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Mitigation Strategies of Agricultural Air Emissions

By

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ABSTRACT

Cattle and their waste products are considered a major source of greenhouse gas (**GHG**) emissions. In the United States, animal agriculture accounts for 38% of methane (**CH_4**) emissions with 28% arising from enteric fermentation, and 10% from manure management. With California being the leading dairy state in the United States, it is increasingly important that strategies for reducing gaseous emissions in lactating dairy cows and management of their waste within the state be investigated. In studies 1 and 2, the objective was to determine the efficacy of various commercially available plant secondary metabolites (**PSM**) at reducing GHG and ammonia (**NH_3**) emissions without negatively impacting the productive performance of dairy cows. The PSM's investigated were: (1) a commercial essential oils blend comprised of eugenol, coriander seed, and geranyl acetate (Agolin® Ruminant; Agolin SA, Bière, Switzerland), and (2) a commercial blend of quebracho and chestnut tannins with saponins (SilvaFeed® BX; SILVATEAM SpA, San Michele Mondovì, Italy). In each study, twenty early- to mid-lactation Holstein dairy cows were blocked by days in milk and parity in a randomized complete block design, and were assigned one of two treatments: PSM or control (n=2/block). Cows were individually fed, group-housed in a free-stall pen, and were milked twice daily. The treatments were administered as a top dress at each of two feedings per day. Cows were sampled for enteric CH4, carbon dioxide (**CO_2**), nitrous oxide (**N_2O**) and NH_3 emissions in head chambers (**HC**) for 12 hours on treatment days 0, 14, 28, 42, and 56 in study 1, and 0, 16, 32, and 48 in study 2. Enteric GHG and NH_3 emissions, energy-corrected milk (**ECM**; kg), milk component yields (kg) and proportions (%), and dry matter intake (**DMI**; kg) were analyzed for pairwise comparison in R. In study 1, supplemental Agolin® tended to decrease enteric N_2O intensity, and significantly decreased enteric CH_4 and NH_3 intensity (g or mg gas/d/kg ECM). Enteric NH_3 production

decreased significantly with Agolin supplementation, though there was no effect on CH_4, CO_2, or N_2O production (g or mg gas/d). Enteric GHG and NH_3 yields (g or mg gas/d/kg HC DMI) did not differ between treatment types in study 1. In study 2, SilvaFeed® BX tended to decrease enteric CH_4 and CO_2 production (g/h), significantly decreased N_2O production (mg/h), and tended to increase NH_3 production (mg/h). Supplementing cows with SilvaFeed BX resulted in an increase in slurry NH_3 emissions (mg/h/m^2), though the GHG's were unaffected. No differences were found in ECM, milk fat yield, milk protein yield, and DMI in PSM-fed cows in both studies 1 and 2. Study 3 investigated the ability of a commercial biological wastewater applicant (BiOWiSH® AQUA; BiOWiSH Technologies Inc., Cincinnati, Ohio) for reducing NH_3 emissions in the effluent from anaerobic digesters. Effluent was collected from an anaerobic digester and was homogenized and distributed equally between 18 steel drums. The drums were placed in a 6x3 (row x column) grid with treatments allocated and applied according to a double Latin square. Treatments were comprised of positive control (aeration but no BiOWiSH), negative control (no aeration and no BiOWiSH), and the experimental treatment (both BiOWiSH and aeration). Gaseous emissions were measured continuously across 56 days, with each column being measured for 24 hours every 3-4 days. Ammonia emissions did not significantly differ between the experimental treatment and the positive control. Both the experimental treatment and the positive control had higher NH_3 emissions than the negative control. Future research should assess the mitigation potentials of each of the feed additives and the applicant at varying dosage levels.

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Livestock occupy an important segment of the global food systems. Contributing tremendously to food security in developed- and developing countries alike, animal products source a large proportion of total dietary protein. It is estimated that animal sourced proteins account for 43% of the global and 46-69% of the United States protein consumption [1]. According to projections by the FAO conducted in 2020, global meat consumption is expected to increase by 14% while the global population is projected to increase by 11% by 2030 [2,3]. In order to meet these growing demands in animal sourced proteins, more efficient and environmentally-focused livestock systems will need to be developed. With a rise in global human population and a consequent rise in demand for more food and animal sourced proteins, it becomes imperative that the agricultural sectors continue to look toward more sustainable systems. Chief among environmental topics of sustainability is the climate impact by means of greenhouse gas (**GHG**) emissions from the livestock industry.

In the 20th century, literature in environmental health began describing the interrelation between global climate change and infectious diseases [4]. Though speculative and hypothetical at the time, parallels between environmental- and climatic disturbances and the incidence and prevalence of various infectious diseases were noted. Many organisms that caused disease were found to be highly influenced by environmental conditions that were affected by climate change including temperature, moisture, and humidity as shifts in seasonality began to unfold [5]. Like other species that are undergoing evolutionary shifts in persistence, pathogens and parasites— in particular vector-borne diseases– have the capacity to undergo climate driven evolutionary changes [6]. Poor air quality and pollution also pose significant challenges to human health. Air toxicants, such as carbon monoxide (**CO**), ozone (**O_3**), nitrogen oxides, particle pollution, and

sulfur oxides, can be toxic to humans and animals and harmful to ecosystems if present at too high of concentrations [7]. Some classes of particle pollution and of particulate matter (**PM**) can be formed by complex chemical reactions while others are directly emitted from specific sources including combustion emissions, wildfires, and fields and livestock operations to some degree. If inhaled, PM of ≤ 2.5 μm can be deposited into the alveolar tissues of human lungs that can lead to respiratory and cardiac issues, changes that may contribute to premature mortality [8].

Animal agriculture has received considerable societal pressure to reduce their carbon footprint. In an effort to regulate climate pollutants, regulatory agencies are beginning to regulate methane (**CH_4**) emissions from the livestock sector. For example, California legislators enacted bills regulating short-lived climate pollutants (**SLCP**) including CH_4 from sources such as landfills and livestock animal and manure management directly calling to reductions by the dairy sector [9]. In this review, topics will be discussed that relate ruminant livestock's contribution to the growing climate crisis. Another focus will be the direct impacts of livestock on GHG and ammonia (**NH_3**) emissions as well as strategies to reduce their impact climate. This review will conclude with an environmental outlook of the future of livestock production.

1.2. Greenhouse Gas and Ammonia Emissions

Greenhouse gases are given the name due to their ability to create a blanket around earth's atmosphere, which allows for solar heat to be trapped and kept at a temperature capable of maintaining and sustaining life. Due to anthropogenic emission of these gases, GHGs have been accumulating beyond sustainable amounts leading to a warming effect on our planet. It is estimated that carbon dioxide (**CO_2**) accounted for 80%, CH_4 for 10%, and nitrous oxide (**N_2O**) for 7% of total United States GHG emissions in 2019 [10]. Carbon dioxide and N_2O are considered long-

lived climate pollutants with an atmospheric lifetime of approximately 1,000 years and 100 years, respectively. By contrast, CH_4 is considered a SLCP with an atmospheric lifetime of 10-12 years [10].

Methane and CO_2 are part of the biogeochemical carbon cycle – through this process, atmospheric CO_2 is assimilated through photosynthesis to then become incorporated into plant material such as in the form of cellulose or starch which is then consumed by animals. Once consumed, the molecules of carbon are either digested and metabolized, pass through the animal undigested and end up in the excreta, or are eructated in the form of CH_4. The CH_4 produced enters the atmosphere, and after a period of 10-12 years is converted back to CO_2 and water; from this point, the cycle can continue. Atmospheric CH_4 is removed through oxidation processes in the atmosphere, with hydroxyl (**OH**) oxidation serving as its largest sink. The process begins in the stratosphere and troposphere where reactions with O_3 result in the formation of the OH radical [11]. In the troposphere, reactions with the OH radical results in 85% destruction of CH_4; CH_4 can also react with oxygen in the atmosphere, being converted to CO_2 and H_2O [11]. In addition to being a sink for atmospheric CH_4, OH is also a sink for CO. Hydroxyl oxidation reactions with CH_4 and CO suppress OH formation; increasing levels of CH_4 or CO in the atmosphere may therefore lead to instability in this dynamic and impede their destruction. Back on earth, methanotrophic bacteria also exists in soil, oxidizing methane from the air into CO_2 and water. According to Moss et al. (2000) the methane produced on earth exceeds the CH_4 sinks – from OH reaction and microbial uptake in soil – by approximately 84 Tg [12].

Approximately 40% of global CH_4 produced from 2000-2017 was attributed to natural sources and the remainder to anthropogenic sources [13]. Natural sources include wetlands, water systems, and geological sources, whereas anthropogenic sources include landfills, oil and gas

drilling, coal mining, and agricultural practices [12]. According to the United States Environmental Protection Agency (**EPA**), animal agriculture accounted for 36% of anthropogenic CH_4 emissions in the United States in 2020. Approximately 75% of that total stemmed directly from enteric fermentation; the remaining 25% was attributed manure management practices [10]; research and implementation efforts are being made to reduce emissions from both sources, which will be highlighted in later sections. In addition to CH_4, agriculture sectors also contribute heavily to N_2O emissions. While the United States EPA attributed 5% of N_2O directly to manure management, another 74% were attributed to agriculture soil management, which includes deposition of livestock manure to soils [10].

Nitrogen also abides by biogeochemical processes and is transferred between ecosystems and the atmosphere following the nitrogen (**N**) cycle. Through this process, N can be introduced into both aerobic- and anaerobic environments. The N cycle is comprised of at least five known flows of N: ammonification, nitrification, denitrification, anaerobic ammonia oxidation (**anammox**), and nitrite-nitrate interconversion [14].

1.3. Enteric Fermentation & Emissions

Methanogenesis is considered a major hydrogen sink in the rumen as it is one of the more thermodynamic processes for the uptake of hydrogen that naturally occurs within the rumen [15]. The vast majority of methane-producing archaea existing within the gastrointestinal tracts of livestock are of the phylum *Euryarcheota* [16]. It is estimated that at least 65% of rumen methanogens are of the genera *Methanobrevibacter* [17]. The other top genera identified include *Methanosphaera* (9.8%), *Methanomicrobium* (7.7%), and *Methanocbacterium* (1.2%) [17]. Methanogens compromise less than 3% of prokaryotic microbiota in the rumen. Ruminal

methanogenesis was found to occur through at least three distinct pathways. The hydrogenotrophic pathway is the pathway predominately used by methanogenic microorganisms, likely due to the thermodynamics in the rumen [18]. Though the production of methane through alternative pathways is less common, ruminal methanogens have been discovered that are capable of producing methane through the acetoclastic or methylotrophic methanogenesis pathways [19,20].

The production of CH_4 also signifies a 2-12% loss in gross energy in ruminants [21]. Additionally, Ruminants are considered to be inefficient at retaining and utilizing fed N for metabolic needs throughout their body. It was estimated that only 20% of dietary-N is utilized by beef animals for growth [22] whereas 25-35% of the dietary-N consumed by dairy cows was secreted in the form of milk [23]. Much of the remaining dietary-N is excreted by the animal in either feces or urine. Literature suggests that ruminal volatilization and enteric emissions of N_2O does occur, though enteric fermentation is a minor source of these emissions [24–26]. Even with this being the case, it remains important that dynamics of ruminal-N be considered due to their downstream impacts. With this in mind, ideal rumen fermentation has the following parameters: rapid fermentation of fiber, efficient and rapid microbial protein production, little accumulation of NH_3, little production of CH_4 and lactate, optimal volatile fatty acid (**VFA**) ratios, and reduced toxin production by ruminal microorganisms [27].

It is important to note that the detailing of this review is inexhaustive. In addition to direct methods to alter ruminal fermentation such as those addressed below, indirect methods such as through improved animal productivity, genetic selection, and animal health, and reduced age when productive or at harvest have been explored to great lengths [28].

1.4. Dietary Strategies for Methane Mitigation

Consideration and proper management of dihydrogen (**H_2**) is very important when working to reduce enteric CH_4 emissions from ruminant livestock [29]. The goal of many CH_4 mitigation strategies is therefore to introduce or promote alternative routes of H_2 utilization in the rumen, or to limit the ruminal production of H_2 by affecting specific microbial populations. Methanogenesis in ruminant livestock is strongly correlated with digestibility of the diet. For instance, high proportions of digestible fiber in a diet may lead to lower rates of fiber fermentation, which in turn leads to further accumulation of H_2 and consequently of CH_4 in the rumen, while easily fermentable diets lead to lower emission intensities. As such, one of the simplest ways of decreasing enteric CH_4 formation and emissions is by altering the basal diet, such as by improving the quality of the feed or adjusting the forage-to-concentrate ratio (**F:C**).

Factors affecting improved forage quality include plant stage of growth and seasonality, with the goal of enhancing the digestibility of the organic matter (**OM**). Increased forage quality promotes increased dry matter intake (**DMI**). Though this has been shown to increase CH_4 emissions in dairy and beef animals, it resulted in decreased CH_4 per unit of DMI (**CH_4 yield**) for a given animal [30]. Methane expressed per unit of productive output (**CH_4 intensity**) may also decrease, as was demonstrated through experiments which provided medium- and higher-quality silages-based forage rations to lactating dairy cows [31].

Research demonstrated that modulating enteric CH_4 by adjusting the F:C of the diet was an effective strategy in dairy [32–34] and moderately effective strategy in beef cattle [33,35]. The rate at which high-concentrate diets were fermented led to increased formation of propionate in the rumen, which could serve as an alternative hydrogen sink. This can thereby result in a depressed availability of hydrogen for methanogenesis. The inverse relationship is suspected for

high-forage diets, which was supported by the findings of Li et al. (2019). Li et al. (2019) measured CH_4 production in real time over the course of 12-h *in vitro* ruminal fermentation experiments, in which donor sheep were fed diets of varying F:C. They found that as the F:C increased the maximum CH_4 concentration decreased. In addition, they noted a 26-27 minute delay in the time it took for the maximum concentration to be reached in high F:C diet conditions [36]. Though adjustment of the F:C is beneficial for CH_4 mitigation, abrupt reductions in F:C may lead to increased lactic acid formation and consequentially a rapid decrease in ruminal pH [37]. The chief concern in this case would therefore be incidence of ruminal acidosis, which can be averted through gradual adjustment of F:C in the ration [38].

1.4.1 Feed Additives

As defined by the European Commission, feed additives are products that are used in animal nutrition in order to achieve effects on the animals, feed itself, food products harvested from the animals which consume the additive, or on the environment [39]. Feed additives can therefore be used to enhance the flavor or palatability of feed, to aid meeting certain dietary or environmental requirements for the animal, or to increase animal performance. For the purpose of CH_4 mitigation, feed additives can fall under two broadly generalized classes based on their modality and general functional properties within the ruminal environment: methane inhibitors, and rumen modifiers. Methane inhibitors impact the methanogenesis pathways directly whereas rumen modifiers modulate the ruminal environment, posing either direct or indirect effects on the ruminal microbiome which includes methanogens. The following sections will examine a subset of the commonly explored feed additives, focusing predominately on those that have demonstrated greatest aggregate potential. The sections will describe the known effects of each feed additive

type on enteric and manure related GHG and NH_3 emissions as well as their effects on factors such as ruminal fermentation, animal health, and animal performance.

1.4.2. 3-Nitrooxypropanol (3NOP)

3-Nitrooxypropanol (**3NOP**) is a synthetic compound formulated with the specific purpose of inhibiting methanogenesis. Possessing conformational similarities with methyl-coenzyme M, 3NOP inactivates methyl-coenzyme M reductase (**MCR**), an important enzyme for catalyzing one of the last steps in the formation of CH_4 via the methanogenesis pathways [40]. The product has withstood the test of both *in vitro* [41–45] and *in vivo* [46–49] experiments, demonstrating CH_4 reduction capabilities of up to 97% [45] and 31% [46], respectively. The variable efficacy is likely associated with factors such as the dosage level of the additive, the proportion of neutral detergent fiber (**NDF**) in the diet, and the duration of supplementation [50]. The direct anti-methanogenic effect of 3NOP was additionally shown to be impacted by both ruminal residence time of the molecule and by feed-related time budgets of cows. Using emissions data collected via Greenfeed and DMI-related time budget information gathered by Niu et al. (2018) and Hristov et al. (2020) estimated that with a mitigation potential of up to 45% immediately following the largest bout of feed consumption in lactating dairy cows [51,52].

Research suggests that 3NOP as a tool for reducing bovine CH_4 emissions may be more effective in dairy cattle than in beef [53]. This finding was recently supported by a meta-analysis evaluating 14 experimental studies which supplemented cattle with 3NOP conducted by Kim et al. (2020). They determined that the slope of CH_4 decrease was greater for dairy than for beef cattle (-0.073 and -0.037, respectively) [54]. Though the particular reason was yet to be determined, dietary differences were the suspected cause of the variability. Diet quality and composition (i.e., F:C) demonstrated profound impacts on 3NOP's effectiveness as a feed additive.

Dijkstra et al. (2018) investigated the relative impact of 3NOP when NDF varied, and found that the efficacy of 3NOP decreased with increasing NDF content, which they attributed to a lower MCR concentration in lower-fiber fed animals [53]. The change in efficacy with varying dietary composition was additionally supported by van Gastelen et al. (2022) who were interested in 3NOP's CH_4 mitigation potential when cows were fed different basal diets [55]. They found that lactating cows fed a corn silage-based diet (lower NDF) or a mixed diet of grass and corn-silage had lower CH_4 yield and intensity than those fed a grass silage-based diet (higher NDF) [55]. It is important to note that the concentrate composition was adjusted for each of the basal diet types to meet maintenance and milk production requirements, and were therefore not the same for the three treatment types. Dry matter intake was lowest in cows fed a grass silage diet, and was greatest in cows fed a corn silage diet [55].

The impact on productive performance of ruminant livestock is worth considering and should be further evaluated. For instance, the meta-analysis results from Kim et al. (2020) showed that increasing inclusion levels resulted in decreased DMI, though DM, OM and NDF digestibility were not affected in beef cattle [54]. In the case of dairy cows, milk yield tended to decrease as inclusion level increased, with no impact to DMI. Kim et al. (2020) did not report on CH_4 intensity or average daily gain (**ADG**) in beef cattle [54]. A recent experiment found that dairy cows had increased DMI in the first 10 h after feed delivery when supplemented with 3NOP at rates of 30, 60, 90, and at 120 mg/kg DM [56]. Experiments focused on consumption preferences in beef cattle, however, found that there is an initial lag in consumption of 3NOP supplemented feeds when an alternative feed choice was offered [57]. However, this preferential selection disappeared after a week on treatment with their animals [57]. A consequential experiment conducted using the animals fed a high forage diet noted 17% reductions in CH_4 production [58].

Melgar et al. (2020) studied the impact of supplementing early-lactation dairy cows with 3NOP at 60 mg/kg dry matter (**DM**) and saw a decrease in CH_4 production (26%), yield (21%), and intensity (25%) [49]. Studying the mitigation potential of 3NOP when supplemented to mid-lactation dairy cows, Melgar et al. (2021) noted comparable reductions in CH_4 production (26%), yield (27%) and intensity (29%) to their aforementioned experimental findings [59]. Taken together, these findings suggest that lactation stage in lactating dairy cows may not heavily impact the CH_4 mitigation potential of 3NOP. Dairy producers may therefore be able to target inclusion of 3NOP in rations for instances that are best suited for their operational needs.

1.4.3. Nitrate

As previously mentioned, the candidate CH_4 mitigation strategies that are typically considered most effective are those that offer alternative means for managing H_2 in the rumen. Nitrate can act as a H_2 sink within the rumen making it a candidate feed additive of particular interest [60]. Among the electron acceptors available in the rumen that are capable of managing the H_2, reducing nitrate to ammonia is thermodynamically more favorable than the converting CO_2 to CH_4. Based on the stoichiometry for hydrogen consumption within the rumen, nitrate has a theoretical capability of reducing ruminal CH_4 production by 25.8 g for every 100 g of nitrate supplemented [60]. In addition to seeing reductions in CH_4 emissions, Zhao et al. (2018) saw 4.47 and 25.82% reductions in methanogen populations when treating beef steers with nitrate at 1% or 2% of DM, respectively [61]. According to a recent meta-analysis by Feng et al. (2020), supplementing dairy cows with nitrate reduced CH_4 production by approximately 17% and CH_4 yield by 15%; beef steers experienced reductions in CH_4 production by 12% and CH_4 yield by 9% [62]. Van Zijderveld (2011) also found that the inhibition of methanogenesis persisted after a 4-week adaptation period and throughout a 24-d test interval [63].

The inhibition of CH_4 formation is largely dependent on the reduction of nitrate to nitrite [64,65]. However, a key consideration when feeding nitrate is the fact that they are largely regarded undesirable as a component of ruminant diets. This is due to the risk of nitrite toxicity which is manifested by induced methemoglobinemia. A number of researchers worked to determine if this toxicity avoidable. To that end, research revealed that reduction of CH_4 without leading to toxicosis in cattle can be accomplished when animals were acclimated to nitrates gradually [66,67], or when ad libitum access to un-treated feed was offered in conjunction with encapsulated nitrate supplementation [68].

Existing naturally in variable concentrations within feeds, nitrates are readily accessible. Nitrates are also considered as possible alternative source of non-protein nitrogen (**NPN**) capable of replacing urea in a ration [69]. Through modeling of scenarios in which nitrates were incorporated into ruminant diets as a source of NPN at a rate of 16.7 g/kg DM, Feng and Kebreab (2020) estimated that California could encounter a total GHG reduction of approximately 4.96% if high protein meals were replaced with nitrate (without adjusting DMI) [70]. In addition to the direct impact on enteric GHG emissions, they additionally found that the California dairy production system could experience a total GHG reduction of 3.82% if all dairy cows consumed nitrate while lactating [70].

Few experiments have addressed how dietary nitrate may impact enteric or ruminal N_2O emissions. An *in vitro* analysis by Parker et al. (2018) found a strong up-ward curvilinear correlation between nitrate inclusion level and N_2O production in ruminal liquor extracted from cannulated beef cattle [24]. Taken together with results from their *in vivo* experiments, their findings suggest that enteric N_2O production may be more correlated with the inclusion of dietary nitrate than with the total N consumed from feed [24]. There is additionally little consensus in

literature regarding the effects on nitrate supplementation on other ruminal fermentation measurements. For instance, while Wang et al. (2018), El-Zaiat et al. (2014), and Guyader et al. (2015) saw reduced ruminal NH_3 with nitrate supplementation [71–73], Olijhoek et al. (2015), Hulshof et al. (2012), and Sharifi et al. (2022) saw increased ruminal NH_3 [66,74,75]. Likewise, while most experiments saw no supplementation impact on total VFA concentrations [42,66,76,77], Guyader et al. (2015) and Patra and Yu (2015) saw reduced and increased total VFA concentrations, respectively [73,78]. These differences may be associated with variations in lactation stage and diet in the two aforementioned experiments.

The impact of nitrate supplementation on milk yield is also unclear. Sharifi et al. (2022) and Veneman et al. (2015) both saw improved milk yield with nitrate supplementation, whereas Klop et al. (2016) and van Zijderveld et al. (2010) saw no effect of treatment [60,75,79,80]. Although it is expected that beef cattle fed nitrates would experience decreased or no changes in ADG [81,82], some experiments have reported increases [83,84]. Beef cattle are also characterized for performance based on their gain-to-feed ratio (also referred to as feed conversion ratio), which most experiments find to be unaffected by supplementation [81] though some have seen improvements with supplementation [82].

1.4.4. Seaweed

Seaweeds, also known as macroalgae, are categorized under three primary classifications: chlorophyta (green), phaeophyta (brown), and rhodophyta (red) seaweeds. Many seaweed species within each of the classifications have been investigated for their anti-methanogenic potential [85,86]. Among the classes of seaweeds investigated, red seaweeds tend to exhibit the greatest anti-methanogenic potential. Experiments investigating the anti-methanogenic action of seaweeds and possible mode of action have suggested that the biological activity of seaweeds are likely due

to the presence of halogenated compounds such as chloromethane and bromoform and to their secondary metabolite compositions [87]. The halogenated compounds present in seaweeds are capable of reacting with vitamin B12 and inhibiting cobamide-dependent transferase at the final step in the methanogenesis pathway [88].

