity, and yield throughout lactation in Holstein Cows. *Journal of Dairy Science*, *106*(6), 4147–4157. https://doi.org/10.3168/jds.2022-22855

Gao, Z., Yuan, H., Ma, W., Li, J., Liu, X., & Desjardins, R. L. (2011). Diurnal and seasonal patterns of methane emissions from a dairy operation in North China Plain. *Advances in Meteorology*, *2011*, 1–7. https://doi.org/10.1155/2011/190234

Gerber, P. J., Hristov, A. N., Henderson, B., Makkar, H., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A. T., Yang, W. Z., Tricarico, J. M., Kebreab, E., Waghorn, G., Dijkstra, J., & Oosting, S. (2013a). Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: A Review. *Animal*, *7*, 220–234. https://doi.org/10.1017/s1751731113000876

Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. and Tempio, G. (2013b). Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities. Page 76. Food and Agriculture Organization of the United Nations (FAO), Rome.

Giesenhagen, J., & Place, S. (2023, July 27). *What is methane?*. AgNext. https://agnext.colostate.edu/2023/07/27/what-is-methane/

Gillespie, J., & Njuki, E. (2026, February 23). *Fewer farms, more milk: The changing structure and costs of U.S. dairy farming*. Fewer Farms, More Milk: The Changing Structure and Costs of U.S. Dairy Farming | Economic Research Service. https://www.ers.usda.gov/amber-waves/2026/february/fewer-farms-more-milk-the-changing-structure-and-costs-of-us-dairy-farming

Goopy, J. P., Donaldson, A., Hegarty, R., Vercoe, P. E., Haynes, F., Barnett, M., & Oddy, V. H. (2014). Low-methane yield sheep have smaller rumens and shorter rumen retention time. *British Journal of Nutrition*, *111*(4), 578–585. https://doi.org/10.1017/s0007114513002936

Grainger, C., & Beauchemin, K. A. (2011). Can enteric methane emissions from ruminants be lowered without lowering their production? *Animal Feed Science and Technology*, *166–167*, 308–320. https://doi.org/10.1016/j.anifeedsci.2011.04.021

Grossi, G., Goglio, P., Vitali, A., and A. G. Williams. 2018. Livestock and climate change: impact of livestock on climate and mitigation strategies. Anim Front. 9:69-76. https://doi.org/10.1093/af/vfy034

Harmon, D. L., Yamka, R. M., & Elam, N. A. (2004). Factors affecting intestinal starch digestion in ruminants: A Review. *Canadian Journal of Animal Science*, *84*(3), 309–318. https://doi.org/10.4141/a03-077

Hatew, B., Podesta, S. C., Van Laar, H., Pellikaan, W. F., Ellis, J. L., Dijkstra, J., & Bannink, A. (2015). Effects of dietary starch content and rate of fermentation on methane production in lactating dairy cows. *Journal of Dairy Science*, *98*(1), 486–499. https://doi.org/10.3168/jds.2014-8427

Hristov, A.N., Price, W. J., & Shafii, B. (2005). A meta-analysis on the relationship between intake of nutrients and body weight with milk volume and milk protein yield in dairy cows. *Journal of Dairy Science*, *88*(8), 2860–2869. https://doi.org/10.3168/jds.s0022-0302(05)72967-2

Hristov, A.N., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A., Yang, W., Tricarico, J., Kebreab, E., Waghorn, G., Dijkstra, J. & Oosting, S. 2013. Mitigation of greenhouse gas emissions in livestock production – A review of technical options for non-CO2 emissions. Edited by Pierre J. Gerber, Benjamin Henderson and Harinder P.S. Makkar. FAO Animal Production and Health Paper No. 177. FAO, Rome, Italy.

