THESIS

IMPACT OF LOW-LEVEL TANNIN SUPPLEMENTATION ON ENTERIC METHANE EMISSIONS, ESTIMATED NITROGEN EXCRETION, OXIDATIVE STRESS, AND ANIMAL PERFORMANCE IN ORGANIC DAIRY HEIFERS

Submitted by

Ashley Schilling

Department of Animal Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2023

Master's Committee:

Advisor: Kim Stackhouse-Lawson

Sara Place
Pablo Pinedo
Juan Velez
Rhyannon Moore-Foster

ABSTRACT

IMPACT OF LOW-LEVEL TANNIN SUPPLEMENTATION ON ENTERIC METHANE EMISSIONS, ESTIMATED NITROGEN EXCRETION, OXIDATIVE STRESS, AND ANIMAL PERFORMANCE IN ORGANIC DAIRY HEIFERS

Heightened attention and concern regarding the role of anthropogenic greenhouse gas (GHG) emissions in climate change has challenged every industry to reduce their environmental impact. In cattle production systems, the importance of feeding the growing human population while minimizing environmental impacts has been given significant attention throughout the 21st century (Steinfeld et al. 2006; Golub et al., 2012; Eisler et al. 2014). In 2020, the United States dairy industry was responsible for approximately 1.4% of total anthropogenic GHG emissions (EPA, 2021). The GHGs with the largest global warming potential (GWP) equivalents in dairy cattle production systems are nitrous oxide (N_2O) and methane (CH_4) (Rotz et al., 2021). The use of tannins as a feed additive in cattle production systems has been explored as a GHG mitigation strategy given their potential to reduce enteric CH_4 and reactive-nitrogen (N) emissions, while also benefiting animal health. Tannins are secondary components of plants comprised of phenolic compounds of diverse molecular weights and of variable complexity (Place et al., 2011). They are classified into two major classes: 1) hydrolysable and 2) condensed tannins and exhibit variable affects depending on their class, concentration/purity, dose, type, and other factors such as animal species, animal physiological state, and diet composition (Makkar 2003; Aboagye and Beauchemin, 2019). When fed to ruminants, such as dairy cattle (*Bos taurus),* tannins act as rumen modifiers by altering protein and carbohydrate degradation in the rumen.

Moreover, tannins have demonstrated anti-microbial, anti-parasitic, antioxidant, anti-inflammatory, and anti-viral effects in animals and the ability to serve as a bloat control mechanism (Mangan, 1988; Jones et al., 1971, Min et al., 2005). Since tannins target rumen microbial populations that assist in fiber degradation, unintended consequences can include reductions in feed intake, digestibility, and rate of BW gain when tannins are supplemented at concentrations greater than 55 g condensed tannins/kg dry matter (DM) (Min et al., 2003). Therefore, the objective of this study was to determine the impact of low-level tannin (< 0.30 g/kg DMI) supplementation on enteric CH_4 emissions, estimated N excretion, oxidative stress, and performance in organic Holstein heifers. Heifers (n=20) were supplemented with Silvafeed® ByPro, a *Schinopsis lorentzii* condensed tannin product, at increasing levels as recommended by the manufacturer: 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of dry matter intake (DMI). Based on animal success to a 28 d acclimation period, 20 certified organic Holstein heifers (BW = 219 ± 17 kg) were randomly assigned into one of the four treatment groups and stratified based on initial body weight (i.e., a completely randomized design). A 7 d pretrial gas analysis was performed prior to study initiation to account for individual animal emission differences. Daily, heifers were supplemented with one kg of sweet feed and tannin in accordance with the assigned treatment in individual feeding stanchions for 45 d and fed a basal total mixed ration (TMR) diet through four SmartFeed Pro intake measurement bunk systems (C-Lock Inc., Rapid City, SD) which allowed for measurement of individual animal feed intake. Additionally, CH_4 and carbon dioxide (CO_2) production was measured using one GreenFeed automated head chamber system (AHCS, C-Lock Inc., Rapid City, SD) for the entirety of the study. Statistical analysis was conducted in R© (R Core Team, 2021, v. 4.1.2). Data were analyzed as a completely randomized design with animal (n=20) as the experimental unit, using

the Type III ANOVA procedure. Post-hoc pairwise comparisons for dependent variables by treatment were performed using the least squared means procedure with the Tukey HSD adjustment applied. Daily CH_4 production ranged from 136.5 to 140.1 g CH_4/hd/d between treatments. No significant difference was observed between treatments for daily CH_4 production (P=0.95), CO_2 production (P=0.95), CH_4 as a percent of gross energy (GE) intake (Y_m; P=0.87), CH_4 yield (MY; g CH_4/kg DMI; P=0.80), and CH_4 emission intensity (EI; g CH_4/kg of BW gain; P=0.70). Similarly, a treatment effect was not observed for DMI (kg/d; P=0.92), average daily gain (ADG; kg BW gain/d; P=0.53), or feed efficiency (G:F; kg of BW gain/kg of DMI; P=0.42). Nitrogen intake ranged from 195 to 214 g/d among treatments (P=0.93). No significant difference was observed among treatments for fecal output (P=0.98), fecal N (FN; P=0.98), fecal neutral detergent fiber (NDF; P=0.33), or fecal acid detergent fiber (ADF; P=0.30). Estimated urine nitrogen (UN) (P=0.77), FN:UN (P=0.93), and N excretion (P=0.86) did not differ among treatments when estimated using methodologies described by Kohn (2005) (Table 5). Similarly, estimated UN (P=0.66), FN:UN (P=0.94), and N excretion (P=0.72) did not differ among treatments when estimated using methodologies described by Reed (2015). Moreover, no significant difference was observed among treatments for serum parameters, blood urea nitrogen (BUN; P=0.99) or creatinine (P=0.20), the common oxidative stress biomarker malondialdehyde (MDA; P=0.63), or antioxidant enzyme biomarkers superoxide dismutase (SOD; P=0.26) and reduced glutathione (GSH; P=0.19). Ultimately, the results of this study would not indicate that low-level tannin supplementation alters CH_4 emissions, estimated N excretion, oxidative stress, or animal performance in organic dairy heifers.

ACKNOWLEDGEMENTS

The completion of this thesis depended on the contribution of many individuals without whom this research would not have been possible. I would like to start by thanking my advisor, Kim Stackhouse-Lawson, Ph.D., for not only her advisement throughout my research and writing but also her mentorship throughout my graduate career by instilling in me a passion for the nexus of sustainability and animal agriculture. I would also like to thank my committee members Sara Place, Ph.D., Pablo Pinedo, Ph.D., Juan Velez, M.V., M.S., Dipl. A.C.T., and Rhyannon Moore-Foster, Ph.D., DVM., for their support in my research and academic achievements.

I would like to extend my sincerest appreciation to Aurora Organic Dairy for donating the cattle, facilities, and additional labor necessary to complete my research. I would like to acknowledge and express my appreciation for the students, staff, and faculty on the AgNext team that have assisted me in the completion of my research, laboratory analyses, and writing. Also, I would like to thank Terry Engle, Ph.D. and the Department of Animal Sciences Nutrition group for their guidance and support in the completion of laboratory analyses.

Lastly, I want to thank Jennie, Kelly, Brittney and Bailey Schilling, Sam Hazlett, Jessica Wiggins, and the rest of my family and friends, who have shown me unwavering encouragement and support throughout my academic achievements.

TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS v
LIST OF TABLES vii
LIST OF FIGURES viii
CHAPTER 1 − REVIEW OF LITERATURE 1
INTRODUCTION 1
SOURCES OF GHG EMISSIONS IN DAIRY PRODUCTION SYSTEMS 2
TANNINS 3
TANNINS AS A GHG MITIGATION STRATEGY 5
TANNINS AS AN ANIMAL HEALTH STRATEGY 6
UNINTENDED CONSEQUENCES OF FEEDING TANNINS 8
CONCLUSION 8
LITERATURE CITED 10
CHAPTER 2 − IMPACT OF LOW-LEVEL TANNIN SUPPLEMENTATION ON ENTERIC METHANE EMISSIONS, ESTIMATED NITROGEN EXCRETION, OXIDATIVE STRESS, AND ANIMAL PERFORMANCE IN ORGANIC DAIRY HEIFERS 15
PROJECT SUMMARY 15
INTRODUCTION 17
MATERIALS AND METHODS 19
CALCULATIONS 25
STATISTICAL ANALYSIS 28
RESULTS 28
DISCUSSION 30
CONCLUSION 37
LITERATURE CITED 53

LIST OF TABLES

Table 1. Diet composition of organic Holstein heifer TMR. 39

Table 2. Nutritive analysis of diet on a dry matter basis. 40

Table 3. Aveage emissions measurment results 41

Table 4. Average animal performance results 42

Table 5. Average estimated nitrogen excretion results.....43

Table 6. Average concentration of oxidative stress and antioxidant enzyme biomarkers and blood parameters.....44

LIST OF FIGURES

Figure 1. Components of the GreenFeed Automated Head-Chamber System (AHCS) 45

Figure 2. Variation in CH_4 production by animal by treatment 46

Figure 3. Average CH_4 production by treatment.....47

Figure 4. Average CH_4 production by treatment by hour of the day.....48

Figure 5. Relationship between CH_4 production and DMI by hour of the day 49

Figure 6. Correlation between average CH_4 production and DMI.....50

Figure 7. Average DMI by treatment 51

Figure 8. Average DMI by treatment by day 52

CHAPTER 1 − REVIEW OF LITERATURE

INTRODUCTION

Atmospheric GHG concentrations have increased since the industrial revolution which has resulted in an increased average global temperature, greater variability in temperature and precipitation extremes, and more frequent severe weather events (IPCC, 2021; USGCRP, 2018). Heightened attention and concern regarding the role of anthropogenic GHG emissions in climate change has challenged every industry to reduce their environmental impact. In cattle production systems specifically, the importance of feeding the growing human population while minimizing environmental impacts of cattle production has been given significant attention throughout the 21st century (Steinfeld et al. 2006; Golub et al., 2012; Eisler et al. 2014). Cattle production contributes to GHG emissions, specifically CH_4 and N_2O emissions (Rotz et al., 2021). When considering cattle production systems, enteric CH_4 is a significant source of GHG emissions with enteric CH_4 comprising approximately 30% of total United States CH_4 emissions (EPA, 2021). This contribution becomes problematic because CH_4 is a potent greenhouse gas, despite being short-lived, that has a global warming potential (GWP) over a 100 year time horizon of 28 times that of CO_2 (IPCC, 2021). In cattle production systems, the majority of enteric CH_4 is produced as a byproduct of the enteric fermentation process that occurs in the rumen, which allows for cattle to utilize complex structural carbohydrates and fibers, such as cellulose and lignin, that are otherwise indigestible by humans (Patra, 2012; Knapp et al., 2014). Additionally, manure management can also serve as a significant source of CH_4 and N_2O emissions from cattle production systems (EPA, 2021). Manure management accounts for approximately 12% of total GHG emissions from the agricultural sector in the United States, serving as a significant, but

manageable, emissions source since different manure treatment and storage methods affect how much CH_4 and N_2O are produced. Therefore, it is critical to assess and identify strategies that mitigate CH_4 and N_2O production in the dairy industry.

SOURCES OF GHG EMISSIONS IN DAIRY PRODUCTION SYSTEMS

In 2020, the United States dairy industry was responsible for approximately 1.4% of total anthropogenic GHG emissions (EPA, 2021). The specific GHGs of interest in dairy cattle production systems are N_2O and CH_4, due to their high GWP equivalence (Rotz et al., 2021). Nitrous oxide is a highly volatile GHG that is formed through microbial processes during denitrification of nitrate to nitrogen gas (Place et al., 2011). Major sources of N_2O on dairy farms are long term manure storage lagoons and emissions from cropland fertilized with N fertilizer or manure (Velthof et al., 1998; de Boer, 2003). Small amounts of N_2O result from nitrate reduction processes in the rumen (Kaspar and Tiedje, 1981), although this source is not considered in analyses of dairy emissions in most cases (Casey and Holden, 2005). Nitrous oxide has a GWP 273 times that of CO_2, over a 100 year time horizon (IPCC, 2021). Therefore, reducing N_2O emissions through manure and nutrition management strategies can have large impacts on the overall GHG emissions contribution from dairies because of the high GWP of N_2O.

The majority of CH_4 production results from enteric fermentation, where CH_4 is a byproduct of enteric fermentation of feed by bacteria, protozoa, and fungi (Janssen and Kirs, 2008). Methane production from dairy cattle represents an energy loss, varying from 2 to 12% of gross energy intake (Moe and Tyrrell, 1979; Johnson and Johnson, 1995). While the majority of enteric CH_4 is produced in the rumen and released through eructation via the mouth, approximately 13% of methanogenesis can occur in the large intestine (Ellis et al., 2008).

Additionally, the amount of CH_4 emitted per animal varies widely due to numerous factors including, but not limited to, DMI, the amount and type of dietary carbohydrate, forage processing and quality, dietary lipids, and the addition of feed additives that can modify rumen microbial populations (Moe and Tyrrell, 1979; Johnson and Johnson, 1995). While there has been considerable research conducted looking for strategies to reduce enteric CH_4 emissions, minimal progress has been made in identifying practical and scalable strategies to mitigate cattle emissions (Beauchemin et al., 2008; Place and Mitloehner, 2010; Hristov et al., 2013; Beauchemin et al., 2020). Therefore, identifying strategies that are capable of reducing GHG emissions from dairy cattle at scale represents a significant need and opportunity for the dairy industry.

TANNINS

A variety of feed additives have been explored in ruminant livestock systems aiming to mitigate CH_4 emissions such as 3-Nitrooxypropanol (3-NOP), *Asparagopsis* (i.e., red algae), microalgae, nitrate, biochar, antibiotic rumen modifiers (i.e., ionophores), direct-fed microbials, essential oils, saponins, and tannins (Hegarty et al., 2021). The feed additive of particular interest to this thesis is tannins. Tannins are secondary components of plants comprised of phenolic compounds of diverse molecular weights and of variable complexity (Place et al., 2011). They are classified into two major classes: 1) hydrolysable and 2) condensed with variable affects depending on the class, concentration/purity, dose, type, and other factors such as animal species, animal physiological state, and diet composition (Makkar 2003; Aboagye and Beauchemin, 2019). When tannins are fed to ruminants, they act as rumen modifiers, but the main mechanism by which they affect methanogenesis has not definitively been isolated in vitro or in vivo (Aboagye and Beauchemin, 2019). Currently, there are multiple hypotheses (or modes of action)

regarding how tannins decrease CH_4 production: 1) tannins act directly on methanogens (Field et al., 1989; Tavendale et al., 2005; Jayanegara et al., 2011; Díaz Carrasco et al., 2017), 2) tannins affect protozoa populations that are associated with methanogens (Makkar et al., 1995; Bhatta et al., 2009), 3) tannins act on fibrolytic bacteria and decrease fiber degradation (Carulla et al., 2005), and 4) tannins act as a H_2 sink (Becker et al., 2014). Aboagye and Beauchemin (2019) suggested tannins may function via all, some, or any of the proposed mechanisms. Tavendale et al. (2005) demonstrated this when tannins reduced H_2 production by lowering feed degradation due to the altering of rumen microbes, encompassing two of the above-proposed modes of action. This non-mutually exclusive explanation by Aboagye and Beauchemin (2019) is founded on the basis that in studies where significant effects of tannins on CH_4 reductions were reported between or among treatments, there has been a large range in the amount by which CH_4 was decreased. Additional studies have affirmed the complex interrelationships that may exist in these proposed mechanisms from tannin supplementation including decreased ruminal fiber digestion related to a decrease in the number of cellulolytic bacteria (McSweeney et al. 2001), formation of tannin-cellulose complexes that are resistant to enzymatic digestion (Makkar et al. 1995), and/or result in the impairment in substrate adhesion by fibrolytic microbes (Bento et al. 2005), all of which could reduce H_2 availability and lessen methanogenesis (Carulla et al. 2005). Furthermore, it should be acknowledged that the antimethanogenic property of tannins has mainly been studied for condensed tannin-rich plants, extracts, and feed additives because of their lower risk of toxicity to the animal when compared to hydrolysable tannins (Beauchemin et al. 2008). Ultimately, there is evidence that suggests that when tannins are included in the diet there is potential for tannins to modify normal rumen function via multiple modes of action, simultaneously.