Recently, two species of red seaweed (*Asparagopsis armata* and *Asparagopsis taxiformis*) have received interest both in research and commercialization. An *in vitro* analysis found that increasing dosage level of *A. taxiformis* was associated with improved CH_4 mitigation potential [89]. Research *in vivo* revealed similar findings; for instance, experiments in dairy cattle demonstrated decreases in CH_4 production by 34.4%, yield by 29.4%, and intensity by 34.5% when cows were with *A. taxiformis* at 0.50% of DM [90]. Likewise, experiments in which dairy cows were supplemented with *A. armata* at 0.5 and 1.0% of OM have also demonstrated reductions of CH_4 yield (g/kg DMI) by 20.5 and 42.6%, respectively [91].

With diet composition differing at various stages in an animal's life cycle, it becomes important to assess possible interrelation or interactions between seaweed inclusion and F:C in the diet. Through an *in vitro* analysis using rumen fluid from Brahman steers, Kinley et al. (2021) noted increasing CH_4 production as F:C decreased [89]. However, studies which applied a similar experimental approach to *in vivo* systems noted the opposite effect. Though CH_4 emissions decreased in all steers supplemented with *A. taxiformis* at 0.50% of OM regardless of the F:C, Roque et al. (2021) observed that steers fed a low-forage diet had the greatest reduction in CH_4 production (g/d), yield (g/kg DMI), and intensity (g/kg ADG) [92]. The increase in CH_4 noted under *in vitro* conditions, is suspected to be associated with the high-levels of concentrate; the high levels of carbohydrates can be rapidly fermented and result in a surge of CH_4 precursors in the static system. Dynamic attributes of the *in vivo* rumen systems such as digestibility and rates of

passage pose major challenges and limitations when trying to replicate or compare *in vitro* versus *in vivo* findings.

To date, most experiments investigating the impact of seaweed supplementation have seen marginal improvements in animal performance. A meta-analysis analyzing cattle whose feed was augmented with *A. taxiformis* found that supplementation had no overall effect on ADG in beef cattle [93]. On the other hand, Kinley et al. (2020) saw an improvement in ADG in beef steers supplemented with *A. taxiformis* at 0.10% of OM over a 60-d treatment period [94]. A similar trend of marginal improvements was noted in terms of productive performance of dairy cows. Lean et al. (2021) conducted a meta-analysis of lactating dairy cows supplemented with seaweed (*Ascophyllum nodosum* or *A. taxiformis*) and found that seaweed-treated dairy cows had greater milk yield based on weighted mean differences, though they also reported that treatment related differences in milk yield were not significant on a standardized mean differences basis [93]. As described by the authors, weighted mean differences compares treatment and reference estimates which allows for inferences of treatment effects to be made using synonymous units, whereas standardized mean differences compare data based on the effect size of a dataset. While most experiments reveal no changes in total VFA concentrations with inclusion of *A. taxiformis*, acetate concentration and acetate-to-propionate ratio are generally found to decrease whereas both propionate and butyrate concentrations increase [89,95].

Bromoform has been classified as a probable human carcinogen (Group B2) which has the potential to negatively affect the kidneys, liver, and central nervous system as indicated by animal studies [96]. One of the primary concerns with feeding of seaweeds is therefore the metabolism and the fate of the halogenated compounds, in particular bromoform, within the animal and its productive outputs (i.e., milk, meat) and their excreta. Experimental findings suggest that

bromoform was detectable in the milk and urine in lactating dairy cows, with greatest concentrations being present in urine [97]. In an *in vivo* experiment with lactating dairy cows, Stefenoni et al. (2021) saw no difference in milk bromoform concentrations between *A. taxiformis* and control cows, though both iodine and bromide concentrations were significantly increased in milk of seaweed-fed cows [90]. Surprisingly, Roque et al. (2019) noted the presence of bromoform in treated and control cows alike [88]. Likewise, Roque et al. (2019) did not report on the impacts of seaweed supplementation on either iodine or bromide concentrations in milk [91]. Strikingly, experiments in beef cattle have not detected bromoform residues in feces collected during chamber measurements, or in samples of meat, organs, or the fat collected after the animals were harvested [94].

In addition to consideration as a candidate CH_4 mitigation strategy, seaweeds have demonstrated antimicrobial and antioxidant characteristics in ruminant models through *in vitro* and *in vivo* investigations, respectively [98,99]. Seaweeds are comprised of highly variable nutrient profiles based on attributes such as the species, geographical habitat, time of collection, and water and environmental conditions when harvested [100]. Seaweed species have exhibited high non-starch polysaccharides, NPN, and ash content [101]; though ruminants may be able to digest intact seaweeds based on the former two characteristics, the high ash content must be considered due to the potential for the seaweed to contain heavy metals. For example, lactating cows fed high seaweed diets (1.5% of DM) were found to have statistically-relevant elevated levels of arsenic in their milk which was attributed to the increased arsenic in the seaweed-supplemented diets [102].

1.4.5. Plant Secondary Metabolites

Plant Secondary Metabolites (**PSM**) encompass a category of over 200,000 phytochemicals that are present in plants [103]. Generally considered as nonessential to the

biochemical processes of plants such as for reproduction, growth and development, PSMs offer the plant protective capabilities. They function to protect the plant in their interactions with environmental factors and stressors including ultraviolet radiation, drought, pathogens, and herbivory [104]. Plant secondary metabolites occupy a wide spectrum of biologically active chemical compounds, with conformations that elicit particular actions on a wide variety of cellular and molecular targets [105], including altering the metabolic activity when ingested by herbivores [106]. Inhibition of CH_4 emissions can be attributed to modes of actions such as: direct inhibition of methanogenesis, anti-protozoal activities, and more broad anti-microbial activity. In the case of protozoa, many methanogens have a symbiotic relationship with ruminal protozoa, which is due to their need for H_2 that protozoa produce via hydrogenosomes [107].

1.4.5.1. Tannins

Polyphenols are among the most broadly distributed PSMs. Tannins are a class of polyphenolic compounds with high molecular weight, which contain functional groups such as hydroxyl and carboxyl groups enabling them to complex with macromolecules under particular environmental conditions, possessing a strong affinity for proteins [108]. Commonly found in high proportions in unripened fruit, leaves, seeds, tree bark, and wood, it is hypothesized that tannins serve to protect the plant from microbial infection and herbivory by animals and insects [109]. Once extracted from the plant, tannins have demonstrated negative traits such as reduced nutrient digestibility, decrease in palatability, and possible decreases in nutrient intake [110,111]. Tannins have also demonstrated positive traits such as antimicrobial, antioxidant, anthelmintic, and radical scavenging effects [112–115]. Various modes of antimicrobial action have been hypothesized, though most suppositions include the following to some capacity: (1) the destabilization of

microbial cell wall and membranes, (2) metal ion depletion, or (3) affecting the enzymes produced by the microorganisms in question [116].

Tannins are classically categorized as either condensed or hydrolysable tannins based on their ability to be broken down via hydrolysis in the presence of the tannase enzymes or hot water, and the ratios of phenolic monomers they contain [109,117]. Condensed tannins, which are high molecular weight compounds, are created through the phenylpropanoid pathway [117,118]. Hydrolysable tannins on the other hand are comprised of polyesters linked to a glucose core. This form of tannin can be subcategorized as either gallotannins or ellagitannins, with the former being formed of gallic and the latter of ellagic acids [117]. Due to their lower risk of toxicity, the anti-methanogenic effects of condensed tannins have been studied to a greater extent than hydrolysable tannins [119].

Experiments supplementing ruminant rations with tannins have revealed varying efficiencies, which were largely dependent on the source of tannin as well as the level of inclusion within the diet. Many *in vitro* tannin supplementation experiments have successfully reduced CH_4 production, regardless of whether the experiment involved either batch or continuous fermentation [120–128]. For instance, Wischer et al. (2013) investigated 10 tannin-rich extracts from various plant sources and saw reductions in CH_4 production (mL/day) in syringes dosed with chestnut, mimosa, myrabolan, quebracho, valonea, oak, or grape seed tannin extract (at both 6 and 12 mg of inclusion). They additionally saw reductions in CH_4 expressed per unit of degradable OM (**dOM**; mL/g dOM) when syringes were treated with chestnut, grape seed, sumach, or valonea tannin extracts [129]. Screening two sources of condensed (acacia and quebracho) and hydrolysable tannins (chestnut and valonea) at varying inclusion levels, Hassanat and Benchaar (2013) found that acacia and both of the hydrolysable tannins effectively reduced CH_4 production (mL) with as

low as 50 g/kg dosed, whereas quebracho tannin required a dose of 100 g/kg or greater to be effective [130]. The researchers did not report findings for CH_4 production expressed per unit of OM [130].

Far fewer experiments have demonstrated their CH_4 mitigation potential *in vivo* [122,131–135] than *in vitro*. *In vivo* inclusion levels within the existing body of literature ranges considerably based on the intended purpose for including tannins in the diet. For instance, tannin treatment on the basis of intended CH_4 mitigation was considerably more variable with literature reporting levels as low as 0.07% in beef and as high as 2.7% of DM in beef and dairy [136,137]. Jayanegara et al. (2012) estimated that for every 1% increase in tannin inclusion within the diet, CH_4 yield consequentially decreased by 0.8 g [137].

Tannins, in general, particularly those of the class *Calliandra*, demonstrated a rather strong inhibitory effect on fibrolytic bacteria including *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefacien*s [138] suggesting that the inhibitory effects on fiber degradation may be an inherent trait of tannin utilization. Although higher rates of inclusion have demonstrated the largest CH_4 mitigation potential, increasing inclusion rates may negatively impact DM digestibility. Supplementing sheep with two commercially available tannin extracts (Silvafeed®ByProX and Silvafeed®Q) at a rate of 3% of DM, Menci et al. (2021) noted reduced CH_4 production and increased CH_4 yield (g/g of degraded DM) [139]. This resulted from the decreasing *in vitro* DM degradability (**IVDMD**) as the tannin dosage-levels increased [139]. Similar trends are noted in Wang et al. (2018) – though feed intake was not affected, they saw decreased apparent digestibility of fiber and OM with increasing dietary tannin levels [140]. A balance must therefore be struck in order to reduce CH_4 emissions while still allowing for optimal productive performance of ruminant livestock. A 2020 meta-analysis which focused on milk

productive performance (milk yield and composition) and N utilization noted that on average, dairy cows across 47 studies were provided with dietary tannins at an average of 183 g/d which translated to a relative dose of 0.95% of DM [141]. This level of inclusion was within the range of inclusion levels reported in the literature, 0.7-4.0% of DM, suggesting that an optimal inclusion level for tannins can be determined as a feed strategy for CH_4 mitigation.

1.4.5.2. Essential Oils

Essential oils (**EOs**) are naturally occurring plant volatile compounds comprised predominately of phenylpropenes and terpenes, involved in the scent and flavor profiles of plants. Similar to other PSMs, EOs are present in varying proportions and compositions between and within plant species [104,142,143]. Once extracted from plants through methods such as steam distillation or organic-solvents [144,145] EOs were found to have an antimicrobial nature against a wide range of microorganisms [146]. Though the specific mode of action of EOs is unclear, it is widely accepted that their lipophilic nature may lead to disruptions of microorganism cellular membranes such as through reducing microbial surface charge or increasing outer-membrane permeability by accumulating within a microorganism's lipid bilayer [147].

Literature suggests that some EOs exhibit modulatory effects on rumen fermentation, which includes reduced methanogenesis [148,149]. Garcia et al. (2020) attributed the reduction of rumen CH_4 by EOs to their chemical compositions, suggesting that mitigation potential could also be influenced by the relative proportion of oxygenated compounds present within each EO [150]. A wide array of EOs from different sources have been studied, with many of these EOs tested using *in vitro* approaches to explore their CH_4 reduction potentials. Screening 18 EOs, Pirondini et al. (2015) found that lemon grass and thymol exhibited the greatest CH_4 reduction potential,

though guaicol, limonen, thyme oil, and vanillin also effectively reduced CH_4 production [151]. Other *in vitro* literature revealed potential for other EOs including anise, citral, clove, cinnamon, eugenol, eucalyptus, garlic, limonene, oregano, thyme, and vanillin oils among others [78,152–156].

It remains challenging to take findings from experiments using *in vitro* systems and successfully replicate the findings in *in vivo* settings. This stems from many factors such as the difficulty in estimating proper *in vivo* dosage-levels from *in vitro* results, and collecting and maintaining proper representation of the ruminal microorganisms including those that are feed-adherent. Though fewer experiments were conducted *in vivo*, a small subset of EOs demonstrated CH_4 reduction potential when supplemented in diets fed to ruminants. These include EO extracts from oregano, garlic, and citrus, as well as EO blends such as a commercial blend comprised of coriander seed oil, eugenol, and geranyl acetate (Agolin) [157–159]. Zhang et al. (2021) saw significant reductions in CH_4 production on a L/d basis in sheep supplemented with 20 g oregano oil/kg DM [122]. These findings were further supported by Wang et al. (2009) who found reductions in CH_4 production on a g/d basis and on a metabolic body weight basis (g/d/kg $BW^{0.75}$) in sheep fed a 75:25 F:C diet were supplemented with a commercial EO of oregano extract (Ropadiar) at 250 mg/d [158]. However, the impacts of oregano extracts on CH_4 appear to be limited by factors such as species, dietary inclusion level, and the ingredient composition of the diet. For instance, Benchaar saw no differences in either CH_4 production (g/d) or yield (g/kg DMI) when feeding lactating dairy cows a 60:40 F:C diet supplemented with 50 mg/kg of oregano oil [160]. Likewise, Olijhoek et al. (2019) saw no differences in CH_4 emissions when supplementing lactating Holstein dairy cows with various oregano inclusion levels ranging from 7 to 53 g/kg DM (18-53 and 7-21 g/kg DM; for *Origanum vulgare* ssp. *vulgare* and ssp. *hirtum*, respectively) [161].

In order to investigate the Agolin's efficacy in reducing CH_4 emissions and its impact on production in dairy cows, Belanche et al. (2020) conducted a meta-analysis which incorporated 19 experimental studies [162]. They found CH_4 production and intensity (g/kg FPCM) to decrease within the first 4 weeks of treatment application, whereas CH_4 yield (g/kg DMI) significantly decreased with long-term treatment inclusion ($\geq$ 28 days) [162].

Mootral, a patent-pending commercial EO blend comprised of garlic and citric extracts, has also demonstrated CH_4 reduction potential primarily through *in vitro* experiments [163–165]. For instance, Ahmed et al. (2021) tested Mootral in an *in vitro* fluid batch culture system added to rumen fluid from donor wethers fed a 50:50 F:C diet; they noted that CH_4 production decreased by 22% and 54%, when Mootral was included at a rate of 10% and 20% of substrate, respectively [165]. *In vivo* research findings are limited in both dairy and beef cattle. In a commercial *in vivo* experiment, Vrancken et al. (2019) compared the differing effect of Mootral supplementation between lactating Holstein and Jersey dairy cows reported CH_4 emission (ppm) reductions by 20.7% and 38.3% for each of the breeds, respectively. It is important to note that the same group of cows that received the Mootral treatment served as the control in this experiment [166]. Each of the two pens (one Holstein and one Jersey pen) used in the experiment had Mootral pellets added directly to a group-fed TMR and enteric CH_4 sampling was conducted from a subset of cows in each pen using a handheld a laser CH_4 detector after 12 weeks on treatment. Methane emissions were once again sampled from a subset of cows within the same two pens 4 weeks after Mootral was removed from their diets [166]. Bitsie et al. (2022) reported no impacts on CH_4 production (g/d), yield (g/kg DMI), or intensity (g/kg $BW^{0.75}$) when feedlot steers were supplemented with Mootral a rate of 0.25% of DM [167].

In addition to the potential positive benefits on the environment, EOs are lucrative due to improved productive performance of animals. Belanche et al. (2020) found that both milk yield and fat-and-protein-corrected milk increased with long-term Agolin inclusion [162]. Other EO compounds and blends demonstrated similar findings. For instance, a blend of clove, oregano, and juniper EOs included in equal proportions demonstrated positive impacts on milk yield and concentration of milk protein and lactose, and serum total protein [168]. Based on their suspected similarities in mode of action, EOs have also been suggested in many cases as an alternative to feeding monensin to ruminant livestock. Torres et al. (2021) conducted a meta-analysis focused on the replacement of monensin by EOs in the diet of beef cattle and found that EOs increased DMI, though CH_4 yield (g/kg DMI) and feed efficiency was unchanged [169]. Additionally, ruminal concentrations of propionate were reduced with EOs supplementation, which has been shown to have impacts on feed efficiency [169,170].

1.4.5.3. Saponins

Saponins are defined as glycosides that have one or more carbohydrate branches connected to a hydrophobic steroid or a triterpenoid sapogenin of high molecular weight. Their biological activity is highly driven by the form and number of sugars existing within their chemical structure, but generally exhibit detergent-like characteristics. Similar to tannins, saponins have an affinity for microorganisms, with a specific inhibitory effect on protozoa, fungi, and on select bacteria [171,172]. It is hypothesized that they act against microorganisms by disrupting the microbial cellular membrane, likely through interactions with the sterols within the cell membrane [173–175]. Literature additionally suggests that saponins may exhibit direct inhibitory actions against methanogens [176,177]. Tea, yucca, and quillaia are among the most widely explored saponins for

their anti-methanogenic impacts. Similar to most other PSMs explored, the largest body of literature involving saponins is in *in vitro* experimentation.

Canul-Solis et al. (2019) conducted *in vitro* batch culture rumen fluid experiments comparing various concentrations and sources of saponins including *Yucca schidigera*, *Gliricidia sepium* foliage, and *Enterolobium cyclocarpum* fruit. Compared with an untreated control, *Yucca schidigera* dosed at 3.5 mg/g DM reduced CH_4 production when expressed on both a fermentable DM (**FDM**; ml CH_4/g FDM) and organic DM basis (**ODM**; ml CH_4/g ODM) [178]. Both IVDMD and *in vitro* OM digestibility (**IVOMD**) were increased with saponin inclusion at 3.5 and 7.0 mg/g DM, suggesting that saponins did not exhibit a negative impact on the fermentability of the feed substrate at these specific saponin inclusion levels. Jayanegara et al. (2014) also investigated the efficacy of the aforementioned saponin sources; they completed a meta-analysis of 23 *in vitro* experiments where the saponins were tested at levels ranging from 0.1 to 480.0 mg/g of substrate. Their results showed that saponins were generally effective at decreasing CH_4 production per unit of substrate, and that yucca saponin exhibited the greatest margin of success and CH_4 reduction potential without negatively impacting IVOMD [179].

The relative effectiveness of reducing CH_4 emissions when supplementing ruminants with saponins *in vivo* remains unclear. Tea saponin reduced methane through *in vitro* experiments, but when used in *in vivo* research with lactating dairy cows demonstrated no effect on CH_4 production, and increases in CH_4 yield and intensity [180]. Increasing the inclusion level of yucca saponin likewise did not alter CH_4 production (g/d), intensity (g/kg $BW^{0.75}$) or yield (g/kg DMI) in sheep fed a forage-based diet [181]. On the other hand, *in vivo* supplementation with tea saponin at a rate of 5 g/kg DM resulted in an 8.5% CH_4 yield (l/kg of DMI) decrease in sheep [182].

Saponins have however demonstrated the ability to serve as gut modifiers within the rumen, leading to reduced serum urea and NH_3, as well the potential to positively impact animal growth rates, feed efficiency, and animal health [172]. In an experiment using a standardized *in situ* (*in sacco*) technique, McMurphy et al. (2014) supplemented steers with a commercially available saponin additive (MicroAid®) at 1.1 and 2.2 g/kg of DM. They report a decrease in particulate rate of passage (calculated from acid detergent insoluble ash concentrations) which further resulted in increased digestibility of NDF and DM in the rumen, suggesting favorable impacts on ruminal fermentation [183].

1.5. Manure-Related Environmental Impacts

Methane is the primary GHG produced during the manure storage stage largely due to anaerobic fermentation of manure OM by microorganisms [184]. The production of CH_4 is substantially greater in manure stored anaerobically rather than aerobically, enabling it to hold tremendous potential energy that can be captured and used similarly to natural gas. Contrary to popular perception, manure from bovines constitutes a minor impact when compared with other livestock species. For instance, a 2004 analysis by Møller et al. (2004) compared manure CH_4 between different livestock systems and found that CH_4 yield is 9.8-12.2% higher in hog production than it is in dairy cattle [185]. A 2016 legislation in California has mandated livestock sector to reduce GHG emissions by the year 2030 to 40% below 2013 estimates. In the six years since its implementation, it has been estimated that GHG emissions from manure management have decreased by 2.2 million metric tons per year due to on-farm shifts from conventional to alternative manure management practices [186].

Considering the global warming potential (**GWP**) of N_2O and the amount of N lost in the form of NH_3 and N_2O through animal housing, proper management, and reduction strategies in this phase of manure management must be carefully considered. As touched upon briefly in previous sections, agricultural-N follows a biogenic carbon cycle; the primary point of N_2O emissions throughout the cycle are during the formation of N_2O through the nitrification and denitrification processes. These processes take place at various steps within the manure management systems, including at the point in which manures are land applied [187,188].

Gaseous NH_3 is created and released through the hydrolysis of urea to carbamic acid by the enzyme urease [189]. In the case of ruminants, the largest point of volatilization is during the reaction between urine-urea and fecal urease [190]. From a manure management perspective, NH_3 volatilization is one of the primary causes of manure-N losses; it is estimated that 19% of excreted-N is lost in the form of gaseous NH_3 emissions during animal housing [191]. The loss of N from manure in the form of NH_3 results in less nutrient to soils in land applied manure for purposes of fertilization, and can indirectly lead to the formation of N_2O; reductions of NH_3 emissions is therefore vastly important. Gaseous NH_3 also poses problems to comfort and health – in addition to being a significant odor nuisance, NH_3 emissions may lead to PM formation which could be harmful to human and animal health [192].

1.6. Manure Management Practices

By definition, manure management includes all activities, components used, and decisions made in order to properly handle, store, and discard of animal excreta. Manure management practices also incorporate efforts made to properly manage and recycle the nutrients through sustainable means [193]. Adapted from Chadwick et al. (2011), manure management is separated into a number of logical categories: animal management (i.e., feeding, efficiencies, animal health),

animal housing, manure storage and processing, and manure application [188]. Animal waste related emissions therefore encompasses the any emissions from the moment in which excreta exit the animal through to when the waste has reached its ultimate destination, such as its land application. As such, many strategies have been explored to reduce GHG and NH_3 from manure both in their gaseous and dissolved forms.

1.6.1. Animal Feeding and Management

Dietary changes have a profound impact on the composition of animal digestate and excreta, and as such continue to have impacts further along in the manure management chain. For instance, N dynamics are heavily influenced by the level and form of dietary-N fed to ruminants. Reductions in crude protein (**CP**) fed to an animal impact manure-related NH_3 emissions – NH_3 emissions are reduced as a result of decreased urinary-N excretion, which may result in downstream reduction of N_2O emissions with little positive or negative impact on CH_4 emissions [194]. The type of CP feed will have an influence on factors such as microbial protein synthesis (**MPS**) and feed degradability which will influence the flow of N in the animal and manure, as well as gaseous emissions. For instance, diets low in rumen degradable protein (**RDP**) have also led to reduced NH_3 emissions. While some experiments noted no impacts when feeding low RDP diets [195,196], others saw decreased on MPS (mg/100 mL) and nutrient digestibility (%; DM disappearance and OM disappearance) when the proportion of RDP:RUP (rumen undegradable protein) was decreased [197]. The aforementioned and discussed feed additives have also demonstrated downstream impacts on manure. The following highlights some of the known literature on excreta and manure-management related impacts:

3-nitrooxypropanol - There is little known about the direct impacts of 3NOP on animal manure; there are a few published studies on the effects of feeding 3NOP to livestock and the subsequent storage or treatment of animal manure. Research conducted by Nkemka et al. (2019) began to elucidate these downstream impacts of 3NOP, looking to 3NOP's ability to influence manure stored and processed using an anaerobic digester (**AD**) approach. They found that 3NOP applied directly to manure reduced CH_4 yield (mL/g volatile solids), while CH_4 yield (mL/g volatile solids) from manure of 3NOP-fed beef cattle did not differ from that of untreated animals [198]. Weber et al. (2021) took an alternative approach, looking at the emissions impact of manure from 3NOP-fed animals when applied to various soil types. Through their work they found that N_2O emissions (μg N_2O-N/kg/h) were highest in soils amended with manure of 3NOP-fed animals, although CH_4 emissions (μg CH_4-C/kg/h) were not impacted [199].

