USING SAPONINS TO REDUCE GASEOUS EMISSIONS FROM STEERS

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ABSTRACT

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Enteric methane (CH_4) production from beef cattle accounts for more than 71% of the total enteric CH_4 fermentation from ruminants. Many nutritional strategies have been investigated *in vitro* to mitigate CH_4 production from ruminants. Saponin is a plant extract that has been demonstrated to be effective *in vitro*. In this thesis, a series of studies were conducted to investigate the effects of dietary inclusion of steroid (*Yucca schidigera*) and triterpenoid (*Quillaja saponaria* and *Camellia sinensis*) saponins on animal and manure-derived CH_4 and other gaseous emissions. In addition, the effects of adding saponin extracts to manure on manure-derived CH_4 and other gaseous emissions were also investigated. Dietary inclusion of up to 1.5% of quillaja and yucca saponins or 0.25% of tea saponin did not change animal-derived CH_4 emissions, while CH_4 emissions were significantly reduced when steers were fed 0.5% tea saponin. The reductions of CH_4 production can be possibly attributed to reduced DMI in 0.5% tea saponin treatment. Manure-derived CH_4 emissions were reduced in steers fed 0.64% yucca saponins, increased in steers fed 1.5% quillaja saponin treatment and not affected in steers fed 0.25% tea saponin treatment compared to the control treatment. However, direct saponin addition to manure showed no effects on CH_4 emissions. Feeding steers up

to 1.5% yucca saponin or 0.5% of tea saponin did not affect animal-derived NH_3 emissions. Manure-derived NH_3 emissions were reduced in 0.64% yucca saponin treatment. Increased animal-derived NH_3 daily emissions were observed in 1.5% quillaja treatment in one of the studies, whereas in another study, 1.5% dietary quillaja saponin supplementation did not change NH_3 emissions. The differences may be explained by variation among animals. Animal-derived H_2S, NMTHC and N_2O emissions were not influenced by dietary saponin inclusion or direct addition. Dietary inclusion of 1.5% of quillaja saponin reduced manure-derived H_2S emissions, increased NMTHC emissions but did not affect N_2O emissions. Both NMTHC and H_2S emissions from manure were reduced as a result of dietary inclusion of 0.64% yucca. Dietary inclusion of 0.25% tea saponin treatments reduced NMTHC, H_2S and N_2O emissions. Overall, dietary inclusion of all saponin sourced failed to change animal-derived CH_4 emissions without affecting growth the performance. Effects of dietary saponin supplementation on manure-derived air emissions were varied by saponin type. Direct application of saponins to manure had no effects on manure-derived air emissions.

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LIST OF ABBREVIATIONS

AAQRF ……………………….……….. Animal Air Quality Research Facility

ADG ……………………..…………... average daily gain

A:P ……………………..…………… acetate to propionate

BCTRC …………………………..…….. Beef Cattle Teaching Research Facility

BW ………………………………….. body weight

CH_4 ……………………………….…... Methane

CO_2 Eq. …………………………….. CO_2 equivalent

CP …………………………………… crude protein

DE …………………………………… digestible energy

DM …………………………..…….. dry matter

DMI …………………………….…... dry matter intake

F:C ……………………………….…... forage to concentrate ratio

GHG ……………………..…………... greenhouse gas

GI……………………….....………….. gastro-intestinal

GWP ……………………..…….…..... global warming potential

EDMI ……………………………….. estimated dry matter intake

H_2S ………………………………….. hydrogen sulfide

MAU ………………………………… Make-up Air Unit

MPS ………………………………… microbial protein synthesis

N_2O ……………………………….…... nitrous oxide

NH_3 ………………………………...... ammonia

NMTHC …………………………….. none-methane total hydrocarbon

RFI ………………………………... residual feed intake

TMR ………………………………… total mixed ration

Tg ………………………………….... tetra gram

VFA ………………………………..... volatile fatty acid

RFI …………………………….…...... Residual feed intake

CHAPTER 1

REVIEW OF LITERATURE

CLIMATE CHANGE AND GREEN HOUSE GAS EMISSIONS

Greenhouse gases (GHG), natural and anthropogenic originated, absorb thermal infrared radiation from the atmosphere and the Earth's surface as a mechanism to maintain the Earth's surface temperature. For those long-lived GHG such as methane (CH_4), carbon dioxide (CO_2) and nitrous oxide (N_2O), the ability to absorb radiation is described by the global warning potential (GWP) index as a comparison to the relative effectiveness of CO_2 based on certain duration of time. The 100 yr GWP for CH_4 and N_2O are 21 and 298 times greater than CO_2 (IPCC, 2007).

Non-anthropogenic and anthropogenic activities produce GHG. Nevertheless, human related GHG emissions, for example agricultural activities, energy use, land use and industrial processes are considered to be the principal contributors to the drastic increase in atmospheric GHG concentrations (US EPA, 2011). Since pre-industrial times, CO_2 concentration has increased by 38%, from 280 parts per million (ppm) to 382 ppm (NOAA, 2008), while CH_4 and N_2O concentration in the atmosphere has increased 148% and 18%, respectively, compared with the pre-industrial levels (IPCC, 2007). Because the complex and interactive global climate system is very susceptible to the atmospheric concentrations of gases, increased atmospheric GHG concentrations can consequently change global climate (IPCC, 2007). Increased atmospheric temperature is the most direct consequence of increased GHG concentration due to the heat-trapping properties of GHG. It is reported that global surface temperature on average was 0.54 °C higher in 2005 than in 1988 (Hansen et al., 2006). Prediction of temperature by 2040 is suggested to be increased by 1 to 2.7 °C based on different scenarios (Allen et al., 2000). The warmed planet changes climatic patterns, resulting in many severe consequences such as increased sea level and oceanic pH and more frequent extreme weather, which in combination, may greatly affect people's livelihood (Allen et al., 2010). Because human activities have overwhelmed natural processes of changing climate, reducing GHG emissions from anthropogenic activity could have important implications in the future climate.

METHANE EMISSIONS FROM RUMINANTS

In the U. S. enteric fermentation from ruminants produces 139.8 Tg CO_2 Eq. annually, accounting for more than 20% of total CH_4 emissions from human activities (US EPA, 2011). Beef (71%) and dairy (23%) cattle are mainly responsible for the enteric CH_4 emissions, which, together represent over 95% of entire enteric CH_4 production, with sheep, swine and goat accounting for the rest 5% (US EPA, 2011). In addition, enteric CH_4 fermentation from ruminants is also responsible for 2-12% of energy loss (Nelson et al., 1960; Czerkawski, 1978).

Methanogenesis in the rumen

Methane is produced by methanogens in the rumen, which are obligate anaerobes belonging to the domain of *Archaea*. Different from bacteria, the cell wall peptidoglycan of methanogens is replaced by pseudomurein (*Methanobrevibacter and Methanobacteruim*), heteropolysaccharide (*Methanosarcina*) and protein (*Methanomicrobium*) (Balch et al., 1979).

Bacteria, protozoa and fungi hydrolyze feed nutrients into amino acids and simple carbohydrates such as sugars, which can be further fermented into volatile fatty acids

(VFA), principally acetic acid, propionic acid and butyric acid, and utilized by animals as the energy sources (McAllister et al., 1996). Meanwhile, reducing equivalents, mainly NADH and H^+, are produced as the electron carriers and need to be oxidized timely to facilitate the process of fiber digestion (Wolin et al., 1997). Although there are several pathways to uptake the reducing equivalents, such as lactic acids, ethanol and H_2S formation, CH_4 production by methanogens is considered as the more effective electron sink in the rumen (Sharp et al., 1998). Bauchop and Dauglas (1981) demonstrated that in the mono-culture of ruminal fungi, concentration of acetate, ethanol, lactate and hydrogen in final products was 73, 37, 67 and 35 mol/100 mol of hexose units, respectively, without CH_4 production. In contrast, when methanogens were co-cultured with fungi, considerable amounts of CH_4 were detected (59 mol/100 mol of hexose units) and no accumulation of hydrogen was observed. Meanwhile, improved rate and extent of cellulose degradation were found in co-culture compared to the mono-culture. Formation of acetate increased to 135 mol/100 mol of hexose units; besides the yields of lactate and ethanol decreased to 3 and 19 mol/100 mol of hexose units, respectively.

Approximately, 82% of the CH_4 formed in the rumen is produced from CO_2 follows the reduction of CO_2 to formyl, formaldehyde and methyl groups and the conversion of methyl group to methane (Ferry, 1992). To yield one mole CH_4, 1 mole of CO_2 and 4 moles of H_2 are involved, generating 103.4 kJ:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

Although, this reaction is major pathway for most methanogens (Jones et al., 1987), other substrates such as formate and acetate can also be utilized by some methanogens (Garcia et al., 2000).

Profiles of methanogens can be highly affected by dietary factors. Grazing sheep were found to have more diversified methanogen populations and strain *Methanobrevibacter M6* was more prevalent compared to sheep fed on oaten or lucerne hay diets (Wright et al., 2004). Zhou et al. (2010) observed a shift of dominant methanogen community from *Methanobrevibacter ruminantium NT7* when fed a low-energy diet to *Methanobrevibacter smithii* and/or *Methanobrevibacter sp. AbM4* when fed a high energy diet in beef cattle. Methanogens from feedlot beef cattle fed on corn-based diets with potato by-product based diets, contained only 67% of total clones were found to exist in both herds (Wright et al., 2007). King et al. (2011) reported that under the same feeding regime, management and environmental conditions, there remained a 15% discrepancy in the combined genome library between Holstein and Jersey cows, suggesting that internal factor from the host breeding genetics also has an effect on methanogen community.

Role of protozoa in ruminal methanogenesis

Protozoa constitute a considerable part of the rumen biomass and are responsible for the extensive production of ruminal ammonia (NH_3) by metabolizing rumen bacteria and proteins. The majority of protozoa present in wild and domestic ruminants belong to the order *Entodiniomorpid* and *Holotrich* (Williams and Coleman, 1992). The population of protozoa is dynamic and subject to many factors, such as host specificity, geographical distribution, feed composition and young ruminant's infection (Williams and Coleman, 1992). Protozoa population is more diversified in ruminants fed high forage diets rather than high concentrate diets (Dehority, 1978), while they are found to be less diversified (Hristov et al., 2001) or even absent (Lyle et al., 1981) when ruminants are under sub-clinical acidosis or fed with high grain diets.

Butyric and acetic acids are the principal end products of carbohydrate fermentation by protozoa (Howard, 1959; Hansen et al., 2006). Reducing equivalents are usually accompanied by butyric and acetic acids production can later be converted to H_2 and used by methanogens to produce CH_4. The symbiosis of methanogens and protozoa provides the advantage for fast removal of the H_2, which is also recognized as interspecies hydrogen transfer.

In the rumen, 20% of the methanogen population is associated with protozoa of which on an average, 43% and 20% are *Methanobrevibacter gottschalkii* and *Methanomicrobium*, respectively (Janssen and Kirs, 2008), contributing 9 to 37% of total CH_4 production in the rumen (Newbold et al., 1995). High methanogenic activity was observed in fractions containing greater density of protozoa *in vitro* (Krumholz et al., 1983) and variations of CH_4 production in calves were in accordance with composition of ciliate population (Itabashi et al., 1994).

Because the presence of protozoa is important to methanogen populations and methanogenic activity, defaunation is suggested to reduce methanogenesis (Newbold et al., 1995). Hu et al. (2005) reported that defaunation resulted in 60% reduction in methanogenesis in rumen fluid. Hess et al. (2003) found 40% to 50% reduction of CH_4

yield from a defaunation treatment compared to a faunated control treatment, *in vitro*. Similar results were also reported from *in vivo* research. Sheep kept under a protozoa-free environment produced 17% to 20% less CH_4 compared to faunated sheep during both short-term (10 wk) and long-term (2 yr) studies (Mosoni et al., 2011).

When using molecular techniques to examine the changes of methanogen population, studies have found that reduced CH_4 production is not always associated with the abundance of methanogens (Guo et al., 2008; Mosoni et al., 2011). Those results indicated that the elimination of protozoa was likely to reduce the amount of hydrogen that is available for methanogens and cause a possible shift from dominant methanogens to less active stains (Denman et al., 2007), as such reducing the ruminal CH_4 production, rather than reducing the biomass of methanogen, directly (Hess et al., 2003).

Dietary factors affecting methane emissions

Decreased forage level or increased forage quality reduces energy losses as CH_4 (Johnson and Johnson, 1995; Boadi et al., 2004). Reduced CH_4 production is the result of a shift of rumen fermentation patterns from acetate to propionate, which favors an in increased rumen fermentation rate (Demeyer and Van Nevel 1975). In addition, easily-fermented carbohydrates often lead to lower rumen pH, which may reduce the activity of rumen methanogens, resulting in reduced CH_4 production (Hristov et al., 2001). However, the reduced CH_4 that results from feeding steers a high concentrate diet only occurs when dietary concentrate levels exceed 70% and CH_4 production is not linearly associated with concentrate levels. Lovett et al. (2003) compared CH_4 emissions from finishing beef heifers fed diets with different forage to concentrate (F:C) ratios (65:35, 40:60 and 10:90). Quadratic responses of CH_4 output to reduced F:C ratio were found as emissions were expressed by daily mass, per kg DMI as well as percentage of gross energy intake. The greatest CH_4 production was observed as a result of feeding the 40:60 (F:C) ratio treatment. Similar results were also reported by Moss et al. (1995). Possible associative effects could have occurred when a grass hay diet was supplemented with small amount of maize (Blaxter and Vainman, 1964).

The effects of dietary crude protein (CP) on CH_4 emissions have also been investigated. Kurihara et al. (1997) reported that when increasing the CP content from 4% to 9% in goats fed at maintenance levels, an 18% increase in CH_4 production per kg DMI was observed in 9% CP treatment without affecting CH_4 production on energy basis. However, when animals were fed above the maintenance level, a negative relationship occurred between dietary CP intake and CH_4 emissions (Sekine et al., 1986a and b).

Legumes usually contain lower fiber but higher crude protein compared to grasses. In addition, the presence of tannins in legume forages has been demonstrated to reduce methanogenesis (Ahn et al. 1989; Puchala et al., 2005). As a result, lower CH_4 yield is generally found when ruminants are fed legume based diets compared with grass based diets (McCaughney et al., 1999). However, the extent of reduction on enteric CH_4 emissions is largely determined by the maturity stage of the legume. Advanced maturity of alfalfa for grazing cattle could result in greater CH_4 production than grass of less maturity (Chaves et al., 2006).

In addition, pelleting and particle size changes CH_4 production from ruminants. Hironaka et al. (1996) reported that pelleted alfalfa hay diet reduced daily CH_4 emissions on a DMI basis without changing the CH_4 emissions on a digestible energy (DE) basis when steers were fed at maintenance level compared to chopped alfalfa hay diet. When increasing the feeding level to 1.6 times maintenance, CH_4 production (both DMI and DE basis) was reduced as a result of feeding the pelleted alfalfa hay diet.

Intake levels

The amount of feed consumed is another factor that is important to determine the daily CH_4 emission from ruminants. The relationship between feed intake and CH_4 emissions has been investigated intensively. Models used to predict CH_4 emissions suggested that DMI accounts for 64% of the daily CH_4 production variation (Boadi and Wittenberg; 2002). Generally, increasing DMI levels results in higher CH_4 production, whereas the CH_4 emissions per unit of DMI decreases or is unaffected (Mills et al., 2001; Mills et al., 2003; Ellis et al., 2007; Ellis et al. 2009). However, poor predictions have been reported when animals are fed low quality diets such as tropical forage and straw (Kurihara et al., 1997; Kurihara et al., 1999).

Herd et al. (2002) found that beef cattle of low residual feed intake (RFI) produced approximately 5% less CH_4 than high RFI beef cattle. Other studies have shown that differences in CH_4 production between low RFI and high RFI beef was 25-

28%, corresponding to approximately 16100 L/yr less CH_4 in low RFI beef cattle (Nkrumah et al., 2006; Hegarty et al., 2007).

Genetic variations

Genetic differences may lead to ecological changes of microbial communities in the rumen, translating into different CH_4 productions (Hackstein et al., 1996; Nkrumah et al., 2006). Persistent difference of CH_4 emissions exists among sheep managed under the same grazing conditions, where 37% more CH_4 on a gross energy intake basis was produced in high emitters compared to low CH_4 emitters (Pinares-Patino et al., 2003). Robertson and Waghorn (2002) compared cows in New Zealand originating from overseas with domestic cows and found no CH_4 production per kg DMI differences at 240 days of lactation, while at 60 and 150 days of lactation, overseas originated cows emitted 15% less CH_4 on a DMI basis compared to domestic cows. Machmüller and Clark (2006) reviewed 32 CH_4 emission trials with grazing animals and concluded that CH_4 emission mass from female cattle was twice more than male cattle. However, because DMI in female cattle was greater than DMI in male cattle, the production of CH_4 was reversed when adjusting the daily mass by estimated dry matter intake (EDMI). In sheep species, although females tended to emit 52% more CH_4 per day than males, neither the daily mass nor adjusted emissions on EDMI basis showed differences between the two genders (Machmüller and Clark, 2006). Variations may exist in terms of management and diets between male and female animals, but the generic differences of gender should no doubt be taken into account when estimating CH_4 emissions.

The genetic differences between animals provide the opportunity for selection of low CH_4 producers in terms of mitigating CH_4 emissions. However, it also reveals the complexity and difficulty in accurate prediction of CH_4 emissions. Future strategies, for a better achievement of CH_4 reduction, need to take these variations into account.

USING PLANT SAPONIN EXTRACTS TO REDUCE ENTERIC METHANE EMISSIONS

Saponins are natural glycosides that occur widely in various parts of plants, including the fruits, roots, stems, leaves and seeds (Vincken et al., 2007). Saponins are characterized by several properties, which, most significantly, are the foaming, haemolytic and emulsifying properties (Oda et al., 2000; Price et al., 1987). Chemically, saponins comprise a large family of structurally related compounds containing a steroid or triterpenoid aglycone (sapogenin) linking to one or more oligosaccharide moieties by glycosidic linkage (Makkar et al., 2007). Usually, the aglycone of a steroid saponin is derived from spirostanol or furostanol (Hostettmann and Marston, 1995a). Triterpenoid saponins, however, are more diversified than steroid saponins. Depending on whether amyrin (α- or β- type) or lupeol group is presented in the sapogenin, the triterpenoid saponins can be classified into three classes (Hostettmann and Marston, 1995a). Although it is suggested that the distribution of sapogenins are not subclass-specific (Vincken et al., 2007), triterpenoid saponins are found to be more prevalent in plants compared to steroid saponins (Hostettmann and Marston, 1995b).