Tannins as a GHG Mitigation Strategy

The use of tannins in dairy systems as a GHG mitigation strategy has the potential to be beneficial by 1) reducing enteric CH_4 emissions and 2) reducing reactive-N emissions. One goal for sustainable dairy production systems should be minimizing GHG emissions while improving efficiency and animal health (Min et al., 2020). Nitrogen excreted by dairy cattle is a main source of N_2O emissions and can impact air and water quality. Therefore, strategies based on changing the composition and concentration of urinary compounds via diet manipulation should be considered as potential options to mitigate urine N emissions and simultaneously improve the sustainability of dairy production systems (Gardiner et al., 2016). Among the currently researched GHG mitigation strategies, tannin-containing plants, extracts, and feed additives have received heightened attention, particularly in ruminants, due to their two-pronged potential for emissions reductions and proposed benefits to animal health. Tannins have the affinity to form complexes with proteins in the rumen which increases bypass protein and generally shifts N excretion from the urine to the feces, since protein degradation in the rumen is altered (Mangan, 1988). The alteration of protein degradation from the rumen to the small intestine, mediates urine-N and fecal-N output, ultimately influencing reactive-N emissions (Mangan, 1988). Therefore, if protein digestion is altered through the addition of tannins in the diet to increase bypass protein, reactive-N emissions should decrease as the proportion of fecal-N to urinary-N increases. Condensed tannins are also thought to alter CH_4 production by forming complexes with digestive enzymes and directly acting on rumen microorganisms, which inhibits fiber degradation, acetate production, and ultimately the amount of H_2 available to methanogens (Barry et al., 1984; Jones et al., 1994; McMahon et al., 2000; Scalbert et al., 1991). If fiber fermentation is decreased as a result of fibrolytic microorganisms being inhibited, tannins may

have the ability to decrease CH_4 emissions. Ultimately, tannins may have the ability to decrease CH_4 emissions and reactive N emissions.

Over the past decade there has been increased interest in using naturally occurring phytochemicals as alternatives to ionophores (Place et al., 2011). Ionophores are a common technology used in dairy systems that can change rumen microbial processes to potentially improve feed efficiency and reduce enteric CH_4 emissions (Tedeschi et al., 2003). Additionally, ionophores have demonstrated the ability to enhance the glucose status of dairy cows through increased production of propionate in the rumen. Unlike ionophores, tannins are not an emissions mitigation strategy that has been scaled for use in production environments. Yet, tannins have the potential to reduce CH_4 emissions and reactive-N emissions, while also benefitting animal health, potentially generating multiple benefits which could increase producer incentivization and adoption. Ultimately, alternatives to ionophores such as probiotics (e.g., yeast), essential oils, and phytochemicals (e.g., tannins) have been shown to mitigate GHG emissions; however, additional research is necessary to evaluate the efficacy of these alternatives and their commercial viability (Calsamiglia et al., 2007; Beauchemin et al., 2009).

Tannins as an Animal Health Strategy

Beyond the potential GHG abatement from utilizing tannins as a CH_4 and reactive-N mitigation strategy, tannins also have the potential to benefit animal health which ultimately influences production efficiency. Place and Mitloehner (2010) describe production efficiency in the dairy industry as minimizing the quantity of inputs (e.g., feed, fossil fuels) and outputs (e.g., ammonia, GHG) to produce a given quantity of milk. Given this context, production efficiency improvements can come from minimizing waste, feed, and GHG emissions while simultaneously enhancing milk production, reproductive performance, and cow longevity without sacrificing

animal health and well-being (Place and Mitloehner, 2010). Ultimately, in the dairy industry, herd health challenges affect production efficiency by decreasing reproductive performance and milk production efficiency, thus necessitating strategies that not only minimize GHG emissions, but synergistically benefit animal health and performance.

As discussed above, over the past decade there has been an interest in using naturally occurring plant compounds as alternatives to ionophores (Place et al., 2011). Some studies have demonstrated animal health benefits associated with ionophore feed additives due to enhancement of the energy status of dairy cows in the transition period and during early lactation, resulting in lower incidence of ketosis and displaced abomasum, reduced loss of body condition, increased milk production, and improved milk production efficiency which impacts GHG emissions yield and efficiency metrics (McGuffey, 1995; McGuffey and Giner-Chavez, 1998; McGuffey et al., 2001). However, tannins appear to have the potential to serve as an alternative to ionophores since tannins could not only be used as a two-pronged GHG mitigation strategy but have shown positive benefits to animal health by demonstrating anti-microbial, anti-parasitic, antioxidant, anti-inflammatory, and anti-viral effects in animals (Huang et al., 2018). Additionally, the potential for tannins to serve as a bloat control mechanism has been demonstrated (Mangan, 1988; Jones et al., 1971), and is likely influenced by the ability for condensed tannins to alter ruminal gas production and degradation of feed protein within the rumen (Min et al., 2005). Yet, minimal research has been conducted in production environments to determine the scalable efficacy of animal health benefits when cattle are fed tannin-containing plants, extracts, or feed additives. Additional research is necessary to determine the ability for tannins to serve as an alternative to ionophores in dairy cattle production systems and other animal health benefits that have not yet been documented.

Unintended Consequences of Feeding Tannins

Due to the hypothesized modes of action for tannins explained above, which target the alteration of rumen microbial populations that assist in the degradation of fiber, unintended consequences that have been reported when tannins have been included in the diet are reductions in fiber fermentation and feed intake. Tannins have been shown to negatively impact feed intake and digestion and absorption of nutrients, ultimately impacting animal performance (Kumar and Singh, 1984). Additionally, Min et al. 2003 proposed that the effect of condensed tannins is dependent upon the concentration and structure of the condensed tannin provided and when included in the diet of ruminant animals at concentrations greater than 55 g condensed tannin per kg DM, a reduction in voluntary feed intake, digestibility, and rate of BW gain could be experienced. Aboagye and Beauchemin (2019) affirmed this proposal by stating that the negative impacts of tannins on ruminants are not specific to tannin type, but rather may depend upon the concentration and dose of tannin in the forage, extract, or feed additive.

CONCLUSION

None of the existing CH_4 mitigation strategies that have been researched provide producers economic incentives to influence adoption and implementation (Hegarty et al., 2021). To date, mitigation strategies that have been researched are still not viable at scale in production environments. This paucity in information is particularly alarming given the numerous decades scientists have spent innovating and researching strategies to reduce enteric CH_4 emissions from cattle production systems (Beauchemin et al., 2020). Therefore, designing research approaches in production environments to explore outcomes that incorporate all three pillars of sustainability (economic, environmental, and social) while also incorporating producer outreach aspects should

aid in incentivizing adoption of mitigation strategies in the future. While the use of tannins as a GHG emissions mitigation strategy has presented great variability in the efficacy of emission reductions, further research should encompass the two-pronged emissions mitigation potential of tannins by measuring N emissions in addition to CH_4. Research should also consider animal health measures to holistically assess the viability, practicality, and scalability of tannins as a GHG emissions mitigation strategy as well as a means to maintain or, ideally, improve animal health.

LITERATURE CITED

Aboagye, I. A., & Beauchemin, K. A. Potential of molecular weight and structure of tannins to reduce methane emissions from ruminants: A Review. *Animals* **2019**, *9*(11), 856. https://doi.org/10.3390/ani9110856

Arias, P.A. et al. Technical summary: In Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. *Cambridge University Press* **2021**, pp. 33-144, doi:10.1017/9781009157896.002.

Barry, T. N., & Manley, T. R. The role of condensed tannins in the nutritional value of lotus pedunculatus for sheep. *Br. J. Nutr.* **1984**, *51*(3), 493–504. https://doi.org/10.1079/bjn19840055

Beauchemin, K. A., Kreuzer, M., O'Mara, F., & McAllister, T. A. Nutritional management for enteric methane abatement: A Review. *Australian Journal of Experimental Agriculture* **2008**, *48*(2), 21. https://doi.org/10.1071/ea07199

Beauchemin, K. A., McAllister, T. A., & McGinn, S. M. Dietary mitigation of enteric methane from cattle. *CABI Reviews* **2009**, *4 (35)*, 1–18. https://doi.org/10.1079/pavsnnr20094035

Beauchemin, K. A., Ungerfeld, E. M., Eckard, R. J., & Wang, M. Review: Fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal* **2020**, *14*. https://doi.org/10.1017/s1751731119003100

Becker, P. M., van Wikselaar, P. G., Franssen, M. C., de Vos, R. C., Hall, R. D., & Beekwilder, J. Evidence for a hydrogen-sink mechanism of (+) catechin-mediated emission reduction of the ruminant greenhouse gas methane. *Metabolomics* **2013**, *10*(2), 179–189. https://doi.org/10.1007/s11306-013-0554-5

Bento, M. H. L., Makkar, H. P. S., & Acamovic, T. Effect of mimosa tannin and pectin on microbial protein synthesis and gas production during in vitro fermentation of 15n-labelled maize shoots. *Animal Feed Science and Technology* **2005**, *123-124*, 365–377. https://doi.org/10.1016/j.anifeedsci.2005.04.022

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., & Kurihara, M. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic Archaea and protozoal populations. *J. Dairy. Sci.* **2009**, *92*(11), 5512–5522. https://doi.org/10.3168/jds.2008-1441

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., & Ferret, A. Invited review: Essential oils as modifiers of rumen microbial fermentation. *J. Dairy. Sci.* **2007**, *90*(6), 2580–2595. https://doi.org/10.3168/jds.2006-644

Carulla, J. E., Kreuzer, M., Machmüller, A., & Hess, H. D. Supplementation of acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Australian Journal of Agricultural Research* **2005**, *56*(9), 961-970. https://doi.org/10.1071/ar05022

Casey, J. W., & Holden, N. M. (2005). Analysis of greenhouse gas emissions from the average Irish Milk Production System. *Agricultural Systems* **2005**, *86*(1), 97–114. https://doi.org/10.1016/j.agsy.2004.09.006

de Boer, I. J. M. Environmental impact assessment of conventional and organic milk production. *Livestock Production Science* **2003**, *80*(1-2), 69–77. https://doi.org/10.1016/s0301-6226(02)00322-6

Díaz Carrasco, J. M., Cabral, C., Redondo, L. M., Pin Viso, N. D., Colombatto, D., Farber, M. D., & Fernández Miyakawa, M. E. Impact of chestnut and quebracho tannins on rumen microbiota of Bovines. *BioMed Research International* **2017**, *2017*, 1–11. https://doi.org/10.1155/2017/9610810

Eisler, M. C., Lee, M. R., Tarlton, J. F., Martin, G. B., Beddington, J., Dungait, J. A., Greathead, H., Liu, J., Mathew, S., Miller, H., Misselbrook, T., Murray, P., Vinod, V. K., Van Saun, R., & Winter, M. Agriculture: Steps to sustainable livestock. *Nature* **2014**, *507*(7490), 32–34. https://doi.org/10.1038/507032a

Ellis, J. L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N. E., McBride, B. W., & France, J. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. *The Journal of Agricultural Science* **2008**, *146*(2), 213–233. https://doi.org/10.1017/s0021859608007752

EPA. *Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2019* (No. EPA-430-R-21-005). United States Environmental Protection Agency, Washington, D.C. USA. 2021.

Field, J. A., Kortekaas, S., & Lettinga, G. The tannin theory of methanogenic toxicity. *Biological Wastes* **1989**, *29*(4), 241–262. https://doi.org/10.1016/0269-7483(89)90016-5

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J., Edwards, G. R., & de Klein, C. A. M. Potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate ruminant urine derived N2O emissions: A Review. *New Zealand Journal of Agricultural Research* **2016**, *59*(3), 301–317. https://doi.org/10.1080/00288233.2016.1190386

Golub, A. A., Henderson, B. B., Hertel, T. W., Gerber, P. J., Rose, S. K., & Sohngen, B. Global climate policy impacts on livestock, land use, livelihoods, and food security. *Proc. Natl. Acad. Sci.* **2012***, U. S. A.*, *110*(52), 20894–20899. https://doi.org/10.1073/pnas.1108772109

Guan, H., Wittenberg, K. M., Ominski, K. H., & Krause, D. O. Efficacy of ionophores in cattle diets for mitigation of enteric methane1. *J. Anim. Sci.***2006**, *84*(7), 1896–1906. https://doi.org/10.2527/jas.2005-652

Hamilton, S. W., DePeters, E. J., McGarvey, J. A., Lathrop, J., & Mitloehner, F. M. Greenhouse gas, animal performance, and bacterial population structure responses to dietary monensin fed to dairy cows. *J. Environ. Qual.* **2010**, *39*(1), 106–114. https://doi.org/10.2134/jeq2009.0035

Hegarty RS, Cortez Passetti RA, Dittmer KM, Wang Y, Shelton S, Emmet-Booth J, Wollenberg E, McAllister T, Leahy S, Beauchemin K, Gurwick N. An evaluation of emerging feed additives to reduce methane emissions from livestock. Edition 1. A report coordinated by

Climate Change, Agriculture and Food Security (CCAFS) and the New Zealand Agricultural Greenhouse Gas Research Centre (NZAGRC) initiative of the Global Research Alliance (GRA). 2021.

Hook, S. E., Northwood, K. S., Wright, A.-D. G., & McBride, B. W. (2009). Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the Lactating Dairy Cow. *Appl. Environ. Microbiol.* **2009**, *75*(2), 374–380. https://doi.org/10.1128/aem.01672-08

Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H. P., Adesogan, A. T., Yang, W., Lee, C., Gerber, P. J., Henderson, B., & Tricarico, J. M. Special topics — mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options1. *J. Anim. Sci.* **2013**, *91*(11), 5045–5069. https://doi.org/10.2527/jas.2013-6583

Huang, Q., Liu, X., Zhao, G., Hu, T., & Wang, Y. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. *Animal Nutrition* **2018**, *4*(2), 137–150. https://doi.org/10.1016/j.aninu.2017.09.004

Janssen, P. H., & Kirs, M. Structure of the Archaeal community of the rumen. *Appl. Environ. Microbiol.* **2008**, *74*(12), 3619–3625. https://doi.org/10.1128/aem.02812-07

Jayanegara, A., Leiber, F., & Kreuzer, M. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. *Journal of Animal Physiology and Animal Nutrition* **2011**, *96*(3), 365–375. https://doi.org/10.1111/j.1439-0396.2011.01172.x

Johnson, K. A., & Johnson, D. E. Methane emissions from cattle. *J. Anim. Sci.***1995**, *73*(8), 2483–2492. https://doi.org/10.2527/1995.7382483x

Jones, G. A., McAllister, T. A., Muir, A. D., & Cheng, K.-J. Effects of sainfoin (Onobrychis viciifolia Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. *Appl. Environ. Microbiol.* **1994**, *60*(4), 1374–1378. https://doi.org/10.1128/aem.60.4.1374-1378.1994

Jones, W. T., & Lyttleton, J. W. Bloat in cattle. *New Zealand Journal of Agricultural Research* **1971**, *14*(1), 101–107. https://doi.org/10.1080/00288233.1971.10421306

Kaspar, H. F., & Tiedje, J. M. Dissimilatory reduction of nitrate and nitrite in the bovine rumen: Nitrous oxide production and effect of acetylene. *Appl. Environ. Microbiol.* **1981**, *41*(3), 705–709. https://doi.org/10.1128/aem.41.3.705-709.1981

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

Kumar, R.; Singh, M. Tannins: Their adverse role in ruminant nutrition. *Journal of Agriculture and Food Chemistry* **1984,** 32, 447–453.

Makkar, H. P. S. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research* **2003**, *49*(3), 241–256. https://doi.org/10.1016/s0921-4488(03)00142-1

Makkar, H. P., Becker, K., Abel, H., & Szegletti, C. Degradation of condensed tannins by rumen microbes exposed to quebracho tannins (QT) in Rumen Simulation Technique (RUSITEC) and effects of qt on fermentative processes in the RUSITEC. *J. Sci. Food Agric.* **1995**, *69*(4), 495–500. https://doi.org/10.1002/jsfa.2740690414

Mangan, J. L. Nutritional effects of tannins in animal feeds. *Nutrition Research Reviews* **1988**, *1*(1), 209–231. https://doi.org/10.1079/nrr19880015

McGuffey, R. K. Potential for improving productive efficiency of lactating dairy cows through use of ionophores. In *Proc. Maryland Nutr. Conf. for Feed Mfgrs. Baltimore* **1995**, (pp. 90-99).