Nitrate – In experiments comparing nitrate- to urea-supplementation as a N source provided to beef heifers, Lee et al. (2015) noted no effect on total fecal-N output (g/d) and a decrease in total urinary-N output (g/d) [200]. They additionally saw a decrease in urine urea-N (g/d) and an increase in NO_3-N (g/d) in both the feces and urine fractions [200]; taken together, we would expect to see reduced NH_3 emissions from their manure if the fractions had been recombined into a manure slurry. This was replicated in a subsequent experiment by the authors, similarly finding increasing NO_3-N levels in urine, feces, and reconstituted manure slurries with increasing dietary inclusion level [201]. They surprisingly did not see differences in manure NH_3, N_2O, and CO_2 emission rates (expressed as mg/m^2/h and mg/head/h) and cumulative emissions (g/m^2 or g/head) when comparing nitrate to an unsupplemented-control, though CH_4 manure emission rates and cumulative emissions (same units as above) were significantly reduced.

However, NH_3 emissions ($mg/m^2/h$) observed on an hourly-emissions basis had a tendency to be lower in manure of nitrate-supplemented heifers from zero through to 12-h incubation time as expected [201].

Seaweeds – Experiments have addressed animal-manure related effects of dietary seaweed supplementation for a variety of experimental objectives and perspectives. A subset of the literature focuses its attention on optimizing the antimicrobial properties of seaweeds. To this end, experiments have investigated the use seaweed as a tool to modulate shedding of pathogenic bacterial strains such as *E. coli* O157:H7 in manure through challenge experiments and through live animal work with feedlot steers [202,203]. Other experiments assess excreta and manures for purposes of answering research questions related to nutrient metabolism such as protein degradation. Antaya et al. (2019) supplemented Jersey cows with 113 g/d of the brown seaweed *A. nodosum* and saw shifts in N-excretion from urine to feces and a consequential 12.6% increase in fecal-N in seaweed supplemented cows [204]. In experiments with lactating Sahiwal cows, Singh et al. (2016) saw no differences in fecal and urine calcium (**Ca**), phosphorous (**P**), iron (**Fe**), zinc (**Zn**), and manganese (**Mn**) when comparing between control and brown seaweed (*Sargassum wightii*) supplemented cows [205]. They additionally reported no treatment differences in fecal-, urinary-, or milk-N (g/d) in a later publication within the same group of cattle as used and mentioned previously [206]. Though the researchers did not report on the anti-methanogenic effects of this strain of seaweed, they saw no differences in DMI between treatment groups, increased milk yield (kg/d), and increased 4% fat corrected milk yield (kg/d) in seaweed-supplemented cows [206].

To our knowledge, there is no published literature that addresses the direct environmental impacts of excreta from animals supplemented with seaweed for the purpose of reducing CH_4 emissions. Partial findings in a conference abstract by Ramin et al. (2022) suggest that CH_4 production was not reduced in feces of dairy cows directly-fed *A. taxiformis*, though CH_4 production was reduced by as much as 50% when *A. taxiformis* was applied directly to feces [207].

Tannins **–** Tannins have a demonstrated record and ability to alter N-utilization within the rumen. Due to this effect, inclusion of dietary tannins generally leads to a decrease in ruminal NH_3 and blood urea-N, and milk urea-N concentrations as well as a consequential shift in N-excretion from urinary-N to fecal-N, measured both as yield and fecal-N:urinary-N proportions [141,208,209]. In addition, meta-analysis results showed decreases in protein digestibility and DM digestibility in beef cattle [210], and N-, DM and OM digestibility to decrease in dairy cattle [141]. The increase in fecal-N levels and decreased urinary-N, noted in these experiments therefore could have been associated with the decrease in protein digestibility.

When crossbred steers were fed a quebracho tannin supplemented diet, urinary-N concentration decreased and fecal-N concentration increased, which led to a 1.4-fold decrease in manure N_2O emissions (µg/g DM) [211]. By contrast, both Norris et al. (2020) and Duval et al. (2016) saw no reduction in N_2O emissions from manure when supplementing steers with quebracho tannin and lactating dairy cows with a quebracho and chestnut tannin blend, respectively [212,213]. Accompanying the reductions in ruminal NH_3 concentration expected with tannin supplementation, Duval et al. (2016) also saw decreased NH_3 emissions at day 45 on treatment [213]. Reduced gaseous NH_3 emissions were also described in feedlot beef cattle which were supplemented with *acacia mearnsii* tannins at a rate of 2.5% of DM [214]. In addition to

reducing NH_3 emissions when passed through the animal's digestive tract, tannins also demonstrated pen-level NH_3 reduction potential when applied directly to manure slurries [215].

Essential oils – Research publications addressing the impact of dietary EO supplementation on manure-related emissions are scarce. We only found one published experiment addressing emissions from manure; the researchers reported no treatment related impacts on manure CH_4 and NH_3 emissions in lactating dairy cows supplemented with a commercial blend of eugenol and cinnamaldehyde EO (Xtract 6965) [216]. There is, however, a sound body of literature addressing N-partitioning in cattle supplemented with EO. Benchaar et al. (2015) saw no differences between control- and eugenol-supplemented lactating dairy cow in fecal-, urinary-, and milk-N (g/d) [217]. Likewise, experiments supplementing with Xtract 6965 [216] and Crina (a commercial blend of thymol, guaiacol, eugenol, vanillin, salicyaldehyde, and limonene) [218] demonstrated no treatment-related differences in urinary- and fecal-excretion parameters or milk protein-N. Though they did not report on the fluctuations in fecal-N, Santos et al. (2010) similarly saw no difference in urinary-N (mg/L) concentration between cows supplemented with control or an EO blend at 1 g/d. They further did not see differences between treatment groups in urinary NH_3-N (mg/L), urea-N (mg/L), or in urea-N:total-N concentrations, suggesting that there would be no impacts on fecal-N concentrations [219].

Saponins - To date, only one experiment has been found addressing the effect of saponin supplementation on manure-related emissions in ruminant livestock. Yucca extracts demonstrated the potential for reducing N_2O emissions (ppm/mL) though it did not impact CH_4 or CO_2 emissions [220]. The little that is known regarding the manure-related impacts of dietary supplementation

with saponin is limited to digestibility-related N impacts. Experiments that supplemented dairy cows with saponins demonstrated no treatment-related differences in fecal-N and urinary-N yield (g/d), regardless of the saponin source [180]. Similar results have been demonstrated in sheep [221,222] and in beef cattle [223].

1.6.2. Animal Housing

Animal housing and manure handling have an influence on GHG and NH_3 emissions. Research suggests that gaseous emission abatement principals can be incorporated into housing systems. For instance, Pereira et al. (2010) reported decreased GHG and NH_3 emissions in animal housing systems that incorporated slatted floors and under floor collecting pits for manure in place of solid floors [224]. Other research has demonstrated the impact of animal housing with respect to N_2O emissions, such as Thorman et al. (2002) who observed greater N_2O emissions in deep litter animal housing systems (i.e., straw-based systems) than in manure slurry-based systems [225]. In a generalized sense, gaseous emission abatement principals incorporate the following: reduction of the manure surface, complete and rapid removal of liquid manure from pits to external storage, cooling of the manure surface, and altering the manure's physiochemical properties such as the pH [224,226]**.**

Infrastructure limitations may influence the manure management strategies that can be implemented at particular operations leading to fluctuations in certain emissions. For instance, the method in which animal-manure is removed from a pen within various housing types has a large impact on the emissions produced. Ross et al. (2021) found that removal of animal waste via scraping resulted in greater NH_3 emissions than removal via flushing [227]. Regardless of animal housing type, the amount of time manure remains in the housing area has a profound impact on

the levels of GHG and NH_3 emitted [228,229]. Hristov et al. (2015) found that NH_3 and CH_4 emissions were greater in barns where manure was kept in the pens for longer periods of time, such as in gravity-flow or gutter-scraped pens versus those with flush lanes [230].

1.6.3. Manure Storage and Processing

Factors such as manure source (i.e., species), nutrient load, intended downstream use, and economic feasibility significantly influence decisions for which methods to implement on farm [231,232]. Assessing CH_4 emissions from varying manure storage methods, Chianese et al. (2009) found that covered manure slurries produced 5.4 kg $CH_4/m^3/yr$, whereas uncovered manure slurries produced 6.5 kg $CH_4/m^3/yr$. Stacked manure by contrast produced substantially less, approximately producing 2.3 kg $CH_4/m^3/yr$ [233]. Nitrous oxide emissions increased in instances where nitrification of ammonium to nitrite was optimized such as when manure was handled in aerobic conditions, while denitrification of nitrite to nitrous and nitric oxide was optimized in anaerobic conditions [234].

In uncovered liquid manure storage – as in holding ponds or manure lagoons – mechanical or intermittent aeration has demonstrated potential for reducing CH_4 emissions. Though this is the case, increases in N_2O emissions may result in situations where the aeration leads to an aerobic environment [235,236]. Acidification of manure may also be an effective strategy for reducing CH_4 and NH_3 emissions [237,238]. Various applicants to manure and wastewater have also been considered, with some showing success. For instance, Naujokienė et al. (2021) directly treated animal waste with a biological applicant comprised of bacteria (*Azospirillium* sp., *F. aurantia* and *B. megaterium*), seaweed, plant hormones, amino acids, and vitamins, which resulted in significant reductions in NH_3 emissions. While NH_3 emissions were reduced in every scenario, they reported

that the degree to which NH_3 emissions were impacted was greatly influenced by factors such as manure storage time and the velocity of air that passed over the manure surface [239]. Such strategies may be best suited for aerobic environments, as in within manure settling basins or in uncovered lagoons for example.

Anaerobic digestion has received considerable attention in the past few decades, both in research and implementation. In the case of covered lagoons, factors such as the cover's degradability, permeability, porosity, management and upkeep, and thickness will tremendously impact the GHG reduction potential [240]. Covers that are not porous or permeable to air allow for the gas that is generated to be captured and subsequently used as biogas. The biogas produced and captured within an AD can then be burned as a source of renewable energy, with CH_4 comprising 55% to 75% of the biogas generated [241]. In an effort to optimize biofuel generation, manure co-digested with other biomass has also been investigated for its ability to increase CH_4 production within AD, with tremendous success. For instance, the co-digestion of cattle manure with seaweeds such as *Laminaria digitata* has demonstrated the potential to increase the amount of CH4 produced [242] while still allowing the digester to operate efficiently for over 20 weeks [243]. Other experiments similarly saw improved CH_4 generation when co-digesting cattle manure with vegetable biomass such as cabbage and potatoes [244].

1.6.4. Manure Post-Processing Treatment and Application

Though AD may help reduce immediate environmental impacts of livestock manure (influent), the further downstream AD digestate (hereinafter referred to as effluent) must also be considered. While carbon is removed, effluents have been shown to maintain high levels of N and P. Due to the ability of N and P to contribute to eutrophication of waterways, concentrations of

nitrogenous compound are heavily regulated from an environmental safety perspective. In an effort to minimize the leaching or release of these nutrients into the environment, researchers are exploring methods of N recovery, which allow for the recycling of this N in the form of nitrogenous fertilizers.

Nitrogen is generally present in AD effluent in the form of NH_3 or ammonium ($\mathbf{NH_4^+}$), which can be present in high concentrations. Effluent is therefore subjected to NH_3-N recovery technologies or processes, which are broadly categorized as: (1) physiochemical processes, (2) biological processes, or (3) a combination of the two. In a general sense, the ammoniacal-N (either in the form of NH_3 or NH_4^+) removed via physiochemical processes can typically be recovered for later use; on the other hand, the ammoniacal-N removed via biological processes cannot be recovered. Among many others, some of the most promising recovery methods that classify as physiochemical processes include ion-exchange and adsorption, stripping procedures, and struvite precipitation.

Ionic-exchange and adsorption are two interrelated NH_4^+ recovery methods. With ion-exchange, ionic compounds (adsorbents) are added to the liquid media in order to exchange ions with NH_4^+ ions (target ions). This exchanging of ions continues until the adsorbent's replaceable ions are fully depleted [245]. Adsorption similarly targets the NH_4^+ ion, but in its case the goal is to optimize intermolecular force instead of ion exchange. When narrowing our search to manure-sourced AD effluents, the literature focused on this treatment form is scarce. However, some commonly considered adsorbents for optimizing ion-exchange include homoionic zoelite [246] and adsorption include biochars and clay materials such as bentonite [247,248].

Stripping procedures can occur in various forms such as gas-permeable membranes, air-stripping, and steam-stripping. Generally speaking, stripping procedures are among the most

highly considered ammoniacal-N recovery procedures, due to their high NH_3 recovery efficiency which results in a saleable form of nitrogenous fertilizer. Due to its superior NH_3 recovery ability – based on the total contact time it has with the liquid media – gas-permeable membrane stripping was likely the most advantageous stripping technologies [249]. In the case of air-stripping, air is passed through the liquid media by direct aeration or via a stripping tower. This allows for NH_3 gas to volatilize, which can then be removed. Aeration has demonstrated similar NH_3 recovery as traditional air stripping methods, though it has been shown to take longer to achieve [250]. Steam-stripping is a similar process to air-stripping, but includes the incorporation of chemical processing methodology. In order for the N to be extracted through this procedure, the temperature of the liquid media must be increased resulting in a distillation reaction. In an experiment which employed the steam-stripping procedure to anaerobically digested and centrifuged cattle manure, Zeng et al. (2006) demonstrated NH_4-N removal efficiencies ranging from 91-96% [251].

Struvite precipitation is a process that involves the crystallization of N and P in the presence of magnesium (**Mg**) into struvite, which is chemically named magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6H_2O$). Struvite precipitation can be implemented as a tool to remove both NH_3 and phosphate. One of the large benefits to struvite precipitation on the part of the treatment facility is the reduction of sludge, which they would need to later dispose of. From a nutrient management perspective, struvite is considered a highly valuable and marketable slow-release fertilizer. Although mono-digested cattle manure has not consistently exhibited high efficiency in struvite formation [252,253], it remains a viable solution, which requires further investigation in order to best optimize its formation [254,255].

In the case of biological removal, the ability of specific classes of microorganisms to breakdown NH_3 is utilized. This category includes processes such as anammox, and conventional

or simultaneous nitrification-denitrification. The anammox process involves Anammox Planctomycete bacteria, which are characterized by their ability to oxidize NH_4^+ to dinitrogen (**N_2**) [256]. Anammox bacteria operate within extremely specific environmental conditions and constraints, functioning optimally within a narrow pH range of 7.5-8.0 [257] and physiologically within a pH range of 6.6 and 8.1 [258]. In addition to pH, temperature also greatly influences anammox potential, with constant high temperatures of 28.8 ± 1.1°C having greatest affect than at 21.2 ± 3.2°C [259]. Literature demonstrating the use of anammox processes in mono-digested manure is limited; the process has, however, been shown to have great potential with other OM sources including co-digested manure [260].

Similar to anammox, nitrification-denitrification processes are better optimized in situations where sludge is activated via agitation or aeration. In this process, the NH_4^+ produced throughout the upstream processes serve as substrate for the ammonia oxidizing bacteria (**AOB**) to oxidize. The nitrification process begins with AOBs converting ammonia to hydroxylamine, which is then converted to nitrite; nitrite oxidizing bacteria can then use nitrite as a substrate to convert to nitrate. Under anaerobic conditions, denitrification then converts nitrate into N_2 [261]. One of the most predominate concerns with the nitrification-denitrification process is the potential for N_2O emissions to increase in situations where the denitrification process is not completed. However, if the operational conditions which are favorable for both nitrification and denitrification to occur are met, both of the processes will occur [262].

1.7. Outlook to the Future

Greenhouse gases are classically categorized based on their GWP. Global warming potential is a method by which to compare the GHGs based on a "GWP conversion factor" and a "time horizon", using CO_2 as the comparative or base gas in the equation. The 100-year scale

(**GWP_{100}**) is traditionally used to make comparisons between gases. By this method, CO_2 is given a GWP_{100} of 1, whereas CH_4 is calculated to have a GWP_{100} of approximately 27, and N_2O of approximately 273 [263]. Recent literature has demonstrated that GWP_{100} overestimates the impact of SLCPs, especially those from constant sources, and therefore can only accurately predict the mitigation effects over a maximum of 20-40 years [264]. Updated methods for quantifying the GWPs of GHGs, known as GWP star (**GWP***) have been proposed [264,265]. Through GWP*, the changing rates in SLCPs would be characterized based on their actual warming potential rather than its relationship to CO_2 emissions. This allows for a more accurate representation of the impact of SLCPs in various emissions scenarios such as if emissions are kept stable or if they decrease over time [266]. Having more accurate metrics would allow for enhanced precision in the accounting of emissions, which could lead to more appropriate GHG mitigation targets and implementation strategies.

The United States livestock system is considered a relatively efficient system and may serve as a case study for addressing questions pertaining to the environmental sustainability of livestock sectors from a global perspective. In the United States, cattle production has become highly specialized; sectors of production have become segmented and specialized for certain fundamental growth- and reproduction-related milestones in an animal's life cycle. Specialization and targeted approaches to improve efficiencies have resulted in historically low herd sizes in the United States, while productive outputs per species type are optimized. For instance, the use of byproducts as a commodity in livestock rations not only results in less organic waste and therefore environmental benefits, but also provides nutritive value to the animal which can lead to improved animal efficiencies. A survey of the United States beef cattle industry found that approximately 89% of feed consumed by beef cattle is human-inedible, 7% of this being associated with

byproducts [267]. The United States dairy industry has also continued to progress their usage of human-inedible feeds, with byproducts accounting for 32% of feed consumed by lactating dairy cows [268].

Livestock operations are often located in close proximity to the feed commodities, which suit the operation-type best. For instance, California's San Joaquin Valley is home to some of the most fertile agricultural lands in the world, and is also home to a high concentration of dairy cattle operations. Byproducts such as almond hulls, grape pomace, and citrus pulp serve as meaningful byproduct commodities found in a California dairy ration [269]. Likewise, corn and cereal grains are found abundant in states within the Great Plains of the United States, which grow the crops for ethanol production; a large concentration of feedlots are also located in those states, feeding byproducts from ethanol production such as distiller's grains [270].

Though the digestive systems of dairy cows, beef cattle, and sheep are similar, the relative effectiveness of various GHG mitigation strategies may depend on factors including diet composition and ruminal physiology [30]. What is apparent is that there will not be a one-size-fits-all solution that meets the needs of various livestock sectors or geographic regions. Instead, GHG mitigation and efficiency solutions should involve aggregation of strategies that suit individual needs best.

CHAPTER 2: THE IMPACT OF ESSENTIAL OIL FEED SUPPLEMENTATION ON ENTERIC GAS EMISSIONS AND PRODUCTION PARAMETERS FROM DAIRY CATTLE

Abstract

Societal pressure to reduce enteric methane emissions from cattle continues to increase. The present study evaluated the efficacy of the commercial essential oil feed additive Agolin® Ruminant on reducing enteric gas emissions and improving milk parameters in dairy cattle. Twenty mid-lactation Holstein cows, blocked by parity and days in milk, were randomly assigned to a top dress treatment with Agolin or an un-supplemented control for a 56-day trial. Cows were group housed and individually fed twice daily. Enteric gas emissions, including methane, carbon dioxide, ammonia, and nitrous oxide, were sampled every 14 days for a 12 h period via head chambers connected to a mobile air quality laboratory. Cows supplemented with Agolin versus the control had less methane intensity (g/period/kg energy-corrected milk (ECM); $p = 0.025$). Ammonia was the most affected gas, with lower ammonia production (mg/period; $p = 0.028$), and ammonia intensity (mg/period/kg ECM; $p = 0.011$) in Agolin-fed versus control-fed cows. All cow performance variables, including dry matter intake, ECM, milk fat, milk protein, or feed efficiency were similar between treatments. Further research should evaluate how Agolin impacts ruminal flora, focusing on mechanistic impacts to fermentation.

Keywords: greenhouse gas; methane; essential oils; dairy cow; enteric emissions; sustainability; feed additive

2.1. Introduction

Air pollutants have strong effects on public and environmental health. Carbon dioxide (**CO_2**), methane (**CH_4**), and nitrous oxide (**N_2O**) are greenhouse gases (**GHGs**) that have received attention due to their contribution to climate change [234]. These GHGs, as well as ammonia (**NH_3**), can be emitted by animal agricultural processes. In an effort to regulate climate change, California passed Senate Bill 1383, mandating a 40% reduction in CH_4 emissions below 2013 levels by the year 2030 [271]. The primary focus of this bill lies on CH_4 emissions from all sources, but within the dairy sector, both CH_4 manure mitigation and enteric fermentation CH_4 can be considered. To date, the majority of research and improvements in CH_4 emissions have been in the area of manure management. With enteric fermentation accounting for 28% of total CH_4 emissions in the United States [234], reducing emissions from this source of CH_4 is important.

In addition to detrimental effects on the environment, CH_4 accounts for a 2–12% loss in gross energy intake in ruminants [21]. Dairy farmers are thus seeking ways to reduce the enteric CH_4 loss on their farms, and to improve cow efficiency [272]. Current strategies for reducing enteric CH_4 include altering the ration formulation and quality of feed [149,273,274], increasing amounts of dietary lipids [272], and including compounds with antimicrobial abilities such as bacteriocins or ionophores, amongst others [104,275]. Although these strategies make an impact on methanogenesis, they each come with limitations with respect to animal performance and efficiency. The use of secondary plant compounds [276], such as essential oils, has been investigated as a novel approach for reducing enteric CH_4 emissions [144].

Essential oils (**EOs**) are plant metabolites consisting of phenylpropenes and terpenes that are extracted by steam distillation or through the use of organic solvents to create a concentrated product [144,145]**.** These naturally occurring metabolites function to protect the plant from abiotic

stress, predation, and competition, and are antimicrobial in nature [104,146]. Research suggests that EOs exhibit desirable effects with respect to rumen fermentation, thereby affecting CH_4 formation [148]. Individual EOs elicit varied effects on rumen fermentation and microbial persistence based on their particular modes of action [277]. Although some may also affect Gram-negative bacteria, EOs as a whole exhibit a large inhibitory effect on Gram-positive bacteria [146,278]. For instance, Chao et al. (2000) found cinnamon bark and tea tree oils inhibited both Gram-positive and -negative bacteria, fungi, and bacteriophages, whereas cedarwood and cumin oils were found to only inhibit Gram-positive bacteria. The inhibition of Gram-positive bacteria may be beneficial, because they are generally associated with the production of hydrogen (H_2), formate, NH_3, and CH_4 [279].

Agolin® Ruminant (**AGO**; Agolin SA, Bière, Switzerland), is a commercially available blend of EOs, containing coriander seed oil, eugenol, and geranyl acetate, that has been shown to reduce CH_4 through in vitro [280–282] and in vivo experiments [157,283,284]. It would be beneficial if AGO could favorably impact CO_2, N_2O, and NH_3, because of their importance with respect to air quality, climate, and energy loss to the animal.

Previous experiments in dairy cattle supplemented with AGO demonstrated improved milk parameters, including increased milk production [157,280] and improved milk fat and protein yield [157]. Few studies, however, have assessed the impacts of AGO on feed efficiency [162,280,285], and no previous work has addressed animal measures such as milk or serum urea nitrogen (**MUN** and **SUN**, respectively).

The present trial aimed to determine whether AGO has the potential to reduce enteric emissions of CH_4, CO_2, N_2O, and NH_3, and the potential to improve production parameters including nitrogen utilization (MUN and SUN), total milk production, and its components in mid-

lactation Holstein dairy cattle. If AGO could reduce GHGs, this and related technologies could be a part of a climate change mitigation program.