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T., Weeks, H., Zimmerman, P. R., Harper, M. T., Hristova, R. A., Zimmerman, R. S., & Branco, A. F. (2015). The use of an automated system (GreenFeed) to monitor enteric methane and carbon dioxide emissions from Ruminant Animals. *Journal of Visualized Experiments*, (103). https://doi.org/10.3791/52904

Hristov, A. N., Bannink, A., Battelli, M., Belanche, A., Cajarville Sanz, M. C., Fernandez-Turren, G., Garcia, F., Jonker, A., Kenny, D. A., Lind, V., Meale, S. J., Meo Zilio, D., Muñoz, C., Pacheco, D., Peiren, N., Ramin, M., Rapetti, L., Schwarm, A., Stergiadis, S., Theodoridou, K., Ungerfeld, E. M., van Gastelen, S., Yáñez-Ruiz, D. R., Waters, S. M. & Lund, P. (2025). Feed additives for methane mitigation: Recommendations for testing enteric methane-mitigating feed additives in ruminant studies. *Journal of Dairy Science*, *108*(1), 322–355. https://doi.org/10.3168/jds.2024-25050

Huhtanen, P., K. Kaustell, and S. Jaakkola. 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. Anim. Feed Sci. Technol. 48:211–227. https://doi.org/10.1016/0377-8401(94)90173-2.

Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483–2492. https://doi.org/10.2527/1995.7382483x.

Jones, D. (2025, December 2). *Cow Efficiency Equals Carbon Efficiency*. Agri-King Key to Profit. https://agriking.com/cow-efficiency-equals-carbon-efficiency/#:~:text=A%20relationship%20exists%20between%20DMI,efficiency%20and%20reduced%20carbon%20emissions.

Kamalanathan, S., Houlahan, K., Miglior, F., Chud, T. C., Seymour, D. J., Hailemariam, D., Plastow, G., de Oliveira, H. R., Baes, C. F., & Schenkel, F. S. (2023). Genetic analysis of methane emission traits in Holstein Dairy Cattle. *Animals*, *13*(8), 1308. https://doi.org/10.3390/ani13081308

Kjeldsen, M. H., de Evan Rozada, T., Noel, S. J., Schönherz, A., Hellwing, A. L., Lund, P., & Weisbjerg, M. R. (2024). Phenotypic traits related to methane emissions from Holstein dairy cows challenged by low or high forage proportion. *Journal of Dairy Science*, *107*(12), 10787–10810. https://doi.org/10.3168/jds.2024-24848

Knapp, J. R., Laur, G. L., Vadas, P. A., Weiss, W. P., & Tricarico, J. M. (2014). Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. *Journal of Dairy Science*, *97*(6), 3231–3261. https://doi.org/10.3168/jds.2013-7234

Knight, R. (2025, July 2). *Cattle & Beef - Statistics & Information*. Cattle & Beef - Statistics & Information | Economic Research Service. https://www.ers.usda.gov/topics/animal-products/cattle-beef/statistics-information

Kolathingal-Thodika, N., Elayadeth-Meethal, M., Dunshea, F. R., Eckard, R., Flavel, M., & Chauhan, Surinder. S. (2026). Harnessing methane proxies to understand and mitigate enteric emissions from ruminant production systems. *Science of The Total Environment*, *1012*, 181258. https://doi.org/10.1016/j.scitotenv.2025.181258

Kurth, J. M., Op den Camp, H. J. M., & Welte, C. U. (2020). Several ways one goal—methanogenesis from unconventional substrates. *Applied Microbiology and Biotechnology*, *104*(16), 6839–6854. https://doi.org/10.1007/s00253-020-10724-7

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen. 2017. lmerTest package: Tests in linear mixed effects models. *J. Stat. Softw.* 82:1–26. https://doi.org/10.18637/jss.v082.i13

Lee, C., A. N. Hristov, T. W. Cassidy, K. S. Heyler, H. Lapierre, G. A. Varga, M. J. de Veth, R. A. Patton, and C. Parys. 2012. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J. Dairy Sci. 95:6042–6056. https://doi.org/10.3168/jds.2012-5581.

Livestock and the environment. Food and Agriculture Organization of the United Nations. (n.d.). https://www.fao.org/livestock-environment/en#:~:text=The%20global%20demand%20and%20production,in%20the%20use%20of%20resources

Løvendahl, P., Difford, G. F., Li, B., Chagunda, M. G. G., Huhtanen, P., Lidauer, M. H., Lassen, J., & Lund, P. (2018). Review: Selecting for improved feed efficiency and reduced methane emissions in dairy cattle. *Animal*, *12*. https://doi.org/10.1017/s1751731118002276

Martínez-Álvaro, M., Auffret, M. D., Duthie, C.-A., Dewhurst, R. J., Cleveland, M. A., Watson, M., & Roehe, R. (2022). Bovine host genome acts on rumen microbiome function linked to methane emissions. *Communications Biology*, *5*(1). https://doi.org/10.1038/s42003-022-03293-0