McGuffey, R. K., & Giner-Chavez, B. Lactation performance of dairy cows receiving monensin and a sustained release formulation of methionyl bovine somatotropin. *J. Dairy. Sci.* **1998**, *81*(Suppl 1), 261.

McGuffey, R. K., Richardson, L. F., & Wilkinson, J. I. D. Ionophores for dairy cattle: Current status and future outlook. *J. Dairy, Sci.* **2001**, *84*. https://doi.org/10.3168/jds.s0022-0302(01)70218-4

McMahon, L. R., McAllister, T. A., Berg, B. P., Majak, W., Acharya, S. N., Popp, J. D., Coulman, B. E., Wang, Y., & Cheng, K.-J. A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. *Canadian Journal of Plant Science* **2000**, *80*(3), 469–485. https://doi.org/10.4141/p99-050

McSweeney, C. S., Palmer, B., McNeill, D. M., & Krause, D. O. Microbial interactions with tannins: Nutritional consequences for ruminants. *Animal Feed Science and Technology* **2001,** *91*(1-2), 83–93. https://doi.org/10.1016/s0377-8401(01)00232-2

Min, B. R., Barry, T. N., Attwood, G. T., & McNabb, W. C. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: A review. *Animal Feed Science and Technology* **2003**, *106*(1-4), 3–19. https://doi.org/10.1016/s0377-8401(03)00041-5

Min, B. R., Pinchak, W. E., Fulford, J. D., & Puchala, R. (2005). Wheat pasture bloat dynamics, in vitro ruminal gas production, and potential bloat mitigation with Condensed Tannins1. *J. Anim. Sci.* **2005**, *83*(6), 1322–1331. https://doi.org/10.2527/2005.8361322x

Min, B. R., Solaiman, S., Waldrip, H. M., Parker, D., Todd, R. W., & Brauer, D. Dietary mitigation of enteric methane emissions from ruminants: A review of plant tannin mitigation options. *Animal Nutrition* **2020**, *6*(3), 231–246. https://doi.org/10.1016/j.aninu.2020.05.002

Moe, P. W., & Tyrrell, H. F. Efficiency of conversion of digested energy to milk. *J. Dairy. Sci.* **1975**, *58*(4), 602–610. https://doi.org/10.3168/jds.s0022-0302(75)84616-9

Moe, P. W., & Tyrrell, H. F. Methane production in dairy cows. *J. Dairy. Sci.* **1979**, *62*(10), 1583–1586. https://doi.org/10.3168/jds.s0022-0302(79)83465-7

Odongo, N. E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M. M., Hook, S. E., Gray, J. T., Kebreab, E., France, J., & McBride, B. W. Long-term effects of feeding monensin on methane production in lactating dairy cows. *J. Dairy. Sci.* **2007**, *90*(4), 1781–1788. https://doi.org/10.3168/jds.2006-708

Patra, A. K. Enteric methane mitigation technologies for ruminant livestock: A synthesis of current research and future directions. *Environmental Monitoring and Assessment* **2011**, *184*(4), 1929–1952. https://doi.org/10.1007/s10661-011-2090-y

Place, S. E., & Mitloehner, F. M. Invited review: Contemporary environmental issues: A review of the dairy industry's role in climate change and air quality and the potential of mitigation through improved production efficiency. *J. Dairy. Sci.* **2010**, *93*(8), 3407–3416. https://doi.org/10.3168/jds.2009-2719

Place, S. E., Stackhouse, K. R., Wang, Q., Mitloehner, F. M. 23. *In Understanding greenhouse gas emissions from Agricultural Management.* American Chemical Society. **2011**; pp. 443–457).

Rotz, A., Stout, R., Leytem, A., Feyereisen, G., Waldrip, H., Thoma, G., Holly, M., Bjorneberg, D., Baker, J., Vadas, P., & Kleinman, P. Environmental assessment of United States dairy farms. *Journal of Cleaner Production* **2021**, *315*, 128153. https://doi.org/10.1016/j.jclepro.2021.128153

Scalbert, A. Antimicrobial properties of tannins. *Phytochemistry* **1991**, *30*(12), 3875–3883. https://doi.org/10.1016/0031-9422(91)83426-l

Steinfeld, H., Gerber, P., Wassenaar, T. D., Castel, V., Rosales, M., Rosales, M., & de Haan, C. *Livestock's long shadow: environmental issues and options*. Food and Agriculture Organization of the United Nations, Rome, Italy, 2006

Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T., & Sivakumaran, S. Methane production from in vitro rumen incubations with lotus pedunculatus and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Animal Feed Science and Technology* **2005**, *123-124*, 403–419. https://doi.org/10.1016/j.anifeedsci.2005.04.037

Tedeschi, L. O., Fox, D. G., & Tylutki, T. P. Potential environmental benefits of ionophores in ruminant diets. *Journal of Environmental Quality* **2003**, *32*(5), 1591–1602. https://doi.org/10.2134/jeq2003.1591

USGCRP. *Impacts, Risks, and Adaptation in the United States: Fourth National Climate Assessment, Volume II*. Reidmiller, D. R., C. W. Avery, D. R. Easterling, K. E. Kunkel, K. L. M. Lewsi, T. K. Maycock, and B. C. Stewart (eds.). U. S. Global Change Research Program. Washington, D. C. 2018. Doi: 10.7930/NCA4.2018

Velthof, G. L., van Beusichem, M. L., & Oenema, O. Mitigation of nitrous oxide emission from dairy farming systems. *Environmental Pollution* **1998**, *102*(1), 173–178. https://doi.org/10.1016/s0269-7491(98)80030-4

CHAPTER 2 − IMPACT OF LOW-LEVEL TANNIN SUPPLEMENTATION ON ENTERIC METHANE EMISSIONS, ESTIMATED NITROGEN EXCRETION, OXIDATIVE STRESS, AND ANIMAL PERFORMANCE IN ORGANIC DAIRY HEIFERS

PROJECT SUMMARY

The objective of this study was to determine the impact of low-level tannin supplementation on enteric methane (CH_4) emissions, estimated nitrogen (N) excretion, oxidative stress, and animal performance in organic dairy heifers. Heifers were supplemented with Silvafeed® ByPro, a *Schinopsis lorentzii* condensed tannin product, at increasing levels as recommended by the manufacturer: 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI. Based on a 28 day (d) acclimation, 20 certified organic Holstein heifers (BW = 219 ± 17 kg) were randomly assigned into one of the four treatment groups and stratified based on initial body weight. A 7 d pretrial gas analysis was performed prior to the study to account for individual animal emission differences. Daily, heifers were supplemented with one kg of sweet feed and tannin in accordance with the assigned treatment in individual feeding stanchions for 45 d and fed a basal total mixed ration (TMR) through four SmartFeed Pro intake measurement bunk systems (C-Lock Inc., Rapid City, SD) which allowed for measurement of individual animal feed intake. Daily, one GreenFeed automated head chamber system (AHCS, C-Lock Inc., Rapid City, SD) was used to continuously evaluate CH_4 and carbon dioxide (CO_2) production throughout the duration of the study. Fecal and blood samples were collected on d 0, 23, and 45 prior to treatment and TMR feeding. Statistical analysis was conducted in R© (R Core Team, 2021, v. 4.1.2), where data were analyzed as a completely randomized design with the individual animal (n=20) as the experimental unit. Data was analyzed using the Type III ANOVA procedure, and a pairwise comparison was analyzed for dependent variables by treatment using

the least squared means procedure with the Tukey HSD adjustment applied. Daily CH_4 production ranged from 136.5 to 140.1 g CH_4/hd/day between treatments. No significant difference was observed between treatments for daily CH_4 production (P=0.95), CO_2 production (g/hd/d; P=0.95), CH_4 as a percent of gross energy (GE) intake (Y_m; P=0.87), CH_4 yield (MY; g CH_4/kg dry matter intake (DMI); P=0.80), or CH_4 emissions intensity (EI; g CH_4/kg of average daily gain (ADG); P=0.70). Similarly, a treatment effect was not observed for DMI (P=0.92), ADG (P=0.53), or feed efficiency (G:F; kg of body weight gain/kg of DMI; P=0.42). Nitrogen intake ranged from 195 to 214 g/d among treatments (P=0.93). No significant difference was observed among treatments for fecal output (P=0.98), fecal N (FN; P=0.98), fecal neutral detergent fiber (NDF; P=0.33), or fecal acid detergent fiber (ADF; P=0.30). Estimated urine N (UN; P=0.77), FN:UN (P=0.93), and N excretion (P=0.86) did not differ among treatments when estimated using methodologies described by Kohn (2005) (Table 5). Similarly, estimated UN (P=0.66), FN:UN (P=0.94), and N excretion (P=0.72) did not differ among treatments when estimated using methodologies described by Reed (2015). No significant difference was observed among treatments for serum parameters, blood urea nitrogen (BUN; P=0.99) or creatinine (P=0.20), the common oxidative stress biomarker malondialdehyde (MDA; P=0.63), or antioxidant enzyme biomarkers superoxide dismutase (SOD; P=0.26) and reduced glutathione (GSH; P=0.19). Ultimately, the results of this study would not indicate that low-level tannin supplementation alters CH_4 emissions, estimated N excretion, oxidative stress, or animal performance in organic Holstein heifers.

INTRODUCTION

Heightened attention and concern regarding the role of anthropogenic greenhouse gas (GHG) emissions in climate change has challenged every industry to reduce their environmental impact. In 2020, the United States dairy industry was responsible for approximately 1.4% of total anthropogenic GHG emissions (EPA, 2021). Methane (CH_4) and nitrous oxide (N_2O), resulting from enteric fermentation and manure, respectively, are the primary components of the dairy industry's GHG footprint (Rotz et al., 2021). Approximately 25% of total GHG emissions from enteric fermentation, in millions of metric tons of carbon dioxide (CO_2) equivalents, and 48% of total GHG emissions from manure management came from dairy cattle production systems (EPA, 2021). While there has been considerable research conducted to identify strategies that reduce enteric CH_4 emissions, minimal progress has been made in identifying practical strategies to mitigate cattle emissions (Beauchemin et al., 2008; Place and Mitloehner, 2010; Hristov et al., 2013; Beauchemin et al., 2020). When innovating strategies to mitigate dairy cattle GHGs, CH_4 and nitrogen (N) emissions should be considered in order to recognize the potential consequences or tradeoffs associated mitigation strategies. Further, animal health and welfare should be considered when assessing mitigation potential. Identifying abatement strategies that are capable of reducing GHG emission from dairy cattle production systems at scale while simultaneously benefiting animal performance and health is a significant opportunity for the dairy industry and could aid in producer adoption.

Tannins have historically been explored due to their potential to reduce CH_4 and reactive-N emissions, while also benefiting animal health. Tannins can be classified based on chemical structure in two major classes 1) hydrolysable and 2) condensed. Hydrolysable tannins are esters of gallic or ellagic acid linked to central carbohydrate core, while condensed tannins consist of

two or more flavan-3-ol subunits linked together to form oligomers and polymers (Makkar et al., 2007; Naumann et al., 2017). However, condensed tannins are more commonly used in livestock systems since hydrolysable tannins can be toxic to animals at high concentrations (Makkar et al., 2007). Currently, there are multiple hypotheses regarding how tannins decrease CH_4 production: 1) tannins act directly on methanogens (Field et al., 1989; Tavendale et al., 2005; Jayanegara et al., 2011; Díaz Carrasco et al., 2017), 2) tannins affect protozoa populations that are associated with methanogens (Makkar et al., 1995; Bhatta et al., 2009), 3) tannins affect fibrolytic bacteria leading to a decrease in ruminal fiber degradation (Carulla et al., 2005), and 4) tannins act as a H_2 sink (Becker et al., 2014). Aboagye and Beauchemin (2019) suggested that tannins may function via all, some, or any of the proposed mechanisms, because in studies where significant effects in CH_4 reductions have been reported there has been a variable range in CH_4 decrease. Tannins also have the potential to negatively impact feed intake, digestion and absorption of nutrients, and animal performance measures (Kumar and Singh, 1984; Min et al., 2003; Aboagye and Beauchemin, 2019). When included in the diet of ruminant animals at concentrations greater than 55 g of condensed tannin per kg dietary dry matter, a reduction in voluntary feed intake, digestibility, and rate of body growth could be experienced in cattle (Min et al., 2003). However, the effect of tannins can be influenced by a variety of factors such as tannin class, concentration/purity, dose, type, and other factors such as animal species, physiological state of the animal, and diet composition (Min et al., 2003; Makkar 2003; Aboagye and Beauchemin, 2019).

Beyond potential emissions mitigation, tannins appear to have positive benefits to animal health by demonstrating anti-microbial, anti-parasitic, antioxidant, anti-inflammatory, and anti-viral effects in animals (Huang et al., 2018). Additionally, the potential for tannins to serve as a

bloat control mechanism has been demonstrated (Mangan, 1988; Jones et al., 1971; Min et al., 2005), and is likely influenced by the ability for condensed tannins to alter ruminal gas production and degradation of feed protein within the rumen (Min et al., 2005). Yet, minimal research has been conducted when cattle are fed tannins to determine how these compounds might impact oxidative stress, which is when an animal's cellular ability for antioxidant enzymes to neutralize free radicals is imbalanced due to the accumulation of oxygen reactive species.

Due to the variety of factors influencing the efficacy of tannin-containing forages (e.g., bird's-foot trefoil [*Lotus corniculatus*] and sainfoin [*Onobrychis viciifolia*]), extracts (e.g., black wattle tree, quebracho, chestnut, mimosa, and pine), and feed additives (e.g., Silvafeed® ByPro and Silvafeed® BX) , there is a need for research that encompasses the two-pronged emissions mitigation potential of tannins by measuring 1) N emissions in addition to 2) CH_4 emissions and considers animal health and performance to quantify the viability, practicality, and scalability of tannins as a GHG emissions mitigation strategy. Moreover, low-level tannin supplementation should be investigated to capitalize on the proposed GHG mitigation and improved animal health potential, while avoiding unintended animal performance consequences imposed by including tannins in diets at high levels. Therefore, the objective of this study was to determine the impact of low-level tannin supplementation on enteric CH_4 emissions, estimated N excretion, oxidative stress, and animal performance in organic Holstein heifers supplemented with Silvafeed® ByPro, a *Schinopsis lorentzii* condensed tannin product.

MATERIALS AND METHODS

This study was approved by the Colorado State University (CSU) Institutional Animal Care and Use Committee (#2341) prior to project initiation and was conducted at a commercial

certified organic dairy in Gill, Colorado (40.537715, -104.506222) from October 2021 through January 2022 in a drylot pen (~ 0.06 hectares; 0.003 hectares/hd).

Twenty-four organic, purebred Holstein heifers (BW = 219 ± 17 kg; age: 9 mo) were acclimated to two SmartFeed Pro trailers each equipped with two feed intake measurement bunk systems in each trailer, one GreenFeed automated head chamber system (AHCS; C-Lock Inc., Rapid City, USA), and individual animal feeding stanchions. During the 28 d acclimation period, the animals were introduced to the AHCS with entrance alley panels removed as described by Gunter and Bradford (2017). Heifer AHCS visitation was not limited during the acclimation period to motivate voluntary acclimation to the AHCS system. To maintain consistent use of the AHCS during the acclimation period, alley panels were put in place on d 21 and gradually narrowed until one animal at a time could enter and use the AHCS. A 7 d pretrial gas analysis period was conducted from d -7 to -1 to account for individual animal emissions differences and serve as a baseline for each individual animal in subsequent analyses. Following acclimation, 20 acclimated heifers were selected to participate in the study based on frequency of visitation to the AHCS. Heifers were randomly assigned to one of four treatments (CON= tannin supplemented at 0% of dry matter intake (DMI), LOW= tannin supplemented at 0.075% DMI, MED= tannin supplemented at 0.15% DMI, and HIG= tannin supplemented at 0.30% DMI), and stratified based on initial body weight (BW = 219 ± 17 kg). Supplemental tannin dosage was recommended by the manufacturer and mixed to a total weight of one kg with sweet feed (Aurora Organic Dairy, Boulder, CO Weaning Calf Feed #3) to increase the palatability of and bind to the tannin supplement to ensure consumption of the tannin offered. Heifers were fed the tannin supplement corresponding to treatment dosage in individual animal feeding stanchions daily at 0700h prior to the delivery of the total mixed ration (TMR) in the SmartFeed Pro feed

bunks for 45 d. Heifers were allotted 30 minutes to consume their assigned treatment, then were returned to the pen and orts were weighed. Throughout the duration of the trial all heifers consumed the entirety of the supplement provided, therefore no orts were present.