2.2 Materials and Methods

2.2.1. Animals and Experimental Design

The present trial was conducted at the University of California, Davis, Dairy Teaching and Research Facility under an approved Institution for Animal Care and Use Committee protocol. The study design was a randomized complete block design with repeated measures over time. Twenty mid-lactation Holstein dairy cows were blocked according to days in milk (150 ± 43) and parity (10 primiparous and 10 multiparous), and were then randomly assigned to one of two treatments. Cows were group housed in a free-stall pen with ad libitum access to water and were milked twice daily at 6:30 a.m. and 6:30 p.m.

2.2.2. Feeding

Cows were randomly assigned to a feed gate and were individually fed using the Calan Broadbent System (American Calan, Northwood, NH, USA). Cows were fed twice daily immediately following milking at approximately 6:45 a.m. and 6:45 p.m.

The diet comprised an 89–90% dry matter (**DM**) total mixed ration (**TMR**; Table 1), with cows being fed to yield 5% refusals. Feed refusals were collected and sampled prior to each feeding at approximately 6:15 a.m. and 6:15 p.m. to determine DM content and daily DM intake (**DMI**). Cows were adapted to the basal diet without supplementation for 30 days prior to the start of emission sampling.

The AGO and control (**CON**) treatments were administered as a top dress. Cows fed AGO treatment received a premix composed of cornmeal + AGO, with AGO being included at a rate of 1 g/head/day (149.5 g cornmeal + 0.5 g AGO per feeding), while CON-fed cows received 150 g of cornmeal only per feeding.

Two cows were paired in each block, with each block comprising one AGO-fed and CON-fed cow. To accommodate two cows sampled for gas emission per day in the two head chambers, cow blocks were stagger-started onto their respective treatments. Blocks were randomly assigned to a respective day 0 emission sampling day and began receiving their treatments on day 1. Treatment with AGO or CON continued for 57 days.

2.2.3. Emission Sampling

On gas emission sampling days, the two cows were each secured in their respective head chamber (**HC**) using neck chains similar to a tie-stall system for a 12 h gas emissions sampling period from approximately 6:45 a.m. to 6:45 p.m. Cows were subjected to three training sessions within the HC prior to the start of the experiment, in order to become habituated to the HC. Cows were sampled every 14 days, on each of their respective days 0, 14, 28, 42, and 56 on treatment. Cows had ad libitum access to feed and water and could stand up or lie down during the HC gas emission sampling period.

Each HC consisted of a head chamber manufactured with clear polycarbonate sheeting, blowers pumping air out of the hoods, and Teflon tubing to extract emission samples. The Teflon tubing was connected from the HC to a mobile agricultural air quality laboratory, which housed all of the necessary equipment [286]. Concentrations of CH_4, CO_2, N_2O, and NH_3 were analyzed using the Innova 1412 photo-acoustic multi-gas analyzer (LumaSense Technologies Inc., Ballerup,

Denmark). The INNOVA 1412 analyzer had minimum detection limits of 0.4 ppm for CH_4, 1.5 ppm for CO_2, 0.03 ppm for N_2O, and 1.0 ppm for NH_3, and a maximum detection limit of 106 ppm. The continuous sampling cycle included the two HCs followed by ambient air, with each being sampled for 15 min intervals in sequence. The HCs in use were validated by Place et al. (2011) [286], and underwent both a pre- and post-trial validation in the present trial.

2.2.4. Emission Calculations

The measured gas concentrations of the outgoing air samples from the HC for each 15 min period were truncated to remove the first five minutes and last two minutes of each sample period for the prevention of carry-over effects. The total flux of gases in mg/h were calculated according to the equation outlined in Peterson et al. (2020) with an ambient air flow rate (**FL**) of 2300–2500 L min^{-1} [287]. The emission rate by cow for the HC (mg/h/head) was the same as the total flux of gases, as there was only one cow housed in each HC.

2.2.5. Milk Yield and Analysis

Milk yield for each cow was recorded daily at both the a.m. and p.m. milking sessions. Samples of milk were collected at consecutive a.m. and p.m. milking sessions on a weekly basis, and were sent for component analysis of milk fat, protein, and MUN (Central Counties Dairy Herd Improvement Association, Atwater, CA, USA). Energy-corrected milk (**ECM**) was calculated according to the following formula [288]:

$$ECM = (0.327 \times \text{milk kg}) + (12.95 \times \text{milk fat kg}) + (7.65 \times \text{milk protein kg}).$$

2.2.6. Blood Sampling

Blood samples were collected from each cow following the morning milking (hour 0) and before feeding on their respective days 1, 15, 29, 43, and 57 on treatment. Animals were secured in a chute and blood samples collected into 9 mL serum separator tubes (Corvac™ Serum Separator Tubes, Covidien, Mansfield, MA, USA) from the coccygeal vein prior to returning them to the free-stall pen. Cow blood samples were immediately centrifuged, and serum was stored at −18° C. Frozen samples were transported to the UC Davis Veterinary Medical Teaching Hospital Clinical Diagnostic Laboratory Services (Davis, CA, USA) for analysis of SUN concentration.

2.2.7. Data Analysis

Gas emissions over the 12 h gas emission sampling period were summed to determine total gas production for the sampling period. Gas emissions were analyzed for intensity by calculating gas production per unit of energy-corrected milk (gas production/ECM), using ECM from the afternoon milking session which immediately followed the gas emission measurements. Gas yield was defined as the total production of the gas (i.e., summative emission measurement) per unit of DMI. Gas yield was calculated using DMI only while the cow was undergoing gas emission sampling in the HC (HC yield). Feed efficiency was calculated as kg ECM/kg daily DMI. Data were analyzed as a randomized complete block design with repeated measures, using the "nlme" and "emmeans" packages in R [289–291]. Blocks refer to each pair of parity and days in milk-matched cows. Gas emissions and production parameters were analyzed according to the following base model:

$$Y_{ijklm} = \mu + \beta_i + \beta_j + \beta_{k(j)} + \beta_l + \beta_m + (\beta_l \times \beta_m) + \varepsilon_{ijklm}$$

where μ = the overall mean of the response variable in question; β_i = overall mean of day 0 for the response variable in question; β_k = cow (random) which was nested within β_j = block (random); β_l = treatment; β_m = day; ε_{ijklm} = the error term. Serial correlation structures and model selection were determined based on the Akaike information criterion, Bayesian information criterion, and log-likelihood [292]. Day 0 afternoon ECM, which was used to calculate gas intensity, was unavailable for one cow; following the model selection criteria and model fit, day 0 was excluded from the gas intensity and the head chamber ECM models. The data for each of the response variables were further verified for assumptions of normality by the Shapiro–Wilk method, with outliers removed accordingly where normality was not met.

All means are presented as least squares means (**LSMs**) based on "emmeans" and comparisons between treatment LSMs were completed using the "anova" function. Test day means were compared using Tukey's test pairwise comparisons using "glht" and "cld" in "multcomp" [293]. Differences were declared significant at $p < 0.05$ and a trend toward significance at $0.05 \leq p < 0.10$.

2.3. Results and Discussion

2.3.1. Effect of AGO on GHG Emissions

Methane production was found to be similar between AGO-treated versus CON-treated cows ($p > 0.05$; Table 2). Methane production differed by day ($p < 0.001$; Table S1), whereas the interaction of treatment by day was not significant. Our findings are dissimilar to those of Hart et al., (2019) who found a significant decrease in enteric CH_4 production when cows were supplemented with AGO [157]. Hart et al. (2019) separated the AGO-treated from the CON-

treated cows in group-fed and group-treated pens, rather than individually feeding and applying the treatment to the cow. The researchers could therefore not ensure that each cow consumed the allocated 1g/head/day of AGO in this model [157], which makes extrapolation of their findings difficult. Similarly, Castro-Montoya et al. (2015) found that enteric CH_4 production tended to decrease when cows were supplemented with AGO [283]. Castro-Montoya et al. (2015) used each cow's respective day 0 as a control in their experiment; it is therefore possible that temporal changes could have affected CH_4 production in each cow. Klop et al. noted a brief reduction in CH_4 production ($p < 0.05$) in the first 14-day period after AGO supplementation began, compared with pre-treatment CH_4 production. Although DMI was unaffected by AGO supplementation, CH_4 production increased and was no longer different from pre-treatment CH_4 production by the third 14-day period in Klop et al. (2017) [284].

In general, a strong positive correlation has been found between CH_4 production and individual animal DMI [21,28]. Cows that consume higher levels of DM have more substrate available for fermentation and more hydrogen available for methanogenesis, and are therefore generally associated with higher daily CH_4 emissions [294,295]. Gas yield (gas emissions/DMI) is therefore an important outcome to measure [295]. In the present trial, AGO- versus CON-fed cows showed similar HC yields for CH_4, CO_2, N_2O, and NH_3 ($p > 0.05$; Table 2). Klop et al. (2017) found a reduction in CH_4 yield in AGO-supplemented cows when comparing the pre-treatment period to the first period (periods were 14 days in length); however, the difference was no longer present when comparing the pre-treatment period to the third or the fifth period [284]. Klop et al. (2017) housed their cows in climate respiration chambers for CH_4 sampling for 2.5 days, taking daily DMI into consideration. Castro-Montoya et al. (2015) found that CH_4 yield tended to decrease in cows supplemented with AGO ($p = 0.07$) [283]. Castro-Montoya et al. (2015)

considered DMI from each of three consecutive days that animals were housed in an open circuit chamber in their calculations.

In the present study, cows supplemented with AGO versus CON showed lower CH_4 intensity ($p = 0.025$; Table 2). The effect of day was found to be significant for CH_4 intensity ($p < 0.001$; Table S1), while the interaction of treatment by day was not significant ($p > 0.05$). Our findings are consistent with those of Hart et al., (2019) who found a reduction in CH_4 intensity in AGO- versus CON-treated cows [157]. Klop et al. (2017) similarly noted a decrease in CH_4 intensity when comparing the period in which cows were on AGO treatment to the cow's respective pre-treatment period [284]. Our findings are contrary to those by Castro-Montoya et al. (2015), who found no differences in CH_4 intensity when cows were supplemented with AGO [283], although they used actual kg milk instead of ECM. A cow could be more productive with respect to CH_4 intensity; however, this is diminished as herd size increases [296].

In the present trial, no differences between AGO- versus CON-treated cows were detected for CO_2 production, CO_2 HC yield, or CO_2 intensity ($p > 0.05$; Table 2). The effect of day was significant for CO_2 production ($p < 0.001$) and intensity ($p < 0.001$; Table 2.4), whereas the interaction of treatment by day was not significant for any of the CO_2 emission measurements ($p > 0.05$). Rumen methanogens have long been regarded nutritionally discriminatory, consuming select substrates such as CO_2 as a source of carbon, and H_2, formate, and acetate as sources of hydrogen [297]. Based on this, we would expect CO_2 emissions to either increase or remain unchanged by AGO supplementation. Although this was not the case, our findings were consistent with those of Melgar et al. (2020), who found decreased CO_2 production and no changes in CO_2 yield when dairy cows were supplemented with 3-nitrooxypropanol (**3NOP**) to reduce CH_4 emissions [298]. Hristov et al. (2015) saw no changes in CO_2 production in instances where CH_4

production was reduced in cows supplemented with 3NOP [298,299]. The dosing level of 3NOP was found to affect the CO_2 emission response, with CO_2 increasing as dosing levels increased [300]. Further research is therefore needed to determine if the dosage level of AGO could similarly affect CO_2 emissions in dairy cows.

No differences were found between AGO- versus CON-treated cows for N_2O production, N_2O HC yield, or N_2O intensity in the present study ($p > 0.05$; Table 2). The effect of day was significant for N_2O HC yield ($p = 0.012$), but not for N_2O production or intensity ($p > 0.05$; Table 2.4). Similar to the other parameters, the interaction of treatment by day was not significant ($p > 0.05$). Despite making a smaller contribution to overall emissions from enteric fermentation, enteric N_2O production has been quantified in the literature [25,301]. However, previous research with EO supplementation in dairy cows has not quantified enteric emissions of N_2O.

Enteric NH_3 was the most impacted gaseous emission in the present trial, with NH_3 production ($p = 0.028$), and NH_3 intensity ($p = 0.011$) being lower among AGO- versus CON-treated cows. No difference was found for NH_3 HC yield (Table 2). The effect of day was highly significant for NH_3 production, NH_3 HC yield, and NH_3 intensity ($p < 0.001$; Table 2.4), and the interaction of treatment by day was not significant for any of the parameters ($p > 0.05$). These findings are consistent with those of Castillejos et al. (2006) who found that the inclusion of eugenol led to a decrease in ruminal ammonia-N concentration when investigated in a batch fermentation system [302]. Coriander seed oil was also found to reduce ruminal ammonia-N concentration when compared to control- and salinomycin-treated cows [303]. The decrease in ammonia could be the result of the sensitivity of hyper-NH_3-producing bacteria to EOs [144]. Working with another commercial EO, McIntosh et al. (2003) found that EOs may specifically affect the deamination of amino acids, which is the final step in protein catabolism [304]. The deamination of amino acids

lead to more NH_3 being produced than can generally be consumed by ruminal microorganisms, resulting in nutritional losses [305]. Although NH_3 was measured within the ruminal fluid content for many of these studies, previous literature noted that NH_3 gas can form and be eructated from the rumen [306]. The reduction of NH_3 gas in the present trial may therefore be due to more nitrogen being retained by the animals, resulting in less nutritional loss.

Essential oils have demonstrated diverse mechanisms of action, which are used to interact with ruminal microorganisms. For example, some EOs interact with the external membranes of bacterial cells, which leads to conformational changes and the loss of stability of the cell membrane [148]. Other EOs act on microorganisms by coagulating the material within the cytoplasm of the cell [307]. The specific mechanism of action of AGO remains unclear. Further research should assess how the blend of EO within AGO individually and collectively interacts with and affects ruminal microorganisms.

2.3.2. Effect of AGO on Production Parameters

In the present study, daily DMI and head chamber DMI were similar between AGO- versus CON-treated cows ($p > 0.05$; Table 3). The effect of day on DMI was significant ($p = 0.003$; Table 2.5), whereas the interaction of treatment by day was not significant. Although Hart et al. (2019) found that AGO increased DMI [157], our present findings are consistent with those of both Elcoso et al. (2019) and Guasch et al. (2016), who saw no differences in DMI between treatment groups [280,285].

In the present trial, all production parameters, such as ECM, head chamber ECM, milk fat, and milk protein, were similar between treatments ($p > 0.05$; Table 3). For each of these production parameters, the effect of day was significant ($p < 0.05$), while the interaction of treatment by day

was not ($p > 0.05$). Although they both focused on actual milk yield instead of ECM, our present findings are consistent with those of Castro-Montoya et al. (2015) and Santos et al. (2010), who found no differences in milk yield with AGO supplementation [219,283]. Effects of increased milk fat (kg/d) [157,219], and protein yield (kg/d) [157] have been found in previous AGO supplementation experiments; however, Castro-Montoya et al. (2015) and Elcoso et al. (2019) found no differences with respect to milk fat or protein, which is in agreement with our present findings [280,283]. In the case of Santos et al. (2010), it should be noted that the AGO treatment was applied to the pen and not the cow and the increase in milk fat yield with EO was just 0.03 kg/cow [219]. A meta-analysis conducted by Belanche et al. (2020) showed that supplementation with 1 g/head/day of AGO to dairy cattle improved ECM (referred to as FPCM) (response ratio = 1.031; $p < 0.001$) across the 20 studies that had addressed this parameter [162]. However, it is important to note that in addition to the published literature, the meta-analysis also incorporated unpublished experiments and information from on-farm trials.

In our trial, feed efficiency was similar between AGO- and CON-treated cows (Table 3). For feed efficiency, the effect of day was found to be highly significant ($p < 0.001$; Table 2.5), while the interaction of treatment by day was not significant ($p > 0.05$). Elcoso et al. (2019) and Guasch et al. (2016) saw increased feed efficiency in AGO- versus CON-treated cows, which is not consistent with our present findings [280,285]. The meta-analysis conducted by Belanche et al. (2020) showed an overall improvement in feed efficiency (response ratio = 1.030; $p = 0.002$) across 16 trials, when dairy cows were supplemented with 1 g/head/day of AGO [162]. This improvement in feed efficiency appears fairly common across various EO supplements. Another commercially available blend of EO, containing eugenol, cinnamaldehyde, and capsicum, was also found to improve feed efficiency in lactating Holstein cows [308]. Supplementing cows with an EO blend

containing eugenol, thymol, and m-cresol and 10 other volatile compounds, Joch et al. (2019) noted a trend towards improved feed efficiency [309]. Braun et al. (2019) also found an increase in feed efficiency when supplementing Holstein dairy cows with a commercial blend of menthol, eugenol, and anethol [310].

In the present trial, MUN was similar between dietary treatments ($p > 0.05$; Table 3). The effect of day was highly significant for MUN ($p < 0.001$; Table 2.5), whereas the interaction of treatment by day was not significant ($p > 0.05$). Previous studies reported varying results when supplementing cows with EOs**.** Benchaar et al. (2015) similarly found no differences with respect to MUN when cows were supplemented with eugenol [217], which was also confirmed by Joch et al. (2019) [309]. However, in a series of experiments where cows were supplemented with Xtract 6965 (consisting of eugenol and cinnamaldehyde) at the same dosage levels, Tekippe et al. (2013) showed increased MUN concentrations when the supplement was mixed into the mineral premix ($p < 0.001$), but not when the supplement was administered as a top dress ($p = 0.50$) [216]. Dairy cow MUN may therefore differ based on the method in which the EO is supplemented.

In the present experiment, SUN concentrations were also found to be unaffected in AGO- versus CON-treated cows (Table 3). Test day was found to be highly significant for SUN ($p < 0.001$), and the interaction of treatment by day was not significant ($p > 0.05$). Experiments conducted on Holstein dairy heifers supplemented with cinnamaldehyde demonstrated no difference in plasma urea nitrogen (**PUN**) between EO- versus control-supplemented cows [311]. Supplementation of eugenol and cinnamaldehyde to multiparous Holstein dairy cows resulted in inconclusive results, with significantly higher PUN concentrations when the EO was mixed into the premix ($p < 0.001$) and significantly lower PUN when the EO was applied as a top dress ($p = 0.03$) [216].

2.4. Conclusions

Societal pressure and legislation have resulted in a need for California's dairy industry to reduce GHG emissions. Our present findings suggest that supplementing lactating dairy cow rations with 1 g/head/day of AGO may be part of an effective enteric CH_4 intensity mitigation strategy. Agolin also demonstrated a potential for reducing nitrogen-based gas emissions in mid-lactation dairy cattle, although additional research is needed to elucidate AGO's impact on nitrogen utilization. In order to form a more comprehensive understanding of the benefits of supplementation, future research should assess AGO's impact on ruminal microorganisms, and determine the EO blend's specific mode of action.

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Conflicts of Interest: The sponsor played no role in the execution and interpretation of the data and preparation of the present manuscript. The authors declare no conflict of interest.

Table 2.1. Composition (%) of the basal total mixed ration (TMR) fed to cows during the 56-day trial period, as fed (89–90% dry matter (DM)).

TMR Composition (%; As Fed)	
Grain Mix [1]	41.47
Alfalfa Hay	32.25
Chopped Wheat	8.06
Cottonseed, Whole	7.68
Almond Hulls	7.68
Mineral Premix	1.15
EnerGII [2]	1.15
Strata [2]	0.32
Salt	0.23
Grain Mix [1]	
Steam Flaked Corn	30.75
Wheat Mill Run	21.95
Dried Distillers Grains	21.04
Beet Pulp	14.1
Rolled Barley	10.25
Soybean Meal	1.91

[1] Detailed composition of the grain mix and percentages of each ingredient; [2] Virtus Nutrition LLC.

Table 2.2. Treatment least squares means (LSMs) for gas production, gas head chamber (HC) yield, and gas intensity of methane (CH_4), carbon dioxide (CO_2), nitrous oxide (N_2O), and ammonia (NH_3) from Holstein dairy cattle to which Agolin (AGO) vs. untreated control (CON) diets were supplemented (n = 10 per treatment).

	Treatment LSM		**SEM**	***P-Value***
	AGO	**CON**		**Treatment**
Gas Production				
CH_4 (g/period)	357	381	12.1	0.15
CO_2 (g/period)	9248	9660	272	0.39
N_2O (mg/period)	1298	1374	39.3	0.11
NH_3 (mg/period)	293	331	12.1	***0.028***
Gas Head Chamber Yield [1]				
CH_4 (g/period/kg)	24.5	24.1	0.56	0.62
CO_2 (g/period/kg)	641	614	17.1	0.18
N_2O (mg/period/kg)	89.1	87.6	2.12	0.54
NH_3 (mg/period/kg)	20.4	21.4	0.83	0.10
Gas Intensity [2]				
CH_4 (g/period/kg)	15.8	17.8	0.71	***0.025***
CO_2 (g/period/kg)	411	452	22.3	0.15
N_2O (mg/period/kg)	56.8	64.7	2.65	**0.05**
NH_3 (mg/period/kg)	13.1	15.6	0.82	***0.011***

Period = 12 h gas emission sampling period; [1] gas production per period × (1/kg dry matter intake (DMI) from the sampling period while in the HC); [2] gas production per period × (1/kg energy-corrected milk from the afternoon milking session).

Table 2.3. Treatment least squares means (LSMs) for feed efficiency, daily dry matter intake (DMI), head chamber DMI, head chamber energy-corrected milk (ECM), ECM, milk fat, milk protein, milk urea nitrogen (MUN), and serum urea nitrogen (SUN) from Holstein dairy cattle fed Agolin (AGO) vs. untreated control (CON) (n = 10 per treatment).

	Treatment LSM		SEM	*P-Value*
	AGO	CON		Treatment
Feed Efficiency [1]	1.57	1.63	0.03	0.28
DMI[2] (kg)	26.4	26.2	0.30	0.60
Head Chamber DMI (kg)	14.8	15.8	0.42	0.14
Head Chamber ECM[3] (kg)	22.9	22.0	1.20	0.49
ECM (kg)	41.1	42.1	0.98	0.47
Milk Fat (kg)	1.65	1.69	0.05	0.56
Milk Protein (kg)	1.11	1.13	0.02	0.60
MUN (mg/dL)	9.67	9.68	0.27	0.97
SUN[4] (mg/dL)	12.2	11.6	0.37	0.36

[1] kg ECM/kg daily DMI; [2] excludes DMI from when the cow was secured in the head chamber; [3] ECM from the afternoon milking session, immediately following the emission sampling period; [4] samples were collected following morning milking session (hour 0).

Table 2.4. Test Day Least Square Means (LSM) for gas production, head chamber yield, and intensity of methane (CH_4), carbon dioxide (CO_2), nitrous oxide (N_2O), and ammonia (NH_3) from all Holstein dairy cattle enrolled in the trial (n = 20).

	Test Day LSM					SEM	*P-Value*
	Day 0 [1]	**Day 14**	**Day 28**	**Day 42**	**Day 56**		**Day**
Gas Production							
CH_4 (g/period)	**399**	338^{a}	390^{b}	367^{b}	380^{b}	11.4	***<.001***
CO_2 (g/period)	**9800**	8747^{a}	9990^{b}	9392^{ac}	9688^{c}	267	***<.001***
N_2O (mg/period)	**1705**	1339	1379	1295	1332	43.3	*0.32*
NH_3 (mg/period)	**423**	331^{a}	358^{a}	285^{b}	274^{b}	11.7	***<.001***
Gas Head Chamber Yield[2]							
CH_4 (g/period/kg)	**26.5**	23.8	25.3	23.6	24.6	0.65	*0.17*
CO_2 (g/period/kg)	**652**	620	652	608	630.7	19.1	*0.18*
N_2O (mg/period/kg)	**113**	94.0^{a}	89.8^{ab}	83.3^{b}	86.4^{ab}	2.66	***0.012***
NH_3 (mg/period/kg)	**28.0**	23.7^{a}	23.3^{a}	18.6^{b}	17.9^{b}	0.97	***<.001***
Gas Intensity[3]							
CH_4 (g/period/kg)	**17.4**	15.0^{a}	17.1^{b}	16.8^{ab}	18.3^{b}	0.7	***<.001***
CO_2 (g/period/kg)	**432.4**	389^{a}	440^{bc}	431^{b}	467^{c}	20.4	***<.001***
N_2O (mg/period/kg)	**74.8**	58.9	60.8	59.1	64.1	2.55	**0.06**
NH_3 (mg/period/kg)	**18.5**	14.9^{a}	16.2^{a}	13.2^{b}	13.2^{b}	0.79	***<.001***

Differences between means determined by Tukey's multiple comparison test; Period = 12-hour gas emission sampling period; [1]Day 0 included as covariate within the model for the four test days (when treatment was applied); [2]gas production × (1/kg DMI from the sampling period while in the HC); [3]gas production × (1/kg energy corrected milk from the afternoon milking session)

Table 2.5. Test Day Least Square Means (LSM) for feed efficiency, daily dry matter intake (DMI), energy corrected milk (ECM), Milk fat, milk protein, milk urea nitrogen (MUN), and serum urea nitrogen (SUN) from all Holstein dairy cattle enrolled in the trial (n = 20).