Saponins are not evenly distributed in plant parts. They are found more abundant in tissues vulnerable to fungi or bacterial infections. For example, in *Bacopa monnieri*, shoots and leaves are responsible for over half of the total saponin content of the plant (Phrompittayarat et al., 2011). Saponin concentration can be determined by the growth stage of plant as well. Generally, concentration increases as the maturity of plants proceeds (Singh et al., 1986; Phrompittayarat et al., 2011). In addition, sowing dates, growing locations and organs of plants can also affect the saponin composition and concentration considerably (Tsukamoto et al., 1995). Different extract method also can be a factor in terms of determining the saponin concentration in plants (Adebayo et al., 2009).

Effects on rumen protozoa population

Saponins are toxic to rumen protozoa by forming an irreversible complex with the steroid in protozoal cell wall (Francis et al., 2002). However, the degree of this effect is dose-dependent and subject to saponin types. When 1.2 mg/ml saponin-rich fraction from *Quillaja saponaria* was added to substrates containing only hay or a 50:50 hay and concentrate mixture, *in vitro*, 38% to 54% reduction of protozoal population was observed and accordingly, ruminal NH_3-N concentration was 12% to 15% lower in *Quillaja saponaria* treatments compared to the control treatment (Makkar et al., 1998). In another study, 8% less protozoa were observed, *in vitro*, in saponin treatment where extract from *Quillaja saponaria* was added at concentrations from 0.1 to 0.4% of DM compared to control treatment (Hristov et al., 2003). The anti-protozoal effects of *Yucca schidigera* and *Camellia sinensis* have also been confirmed. When 1 or 10 mg/ml yucca saponin was added to rumen fluid with no substrate, a 22% reduction of protozoal counts was observed in the 1 mg/ml treatment and protozoa were totally eliminated in the 10 mg/ml treatment (Wallace et al., 1994). Linearly dose-dependent effects on protozoa population reduction of yucca saponin was reported by Lovett et al. (2006) and Hristov et al. (1999), *in vitro*. Protozoal population, measured by real-time PCR was 50% (*in vitro*; sheep) and 40% (*in vivo*; sheep and lamb) lower in *Camellia sinensis* saponin treatments compared to no saponin treatments (Guo et al., 2008; Mao et al., 2010; Zhou et al., 2011). Similar results were also reported from *in vivo* studies (Hristov et al., 1999; Hess et al., 2004; Lovett et al., 2006). Nasri et al. (2011) reported that administrating saponin extract from *Quillaja saponaria* to male lambs fed a hay diet with daily supplementation of 400 mg concentration reduced protozoal population by 30 to 47% compared with the control diet.

Inclusion of saponins does not always reduce protozoa populations *in vivo*. Holtshausen et al. (2009) reported that when diets containing 10 g/kg DM saponin extract from *Yucca schidigera* or *Quillaja saponaria* were fed to dairy cows, protozoa population was not affected. Both saponin extracts in their study were added at lower concentration *in vivo* than the lowest concentration tested *in vitro*, hence the lack of effect can possibly be attributed to the low doses offered.

Protozoal communities are significantly influenced by dietary composition and animal species (Williams and Coleman, 1992). This may help to explain the discrepancies between studies in saponin's efficacy in reducing protozoal population. The inconsistency of saponin's effects may also arise from different extraction methods. A more pronounced effect of saponin of *Acacia concinna* against protozoa was observed by methanol extracts compared with water or ethanol extracts (Patra et al., 2006). In addition, it is suggested that saponin's anti-protozoal effects may be selective towards individual protozoa species. Saponin-rich fruit of *Sapindus saponaria* was found to reduce *Entodiniomorphs* numbers without affecting *Holotrichs* (Abreu et al., 2004). However, this finding is not confirmed in other plant species such as *Enterolobium cyclocarpum* (Ivan et al., 2004) or *Yucca schidigera* (Benchaar et al. 2008), suggesting that variations may exist among plant species.

Effects on N metabolism

Protozoa account for approximately half of the total microbial biomass in the ruminant. One of the major activities of protozoa is the proteolytic effect towards rumen bacteria with NH_3 produced as the end product. Inhibition of protozoal populations can therefore prevent the degradation of bacteria, reducing ruminal NH_3 concentration and increase the net biosynthesis of microbial crude protein (MCP) in the rumen (Mao et al., 2010; Zhou et al., 2011). The positive relationship between ruminal protozoa population and NH_3 concentration has been well established both *in vitro* and *in vivo* (Hart et al., 2005, review; Wina et al., 2005, review).

Reduction of protozoa is not always accompanied by decreased ruminal NH_3 concentrations. In studies with sheep species, dietary inclusion of *Enterolobium cyclocarpum* saponin at 200 g/d (Ivan et al., 2004) or intra-ruminal (8 g/kg $BW^{0.75}$) or oral (5 g/kg $BW^{0.75}$) addition of saponin-rich fruit from *Sapindus saponaria* (Abreu et al., 2004; Hess et al., 2004) suppressed protozoa population but failed to reduce NH_3 concentration in the rumen. This can be attributed to low concentrations of saponin. Hristov et al., (1999) reported that, in heifers, association of decreased NH_3 with reduced protozoa population only occurred when treating with higher *Yucca schidigera* saponin concentrations (5.83 g/kg DM), whereas in lower concentration (1.96 g/kg DM) treatment, protozoa population was reduced by *Yucca schidigera* saponin without affecting ruminal NH_3 concentrations.

The inhibition of protozoa may also reduce the protozoal predation activity of bacteria, leading to increased efficiency of microbial protein synthesis (MPS). Saponin extracts from *Camellia sinensis* (Mao et al., 2010; Zhou et al., 2011), *Biophytum petersianum* (Santoso et al., 2007), *Sapindus saponaria* (Abreu et al., 2004) and *Yucca schidigera* (Santoso et al., 2006) have been identified to improve ruminal MPS. Zhou et al. (2011) noted that inclusion of *Camellia sinensis* saponin into both faunated and defaunated sheep increased rumen MPS by 16% and 36%, respectively, indicating the lack of interaction between saponin and defaunation. The lack of saponin's effects on MPS suggests that improved efficiency of MPS in saponin treatments could be due to a greater amount of digested substrate partitioned in to microbial mass synthesis (Makkar et al., 1998). In addition, diet composition can also play a role in MPS (Lu and Jorgensen; 1987). However, the benefits of MPS as a result of saponin inclusion may be compromised at high saponin concentration by suppression of bacteria and fungi population (Wang et al., 2000, Guo et al., 2008).

When examined on the whole animal basis, saponin usually did not affect N metabolism. *Quillaja saponaria* administrated at 30 to 90 mg/kg DM to lambs showed no effect on N intake or losses, suggesting N retention was not affected by saponin treatment (Nasri et al., 2011). Similar results were reported by Hristov et al. (1999) when offered 20 or 60 g/d *Yucca schidigera* to heifers fed barley grain and alfalfa hay based diet

(61:39). Urinary N was not affected by supplementing 120 mg/kg DM of *Yucca schidigera* saponin (Santoso et al., 2004), while a 15% reduction of urinary N was reported when the dose increased to 240 mg/kg DM in sheep species (Santoso et al., 2006), indicating N metabolism may be changed by saponin at higher concentrations.

Effects on rumen VFA fermentation and animal production

Regardless of the sources, inclusion of saponins usually does not change total VFA production *in vitro*, whereas the acetate to propionate ratio (A:P) almost always declines in saponin treatments albeit sometimes propionate concentration does not increase (Hess et al., 2004; Goel et al., 2008; Guo et al., 2008; Pen et al., 2008; Holtshausen et al., 2009; Wang et al., 2011; Zhou et al., 2011). The shift of VFA products from acetate to propionate can probably be explained by the reduction of protozoa population. In some cases, propionate concentration was found to be decreased in rumen fluid containing 100 g/kg *Enterolobium cyclocarpum* or *Pithecellobium saman* (Hess et al., 2003). However, it should be noted that the decline of propionate production was due to increased protozoa population.

Unlike *in vitro* studies, rumen VFA production from *in vivo* experiments showed great variation. *Yucca schidigera* extract had no influence on total VFA production when fed at 0.075, 10 and 5.83 g/kg DM to steers (Hussain and Cheeke, 1995), dairy cows (Holtshausen et al., 2009) and heifers (Hristov et al., 1999), respectively. *Biophytum petersianum* (Santoso et al., 2007) reduced total rumen VFA production at the dose of 19.5 mg/kg BW, whereas at a higher dose (26 mg/kg BW) it failed to affect ruminal VFA production. Lu and Jorgensen (1987) reported that 20 g/kg DM lucerne saponin reduced total rumen VFA concentration when diets containing 40% forage and 60% concentrate were fed, but not when diets containing 60% forage and 40% concentrate. Interaction between feed type and saponin concentration has also been reported by Singer et al. (2008), suggesting that response to saponin in terms of rumen VFA production is diet-dependent, therefore conclusions should be made diet and saponin concentrations specifically.

Reduced rumen A:P ratios, in most cases, were observed in sheep and goat species (Lu and Jorgensen, 1987; Abreu et al., 2004; Hess et al., 2004; Santoso et al., 2006; Santoso et al., 2007). In large ruminants, such as cows, heifers and steers, rumen A:P ratios were usually not changed by saponin treatments (Hussain and Cheeke, 1995; Lovett et al., 2006; Holtshausen et al., 2009), indicating responses to saponin inclusion maybe species-dependent.

Fewer studies have investigated effects of saponin containing-diets on animal performance (growth, meat quality, wool and milk production). Saponin extracts from *Yucca schidigera*, *Quillaja saponaria* and *Camellia sinensis* are the few commercialized saponin-rich products and have therefore been used as the main sources of saponin in performance studies.

Dairy cows (BW = 586 kg; 69 d post calving) offered 25 g/d *Yucca schidigera* saponin had greater BW but the milk yield and composition did not differ from that of non-saponin treatment cows (Lovett et al., 2006). Similar results were reported by Singer et al. (2008), where dairy cows (BW = 810 kg; late lactation) fed up to 150 g/d of *Yucca schidigera* showed no difference on milk production. Milk efficiency of dairy cows (BW = 627; early lactation), when expressed as per kg DMI, was improved when 10 g/kg DM *Yucca schidigera* (equivalent 230 g/d) and *Quillaja saponaria* (equivalent 225 g/d) treatments were fed, although milk production was not affected in these treatments (Holtshausen et al., 2009). It needs to be noted that these comparisons are based on crude saponin extracts rather than the actual saponin content, which is affected by the plant source and product. Because animals used in every experiment are not at the same BW and lactation stages, conclusions should be made carefully regarding the effect of saponin-containing diets on milk production.

In small ruminants, differences in growth performance and meat quality in lambs were not observed among *Quillaja saponaria* and control treatments at up to 90 mg/kg DM inclusion (Nasri et al., 2011). Lambs fed 3 g/d *Camellia sinensis* showed no difference in growth performance (Mao et al. 2011). However, a dose-dependent response of increased BW change was reported when goats were offered 90 to 160 mg/kg DM *Yucca schidigera* (Aregheore, 2005), suggesting that the lack of effect observed in Mao et al. (2011) and Nasri et al. (2011) could be due to the low doses. Greater DMI and enhanced nutrient digestibility in *Yucca schidigera* treatment might explain the better growth performance (Aregheore, 2005). In sheep fed a forage-only diet, both 100 g/d and 300 g/d *Enterolobium cyclocarpun* dietary inclusion improved ADG and wool growth, although in the 300 g/d treatment, DM digestibility was reduced (Navas-Camacho et al., 1993).

Effects on methane production

A number of studies have demonstrated that ruminal methanogenesis is reduced by dietary inclusion of saponin *in vitro*. Saponins from *Yucca schidigera* and *Quillaja saponaria* have been the most extensively studied and both saponins are suggested to reduce ruminal methanogenesis (Makkar et al., 1998; Wang et al. 1998; Pen et al., 2008).

Two studies compared the effects of *Yucca schidigera* and *Quillaja saponaria* on methanogenesis and found *Yucca schidigera* was more effective than *Quillaja saponaria*. In the first study (Pen et al., 2006), *Yucca schidigera* extract and *Quillaja saponaria* extract, at 0, 2, 4, and 6 mL/L, were administered to culture media containing oat hay and concentrate (50:50). Methane was reduced in a dose-dependent manner by up to 42% in *Yucca schidigera* treatment, while no effect was observed in *Quillaja saponaria*. In the second study (Holtshausen et al., 2009), saponin extract of *Yucca schidigera* (6% saponin) and *Quillaja saponaria* (3% saponin) was added to rumen fluid at 1.5%, 3.0% and 4.5% of substrate DM. Methanogenesis was reduced by 8%, 12% and 26%, respectively in *Yucca schidigera* and 6%, 11% and 12%, respectively, in *Quillaja saponaria*. When the reduction of CH_4 production was corrected by actual saponin content in both extracts, *Yucca schidigera* still had a stronger effect on CH_4 production over *Quillaja saponaria*.

Guo et al. (2008) found that 5.3 g/kg DM crude *Camellia sinensis* saponin extract significantly reduced methane production by 8% from sheep. In another study, Hu et al. (2005) observed that methanogenesis was suppressed up to 26% when *Camellia sinensis* saponin concentration was increased to 40 g/kg DM. However, the suppressive effect of *Camellia sinensis* on ruminal methanogenesis only occurred in faunated rumen fluid rather than defaunated rumen fluid. Guo et al. (2008) used mcrA gene to monitor the methanogen population and found that in saponin treatment the abundance of methanogens were not affected. This evidence indicates that rather than direct targeting methanogens, declined CH_4 production as a result of saponin inclusion could because of their toxicity towards protozoa population, which in turn reduces the availability of hydrogen available for CH_4 formation.

In other *in vitro* studies, saponin-rich tropical fruit *Sapindus saponaria* was found to inhibit methanogenesis by 14% when supplemented at 100 g/kg DM to a forage-based diet containing low quality meadow grass hay (Hess et al., 2003). Methanogenesis was increased in the *Enterolobium cyclocarpum* treatment but was not affected when *Pithecellobium saman* was added. The authors suggested that this was because the crude saponin in *Sapindus saponaria* (120mg/g) is higher than in *Enterolobium cyclocarpum* (19 mg/g) and *Pithecellobium saman* (17 mg/g), therefore the lack of effect could be attributed to a dose dependent response (Hess et al., 2003).

Given the amount of work conducted *in vitro,* unfortunately, information relating to the effect of saponins on CH_4 production from *in vivo* studies is relatively sparse. Sheep fed roughage based diet (roughage:concentrate, 70:30) with 120 mg/kg DM supplementation of *Yucca schidigera* were found to have lower CH_4 emissions when expressed as metabolic body weight (per kg $BW^{0.75}$) and g/kg DMI but not as g per digestible organic matter intake (Santoso et al., 2004). In other studies, neither supplementation of the *Yucca schidigera* nor *Quillaja saponaria* reduced CH_4 emissions in sheep or dairy cows (Pen et al., 2006, Holtshausen et al., 2009), although in both of those studies, numerical reductions were found in saponin treatments.

Considerable reduction of CH_4 emissions was found in lambs receiving diets containing 3 g/d *Camellia sinensis* saponin, where CH_4 production mass was 27.2%

lower in saponin treatment compared to control treatment (Mao et al., 2010). Similar results (8.71% reduction) were observed in sheep fed 5 g/d *Camellia sinensis* saponins for 21 days (Yuan et al., 2007).

The variability in response to saponin inclusion from *in vivo* studies could be partially attributed to the sources which may contribute to the variation of saponin concentration in the diets. On the other hand, low dietary saponin concentration offered in some studies in order to avoid negative effects of saponins have on animal performance may also explain the variation.

Metabolism and adaptation of saponins in the rumen

One of the challenges that impedes application of saponin is the microbial degradation of saponins in rumen. Gestetner et al. (1968) found microorganisms in the cecum and colon of mice were able to deglycosylate soybean saponins. Similar results were also observed in *in vitro* cultures of both steroid and triterpenoid saponins with rumen fluid (Wang et al., 1998, Makkar et al., 1998). Bacteria that were capable of attacking soluble *Medicago sativa* (alfalfa) saponin were isolated from steers fed fresh cut alfalfa diet (Gutierrez et al., 1959), suggesting some rumen bacteria might be able to use the sugar moiety of saponin leaving the intact sapogenin part in the rumen. The structure of sapogenin (steroid or triterpenoid) matters more than sugar moieties in the aspect of saponin's haemolytic activity on bacteria cell membranes (Segal et al., 1966), suggesting that deglycosylation of saponin can enhance the biological activities of saponins in the rumen. Hydrolyzed sapogenin moieties of *Narthecium ossifragum* underwent oxidative and reductive reactions into epismilagenin, smilagenone, smilagenin and tigogenin in the rumen (Flaoyen and Wilkins, 1997). However, the microbial degradation of sapogenin was found to be limited in *Yucca schidigera* saponins, even when microbes were pre-exposed to saponin for 20 days (Wang et al, 2008).

Another challenge lies in the transient characteristic of the antiprotozoal effect of saponins. Ivan et al. (2004) found that when *Enterolobium cyclocarpum* was fed to sheep, protozoa population was significantly reduced, but only during the first 11 days, whereas the population increased to almost the same level compared to non-saponin treatment after 14 d. Newbold et al. (1997) noted that protozoa counts in *Sesbania sesban* saponin treatment recovered after 10 d of feeding. The microbial degradation of saponin was suggested to be one of the explanations and the increased glycosidase activity was considered as one of the adaptation processes (Newbold et al., 1997). In addition, Wang et al. (2000) proposed that rumen protozoa may also adapt to saponin by developing their cell morphology for a better resistance of saponins' toxicity. Nevertheless, Wina et al. (2006) found the protozoa population did not recover over the 3-months study when *Sapindus rarak* was fed to sheep, suggesting no adaptation occurred over the long term. To explain the differences in the aspect of microbial adaptation, Teferedegne et al. (1999) compared sheep bred in Scotland and Ethiopia, suggesting that species differences and environmental factors might both contributed to animal's tolerance to the presence of saponin in the diet and the adaptation might not happen in all animal species.

USING DYNAMIC ROOMS TO MEASURE GASEOUS EMISSIONS FROM RUMINANTS

A number of techniques have been developed to measure CH_4 emissions from ruminants including meteorological techniques, ventilated hood techniques, static or dynamic room techniques and tracer gas technique. The tracer gas and ventilated hood techniques are designed more specifically for enteric CH_4 measurement from ruminants, whereas other techniques are also capable of measuring other gaseous emissions, for example ammonia, nitrous oxide, hydrogen sulfide from various sources.