Enteric CH_4 and CO_2 Emissions

Daily CH_4 and CO_2 production was measured using one AHCS (Figure 1) to determine individual animal CH_4 and CO_2 emissions, which naturally exhibit some degree of variability (Figure 2). Heifers voluntarily utilized the AHCS system and emissions data was recorded upon recognition of a radio frequency identification (RFID) ear tag placed in the left ear. Organic alfalfa pellets (Alfalfa Pellets (#5863); Modesto Milling Inc., Empire, CA) were used in the AHCS as a bait feed that was dispensed at each drop during an animal visit. The AHCS was programmed to dispense 6 drops of organic alfalfa pellets as bait at 30 s intervals when an animal was present. This distribution routine encouraged the animal to stay at the AHCS while exhalations were sampled (Gunter et al., 2017). Only measurements from animals sampled from three to eight minutes were considered sufficient for statistical analysis (Gunter et al., 2017). Three minute samples have been standardized as the minimum time required to capture at least three eructations during a sampling event, which minimizes variation in CH_4 emission estimates (Huhtanen et al., 2015; Velazco et al., 2016; Arthur et al., 2017). Additionally, airflow through the AHCS is an important factor, therefore visits reporting airflow below 26 L/s were discarded (Gunter et al., 2017). Heifers were limited to 4 visits per day, with a minimum 4 h interval between visits. Visits were limited to capture diurnal variation in CH_4 emissions by encouraging an even distribution of visits throughout a 24 h period (Gunter and Bradford, 2017). The CH_4 and CO_2 sensors were calibrated daily by a manufacturer installed automatic calibration system (C-Lock Inc., Rapid City, USA). During the experiment, two CO_2 recoveries were performed (d -8

and 45) to gravimetrically calibrate the air flux sensor by releasing CO_2 into the AHCS with a 90 g prefilled CO_2 cylinder supplied by the manufacturer. During each CO_2 recovery three release intervals were conducted where approximately 30 g of CO_2 was released into the AHCS. Each release interval was three minutes in duration with a three minute waiting period between each release interval. Gravimetric release was compared with the AHCS calculated capture using an internet portal. A clean air filter was fitted to the AHCS when air flow was < 33.1 L/s.

Animal Feed Intake

Daily feed intake was measured using two SmartFeed Pro trailers, each equipped with two intake measurement bunk systems, for a total of four intake measurement bunk systems (C-Lock Inc., Rapid City, USA). Heifers voluntarily utilized the SmartFeed Pro feed bunks and data was recorded upon recognition of a RFID ear tag placed in the left ear. A TMR was delivered into the SmartFeed Pro bunks daily following treatment feeding at 0700h.

Feed Samples

Fresh and residual TMR samples were collected daily from each SmartFeed Pro feed bunk and preserved in WhirlPak (Filtration Group, Madison, USA) sampling bags at -20°C until the completion of the study. At study completion, samples were thawed, dried at 65°C for 72 h to determine analytic DM. Dried samples were then ground to pass a 1 mm screen (Wiley Mill, Model 4; Arthur H. Thomas Co., Philadelphia, PA). Following grinding, samples were composited by week. Weekly composites were then composited into one sample per TMR formulation and sent to a commercial laboratory (Dairy One Inc., Ithaca, NY) for nutritive analysis (Table 2). Two TMR formulations were fed during the duration of the study. The TMR formulation changed on d 26 of the study. The TMR formulations were classified as TMR^A, a

growing heifer ration, consisting of 57.4% roughage and 38.9% concentrate and TMR[B], a pre-breeding ration, consisting of 87.4% roughage and 12.1% concentrate (Table 1).

Samples of the Silvafeed® ByPro tannin product were taken on d 0, 23, and 45, composited, and sent to a commercial laboratory (Dairy One Inc., Ithaca, NY) for nutritive analysis (Table 2). Organic alfalfa pellets and sweet feed were collected on d 0, 23, and 45, weighed and dried at 65°C for 72 h to determine analytic DM. Dried samples were ground to pass a 1 mm screen (Wiley Mill, Model 4; Arthur H. Thomas Co., Philadelphia, PA). Following grinding, samples were composited and sent to a commercial laboratory (Dairy One Inc., Ithaca, NY) for nutritive analysis (Table 2). A 240 h *in situ* analysis was performed using a fistulated steer, followed by neutral detergent fiber (NDF) and acid detergent fiber (ADF) analyses for composited TMR, organic alfalfa pellets, and sweet feed samples using an Ankom 200 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY) using methodologies derived from Van Soest et al. (1991) to determine digestibility.

Animal Body Weights

Heifers were weighed (unshrunk) on d -15, 0, 8, 15, 23, 29, 36, 43, and 45 before treatment feeding at 0700h on a validated scale (Tru-Test AP600 platform and Tru-Test ID5000 scale indicator) using methodologies described by Thompson et al. (2019). Average daily gain (ADG) was calculated using weekly weights collected from d 0 to 45.

Blood Samples

For the purposes of this study, malondialdehyde (MDA), a product of lipid peroxidation, was used as an indicator of oxidative stress (Armstrong and Browne, 1994). Antioxidant enzyme activity was assessed through the analysis of superoxide dismutase (SOD) and reduced glutathione (GSH) concentrations in serum due to historic research using these two enzymes and

the role they play in antioxidative status, despite several endogenous antioxidant enzymes having the ability to convert oxygen-derived free radicals into less dangerous forms. Moreover, blood urea nitrogen (BUN) and creatinine were assessed as additional blood parameters used to evaluate additional N impacts of tannin supplementation.

Blood was collected in two BD Vacutainer® blood collection tubes (Fisher Scientific, Pittsburgh, PA) from the jugular vein on d 0, 23, and 45, placed on ice, and transported to CSU laboratory facilities. Blood was centrifuged in a Thermo IEC Centra® GP8 Centrifuge for 12 min at 3400 RPM. Following centrifuging, serum was pipetted into Eppendorf Tubes® (Eppendorf AG, Hamburg, Germany) and stored at -20°C. Following study completion, serum was used to draw inference on oxidative stress by deriving MDA, SOD, and GSH concentrations using the TBARS Assay Kit, Superoxide Dismutase Assay Kit, and Glutathione Assay Kit, respectively (Cayman Chemical Company, Ann Arbor, MI). The TBARS Assay Kit was a colorimetric assay used to determine MDA concentration which is a commonly used biomarker for oxidative stress. The Superoxide Dismutase Assay Kit was a fluorometric analysis that analyzed SODs, which are crucial components in the cellular antioxidant defense mechanism (Malstrom et al., 1975). The Glutathione Assay Kit was a fluorometric analysis used to analyze reduced glutathione. Glutathione occurs in two states in healthy cells, GSH and oxidized glutathione (GSSG), with the majority being in the GSH form. Glutathione was evaluated since it is a well-established antioxidant in cells and plays a critical role in the maintenance and regulation of proper physiological functioning (Aquilano et al., 2014).

Following study completion, serum samples from each heifer at d 0, 23, and 45 were sent to the CSU Diagnostic Laboratory to assess additional blood parameters blood urea nitrogen

(BUN) and creatinine, to draw further inference on how tannin supplementation might impact N use in growing heifers.

Fecal Samples

Fecal samples were collected on d 0, 23, and 45, placed in quart-sized Ziploc bags, and transported to CSU laboratory facilities. Samples were then weighed and dried at 65°C for 72 h to determine analytic DM. Dried fecal samples were ground to pass a 1 mm screen (Wiley Mill, Model 4; Arthur H. Thomas Co., Philadelphia, PA) and placed in WhirlPak sample bags to prevent sample contamination until further analysis occurred. A 240 h *in situ* analysis was conducted in a fistulated steer followed by NDF and ADF analyses using an Ankom 200 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY) using methodologies derived from Van Soest et al. (1991) to determine digestibility.

CALCULATIONS

Neutral Detergent Fiber Equation

Neutral detergent fiber from fecal and feed samples was determined using Equation 1 below, provided by Ankom Technology Method 13.

Equation 1

$$NDF = (100 * (W_3 - (W_1 * C_1))) / W_2$$

In Equation 1, W_3 represents the dry weight of the Ankom F57 filter bag including sample after the extraction process, W_2 represents the initial sample weight, W_1 represents the initial weight of the Ankom F57 filter bag, and C_1 represents the blank bag correction factor.

Acid Detergent Fiber Equation

Acid detergent fiber from fecal and feed samples was determined using Equation 2 below, provided by Ankom Technology Method 12.

Equation 2

$$ADF = (100 * (W_3 - (W_1 * C_1))) / W_2$$

In Equation 2, W_3 represents the dry weight of the Ankom F57 filter bag including sample after the extraction process, W_2 represents the initial sample weight, W_1 represents the initial weight of the Ankom F57 filter bag, and C_1 represents the blank bag correction factor.

Digestibility

Digestibility was determined using Equation 3 below, from Velásquez et al. (2018) adapted from Kartchner (1980).

Equation 3:

Digestibility (g/kg DM) = 1- ((ADF in feed/ feed DM)/(ADF in feces/ fecal DM))

In Equation 3, ADF in feed and feces was determined using methodologies explained in Equation 2.

Urinary N Excretion Estimation

Daily urinary N (UN) excretion in grams was estimated using Equation 4 below, from Kohn (2005).

Equation 4

$$UN (g/d) = CR \times BUN \times BW$$

In Equation 4, CR stands for N clearance rate in L of blood cleared of BUN per d per kg body weight (BW) and BUN stands for blood urea N in g/L.

Daily urinary N (UN) excretion in grams was also estimated using Equation 5 and below, from Reed et al. (2015).

Equation 5

$$UN\ (g/d) = 14.3 + (0.510 \times NI)$$

In Equation 5, NI is nitrogen intake in grams per day

Fecal N Excretion Estimation

Daily fecal N (FN) excretion in grams was estimated by first calculating fecal output using Equation 6 below, from Kartchner (1980).

Equation 6

$$\text{Fecal output} = \text{Intake} * (1\text{-Digestibility})$$

In Equation 6, intake was determined per experimental unit, the individual animal, using four SmartFeed Pro intake measurement bunk systems and digestibility was calculated using Equation 3.

Daily FN was derived using fecal output calculated with Equation 6 and multiplying fecal output by the percent N in feces collected on d 0, 23, and 45 of the study using Equation 7 below, developed from methodologies described by Peripolli et al. (2011).

Equation 7

$$FN\ (g/d) = \text{Fecal output}\ (g/d) * \text{fecal \% N}$$

In Equation 7, FN excretion in grams per d was estimated by multiplying fecal output by percent N from fecal samples collected on d 0, 23, and 45.

STATISTICAL ANALYSIS

JMP® Pro (JMP®, v. 16.2.0. SAS Institute Inc., Cary, NC, 1989–2021) software was used for initial data visualization and correlation analyses. R© (R Core Team, 2021, v. 4.1.2) software was used for all subsequent analysis of data. Data were analyzed as a completely randomized design. The experimental unit was animal (n=20). Treatment was included in the model as a fixed effect. Data was analyzed using the Type III ANOVA procedure, *aov()*, in the 'car' package (Fox and Weisberg, 2019), a pairwise comparison was analyzed for dependent variables by treatment using the least squared means procedure in the 'lsmeans' package, of R© (R Core Team, 2021, v. 4.1.2) with the Tukey HSD adjustment applied (Lenth, 2016). Dependent variables were ADG, DMI, G:F (kg BW gain/ kg DMI), daily CH_4 production (g CH_4/hd/d), CH_4 as a percent of gross energy (GE) intake (Y_m), CH_4 yield (MY; g CH_4/kg DMI), CH_4 emission intensity (EI; g of CH_4/kg ADG gain), CO_2 production (g CO_2/hd/d), N intake (g/d), N excretion (g/d), UN (g/d), fecal output (kg/d), FN (g/d), FN:UN, fecal NDF (%), fecal ADF (%), BUN (mg/dL), creatinine (mg/dL), MDA (μM), SOD (units/mL), and GSH (μM). Daily CH_4 and CO_2 production was analyzed using the 7 d pretrial emissions rate as a covariate (Hristov et al., 2015). Average daily gain was determined via the slope coefficient of a linear regression as a function of gain and day using *lm()* (R Core Team, 2021, v. 4.1.2) using weights collected from d 0 to 45 (Ahlberg et al., 2018). The effect of treatment was determined significant at $P < 0.05$.

RESULTS

Individual animal CH_4 production in g/hd/d naturally exhibits some degree of variation (Figure 2). Between animal variation across all treatments from d 0 to 45 for average CH_4

production in g/hd/d had a coefficient of variation (CV) of 39%. Between animal treatment CVs for average CH_4 production in g/hd/d were CON=39%, LOW=42%, MED=37%, and HIG=37%. Daily CH_4 production ranged from 136.5 to 140.1 g CH_4/hd/d among treatments (Figure 3). Daily CH_4 production by treatment was averaged over the entire study by the hour of the day and exhibited an expected diurnal pattern across treatments (Figure 4). Daily CH_4 production was correlated to DMI (Figure 5), with 42% of the variation in daily CH_4 production explained by DMI (R^2=0.42; Figure 6). No significant difference was observed among treatments for daily CH_4 production (P=0.95), CO_2 production (P=0.95),Y_m (P=0.87), MY (P=0.80), or EI (P=0.70) (Table 3). Daily DMI ranged from 6.32 to 7.00 kg/d among treatments (Figure 7). Average daily DMI by treatment by day also increased from d 0 to 45 (Figure 8). A treatment effect was not observed for DMI (P=0.92), ADG (P=0.53), or G:F (P=0.42) (Table 4). No significant difference was observed among treatments for N intake (P=0.93), estimated fecal output (P=0.98), FN (P=0.98), fecal NDF (P=0.33) or fecal ADF (P=0.30; Table 5). Estimated UN (P=0.77), FN:UN (P=0.93), and N excretion (P=0.86) did not differ among treatments when estimated using methodologies described by Kohn (2005) (Table 5). Similarly, estimated UN (P=0.66), FN:UN (P=0.94), and N excretion (P=0.72) did not differ among treatments when estimated using methodologies described by Reed (2015) (Table 5). No significant difference was observed among treatments for common oxidative stress biomarker MDA (P=0.63) or antioxidant enzyme biomarkers SOD (P=0.26) and GSH (P=0.19) (Table 6). Blood parameters BUN (P=0.99) and creatinine (P=0.20) did not significantly differ among treatments (Table 6).