	Test Day LSM					SEM	*P-value*
	Day 0 [1]	**Day 14**	**Day 28**	**Day 42**	**Day 56**		**Day**
Feed Efficiency [2]	**1.64**	1.69[a]	1.61[b]	1.56[bc]	1.54[c]	0.03	***<.001***
DMI (kg)	**25.9**	26.1[a]	26.7[b]	26.4[a]	26.0[a]	0.24	***0.003***
ECM (kg)	**42.4**	43.6[a]	42.5[a]	40.7[b]	39.6[b]	0.85	***<.001***
Milk Fat (kg)	**1.71**	1.81[a]	1.71[b]	1.61[c]	1.56[c]	0.04	***<.001***
Milk Protein (kg)	**1.11**	1.10[a]	1.14[b]	1.12[ab]	1.12[ab]	0.02	***0.002***
MUN (mg/dL)	**10.1**	10.9[a]	9.20[b]	9.23[b]	9.38[b]	0.30	***<.001***
SUN[3] (mg/dL)	**11.7**	12.5[a]	12.3[a]	10.9[b]	11.9[a]	0.36	***<.001***

Within rows, means with different superscript differ ($p < 0.05$). Differences between means determined by Tukey's multiple comparison test; [1]Day 0 included as covariate within the model for the four test days (when treatment was applied); [2]kg ECM/kg daily DMI; [3]Samples were collected following morning milking session (hour 0)

CHAPTER 3: A TANNIN AND SAPONIN BLEND FEED ADDITIVE IMPACTS METHANE PRODUCTION IN LACTATING DAIRY COWS

Abstract

The objective of this study was to determine if a commercial feed additive blend comprised of quebracho and chestnut tannins and saponins (**TAN**; SilvaFeed® BX) could reduce enteric greenhouse gas (**GHG**) and ammonia (**NH_3**) emissions without negatively impacting the productive performance of dairy cows. Twenty early- to mid-lactation Holstein dairy cows were blocked by days in milk and parity in a randomized complete block design, and were assigned to one of two treatments: TAN or control (**CON**; n=10). Cows were individually fed, group-housed in a free-stall pen, and milked twice daily. The treatments were administered as a top dress at each of two feedings per day with TAN supplemented at a rate of 0.07% of DM. Cow blocks were sampled for enteric gaseous emissions in head chambers for 12-h on their respective treatment d 0, 16, 32, and 48. All urine and manure produced by each cow during enteric emission sampling were collected and stored. After the conclusion of enteric emission sampling, urine and manure were homogenized separately and were then combined into a slurry at 1:1.7 (urine wt: feces wt) per cow; slurry methane (**CH_4**), carbon dioxide (**CO_2**), nitrous oxide (**N_2O**) and NH_3 emissions were measured for 24-h. Supplemental TAN decreased enteric production (g or mg/h gas) of N_2O ($p = 0.03$), tended to decrease CH_4 ($p = 0.07$) and CO_2 ($p = 0.09$), and tended to increase NH_3 ($p = 0.07$) production in TAN-fed cows. Gaseous emission yield (g or mg gas/h/kg DMI) did not differ between TAN- vs CON -fed cows for CH_4, CO_2, or N_2O, though TAN-fed cows had higher NH_3 yield ($p = 0.04$). Gas intensity (g or mg gas/h/kg ECM) was similar between TAN vs CON fed cows for CH_4, CO_2, N_2O, or NH_3 intensity. Supplemental TAN did not impact slurry GHG emissions, though it increased NH_3 emissions. No differences were found in energy-corrected milk, milk fat yield, milk protein yield, and dry matter intake in TAN- vs CON-fed cows.

Keywords: greenhouse gas; methane; ammonia; tannins; saponins; dairy cow; enteric emissions; slurry emissions; manure emissions; sustainability; feed additive

3.1. Introduction

The livestock sector is a contributor of anthropogenic greenhouse gas (**GHG**) emissions and air pollutants. According to the United States Environmental Protection Agency, animal agriculture accounted for over one-third of methane (**CH_4**) emissions in the United States in 2020; 27% was associated with enteric fermentation and the remaining 9% with manure management practices [10]. In addition to CH_4, dairy cattle manure leads to nitrous oxide (**N_2O**) emissions when land applied, due to the nitrification and denitrification processes conducted by soil microorganisms. These events pose short-term environmental warming as CH_4 traps atmospheric heat 27 times more efficiently than carbon dioxide (**CO_2**) over its 12-year lifetime, and long-term as N_2O traps heat 273 times more effectively over its 114-year atmospheric lifetime [263].

Animal agriculture has additionally received societal pressure to reduce their carbon footprint with particular emphasis being placed on the ruminant industry. In an effort to regulate short-lived climate pollutants such as CH_4, California passed Senate Bill 1383 in 2016, mandating that the dairy sector reduce CH_4 emissions by 40% below 2013 levels by the year 2030 [9]. In addition to GHG emissions, dairy production also pollutes the air through ammonia (**NH_3**) emissions, which is a major precursor to particulate matter (**PM**) formation. These two compounds can form criteria pollutants, which are harmful to public health and the environment, and are currently regulated by the Clean Air Act [312].

Production of CH_4 within the rumen also accounts for a 2-12% loss in gross energy intake to the animal [21,272]. Reducing enteric CH_4 emissions may therefore lead to improved cow efficiency. Current candidate CH_4 mitigation strategies include altering the ration formulation and quality of animal feeds, and the use of feed additives such as 3-nitrooxypropanol, nitrates, ionophores, and plant secondary metabolites (**PSM**) such as essential oils, saponins, and tannins

[28]. Plant secondary metabolites were found to help the plant modulate cellular and molecular targets [105]. Saponins are comprised of glycosides of high molecular-weight, whose saccharide chain units are linked to a triterpene or a steroidal group, forming triterpene saponins or steroid saponins, respectively [313]. It was hypothesized that saponins may reduce enteric CH_4 by inhibition of ruminal protozoa [177,314,315]. Tannins are water soluble polyphenolic compounds which exist in condensed and hydrolysable forms within plant leaves, roots, trunks, barks, and other plant elements [316,317]. The ecological function of tannins is to protect plants against herbivory by reducing cell wall and protein degradation through the precipitation of proteins [318,319]. This precipitation occurs in aqueous solutions that are either acidic or slightly basic, such as when they are in the gut of an animal [320]. Tannins have demonstrated positive impacts as ruminal environment modifiers, particularly by improving nitrogen (**N**) utilization efficiencies when they were included in ruminant rations at small dosage levels [317]. They have also demonstrated antimicrobial and anthelmintic effects [118,321,322] and the ability to serve as a natural antioxidant when fed to ruminants [323]. Tannins were additionally found to mitigate CH_4 emissions through numerous *in vitro* and in some *in vivo* experiments [108,324–326] which could result from inhibitory effects against methanogen, fibrolytic bacteria, or protozoal numbers [313]. Though this is the case, tannins have been criticized for their anti-nutritional qualities when included at high dosage levels [327], in some cases demonstrating negative effects on fiber degradation which could result in decreased DMI (kg/d) [328] as well as decreased digestible and metabolizable energy (MJ/d) [329,330].

Quebracho and chestnut tannin have demonstrated promising effects on CH_4 emissions when fed to ruminants either separate from each other [331,332] or in combination with one another [213,333–335]. These tannin sources also favorably impacted feed efficiency [333], antioxidant

capacity [115,336], and milk quality [337] when supplemented at varying dosage levels. This makes tannins a feed additive of interest from an industry- and producer-perspective. Experiments in beef steers supplemented with as low as 0.07% and 0.1% of DM of Silvafeed Bypro, a commercially available blend of quebracho and chestnut tannins demonstrated favorable results with respect to productive performance [136,338]. Though tannins carry significant benefits when fed on their own, some experiments have demonstrated additive and enhanced effects when fed in combination with saponins [125,339].

There is no published literature addressing the effects of quebracho and chestnut tannin supplemented in combination with saponins to dairy cows. The aim of the present study was to investigate the impact of a commercial blend of quebracho and chestnut tannin extracts along with the inclusion of saponins (SilvaFeed BX, Silvateam) on enteric and manure gaseous emissions and productive performance of dairy cows supplemented at a low inclusion level. We hypothesize that the dietary supplementation of Silvafeed BX (**TAN**) at a rate of 0.07% of DM will lead to reduced enteric and manure GHG and NH_3 emissions without compromising the productive performance of lactating dairy cows.

3.2. Materials and Methods

3.2.1. Animals and Experimental Design

The present research trial was conducted at the University of California, Davis, Dairy Teaching and Research Facility under an approved Institution for Animal Care and Use Committee protocol. Twenty mid-lactation Holstein dairy cows were enrolled in a randomized complete block design experiment with repeated measures over time, and were blocked according to days in milk

(138 ± 69 at Day 0) and parity (10 primiparous and 10 multiparous) (n = 2 cows/block). Cows were split into two treatment groups TAN and CON, allowing for a n=10, with one cow assigned to each of the two treatments within a block. While not undergoing emission sampling, cows were group housed in a free-stall pen where they received *ad libitum* access to water, and were milked and fed twice daily at 06:30 and 18:30.

3.2.2. Feeding and treatments

Cows were individually fed to target 105% of their previous day's intake using the Calan Broadbent System (American Calan, Northwood, NH, USA). Cows returned to fresh feed immediately following milking at approximately 06:45 and 18:45. Their basal diet comprised a 90% dry matter (**DM**) alfalfa-based total mixed ration (**TMR**; Table 3.1), to which cows were adapted without supplementation for 30 d prior to the start of emission sampling. Feed refusals were collected and sampled prior to each feeding at 06:15 and 18:15 to determine DM content and daily DM intake (**DMI**).

Two cows were paired in each block, and were randomly assigned one of the two treatments: TAN (Silvafeed BX, Silvateam) or Control (**CON**; n=10). Treatments were administered as a top dress. Control-assigned cows received a top-dress containing 50 g of cornmeal only per feeding, while TAN-assigned cows received a premix top-dress composed of TAN included at a rate of 0.07% of DM plus cornmeal to total 50 g of total top dress. To best target 0.07% of DM for each cow, a categorical "binning" approach was implemented with the TAN top dress premix. The concentration of TAN targeted the average cow for each bin-category, based on the following feed call ranges: 10-14, 15-19, 20-24, and 25-29 kg of feed per feeding. The corresponding bin-category was readjusted for each cow at each feeding, based on her individual feed call.

3.2.3. Enteric Emission Sampling

Two head chambers (**HC**) were used for gas emission sampling - to accommodate two respective cows sampled for gas emission per day in the two HCs, cows were stagger-started onto their treatment. Prior to the start of the experiment each cow was subjected to at least three HC training sessions in order to become accustomed to the area and the HC. Cows underwent baseline emission sampling (day 0) which was used as a covariate within the model, and began receiving their assigned treatments on day 1. Cows were then sampled every 16 d on each of their respective treatment d 16, 32, and 48 on treatment.

Immediately following the morning milking session on their assigned gas emission sampling days, cows were moved to the sampling area and were secured in their respective HC to begin their 12-h emission sampling period. While in the HC, cows were fed 105% of their previous day's intake and had *ad libitum* access to water. Cows were secured in the HC using neck chains similar to a tie-stall system and were able to stand up or lie down during the HC gas emission sampling period. Once the emission sampling period had concluded, cows were moved directly to the milking parlor where they rejoined the other cows within the experimental pen.

Each HC system consisted of a HC manufactured with clear polycarbonate sheeting, blowers which pumped air out of the chamber, a neck sleeve attached to the chamber, which was secured to the animal in order to minimize air leakage, and Teflon tubing for extraction of the emission sample. The Teflon tubing was connected from the HC to a mobile agricultural air quality laboratory (**MAAQL**), which housed all of the necessary analytical air equipment [286]. The HCs in use were validated by Place et al. (2011), and underwent further validation both before and after the conclusion of the present trial [286].

3.2.4. Manure Slurry Emission Sampling

Total urine and feces produced by each cow while secured in the HC were collected and stored for consequent manure slurry gas emission sampling. Urine was collected via manual stimulation of the vulva and feces was collected at each defecation event by the cow.

After the 12-h enteric emission sampling concluded, urine and feces were combined into a manure slurry at a ratio of 1:1.7 (urine wt: feces wt) per cow [340]. The slurry was homogenized for 120 s, and a subsample of 680 g of manure slurry was allocated to a circular ceramic tray (26 cm internal diameter) to undergo manure slurry emission sampling for a 24-h period. Each tray was covered with an OdoFlux flux chamber (**FC**; Odotech Inc. Montreal, Quebec, Canada). The 64.5 L FC was made of acrylic resin and consisted of a cylindrical enclosure with a spherical top. Each FC contained three holes that allowed the samples to remain at constant pressure. The inside circumference of each FC was lined with perforated Teflon tubing to allow for continuous ambient airflow through the system when connected to air pumps, with an additional Teflon tube attached to the top of the FC for extraction of emission sample to the MAAQL. Further details of the OdoFlux FC setup are described in Burgos et al. (2010) [341].

3.2.5. Emission Measurements

The MAAQL was equippeds with air pumps, mass flow controllers, a rotary valve and manifold, a continuous gas analyzer, and a computer system to control sample timing and switching from each chamber and to acquire emission data. Air samples from each HC and FC were transferred through a manifold to an Innova 1412 photo-acoustic multi-gas analyzer (LumaSense Technologies Inc., Ballerup, Denmark) to determine the concentrations of CH_4, CO_2, N_2O, and NH_3. The INNOVA 1412 analyzer had the following minimum detection limits: 0.4 ppm

for CH_4, 1.5 ppm for CO_2, 0.03 ppm for N_2O, and 1.0 ppm for NH_3, and a maximum detection limit of 106 ppm. The HC and FC along with ambient air were sampled for 15 min intervals according to the following sequence, which was repeated continuously: HC-1, HC-2, HC-ambient, HC-1, HC-2, FC-1, FC-2, FC-ambient.

3.2.6. Emission Calculations

To prevent carry-over effects between sampling source, the measured gas concentrations of the outgoing air samples for each 15 min sampling period were truncated, removing the first five minutes and the final two minutes of each sample period. Total flux in mg/h was calculated using the following equations:

$$Total\ HC\ flux\ = \frac{MIX \times FL \times 60}{\mathrm{MV}} \times MW \times Conv$$

$$Total\ FC\ flux = \frac{MIX \times FL \times 60}{V} \times MW \times Conv$$

where *MIX* is the concentration in either ppm or ppb, *FL* is the ambient air flow rate varying from 2300 to 2500 L/min for HCs and 8 L/min for FCs, 60 is the conversion from minute to hour, *MW* is the molecular weight in grams per mole, *Conv* is a conversion factor of 10^{-3} for concentration in ppm and 10^{-6} for concentration in ppb. For the HC, *MV* is 24.04 (liter/mole), the volume of one molar gas at temperature 20 °C. For the FC, *V* is the volume of one molar gas at temperature *T* in liter/mole and is calculated by the following:

$$V = \frac{V_s \times T}{T_s}$$

where *Vs* is the standard volume 22.4 liters at 0 ºC, T_S is the standard temperature 0 ºC that equals to 273.15 K, *T* is the air temperature in K equaling to *T* in ºC + 273.15.

The emission rate by animal heads for the HCs (mg or g/h/head) was calculated by:

$$Emission\ Rate = \frac{Total\ Flux}{number\ of\ animal\ heads}$$

where "number of animal head" is 1 because only one animal was housed in each head chamber at each sampling period. The emission rate by surface for the flux chambers (mg/h/m^2) was calculated by:

$$Emission\ rate = \frac{Total\ Flux}{Surface\ area}$$

where *surface area* was calculated by the following equation:

$$Surface\ area = \pi r^2 = 3.14 \times \left(\frac{26}{2} \times \frac{1}{100}\right)^2$$

where *26* is the diameter of the sample in cm and *100* is the conversion from cm to m.

3.2.7. Milk Sampling and Analysis

Milk yield for each cow was recorded at both the a.m. and p.m. milking sessions each day. Milk was sampled once a week at consecutive a.m. and p.m. milking sessions, and samples were sent for component analysis (Central Counties Dairy Herd Improvement Association, Atwater, CA, USA) to determine milk fat, milk protein, and milk urea N (**MUN**). Energy-corrected milk (**ECM**) was then calculated according to the following equation:

$$ECM = (0.327 \times milk\ kg) + (12.95 \times milk\ fat\ kg) + (7.65 \times milk\ protein\ kg).$$

3.2.8. Rumen Fluid Sampling and Analysis

Samples of rumen fluid were collected from each cow via an oro-ruminal probe [342] at approximately 120 mins after the morning feeding on each cow's respective treatment d 1, 17, 33,

and 49. Once collected, the rumen fluid was passed through a strainer to remove large particles, collected into four 15-mL polypropylene centrifuge tubes per cow, and were flash frozen using liquid N, and stored frozen at -20°C for consequent analyses. Samples from 10 cows (n = 5/treatment) were used to analyze volatile fatty acid (**VFA**) and for ruminal-NH_3 concentrations.

3.2.9. Volatile Fatty Acids Analysis

A sample of rumen fluid from each cow and sample point was used for VFA analyses. After each sample was thawed, samples were homogenized using a vortex for 30 s followed by 5 hand inversions to displace and distribute larger particles; a 1 mL subsamples per sample was then aliquoted into sterile 1.5 mL microcentrifuge tubes. Subsamples were centrifuged for 6 mins at 8000 rpm. The supernatant was retained and filtered through a 0.2 µm filter into HPLC vials, treated with one-fifths volume of 25% metaphosphoric acid, mixed, and stored at 4°C until analyzed. Just prior to GC analyses, samples were diluted by a factor of 5 in DI water. Rumen fluid samples were analyzed in duplicate for acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids (ppm) and additional VFA profile information using the Thermo TriPlus Autosampler and Thermo Trace GC Ultra (Thermo Electron Corporation, Rodano Milan, Italy) which is a Gas Chromatography-Flame Ionization Detection (GC-FID). The conditions of the GC were as follows: analytical column RESTEK Rxi® – 5 ms (30 m × 0.25 mm I.D. × 0.25 µm) film thickness. The temperature of the oven was set to 80°C for 0.50 mins, followed by a ramp rate of 20°C/min until 200°C was reached, which was held for 2 mins. High purity helium served as the carrier gas, and was administered at a 2.0 mL/min flow rate while the FID was held at 250°C. A 1 µL sample was injected through Split/Splitless Injectors with the injector base temperature set to 250°C. Split

flow was programmed to 200 mL/min, and split ratio to 100 mL/min. Certified reference standards (RESTEK, Bellefonte, PA, USA) were used to establish the calibration curves.

3.2.10. Ruminal Ammonia Analysis

A second sample of rumen fluid from each animal and sample point was used to determine ruminal NH_3 concentrations. After samples were homogenized using a vortex for 60 s, 10 mL of rumen fluid were aliquoted into a separate tube and treated with 200 µL of a pH adjusting low-level ionic-strength adjusting buffer solution for NH_3-ion selective electrodes (ORI-951210; Orion® Thermo Scientific, Pittsburgh, PA, USA). Samples were left to acclimate for 2 mins and were then vortexed for 30 s. Ammonia ppm and mV were measured from each ruminal sample in triplicate using an Orion Star^{TM} A214 pH and ISE benchtop meter (STARA2146; Thermo Fisher Scientific).

3.2.11. Data and Statistical Analysis

Gas emissions (GHG and NH_3), productive performance parameters (milk yield and components, and DMI), and ruminal concentrations (NH_3 and VFA) data were analyzed as a randomized complete block design with repeated measures over time, using the "nlme" and "emmeans" packages in R (V4.1.1) [289,343,344] according to the following base model:

$$Y_{ijklm} = \mu + \beta_i + \beta_j + \beta_{k(j)} + \beta_l + \beta_m + (\beta_l \times \beta_m) + \varepsilon_{ijklm}$$

where μ = the overall mean of the response variable; βi = overall mean of day 0 for the response variable; βk = cow (random) which was nested within βj = block (random); βl = treatment; βm = day; $\varepsilon ijklm$ = the error term. Block refers to each pair of parity and days in milk-

matched cows. Model selection for each of the aforementioned parameters was made based on Akaike information criterion, Bayesian information criterion, and log-likelihood [292]. The data for each of the response variables were further verified for assumptions of normality by the Shapiro–Wilk method, with outliers removed accordingly when normality was not met. All means are presented as least squares means (**LSM**) based on "emmeans". Comparisons between LSMs were completed using the "anova" function. Treatment by test day means were compared using Tukey's test pairwise comparisons using "cld" in "multcomp" [345]. Differences were declared significant at $p \leq 0.05$ and a trend toward significance at $0.05 < p < 0.10$.

3.3. Results and Discussion

3.3.1. Gaseous Emissions

Gaseous production quantifies the enteric gas directly emitted by the animal, and is represented by the average amount of gas produced per hour during the sampling period; Least squares means for production of greenhouse gases and NH_3 are shown in Table 3.3. In the present trial, both enteric CH_4 and CO_2 production tended to be lower in TAN- vs. CON- supplemented cows (Figure 3.1). To our surprise, enteric N_2O production was lower in TAN- vs CON-supplemented cows, while enteric NH_3 production tended to be higher (Figure 3.1). Though average CH_4 and N_2O production significantly differed across test days ($p < 0.043$), the interaction of treatment and test day was not found to be significant in this analysis. The interaction of treatment and test day was also non-significant for enteric CO_2, and NH_3 production.

The literature detailing the impact of quebracho and chestnut tannins on enteric GHG and NH_3 production in dairy cows is limited. No effect on CH_4 production was noted in experiments by

Aboagye et al. (2018) in beef cattle and Adejoro et al. (2019) in sheep supplemented with quebracho and chestnut tannins (Bypro) [335,346]. Through *in vitro* experiments where rumen fluid sourced from rumen-fistulated cows was treated with tannins and saponins sourced from soapberry fruit-mangosteen, Poungchompu et al. (2009) noted decreased CH_4 production and protozoal population; the latter consequently resulted in a decrease in the proportion of methanogens present [131]. Likewise, *in vitro* experiments conducted by Chen et al. (2021) demonstrated reduced CH_4 production (mL/g) when supplementing ruminal fluid with chestnut tannin alone or combined with quebracho tannin at doses ranging between 2-5% of DM [121]. Though one of the primary objectives of our present experiment was to determine if a low dosage levels of the tannin and saponin blend would lead to favorable effects on CH_4 emissions, it is possible that more pronounced effects could have been seen if cows were maintained on the treatment for a longer period of time. For instance, Duval et al. (2016) noted decreased CH_4 production (g/d) at 90 d but not on 45 d on treatment when supplementing dairy cows with Bypro. They additionally saw no treatment or test day differences for both N_2O and NH_3 production [213].