Two types of rooms have been used in measuring CH_4 emissions from ruminants, static rooms and dynamic rooms.

Static rooms are more often applied in measuring CH_4 emissions from soil, crop, landfill or manure (Raich et al., 1990). In order to monitor the CH_4 emissions, an area needs to be enclosed by a room for a duration of time to allow the accumulation of gaseous concentration in this area, therefore any leakage can contributed to the error of measurement. Commonly, gas samples are collected by vacuumed gas bulbs or syringes and analyzed by GC later. Cheap and easy to use are the two most significant advantages of static rooms. However, when restricting animals in an enclosed area without ventilation, they are very likely to suffer from stress, resulting in inaccuracy of measurement. In addition, temperatures in static rooms usually increase during the enclosed period, uncontrollably (Livingston and Hutchinson, 1995); the lack of temperature regulation is prone to affect the volume of gas samples, causing changes in animal's metabolism and welfare issues.

In dynamic rooms, animals are confined in sealed rooms with inlets to supply fresh air and outlets for exhausted gases. Rooms, depending on the size, are usually capable of housing one or more ruminants, with fixed or regulated temperatures (McGinn et al., 2004; Li et al., 2011). Concentrations of targeting gases are calculated by the difference of exhausted and background ambient air. To account for the accuracy of emitted gaseous concentrations from animals, air flow rates and sophisticated instruments calibrated on a regular basis are required. The extent of control allows dynamic rooms to be less susceptible to be affected by ambient changes compared to other techniques, providing the benefit of using them to compare treatment effects on gaseous emissions from ruminants. Nevertheless, the spatial limitation may change animal's behaviors and activities thus affecting their emissions.

The Animal Air Quality Research Facility (AAQRF) at Michigan State University facilitates 12 dynamic rooms (height = 2.14 m, width = 3.97 m, length = 2.59 m), capable of occupying different animal species, such as swine (6 head/room), laying hens (7 bird/cage, 8 cage/room), boilers (50 bird/room), turkeys (10 bird/room), heifers (1 head/room) and steers (1 head/room). Since 2007, more than 10 research projects investigating the air emissions from different animal species and manure management have been conducted at AAQRF.

Each room is constructed with an individual inlet for incoming fresh air and an outlet for exhausting air. Incoming air ducts are designed at the same length and size with one end attached to the room and the other end linked to a main duct which is responsible for the overall supply of ambient air. Through the ceiling on one side of the room enters the ambient air through a tri-directional vent and an exhaust duct, 12 cm above floor level, is located at the corner of the room on the diagonal side to expel the room air. This design allows room air to be well-mixed, providing homogenous gas samples for analysis. Temperatures are controlled through a Make-up Air Unit (MAU) system. Within the desired range, temperatures can be adjusted automatically by air flow rate through a dedicated fan in the main duct.

A standard sampling cycle is 195 min. During every cycle, each of the 12 rooms is sampled for a 15-min period. Within the 15-min sampling period, the line is purged for the first 10 min and then data are saved for the remaining 5 min of the sampling period (concentration readings every half second and averaged over the 10 readings). After each of the 12 rooms is sampled, a background sample is collected to obtain baseline readings. Through software control (LabVIEW Version 8.2; National Instruments Corp., Austin, TX), gaseous concentration monitoring of each room occurs in a sequential manner. Daily mass of emitted gas is calculated by summing the mass emitted during each sampling period for that day (7 to 8 daily observations per room (Powers et al., 2007). All emission factors are calculated from emission mass, which is calculated based on the emission rate (the product of concentration and airflow). Gas emission rates are calculated as the product of ventilation rates and concentration differences between exhaust and incoming air using the following equation:

$$\mathrm{ER} = \mathrm{Q}\frac{273}{\mathrm{T}} \times (\mathrm{C_o} - \mathrm{C_i}) \times 10^{-6} \times \frac{\mathrm{MW}}{\mathrm{V_m}}$$

where ER is emission rate, g/min; Q is ventilation rate at room temperature and pressure, L/min; T is air temperature in room exhaust, in Kelvin; C_o is gas concentration in room exhaust, ppm; C_i is gas concentration in the incoming air, ppm; MW is molecular weight of the gas, g/mol; V_m is molar volume of gas at standard condition (22.414 L/mol).

OBJECTIVES AND RATIONALE STATEMENT

Due to facility limitations, available data from research efforts regarding the nutritional impacts on enteric fermentation from ruminants are primarily from *in vitro* studies. Significant knowledge gaps of the relationship between *in vitro* and *in vivo* studies and nutritional impacts *in vivo* still exist. In fact, product and diet-specific impacts on methane emissions in ruminants *in vivo* are still unknown. Regardless of the efficiencies of saponins in reducing methane emission *in vitro*, *in vivo* evidence is required before products can be applied to animal industries.

The overarching aim of this project is to investigate potential application of dietary saponin inclusion to reduce CH_4 emissions from steers. Because the detrimental effect of saponin against protozoa population has been well established *in vitro*, we hypothesize that CH_4 emissions will be reduced as a result of dietary saponins inclusion, *in vivo*.

This study will combine *in vitro* approaches with utilization of environmental-controlled facilities for air sampling/monitoring at MSU's AAQRF.

The specific objectives of the study were to:

- Establish the dose-dependent response of ruminal fermentation and methanogenesis to saponin inclusion, *in vitro*.
- Determine the effects of dietary saponin inclusion on animal-derived methane and other air emissions, *in vivo*;
- Determine the effects of dietary supplementation of saponin and direct application to manure on manure-derived methane and other emissions.

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CHAPTER 2

USING DIETARY SAPONIN EXTRACTS TO REDUCE METHANE EMISSIONS FROM HOSTEIN STEERS

Formats in Chapter 2 to 4 are modified according to the requirements of Journal of Animal Science

Using dietary saponin extracts to reduce methane emissions from steers

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ABSTRACT A total of 3 experiments (Exp), *in vitro* and *in vivo*, were conducted to investigate the effects of saponin extracts from *Quillaja saponaria* (QS), *Yucca schidigera* (YS) and *Camellia sinensis* (TS) on CH_4 emissions from steers. The *in vitro* Exp was carried out to determine the effects of saponin inclusions on ruminal methanogenesis and fermentation parameters. Two doses (0.5% and 2.0% of substrate DM) of each saponin were added to the mixture of rumen fluid and buffer for 0, 4, 8, 12 and 24 hr incubation. During the *in vivo* experiments (Exp 1 & 2), concentrations of saponin extracts added to the diets were determined based on the actual saponin content in the extract to provide 0.54 g/kg DM saponin in the diets. Exp 1 used a 3 × 3 Latin Square design with 4 replicates for each treatment, to compare the effects of saponin containing diets, QS (QS1, 1.5% DM) and YS (YS1, 0.64% DM), to a corn and corn silage based control (C1) treatment on enteric CH_4 emissions. The second experiment designed using a Latin Square (2 × 2, 6 replicates) to evaluate the effect of TS (TS2, 0.25%) on enteric CH_4 emissions, by comparing it to a corn and corn silage based control diet (C2). For each study, 12 Holstein steers were individually-housed in environmental rooms for 14 d per period. Methane concentrations were monitored in room exhaust air. During *in vitro* experiment, CH_4 production was reduced in all saponin treatments at 24 hr incubation ($P < 0.01$). Gas production was reduced in TS0.5%, TS2.0% and YS2.0% treatments, but was not affected in the other saponin treatments. The NH_4^+-N production was reduced in all saponin treatments expect QS0.5%. Acetate concentration was reduced in all treatments but 0.5% QS and 0.5% YS ($P < 0.01$). Except the QS0.5% treatment, all saponin treatments reduced A:P ratio compared to the control treatment, at 24hr

incubation ($P < 0.01$). During both *in vivo* Exp, feeding saponins to steers did not change ADG or manure excretion characteristics ($P > 0.05$), but feeding steers TS2 decreased DMI compared to C2 ($P < 0.01$). Methane emission mass, emission factors and manure excretions were not affected by dietary saponin inclusion. Results indicated that dietary supplementation of 0.54 g/kg DM saponin did not affect CH_4 emissions.

Key words: *Quillaja saponaria*, *Yucca schidigera*, *Camellia sinensis*, CH_4 emissions, rumen fermentation, Holstein steer

INTRODUCTION

Enteric methane (CH_4) fermentation from ruminants represents a substantial loss of feed energy and contributes to global climate change. According to US EPA, approximately 28% of CH_4 emissions from the U.S. originate from ruminants (US EPA, 2011). Reducing ruminal enteric CH_4 production will thus be significant in terms of improving feed efficiencies and moderate the impact of ruminant production on global climate change.

Because 20% of the ruminal methanogen population is associated with protozoa, which aplay a critical role in interspecies hydrogen transfer (Newbold et al., 1995), reduction of protozoa population is often accompanied by lower CH_4 production in the rumen (Itabashi et al., 1994). Plant saponins from *Quillaja saponaria*, *Yucca schidigera* and *Camellia sinensis* have been shown to suppress ruminal methanogenesis *in vitro* (Makkar et al., 1998; Wang et al. 1998; Pen et al., 2006; Pen et al., 2008; Guo et al., 2008; Holtshausen et al., 2009), mainly due to their toxicity against rumen protozoa (Guo et al., 2008). However, results from animal studies are not consistent with *in vivo* studies

(Santoso et al., 2004; Holtshausen et al., 2009; Mao et al., 2010). In addition, very few studies have examined the efficacy of dietary saponin inclusion to reduce CH_4 emissions from beef steers.

We hypothesized that CH_4 emissions from beef steers could be reduced as a result of feeding saponin-containing diets. The objectives of current study were to: 1) determine the effects of saponin extracts from *Quillaja saponaria*, *Yucca schidigera* and *Camellia sinensis* on ruminal CH_4 production and fermentation *in vitro*; and 2) compare the effects of saponin extracts from *Quillaja saponaria*, *Yucca schidigera* and *Camellia sinensis* on CH_4 production and animal growth performance, *in vivo*.

MATERIALS AND METHODS

All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee. Three experiments (Exp), one *in vitro* and two *in vivo*, were conducted at the Animal Air Quality Research Facility (AAQRF) at Michigan State University.

Saponin Sources

The 3 saponin extracts used in the Exp were yucca saponin (YS) which is a powder made entirely from the stem of the *Yucca schidigera* plant and rich in steroidal *saponin* (contains 8.5% saponin; Desert King International, San Diego, CA, USA); quillaja saponin (QS), which is a triterpenic saponin enriched extract from pure Chilean soap bark tree *Quillaja saponaria* (contains 3.6% saponin; Desert King International, San Diego, CA, USA); and tea saponin (TS), which is the whole plant saponin extract from *Camellia sinensis* and rich in triterpenoid saponin (contains 21.6% saponin; Ningbo Good

Green Science & Technology, Ningbo, ZJ, China**).** Saponin concentration from each product was measured by Desert King International (San Diego, CA, USA).

***In vitro* study**

Experimental design. The *in vitro* study was conducted as a repeated completely randomized experimental design. Two doses of each saponin (0.5% and 2.0% subtract DM) were compared to a Control treatment with no saponin addition. Rumen fluid was collected from a fistulated dry cow fed on hay diet. The substrates (corn and hay) for *in vitro* incubation were dried at 55°C and grounded through a 1-mm screen Wiley mill. (Thomas Scientific, NJ).

Prior to the incubation, 400 mg dry substrates (50 : 50, corn and hay) and saponins (0%, 0.5% or 2.0% of substrate DM) were added to a 165 ml serum bottle (15 replicates per treatment, 105 bottles in total). In addition, 15 serum bottles, three for each time point, without substrates were prepared as blanks. All bottles were pre-warmed in a water bath at 39 °C before incubation. Rumen fluid and ingesta were obtained in the morning 2 hr after feeding of the dry cow, blended and filtered by 2 layers of cheesecloth. Two volumes of buffer solution contained bicarbonate (Goering and Van Soest, 1970) was then mixed with the strained rumen fluid and maintained in a 39 °C water bath with continuous CO_2 flow**.** The mixture, 30 ml (10 ml rumen fluid and 20 ml buffer fluid), was transferred to the pre-warmed serum bottle and flushed with CO_2. Bottles, sealed with a rubber stopper and crimped by an aluminum cap to prevent any gas leakage, were incubated in a 39 °C water bath for 0, 4, 8, 12 and 24 hr. Bottles were hand-shaken every 2 hr .

At each time point, 3 bottles from each treatment, including blanks (24 total) were randomly chosen to terminate fermentation for gas and CH_4 production (except 0 hr), ruminal NH_4^+-N concentration, pH and individual VFA concentration analysis. Total VFA production was calculated as the sum of acetate, propionate and butyrate.

Gas and methane production. Gas production at 4, 8, 12 and 24 hr incubation was measured using a relative pressure gauge (Model Media Gauge, SSI Technologies, WI) by inserting the attached 24 gauge needle through the rubber stopper. After the pressure measurement, approximately 10 ml gas sample from each bottle was taken from the head space through a 24 gauge needle and sealed in a syringe for immediate CH_4 analysis by gas chromatography (GC, Model 2010, Shimazu, Japan). The calculation for gas production was:

$$V_{gas} = \frac{V_{BT} - V_R}{P_{EL}} * P_{BT}$$

where V_{gas} is the volume of gas production at each time point, ml; V_{BT} is the volume of serum bottle, ml; V_R is the volume of rumen fluid and buffer solution, 30 ml; P_{EL} is the atmospheric pressure in East Lansing, MI, psi and P_{BT} are the pressure measurements from the gauge, psi.

Volatile fatty acids, ammonium-N and pH. At each time point (0, 4, 8, 12, 24 hr), the pH of the incubation mixture was measured by a pH meter (Model HQ40d Portable pH meter, HACH, CO). About 5 ml of the contents from each bottle was centrifuged at 26,000 × g for 20 min, 1ml of the supernatant was saved at -20 °C for VFA analysis on high performance liquid chromatography (HPLC, Model Water 712 WISP, Millipore, MA). The remaining 25 ml were transferred to a pre-weighed tube for immediate NH_4^+-N analysis (FOSS Tecator, MN).

***In vivo* study**

*General Animal Housing and Management***.** During each of the 2 *in vivo* experiments (Exp), 12 Holstein steers were housed, individually, in 12 environment-controlled rooms at the Animal Air Quality Research Facility at Michigan State University. Temperature was maintained at 13.75 ± 1.38 °C (Exp 1, period 1), 12.77 ± 1.17 °C (Exp 1, period 2), 18.09 ± 1.05 °C (Exp 1, period 3), 20.97 ± 0.85 °C (Exp 2, period 1) and 19.91 ± 0.58 °C (Exp 2, period 2) to remain within the thermoneutral zone of the animals. In each room, steers were confined in a 106.7 cm long × 182.9 cm wide raised stall covered with a rubber matt surface. A fiberglass feeder was placed at the front of the stall and a pan of the same width as the stall was placed at the rear side to collect both urine and feces. Fresh total mixed ration (TMR) feed was sampled by treatment and offered once daily at 16:00 h at 10% above expected DMI. Prior to feeding, orts were removed, weighed and sampled for each steer. Manure was removed and pans were cleaned once daily at 06:00 h to minimize contribution of manure-derived CH_4 emissions to total CH_4 emissions. A homogenous sub-sample was collected each time manure was removed. Samples for feed, orts and manure were stored at -20 °C until the end of each experimental period. At the end of each period, feed samples were composited by treatment for feed analysis. Orts and manure samples were composited by room for N content analysis. N intake was calculated for each steer as the difference between N offered in diet and N left in the orts. Steers were weighed on 2 consecutive mornings prior to feeding before arriving and after leaving the rooms.

Experimental Design and Dietary Treatment. Experiment 1 used a 3 × 3 Latin Square experimental design with 4 replicates for each treatment, while Exp 2 was a 2 × 2 Latin Square experimental design with 6 replicates per treatment. The length of each period was 21 d. All diets offered throughout the 2 Exp were corn-corn silage based (Table 2.1). On d 7 of each period, steers were moved into environmental-controlled rooms for 14 consecutive days for enteric CH_4 measurements. Starting BW was 285 ± 9 kg (Exp 1, period 1), 305 ± 10 kg (Exp 1, period 2), 334 ± 9 kg (Exp 1, period 3), 390 ± 9 kg (Exp 2, period 1) and 411 ± 10 (Exp 1, period 2).

During Exp 1 and 2, inclusion levels of the 3 products were adjusted to similar saponin concentration in order to compare the effects of different saponins at the same dietary saponin concentration, providing 0.54 g/kg dietary DM of saponin. The experimental diets in Exp 1 used a corn and corn-silage based control diet (C1), a diet containing 1.5% quillaja saponin extract of diet DM (QS1) and a third diet containing 0.64% yucca saponin extract of diet DM (YS1); 4 replicates per treatment. During Exp 2, a diet containing 0.25% tea saponin extract of diet DM (TS2) was compared to a corn-silage based control diet (C2); providing 6 replicates per treatment.

Measurements of Gaseous Concentrations

Twelve rooms (height = 2.14 m, width = 3.97 m, length = 2.59 m) were designed to continuously monitor incoming and exhaust concentrations of gases (Li et al., 2011). Concentrations of CH_4 were measured by Innova 1412 photoacoustic analyzer (Lumasense Technologies, Ballerup, Denmark) with a detection limit at 1000 ppm.

Gaseous concentration monitoring of each room occurred in a sequential manner. All emission factors were calculated from emission mass which is calculated based on the emission rate. Gas emission rates were calculated as the product of ventilation rates and concentration differences between exhaust and incoming air using the following equation:

$$\mathrm{ER} = \mathrm{Q}\frac{273}{\mathrm{T}} \times (\mathrm{C_o} - \mathrm{C_i}) \times 10^{-6} \times \frac{\mathrm{MW}}{\mathrm{V_m}}$$

where ER is emission rate, g/min; Q is ventilation rate at room temperature and pressure, L/min; T is air temperature in room exhaust, in Kelvin; C_o is gas concentration in room exhaust, mg/kg; C_i is gas concentration in the incoming air, mg/kg; MW is molecular weight of the gas, g/mol; V_m is molar volume of gas at standard condition (22.414 L/mole). Emissions in 1 full measurement cycle were estimated by multiplying the ER (g/min) with 195 min. Daily emissions were calculated as sum of the emissions in the 7 or 8 measurement cycles (as described by Li et al., 2011).