DISCUSSION

Daily CH_4 production naturally exhibits some degree of variation (Garnsworthy et al., 2012; Yan et al., 2010; Ellis et al., 2010; Grainger et al., 2007; Figure 2). Between heifer variation from d 0 to 45 across all treatments for average CH_4 production in g/hd/d had a coefficient of variation (CV) of 39%. A similar coefficient of variation (30%) was reported by Garnsworthy et al. (2012) for CH_4 emissions in g/min for lactating Holstein cows using CH_4 analyzers installed in on-farm robotic milking stations. Yan et al. (2010) analyzed data from 20 energy metabolism studies using lactating dairy cows fed *ad libitum* in respiration chambers and reported a between animal CV for CH_4 production measured in L/hd/d of 17.1%. Ellis et al. (2010) summarized results from 16 studies evaluating CH_4 production in g/hd/d using calorimetry techniques for dairy cows which exhibited CVs ranging from 3 to 34%. Moreover, in a study conducted by Grainger et al. (2007) using both respiration chambers and the SF_6 tracer technique to quantify CH_4 production in g/hd/d for lactating dairy cows fed *ad libitum*, reported variability between cows was higher for the SF_6 tracer technique (CV = 19.6%) than for respiration chambers (CV = 17.8%). However, when comparing observations from Yan et al. (2010), Ellis et al. (2010), and Grainger et al. (2007) to the present study and Garnsworthy et al. (2012), differences in methodologies used to quantify CH_4 production might have contributed to greater variation reported using on-farm techniques described in the present study and by Garnsworthy et al. (2012). Respiration chambers and SF_6 tracer technique methodologies have been used extensively to quantify CH_4 production from smaller numbers of animals and are rarely used in on-farm production settings. Respiration chambers provide accurate measures of CH_4 production for limited numbers of individual animals under controlled conditions but are impractical for use in production environments and have reported decreases in animal

performance due to reductions in voluntary feed intake (Alemu et al., 2017; Llonch et al., 2018; Moate et al., 2013). The SF_6 tracer technique requires a gas collection apparatus attached to the cow, insertion of rumen boluses to release SF_6, and frequent cow handling, all of which can interfere with cow behavior. Using on-farm techniques described in the present study and by Garnsworthy et al. (2012) may more accurately describe the natural individual animal varication observed on-farm. Ultimately, further investigation quantifying the variation in CH_4 production is needed in order to accurately account for between animal variation when designing experiments capable of detecting treatment differences when testing CH_4 mitigation strategies.

In the present study, daily CH_4 production values by treatment were averaged over the entire study by hour of the day and exhibited expected diurnal variation across treatments (Figure 4). Garnsworthy et al. (2012) also reported distinct diurnal variation in CH_4 emissions when lactating Holsteins were fed a partial mixed ration (PMR) between 0700 and 0900h daily, which is when peak feeding activity was observed, even though PMR was available *ad libitum* throughout the day (24h). Garnsworthy et al. (2012) observed CH_4 emission rates increase sharply between 0800 and 1000h, remain relatively steady throughout the day, and decrease between 1800 and 0600h, which is consistent with observations reported in the present study (Figure 4). Grainger et al. (2007) observed a diurnal pattern in CH_4 production for individual dairy cows in respiration chambers, when cattle were fed twice daily.

Daily CH_4 production was correlated to DMI in the present study (Figure 5). The majority of feed was consumed between 0800 and 1700h, while CH_4 emission rate increased sharply beginning at 0800h and remained relatively steady until 1700h, which is consistent with observations reported by Garnsworthy et al. (2012). In the present study, 42% of the variation in daily CH_4 production was explained by DMI (R^2=0.42; Figure 6). Similar results were observed

by Grainger et al. (2007) where 39% of the variation in CH_4 was explained by DMI ($R^2 = 0.39$) using respiration chambers and 56% of the variation in CH_4 was explained by DMI ($R^2 = 0.56$) when using the SF_6 tracer technique.

Pre-existing literature has not explored the inclusion of tannins in a diet fed to growing dairy heifers in an organic dairy system. The present study did not find low-level tannin supplementation to alter CH_4 production, CO_2 production, Y_m, MY, or EI among treatments. Similar results to the present study were observed by Beauchemin et al. (2007) when quebracho tannin extract containing 91% condensed tannins was included in a roughage-based diet (70% roughage) fed to beef steers and heifers at 0%, 1%, and 2% of dietary DM, yielding no effect on CH_4 production or Y_m. Ebert et al. (2017) reported similar findings to the present study for CH_4 production and Y_m when finishing beef steers fed a high concentrate diet (85.3% concentrate) supplemented with a quebracho tannins, manufactured by Silvafeed®, at 0%, 0.5%, and 1.0% of dietary DM, observing no effect of tannin supplementation. However, Piñeiro-Vázquez et al. (2017) observed significant reductions in CH_4 production and MY at 3% and 4% tannin inclusion rates compared to 0% and 1% tannin inclusion rates in crossbred *Bos taurus* x *Bos indicus* heifers supplemented with quebracho tannin extract at 0%, 1%, 2%, 3%, and 4% of DMI. Piñeiro-Vázquez et al. (2017) also reported DMI decreased, with DMI being significantly lower at the 4% inclusion rate compared to the 0% inclusion rate. Beauchemin et al. (2007) and Piñeiro-Vázquez et al. (2017) utilized respiration chambers to quantify CH_4 production whereas the current study and Ebert et al. (2017) utilized an AHCS. Despite the variability in the efficacy of quebracho tannin products and extracts to reduce CH_4 emissions, the lack of evidence that CH_4 was reduced in the present study could suggest that the level of condensed tannin supplementation may have been below the threshold required to reduce CH_4 in growing cattle.

Evidence has reported that tannins negatively, positively, and have no impact on DMI. In the present study, the inclusion of tannins did not alter DMI, ADG, or G:F among treatments. Similar results to the present study were reported by Beauchemin et al. (2007) where tannin supplementation yielded no effect on BW, ADG, or DMI. Ebert et al. (2017) observed similar findings to the present study and Beauchemin et al. (2007), reporting no effect of tannin supplementation on BW, ADG, DMI, or G:F. However, a study conducted by Norris et al. (2020) reported positive effects on DMI when quebracho tannin extract was included in a roughage-based diet (56.5% roughage) fed to beef steers at 0%, 1.5%, 3%, and 4.5% of DM. Norris et al. (2020) observed steers fed a diet including tannins at 4.5% DM exhibited higher DMI compared to the 0% of dry matter inclusion rate. Aguerre et al. (2020) also noted positive effects on DMI when lactating dairy cattle fed a quebracho-chestnut tannin extract at 0%, 0.45%, or 1.80% of dietary DM and had significantly higher DMI at the 1.8% of DM tannin inclusion rate compared to the 0% of DM tannin inclusion rate when lactating dairy cows were fed a high roughage diet (54% roughage). Aguerre et al. (2016) reported lactating dairy cattle fed a quebracho-chestnut tannin extract at 0%, 0.45%, 0.90%, or 1.80% of dietary DM and had significantly lower DMI at the 1.8% of DM tannin inclusion rate compared to the 0% of DM tannin inclusion rate. Piñeiro-Vázquez et al. (2017) observed that DMI was significantly reduced at 3% and 4% tannin inclusion rates compared to 0% and 1% tannin inclusion rates. Additionally, heifers used in the study conducted by Piñeiro-Vázquez et al. (2017) were fed a diet consisting of low-quality tropical grass consisting of approximately 11% crude protein (CP), whereas heifers in the present study were fed a diet consisting of approximately 19% CP. Therefore, diet quality differences could contribute to the variable results reported amongst the

studies. Future research should include how diet quality impacts DMI and how tannin type, purity, and dose may be related to DMI and other animal performance measures.

Since tannins bind proteins in the rumen, increasing bypass protein, it is logical to predict that the addition of tannins in the diet could result in a decrease in N excreted in urine and increase N excreted in the feces as the degradation of protein is altered. The present study observed low-level tannin supplementation did not alter N intake, estimated N excretion, estimated fecal output, FN: UN, UN, FN, NDF, or ADF. In the present study, when utilizing methodologies described by Kohn (2005), non-biologically explained N retention was observed based on animal intake data (Figure 8). Discrepancies in results could be due to the timing of sample collection, which based on animal visitation and intake data (Figure 5), might have occurred in a time period preceded by minimal feed intake. As explained by Lavery and Ferris (2021) BUN concentration in blood can fluctuate throughout the day, with the highest levels normally detected 4 to 6 h after feeding. Additional evidence has observed that as N intake increases BUN concentration in blood increases (Ordway et al., 2002; Lavery and Ferris, 2021). Thus, due to the relationship between N intake and BUN, blood samples for BUN analysis may have been collected at a time when BUN concentration was low due to minimal feed intake. Future studies should consider collecting multiple samples throughout a 24 h period when sampling to estimate N excretion, in order to improve the quantification of daily UN excretion. When using methodologies described by Reed et al. (2015), biologically explainable N excretion was quantified, but a treatment effect beyond the impact treatments had on DMI and NI is not quantifiable using this method. Therefore, this study has identified a gap in the literature for predicting N excretion in production environments, that can identify a treatment effect. Based on

current methodologies, on-farm research assessing the practicality of emissions mitigation strategies and the quantification on N emissions is not practical or feasible.

Similar N excretion results to the present study were reported by Ebert et al. (2017). Ebert et al. (2017) reported that UN excretion and retained N were not different among treatments when supplemented at 0%, 0.5%, and 1% of DM, but UN as a proportion of total N excretion decreased and FN as a proportion of total N excretion increased significantly when tannins were included in the diet at 1% of DM compared to 0% of DM. However, it should be noted that Ebert et al. (2017) observed the apparent total tract digestibility of starch linearly decreased as the dose of supplemental tannin increased. Dschaak et al. (2011) investigated the effect on supplementing condensed quebracho tannin extract at 0% and 3% of DM to lactating dairy cows fed a high forage and a low forage diet. Unlike the present study, Dschaak et al. (2011) observed diets including tannins at 3% had decreased ruminal ammonia-N and MUN concentrations, which suggested less ruminal N was lost as ammonia due to tannins altering protein degradation in the rumen.

Dschaak et al. (2011) and Ahnert et al. (2015) demonstrated that as quebracho tannin extract dosage increased, fecal N increased, and urinary N decreased from six cannulated heifers infused with quebracho tannin extract at 0%, 1%, 2%, 3%, 4%, 5%, and 6% of DMI. These findings were consistent with Aguerre et al. (2016) and Aguerre et al. (2020) in lactating dairy cows using a quebracho-chestnut tannin extract. Aguerre et al. (2016) observed that tannin extract supplementation at 1.8% DM resulted in lower UN, but also had detrimental effects on DMI. Aguerre et al. (2020) observed tannin extract supplemented a 1.8% of DM reduced estimated urinary N excretion by 11%, but lowered feed efficiency. Norris et al. (2020) observed tannins altered the N excretion route at the inclusion rate of 4.5% of DM, evident by a 14%

increase in the average FN:total N excreted and 38% increase in the FN:UN, without altering retained N. Furthermore, Norris et al. (2020) reported tannin extract reduced digestibility of N and tended to impact fiber digestibility, similar to findings reported by Aguerre et al. (2016) and Aguerre et al. (2020). Contradictory results to the present study reported by Dschaak et al. (2011), Ahnert et al. (2015), Aguerre et al. (2016), Aguerre et al. (2020), and Norris et al. (2020) could manifest due to a difference in type or dose of tannin supplementation being tested, diet composition, differences in physiological status of animals, and/or differences in methods used to derive UN and FN values.

The present study did not find BUN or creatinine values to differ among treatments. Assessing blood parameters BUN and creatinine could provide further insight into degradation of N in dairy cattle. Marshall et al. (2022) found in Holstein-Friesian × Jersey heifers supplemented with a mixture of quebracho condensed and chestnut hydrolysable tannins at 0.15% DMI a 12% reduction in BUN. Similar reductions in BUN were observed by Stewart et al. (2019) when feeding bird's-foot trefoil (Lotus corniculatus) and sanfoin (Onobrychis viciifolia) containing condensed tannins in hay at 2.5% and 0.6% of DM, respectively to beef cows and heifers. Based on findings from Marshall et al. (2022) and Stewart et al. (2019), level of tannin supplementation in the present study may have been below the threshold necessary to reduce BUN and creatinine.

When considering the proposed benefits of condensed tannins supplementation, evaluations of oxidative stress in cattle have been evaluated to a lesser extent when compared to evaluations of N-excretion and CH_4 emissions. Most evaluations regarding changes in oxidative stress and concomitant antioxidant enzyme activity have been conducted in transition and lactating dairy cattle. This is likely due to the physiological strain and stress experienced by transition and lactating dairy cattle. However, it is also relevant to explore impacts of tannin

supplementation on antioxidative status in heifers. The present study did not find low-level tannin supplementation to alter common oxidative stress (MDA) or antioxidant enzyme (SOD and GSH) biomarkers. Contradictory to these findings, Liu et al. (2013) reported that when a chestnut tannin was included in the diet at 10 g/kg DM, SOD and glutathione peroxidase (GSH-Px) increased while MDA decreased with tannin supplementation, suggesting that supplementing tannins in the diet might inhibit lipid peroxidation and increase antioxidant enzymes activities in plasma of transition dairy cows. Santillo et al. (2022) observed a blended chestnut-quebracho tannin supplemented at 60:40 ratio at 50 g/hd/d to lactating dairy cows to alter oxidative stress markers in plasma, with lower values reported for relative oxygen metabolites, and oxidative stress index (reactive oxygen metabolites/biological antioxidant potential). However, for both Liu et al. (2013) and Santillo et al. (2022) the physiological state of experimental lactating cows should be considered, since physiological state may alter the ability for tannins to impact oxidative stress and antioxidant enzyme activity, when compared to heifers used in the present.

CONCLUSION

The impact of tannin supplementation has been reported with variable efficacy in cattle production systems, which could be due to differences in tannin concentration/purity, dose, type, and other factors such as animal species, physiological state of the animal, and diet quality and composition across studies as suggested by Makkar (2003) and Aboagye and Beauchemin (2019). Despite variable results amongst observations reported in published literature, the results of this study suggest that low-level tannin supplementation, at 0%, 0.075%, 0.15%, and 0.30% of DMI, did not alter CH_4 emissions, estimated N excretion, oxidative stress, or animal performance in organic Holstein heifers. Further investigation is needed to determine a range in

which quebracho tannins can be supplemented to mitigate emissions from dairy systems, but these investigations should quantitatively consider other economic, environmental, and social implications. Investigations with higher replication, that account for between variation differences based on emissions measurement methodology, use whole animal collection measurements for N, and greater tannin dosage would likely benefit understanding of tannin efficacy when supplemented in the diet.

Table 1. Diet composition of organic Holstein heifer TMR on a dry matter basis fed in northeastern Colorado from November 2021 to January 2022

Diet Composition[1]		
Ingredients	TMR[A]	TMR[B]
Alfalfa Hay, %	4.8	36.8
Oat Hay, %	9.1	9.8
Straw, %	4.4	0.0
Haylage, %	15.2	23.0
Corn Silage, %	23.9	17.8
Mixed Grains, %	30.6	7.4
Ground Corn, %	8.3	4.7
Mineral, %	3.8	0.56

[1] Total mixed ration (TMR), TMR[A]= composite samples of total mixed ration fed from d 0 to 25, TMR[B]= composite samples of total mixed ration fed from d 26 to 45

Table 2. Nutritive analysis of organic Holstein heifer diet on a dry matter basis fed in northeastern Colorado from November 2021 to January 2022

	Feed Nutritive Analysis[1]				
Item[2]	TMR[A]	TMR[B]	AP	SF	TAN
DM, %	45.8	45.0	92.0	93.0	91.6
CP, %	19.0	18.6	20.0	21.1	1.8
TDN, %	72	70	61	81	90
GE, cal/g	4,571	4,603	4,668	4,841	5,341
% ADF[3]	20.9	27.7	30.8	7.6	0.2
% NDF[4]	29.7	36.7	35	17.6	0.3

[1] Total mixed ration (TMR), TMR[A]= composite samples of total mixed ration fed from d 0 to 25, TMR[B]= composite samples of total mixed ration fed from d 26 to 45, AP= composite sample of organic alfalfa pellets, SF= composite sample of AOD Weaning Calf Feed #3, and TAN= composite sample of tannin supplement

[2] Dry matter (DM), crude protein (CP), total digestible nutrients (TDN), gross energy (GE), acid detergent fiber (ADF), and neutral detergent fiber (NDF)

[3] ADF determined by wet chemistry

[4] Amylase and sodium sulfite treated NDF determined by wet chemistry

Table 3. Average emissions measurements for organic Holstein heifers supplemented with Silvafeed® ByPro from d 0 to 45 fed in northeastern Colorado from November 2021 to January 2022

	Treatment [1]				Standard Error [2]	P-value [3]
Item [4]	CON	LOW	MED	HIG	SE	P
CH_4 Production, g CH_4/hd/d	136.5	140.1	137.0	139.0	5.1	0.95
CO_2 Production, g CO_2/hd/d	5413.9	5515.0	5418.2	5494.1	155.0	0.95
Y_m, % GE intake	6.3	6.7	6.1	6.0	0.6	0.87
MY, g CH_4/kg DMI	21.9	23.3	21.1	20.9	2.2	0.80
EI, g CH_4/kg ADG gain	403.0	310.7	380.6	382.6	57.8	0.70