Martins, L. F., Cueva, S. F., Wasson, D. E., Almeida, C. V., Eifert, C., de Ondarza, M. B., Tricarico, J. M., & Hristov, A. N. (2024). Effects of dose, dietary nutrient composition, and supplementation period on the efficacy of methane mitigation strategies in dairy cows: A meta-analysis. *Journal of Dairy Science*, *107*(11), 9289–9308. https://doi.org/10.3168/jds.2024-24783

Marques, R. da, & Cooke, R. F. (2021). Effects of ionophores on ruminal function of beef cattle. *Animals*, *11*(10), 2871. https://doi.org/10.3390/ani11102871

McFadden, J., Amburgh, M. V., Overton, T., Oliver, J., & Reed, K. (n.d.). *Enteric methane mitigation*. Cornell CALS. https://cals.cornell.edu/about/our-values-impact/roadmap-2050/transdisciplinary-moonshots/livestock-innovations-for-sustainability/enteric-methane-mitigation

Min, B.-R., Lee, S., Jung, H., Miller, D. N., & Chen, R. (2022). Enteric methane emissions and animal performance in dairy and beef cattle production: Strategies, opportunities, and impact of reducing emissions. *Animals*, *12*(8), 948. https://doi.org/10.3390/ani12080948

Negussie, E., de Haas, Y., Dehareng, F., Dewhurst, R. J., Dijkstra, J., Gengler, N., Morgavi, D. P., Soyeurt, H., van Gastelen, S., Yan, T., & Biscarini, F. (2017). Invited review: Large-scale indirect measurements for enteric methane emissions in Dairy Cattle: A review of proxies and their potential for use in management and breeding decisions. *Journal of Dairy Science*, *100*(4), 2433–2453. https://doi.org/10.3168/jds.2016-12030

Pelletier, N., Pirog, R., & Rasmussen, R. (2010). Comparative life cycle environmental impacts of three beef production strategies in the Upper Midwestern United States. *Agricultural Systems*, *103*(6), 380–389. https://doi.org/10.1016/j.agsy.2010.03.009

Peña, M., & Gartman, L. (n.d.-b). *What are the different types of fiber and how do they support milk production?*. Dairy Division of Extension. https://dairy.extension.wisc.edu/articles/what-are-the-different-types-of-fiber-and-how-do-they-support-milk-production/#:~:text=Effective%20fiber%20is%20the%20portion,and%20hemicellulose%20(blue%20network).

Pfau, A. P. (n.d.). *How can we measure methane emissions from commercial farms?*. University of Wisconsin-Madison Dairy Division of Extension. https://dairy.extension.wisc.edu/articles/how-can-we-measure-methane-emissions-from-commercial-farms/

Rooke, J. A., Wallace, R. J., Duthie, C.-A., McKain, N., de Souza, S. M., Hyslop, J. J., Ross, D. W., Waterhouse, T., & Roehe, R. (2014). Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. *British Journal of Nutrition*, *112*(3), 398–407. https://doi.org/10.1017/s0007114514000932

Salisbury, C. M., Warner, J. M., & Thompson, L. (2026). Interactive Effects of Body Weight and Body Condition Score on Enteric Methane Emissions in Grazing Beef Cows. *Kansas Agricultural Experiment Station Research Reports*: Vol. 12: Iss. 1.

Santander, D., Clariget, J., Banchero, G., Alecrim, F., Simon Zinno, C., Mariotta, J., Gere, J., & Ciganda, V. S. (2023). Beef steers and enteric methane: Reducing emissions by managing forage diet fiber content. *Animals*, *13*(7), 1177. https://doi.org/10.3390/ani13071177

Shah, A. M., Zhang, H., Shahid, M., Ghazal, H., Shah, A. R., Niaz, M., Naz, T., Ghimire, K., Goswami, N., Shi, W., Xia, D., & Zhao, H. (2025). The vital roles of agricultural crop residues and agro-industrial by-products to support sustainable livestock productivity in subtropical regions. *Animals*, *15*(8), 1184. https://doi.org/10.3390/ani15081184

Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H., Kelly, W. J., Atua, R., Sang, C., Soni, P., Li, D., Pinares-Patiño, C. S., McEwan, J. C., Janssen, P. H., Chen, F., Visel, A., Wang, Z., Attwood, G. T., & Rubin, E. M. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Research*, *24*(9), 1517–1525. https://doi.org/10.1101/gr.168245.113

Schneider, H., Lajevardi, R. K., Bjerring, M., L⊘vendahl, P., Lund, M. S., & Villumsen, T. M. (2025). Genetic parameters for enteric methane traits and their genetic connection with milk production in Danish Holstein cattle. *Journal of Dairy Science*, *108*(11), 12519–12530. https://doi.org/10.3168/jds.2025-26853

Sjaunja, L. O., L. Baevre, L. Junkkarinen, J. Pedersen, and J. Set.l.. 1990. A Nordic proposal for an energy corrected milk (ECM) formula. Pages 156–157 in 27th Session of the International Commission for Breeding and Productivity of Milk Animals, Paris, France. Wageningen Academic Publishers.

Souza, V. C., Almeida, K. V., Rosenstein, P. K., Desrues, O., Panciroli, N., & Kebreab, E. (2026). Impact of tannin-based additives on animal performance and enteric methane emissions in dairy and beef cattle: A meta-analysis. *Journal of Dairy Science*, *109*(3), 2383–2399. https://doi.org/10.3168/jds.2025-27254

Stefenoni, H. A., Räisänen, S. E., Cueva, S. F., Wasson, D. E., Lage, C. F. A., Melgar, A., Fetter, M. E., Smith, P., Hennessy, M., Vecchiarelli, B., Bender, J., Pitta, D., Cantrell, C. L., Yarish, C., & Hristov, A. N. (2021). Effects of the Macroalga asparagopsis taxiformis and oregano leaves on methane emission, rumen fermentation, and lactational performance of Dairy Cows. *Journal of Dairy Science*, *104*(4), 4157–4173. https://doi.org/10.3168/jds.2020-19686

Stepanchenko, N., Stefenoni, H., Hennessy, M., Nagaraju I., Wasson, D. E., Cueva, S. F., Räisänen, S. E., Dechow, C. D., Pitta, D. W., and A. N. Hristov. 2023. Microbial composition, rumen fermentation parameters, enteric methane emissions, and lactational performance of phenotypically high and low methane-emitting dairy cows. J. Dairy Sci. 106:6146–6170. https://doi.org/10.3168/jds.2022-23190.

Sullivan, H. M. (2005, April). *Increasing Milk Production by Increasing Milking Frequency and Reducing Dry Period Length*. Increasing Milk Production by Increasing Milking Frequency and Reducing Dry Period Length | New Mexico State University - BE BOLD. Shape the Future. https://pubs.nmsu.edu/_d/D109/index.html#:~:text=Research%20has%20shown%20that%20milking%20cows%20four,**More%20frequent%20milking%20may%20flush%20out%20microorganisms**

Szuhaj, M., Kakuk, B., Wirth, R., Rákhely, G., Kovács, K. L., & Bagi, Z. (2023). Regulation of the methanogenesis pathways by hydrogen at transcriptomic level in time. *Applied Microbiology and Biotechnology*, *107*(20), 6315–6324. https://doi.org/10.1007/s00253-023-12700-3

United Nations. (2022). *Goal 2 | Department of Economic and Social Affairs*. United Nations. https://sdgs.un.org/goals/goal2

USDA. (2025, July 25). United States Cattle Inventory Report. Retrieved February 22, 2026, from https://www.nass.usda.gov/Newsroom/2025/07-25-2025.php.

U.S. Environmental Protection Agency. 2024. Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2022. Accessed February 4, 2026. https://www.epa.gov/ghgemissions/inventory-us-greenhouse-gas-emissions-and-sinks-1990-2022

van Gastelen, S., Jan van Dooren, H., & Bannink, A. (2023). Enteric and manure emissions from Holstein-Friesian dairy cattle fed grass silage–based or corn silage–based diets. *Journal of Dairy Science*, *106*(9), 6094–6113. https://doi.org/10.3168/jds.2022-22378

Vargas, J. E., Andrés, S., López-Ferreras, L., Snelling, T. J., Yáñez-Ruíz, D. R., García-Estrada, C., & López, S. (2020). Dietary supplemental plant oils reduce methanogenesis from anaerobic microbial fermentation in the rumen. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-58401-z