Gaseous yield is a measure of the enteric gas produced for a given quantity of DM consumed, estimated using the DMI (kg) consumed throughout the duration of the enteric emission measurement period (12-h DMI). No treatment-related differences were noted in the present trial for CH_4, CO_2, N_2O, or NH_3 yield (Figure 3.2). Though this was the case, gaseous yield decreased significantly over time in TAN-supplemented cows for each of the four gases measured (Table 3.3). To this end, in TAN-supplemented cows, CH_4 yield was 20.5% lower on d 48 vs. d 16 on treatment, suggesting that there may be longer-term impacts of supplementation. There is not a clear consensus in literature regarding the effects of tannin supplementation on gaseous yield. Similar to our findings, Duval et al. (2016) saw no impact on CH_4, N_2O, and NH_3 yield when

supplementing cows with either 0.45% or 1.8% of DM with Bypro [213]. By contrast when supplementing beef steers fed a high forage diet with 1.5% of DM of a quebracho and chestnut tannin blend, Aboagye et al. (2018) found that CH_4 yield tended to decrease by 6.4% without affecting overall DMI (kg/d) [335]. A meta-analysis of 70 publications where tannins from varying sources were supplemented to ruminants found that tannins have more profound impact on CH_4 yield as inclusion level increased [111]. This suggests that higher inclusion levels of TAN may lead to favorable results with respect to gaseous yield. In the present trial, cows supplemented with TAN tended to have lower DMI during the emission sampling period (Table 3.5), which could critically affect and skew the calculations for yield as well as the consequential interpretation. Through work with sheep, Robinson et al. (2014) noted that the DM consumed by sheep up to 48 hours prior to emission measurements still had an impact on CH_4 emissions on the sampling day. Though the degree to which previous days intake impact the gas produced by an animal is unclear for cattle [347], it is apparent that yield as calculated here does not capture the complete effect of DMI on CH_4 produced.

Gaseous intensity is a measure of enteric gas produced for a given quantity of productive output from the cow, which in this case was indicated by energy-corrected milk (kg) from the milking session immediately following the enteric emission measurement period (evening milking). Least squares means for CH_4, CO_2, N_2O, and NH_3 intensity are shown in Table 3.3. In the present trial, no treatment-related differences were found between TAN- and CON-supplemented cows for enteric CH_4, CO_2, N_2O, or NH_3 intensity (Figure 3.3). Likewise, the effect of test day and the interaction between treatment and test day were not different for each of the four gases measured in the present trial (Table 3.3). Duval et al. (2016) similarly saw no treatment differences in CH_4 and N_2O on a kg milk basis. For NH_3 intensity, Duval et al. (2016) noted an

increase in the control group over time, and a slight decrease at 45 d on treatment in the group supplemented with quebracho and chestnut tannin (Bypro) at 1.8% of DM [213].

Slurry-related CH_4, N_2O, and CO_2 emissions were similar between TAN- and CON-supplemented cows in the present trial (Table 3.4). Contrary to our hypothesis and what was found in the literature [348], TAN-supplemented cows had greater slurry NH_3 emissions than the control-supplemented cows in our present findings (p = 0.005; Figure 3.4). The effect of test day was significant for slurry N_2O emissions (p = 0.0003), but was not significant for the other three gases measured. The interaction between treatment and test day was non-significant for slurry CH_4, CO_2, N_2O, and NH_3 emissions (Table 3.4). When supplementing dairy cows with Bypro, Duval et al. (2016) noted a similar trend to ours in CH_4, N_2O, and NH_3. Though NH_3 emissions in the dairy barn of Bypro supplemented cows was greater than that of the control supplemented cows at 45 d on treatment, they noticed a decline in emissions after 90 d on treatment [213]. Building upon this observation of time on tannin supplementation, the reduced manure NH_3 emissions expected with tannin supplementation might have been apparent if our study had been carried out for a longer-term.

3.3.2. Productive Performance

No treatment-related differences were found between TAN- and CON-supplemented cows for ECM, MUN, and for average DMI in the present trial (Table 3.5). Though no treatment differences were found for milk fat and milk protein yield and percentage, milk fat yield and milk protein percentage had significant test day effects (p = 0.093 and $p < 0.0001$, respectively). The interaction between treatment and test day was significant for MUN (p = 0.019) but was not significant for the other productive performance parameters (ECM, DMI, milk fat yield and percentage, and milk

protein yield and percentage). The ECM results in the present study are consistent with others in literature in which lactating cows were also supplemented with quebracho and chestnut tannin [115,334]. In contrast, there is no consensus for the impacts of quebracho and chestnut tannin supplementation on milk protein concentration, milk protein yield, and MUN concentration. For example, while Aguerre et al. (2016) noted decreased milk protein concentration, milk protein yield, and MUN in quebracho and chestnut tannin supplemented cows at inclusion levels of 0.90 and 1.8% of DM [334], Menci et al. (2021) saw no differences in each of the aforementioned parameters in cows supplemented at a rate of 150 g/head [115].

There was no effect of treatment on feed efficiency in the present study (Table 3.5). As previously mentioned, tannins are generally regarded as anti-nutritional; when fed at too high of a concentration, tannins may reduce palatability and digestibility thereby decreasing DMI. There is little agreement in the literature as to what the optimal inclusion level would be to reduce environmental impacts without negatively impacting feed intake and productive performance of cattle. For instance, while Aboagye et al. (2018) saw no effect on DMI and feed efficiency when supplementing cattle with quebracho and chestnut tannin at a rate of 1.5% of DM. In contrast, Aguerre et al. (2016) noted a decrease in DMI and an increase in feed efficiency when supplementing with quebracho and chestnut tannin at a rate of 0.90 and 1.8% DM [334,335]. Though inconsistencies are noted, it is apparent that higher inclusion levels of tannin are predominately more astringent and result in feed intake and palatability concerns [134]. Our present findings confirm that supplementing TAN at a very low inclusion level has no negative impacts on DMI and therefore leads to fewer issues in astringency and palatability of the feed.

3.3.3. Ruminal Analyses

Ruminal NH_3 was similar between treatments ($p > 0.05$; Table 3.6) and across test days (Table 3.9) in the present trial. Due in part to their strong affinity for binding proteins, tannins are known for their ability to reduce the rate of protein degradation in the rumen. This would lead us to expect cows supplemented with tannin to have reduced ruminal NH_3, conditions which has been demonstrated through numerous research experiments [349,350]. Results from a meta-analysis focusing on the effect of tannins on N-partitioning in lactating dairy cows found ruminal NH_3 to be highly influenced by dose quantity (g/d), dose concentration (g/kg DMI), and the forage-to-concentrate ratio of the diet [141]. Aboagye et al. (2018), Aguerre et al. (2016) and Dschaak et al. (2011) noted reduced ruminal NH_3 concentrations when supplementing cattle with between 0.25-3% of DM of quebracho or chestnut tannins or a combination of the two [334,335,351]. In addition to what was discussed in sections above regarding NH_3 emissions, it is possible that the addition of saponins in the feed additive might play a role in the levels of NH_3 noted, as impacts on ruminal NH_3 are heavily influenced by the saponin source [177].

Total VFA concentrations were not different between TAN- vs. CON-supplemented cows in the present study (Table 3.7). For the individual VFA concentration, isobutyric acid tended to be lower in TAN- vs. CON-supplemented cows in the present study, whereas there were no treatment-related differences for any of the remaining VFAs. There was likewise no difference between treatment-groups with respect to the acetate-to-propionate ratio (**A:P**; Table 3.7). The effect of test day was significant only for isobutyric, valeric, and isovaleric acid concentrations (Table 3.8). The interaction between treatment and test day tended to be significant for total VFA ($p = 0.078$), acetic acid ($p = 0.072$), and propionic acid ($p = 0.081$) concentrations.

Our findings with respect to total VFA concentration, acetic acid, propionic acid, and A:P are consistent with that of the literature [335]. The present discovery regarding isobutyric acid, supports those of Aguerre et al. (2016) who supplemented lactating dairy cows with a similar additive [334]. Based on the literature, it is unclear what the expected impact of tannin supplementation would be on butyric acid, valeric acid, and the two studied iso-acids. For example, Aboagye et al. (2018) found that valeric and isovaleric acids tended to decrease with Bypro supplementation [335], and Norris et al. (2020) observed an increase in propionic acid and a consequential reduction in A:P with quebracho tannin supplementation [332]. Through a meta-analysis focused on the effects of tannins on ruminal fermentation, Jayanegara et al. (2012) found that with increasing tannin inclusion levels (range: 0-250 g/kg DM) total VFA concentrations decreased, both A:P and acetic acid tended to decrease, and propionic acid tended to increase *in vitro* [137]. When investigating the effects of tannin supplementation through *in vivo* experiments, Jayanegara et al. (2012) saw no significant relationship between tannins and any of the VFA variables with increasing dietary tannin levels (range: 0-177 g/kg DM) [137]. Branched chain VFAs, isobutyric and isovaleric acids, are anticipated end product of feed amino acid deamination by microbes including ruminal protozoa or proteolytic bacteria. It is suspected that the mode of action of supplemental tannins and saponins was primarily through inhibiting the growth of ruminal protozoa. Ruminal methanogens benefit from a symbiotic relationship with protozoa, as they can gain access to the hydrogen that the protozoa produce [107]. We would therefore postulate that the trend towards decreased isobutyric acid is associated with restricted growth of ruminal protozoa.

3.4. Conclusion

The present experiment assessed the ability of a commercially available blend of quebracho and chestnut tannins plus saponins (SilvaFeed BX) to mitigate GHG and NH_3 emissions and to modulate cow productive performance when supplemented to early- to mid-lactation Holstein dairy cows at a low inclusion level (0.07% of DM). Supplemental-TAN tended to decrease enteric CH_4 production and CO_2 production by 6.5% and 5.6%, respectively, and to significantly decrease N_2O production by 9.6%. This suggests that tannins have the potential serve to as a viable tool for on-farm GHG mitigation for producers, even when included at low dietary concentration within livestock rations. The enteric GHG mitigation potential was not offset by an increase in slurry GHG emissions, though NH_3 emissions did significantly increase with TAN inclusion to the diet. Productive performance of lactating dairy cows did not change when TAN was supplemented at the low dose of 0.07% of DM; we therefore recommend that future experiments explore higher inclusion levels. To our knowledge, this experiment is the first to assess the impacts of feeding this particular tannin and saponin blend. Therefore, more research with this specific additive is needed in order to build a sound body of literature. Future experiments should also investigate the duration of feeding tannins in lactating dairy cattle.

Table 3.1. Total mixed ration (TMR) formulation. All cows were adapted to the control diet for 30 days prior to the start of the trial.

TMR Ingredients (%, As fed)	
Concentrate mix	41.54
Alfalfa hay, chopped	30.77
Wheat hay, chopped	9.62
Cottonseed, whole linted	7.69
Almond hulls	7.69
Mineral	1.15
Energy III	1.23
Strata	0.08
Salt	0.23
% Concentrate Mix	
Steam-flaked corn	39.15
Wheat mill run	22.44
Distillers' grains, dried	14.63
Beet pulp, shredded	14.11
Soybean meal	6.48
Wheat, ground	1.85
Molasses, cane	1.33

Table 3.2. Composition of total mixed-ration (TMR) fed.

Composition	
% Dry Matter	
Crude protein	17.0
Acid detergent fiber	24.7
Neutral detergent fiber	35.9
Total digestible nutrients	67.5
Ash	8.02
Crude fat	4.44
Calcium	0.81
Phosphorous	0.42
Magnesium	0.52
Potassium	1.57
Sodium	0.26
Parts Per Million	
Iron	391
Manganese	62
Zinc	45
Copper	13

Table 3.3. Least squares means (LSM) and standard error means (SEM) of enteric gaseous production, yield, and intensity for Holstein dairy cattle fed Silvafeed BX (TAN) vs the control (CON) over the 3 treatment periods measured using head chamber (n = 10 per treatment).

	Head Chamber LSM						SEM	*P-value*		
	CON Day 16	**TAN Day 16**	**CON Day 32**	**TAN Day 32**	**CON Day 48**	**TAN Day 48**		**Trt**	**Day**	**Trt × Day**
Enteric Gas Production[1]										
CH_4 (g)	16.1	15.8	15.8	14.2	15.6	14.6	0.48	**0.07**	***0.043***	0.24
N_2O (mg)	60.7 ^{a}	58.0 ^{ab}	56.6 ^{abc}	50.4 ^{cd}	52.2 ^{bcd}	45.5 ^{d}	1.98	***0.032***	***<.001***	0.36
CO_2 (g)	454.9	448.3	460.8	425.8	464.3	431.8	12.0	**0.09**	0.65	0.22
NH_3 (mg)	31.2	41.0	36.7	43.1	39.0	41.9	3.99	**0.07**	0.29	0.52
Enteric Gas Yield[2]										
CH_4 (g)	1.25 ^{ab}	1.45 ^{a}	1.26 ^{ab}	1.40 ^{ab}	1.21 ^{ab}	1.18 ^{b}	0.08	0.34	***0.007***	**0.07**
N_2O (mg)	5.42 ^{ab}	6.42 ^{a}	5.43 ^{ab}	6.56 ^{a}	5.21 ^{ab}	5.20 ^{b}	0.39	0.13	***0.014***	0.10
CO_2 (g)	34.99 ^{ab}	41.70 ^{a}	35.13 ^{ab}	40.10 ^{ab}	33.67 ^{ab}	33.76 ^{b}	2.20	0.15	***0.010***	**0.09**
NH_3 (mg)	3.86 ^{ab}	5.39 ^{a}	3.99 ^{ab}	5.08 ^{a}	3.77 ^{ab}	4.14 ^{b}	0.38	**0.07**	***0.003***	***0.017***
Enteric Gas Intensity[2]										
CH_4 (g)	0.77	0.72	0.74	0.75	0.78	0.76	0.03	0.48	0.54	0.32
N_2O (mg)	3.33	3.14	3.17	3.28	3.34	3.31	0.12	0.78	0.52	0.30
CO_2 (g)	21.70	20.40	20.72	21.33	21.76	21.51	0.78	0.72	0.54	0.31
NH_3 (mg)	2.63	2.41	2.54	2.53	2.61	2.52	0.09	0.35	0.81	0.32

[1] Production = average gas produced (g or mg) per h; [2] Yield = gas produced per h × (1/kg dry matter intake from the sampling period while in the head chamber); [3] Intensity = gas produced per h × (1/kg energy-corrected milk from the afternoon milking session).

Table 3.4. Least squares means (LSM) and standard error means (SEM) of the slurry gaseous emissions for Holstein dairy cattle fed Silvafeed BX (TAN) vs the control (CON) over the 3 treatment periods, measured using flux chambers over 24 hours (n = 10 per treatment).

	Flux Chamber LSM						SEM	*P-value*		
	CON Day 16	**TAN Day 16**	**CON Day 32**	**TAN Day 32**	**CON Day 48**	**TAN Day 48**		**Trt**	**Day**	**Trt × Day**
CH_4 (mg/h/m^2)	28.21	29.36	23.76	26.77	26.46	24.59	2.27	0.69	0.24	0.56
N_2O (mg/h/m^2)	1.66 ^{ab}	1.26 ^{a}	1.79 ^{ab}	1.89 ^{ab}	2.26 ^{b}	2.21 ^{b}	0.18	0.61	***<.001***	0.34
CO_2 (mg/h/m^2)	1513.02	1653.94	1409.70	1508.68	1451.13	1527.27	103.84	0.27	0.42	0.94
NH_3 (mg/h/m^2)	275.59 ^{ab}	343.48 ^{ab}	259.49 ^{a}	357.79 ^{b}	305.62 ^{ab}	330.95 ^{ab}	20.16	***0.005***	0.83	0.13

Period = 24 h slurry gas emission sampling period

Table 3.5. Least squares means (LSM) and standard error means (SEM) of average daily energy-corrected milk (ECM) yield, milk fat yield, milk fat concentration (%), milk protein yield, milk protein concentration (%), milk urea nitrogen (MUN) concentration, average daily dry matter intake (DMI), head chamber DMI, and feed efficiency for Holstein dairy cattle fed Silvafeed BX (TAN) vs the control (CON) over the 3 treatment periods (n = 10 per treatment).

	Performance Parameters LSM						**SEM**	***P-value***		
	CON Day 16	**TAN Day 16**	**CON Day 32**	**TAN Day 32**	**CON Day 48**	**TAN Day 48**		**Trt**	**Day**	**Trt × Day**
ECM[1] (kg)	58.20	59.18	59.46	56.40	54.60	56.52	2.13	0.98	0.16	0.30
HC ECM[2] (kg)	24.26	24.92	25.62	23.83	24.30	23.81	0.83	0.53	0.65	0.28
Fat yield (kg)	3.13	3.15	3.18	2.96	2.87	2.96	0.14	0.80	0.09	0.30
Protein yield (kg)	1.92	1.91	2.00	1.87	1.89	1.88	0.06	0.37	0.65	0.39
Fat composition (%)	4.87	5.13	4.81	4.97	4.75	4.98	0.16	0.32	0.18	0.78
Protein composition (%)	3.08 [ab]	3.04 [ac]	3.10 [ab]	3.10 [abcd]	3.18 [cd]	3.16 [bc]	0.04	0.74	***<.001***	0.63
MUN (mg/dL)	11.12 [ab]	12.02 [ab]	11.09 [ab]	11.47 [a]	10.87 [ab]	12.09 [b]	0.38	0.13	0.13	***0.019***
DMI[3] (kg)	27.93	28.59	28.17	28.28	27.92	28.33	0.35	0.38	0.80	0.41
HC DMI (kg)	15.33	12.93	14.96	12.54	15.40	15.02	0.80	**0.09**	***0.018***	**0.08**
Feed efficiency[4]	2.09	2.08	2.10	2.00	1.95	2.00	0.06	0.72	0.13	0.38

[1] Average daily total ECM (AM+PM milking sessions); [2] ECM from the PM milking session immediately following the emission sampling period; [3] Excludes DMI from when the cow was secured in the head chamber; [4] kg daily ECM/kg daily DMI

Table 3.6. Least squares means (LSM) and standard error means (SEM) of ruminal ammonia (rumen-NH_3) concentration (ppm) and conductivity (mV) for Holstein dairy cattle fed Silvafeed BX (TAN) vs the control (CON) (n = 10 per treatment).

	Treatment LSM		**SEM**	***P-value***
	CON	**TAN**		
Rumen-NH_3 (ppm)	271.02	320.12	24.18	0.22
Rumen-NH_3 (mV)	1.30	-0.50	0.83	0.19

Table 3.7. Least squares means (LSM) and standard error means (SEM) of total volatile fatty acid (VFA) concentration, acetic acid concentration, propionic acid concentration, acetate-to-propionate (A:P) ratio, butyric acid concentration, isobutyric acid concentration, valeric acid concentration, and isovaleric acid concentration for Holstein dairy cattle fed Silvafeed BX (TAN) vs the control (CON) over the 3 treatment periods (n = 10 per treatment).

	Ruminal Fluid LSM						**SEM**	***P-value***		
	CON Day 16	**TAN Day 16**	**CON Day 32**	**TAN Day 32**	**CON Day 48**	**TAN Day 48**		***Trt***	***Day***	***Trt* × *Day***
Total VFA (ppm)	1223.34	1323.27	1302.10	1088.89	1252.86	1170.77	71.96	0.42	0.46	**0.08**
Acetic acid (ppm)	548.69	594.33	608.55	468.83	585.15	525.65	42.58	0.31	0.68	**0.07**
Propionic Acid (ppm)	258.20	275.94	278.18	226.34	270.34	243.46	17.98	0.37	0.59	**0.08**
A:P ratio	2.11	2.17	2.17	2.11	2.15	2.17	0.06	0.94	0.90	0.50
Butyric acid (ppm)	184.61	196.39	190.69	162.61	181.91	172.39	9.93	0.42	0.26	0.13
Isobutyric Acid (ppm)	73.70 [a]	70.16 [ab]	70.12 [ab]	68.89 [ab]	67.80 [b]	67.93 [b]	1.03	**0.07**	***0.002***	0.28
Valeric Acid (ppm)	89.93	88.81	88.67	82.67	84.37	82.19	1.86	0.15	***0.005***	0.26
Isovaleric Acid (ppm)	75.50	76.31	73.12	72.80	70.56	72.42	1.29	0.55	***0.013***	0.69

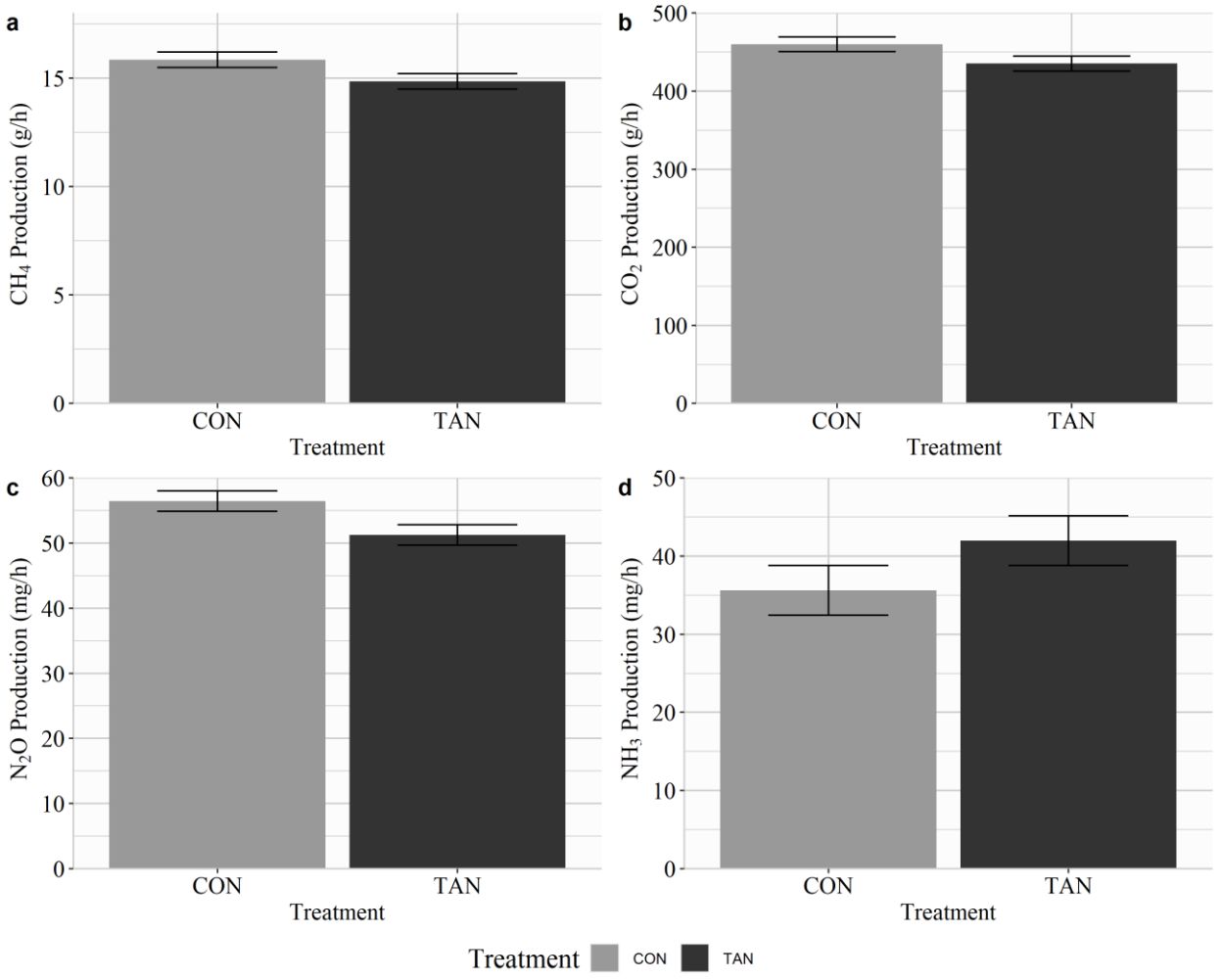

Figure 3.1. Enteric gaseous production of (a) methane (CH_4; g/h), (b) carbon dioxide (CO_2; g/h), (c) nitrous oxide (N_2O; mg/h), and (d) ammonia (NH_3; mg/h) from Holstein dairy cattle fed Silvafeed BX (TAN) vs. untreated control (CON) (n = 10 per treatment) measured using head chambers.