[1] Treatments for tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI

[2] Largest standard error reported of the four treatments

[3] Significance $P<0.05$

[4] Methane (CH_4) production, carbon dioxide (CO_2), average daily gain (ADG), dry matter intake (DMI), CH_4 as a % of GE intake (Y_m), CH_4 yield (MY), CH_4 emissions intensity (EI), feed efficiency (G:F)

Table 4. Average performance measurements for organic Holstein heifers supplemented with Silvafeed® ByPro from d 0 to 45 fed in northeastern Colorado from November 2021 to January 2022

	Treatment [1]				Standard Error [2]	P-value [3]
Item [4]	CON	LOW	MED	HIG	SE	P
ADG, kg BW gain/d	0.4	0.5	0.4	0.4	0.10	0.53
DMI, kg DMI/d	6.32	6.42	6.62	7.00	0.74	0.92
TMR, kg TMR DMI/d	5.06	5.06	5.34	5.68	0.74	0.92
SF, kg SF DMI/d	0.93	0.93	0.93	0.93	0.0	
GF, kg GF DMI/d	0.36	0.44	0.38	0.38	0.03	0.23
G:F, ADG/DMI	0.06	0.08	0.06	0.06	0.01	0.42

[1] Treatments for tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI

[2] Largest standard error reported of the four treatments

[3] Significance $P<0.05$

[4] Average daily gain (ADG), dry matter intake (DMI), total mixed ration (TMR), sweet feed (SF), alfalfa pellets (AP), CH_4 emissions intensity (EI), feed efficiency (G:F)

Table 5. Average estimated nitrogen excretion results from fecal samples taken on d 0, 23, and 45 for organic Holstein heifers supplemented with Silvafeed® ByPro from d 0 to 45 fed in northeastern Colorado from November 2021 to January 2022

	Treatment [1]				Standard Error [2]	P-value [3]
Item [4]	CON	LOW	MED	HIG	SE	P
N intake, g/d	195.1	197.7	204.2	214.4	21.80	0.93
Fecal ADF, %	29.4	31.7	32.2	31.1	0.01	0.30
Fecal NDF, %	46.9	48.8	49.6	48.0	0.01	0.33
Kartchner (1980)[5]						
Fecal output, g/d	719.0	722.7	723.0	747.9	51.2	0.98
FN, g/d	19.8	19.6	19.9	20.6	1.66	0.98
Kohn (2005)[6]						
UN, g/d	45.1	48.1	45.4	47.3	2.37	0.77
FN:UN	0.5	0.4	0.4	0.5	0.04	0.93
N excretion, g/d[7]	64.9	67.7	65.3	67.9	3.08	0.86
Reed et al. (2015)[8]						
UN, g/d	116.0	117.1	119.3	126.4	6.39	0.66
FN:UN	0.2	0.2	0.2	0.2	0.94	0.94
N excretion, g/d[7]	135.8	136.7	139.2	147.0	7.59	0.75

[1] Treatments for tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI

[2] Largest standard error reported of the four treatments

[3] Significance $P<0.05$

[4] Nitrogen (N) intake, fecal neutral detergent fiber (NDF), and fecal acid detergent fiber (ADF), urinary N (UN), fecal N (FN)

[5] Estimation of FN using methodologies from Kartchner (1980)

[6] Estimation of UN using methodologies from Kohn (2005)

[7] Estimation of N excretion used FN estimation methodologies derived from Kartchner (1980)

[8] Estimation of UN using methodologies from Reed et al. (2015)

Table 6. Average concentration of common oxidative stress and antioxidant enzyme biomarkers and blood parameters derived from serum taken on d 0, 23, and 45 for organic Holstein heifers supplemented with Silvafeed® ByPro from d 0 to 45 fed in northeastern Colorado from November 2021 to January 2022

	Treatment [1]				Standard Error [2]	P-value [3]
Item [4]	CON	LOW	MED	HIG	SE	P
BUN, mg/dL	14.2	14.3	14.3	14.2	0.600	0.99
Creatinine, mg/dL	0.6	0.5	0.5	0.6	0.030	0.20
MDA, μM	0.5	0.4	0.8	0.8	0.230	0.63
SOD, units/mL	0.03	0.03	0.03	0.02	0.003	0.26
GSH, μM	7.2	8.9	11.8	11.0	1.600	0.19

[1] Treatments for tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI

[2] Largest standard error reported of the four treatments

[3] Significance $P<0.05$

[4] Blood urea nitrogen (BUN), malondialdehyde (MDA), superoxide dismutase (SOD) and reduced glutathione (GSH)

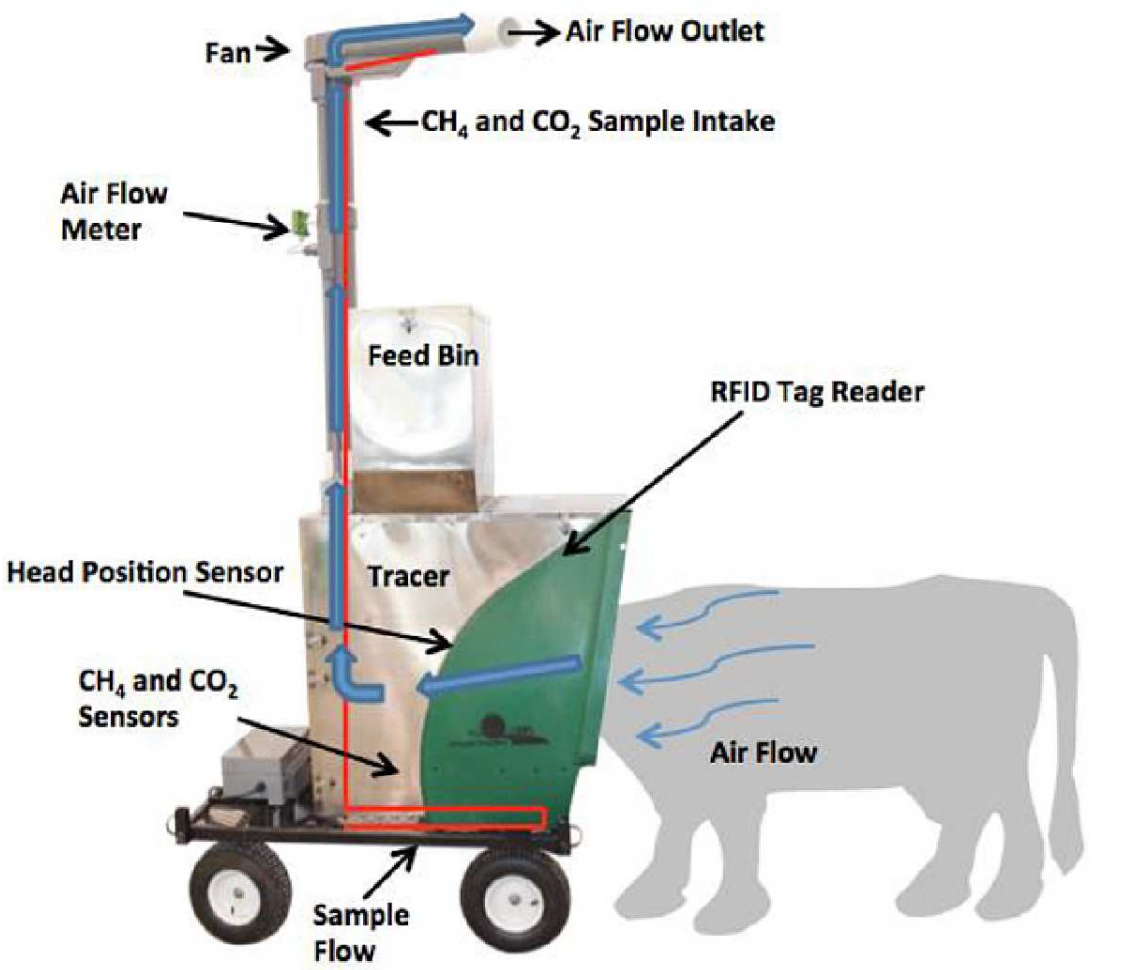

Figure 1. Components of the GreenFeed Automated Head-Chamber System (AHCS) used for measuring daily CH_4 production from Holstein heifers (Modified from Hristov et al., 2015).

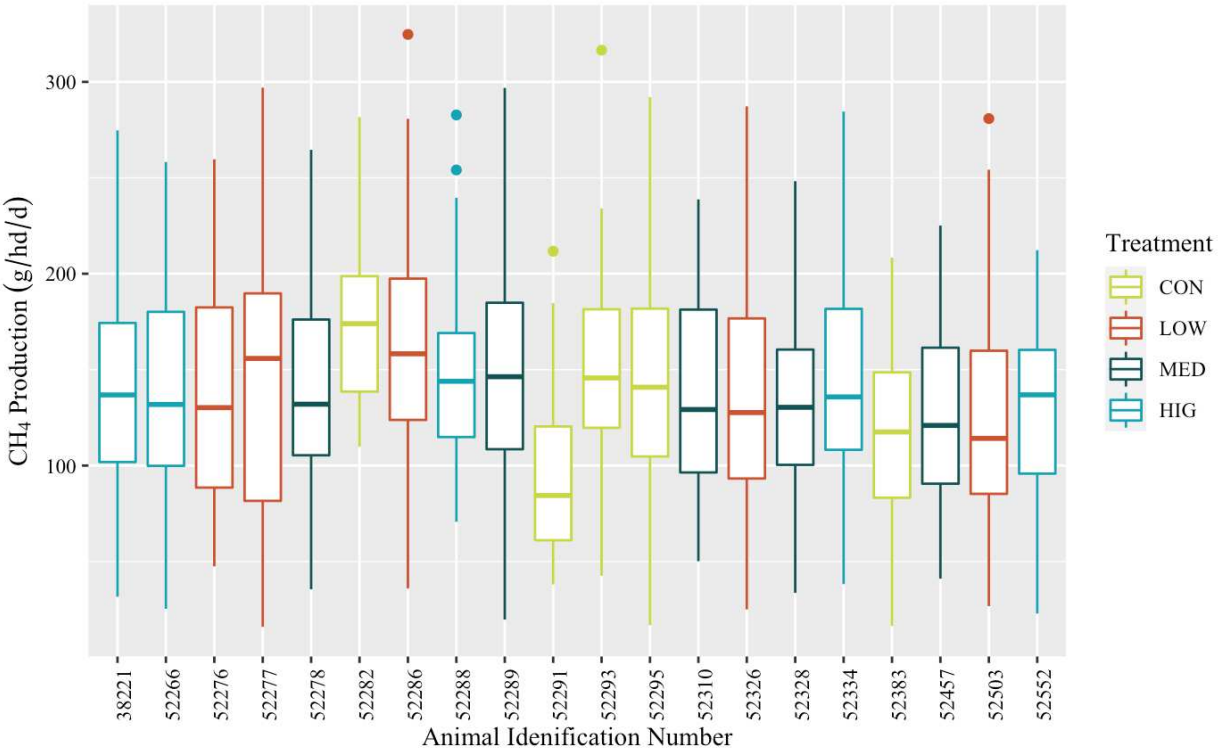

Figure 2. Variation in individual organic Holstein heifer (n=20) CH_4 production in g/hd/d by treatment with tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI from d 0 to 45.

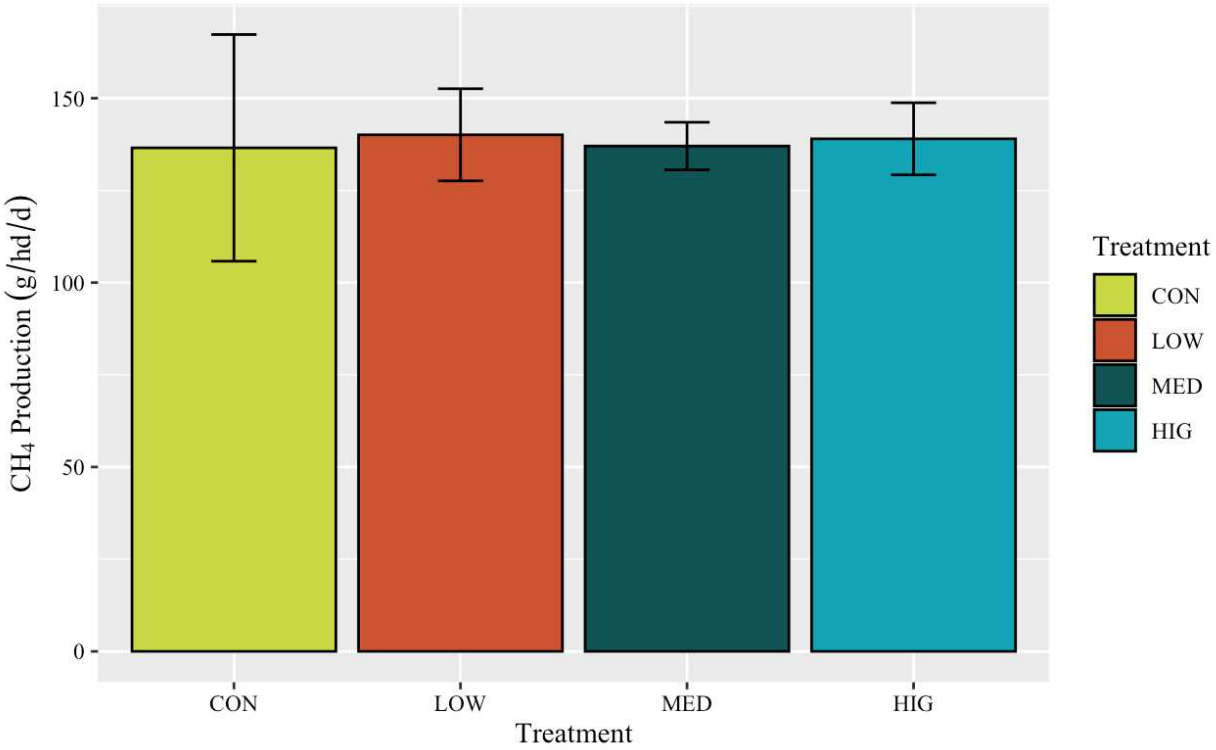

Figure 3. Average CH_4 production of organic Holstein heifers (n=5 per treatment) in g/hd/d by treatment with tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI from d 0 to 45.

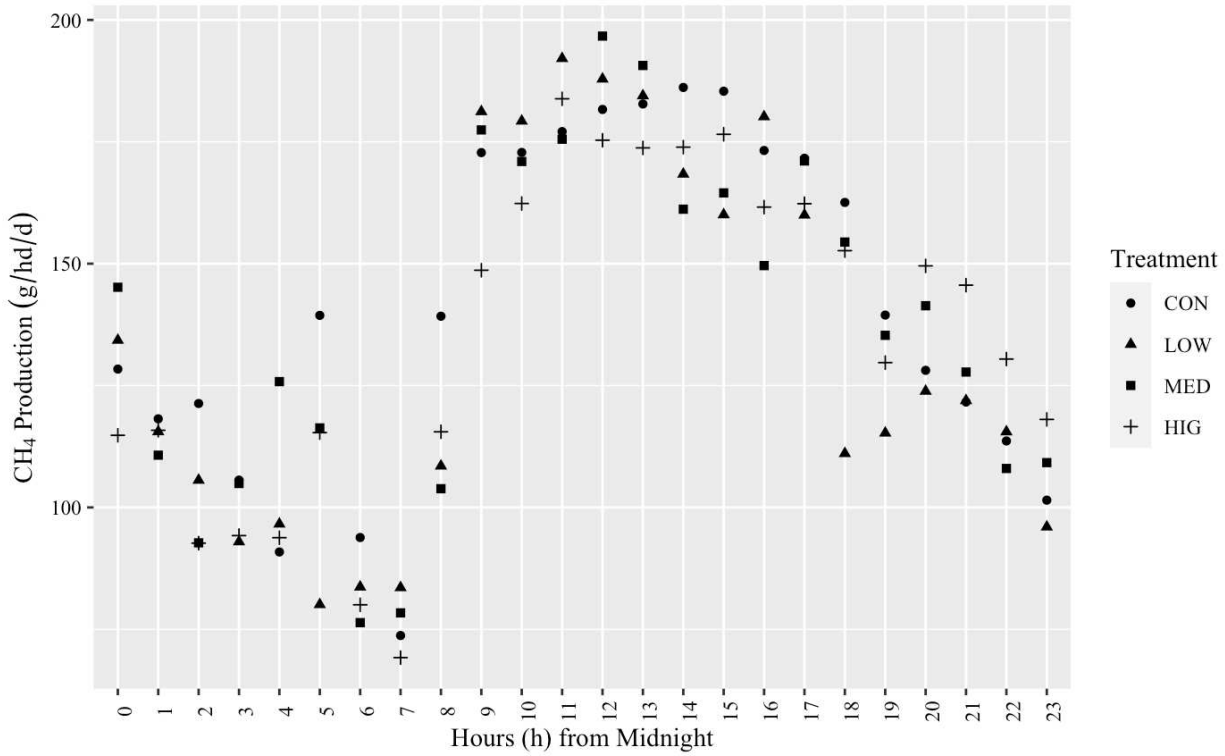

Figure 4. Average daily CH_4 production from organic Holstein heifers (n=5 per treatment) in g/hd/g by treatment with tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI by hour of the day, represented in hours from midnight.