Wang, K., Xiong, B., & Zhao, X. (2023). Could propionate formation be used to reduce enteric methane emission in ruminants? *Science of The Total Environment*, *855*, 158867. https://doi.org/10.1016/j.scitotenv.2022.158867

Wasson, D. E., Almeida, C., Cueva, S. F., Martins, L. F., Richards, A., Stepanchenko, N., Schmidt, W. E., Fredericq, S., & Hristov, A. N. (2026). Effects of the Macroalga laurencia snackeyi on enteric methane emission and lactational performance in dairy cows. *Journal of Dairy Science*, *109*(3), 2514–2526. https://doi.org/10.3168/jds.2025-27251

Wright, A. D., Kennedy, P., O'Neill, C. J., Toovey, A. F., Popovski, S., Rea, S. M., Pimm, C. L., & Klein, L. (2004). Reducing methane emissions in sheep by immunization against rumen methanogens. *Vaccine*, *22*(29-30), 3976–3985. https://doi.org/10.1016/j.vaccine.2004.03.053

Yin, Q., Wu, S., Lei, X., Zhang, J., Wang, D., Li, X., & Yao, J. (2026). Revisiting starch degradability in dairy ruminants: From feed processing to gut microbiota and Metabolic Regulation. *Journal of Animal Science and Biotechnology*, *17*(1). https://doi.org/10.1186/s40104-026-01365-3

Zetouni, L., Difford, G. F., Lassen, J., Byskov, M. V., Norberg, E., & Løvendahl, P. (2018). Is rumination time an indicator of methane production in dairy cows? *Journal of Dairy Science*, *101*(12), 11074–11085. https://doi.org/10.3168/jds.2017-14280

Zhao, Y., Nan, X., Yang, L., Zheng, S., Jiang, L., & Xiong, B. (2020). A review of enteric methane emission measurement techniques in ruminants. *Animals*, *10*(6), 1004. https://doi.org/10.3390/ani10061004

APPENDIX A Sampling Schedules

Table 6: TMR, Refusals, and Feed Components Sampling Schedule

H0425 Screening - Phase 1 - Tie-Stall Barn		
Date	**Time**	**Sample Type**
Sampling Week 1		
10/08/2025	5:00 AM	TMR, Refusals, Feed Components
10/10/2025	5:00 AM	TMR, Refusals, Feed Components
Sampling Week 2		
10/12/2025	5:00 AM	TMR, Refusals, Feed Components
10/14/2025	5:00 AM	TMR, Refusals, Feed Components
H0425 Screening - Phase 2 - Tie-Stall Barn		
10/19/2025	5:00 AM	TMR, Refusals, Feed Components
10/21/2025	5:00 AM	TMR, Refusals, Feed Components
Sampling Week 1		
10/26/2025	5:00 AM	TMR, Refusals, Feed Components
10/28/2025	5:00 AM	TMR, Refusals, Feed Components
Sampling Week 2		
11/01/2025	5:00 AM	TMR, Refusals, Feed Components
H0425 Screening - Phase 3 - Tie-Stall Barn		
11/06/2025	5:00 AM	TMR, Refusals, Feed Components
11/08/2025	5:00 AM	TMR, Refusals, Feed Components
Sampling Week 1		
11/13/2025	5:00 AM	TMR, Refusals, Feed Components
11/15/2025	5:00 AM	TMR, Refusals, Feed Components
Sampling Week 2		
11/17/2025	5:00 AM	TMR, Refusals, Feed Components
11/19/2025	5:00 AM	TMR, Refusals, Feed Components

Sampling Schedule

H0425 Screening - Phase 1 - Tie-Stall Barn		
Date	**Time**	**Sample Type**
Sampling Week 2		
10/13/2025	5:00 AM	Milk
10/13/2025	5:00 PM	Milk
10/14/2025	5:00 AM	Milk
10/14/2025	5:00 PM	Milk
H0425 Screening - Phase 2 - Tie-Stall Barn		

Sampling Week 2		
10/30/2025	**5:00 PM**	**Milk**
10/31/2025	**5:00 AM**	**Milk**
10/31/2025	**5:00 PM**	**Milk**
H0425 Screening - Phase 3 - Tie-Stall Barn		
Sampling Week 2		
11/17/2025	**5:00 PM**	**Milk**
11/18/2025	**5:00 PM**	**Milk**
11/19/2025	**5:00 AM**	**Milk**