Chemical Analyses

Feed and orts samples were analyzed by Dairy One Forage Testing Laboratory (Dairy One, Inc. Ithaca, NY) for compositional analysis. Feed DM content was analyzed by Near Infrared Reflectance Spectroscopy (NIRS) (AOAC-991.01, 1995). Crude protein, degradable protein, NDF and ADF were analyzed by Foss NIRS systems Model 6500 with Win ISI II v1.5 (AOAC-989.03, 1996). Minerals were analyzed by microwave digestion followed by Inductively Coupled Plasma Mass Spectrometry (ICP). Energy content was determined by an IKA C2000 basic Calorimeter System (IKA Works, NC). Manure NH_4-N (AOAC-928.08, 2000) and total Kjeldahl N (TKN; FOSS Tecator, 1987) content was measured by distillation and digestion followed by distillation, respectively, in a Michigan State University laboratory.

Statistical Analyses

Data from both *in vitro* and *in vivo* studies were analyzed using mixed model procedures (SAS Institute, 2008). Results from the repeated *in vitro* experiments were pooled. The different experiments and serum bottles were treated as random variables. For the *in vitro* study, dietary effect of saponins and different concentrations were the fixed effects and the interaction between concentration and saponin types was tested in the model. A contrast statement was used to compare the least squares means differences between the Control and each saponin treatment. Orthogonal polynomial contrasts were applied to determine a linear response to saponin concentrations (0, 1.5% and 2.0% DM) for CH_4 and gas production. For *in vivo* study, performance (DMI and ADG), excretion (wet mass, DM mass, NH_4^+-N and TKN) and CH_4 emissions data was analyzed using a mixed model testing the fixed effects of diet and random effects of steers and period. Day was considered as a repeated measure for DMI and manure excretion (wet mass, DM mass, NH_4^+-N and TKN). Tukey's test was applied to compare treatment differences. Significant differences among the least squares means were declared at $P < 0.05$.

RESULTS

In vitro fermentation

Inclusion of QS at both concentrations did not affect gas productions during 24 hr incubation, except that at 12 hr incubation the gas production was reduced by 4% as a result of including 0.5% and 1.5% of QS (Table 2.2). At the inclusion of 0.5% of TS, reduced gas production was observed only at the end of 24 hr incubation ($P < 0.01$) but not at other incubation periods. When TS concentration increased to 1.5%, lower gas production was observed at 12 and 24 hr incubation periods compared to Control treatment ($P < 0.01$). Yucca saponin, when included at 0.5%, had no effect on gas production during 24 hr incubation compared to Control treatment, while inclusion of 1.5% YS reduced gas production at 4, 12 and 24 hr incubation period ($P < 0.01$).

Tea saponin and YS reduced CH_4 production at both inclusion concentrations during every time point (Table 2.2), while QS included at 0.5% did not affect CH_4 production within the first 8 hr of incubation. Linear regression relationships between saponin concentrations and CH_4 productions were observed for all saponin types.

Saponins inclusion had an immediate effect on ruminal NH_4^+-N concentrations (Table 2.3). When NH_4^+-N concentrations were analyzed at 0 hr, all saponins reduced NH_4^+-N concentration by approximately 7% (Table 2.3). After 24 hr incubation, concentrations of NH_4^+-N in all saponin treatments, except QS 0.5%, were lower compared to the Control treatment ($P < 0.01$). The pH values were not affected by saponin inclusions (Table 2.3). Across all treatments, pH was gradually decreased from the beginning of incubation to the end. Average pH values across all treatments were 7.48, 7.36, 6.97, 6.78 and 6.77 at 0, 4, 8, 12 and 24 hr incubation, respectively.

At every time point, except 0 hr, 2.0% saponin treatment decreased acetate concentration and the acetate : propionate (A:P) ratio ($P < 0.05$), and increased propionate concentrations compared to the Control treatment ($P < 0.05$; Table 2.4 to 2.6).

At the end of 24 hr incubation, total VFA production was not affected by saponin inclusion compared to Control treatment, except in 1.5% TS total VFA was reduced by 3% (Table 2.6). Butyrate production at the end of 24 hr incubation was not affected as a result of saponin inclusion (Table 2.6).

Growth performance and manure excretion

Inclusion of saponin did not affect steer ADG during Exp 1 and Exp 2, respectively (*P* > 0.05; Table 2.7). Dry matter intake was not affected by dietary supplementation of QS or YS (*P* > 0.05), but was reduced as a result of TS inclusion (*P* < 0.01). Manure characteristics were not affected by dietary saponin during both Exp. Average daily NH_4^+-N and TKN excretion mass was 33.58 and 67.68 g, respectively in Exp 1 (*P* > 0.05; Table 2.8) and 46.65 and 94.91 g, respectively, in Exp 2 (*P* > 0.05).

Methane emissions

Diets containing 0.54 g/kg QS or YS did not change daily CH_4 emissions from steers compared to steers fed C1 diet (Exp 1). Across all treatments, average CH_4 daily concentration, emission rate and mass from steers was 8.52 mg/kg, 50.70 mg/min and 79.11 g/d, respectively (*P* > 0.05; Table 2.9). Similarly, no differences were observed in average daily CH_4 concentration (7.89 mg/kg), emission rate (57.86 mg/min) or emission mass (90.27 mg) from rooms where steers fed TS2 treatment compared to C2 (Exp 2).Although the DMI decreased in steers as a result of feeding TS2, CH_4 daily emission mass per unit DMI from steers in TS2 treatment showed no difference compared to the C2 treatment.

DISCUSSION

Ruminal fermentation and in vitro methanogenesis

The well-established dose-dependent response to saponin inclusions on ruminal methanogenesis *in vitro* was confirmed in this study. However, not all saponins were equally effective in reducing ruminal methanogenesis. At 0.5% inclusion level, QS and YS reduced CH_4 production by 9% and 8%, respectively, while TS reduced CH_4 production by 14 % by the end of 24 hr incubation. At 2.0% inclusion level, supplementation of QS, TS and YS resulted in 14%, 20% and 19% less CH_4 compared to Control treatment at 24 hr incubation period. The difference in CH_4 reductions can be partially explained by the different saponin concentrations in the extract. Holtshausen et al. (2009) found that when saponin extracts from *Yucca schidigera* (6% saponin) and *Quillaja saponaria* (3% saponin) were added to rumen fluid at 1.5%, 3.0% and 4.5% of substrate DM, ruminal CH_4 production was reduced by 8%, 12% and 26%, respectively in *Yucca schidigera* treatments and 6%, 11% and 12%, respectively, in *Quillaja saponaria* treatments. When the reduction of CH_4 was corrected by actual saponin content in both extracts, *Quillaja schidigera* showed stronger effect against CH_4 production over *Yucca saponaria* at 1.5% and 3.0% inclusion level. Findings from our study are in agreement with Holtshausen et al. (2009) that at both 0.5% and 2.0% inclusion level, QS was more effective in reducing CH_4 than TS and YS when the reductions were adjusted by the saponin concentration in the extract.

Guo et al. (2008) found that 5.3 g/kg DM crude *Camellia sinensis* saponin extract significantly reduced methane production by 8%, *in vitro*. In another study, Hu et al.

(2005) observed up to 26% methanogenesis was suppressed when *Camellia sinensis* saponin concentration increased to 40 g/kg DM. Our results are similar with both Hu et al. (2005) and Guo et al. (2008).

Our results showed that adding QS and YS to rumen fluid at 0.5% did not negatively affect gas production at the end of 24 hr incubation, *in vitro*, which is in agreement with Lila et al. (2003), Hu et al. (2005) and Guo et al. (2008). However, reduced gas production was observed as a result of including 0.5% TS, 2.0% TS and YS. This may be explained as a toxic effect of saponin against rumen protozoa and some fibrolytic bacterium at high saponin concentrations (Hu et al., 2005; Holtshausen et al., 2009).

Our results showed that over 24 hr incubation, all saponin treatments, except QS 0.5%, produced less NH_4^+-N compared to Control, which is supported by many other studies (Hart et al., 2005, review; Wina et al., 2005, review). Zhou et al. (2011) found that protozoal concentration was decreased from 4.68% of total bacteria to2.66% when dietary saponin concentration increased from 0 to 3g/d. In addition, results from Valdez et al. (1986) showed that protozoa count reduced by 19% in treatment containing 77 mg/kg saponin. The predation activity of rumen protozoa proteolyzes bacteria protein, releases NH_4^+-N as the end product. In addition, a reduction in protozoal number usually leads to decreased NH_4^+-N concentration (Hart et al., 2005, review; Zhou et al., 2011). Although the protozoa population was not examined in the current study, declined NH_4^+-N concentration implies that the protozoa population is possibly reduced as a result of saponin inclusion. It is interesting to see that all saponins exerted immediate effects on ruminal NH_4^+-N concentrations. Similar results were also reported by Wu et al. (1994) who found both quadratic and cubic effects of yucca saponin concentration on ruminal NH_3 concentrations. Yucca saponin is known to bind NH_3 (Wu et al., 1994), whereas the present study suggests that both quillaja and tea saponin are also capable of binding ruminal NH_3. Because of the CH_4 reduction, propionate concentration and decreased A:P ratio are often observed (Makkar et al., 1998; Hu et al., 2005; Holtshausen et al., 2009). Hu et al. (2005) found that at a low saponin dose, total VFA production was increased compared to a control treatment which was in agreement with our observations during the 24 hr incubation. However, at higher concentration, TS reduced total VFA production at the end of 24 hr incubation, while VFA production in QS and YS treatment was not different from control treatment. The TS extract used in our study has a higher concentration of triterpenoid saponin (21.6%) which is 2.5 and 6 times greater than the saponin concentration in QS (8.5% of triterpenoid saponin) and YS (3.6% of steroid saponin) extracts, therefore, the stronger negative effect of TS on acetate and total VFA production could possibly be attributed to its greater concentration.

In vivo study

The objective of the current study was to mitigate enteric CH_4 by dietary supplementation of saponins without impairing animal growth performance. Many studies have shown that dietary saponin inclusion would not affect animal performance (Aregheore, 2005; Nasri et al., 2011; Santoso et al., 2004; Hristov et al., 1999; Holtshausen et al., 2009; Depenbusch et al., 2007). In our study, ADG during either study was not affected, although the DMI was decreased by feeding TS2 treatments, suggesting TS improved the feed efficiency in steers.

One study from Hristov et al. (1999) demonstrated that supplementation of 20 g and 60 g of yucca saponin to heifers did not change N concentration in either urine or feces which was also observed in our experiments. Degradation of microorganisms may be decreased as a consequence of saponin inclusion, while the increased flow of microbial protein could lead to better absorption in the small intestine, resulting in no net changes in N metabolism (Lu et al. 1987). Therefore, the lack of an effect on N excretion could be due to the comprehensive microbiological and physiological effects in the rumen and lower GI tract digestion (Newbold et al., 1997; Holtshausen et al., 2009).

Although a number of studies have demonstrated that ruminal methanogenesis can be reduced by dietary inclusion of saponin *in vitro*, results from *in vivo* studies are not consistent. In small ruminants, supplementation of 120 mg/kg DM *Yucca schidigera* to roughage-based diets in sheep (roughage:concentrate, 70:30) reduced CH_4 emissions when expressed as metabolic body weight (per kg $BW^{0.75}$) and g/kg DMI (Santoso et al., 2004). Considerable reductions in CH_4 emissions was also found in lambs receiving diet containing 3 g/d *Camellia sinensis* saponin, where CH_4 production mass was 27.2% lower in saponin treatment compared to control treatment (Mao et al., 2010). Similar results (8.71% reduction) were reported in sheep fed 5 g/d *Camellia sinensis* saponins for 21 days (Yuan et al., 2007).However, in large ruminants, dietary saponin inclusions generally produce no effects on enteric CH_4 production. Holtshausen et al. (2009) fed dairy cows with 1.0% quillaja or yucca saponin and observed no changes in CH_4

emissions compared to control treatment. Likewise, in our study, saponin when included at 0.54 g/kg DM (or 10 g/kg DM quillaja saponin extract, 6.4 g/kg DM yucca saponin extract and 2.5% tea saponin extract),did not change enteric CH_4 emissions. The variation among large and small ruminants may be attributed to dietary compositions where in sheep or lamb species, experimental diets were usually high in roughage while roughage made up a relatively smaller proportion of the diets in large ruminants. In addition, genetic variations among ruminant species, saponin sources and extraction methods of saponin may also be the factors contributing to the discrepancies.

Comparing methane emissions from in vitro and in vivo studies

Although saponins were effective in reducing CH_4 production *in vitro*, unfortunately, the effectiveness *in* was not confirmed from the animal study. The divergences between *in vitro* and *in vivo* results may be explained by several factors.

The *in vitro* fermentation technique was primarily developed to evaluate the feed digestion and N utilization in the rumen (Johnson, 1966) and isa wildly used tool for various research purposes not limited to the rumen. The validation of the *in vitro* technique was challenged by Moss and Givens (1997) because of the poor correlations (R^2 = 0.264) between *in vitro* and *in vivo* results. However, more recently Blümmel et al. (2005) demonstrated that CH_4 production calculated from the efficiency of microbial production was well correlated (R^2 = 0.89) to measured CH_4 emissions. Further study from Getachew et al. (2005) suggested the possibility of applying the *in vitro* fermentation technique to estimate CH_4 productions under commercial conditions.

Unlike animal studies, *in vitro* experiments offer better opportunities to control error. For example, rumen fluid, obtained from one or more fistulated animals, is often composited to provide homogenous samples for *in vitro* incubation rather than contributing to variation, as such, genetic related variations which are commonly seen in animal studies are eliminated. In addition, substrates for fermentation are provided equally for all treatments and well-mixed in the *in vitro* incubation system, hence the possible differences of DMI among animals are not considered. By controlling the factors that contribute to experimental errors in animal studies, the *in vitro* system is very sensitive to small treatment differences.

In order to avoid the influence of ingesta from donor animals, rumen fluid used in *in vitro* studies is usually subjected to several steps, such as filtration and straining, before mixing with the buffer solution and substrates. Elimination of some microbial species especially those attached to the ingesta is inevitable during these procedures. The *in vitro* system could possibly enrich certain microbial species while leaving some species uncultivable (Johnson, 1966). The changes in microbial communities from the donor animal to the *in vitro* system will possibly affect the microbial fermentation.

In animals, rumen contents are subject to continuous wash out to lower GI tract, the dilution of substrate may therefore reduce the biological effects some dietary supplements especially those whose primarily biological effects are in the rumen, such as saponins (Lu et al., 1987). In contrast, the majority of *in vitro* experiments conducted to investigate enteric CH_4 emissions use closed systems, thereby preventing the outflow of rumen digesta and allowing the accumulation of fermentation end products, which together might amplify the dietary effects on methanogenesis.

Another difference between *in vitro* studies and *in vivo* experiments is the short experimental period. Very often, the *in vitro* experiments are completed within 1 d. Comparably, the experimental period in animal studies is usually more than 21 d with at least 14 d of adaptation period. The short experimental duration of *in vitro* studies limits the day-by-day variation in CH_4 production, making the system more vulnerable to small differences among dietary treatments.

The acute exposure of rumen microorganisms to treatment diets during *in vitro* incubations could lead to drastic changes in microbial communities and CH_4 production. On the other hand, in animal studies, adaptation may occur during prolonged exposure to treatment diets. In the case of dietary saponin inclusion, the adaptation of ruminal microorganisms is suggested to be one of the major reasons for the lack of effects on methanogenesis, *in vivo* (Wang et al., 1998, Makkar et al., 1998).

In summary, the nature of *in vitro* systems, better control experimental errors, provide simplified fermentation conditions, offer short duration of data collections and prevent adaptations to treatments, rendering the system to be more sensitive to minor differences among treatments compared to animal studies. *In vitro* results are not transferable to *in vivo* effects.

CONCLUSION

The effect of saponins on CH_4 emissions were not confirmed during the short-term animal study. However, the dose-dependent response of ruminal fermentation parameters to saponin inclusion was confirmed in the current study, *in vitro*. Possibly explanations may be related to dose and species variations. Dietary inclusion of saponin did not affect growth performance or excretion characteristics in steers.

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Table 2.1. Diet and nutrient composition from experimental diets (DM basis)

	Exp 1[*]			Exp 2	
	C1	YS1	QS1	C2	TS2
Ingredients (%) DM					
High moisture corn	46	46	46	46	46
Corn silage	46	46	46	46	46
Soybean meal	3	3	3	3	3
Supplement 50[#]	5	5	5	5	5
Saponins					
yucca saponin	--	0.64	--	--	--
quillaja saponin	--	--	1.5	--	--
tea saponin	--	--	--	--	0.25
Supplement 50, % of DM					
Akey TM premix #4[TM¶]				1.4	
Limestone				24.9	
Soybean meal, 48% N				48.3	
Rumensin[TM] 80				0.3	
TM salt				9.6	
Vitamin E, 5%				0.2	
Urea, 45% N				9.6	
Potassium chloride				5.1	
Selenium 90				0.7	
Total			100		
Analyzed composition, % DM					
DM	46.9	46.2	46.8	50.5	50.1
CP	11.6	11.8	12.1	12.2	11.9
ADF	16.5	17.0	16.4	10.1	11.5
NDF	27.4	27.8	28.1	27.8	26.4
P	0.27	0.28	0.27	0.29	0.29
ME[§] (Mcal/kg)	1.82	1.79	1.83	1.80	1.82

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from *Camellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1.

Table 2.1. Diet and nutrient composition from experimental diets (DM basis) con't

[#] Middle section of the table lists the ingredients for BFS50 supplement for all treatments
[¶] Akey TM premix # 4 composition: 9% Mg, 4% S, 0.02% Co, 1% Cu, 0.09% I, 2% Fe, 4% Mn, 0.03% Se, 4% Zn, 4,400,000 IU vitamin A, 550,000 IU vitamin D, and 5,500 IU vitamin E/kg (Akey Inc., Lewisburg, OH).
[§] Calculated value.