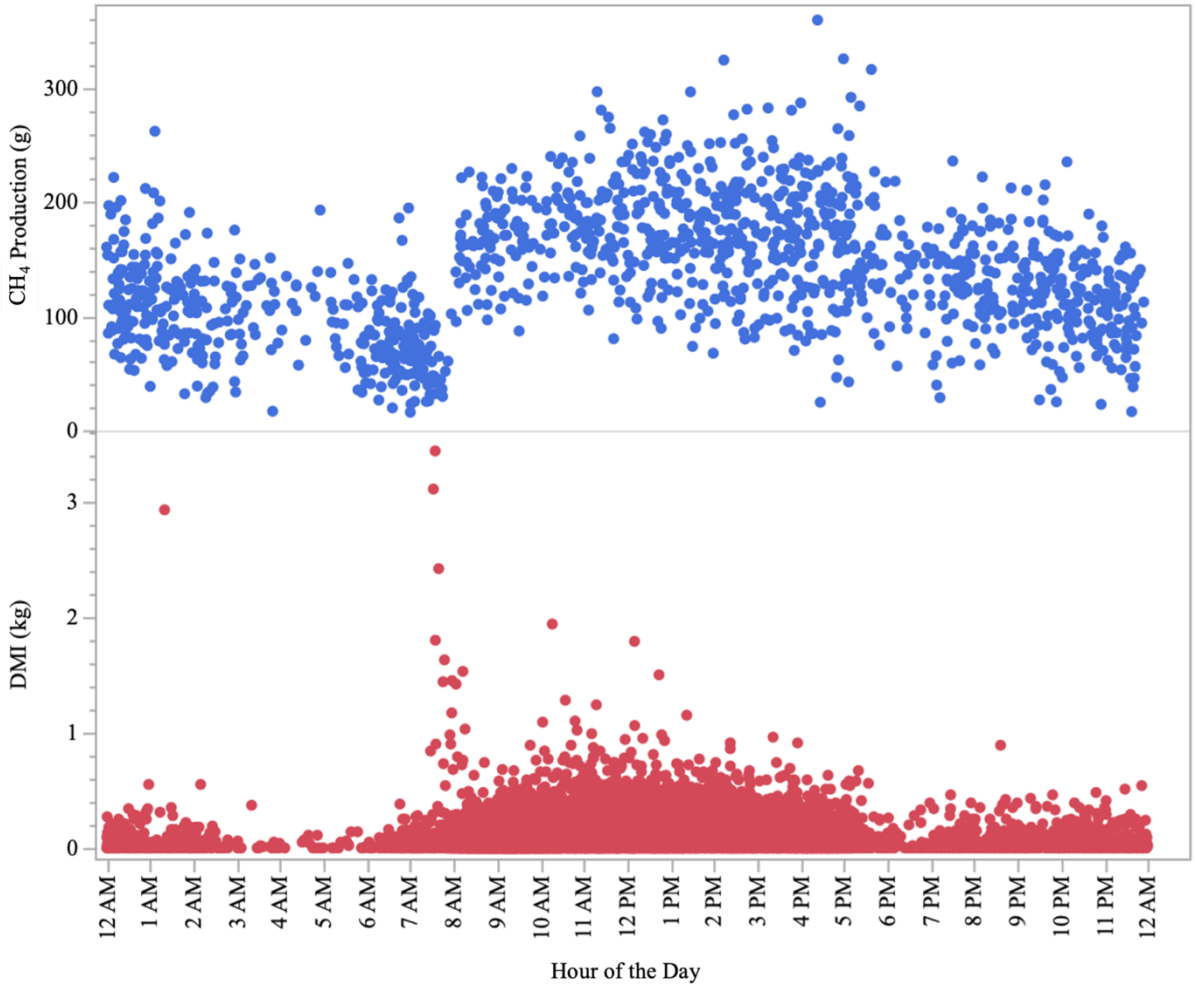

Figure 5. Relationship between CH_4 production and total mixed ration (TMR) dry matter intake (DMI) depicted by displaying animal visitation data by hour of the day where CH_4 production (g) and individual animal feed intake (kg) were measured for organic Holstein heifers (n=20) using one Greenfeed automated head chamber system (AHCS) and four SmartFeed Pro intake measurement bunk systems, respectively.

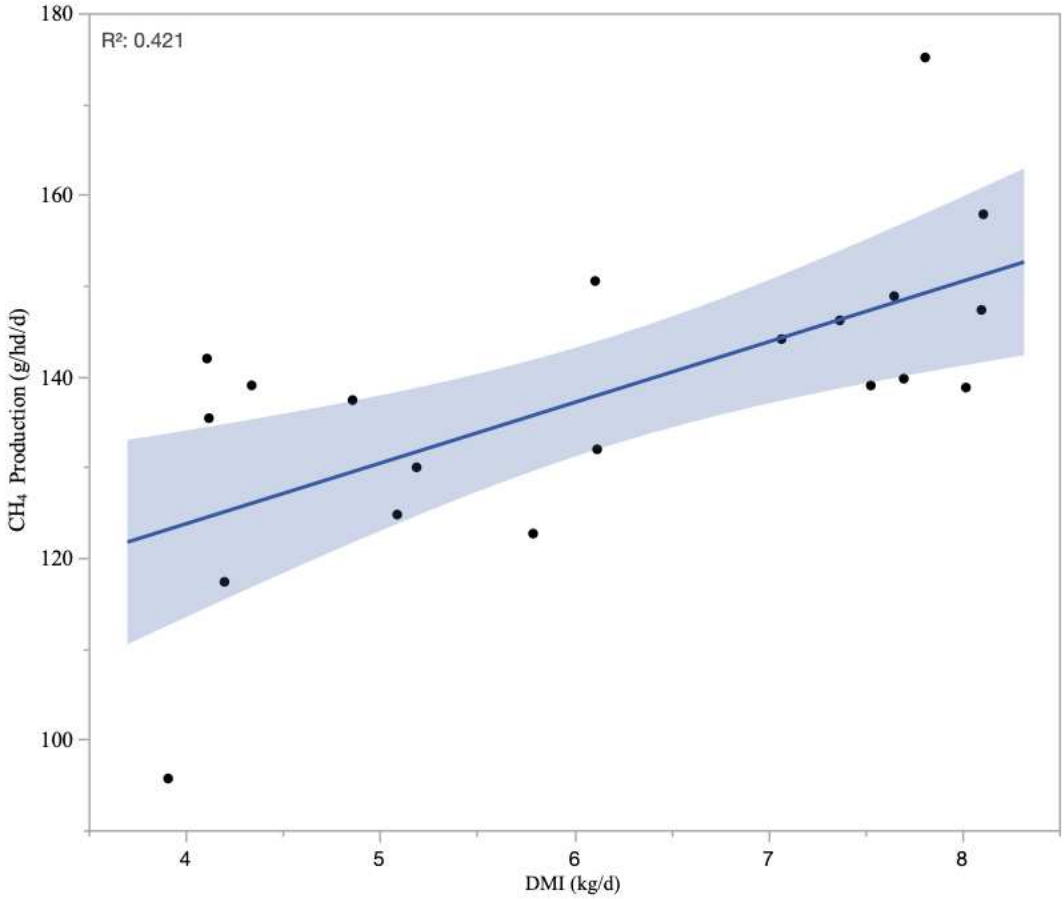

Figure 6. Correlation between average methane (CH_4) production and dry matter intake (DMI) for organic Holstein heifers (n=20) receiving a supplemental tannin product for 45 d in northeastern Colorado from November 2021 to January 2022.

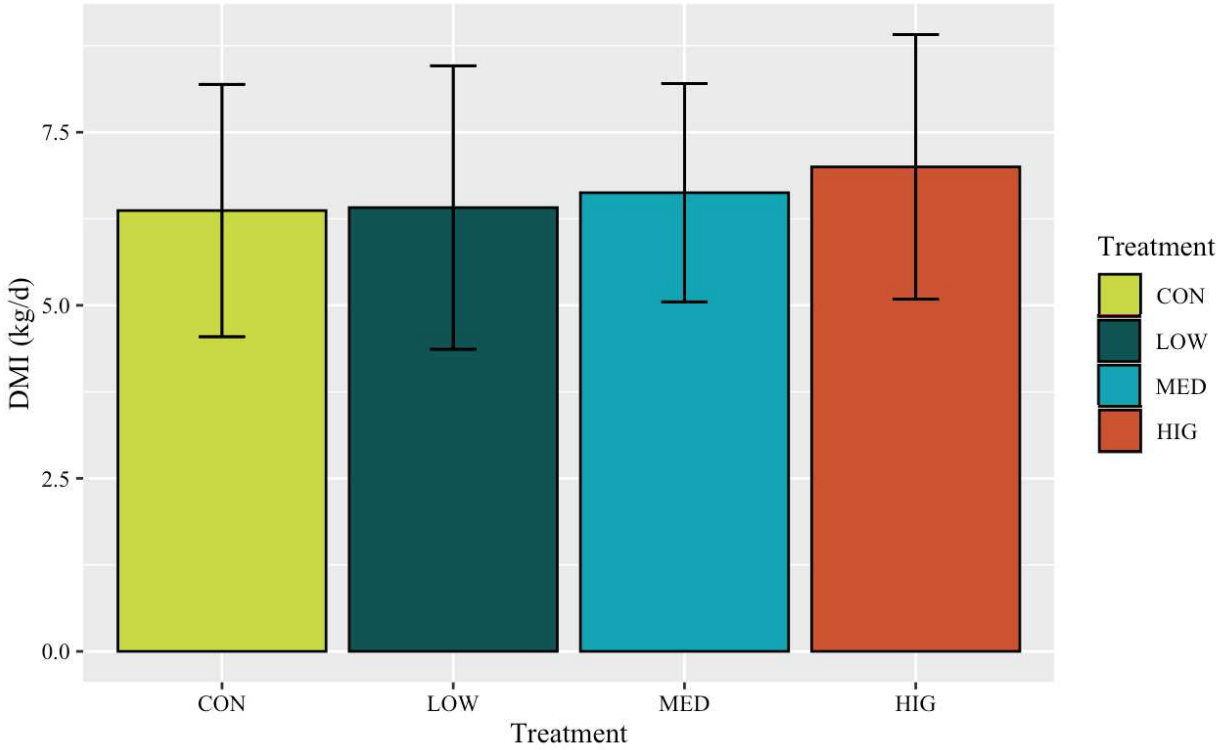

Figure 7. Average dry matter intake (DMI) of organic Holstein heifers (n=5 per treatment) in kg/d by treatment with tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI from d 0 to 45.

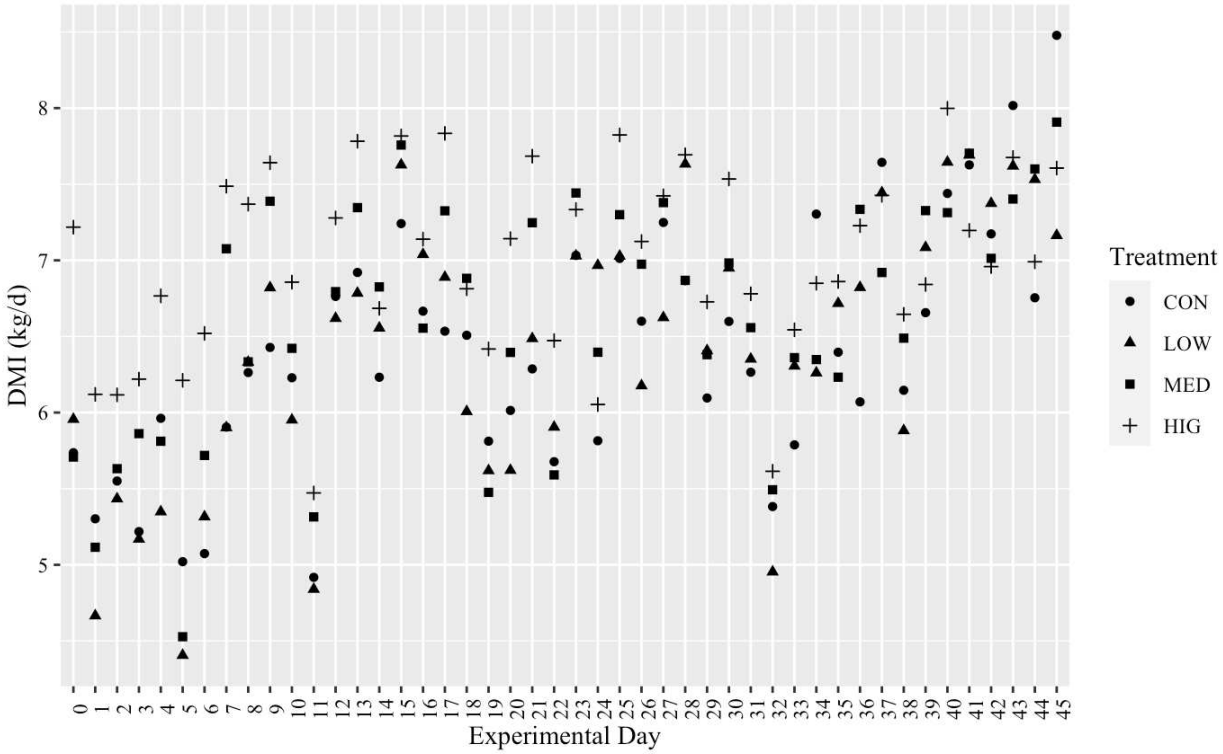

Figure 8. Average daily dry matter intake (DMI) from organic Holstein heifers (n=5 per treatment) in kg/d by treatment with tannin supplementation at 0% (CON), 0.075% (LOW), 0.15% (MED), and 0.30% (HIG) of DMI from d 0 to 45.