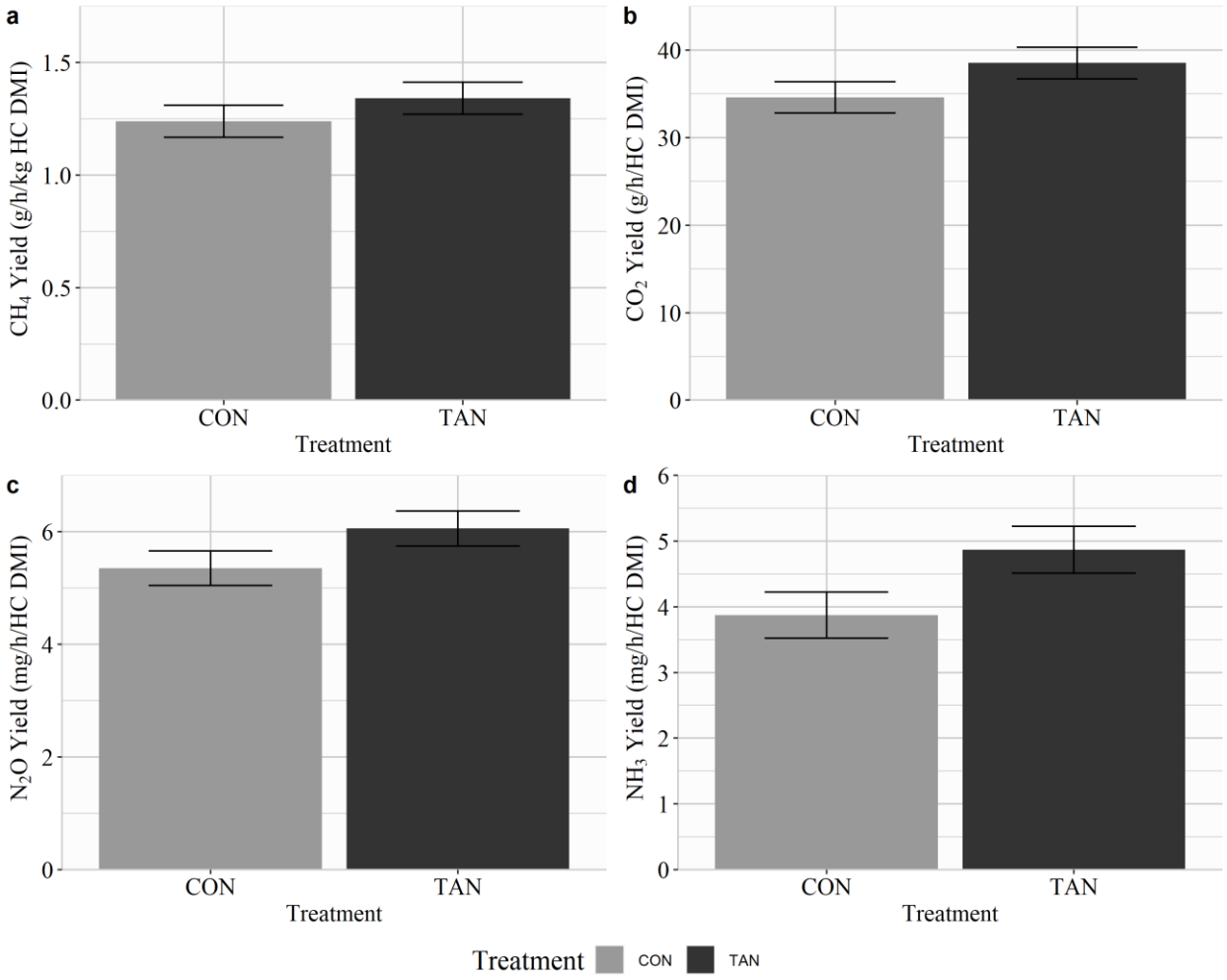

Figure 3.2. Enteric gaseous yield of (a) methane (CH_4; g/h/HC DMI), (b) carbon dioxide (CO_2; g/h/HC DMI), (c) nitrous oxide (N_2O; mg/h/HC DMI), and (d) ammonia (NH_3; mg/h/HC DMI) from Holstein dairy cattle fed Silvafeed BX (TAN) vs. untreated control (CON) (n = 10 per treatment). Emissions were measured using head chambers, and dry matter intake (DMI) corresponds to the feed consumed while in the head chambers.

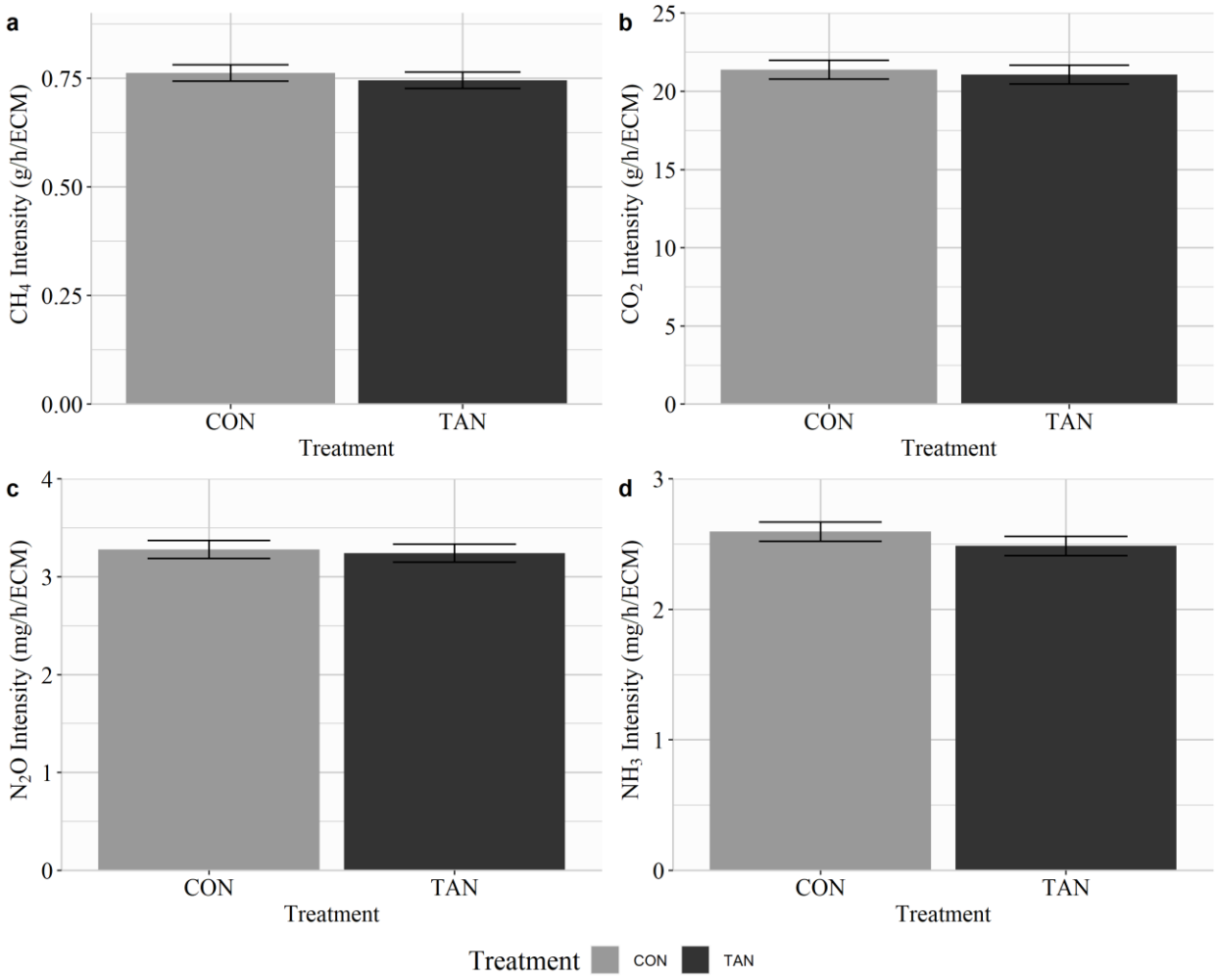

Figure 3.3. Enteric gaseous intensity of (a) methane (CH_4; g/h/PM ECM), (b) carbon dioxide (CO_2; g/h/ PM ECM), (c) nitrous oxide (N_2O; mg/h/ PM ECM), and (d) ammonia (NH_3; mg/h/ PM ECM) from Holstein dairy cattle fed Silvafeed BX (TAN) vs. untreated control (CON) (n = 10 per treatment). Emissions were measured using head chambers, and energy corrected milk (ECM) corresponds to the milk yield at the afternoon milking session immediately following enteric emission measurements.

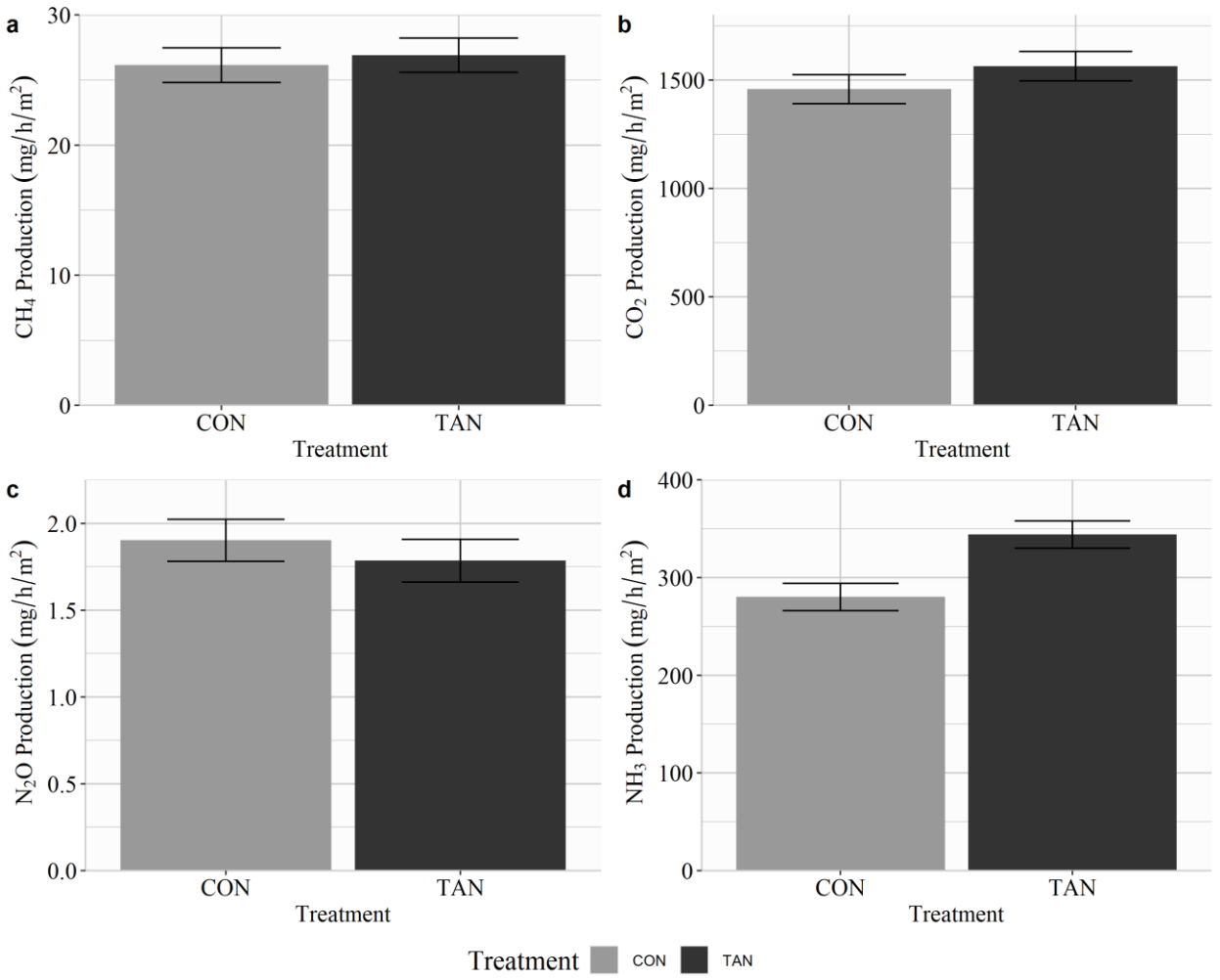

Figure 3.4. Slurry gaseous production of (a) methane (CH_4; mg/h/m^2), (b) carbon dioxide (CO_2; mg/h/m^2), (c) nitrous oxide (N_2O; mg/h/m^2), and (d) ammonia (NH_3; mg/h/m^2) from Holstein dairy cattle fed Silvafeed BX (TAN) vs. untreated control (CON) (n = 10 per treatment). Slurry for each cow was produced by combining urine and feces at 1:1.7 (urine wt:feces wt); gaseous emissions were measured for 24 h using flux chambers.

CHAPTER 4: EXAMINING A BIOLOGICAL APPLICANT FOR ITS ABILITY TO REDUCE GASEOUS AND DISSOLVED AMMONIA FROM ANAEROBIC DIGESTER EFFLUENT

Abstract

Anaerobic digesters (**AD**) are gaining in momentum due to their unrivaled ability to convert organic waste including food waste and animal manure into biogas. However, effluent from ADs is high in nitrogenous content, which can lead to environmental issues that can affect air quality. Here we investigate the ability of a commercial wastewater applicant (BiOWiSH® AQUA; BiOWiSH Technologies Inc., Cincinnati, Ohio) to reduce ammonia (**NH_3**) and greenhouse gas (**GHG**) emissions from aerated effluent of anaerobic digesters. Effluent from an AD was homogenized and distributed equally between 18 steel drum barrels which were placed in a 6x3 (row x column) grid. Treatments were a positive control (**PC**; aeration but no BiOWiSH), negative control (**NC**; no aeration and no BiOWiSH), and the BiOWiSH treatment (BiOWiSH and aeration; n = 6). Treatments were applied according to a replicated Latin Square design, with each treatment duplicated in each row. Gaseous emissions were measured continuously across 56 days, with each column being measured for 24 h every 3 d. Ammonia emissions were similar between BiOWiSH vs. PC-drums. Drums treated with both BiOWiSH and PC had higher NH_3 emissions than that of the NC-drums. Likewise, though NC-drums significantly differed from each of the other two treatments, there were no differences between BiOWiSH- and PC-treated drums in dissolved effluent nitrogen (**N**) parameters including total-N, total ammoniacal-N, ammonia-N, and nitrate-N. Future research should assess the applicant at varying dosage levels.

Keywords: ammonia; nitrogen; methane; greenhouse gas; biowish; anaerobic digester; digestate; effluent; emissions; sustainability; applicant

4.1. Introduction

California is the top agriculture producing state in the United States, occupying 13.5% of U.S. agricultural cash receipts in 2020 [352]. The state leads the nation in the production of numerous agricultural commodities including but not limited to dairy, almond, grape and pistachio production and sales [352]**.** The USDA estimated that 30-40% of the available food at the consumer and retail level in the United States was ultimately wasted [353,354]. Nearly 6 million tons of food is wasted annually in California [355]. Food waste generally enters landfills, accounting for nearly 25% of the municipal solid waste that was being landfilled in 2018 throughout the United States [356]. Alarmingly, landfills constituted the 3rd largest source of methane (**CH_4**) emissions within the United States, accounting for an estimated 17% of CH_4 emissions [10]. Beyond this, livestock waste poses challenges with respect to manure nutrient management; for instance, dairy waste was estimated to contain an average of 1.5 kg of phosphorous (**P**) and 7.6 kg of nitrogen (**N**) per ton of manure generated, as is [357]. Additionally, livestock manure accounts for 10% of CH_4 emissions in the United States.

Recent California legislation (Senate Bill 1383) is urging businesses and residents to divert organic food waste from landfills and additionally calls for a reduction in dairy methane by 40% by the year 2030 as compared with 2013 values [9]. Food waste and livestock manure can be diverted to anaerobic digesters (**AD**) – through promoting the anerobic digestion of nutrients within the waste, the system capitalizes on the generation of biogases, capturing them to be used as a source of renewable energy. Ebner et al. (2015) estimated a 71% reduction in GHG emissions when food waste and manure were diverted to AD systems in contrast to conventional methods of treatment or storage [358].

Organic waste, including municipal food waste, contain a good balance of nutrients needed to produce high levels of CH_4 biogas and volatile solids destruction through the AD process [359]. Though AD substantially reduces both CH_4 and carbon dioxide (**CO_2**) emissions, ammonia (**NH_3**) and ammonium (**NH_4^+**) are known inhibitors of the methanogenic process and are therefore suppressed in the AD process by carefully controlling the pH [360]. This results in high concentrations of total ammoniacal nitrogen (**TA-N**; either NH_3 or NH_4^+) to be present within AD effluent (**ADE**) [361]. Ammonia can be toxic to aquatic organisms and can lead to eutrophication [362] and when volatilized plays a critical role in atmospheric particulate matter formation. Particulate matter results in negative impacts to both public and environmental health [363,364]. Although TA-N can be removed in a recoverable form from ADE through physiochemical processes such ammonia stripping or membrane filtration [365,366], these strategies may be cost and labor intensive, and may yield nitrogen gas and nitrous oxide (**N_2O**), a greenhouse gas that can lead to further climate impacts [361]. Total ammoniacal-N can also be removed from ADE through biological processes such as through anammox based processes (i.e., ELAN®) or nitrification/denitrification [367–369].

Likewise, though the general purpose of anaerobic digestion is to trap and recover CH_4 as a form of biogas, as much as 50% of the CH_4 produced can escape the system in the effluent fraction in the form of dissolved CH_4 [370]. Effluent storage is a known source of GHG emissions, due to the high concentration of OM remaining [371–373]. Physiochemical strategies have been explored for the removal of CH_4 from ADE, including CH_4 stripping followed by combustion and down-flow hanging sponge reactors [374], though they too are regarded as time- or cost-intensive strategies. Aeration was also explored as a plausible strategy for further stripping CH_4 and NH_3 from the effluent [375,376].

Here we investigate a novel biological approach of NH_3 removal from ADE. BiOWiSH® Aqua (BiOWiSH® Technologies, Cincinnati, OH, USA) is a biological applicant, which claims to reduce ammonia and nitrates and biological oxygen demand, thereby reducing the need for chemically-based applicants in the elimination processes [377]. Though BiOWiSH® Aqua (hereinafter referred to as **BiOWiSH**) is commercially available, there is only one known peer-reviewed publication that has investigated the applicant's impact on wastewater [378]. The objective of the present experiment was to investigate BiOWiSH for its impact on GHG (CH_4, N_2O, and CO_2) and NH_3 emissions, and nitrogenous contents in ADE. We hypothesize that applying BiOWiSH in combination with aeration will reduce both CH_4 and NH_3 emissions originating from ADE.

4.2. Materials and Methods:

4.2.1. Experimental Design & Set Up

The present study was conducted within an environmentally controlled cattle pen enclosure (**CPE**) located at the University of California Davis Feedlot Facility. The CPE was semi-permanent (L: 22.0 m, W: 11.3 m, maximum H: 6 m) hoop-house shaped structure, constructed with a steel frame (11 m Legend Series Cover-All Building, Saskatoon, Saskatchewan, Canada; Fig. 3.1) and covered with a double stacked Dura-Weave cover (Intertape Polymer Group, Montreal, Quebec, Canada). The enclosure was equipped with a roll up door and fans which allowed for constant air flow within the study site. The use of the CPE allowed for continual control and monitoring of the ambient environmental conditions throughout the duration of the experiment. Within the CPE, 18 208 L open top steel drums (D: 0.57 m, H: 0.88 m; Uline, Pleasant Prairie, WI) were configured in a replicated 3 × 3 Latin Square (2 repetitions; Figure 4.1) resulting

in n=6 per treatment. The drums were arranged into 3 rows of 6 drums, with 1.25 m of separation between each neighboring drum in order to diminish the possibility of carry-over emissions from adjacent drums.

Effluent was collected from the final tank of the AD system located at the University of California, Davis, Renewable Energy Anaerobic Digester (READ; Davis, CA, USA) over 3 consecutive days. The AD was comprised of a stainless-steel tank high solids system with the capability of processing 50 tons of organic waste on a daily basis. Once the effluent was collected, it was transported to the study site within the CPE. The ADE was homogenized, weighed, and distributed equally amongst the six steel drums within a single row on each day to total 75.7 L (20 gallons) per drum, and homogenized using a paint mixer for 60 s. Samples of effluent were collected from each of the drums, and re-constituted treatments were applied to the effluent according the methods outlined in the following section. In order accommodate for the 6 flux chambers, the start dates and gas sampling days were staggered by rows (i.e., drums 1-6 were in row 1), with each row of drums being sampled for emissions on d 1, 4, 8, 11, 15, 18, 22, 25, 29, 32, 36, 39, 43, 46, 50, 53 (Figure 4.1). Day 1 was both the effluent collection day and the start of treatment application.

4.2.2. Treatment Preparation & Application

Treatments included: (1) the experimental treatment, which was aerated and received BiOWiSH® Aqua (BiOWiSH® Technologies, Cincinnati, OH, USA), (2) the positive control (**PC**) which was aerated and received water in place of BiOWiSH, and (3) the negative control (**NC**) which was not aerated and received water in place of BiOWiSH. One hour prior to the application, 500 g of packed BiOWiSH were diluted in 2.0 L of water to produce a re-constituted

stock solution of BiOWiSH for consequential application. Beginning on d 1 of the trial, 15.4 mL of the stock solution were applied to the BiOWiSH-drums, and 15.4 mL of water were applied to the NC and PC-drums. The BiOWiSH treatment was reapplied to the drums every 7 days on respective study d 8, 15, 22, 29, 36, 43, and 50. All BiOWiSH and PC drums underwent continuous aeration throughout the duration of the trial, which was supplied using a Commercial Air Pump (ACO-102; VivoSun, Ontario, CA, USA) with 1750 GPH output pumping 0.44 psi of air into each drum. Aeration was delivered to the bottom of each BiOWiSH- and PC-drum through rubber cylindrical fine-bubble diffusers (0.05 m diameter × 0.21 m length).

4.2.3. Liquid and Emission Sampling

A 250 mL sample of liquid ADE was sampled from each of the drums on a weekly basis, immediately preceding the weekly treatment application. Samples were sent to a commercial laboratory for chemical analysis to determine moisture (%), total nitrogen (**TN**; mg/L), TA-N (mg/L), ammonia nitrogen (**NH_3-N**; mg/L), ammonium nitrogen (**NH_4-N**; mg/L), and nitrate nitrogen (NO_3-N; mg/L) (JM Lord, Inc., Fresno, CA, USA). Temperature, pH, dissolved oxygen (**DO**) and oxidation reduction potential (**ORP**) were measured from each of the drums on a daily basis.

Following the effluent sampling and daily observation measurements, treatments were then applied every 7 days to row of six drums that were going to begin their 24 h emission sampling period. In preparation for emission sampling, a plywood sheet with a 0.381 m (15”) diameter hole cut out of the middle was placed over the drums; a gasket made of weather stripping was used in between the drum and plywood in order to minimize the fugitive escape of gases. On top of the plywood was placed an OdoFlux flux chambers (Odotech Inc. Montreal, Quebec, Canada), which

was used to sample emissions. Drums that were not being sampled for emissions were covered with a loose-fitted lid in order to minimize the loss of water due to aeration and evaporation and to prevent the entry of any foreign debris.

The flux chambers were comprised of a 64.5 L volume cylindrical enclosure with a spherical top constructed of acrylic resin. A small hole located at the top of the sphere allowed for constant pressure to be maintained within the chamber. The inside of each flux chamber was lined with perforated Teflon tubing allowing for continuous ambient airflow. The flux chambers were sampled in sequence for 15 min each, followed by an ambient air sampling which was used to determine net emissions for each of the drums. Teflon tubing attached to the top of the flux chambers served as the point of emission sample extraction from the chambers to the nearby mobile agricultural air quality laboratory (**MAAQL**).

The MAAQL housed mass flow controllers, air pumps, a rotary valve and manifold, and a continuous gas analyzer. The concentration of CH_4, CO_2, N_2O, and NH_3 were analyzed from air samples via an INNOVA 1412 photo-acoustic multi-gas analyzer (LumaSense Technologies Inc., Ballerup, Denmark). The analyzer has a maximum detection limit of 10^6 ppm, and minimum detection limits of 0.4 ppm for CH_4, 1.5 ppm for CO_2, 0.03 ppm for N_2O, and 1.0 ppm for NH_3.