Table 2.2. Effects of Yucca schidigera (YS), Quillaja saponaria (QS) and Camellia sinensis (TS) on gas production, methane concentration at 0 and 4 hr incubation, *in vitro*

Saponin	Level % DM	Time (hr) 4 Gas (ml)	4 CH_4 (ml)	8 Gas (ml)	8 CH_4 (ml)	12 Gas (ml)	12 CH_4 (ml)	24 Gas (ml)	24 CH_4 (ml)
Control[#]	0	21.83	3.05	34.11	4.71	62.95	7.80	83.95	9.26
QS	0.5	21.72	2.88	34.25	4.54	60.43*	7.34*	83.45	8.45*
	2.0	21.66	2.65*	33.73	3.92*	60.41*	6.60**	83.04	8.00*
TS	0.5	21.51	2.81*	34.08	4.25*	62.11	6.83*	82.67*	8.19*
	2.0	21.35	2.39**	33.85	3.77**	61.29*	5.91**	82.61*	7.40**
YS	0.5	21.53	2.83*	33.48	4.26*	62.53	7.04*	83.06	8.53*
	2.0	20.54*	2.59**	33.00	3.80**	60.62*	6.68**	82.27*	7.51**
SEM		1.17	0.19	4.30	0.49	2.44	0.64	2.14	0.41
Source of variation									
Saponin		0.21	0.12	0.32	0.01	0.04	<0.01	0.39	0.25
Level		0.04	<0.01	0.29	<0.01	<0.01	<0.01	<0.01	<0.01
Saponin × Level		0.36	0.33	0.86	0.16	0.02	0.02	0.51	0.49
Linear regression									
	QS	0.69	<0.01	0.41	<0.01	<0.01	<0.01	0.12	<0.01
	TS	0.27	<0.01	0.63	<0.01	0.19	<0.01	0.22	<0.01
	YS	<0.01	<0.01	0.95	<0.01	<0.01	<0.01	0.02	<0.01

[#] Treatments were corn and hay based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). All saponins were added on substrate DM basis.

[*] Representing the significant difference between Control and saponin treatments ($P < 0.05$).

Table 2.2. Effects of Yucca schidigera (YS), Quillaja saponaria (QS) and Camellia sinensis (TS) on gas production, methane concentration at 0 and 4 hr incubation, *in vitro*, con't

** Representing the significant difference within 2 concentrations of one saponin type and between Control and saponin treatments ($P < 0.05$).

Table 2.3. Effects of Yucca schidigera (YS), Quillaja saponaria (QS) and Camellia sinensis (TS) on gas production, methane concentration at different time points during 24hr incubation period, *in vitro*

Saponin	Level % DM	NH_4^+-N (mg/dl)					pH				
		Time (hr)					Time (hr)				
		0	4	8	12	24	0	4	8	12	24
Control#	0	17.92	19.70	21.96	25.52	30.09	7.46	7.33	6.94	6.79	6.78
QS	0.5	16.59*	20.25*	22.20	24.21*	29.40	7.48	7.37	7.00	6.82	6.80
	2.0	17.15*	19.74	21.28*	23.26**	27.81*	7.49	7.35	6.95	6.78	6.76
TS	0.5	16.71*	20.09*	21.93	23.41*	28.31*	7.46	7.33	6.91	6.81	6.80
	2.0	16.42*	19.23**	20.97*	22.34**	27.90*	7.48	7.40	7.01	6.72	6.73
YS	0.5	16.88*	19.82	21.65	24.06*	28.62*	7.49	7.38	7.02	6.81	6.81
	2.0	16.65*	19.32*	21.01*	22.88**	26.16**	7.51	7.34	6.97	6.76	6.73
SEM		1.26	1.38	0.57	0.96	1.14	0.21	0.33	0.33	0.08	0.29
Source of variation											
Saponin		0.52	<0.01	0.17	0.03	0.02	0.34	0.98	0.88	0.36	0.97
Level		<0.01	<0.01	<0.01	<0.01	<0.01	0.12	0.15	0.66	0.01	0.32
Saponin × Level		0.29	<0.01	0.62	0.43	0.01	0.81	0.22	0.58	0.66	0.99

Treatments were corn and hay based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). All saponins were added on substrate DM basis.

* Representing the significant difference between Control and saponin treatments (*P* < 0.05).

** Representing the significant difference within 2 concentrations of one saponin type and between Control and saponin treatments (*P* < 0.05).

Table 2.4. Effects of *Yucca schidigera* (YS), *Quillaja saponaria* (QS) and *Camellia sinensis* (TS) on VFA concentration at 4 hr incubation period, *in vitro*

Saponin	Level % DM	0 hr					4 hr				
		Total (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	A:P ratio	Total (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	A:P ratio
Control#	0	17.90	12.60	3.46	1.84	3.66	28.39	19.49	6.55	2.35	2.97
QS	0.5	18.07	12.66	3.46	1.95	3.66	28.14*	19.21*	6.59	2.34	2.91*
	2.0	17.78	12.54	3.44	1.80	3.66	28.20	18.81**	7.10*	2.29*	2.65**
TS	0.5	18.10	12.86	3.42	1.81	3.75	28.25	19.27	6.63*	2.35	2.90*
	2.0	18.16	12.87	3.47	1.81	3.71	28.59	19.09*	7.10**	2.41*	2.69**
YS	0.5	17.28	11.98	3.44	1.85	3.49	28.43	19.47*	6.60	2.37	2.95
	2.0	17.94	12.63	3.46	1.84	3.66	28.36	18.93*	7.05*	2.37	2.68**
SEM		1.08	0.61	0.22	0.28	0.11	1.84	1.52	0.24	0.08	0.12
Source of variation											
Saponin		0.16	0.07	1.00	0.50	0.40	0.04	0.14	0.39	0.03	0.51
Level		0.72	0.51	0.96	0.43	0.89	0.20	<0.01	<0.01	0.91	<.01
Saponin × Level		0.17	0.13	0.99	0.43	0.67	0.06	0.18	0.64	0.03	0.77

Treatments were corn and hay based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). All saponins were added on substrate DM basis.

* Representing the significant difference between Control and saponin treatments ($P < 0.05$).

** Representing the significant difference within 2 concentrations of one saponin type and between Control and saponin treatments ($P < 0.05$).

Table 2.5. Effects of *Yucca schidigera* (YS), *Quillaja saponaria* (QS) and *Camellia sinensis* (TS) on gas production, methane concentration at 8 and 12 hr incubation period, *in vitro*

Saponin	Level % DM	8 hr					12 hr				
		Total (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	A:P ratio	Total (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	A:P ratio
Control#	0	43.22	31.55	8.36	3.31	3.79	68.40	46.03	16.39	5.98	2.81
QS	0.5	41.86*	29.99*	8.53	3.34	3.54*	64.92	41.62*	18.32*	4.99*	2.29*
	2.0	42.60	30.04*	9.00*	3.56*	3.34**	60.88*	40.16*	15.90	4.82*	2.53**
TS	0.5	42.64	30.64	8.56	3.43	3.59*	60.15*	39.97*	15.44*	4.74*	2.59*
	2.0	42.91	30.40*	9.08*	3.43	3.35*	59.52**	39.57**	15.59	4.36**	2.54*
YS	0.5	42.78	30.69	8.68*	3.41	3.55*	65.35*	43.50	16.33	5.53	2.68
	2.0	42.43	29.83*	8.89*	3.72*	3.37**	64.42*	42.11*	16.83	5.48	2.51*
SEM		3.35	2.20	0.92	0.25	0.13	1.85	1.23	0.54	0.28	0.07
Source of variation											
Saponin		0.47	0.45	0.85	0.19	0.85	<0.01	<0.01	0.02	<0.01	<0.01
Level		0.03	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.35	<0.01	<0.01
Saponin × Level		0.55	0.58	0.35	0.09	0.97	0.05	0.05	0.04	0.14	0.04

Treatments were corn and hay based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). All saponins were added on substrate DM basis.

* Representing the significant difference between Control and saponin treatments ($P < 0.05$).

** Representing the significant difference within 2 concentrations of one saponin type and between Control and saponin treatments ($P < 0.05$).

Table 2.6. Effects of *Yucca schidigera* (YS), *Quillaja saponaria* (QS) and *Camellia sinensis* (TS) on gas production, methane concentration at 24 hr incubation period, *in vitro*

Saponin	Level % DM	24 hr				
		Total (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	A:P ratio
Control#	0	87.78	58.68	21.29	7.81	2.75
QS	0.5	88.26	58.65	21.59	8.02	2.71
	2.0	86.97	56.21*	23.31*	7.45	2.41*
TS	0.5	87.22	57.73*	22.10	7.39	2.60*
	2.0	85.55*	54.89**	23.51*	7.15	2.34**
YS	0.5	88.02	58.22	22.13	7.66	2.63*
	2.0	87.10	55.77*	23.69*	7.64	2.35**
SEM		0.46	6.02	0.91	0.74	0.17
Source of variation						
Saponin		0.28	0.19	0.48	0.26	0.17
Level		0.05	<0.01	<0.01	0.14	<0.01
Saponin × Level		0.81	0.74	0.91	0.62	0.73

Treatments were corn and hay based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). All saponins were added on substrate DM basis.

* Representing the significant difference between Control and saponin treatments ($P < 0.05$).

** Representing the significant difference within 2 concentrations of one saponin type and between Control and saponin treatments ($P < 0.05$).

Table 2.7. Growth performance from Holstein steers fed corn-corn silage based diets with different saponin sources

Diets	BW kg						DMI kg	ADG kg
	Exp 1[¶]							
	Period 1		Period 2		Period 3			
	Start	End	Start	End	Start	End		
C1[*]	283	286	304	318	338	363	6.47	0.95
QS1	288	290	308	324	332	352	6.46	0.85
YS1	284	293	302	319	331	346	6.51	0.96
SEM	9	9	10	9	9	10	0.06	0.30
	Source of variation							
Diet	0.91	0.86	0.91	0.88	0.83	0.48	0.80	0.62
	Exp 2							
	Period 1		Period 2					
	Start	End	Start	End				
C2	400	411	409	428			7.71^{b}	1.05
TS2	380	399	414	429			7.16^{a}	1.21
SEM	9	9	10	10			0.08	0.21
	Source of variation							
Diet	0.14	0.36	0.76	0.96			<.01	0.55

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). Numbers following treatment identify the Exp. eg. LC1 = Control diet in Exp 1. Exp 1, 1.5% DM of quillaja and 0.64% DM of yucca saponin; Exp 2, 0.25% DM of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 2.8. Daily manure excretion from Holstein steers fed corn-corn silage based diets with different saponin sources

Diets	Wet, kg		DM, kg		NH_4^+-N, g		TKN[§], g	
	Total	Daily	Total	Daily	Total	Daily	Total	Daily
				Exp 1[¶]				
C1[*]	159.12	11.86	29.88	2.22	465.47	34.53	890.58	65.99
QS1	167.32	12.44	33.23	2.47	477.24	35.47	949.66	70.34
YS1	151.32	11.31	30.18	2.25	411.11	30.73	900.53	67.01
SEM	6.84	0.50	2.07	0.15	28.63	2.09	50.07	3.72
				Source of variation				
Diet	0.28	0.28	0.46	0.46	0.26	0.26	0.67	0.67
				Exp 2				
C2	221.68	15.83	45.19	3.23	627.36	44.81	1339.45	95.68
TS2	223.68	15.97	44.68	3.19	650.81	46.49	1318.00	94.15
SEM	10.87	0.78	2.49	0.18	38.50	2.70	65.72	4.69
				Source of variation				
Diet	0.89	0.89	0.88	0.88	0.67	0.67	0.82	0.82

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% of quillaja and 0.64% of yucca saponin; Exp 2, 0.25% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

[§] TKN = total kjeldahl N.

Table 2.9. Least squares means from CH_4 emissions from Holstein steers fed corn-corn silage based diets with different saponin sources

Diets	Daily concentration mg/kg	Daily emission rate mg/min	Daily emission mass g/d	Emission factors mg/kg BW	Emission factors g/kg DMI
			Exp 1[¶]		
C1[*]	8.41	49.52	77.26	252.28	12.10
QS1	8.61	51.43	80.24	263.97	12.31
YS1	8.53	51.17	79.82	261.44	12.23
SEM	0.55	3.08	4.82	16.82	0.65
			Source of variation		
Diet	0.70	0.22	0.22	0.13	0.77
			Exp 2		
LC2	7.91	57.95	90.40	229.06	11.78
LTS2	7.87	57.78	90.14	221.66	12.31
SEM	0.38	3.80	5.93	5.92	0.28
			Source of variation		
Diet	0.71	0.91	0.91	0.37	0.23

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from *Camellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% of quillaja and 0.64% of yucca saponin; Exp 2, 0.25% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment.

[a, b, c] Significant differences observed at the P < 0.05 probability level.

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CHAPTER 3

EFFECTS OF SAPONIN EXTRACTS ON GASEOUS EMISSIONS FROM STEERS

Effects of saponin extracts on air emissions from steers

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ABSTRACT A series of experiments (Exp) were conducted to quantify the effects of saponin extracts from *Quillaja saponaria* (QS), *Yucca schidigera* (YS) and *Camellia sinensis* (TS) on gaseous emissions from steers. During Exp1, a control diet (C1; corn-corn silage basal diet) was compared to YS1 (C1 + 0.64% dietary DM of YS) and QS1 (C1 + 1.5% dietary DM of QS); 4 replicates per treatment. During Exp 2, the control diet (C2; corn-corn silage basal diet) was compared to TS2 (C2 + 0.25% dietary DM of TS). Product inclusion levels were established to provide the same concentration of saponin compounds across studies for Exp 1 and 2. Experiment 3 compared C3 (corn-corn silage basal diet), QS3 (C3 + 1.5% QS), YS3 (C3 + 1.5% YS) and TS3 (C3 + 0.5% TS). Holstein steers (n = 12) at initial BW of 354 ± 10 kg (Exp 1), 429 ± 10 kg (Exp 2), 382 ± 16 kg (period 1, Exp 3) and 400 ± 12 kg (period 2, Exp 3) were housed, individually, in environmental rooms for 22 d per study. Gaseous emissions including methane (CH_4), ammonia (NH_3), hydrogen sulfide (H_2S), nitrous oxide (N_2O) and non-methane total hydrocarbon (NMTHC) were monitored in room exhaust air. No differences in DMI (7.54 ± 0.09 kg) and ADG (1.16 ± 0.19 kg) were observed in Exp 1 ($P > 0.05$). Adding TS2 to the diet improved DMI in Exp 2 (8.94 kg in TS2 vs. 8.53 in C2; $P < 0.01$), while ADG was not affected by diet. During Exp 3, steers fed the TS3 diet ate less (6.36 kg/d) and gained less (0.31 kg/d) compared to the other 3 treatments. Saponin inclusion did not alter daily CH_4 emission per unit DMI (13.17, 10.90 and 13.21 g/kg DMI, for Exp 1, 2, and 3, respectively). Emissions of NH_3 per unit N intake were not affected by diets in Exp 1 (134.89 mg/g N consumed) and Exp 3 (134.99 mg/g N consumed). Feeding TS2 reduced NH_3 emission per unit of N consumed by 30% compared to C2 ($P < 0.01$).

Feeding up to 0.5% of TS failed to reduce CH_4 emissions without impairing steer growth. Other gaseous emissions were not affected by TS addition. Air emissions were not affected by feeding steers with up to 1.5% YS. Feeding 1.5% QS to steers had an inconsistent effect upon NH_3 emissions and no other effects upon gaseous emissions from steers in this study.

Key words: *Quillaja saponaria*, *Yucca schidigera*, *Camellia sinensis*, air emissions, Holstein steer

INTRODUCTION

Environmental issues related to animal agriculture are becoming increasingly important, especially as they are related to impacts on global climate change. Enteric fermentation from ruminants produces 139.8 Tg CO_2 equivalents annually, representing 28% of the total GHG emissions from the agriculture sector, according to the greenhouse gas (GHG) emission inventory from US EPA (US EPA, 2011). Beef cattle are estimated to generate 71% of total enteric CH_4 fermentation from animals (US EPA, 2011). Mitigation strategies to reduce CH_4 emissions from beef cattle are needed.

Nutritional studies have been conducted to investigate the potential for different feed additives such as fatty acids and oils **(**Beauchemin et al., 2009; Mao et al., 2010), yeast products (Chung et al., 2011) and plant extract compounds (Zhou et al., 2011) to reduce enteric CH_4 production. Among the category of plant extract compounds, saponins are more often studied and their suppression effects on methanogenesis via inhibition of protozoa populations have been confirmed**,** *in vitro* **(**Hess et al., 2003; Pen et al., 2006; Guo et al., 2008). However, *in vivo* findings are not consistent (Santoso et al., 2004; Pen et al., 2006; Holtshausen et al., 2009; Zhou et al., 2011).

The U.S. beef industry produces 25.2 billion kg of red meat, representing $51.5 billion sales during 2010 (USDA National Agricultural Statistics Service, 2011). Beef production generates approximately 500 thousand tons of N in excreta (De Wit et al., 1996), of which up to 70% of the N excreted is volatized into atmosphere as ammonia (NH_3; Muck and Richards, 1983; Moreira and Satter, 2006; Hristov et al., 2009). Ammonia, along with nitrous oxide (N_2O; another important greenhouse gas) and particle matter (PM) cause environmental, health and welfare issues to both human and animals (Lipfert, 1994; Pope and Dockery, 2006). In addition, the deposition of NH_3 will result in soil acidification (Falkengren-Grerup, 1986). Much is known about the impacts of dietary CP concentration on NH_3 emissions, but relatively less information is available about the effects of dietary saponin additions on air emissions from ruminants.

It has been established *in vitro* that by inhibiting the protozoa population, ruminal NH_4^+-N concentration decreases and microbial protein synthesis increases (Guo et al., 2008; Zhou et al., 2011). Saponin has detrimental effects on protozoa population, therefore we hypothesized that both CH_4 and NH_3 emissions reduced as a result of dietary inclusion of saponins. The objectives of the study were to investigate the effects of feeding steers 3 different saponin extracts from *Quillaja saponaria*, *Yucca schidigera* and C*amellia sinensis* on 1) CH_4 and NH_3 emission, *in vivo*; 2) potential changes in hydrogen sulfide (H_2S), N_2O and non-methane total hydrocarbon (NMTHC) emissions that were unintended and, 3) excretion characteristics.

MATERIALS AND METHODS

All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (protocol # 01/10-004-00).

General Animal Housing and Management

During each of 3 experiments (Exp), Holstein steers were housed, individually, in 12 environment-controlled rooms at the Animal Air Quality Research Facility at Michigan State University. Temperature was maintained at 19.96 ± 0.91 °C during Exp 1 and 2 and was 16.69 ± 1.25 °C during Exp 3 to remain within the thermoneutral zone of the animals. In each Exp, 12 steers were used. The same steers were fed during Exp 1 and 2. Experiment 3 was conducted 3 months later therefore a new group of steers were fed. Steers, each housed individually, were confined in a 106.7 cm long × 182.9 cm wide raised stall covered with a rubber matt surface. A fiberglass feeder was placed at the front of the stall and a pan of the same width as the stall was placed at the rear to collect both urine and feces.

Fresh TMR feed was sampled by treatment and offered once daily at 16:00 h at 10% above expected DMI. Prior to feeding, orts were removed, sampled by room and weighed. Manure was mixed thoroughly every morning and removed partially to maintain an equal depth of 5 cm so as to provide an emissions surface while preventing overflow of the pan. A homogenous sub-sample was collected each time manure was removed. Samples for feed, orts and manure were stored at -20 °C until the end of each Exp. Procedures minimized volatilization of manure N compounds that may have occurred during storage and thawing processes. At the end of each study, feed samples were composited by treatment. Orts and manure samples were composited by room.