LITERATURE CITED

Aboagye, I. A., & Beauchemin, K. A. Potential of molecular weight and structure of tannins to reduce methane emissions from ruminants: A Review. *Animals* **2019**, *9*(11), 856. https://doi.org/10.3390/ani9110856

Aguerre, M. J., Capozzolo, M. C., Lencioni, P., Cabral, C., & Wattiaux, M. A. Effect of quebracho-chestnut tannin extracts at 2 dietary crude protein levels on performance, rumen fermentation, and nitrogen partitioning in dairy cows. *J. Dairy. Sci.* **2016**, *99*(6), 4476–4486. https://doi.org/10.3168/jds.2015-10745

Aguerre, M. J., Duval, B., Powell, J. M., Vadas, P. A., & Wattiaux, M. A. Effects of feeding a quebracho–chestnut tannin extract on lactating cow performance and nitrogen utilization efficiency. *J. Dairy. Sci.* **2020**, *103*(3), 2264–2271. https://doi.org/10.3168/jds.2019-17442

Ahlberg, C. M., Allwardt, K., Broocks, A., Bruno, K., McPhillips, L., Taylor, A., Krehbiel, C. R., Calvo-Lorenzo, M., richards, C. J., Place, S. E., DeSilva, U., VanOverbeke, D. L., Mateescu, R. G., Kuehn, L. A., Weaber, R. L., Bormann, J. M., & Rolf, M. M. (2018). Test duration for water intake, ADG, and DMI in beef cattle1. *J. Anim. Sci.* **2018**. https://doi.org/10.1093/jas/sky209

Ahnert, S., Dickhoefer, U., Schulz, F., & Susenbeth, A. Influence of ruminal quebracho tannin extract infusion on apparent nutrient digestibility, nitrogen balance, and urinary purine derivatives excretion in heifers. *Livestock Science* **2015**, *177*, 63–70. https://doi.org/10.1016/j.livsci.2015.04.004

Alemu, A. W., Vyas, D., Manafiazar, G., Basarab, J. A., & Beauchemin, K. A. Enteric methane emissions from low– and high–residual feed intake beef heifers measured using GreenFeed and respiration chamber techniques1,2. *J. Anim. Sci.* **2017**, *95*(8), 3727–3737. https://doi.org/10.2527/jas.2017.1501

Aquilano, K., Baldelli, S., & Ciriolo, M. R. Glutathione: new roles in redox signaling for an old antioxidant. *Frontiers in Pharmacology* **2014**, *5*. Pages https://doi.org/10.3389/fphar.2014.00196

Armstrong, D., & Browne, R. The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the Clinical Chemistry Laboratory. *Free Radicals in Diagnostic Medicine* **1994**, volume, 43–58. https://doi.org/10.1007/978-1-4615-1833-4_4

Arthur, P. F., Barchia, I. M., Weber, C., Bird-Gardiner, T., Donoghue, K. A., Herd, R. M., & Hegarty, R. S. Optimizing test procedures for estimating daily methane and carbon dioxide emissions in cattle using short-term breath MEASURES1,2. *J. Anim. Sci.* **2017**, *95*(2), 645–656. https://doi.org/10.2527/jas.2016.0700

Beauchemin, K. A., Kreuzer, M., O'Mara, F., & McAllister, T. A. Nutritional Management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture* **2008**, *48*(2), 21. https://doi.org/10.1071/ea07199

Beauchemin, K. A., McGinn, S. M., Martinez, T. F., & McAllister, T. A. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. *J. Anim. Sci.* **2007**, *85*(8), 1990–1996. https://doi.org/10.2527/jas.2006-686

Beauchemin, K. A., Ungerfeld, E. M., Eckard, R. J., & Wang, M. Review: Fifty Years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal* **2020**, *14*. https://doi.org/10.1017/s1751731119003100

Becker, P. M., van Wikselaar, P. G., Franssen, M. C., de Vos, R. C., Hall, R. D., & Beekwilder, J. Evidence for a hydrogen-sink mechanism of (+) catechin-mediated emission reduction of the ruminant greenhouse gas methane. *Metabolomics* **2013**, *10*(2), 179–189. https://doi.org/10.1007/s11306-013-0554-5

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., & Kurihara, M. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic Archaea and protozoal populations. *J. Dairy. Sci.* **2009**, *92*(11), 5512–5522. https://doi.org/10.3168/jds.2008-1441

Carulla, J. E., Kreuzer, M., Machmüller, A., & Hess, H. D. Supplementation of *acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Australian Journal of Agricultural Research* **2005**, *56*(9), 961. https://doi.org/10.1071/ar05022

Díaz Carrasco, J. M., Cabral, C., Redondo, L. M., Pin Viso, N. D., Colombatto, D., Farber, M. D., & Fernández Miyakawa, M. E. Impact of chestnut and quebracho tannins on rumen microbiota of Bovines. *BioMed Research International* **2017**, 1–11. https://doi.org/10.1155/2017/9610810

Dschaak, C. M., Williams, C. M., Holt, M. S., Eun, J.-S., Young, A. J., & Min, B. R. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. *J. Dairy. Sci.* **2011**, *94*(5), 2508–2519. https://doi.org/10.3168/jds.2010-3818

Ellis, J. L., Bannink, A., France, J., Kebreab, E., & Dijkstra, J. Evaluation of enteric methane prediction equations for dairy cows used in whole farm models. *Global Change Biology* **2010,** *16*(12), 3246–3256. https://doi.org/10.1111/j.1365-2486.2010.02188.x

Ebert, P. J., Bailey, E. A., Shreck, A. L., Jennings, J. S., & Cole, N. A. Effect of condensed tannin extract supplementation on growth performance, nitrogen balance, gas emissions, and energetic losses of beef steers1,2,3. *J. Anim. Sci.* **2017**, *95*(3), 1345–1355. https://doi.org/10.2527/jas.2016.0341

EPA. *Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2019* (No. EPA-430-R-21-005). United States Environmental Protection Agency, Washington, D.C. USA. 2021.

Field, J. A., Kortekaas, S., & Lettinga, G. The tannin theory of methanogenic toxicity. *Biological Wastes* **1989**, *29*(4), 241–262. https://doi.org/10.1016/0269-7483(89)90016-5

Fox J, Weisberg S. *An R Companion to Applied Regression*, Third edition. 2007. Thousand Oaks CA.

Garnsworthy, P. C., Craigon, J., Hernandez-Medrano, J. H., & Saunders. Variation among individual dairy cows in methane measurements made on farm during milking. *J. Dairy. Sci.* **2012**, *95*(6), 3181–3189. https://doi.org/10.3168/jds.2011-4606

Grainger, C., Clarke, T., McGinn, S. M., Auldist, M. J., Beauchemin, K. A., Hannah, M. C., Waghorn, G. C., Clark, H., & Eckard, R. J. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF6) tracer and chamber techniques. *J. Dairy. Sci.* **2007**, *90*(6), 2755–2766. https://doi.org/10.3168/jds.2006-697

Gunter, S. A., & Bradford, J. A. Technical note: Effect of bait delivery interval in an automated head-chamber system on respiration gas estimates when cattle are grazing rangeland. *The Professional Animal Scientist* **2017**, *33*(4), 490–497. https://doi.org/10.15232/pas.2016-01593

Gunter, S. A., Duke, S. E., & Beck, M. R. Measuring the respiratory gas exchange of grazing cattle using the GreenFeed emissions monitoring system. *J. Anim. Sci.* **2017**, *95*(suppl_4: 739), 359–359. https://doi.org/10.2527/asasann.2017.739

Henke, A., Dickhoefer, U., Westreicher-Kristen, E., Knappstein, K., Molkentin, J., Hasler, M., & Susenbeth, A. Effect of dietary quebracho tannin extract on feed intake, digestibility, excretion of urinary purine derivatives and milk production in dairy cows. *Archives of Animal Nutrition* **2016**, *71*(1), 37–53. https://doi.org/10.1080/1745039x.2016.1250541

Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H. P., Adesogan, A. T., Yang, W., Lee, C., Gerber, P. J., Henderson, B., & Tricarico, J. M. Special topics — mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options1. *J. Anim. Sci.* **2013**, *91*(11), 5045–5069. https://doi.org/10.2527/jas.2013-6583

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

Huhtanen, P., Ramin, M., & Hristov, A. N. Enteric methane emission can be reliably measured by the GreenFeed monitoring unit. *Livestock Science* **2019**, *222*, 31–40. https://doi.org/10.1016/j.livsci.2019.01.017

Jayanegara, A., Leiber, F., & Kreuzer, M. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. *Journal of Animal Physiology and Animal Nutrition* **2011**, *96*(3), 365–375. https://doi.org/10.1111/j.1439-0396.2011.01172.x

Kartchner, R. J. Effects of protein and energy supplementation of cows grazing native winter range forage on intake and digestibility. *J. Anim. Sci.* **1980,** *51*(2), 432–438. https://doi.org/10.2527/jas1980.512432x

Kohn, R. A., Dinneen, M. M., & Russek-Cohen, E. Using blood urea nitrogen to predict nitrogen excretion and efficiency of nitrogen utilization in cattle, sheep, goats, horses, pigs, and rats. *J. Anim. Sci.* **2005**, *83*(4), 879–889. https://doi.org/10.2527/2005.834879x

Kumar, R.; Singh, M. Tannins: their adverse role in ruminant nutrition. *J. Agric. Food Chem.* **1984**. 1984, 32, 447–453.

Lavery, A., & Ferris, C. Proxy measures and novel strategies for estimating Nitrogen Utilization Efficiency in dairy cattle. *Animals* **2021**, *11*(2), 343. https://doi.org/10.3390/ani11020343

Lenth RV (2016). "Least-Squares Means: The R Package lsmeans." Journal of Statistical Software, 69(1), 1–33. https://doi.org/10.18637/jss.v069.i01.

Liu, H. W., Zhou, D. W., & Li, K. Effects of chestnut tannins on performance and antioxidative status of transition dairy cows. *J. Dairy. Sci.* **2013**, *96*(9), 5901–5907. https://doi.org/10.3168/jds.2013-6904

Llonch, P., Troy, S. M., Duthie, C.-A., Somarriba, M., Rooke, J., Haskell, M. J., Roehe, R., & Turner, S. P. Changes in feed intake during isolation stress in respiration chambers may impact methane emissions assessment. *Animal Production Science* **2018**, *58*(6), 1011. https://doi.org/10.1071/an15563

Makkar, H. P., Becker, K., Abel, H., & Szegletti, C. Degradation of condensed tannins by rumen microbes exposed to quebracho tannins (QT) in Rumen Simulation Technique (RUSITEC) and effects of qt on fermentative processes in the RUSITEC. *J. Sci. Food Agric.* **1995,** *69*(4), 495–500. https://doi.org/10.1002/jsfa.2740690414

Makkar, H. P., Siddhuraju, P., & Becker, K. *Tannins*. Plant Secondary Metabolites 2007, 67–81. https://doi.org/10.1007/978-1-59745-425-4_13

Malmstrom, B. G., Andreasson, L.-E., & Reinhammar, B. Copper-containing oxidases and superoxide dismutase. *The Enzymes* **1975**, vol, 507–579. https://doi.org/10.1016/s1874-6047(08)60231-0

Marshall, C. J., Beck, M. R., Garrett, K., Castillo, A. R., Barrell, G. K., Al-Marashdeh, O., & Gregorini, P. The effect of feeding a mix of condensed and hydrolysable tannins to

heifers on rumen fermentation patterns, blood urea nitrogen, and amino acid profile. *Livestock Science* **2022**, *263*, 105034. https://doi.org/10.1016/j.livsci.2022.105034

Min, B. R., Barry, T. N., Attwood, G. T., & McNabb, W. C. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Animal Feed Science and Technology* **2003**, *106*(1-4), 3–19. https://doi.org/10.1016/s0377-8401(03)00041-5

Min, B. R., Pinchak, W. E., Fulford, J. D., & Puchala, R. (2005). Wheat pasture bloat dynamics, in vitro ruminal gas production, and potential bloat mitigation with Condensed Tannins1. *J. Anim. Sci.* **2005**, *83*(6), 1322–1331. https://doi.org/10.2527/2005.8361322x

Moate, P. J., Williams, S. R. O., Hannah, M. C., Eckard, R. J., Auldist, M. J., Ribaux, B. E., Jacobs, J. L., & Wales, W. J. Effects of feeding algal meal high in docosahexaenoic acid on feed intake, milk production, and methane emissions in dairy cows. *J. Dairy. Sci.* **2013**, *96*(5), 3177–3188. https://doi.org/10.3168/jds.2012-6168

Naumann, H. D., Tedeschi, L. O., Zeller, W. E., & Huntley, N. F. The role of condensed tannins in ruminant animal production: Advances, limitations and future directions. *Revista Brasileira De Zootecnia* **2017**, 46(12), 929–949. https://doi.org/10.1590/s1806-92902017001200009

Norris, A. B., Crossland, W. L., Tedeschi, L. O., Foster, J. L., Muir, J. P., Pinchak, W. E., & Fonseca, M. A. Inclusion of quebracho tannin extract in a high-roughage cattle diet alters digestibility, nitrogen balance, and energy partitioning. *J. Anim. Sci.* **2020**, *98*(3). https://doi.org/10.1093/jas/skaa047

Ordway, R. S., Ishler, V. A., & Varga, G. A. (2002). Effects of sucrose supplementation on dry matter intake, milk yield, and blood metabolites of periparturient Holstein Dairy Cows. *J. Dairy. Sci.* **2002**, *85*(4), 879–888. https://doi.org/10.3168/jds.s0022-0302(02)74146-5

Peripolli, V., Prates, Ê. R., Barcellos, J. O., & Neto, J. B. Fecal nitrogen to estimate intake and digestibility in grazing ruminants. *Animal Feed Science and Technology*. **2011**. *163*(2-4), 170–176. https://doi.org/10.1016/j.anifeedsci.2010.11.008

Piñeiro-Vázquez, A. T., Jiménez-Ferrer, G., Alayon-Gamboa, J. A., Chay-Canul, A. J., Ayala-Burgos, A. J., Aguilar-Pérez, C. F., & Ku-Vera, J. C. Effects of quebracho tannin extract on intake, digestibility, rumen fermentation, and methane production in crossbred heifers fed low-quality tropical grass. *Tropical Animal Health and Production* **2017**, *50*(1), 29–36. https://doi.org/10.1007/s11250-017-1396-3

Place, S. E., & Mitloehner, F. M. Invited review: Contemporary environmental issues: A review of the dairy industry's role in climate change and air quality and the potential of mitigation through improved production efficiency. *J. Dairy. Sci.* **2010**, *93*(8), 3407–3416. https://doi.org/10.3168/jds.2009-2719

Rotz, A., Stout, R., Leytem, A., Feyereisen, G., Waldrip, H., Thoma, G., Holly, M., Bjorneberg, D., Baker, J., Vadas, P., & Kleinman, P. Environmental assessment of United States dairy farms. *Journal of Cleaner Production* **2021**, *315*, 128153. https://doi.org/10.1016/j.jclepro.2021.128153

Santillo, A., Ciliberti, M. G., Ciampi, F., Luciano, G., Natalello, A., Menci, R., Caccamo, M., Sevi, A., & Albenzio, M. Feeding tannins to dairy cows in different seasons improves the oxidative status of blood plasma and the antioxidant capacity of cheese. *J. Dairy. Sci.* **2022**, *105*(11), 8609–8620. https://doi.org/10.3168/jds.2022-22256

Stewart, E. K., Beauchemin, K. A., Dai, X., MacAdam, J. W., Christensen, R. G., & Villalba, J. J. Effect of tannin-containing hays on enteric methane emissions and nitrogen partitioning in beef cattle. *J. Anim. Sci.* **2019**, *97*(8), 3286–3299. https://doi.org/10.1093/jas/skz206

Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T., & Sivakumaran, S. Methane production from in vitro rumen incubations with *lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Animal Feed Science and Technology* **2005**, *123-124*, 403–419. https://doi.org/10.1016/j.anifeedsci.2005.04.037

Thompson, L. R., Beck, M. R., Gunter, S. A., Williams, G. D., Place, S. E., & Reuter, R. R. An energy and monensin supplement reduces methane emission intensity of Stocker Cattle Grazing Winter Wheat. *Applied Animal Science* **2019**. *35*(4), 433–440. https://doi.org/10.15232/aas.2018-01841

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy. Sci.* **1991**, *74*(10), 3583–3597. https://doi.org/10.3168/jds.s0022-0302(91)78551-2

Velásquez, A. V., da Silva, G. G., Sousa, D. O., Oliveira, C. A., Martins, C. M. M. R., dos Santos, P. P. M., Balieiro, J. C. C., Rennó, F. P., & Fukushima, R. S. Evaluating internal and external markers versus fecal sampling procedure interactions when estimating intake in dairy cows consuming a corn silage-based diet. *J. Dairy. Sci.* **2018**, *101*(7), 5890–5901. https://doi.org/10.3168/jds.2017-13283

Velazco, J. I., Mayer, D. G., Zimmerman, S., & Hegarty, R. S. Use of short-term breath measures to estimate daily methane production by cattle. *Animal* **2016**, *10*(1), 25–33. https://doi.org/10.1017/s1751731115001603

Yan, T., Mayne, C. S., Gordon, F. G., Porter, M. G., Agnew, R. E., Patterson, D. C., Ferris, C. P., & Kilpatrick, D. J. Mitigation of enteric methane emissions through improving efficiency of energy utilization and productivity in lactating dairy cows. *J. Dairy. Sci.* **2010**, *93*(6), 2630–2638. https://doi.org/10.3168/jds.2009-2929