Table 7: Enteric Gas Emissions Sampling Schedule

H0425 Screening - Phase 1 - Tie-Stall Barn		
Date	**Time**	**Sample Type**
Sampling Week 1		
10/08/2025	**4:00 AM**	**Gas**
10/08/2025	**10:00 AM**	**Gas**
10/09/2025	**4:00 PM**	**Gas**
10/09/2025	**10:00 PM**	**Gas**
10/10/2025	**2:00 PM**	**Gas**
10/10/2025	**8:00 PM**	**Gas**
10/11/2025	**2:00 AM**	**Gas**
10/11/2025	**8:00 AM**	**Gas**
Sampling Week 2		
10/12/2025	**4:00 AM**	**Gas**
10/12/2025	**10:00 AM**	**Gas**
10/13/2025	**4:00 PM**	**Gas**
10/13/2025	**10:00 PM**	**Gas**
10/14/2025	**8:00 AM**	**Gas**
10/14/2025	**2:00 PM**	**Gas**
10/14/2025	**8:00 PM**	**Gas**
10/15/2025	**2:00 AM**	**Gas**
H0425 Screening - Phase 2 - Tie-Stall Barn		
Sampling Week 1		
10/26/2025	**4:00 AM**	**Gas**
10/26/2025	**10:00 AM**	**Gas**
10/27/2025	**10:00 PM**	**Gas**
10/28/2025	**8:00 AM**	**Gas**
10/28/2025	**2:00 PM**	**Gas**
10/28/2025	**8:00 PM**	**Gas**
10/29/2025	**2:00 AM**	**Gas**
Sampling Week 2		
10/30/2025	**4:00 AM**	**Gas**
10/30/2025	**10:00 AM**	**Gas**
10/31/2025	**4:00 PM**	**Gas**
10/31/2025	**10:00 PM**	**Gas**
11/01/2025	**8:00 AM**	**Gas**
11/01/2025	**2:00 PM**	**Gas**

11/01/2025	**8:00 PM**	**Gas**
11/02/2025	**2:00 AM**	**Gas**
H0425 Screening - Phase 3 - Tie-Stall Barn		
Sampling Week 1		
11/13/2025	**4:00 AM**	**Gas**
11/14/2025	**4:00 PM**	**Gas**
11/14/2025	**10:00 PM**	**Gas**
11/15/2025	**8:00 AM**	**Gas**
11/15/2025	**2:00 PM**	**Gas**
11/15/2025	**8:00 PM**	**Gas**
11/16/2025	**2:00 AM**	**Gas**
11/16/2025	**10:00 AM**	**Gas**
Sampling Week 2		
11/24/2025	**10:00 AM**	**Gas**
11/25/2025	**4:00 PM**	**Gas**
11/25/2025	**10:00 PM**	**Gas**
11/26/2025	**8:00 AM**	**Gas**
11/26/2025	**2:00 PM**	**Gas**
11/26/2025	**8:00 PM**	**Gas**
11/27/2025	**2:00 AM**	**Gas**
11/28/2025	**4:00 AM**	**Gas**

Table 8: Fecal and Urine Sampling Schedule

H0425 Screening - Phase 1 - Tie-Stall Barn		
Date	**Time**	**Sample Type**
Sampling Week 2		
10/16/2025	**4:00 AM**	**Fecal & Urine**
10/16/2025	**10:00 AM**	**Fecal & Urine**
10/16/2025	**4:00 PM**	**Fecal & Urine**
10/16/2025	**10:00 PM**	**Fecal & Urine**
H0425 Screening - Phase 2 - Tie-Stall Barn		
Sampling Week 2		
11/02/2025	**4:00 AM**	**Fecal & Urine**
11/02/2025	**10:00 AM**	**Fecal & Urine**
11/02/2025	**4:00 PM**	**Fecal & Urine**
11/02/2025	**10:00 PM**	**Fecal & Urine**
H0425 Screening - Phase 3 - Tie-Stall Barn		
Sampling Week 2		
11/29/2025	**4:00 AM**	**Fecal & Urine**
11/29/2025	**10:00 AM**	**Fecal & Urine**
11/29/2025	**4:00 PM**	**Fecal & Urine**
11/29/2025	**10:00 PM**	**Fecal & Urine**