4.2.4. Emission Calculation

Gas concentrations from each of the flux chamber measurements were truncated removing the initial 7 mins and final 2 mins of each sample period in order to omit carry-over effects from other chambers. Total flux was calculated by the following methodology, further described in Peterson et al. (2020) [287]:

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

MIX signifies the net concentration equal to the gas concentration in the air that is being sampled minus the background concentration in the fresh inlet air in either ppm or ppb; FL signifies the ambient air flow rate (8 L/min); 60 is the conversion from minute to hour; MW is the molecular weight of the gas in g/mol; Conv is a conversion factor (10^{-3} for concentrations in ppm, 10^{-6} for concentrations in ppb); MV is the volume of one molar gas at temperature 20 °C (24.04 L/mol).

Surface-emission rate (mg/h/m2) of the sample for each drum was calculated by the following:

$$Surface\ Emission\ Rate = \frac{Total\ Flux}{Surface\ Area}$$

where the surface area is the cross-section area of the steel drum under the flux chambers (approximately 0.25 m^2). The emission rate per each m^3 of effluent was calculated based on the surface emission rate, the surface area of the drum and the amount of effluent in each drum.

4.2.5. Statistical Analysis

Greenhouse gas and NH_3 emissions over time were statistically analyzed using a generalized linear mixed-effects model with negative binomial distribution using the function "glmer.nb" within the "lme4" package in R [344] according to the following base model:

$$Y_{dtw} = \mu + \beta_d + \beta_t * \beta_w + \varepsilon_{dtw}$$

Cumulative study-wide GHG and NH_3 emissions were statistically analyzed using linear models using "lm" function in R according to the following base model:

$$Y_{bt} = \mu + \beta_b + \beta_t + \varepsilon_{bt}$$

Nitrogen panels from effluent samples and daily measurements (pH, temperature, ORP and DO) were statistically analyzed using a linear model with random effects in R using the "lmer" function within "lme4" package, according to the following base model:

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

where μ = the overall mean of the response variable in question; β_d = drum (experimental unit; random variable); β_b = block (i.e., row); β_t = treatment; β_w = study week; ε_{dbtw}, ε_{dtw} and ε_{bt}= the error terms for the models in question. Model selection were determined based on the Akaike information criterion, Bayesian information criterion, and log-likelihood [292].

Differences were declared significant at $P \leq 0.05$. Means are presented as least squares means (LSM) which were determined using the "emmeans" and comparisons between treatment LSMs were completed using the "anova" function in R. Pairwise comparisons of treatment LSMs were determined using the Tukey post hoc analyses. The "cld" function in the "multcomp" package was used to visualize the pair comparison of the treatment groups. In order to determine potential treatment-related differences in slopes in a method similar to Hothorn et al. (2008), contrast matrices were constructed for pH, temperature, DO, and ORP using the "glth" function in within the "multcomp" package in R [345].

4.3. Results & Discussion

In the present experiment, gaseous NH_3 emissions of aerated-drums were greater than that of their non-aerated counterparts as anticipated (Table 4.1). When coupled with a method for which to strip the air of volatilized NH_3, aeration was a highly regarded tool for NH_3-recovery methods [250]. The expectation was that the microorganisms present in the re-constituted BiOWiSH

applicant would effectively counteract the influx in NH_3 present, which resulted by aeration of the media. Our findings revealed no differences between the PC- and the BiOWiSH-drums for NH_3 air emissions when expressed both over time and cumulative throughout the present study (Figure 4.2; Table 4.1), based on both pairwise comparisons and contrast matrices. We likewise saw no differences between PC- and BiOWiSH-drums in gaseous CH_4, N_2O, and CO_2 emissions when expressed over time or as a cumulative effect.

Gas emissions differed between aerated (PC and BiOWiSH) and non-aerated NC drums for each of the GHG's, though the interaction between treatment and week was significant (Figure 4.2; Table 4.1). Methane was reduced by 34% in the aerated compared with non-aerated drums in the present study. Our findings regarding the positive impacts of aeration on gaseous emissions are consistent with others in literature, though they were not to the extent of some which found reductions of up to 95% in CH_4 and 98% in NH_3 emissions when effluent was aerated [379,380]. Consistent with the relationships noted in the present study, Cakir et al. (2005) found the concentration of dissolved nitrogen (N_2) in wastewater to be inversely related with CH_4. They attributed the low N_2 concentrations in high strength wastewater to elevated CO_2 and CH_4 production [381].

In the aerated PC- and BiOWiSH-drums, CH_4, N_2O and CO_2 emissions were highest at the start of the experiment, and reduced substantially as the experiment continued. Methane emissions were elevated for each of the treatment types at the start of the experiment. Consistent with the findings of Wang et al. (2014), CH_4 emissions in the aerated drums (PC and BiOWiSH) decreased after week 1 whereas the non-aerated drums (NC) did not decrease in CH_4 emissions until week 3 of the experiment [371].

Dissolved oxygen and ORP were greater in aerated vs. non-aerated drums in the present study (Table 4.2); though the treatment by test day interactions were significant for both of these measures, both groups of aerated drums had positive slopes whereas non-aerated NC-drums had negative slopes (Table 4.3). No differences were found for DO or for ORP between BiOWiSH-treated (5.3 mg/L) and PC-drums for each of the parameters.

It is known that pH and temperature are two of the most important factors influencing the speciation of TA-N, with higher NH_3 concentrations as pH and temperature increase [382]. This is confirmed by Zhao et al. (2015), who similarly noted an interrelationship between temperature, pH, and aeration time in converting TA-N to free NH_3 [383]. In the present study, the non-aerated NC-drums had significantly lower pH than both the PC- and the BiOWiSH-drums (Table 4.2 & 4.3), though the treatment by test day interactions was found to be significant for this parameter. Temperature of the ADE did not differ between treatment groups ($P > 0.05$), with a study-wide average of 14.1°C for drums in all treatment groups. Though pH was sustained above 9.0 throughout the entire study duration in each of the aerated drums, it is possible that temperature was too low for a sufficient conversion rate of TA-N to free NH_3 in order for differences between the two aerated groups to be detected. Zhao et al. (2015) determined aeration to be a highly effective method of CO_2 removal from ADE, with removal of dissolved inorganic carbon reaching up to 80% after 12 h of aeration. This rapid release of CO_2 leads to a rapid increase in pH, which resulted in an increased concentration of free NH_3 [383]. Future experiments should look at determining the optimal pH, temperature, and aeration parameters when BiOWiSH is used for NH_3 removal. Though they noted recovery of NH_3 over their 30-d experiment, Dube et al. (2016) similarly found an increase in pH with aeration from 8.6 to 9.2 [380]. Test week was found to be highly significant for pH, temperature, DO, and ORP ($P < 0.001$; Table 4.2).

Total nitrogen, TA-N, NH_3-N, NH_4-N, and NO_3-N did not differ between BiOWiSH- and PC-treated drums ($P > 0.05$; Table 4.4). Likewise, there were no differences between aerated and non-aerated drums for total nitrogen, TA-N, NH_4-N, and NO_3-N. Dissolved TA-N in effluent was greater and NH3-N was lower in NC-drums versus both the PC- and BiOWiSH-drums (Table 4.4). Total nitrogen, TA-N, NH_3-N, and NH_4-N significantly differed between the aerated- (PC and BiOWiSH) and non-aerated NC drums, though NO_3-N did not (Table 4.4).

Nitrifying bacteria are highly sensitive to free NH_3 and NH_4^+. Xu et al. (2014) found the nitrification process in ADE to be severely inhibited when the initial NH_4-N concentration was greater than 800 mg/L [384]. Similarly, Wei et al. (2012) determined that the concentration of ammonium in the influent highly influenced the nitrification products that resulted, with declining ammonium removal efficiency due to nitrates and nitrite accumulation [385]. The increase in pH throughout the experiment over time could lead to an increase in the bacterial ammonification process and production of both dissolved and gaseous NH_3 [386]. This could therefore explain the notably higher levels of gaseous and aqueous NH_3 in the aerated compared with the non-aerated drums in the later segment of the experiment. The initial NH_4-N concentration in the present trial was far greater than this threshold, with an average of 3135 mg/L, which could in part explain why NO_3-N increased and NH_4-N and NH_3-N decreased for the aerated groups throughout the 8-week experiment. In addition to this, speciation of TA-N from NH_3 to NH_4^+ is influenced by pH, temperature, and ionic strength and contents of the liquid media. Small changes in each of the aforementioned parameters can result in large concentration shifts in NH_3 [387,388]. For instance, a unit change in pH between 5.0-9.0 shifts nitrification efficiency by 13% [389]. As the ADE in the aerated drums had significantly higher pH as well as NH_3-N and lower NH_4^+-N in the liquid fraction when compared with the non-aerated negative control drums, it is probable that TA-N

speciation was enhanced to a capacity that could not be handled with the dosage level of BiOWiSH used. Though extended aeration was shown to enhance the growth of nitrite oxidizing bacteria [390], there have been reports of delayed growth of nitrite-oxidizing bacteria if aeration was continued for greater than 12-h per day [384]. The level of nitrite nitrifiers were sensitive to both low dissolved oxygen and high concentrations of free ammonia and could accumulate when either of these conditions were met [384,391]. Mote et al. (2005) found differences in the nitrate and nitrite concentrations between aeration periods, which suggested that NOB nitrite-oxidizing bacteria could be more sensitive to non-aeration than ammonia-oxidizing bacteria [391].

4.4. Conclusion

Aeration was a highly effective strategy for increasing both gaseous and dissolved NH_3 of effluent, which in combination with an NH_3 recovery method could favorably support the production of ammoniacal fertilizers which can be subsequently used for crop production. Applying a BiOWiSH stock solution at a rate of 15.4 mL weekly to aerated high-solids ADE did not demonstrate an impact on gaseous NH_3 or GHG emissions, or on dissolved N contents in the effluent including total N, TA-N, NH_3-N, NH_4-N, or NO_3-N. Though this was the case, there was a pattern toward reduced gaseous NH_3 emissions near the end of the experiment, suggesting that longer-term experiments may determine an impact with BiOWiSH application. Future experiments should consider various doses of BiOWiSH inclusion and duration time of the treatment.

Table 4.1. Study-wide cumulative gaseous emissions measured using flux chambers from drums containing anaerobic digester effluent of ammonia (NH_3; mg/h/m^2), methane (CH_4; mg/h/m^2), nitrous oxide (N_2O; mg/h/m^2), and carbon dioxide emissions (CO_2; mg/h/m^2) by treatment type (n=6): BiOWiSH® Aqua (BA; 15.4 mL of BA stock solution and aerated effluent), negative control (NC; 15.4 mL of water; effluent not aerated), and positive control (PC; 15.4 mL of water and aerated effluent).

	BA	**NC**	**PC**	**SEM**	***P-Value***
NH_3 (mg/h/m^2)	5133[a]	3810[b]	5384[a]	214	**<*.001***
CH_4 (mg/h/m^2)	258[b]	771[a]	277[b]	108	**<*.001***
N_2O (mg/h/m^2)	5.23[a]	2.26[b]	5.35[a]	0.33	**<*.001***
CO_2 (mg/h/m^2)	32095[a]	18939[b]	33175[a]	1962	**<*.001***

Analysis conducted using linear modeling; means with the same letter are not significantly different (P > 0.05).

Table 4.2. Least squares means (LSM) of pH, temperature, dissolved oxygen (DO; mg/L), and oxidation reduction potential (ORP; mV) across the 8-week study. Treatment types were (n=6): BiOWiSH® Aqua (BA; 15.4 mL of BA stock solution and aerated effluent), negative control (NC; 15.4 mL of water; effluent not aerated), and positive control (PC; 15.4 mL of water and aerated effluent).

	Treatment LSM			**SEM**	***P-Value***		
	BA	**NC**	**PC**		**Trt**	***Test Day***	***Trt × Day***
pH	9.315	8.583	9.312	0.017	***<.001***	***<.001***	***<.001***
Temperature (°C)	14.1	14.1	14.1	0.0881	0.98	***<.001***	0.91
DO (mg/L)	5.829	0.453	5.362	0.34	***<.001***	***<.001***	***<.001***
ORP (mV)	-99.7	-448.4	-113.4	9.15	***<.001***	***<.001***	***<.001***

Analysis conducted using linear modeling; means with the same letter are not significantly different (P > 0.05).

Table 4.3. Average slope of pH, temperature (°C), oxidation reduction potential (ORP; mV), and dissolved oxygen (DO; mg/dL) over duration of study by treatment and corresponding slope contrasts with average slope difference (estimate). Treatment types were (n=6): BiOWiSH® Aqua (BA; 15.4 mL of BA stock solution and aerated effluent), negative control (NC; 15.4 mL of water; effluent not aerated), and positive control (PC; 15.4 mL of water and aerated effluent).

	Slopes	**Contrasts**	**Estimate**	***P-Value***
pH				
BA	0.005226	PC-BA	-0.00059	0.848
NC	0.009713	NC-BA	0.004487	***<.001***
PC	0.004631	NC-PC	0.005082	***<.001***
Temperature (°C)				
BA	-0.109769	PC-BA	-.002744	0.922
NC	-0.109846	NC-BA	-.00007684	1.00
PC	-0.112513	NC-PC	.002667	0.926
ORP (mV)				
BA	3.9219	PC-BA	0.2107	0.689
NC	-1.1541	NC-BA	-5.0759	***<.001***
PC	4.1326	NC-PC	-5.2867	***<.001***
DO (mg/L)				
BA	0.144874	PC-BA	0.01396	0.368
NC	-0.018272	NC-BA	-0.16315	***<.001***
PC	0.158829	NC-PC	-0.17710	***<.001***

Means with the same letter are not significantly different (P > 0.05).

Table 4.4. Least squares means (LSM) of total nitrogen (TN; mg/L), nitrate nitrogen (NO_3-N; mg/L), total ammoniacal nitrogen (TA-N; mg/L), ammonia nitrogen (NH_3-N; mg/L), ammonium nitrogen (NH_4-N; mg/L), and moisture (%), from each drum on a bi-weekly basis. Chemical composition within a 500 mL sample of anaerobic digester effluent by treatment type; statistical analysis was conducted using a linear mixed effects model. Treatment types were (n=6): BiOWiSH® Aqua (BA; 15.4 mL of BA stock solution and aerated effluent), negative control (NC; 15.4 mL of water; effluent not aerated), and positive control (PC; 15.4 mL of water and aerated effluent).

	Week 2			**Week 4**			**Week 6**			**Week 8**			**SEM**	***P-Value***		
	BA	**NC**	**PC**	**BA**	**NC**	**PC**	**BA**	**NC**	**PC**	**BA**	**NC**	**PC**		***Week***	***Trt***	***Week × Trt***
TN (mg/L)	3210^{bc}	3832^{a}	3428^{ab}	2723^{de}	3723^{ab}	2940^{cd}	2310^{f}	3465^{abc}	2372^{ef}	2240^{f}	3437^{bc}	2312^{ef}	109	***<.001***	0.16	***<.001***
NO_3-N (mg/L)	0.184	0.17	0.144	0.148	0.247	0.169	0.212	0.166	0.19	0.193	0.121	0.201	0.03	0.75	0.21	0.17
TA-N (mg/L)	2581^{a}	2200^{ab}	2272^{ab}	1148^{c}	1232^{c}	1175^{c}	118^{c}	2160^{b}	1260^{c}	1068^{c}	1941^{b}	1209^{c}	84.6	***<.001***	***0.006***	***<.001***
NH_3-N (mg/L)	946^{a}	217^{c}	809^{a}	507^{b}	155^{c}	500^{b}	522^{b}	299^{c}	548^{b}	456^{b}	293^{c}	516^{b}	31	***<.001***	***<.001***	***<.001***
NH_4-N (mg/L)	1635^{ab}	1983^{c}	1463^{a}	641^{d}	1077^{e}	675^{d}	658^{d}	1861^{bc}	712^{d}	613^{d}	1648^{ab}	693^{d}	64	***<.001***	0.60	***0.002***
Moisture (%)	97.7	97.5	97.7	97.9	98.0	98.0	98.0	97.8	98.0	98.0	98.0	98.0	0.02	***<.001***	0.48	0.99

Means with the same letter are not significantly different (P > 0.05).

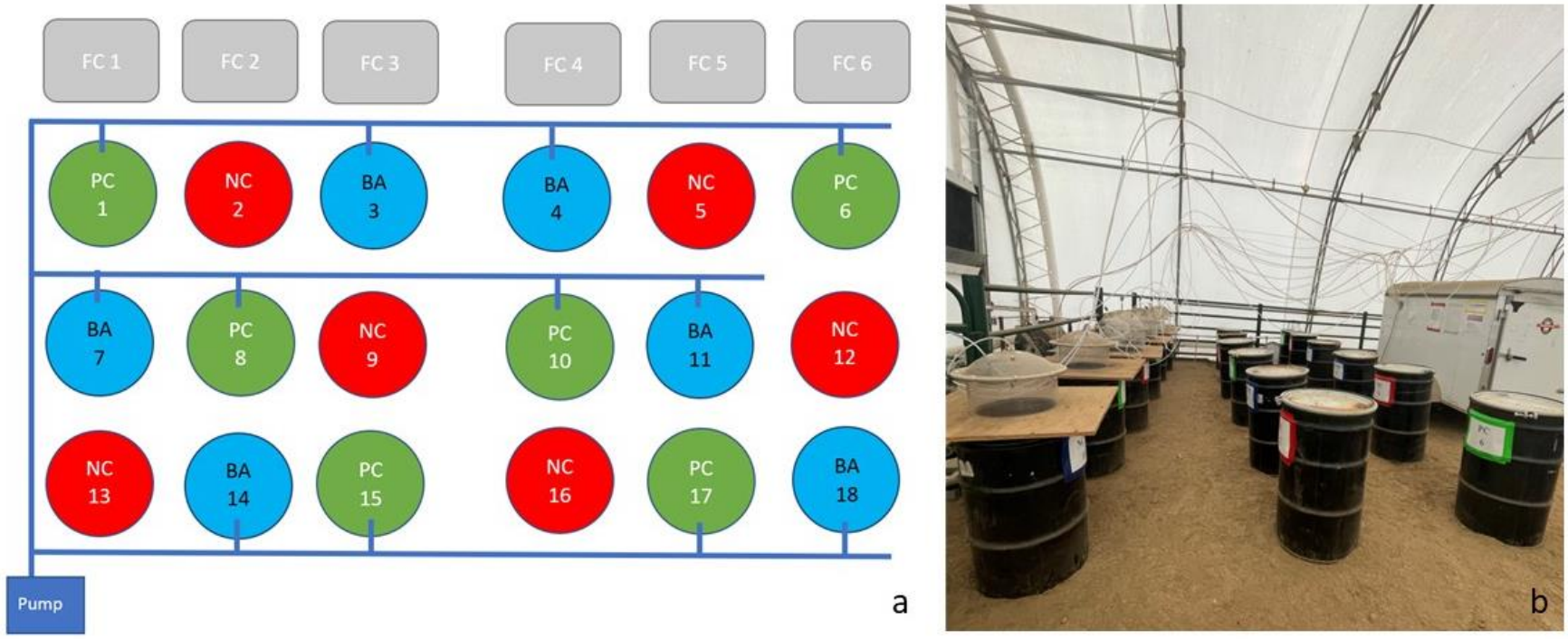

Figure 4.1. (a) schematic of the drums and drum order – effluent was added to each row of drums on three consecutive days; flux chambers (FC) were moved between drums (columns) after 24 h sampling concluded. (b) Experimental set up including drums, flux chambers, and mobile air quality laboratory. Treatment types were (n=6): BiOWiSH® Aqua (BA; 15.4 mL of BA stock solution and aerated effluent), negative control (NC; 15.4 mL of water; effluent not aerated), and positive control (PC; 15.4 mL of water and aerated effluent).

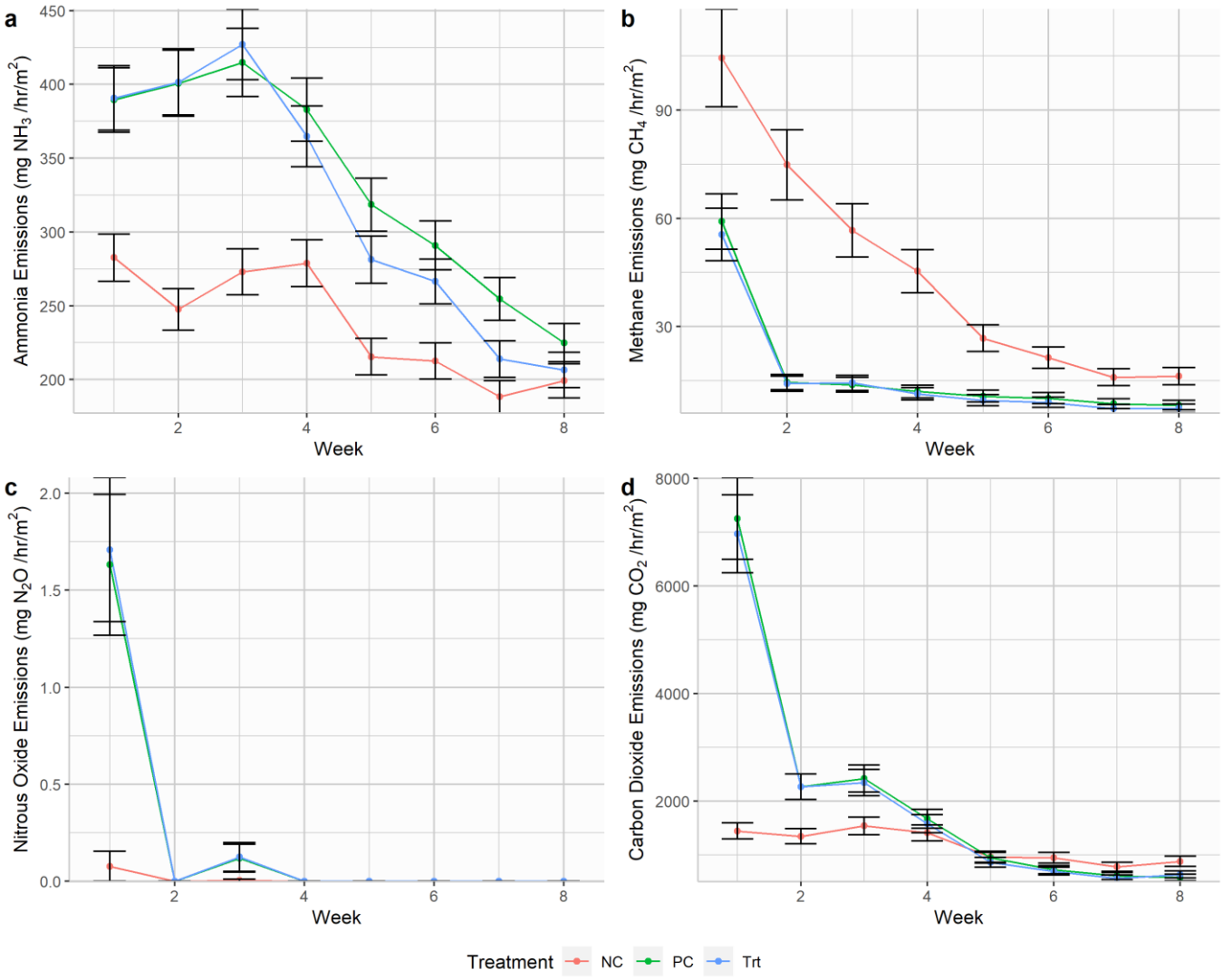

Figure 4.2. Average weekly (a) ammonia (NH_3; mg/hr/m^2), methane (CH_4; mg/hr/m^2), nitrous oxide (N_2O; mg/hr/m^2), and carbon dioxide (CO_2; mg/hr/m^2) emissions from anaerobic digester effluent treated with either negative control (NC; no aeration + water), positive control (PC; aeration + water), or BiOWiSH® Aqua (Trt; aeration + BiOWiSH) over the 8-week experiment (treatment n = 6). Emissions measured using flux chambers for 24h twice weekly; statistical analysis was conducted using a generalized linear mixed effects model with a negative binomial distribution.

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