Saponin Sources

The 3 saponin products used in the Exp were yucca saponin (YS) which is a powder made entirely from the stem of the *Yucca schidigera* plant and rich in steroidal *saponin* (contains 8.5% saponin; Desert King International, San Diego, CA, USA); quillaja saponin (QS), which is a triterpenic saponin enriched extract from pure Chilean soap bark tree *Quillaja saponaria* (contains 3.6% saponin; Desert King International, San Diego, CA, USA); and tea saponin (TS), which is the whole plant saponin extract from *Camellia sinensis* and rich in triterpenic saponin (contains 21.6% saponin; Ningbo Good Green Science & Technology, Ningbo, ZJ, China**).**

Experimental Design and Dietary Treatments

Both Exp 1 and 2 were randomized one-factorial designs with 2 treatments and Exp 3 was a repeated randomized one-factorial study with 3 treatments. All diets offered throughout the 3 studies were corn-corn silage based (Table 3.1). In all Exp, steers were fed 2 wk prior to entering rooms to allow for adaptation to the new diets. Prior to starting the 2nd period of Exp 3, all animals were re-inoculated once a week for 2 consecutive weeks with rumen fluid collected from 2 dry cows fed with hay diet. Steers were fed the corn-corn silage based control diet during the inoculation period and another 2 wk to eliminate any carryover effects from the first period. Then steers were assigned, randomly, to new treatment groups and acclimated to the new treatment diets for 2 wks. Steers were weighed on 2 consecutive mornings before arriving and after leaving the rooms prior to feeding. Starting BW for the 3 Exp were 354 ± 10 kg (Exp 1), 429 ± 10 kg (Exp 2), 382 ± 16 kg (period 1, Exp 3) and 400 ± 12 kg (period 2, Exp 3).

The experimental diets in Exp 1 were a corn and corn-silage based control diet (C1), a diet containing C1 + 1.5% QS of diet DM (QS1), and a third diet containing C1 + 0.64% YS of diet DM (YS1); 4 replicates per treatment. During Exp 2, a corn-silage based control diet (C2) was compared to a diet containing C2 + 0.25% TS of diet DM (TS2); providing 6 replicates per treatment. Inclusion levels of the 3 products were adjusted to similar saponin concentration (0.54 g/kg dietary DM of saponin) during Exp 1 and 2 in order to compare the effects of different saponins at the same dietary saponin concentration. During Exp 3, in addition to the corn-corn silage based control diet (C3), QS (QS3) and YS (YS3) were added to the diet at the maximum inclusion rate (1.5% of dietary DM for QS and YS) and 0.5% TS (TS3) was added because steers rejected feed at the higher inclusion levels (Li et al., unpublished pre-feeding study); there were 3 replicates of each treatment.

Daily N intake was calculated as the difference between N offered in diet and N remaining in orts. Nitrogen loss (N loss) was defined as the sum of N mass from manure total Kjeldahl N (TKN), gaseous ammonia (NH_3-N) and gaseous nitrous oxide (N_2O-N). Nitrogen loss from NO and NO_2 emissions was ignored because of their minor contribution to total N losses.

Measurements of Gaseous Concentrations

Twelve rooms (height = 2.14 m, width = 3.97 m, length = 2.59 m) were designed to continuously monitor incoming and exhaust concentrations of gases (Li et al., 2011). During Exp 1, 2 and 3, the average ventilation rate was 298.3, 295.5 and 289.5 L/s, respectively. Concentrations of NH_3 was measured using a chemiluminescence NH_3 analyzer with a detection limit of 0.001 ppm (Model 17i, Thermo Fisher, Franklin, MA), which is a combination NH_3 converter and NO-NO_2-NO_x analyzer. Hydrogen sulfide (H_2S) was analyzed using pulsed fluorescence SO_2-H_2S analyzer with a detection limit of 0.003 ppm (TEI Model 450i, Franklin, MA; error = 1% of full-scale at 1 ppm). Concentrations of CH_4 (range = 0 to 100 ppm; detection limit = 0.05 ppm) and NMTHC (range = 0 to 10 ppm; detection limit = 0.02 ppm) were determined by a back-flush gas chromatography system (TEI Model 55i, Franklin, MA). Concentration of N_2O (range = 0 to 50000 ppm; detection limit = 0.03 ppm) was measured using an INNOVA 1412 photoacoustic analyzer (Lumasense Technologies, Ballerup, Denmark).

Through software control (LabVIEW Version 8.2; National Instruments Corp., Austin, TX), gaseous concentration monitoring of each room occurred in a sequential manner. All emission factors were calculated from emission mass which is calculated based on the emission rate. Gas emission rates were calculated as the product of ventilation rates and concentration differences between exhaust and incoming air using the following equation:

$$\mathrm{ER} = \mathrm{Q}\frac{273}{\mathrm{T}} \times (\mathrm{C_o} - \mathrm{C_i}) \times 10^{-6} \times \frac{\mathrm{MW}}{\mathrm{V_m}}$$

where ER is emission rate, g/min; Q is ventilation rate at room temperature and pressure, L/min; T is air temperature in room exhaust, in Kelvin; C_o is gas concentration in room exhaust, mg/kg; C_i is gas concentration in the incoming air, mg/kg; MW is molecular weight of the gas, g/mol; V_m is molar volume of gas at standard condition (22.414

L/mole). Emissions in 1 full measurement cycle were estimated by multiplying the ER (g/min) with 195 min. Daily emissions were calculated as sum of the emissions in the 7 or 8 measurement cycles (Li et al., 2011).

Chemical Analyses

Feed and orts samples were analyzed by Dairy One Forage Testing Laboratory (Dairy One, Inc. Ithaca, NY) for compositional analysis. Feed DM content was determined with oven drying at 55 ºC until a constant weight of sample was obtained. Feed composition was analyzed by Near Infrared Reflectance Spectroscopy (NIRS) (AOAC-991.01, 1995). Crude protein, degradable protein, NDF and ADF were analyzed by Foss NIRS systems Model 6500 with Win ISI II v1.5 (AOAC-989.03, 1996). Minerals were analyzed by microwave digestion followed by Inductively Coupled Plasma Mass Spectrometry (ICP). Energy content was determined by an IKA C2000 basic Calorimeter System (IKA Works, NC). Manure NH_4^+-N laboratory (AOAC-928.08, 2000) and total Kjeldahl N (TKN; FOSS Tecator, 1987) content was measured by distillation and digestion followed by distillation, respectively, in a Michigan State University laboratory.

Statistical Analyses

In all Exp, performance (DMI, N intake and ADG), excretion and air emissions data were analyzed using a mixed model testing the fixed effects of diet and random effects of steers. Day was considered as a repeated measure for DMI, period within Exp 3 was treated as a random effect (SAS Institute, 2008). Tukey’s test was applied to compare treatment versus control differences. Significant differences between treatment and control least squares means were declared at $P < 0.05$.

RESULTS

Growth performance

Feeding steers 1.5% QS (QS1) or 0.64% YS (YS1) resulted in no difference in DMI or ADG compared to steers fed C1 (Table 3.2). Across all treatments, in Exp 1, average DMI and ADG was 7.54 ± 0.09 kg and 1.16 ± 0.19 kg, respectively. The N intake of steers was not affected by dietary QS1 inclusion (140.66 g), but decreased as a result of YS1 inclusion (129.42 g; $P < 0.01$) compared to steers fed C1 treatment (134.26 g; Table 3.2).

Similar results were observed in steers fed 1.5% QS treatment (QS3); DMI and ADG was not different between steers fed C3 and QS3 (Table 3.2).Increasing the dietary concentration of YS to 1.5% (YS3) did not change DMI or ADG compared to feeding C3. Feeding steers QS3 resulted in a lower N intake, while N intake was not affected by feeding YS3 (Table 3.2).

Dry matter intake was increased by 5% when steers were fed diets containing 0.25% TS (TS2) compared to C2 ($P < 0.01$; Table 3.2). Accordingly, N intake was increased by 16% in TS2 treatment ($P < 0.01$). Although increased DMI and N intake was observed in steers fed TS2 treatment, the ADG of steers fed TS2 did not differ from those fed C2 (Table 3.2).

Decreases were observed in DMI, N intake and ADG of steers as a result of feeding TS3, while no differences were observed among steers fed QS3, YS3, and C3. Steers fed TS3 had 27% less DMI and 80% less ADG compared to steers fed the C3 diet. The N intake was 80% lower in steer fed the TS3 treatment compared to cattle fed C3 as a result of reduced DMI.

Excretion mass and composition

When examining saponins' effects at the same dietary concentration (0.54 g/kg DM saponins), feeding steers QS1, YS1, or TS2 did not change manure characteristics compared to control diets (Exp 1 and 2; Table 3.3). Average daily fecal DM mass was 2.96 ± 0.21 kg for Exp 1 and 2.97 ± 0.24 kg for Exp 2; NH_4^+-N was 43.98 ± 2.45 g for Exp 1 and 48.66 ± 4.10 g for Exp 2; and TKN was 79.46 ± 4.83 g for Exp 1 and 98.09 ± 9.52 g for Exp 2, respectively.

Feeding steers with increased concentration of YS (1.5%; YS3) or 1.5% QS did not change manure excretion characteristics during Exp 3 (Table 3.3). However, daily manure DM mass excreted was reduced approximately by 27% when TS3 was fed (0.5%; $P = 0.02$) compared to C3. This effect is explained by the reduced DMI observed when the TS3 treatment was fed. Manure NH_4^+-N concentration was increased when steers were fed TS3. However, because TS3 treatment produced less manure DM mass, daily NH_4^+-N mass excreted from steers fed TS3 was not different from steers fed C3 diet (Table 3.3). Average total Kjeldahl N (TKN) remaining in manure was not affected by dietary saponin inclusion.

Nitrogen emissions

<u>Yucca and quillaja saponins.</u> Ammonia emissions from rooms where cattle were fed YS1, QS1, and C1 diets were not different (Table 3.4). Across all treatments, average daily NH_3 emission, concentration, emission rate and daily mass were 1.10 mg/kg, 11.57 mg/min and 18.04 g/d, respectively. Ammonia emission factors calculated based on BW and DMI were not affected by dietary saponin inclusion. Average daily NH_3 emission

factors, across all treatments were 49.12 mg/kg BW and 2.40 g/kg DMI. When daily NH_3 emission mass was adjusted by N intake, an 18% reduction in NH_3 daily emissions was observed for the QS1 treatment compared to C1 (107.70 mg/g N consumed in QS1 vs. 131.29 mg/g N consumed in C1; $P = 0.08$).

Feeding steers the YS3 diet did not influence NH_3 emissions compared to the C3 diet (Table 3.4). Contrary to Exp 1, manure from cattle fed QS3 had 32% higher daily NH_3 emission mass than cattle fed the C3 diet. The NH_3 emission factor calculated on a BW basis was 32% greater from steers fed QS3 treatment than C3 treatment, whereas no differences between these treatments were observed when daily emission mass was adjusted by DMI or N consumption.

Tea saponin. Feeding TS2 reduced NH_3 concentration and emission factors based on BW, DMI and N consumption without affecting manure N composition compared to steers fed C2. Daily NH_3 emission mass from steers fed TS2 was reduced by19% compared to C2 ($P = 0.06$). The NH_3 emissions adjusted by BW ($P = 0.03$), DMI ($P < 0.01$) and N consumption ($P < 0.01$) were 20 to 30% lower from steers where TS2 was fed compared to C2. Ammonia emissions were similar when TS3 and C3 were fed to steers.

Dietary inclusion of saponins showed no treatment effects on steers' N_2O emissions from animal rooms regardless of concentration or saponin type. Average daily N_2O emission mass from Exp 1, 2 and 3 was 3.64, 5.37 and 1.34 mg/d, respectively (Table 3.5).

N balance

Quillaja saponin. Feeding steers QS1 or QS3 did not change manure NH_4^+-N concentrations as a proportion of TKN, TKN excreted per day or N losses from N_2O emissions as a fraction of N excreted compared to control treatments (Table 3.6). When expressed as a proportion of total N excreted, less N was volatized as NH_3 when QS1 was fed compared to C1. On the contrary, N loss as NH_3 emissions in QS3 treatment (12.20%) accounted for a greater proportion of total N excreted compared to C3 treatment (8.89%; Exp 3; $P < 0.01$).

Yucca saponin. Feeding YS1 to steers did not change daily manure TKN mass (Table 3.3) or NH_4^+-N concentration as a proportion of TKN (Table 3.6) but increased the percentage excreted N volatized as NH_3 and the volatilization of NH_3 as a fraction of N intake compared to C1 (Table 3.6). Feeding YS3 reduced NH_4^+-N concentration as a fraction of TKN but did not affect total daily N excretion or N losses as a proportion of N intake compared to C3. However, excreted N remaining in manure as TKN was reduced and N emissions as N_2O-N was increased in the YS3 treatment.

Tea saponin. When TS2 was fed to steers, no differences in N balance were observed between TS2 and C2 treatments except that feeding TS2 resulted in a smaller proportion of N lost as NH_3 relative to N intake. Increasing dietary TS concentration in TS3 treatment reduced manure TKN as a percentage of total N excreted by 2 percentage units, while 30% more N was lost as NH_3 by feeding steers TS3 compared to C3.

CH_4 emissions

Feeding steers up to 1.5% of QS or YS failed to reduce CH_4 emissions compared to control treatment (Table 3.7). When steers were fed TS2, daily CH_4 emissions showed no differences compared to C2 (Table 3.7). However, when a diet containing higher TS concentration (TS3) was fed to steers, CH_4 emission mass was reduced by 31% compared to C3. Because DMI was reduced by 27% in steers fed TS3 treatment, adjusting emissions to DMI basis produced no differences in emissions between TS3 and C3 fed steers. Steers fed TS3 treatment emitted 24% less CH_4 per day per kg BW compared to steers fed C3 diet.

H_2S and NMTHC emissions

Dietary saponin inclusion did not affect H_2S (Table 3.8) or NMTHC (Table 3.9) emissions regardless of inclusion concentration. Average emission mass of H_2S and NMTHC was 91.15 and 1.45 mg/d, respectively.

DISCUSSION

Several studies have reported that feeding ruminants low concentrations of saponin extracts from *Quillaja saponaria* or *Yucca schidigera* have not caused adverse effects on animal performance (Aregheore, 2005 (goats); Nasri et al., 2011 (lamb); Santoso et al., 2004 (sheep); Hristov et al., 1999 (heifers); Holtshausen et al., 2009 (dairy cows): Depenbusch et al., 2007 (steers)). Our findings agree with those studies when we fed lower concentrations of saponins in Exp 1 and 2.

The intent of our study was to investigate the effects of saponins on air emissions by feeding the highest possible concentration to steers. Our results showed that steers can be fed as high as 1.5% of yucca (1.27 g/kg steroidal saponin from *Yucca schidigera*) or quillaja (0.54 g/kg of triterpenic saponin from *Quillaja saponaria*) saponin without changes in growth performance. However, it should be noted that although both Exp 1 and 3 showed that feeding steers with 1.5% QS would not affect DMI, N intake in steers fed QS3 treatment was reduced compared to the control diet while feeding steers with QS1 did not affect N intake. This effect may be the result of the standard error differences between DMI and N observations.

Feeding TS2 increased DMI and decreased N excretion suggesting a possible improvement in N efficiency as proposed by Francis et al., (2002). However, DMI was 27% less when TS3 was fed to steers compared to the C3 diet. Accordingly, an 80% reduction in ADG was also observed in steers fed the TS3 diet. A similar finding was reported by Hu et al. (2006), who found that feeding Boer goats diets containing 3 g/d DM TS improved DMI and ADG, while 6 g/d DM supplementation of TS in the diets reduced DMI. Tea saponin used in our study had a strong bitter taste and is very soluble in water, thus, the reduced DMI observed in TS3 treatment could be due to its palatability (Li et al., unpublished observations).

Ruminal protozoa play an important role in fiber digestion. Reduction of protozoa population as a result of inhibition by saponins leads to impaired fiber digestion in rumen, resulting in reduced ADG (Ushida and Jouany, 1990; Guo et al., 2008). Protozoa may utilize lactic acid and contribute to the buffering capacity in the rumen, preventing an abrupt drop in rumen pH (Williams and Coleman, 1992). Inhibition of the protozoa population by TS may decreases rumen pH, impairing microbial digestion and limiting nutrients available to the animal (Grummer et al., 1983). Because we did not analyze ruminal pH and microbial communities in this study these observations were not verified.

Emission of NH_3 from beef steers are estimated between 0.9 and 19.3 kg/head/yr (Todd et al., 2007). U.S. EPA estimated that the emission factor from dry lot-housed beef steers is 11.4 kg/head/year. Comparably, our results showed that daily NH_3 emissions from steers weighing between 340 kg to 450 kg ranged from 4.68 kg/head/year to 11.16 kg/head/yr.

Studies with dietary supplementation of yucca saponin have achieved a 20 to 50% reduction in NH_3 emissions in poultry and swine (McCrory and Hobbs, 2001) due to its NH_3 binding ability (Wallace et al., 1994). However, less information is available for ruminants. Hristov et al. (1999) demonstrated that supplementation of 0.2% and 0.6% of yucca saponin containing 4.4% of steroid saponins to heifers did not change N concentration in either urine or feces. The present study fed a higher concentration of YS to steers, but neither manure NH_4^+-N concentration nor NH_3 emissions were affected (YS1 and YS3). This indicated that yucca saponin was inefficient in binding NH_3. The lack of effect of yucca saponin on NH_3 emissions could be explained by the comprehensive microbial interactions and microbial adaptation to saponins that may have occurred during our study (Newbold et al., 1997; Holtshausen et al., 2009). Because protozoa play an important role in digesting microbial cell walls, if protozoa population was reduced in the rumen as a result of dietary saponin supplementation, the amount of undigested microbial protein escaping from the rumen would be greater, leading to poorer protein digestibility in the lower GI tract, and thus outweighing the benefit of reducing protozoa populations (Van Soest, 1994).

The effect of feeding 1.5% QS to steers on NH_3 emissions was inconsistent between Exp; NH_3 emissions from steers were not affected as a result of feeding QS1 but feeding QS3 increased NH_3 emission mass from steers. Steers used in Exp 3 responded differently to QS inclusion; fed at the same QS concentration the QS had different effect on N metabolism. While the animal variation is difficult to explain the N balance data showed that, as a proportion of N excreted, less N was volatized as NH_3 but more N was retained in the manure (TKN) in steers fed QS1 compared to C1, while more N was lost as NH_3 and less N was retained as TKN when steers were fed QS3 compared to C3. However, because we did not analyze the microbial community from rumen and lower GI tract or the digestibility of feed, the mechanism behind the observations is unclear.

Less dietary N was lost and NH_3 emissions per unit of N intake were reduced when TS2 was fed to steers compared to C2 treatment, suggesting an improvement in N efficiency when TS2 was fed. Finding of Hu et al. (2006) support the explanation. Boer goats fed 3 g/d tea saponin had increased protein concentration in their blood, indicating more protein was absorbed by animals. When dietary TS concentration increased to 0.5%, manure DM mass was reduced in steers fed TS3 compared to C3, attributable to the reduced DMI. Steers fed TS3 had lower DMI and N intake while daily NH_3 emissions from steers were not different from steers fed C3 (Table 3.5). The N balance showed that compared to steers fed C3, a greater proportion of N was emitted as NH_3 when steers were fed TS3 and more N in the manure was in the form of NH_4^+-N (Table 3.6). In our study, it is very likely that N metabolism was impaired when TS3 was fed, because ruminal microbial digestion declined due to lack of N and energy to support body functions, leading to greater N loss (Van Soest, 1994).

Nitrous oxide emissions were not affected by dietary saponin inclusion, but N_2O emissions when expressed as the proportion of N excreted (Table 3.6) during Exp 3 was increased in TS3 and YS3. Nitrous oxide is the intermediate product of nitrification from nitrate or denitrification from NH_3/NH_4^+. Studies show a positive correlation between N_2O emission and NH_4^+ availability (Fukumoto et al., 2003; Heller et al., 2010). The greater concentration of NH_4^+-N in the manure as a result of feeding steers with TS3 could possibly explain the increased proportion of N_2O emissions compared to steers fed C3 diet. However, it is difficult to explain why steers fed YS3 had greater proportion of N_2O emitted while less TKN was retained in the manure.

We observed that 77% of N ingested was excreted. Twelve percent of total N intake was emitted as NH_3, 51% of consumed N was retained in manure as TKN, 53% of TKN was in the form of NH_4^+-N. Nitrogen lost as N_2O accounted for less than 2% of total N excreted. Even if all N that was not lost was considered as retained by the animal, retained N only accounted for 23% of the total N intake. Ruminants are less efficient than monogastric animals such as swine and poultry regarding to utilize dietary CP. In beef cattle, only about 20% of total N ingested can be retained, with the rest 80% excreted in urine and feces (Farran et al., 2006; Cole and Todd, 2009). The low efficiency of N utilization is confirmed in our study. Our results are in agreement with other reports

(Todd et al., 2006; Todd et al., 2007; Cole and Todd 2009). However, in the current study, the N losses due to NH_3 emissions were smaller (12%) and a greater portion of N was retained in manure as NH_4^+-N (33%) compared to other studies where 40 to 60% of dietary N was emitted as NH_3 (Todd et al., 2006; Todd et al., 2007; Cole and Todd 2009). The difference may be explained by the duration of the current study (22 d) with continuous emissions throughout the duration contributing to the mass balance. Shorter term studies may present results favoring a greater portion of excreted N emitted as NH_3 due to the rapid conversion of urea N to NH_3 whereas longer studies consider the mineralization of organic-bound N to inorganic N.

Saponins inhibit methanogenesis *in vitro* (Takahashi et al., 2000; Hu et al., 2005; Holtshausen et al., 2009). Because approximately 20% of methanogens are associated with protozoa which also play an important role in inter-species hydrogen transfer for methanogenesis (Tokura et al., 1997), reduced CH_4 emissions are thought to be the consequence of saponin's toxicities towards protozoa population (Guo et al., 2008; Holtshausen et al., 2009). However, results from *in vivo* studies have not always been consistent, with reduced CH_4 emissions observed in sheep species, only (Patra and Saxena, 2009; review). Our results indicate that dietary saponin supplementation failed to reduce CH_4 emissions, except when fed at concentrations that inhibited performance. The effects of saponins are suggested to be non-permanent because of microbial adaptation or degradation of saponins in the rumen (Newbold et al., 1997; Teferedegne et al., 1999; Ivan et al., 2004). Because steers were acclimated to diets for 2 wks prior to the start of the air emissions measures ruminal microbes may have already adapted to the presence of dietary saponins.

In studies where 0.27% of TS (3 g/d, average DMI = 750 to 900 g) was fed to sheep and lambs, CH_4 production was reduced 10% and 28%, respectively (Mao et al., 2010; Zhou et al., 2011). Feeding 0.25% TS did not change CH_4 emissions in the present study, possibly due to species variation in rumen microbe populations or feeding different forage:concentrate ratios. Sheep or lambs are often fed diets containing > 60% forage (Yuan et al., 2007; Mao et al., 2010; Zhou et al., 2011), while steer and dairy cow diets contain 37 to 40% forage. Despite that, lack of CH_4 emission response to saponin inclusions have occurred when steers and dairy cows were fed high forage diets (Zinn et al., 1998). Species differences between large ruminants and small ruminants should be considered the primary reason for the different results observed, not dietary forage concentration. Plant maturity, geographical region of production (Ndamba et al., 1993), and efficiency of extraction methods (Vongsangnak et al., 2004) all affect the concentration of saponins in extracts. It is impossible to compare results among studies unless actual dietary saponin concentrations or a measure of activity are provided.

Results from pure-culture studies show that some rumen microbes capable of utilizing saponin can produce small amount of H_2S (Gutierrex et al., 1959). Feeding saponin to steers may produce a small increase in H_2S production. However, throughout the study, H_2S emissions were not affected by dietary saponin inclusion perhaps because bacterial H_2S production was too low for differences to be detected. Emissions of H_2S

are considered to be correlated to the dietary S concentration (Li et al., 2011). In our study, S concentration was not different among treatments within an Exp (data not shown).

Our study suggests that, in large ruminants, high dietary saponin concentrations are necessary in order to achieve a significant inhibitive effect on CH_4 emissions. However, palatability of saponin may affect intake or microbial digestion thereby impairing growth performance.

CONCLUSION

Saponins failed to reduce CH_4 emissions without affecting animal performance in. Although the response to saponin concentration in terms of CH_4 production is dose dependent, higher dietary inclusion level may pose a challenge to animal's performance as observed in steers fed TS3 treatment. Ammonia emissions adjusted for N intake were not affected by either yucca or quillaja saponin saponins however N balance and form in which N losses occurred was impacted.

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Table 3.1. Diet and nutrient composition from experimental diets (DM basis)

	Exp 1			Exp 2		Exp 3			
	C1*	YS1	QS1	C2	TS2	C3	YS3	QS3	TS3
Ingredients (%) DM									
High moisture corn					46				
Corn silage					46				
Soybean meal					3				
Supplement 50#					5				
Total					100				
Saponins									
yucca saponin	--	0.64	--	--	--	--	1.5	--	--
quillaja saponin	--	--	1.5	--	--	--	--	1.5	--
tea saponin	--	--	--	--	0.25	--	--	--	0.5
Supplement 50, % of DM									
Akey TM premix # 4TM¶					1.4				
Limestone					24.9				
Soybean meal, 48% N					48.3				
RumensinTM 80					0.3				
TM salt					9.6				
Vitamin E, 5%					0.2				
Urea, 45% N					9.6				
Potassium chloride					5.1				
Selenium 90					0.7				
Total					100				
Analyzed composition, % DM									
DM	48.9	48.5	47.9	50.3	49.6	45.7	45.2	46.2	45.9
CP	11. 0	11.3	10.8	12.5	12.9	10.0	10.4	10.5	9.9
ADF	14.1	14.5	14.3	16.7	17.0	12.3	11.85	12.1	12.2
NDF	26.1	27.0	27.4	25.6	26.1	22.7	22.00	21.6	22.4
P	0.27	0.27	0.28	0.31	0.29	0.28	0.27	0.28	0.28
ME (Mcal/kg)	1.83	1.80	1.85	1.78	1.78	1.76	1.75	1.78	1.75

* Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from *Camellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1.

Table 3.1. Diet and nutrient composition from experimental diets (DM basis), con't

[#] Middle section of the table lists the ingredients for BFS50 supplement for all treatments

[¶] Akey TM premix # 4 composition: 9% Mg, 4% S, 0.02% Co, 1% Cu, 0.09% I, 2% Fe, 4% Mn, 0.03% Se, 4% Zn, 4,400,000 IU vitamin A, 550,000 IU vitamin D, and 5,500 IU vitamin E/kg (Akey Inc., Lewisburg, OH).

Table 3.2. Growth performance from Holstein steers fed corn-corn silage based diets with and without saponin addition[¶]

Diets	BW, kg Starting		BW, kg Ending		DMI, kg	N intake, g	ADG, kg
			Exp 1				
C1[*]	363		382		7.62	134.26[b]	0.90
QS1	352		379		7.51	140.66[b]	1.27
YS1	346		374		7.49	129.42[a]	1.31
SEM	10		10		0.09	1.58	0.19
			Source of variation				
Diet	0.48		0.84		0.52	< 0.01	0.31
			Exp 2				
C2	428		453		8.53[a]	159.72[a]	1.18
TS2	429		454		8.94[b]	184.56[b]	1.18
SEM	10		10		0.09	1.85	0.21
			Source of variation				
Diet	0.97		0.96		0.01	< 0.01	1.00
			Exp 3				
	Period 1 (kg) Start	Period 1 (kg) End	Period 2 (kg) Start	Period 2 (kg) End			
C3	390	401	398	448	8.71[b]	132.04[c]	1.53[b]
QS3	366	373	401	446	8.67[b]	119.68[b]	1.27[b]
TS3	389	392	401	410	6.36[a]	96.98[a]	0.31[a]
YS3	384	394	400	435	8.58[b]	134.51[c]	1.11[b]
SEM	16	16	12	13	0.42	2.89	0.54
			Source of variation				
Diet	0.72	0.64	1.00	0.24	< 0.01	< 0.01	0.04

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from *Camellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% quillaja and 0.64% of yucca saponin; Exp 2, 0.25% tea saponin; Exp 3, 1.5% yucca and quillaja saponin, 0.5% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 3.3. Daily manure excretion from Holstein steers fed corn-corn silage based diets with and without saponin addition[¶]

Diets	Wet, kg		DM, daily		NH_4^+-N, daily		TKN[§], daily	
	Total	Daily[#]	%	kg	% DM	g	% DM	g
				Exp 1				
C1[*]	297.72	13.53	21.05	2.83	1.65	46.62	2.78	78.59
QS1	304.31	13.83	21.68	2.99	1.47	43.44	2.68	79.67
YS1	306.04	13.91	21.15	3.06	1.40	41.88	2.62	80.12
SEM	26.80	1.21	0.67	0.21	0.10	2.45	0.07	4.83
				Source of variation				
Diet	0.97	0.97	0.53	0.76	0.22	0.41	0.32	0.97
				Exp 2				
C2	354.59	16.12	19.29	3.11	1.58	49.38	3.22	100.95
TS2	329.34	14.97	18.83	2.82	1.73	47.94	3.39	95.22
SEM	23.46	1.16	1.00	0.24	0.11	4.10	0.15	9.52
				Source of variation				
Diet	0.48	0.48	0.59	0.39	0.36	0.80	0.46	0.66
				Exp 3				
C3	330.07	15.00	20.52	3.00[b]	1.33 [b]	39.48	3.31	91.96[b]
QS3	307.94	13.99	21.77	3.00[b]	1.28 [b]	37.58	3.10	94.62[b]
TS3	256.42	11.66	18.77	2.18[a]	1.66[a]	35.84	3.41	74.98[a]
YS3	296.39	13.47	21.70	2.85[b]	1.34 [b]	38.00	3.28	91.22[b]
SEM	40.29	1.83	1.13	0.32	0.02	2.49	0.14	7.60
				Source of variation				
Diet	0.25	0.25	0.23	0.02	0.01	0.78	0.27	0.04

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% quillaja and 0.64% of yucca saponin; Exp 2, 0.25% tea saponin; Exp 3, 1.5% yucca and quillaja saponin, 0.5% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

[§] TKN = total Kjeldahl N.

[#] Duration was 22 d in Exp1 and 2; each period in Exp 3 was 22 d. Therefore, daily excretion was calculated based on a 22 d average of total manure excreted.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 3.4. Least squares means from NH_3 emissions from Holstein steers fed corn-corn silage based diets with and without saponin addition[¶]

Diets	Daily concentration mg/kg	Daily emission rate mg/min	Daily emission mass g/d	Emission factors		
				mg/kg BW	g/kg DMI	mg/g N consumed
			Exp 1			
C1[*]	1.10	11.41	17.80	48.92	2.31	131.29
QS1	0.95	9.61	14.99	40.26	2.02	107.70
YS1	1.25	13.68	21.34	58.17	2.86	165.69
SEM	0.11	1.47	2.30	6.27	0.32	18.32
			Source of variation			
Diet	0.15	0.17	0.15	0.13	0.17	0.08
			Exp 2			
C2	2.11[b]	19.61	30.59	69.31[b]	3.59[b]	192.03[b]
TS2	1.80[a]	15.90	24.80	55.70[a]	2.78[a]	134.63[a]
SEM	0.11	1.37	2.14	4.49	0.20	10.14
			Source of variation			
Diet	0.04	0.06	0.06	0.03	< 0.01	< 0.01
			Exp 3			
C3	0.96[a]	9.36[a]	14.60[a]	35.15[a]	1.67	114.42
QS3	1.19[b]	12.33[b]	19.23[b]	46.58[b]	2.20	158.80
TS3	0.85[a]	8.07[a]	12.58[a]	32.26[a]	2.07	144.21
YS3	0.99[a]	9.98[ab]	15.56[a]	38.47[ab]	1.82	122.54
SEM	0.11	1.26	1.97	3.52	0.20	36.57
			Source of variation			
Diet	0.03	0.02	0.02	0.02	0.14	0.26

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from *Camellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% quillaja and 0.64% of yucca saponin; Exp 2, 0.25% tea saponin; Exp 3, 1.5% yucca and quillaja saponin, 0.5% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 3.5. Least squares means from N_2O emissions from Holstein steers fed corn-corn silage based diets with and without saponin addition¶

Diets	Daily concentration mg/kg	Daily emission rate mg/min	Daily emission mass g/d	Emission factors		
				mg/kg BW	g/kg DMI	mg/g N consumed
			Exp 1			
C1*	0.51	2.28	3.56	9.87	460.96	26.19
QS1	0.49	2.39	3.73	10.04	487.54	26.04
YS1	0.50	2.33	3.64	10.05	494.90	28.64
SEM	0.04	0.19	0.29	0.84	47.71	2.70
			Source of variation			
Diet	0.95	0.92	0.92	0.99	0.87	0.74
			Exp 2			
C2	0.50	3.41	5.33	11.93	603.74	32.25
TS2	0.50	3.47	5.41	12.16	574.36	27.83
SEM	0.03	0.64	1.00	2.23	106.87	5.50
			Source of variation			
Diet	0.94	0.95	0.95	0.94	0.85	0.57
			Exp 3			
C3	0.68	0.84	1.31	3.15	145.91	9.77
QS3	0.68	0.88	1.37	3.32	160.29	11.79
TS3	0.68	0.78	1.22	3.11	205.53	12.68
YS3	0.68	0.93	1.45	3.57	175.08	17.71
SEM	0.17	0.19	0.30	0.61	21.08	1.40
			Source of variation			
Diet	0.99	0.59	0.59	0.68	0.29	0.45

* Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from *Camellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% quillaja and 0.64% of yucca saponin; Exp 2, 0.25% tea saponin; Exp 3, 1.5% yucca and quillaja saponin, 0.5% of tea saponin.

¶ Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 3.6. Nitrogen balance from Holstein steers fed corn-corn silage based diets with and without saponin addition[¶]

Diet	N intake[§]	N excreted, g/d			NH_4^+-N	N losses, % of N intake			N losses, % of N excreted[†]		
	g/d	TKN	NH_3-N	N_2O-N	% of TKN	TKN	NH_3-N	N_2O-N	TKN	NH_3-N	N_2O-N
					Exp 1						
C1[*]	134.26^{b}	78.59	14.81^{b}	2.95	59.46	58.79^{a}	10.92^{b}	3.04	84.40^{ab}	11.70^{b}	2.16
QS1	140.66^{b}	79.67	12.34^{a}	3.06	54.53	56.89^{a}	8.86^{a}	3.24	86.63^{b}	9.85^{a}	2.14
YS1	129.42^{a}	80.12	17.33^{b}	2.99	53.01	61.90^{b}	13.46^{c}	3.01	82.20^{a}	14.01^{c}	2.36
SEM	1.58	4.83	0.54	0.22	2.30	0.91	0.42	0.24	0.74	0.49	0.18
					Source of variation						
Diet	< 0.01	0.97	0.11	0.91	0.32	0.01	< 0.01	0.76	< 0.01	< 0.01	0.64
					Exp 2						
C2	159.72^{a}	100.95	25.14	4.37	49.69	64.95	15.79^{b}	2.65	77.20	12.43	3.10
TS2	184.56^{b}	95.22	20.89	4.34	51.18	48.99	11.45^{a}	2.21	77.93	10.14	3.49
SEM	1.85	9.52	1.99	0.81	3.37	7.22	1.00	0.44	2.89	1.21	0.58
					Source of variation						
Diet	< 0.01	0.66	0.13	0.98	0.77	0.12	< 0.01	0.48	0.86	0.18	0.64
					Exp 3						
C3	132.04^{c}	93.16^{b}	12.00^{b}	0.96	52.74^{b}	75.46^{a}	9.41^{a}	0.72	88.28^{c}	8.89^{a}	0.87^{a}
QS3	119.68^{b}	90.82^{b}	15.81^{c}	1.05	53.51^{b}	78.84^{ab}	13.05^{b}	0.90	84.69^{a}	12.20^{b}	0.97^{ab}
TS3	96.98^{a}	75.13^{a}	10.54^{a}	0.93	54.83^{c}	86.98^{b}	12.26^{b}	0.97	86.62^{b}	14.97^{c}	1.15^{b}
YS3	134.51^{c}	99.07^{b}	12.79^{b}	1.13	50.47^{a}	72.85^{a}	10.07^{a}	0.89	86.93^{b}	9.56^{a}	1.14^{bc}
SEM	2.89	1.50	0.46	0.13	2.37	4.55	0.62	0.11	0.59	0.63	0.14
					Source of variation						
Diets	0.04	< 0.01	< 0.01	0.20	< 0.01	< 0.01	< 0.01	0.11	< 0.01	< 0.01	0.02

Table 3.6. Nitrogen balance from Holstein steers fed corn-corn silage based diets with and without saponin addition[¶], con't

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% quillaja and 0.64% of yucca saponin; Exp 2, 0.25% tea saponin; Exp 3, 1.5% yucca and quillaja saponin, 0.5% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

[§] N intake = dietary N offered – N in orts. N excreted = TKN + NH_3-N + N_2O-N; TKN, total Kjeldahl N remained in manure; NH_3-N, N emitted as gaseous NH_3; N_2O-N, N emitted as gaseous N_2O. The difference between N intake (g) and N excreted (g) is N retained by the growing steer.

[†] Percentages reflected the estimated least squares means of every form of excreted N accounted; sum of the TKN, NH_3-N and N_2O-N did not equal to 100% due to the contributions of within treatment errors.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 3.7. Least squares means from CH_4 emissions from Holstein steers fed corn-corn silage based diets with and without saponin addition[¶]

Diets	Daily concentration mg/kg	Daily emission rate mg/min	Daily emission mass g/d	Emission factors	
				mg/kg BW	g/kg DMI
			Exp 1		
C1[*]	8.88	71.96	112.26	310.54	14.71
QS1	8.08	63.37	98.86	264.32	13.17
YS1	7.52	55.85	87.13	239.49	11.63
SEM	0.55	6.93	10.81	31.30	1.36
		Source of variation			
Diet	0.22	0.26	0.26	0.27	0.28
			Exp 2		
C2	9.12	60.32	94.10	211.78	11.15
TS2	9.07	61.26	95.57	215.96	10.66
SEM	0.41	4.80	7.49	14.71	0.89
		Source of variation			
Diet	0.94	0.89	0.89	0.84	0.70
			Exp 3		
C3	9.77[b]	67.76	103.50[b]	256.97[b]	13.03
QS3	10.28[b]	73.55	112.31[b]	281.21[b]	13.77
TS3	8.05[a]	49.31	73.23[a]	196.18[a]	12.96
YS3	9.67[b]	69.45	105.67[b]	267.23[b]	13.08
SEM	0.58	9.83	9.68	26.28	0.83
		Source of variation			
Diets	<0.01	0.07	0.03	0.05	0.90

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from *Camellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% quillaja and 0.64% of yucca saponin; Exp 2, 0.25% tea saponin; Exp 3, 1.5% yucca and quillaja saponin, 0.5% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 3.8. Least squares means from H_2S emissions from Holstein steers fed corn-corn silage based diets with and without saponin addition[¶]

Diets	Daily concentration mg/kg	Daily emission rate mg/min	Daily emission mass mg/d	Emission factors	
				mg/kg BW	mg/kg DMI
			Exp 1		
C1[*]	0.005	0.077	119.53	0.33	15.03
QS1	0.004	0.040	61.75	0.17	8.37
YS1	0.004	0.059	91.64	0.25	12.04
SEM	0.001	0.023	35.78	0.10	4.22
			Source of variation		
Diet	0.42	0.52	0.52	0.50	0.53
			Exp 2		
C2	0.008	0.080	125.98	0.29	14.11
TS2	0.008	0.083	129.64	0.29	14.29
SEM	0.001	0.010	19.56	0.04	1.82
			Source of variation		
Diet	0.93	0.90	0.90	0.94	0.95
			Exp 3		
C3	0.005	0.051	78.83	0.19	8.64
QS3	0.005	0.049	75.69	0.19	8.60
TS3	0.005	0.055	86.19	0.22	13.32
YS3	0.004	0.033	51.09	0.13	5.79
SEM	0.0004	0.014	21.73	0.06	2.60
			Source of variation		
Diets	0.77	0.57	0.57	0.56	0.11

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% quillaja and 0.64% of yucca saponin; Exp 2, 0.25% tea saponin; Exp 3, 1.5% yucca and quillaja saponin, 0.5% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

Table 3.9. Least squares means from non-methane total hydrocarbon (NMTHC) emissions from Holstein steers fed corn-corn silage based diets with and without saponin addition[¶]

Diets	Daily concentration mg/kg	Daily emission rate mg/min	Daily emission mass g/d	Emission factors	
				mg/kg BW	g/kg DMI
			Exp 1		
C1[*]	0.07	0.95	1.48	4.09	191.07
QS1	0.07	0.88	1.38	3.70	181.04
YS1	0.07	1.00	1.56	4.25	206.50
SEM	0.003	0.05	0.07	0.23	9.84
		Source of variation			
Diet	0.21	0.23	0.23	0.23	0.18
			Exp 2		
C2	0.05	0.82	1.28	2.92	149.75
TS2	0.05	0.83	1.29	2.92	144.27
SEM	0	0.07	0.11	0.29	9.89
		Source of variation			
Diet	0.99	0.95	0.95	0.99	0.69
			Exp 3		
C3	0.06	1.01	1.58	3.80	178.11
QS3	0.06	1.01	1.57	3.82	181.53
TS3	0.06	0.87	1.35	3.43	220.76
YS3	0.06	0.98	1.52	3.76	179.71
SEM	0.01	0.26	0.41	0.85	10.23
		Source of variation			
Diets	0.15	0.35	0.35	0.58	0.08

[*] Treatments were corn and corn silage based Control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from C*amellia sinensis* (TS). Numbers following treatment identify the Exp. eg. C1 = Control diet in Exp 1. Exp 1, 1.5% quillaja and 0.64% of yucca saponin; Exp 2, 0.25% tea saponin; Exp 3, 1.5% yucca and quillaja saponin, 0.5% of tea saponin.

[¶] Exp 1, n=12, 4 replicates per treatment; Exp 2, n=12, 6 replicates per treatment; Exp 3, n=12, 3 replicates per treatment per period.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

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CHAPTER 4

EFFECT OF SAPONIN EXTRACTS, IN THE DIET OF HOLSTEIN STEERS OR ADDED DIRECTLY TO THEIR MANURE, ON GASEOUS EMISSIONS FROM THE MANURE

Effects of saponin extracts, in the diet of Holstein steers or added directly to their manure, on gaseous emissions from that manure

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Abstract A series of experiments (Exp) were conducted to investigate the effects of saponin extracts, in the diet of Holstein steers or added directly to their manure, on gaseous emissions from that manure. Saponin extracts added to the feed or manure were from *Quillaja saponaria* (quillaja saponin), *Yucca schidigera* (yucca saponin) and *Camellia sinensis* (tea saponin). During Exp 1, manure from Holstein steers fed corn and corn silage based control diet (C1) was compared to manure from steers fed control diets plus 1.5% quillaja (QS) or 0.64% yucca (YS) saponins. In Exp 2, the impact of direct application of 2% yucca (CYS, wet basis) or quillaja saponin (CQS, wet basis) to manure collected from steers fed corn and corn silage based diet (C2) on manure air emissions was investigated. In Exp 3 the effects of dietary tea saponin supplementation (TS, 0.25% DM) and direct addition (CTS, 2% wet basis) to manure collected from steers fed corn and corn-silage based diet (C3) on manure air emissions were compared in the same experiment. Gaseous emissions including methane (CH_4), ammonia (NH_3), hydrogen sulfide (H_2S), nitrous oxide (N_2O) and non-methane total hydrocarbons (NMTHC) were reported. When saponin extracts were fed, daily manure CH_4 emission mass was 40.97, 58.12, and 71.49 mg/d, for YS, C1, and QS, respectively ($P < 0.01$). Feeding YS resulted in less ($P < 0.01$) daily manure NH_3 emission mass than C1 and QS (318.18 vs. 391.62 and 365.54 mg/d, respectively). Daily manure H_2S emission mass differed ($P < 0.01$) among dietary treatments (10.63, 15.16 and 21.10 mg/d for YS, C1, and QS respectively). In Exp 2 the addition of saponin extracts directly to manure did not affect any emissions monitored. Average daily emission mass of CH_4, NH_3 and H_2S from manure was 11.92,

424.25 and 19.36 mg/d, respectively. Overall, the results of these experiments indicate that manure-derived gaseous emissions are altered by dietary inclusion of saponins rather than direct addition to manure.

Key words: *Quillaja saponaria*, *Yucca schidigera*, *Camellia sinensis*, air emissions, manure

INTRODUCTION

The U.S. beef industry produces 25.2 billion kg of meat annually (USDA National Agricultural Statistics Service, 2011), while generating approximately 500 thousand tons of N in excreta (De Wit et al., 1996). Of this, 0-70% is emitted into the atmosphere as ammonia (NH_3; Muck and Richards, 1983; Moreira and Satter, 2006; Hristov et al., 2009). Volatilization of NH_3 from livestock manure accounts for 65% of annual NH_3 emissions and is considered as the largest anthropogenic source (NRC, 2002).

Ammonia (NH_3) has received considerable attention because of its unpleasant smell, health and welfare issues for both human and animals and its contribution to fine particle matter formation (PM; Lipfert, 1994; Pope and Dockery, 2006). In addition, deposition of NH_3 contributes to soil acidification (Falkengren-Grerup, 1986).

Microbial fermentation from manure contributes to atmospheric greenhouse gas (GHG) emissions. According to U.S. GHG emissions inventory (U.S. EPA, 2011), the overall GHG emissions from manure has increased by 46% since 1990. In 2009, the manure-derived methane (CH_4) and nitrous oxide (N_2O) was 49.5 Tg CO_2 and 17.9 Tg CO_2, respectively.

Because of the concerns of air quality problems stemming from manure gaseous emissions, extensive research has been conducted to investigate possible strategies to alleviate the environmental impacts from manure, particularly for NH_3 and CH_4 emissions. Unfortunately, most often, only one or two gases are targeted and reported while the responses of other gases such as N_2O, hydrogen sulfide (H_2S) and non-methane total hydrocarbon (NMTHC) remain unknown. In addition, dietary strategies design to mitigate enteric CH_4 emissions overlook the manure–derived gaseous emissions making it impossible to determine if reductions in enteric CH_4 are offset by CH_4 emissions post-excretion, from stored manure (Kreuzer and Hindrichsen, 2006).

Saponins are glycosides of plants that can be classified into the categories of triterpenoids and steroids. Triterpenoid saponin extracts from *Quillaja saponaria* and *Camellia sinensis* have reduced CH_4 emissions and NH_3 concentration by ruminants via the detrimental effects on rumen protozoa (Hess et al., 2003; Pen et al., 2006; Guo et al., 2008). Steroid saponin from *Yucca schidigera* has been shown to reduce ruminal NH_3 concentration by directly binding ruminal NH_3 (Wallace et al., 1994), and inhibit ruminal methanogenesis, *in vitro* by indirect toxicity towards protozoa results (Wallace et al., 1994). Despite the abundant information regarding saponin effects on ruminal methanogenesis and NH_3 production, little research has focused on the effects of manure-derived CH_4 and NH_3 emissions from dietary inclusion of saponins.

The objectives for the current study were to investigate 1) the effects of dietary supplementation of saponin extracts from *Quillaja saponaria* (quillaja saponin), *Yucca schidigera* (yucca saponin) and *Camellia sinensis* (tea saponin) on manure-derived CH_4 and NH_3 emissions and 2) the effects of adding saponin extracts to manure on manure-derived CH_4 and NH_3 emissions.

MATERIAL AND METHODS

Experimental design

A total of 3 experiments (Exp 1, Exp 2 and Exp 3) were designed to investigate the effects of saponin extracts on gaseous emissions from manure. All Exp employed 12 57 L barrels for manure storage and examined the effects of treatments and day. Exp 1 and 2 were conducted as repeated studies, while Exp 3 was a single study. Fresh manure for all experiments was collected from Holstein steers who were housed individually in environmental rooms. Every morning, manure collection pans placed behind each steer were emptied and manure was composited by dietary treatments and mixed well to provide homogenous compositions. Mixed manure for each treatment was loaded for 5 consecutive days into each barrel (5.5 kg/barrel); this was followed by a 17-d emissions monitoring period (22 d total). Air flow rate was maintained at 7.22 L/min throughout all studies. Average temperature of the barrels was 15.28 °C (11.82 – 21.17 °C), 20.06 °C (17.65 – 23.20 °C) and 23.26 °C (20.82 – 25.62 °C) in Exp 1, 2 and 3, respectively.

During Exp 1, manure was collected from 12 Holstein steers which a corn and corn silage based diet was fed as the control treatment (C1), a QS treatment where 1.5% DM of quillaja saponin was added to the base diet and a third diet contained 0.64% DM of yucca saponin (YS) in the basal diet (Table 4.1). Manure treatments were C1, QS and YS which represented manure from steers fed C1, QS and YS diet, respectively; providing 4 replicates of each dietary treatment.

Manure used in Exp 2 was collected from 4 Holstein steers where a corn and corn silage base diet was fed (Table 4.1). A total of 12 barrels were randomly assigned to one of the three treatments: 1) C2, fresh manure treatment; 2) CQS, treatment of fresh manure mixed with 2% (on wet basis) of quillaja saponin and 3) CYS, treatment of fresh manure mixed with 2% (on wet basis) of yucca saponin; 4 replicates per treatment. Manure was pre-mixed with yucca/quillaja saponin individually every day for each barrel prior to being loaded to CYS/CQS treatments.

Manure used in Exp 2 was collected from 4 Holstein steers which were fed a corn and corn silage based diet (Table 4.1). A total of 12 barrels were randomly assigned to one of the three treatments: 1) C2, fresh manure treatment; 2) CQS, treatment of fresh manure mixed with 2% (on wet basis) of quillaja saponin and 3) CYS, treatment of fresh manure mixed with 2% (on wet basis) of yucca saponin; 4 replicates per treatment. Manure was pre-mixed with yucca/quillaja saponin individually every day for each barrel prior to being loaded in CYS/CQS treatments.

In Exp 3, the effects of tea saponin on manure air emissions were compared to control treatment (C3) through dietary inclusion (0.25% DM; TS) and direct application (2% wet basis; CTS) of tea saponin to fresh manure (4 replicates per treatment; Table 4.1). Manure for both the C3 and the CTS treatments was collected from 6 Holstein steers fed a corn and corn silage based diet. Manure for the TS treatment was collected from 6 Holstein steers fed a corn and corn silage based diet with 0.25% (DM) tea saponin supplementation. To construct the CTS treatment, manure was pre-mixed with tea saponin daily prior to filling barrels; similar methods to those used in Exp 2.

Saponin sources

The 3 saponin products used in the Exp were yucca saponin (YS) which is a powder made entirely from the stem of the *Yucca schidigera* plant and rich in steroid *saponin* (contains 8.5% saponin; Desert King International, San Diego, CA, USA); quillaja saponin (QS), which is a triterpenoid saponin enriched extract from pure Chilean soap bark tree *Quillaja saponaria* (contains 3.6% saponin; Desert King International, San Diego, CA, USA); and tea saponin (TS), which is the whole plant saponin extract from *Camellia sinensis* and rich in triterpenoid saponin (contains 21.6% saponin; Ningbo Good Green Science & Technology, Ningbo, ZJ, China**).**

Air sampling

Twelve plastic 57 L barrels (Interior dimensions: diameter = 30.48 cm, height = 60.96 cm) with black lids which were modified to continuously monitor incoming and exhaust concentrations of gases were used (Fogiel and Powers, 2009). Ammonia (NH_3) was measured using a chemiluminescence NH_3 analyzer with a detection limit of 0.001ppm (Model 17i, Thermo Fisher, Franklin, MA). Hydrogen sulfide (H_2S) was analyzed using pulsed fluorescence SO_2-H_2S Analyzer with a detection limit of 0.003ppm (TEI Model 450i, Franklin, MA; error = 1% of full-scale at 1 ppm). Concentration of CH_4 (detection limit = 0.1 ppm), NMTHC (range = 0 to 10 ppm; detection limit = 0.02 ppm) and N_2O (0.03 ppm detection limit at 50,000 ppm range) was measured using an INNOVA 1412 photoacoustic analyzer (Lumasense Technologies, Ballerup, Denmark).

Through software control (LabVIEW Version 8.2; National Instruments Corp., Austin, TX), gaseous concentration monitoring of each barrel occurred in a sequential manner. Emission mass was calculated based on emission rate. Gas emission rates were calculated as the product of ventilation rates and concentration differences between exhaust and incoming air using the following equation:

$$\mathrm{ER} = \mathrm{Q}\frac{273}{\mathrm{T}} \times (\mathrm{C_o} - \mathrm{C_i}) \times 10^{-6} \times \frac{\mathrm{MW}}{\mathrm{V_m}}$$

where ER is emission rate, g/min; Q is ventilation rate at room temperature and pressure, L/min; T is air temperature in room exhaust, in Kelvin; C_o is gas concentration in room exhaust, mg/kg; C_i is gas concentration in the incoming air, mg/kg; MW is molecular weight of the gas, g/mol; V_m is molar volume of gas at standard condition (22.414 L/mole). Emissions in one full measurement cycle were estimated by multiplying the ER (g/min) with 195 min. Daily emissions were calculated as the sum of the emissions in the 7 or 8 measurement cycles. Daily emission mass, emission rate and concentration were reported in all studies (Li et al., 2011).

Manure composition analyses

Manure was sampled every day by treatment during the first five days of loading; a representative sample was taken for each barrel at the end of every experiment and stored at -20 °C until analyzed. Samples were prepared in triplicates; manure NH_4^+-N

(AOAC, 2000) and total kjeldahl N (TKN) contents were determined by distillation and (FOSS Tecator, 1987) in a Michigan State University laboratory.

Statistical Analyses

In all experiments, emissions data were analyzed using a MIXED model of SAS 9.2 (SAS Institute, 2008). The model tested fixed effects of treatment, day, and the treatment × day interaction, by using period as a random variable. Exceptions were made in analyzing Exp 1 when N_2O concentrations were small and close to the detection limit of analyzer, data points collected were insufficient for examining the day effect, therefore only the treatment effect was examined; and during Exp 2, when instrumental malfunction resulted in no data for N_2O emissions. Tukey's test was applied in comparing treatments differences. Significant differences among the means were declared at $P < 0.05$.

RESULTS

Methane emissions

Feeding steers diets containing YS reduced manure-derived daily CH_4 emission rate, while manure from steers fed QS resulted in a greater emission rate compared to the C1 (Exp 1; Table 4.2). Accordingly, the daily concentration in C1 barrels (13.77 mg/kg) was lower than QS (15.36 mg/kg) but higher than YS barrels (11.76 mg/kg; $P < 0.01$). Average daily emission mass in C1, QS and YS was 58.12, 71.49 and 40.97 mg/d, respectively ($P < 0.01$).

Overall, manure CH_4 emissions were not affected by direct applications of yucca (CYS) and quillaja saponins (CQS; Exp 2). Across all treatments, average CH_4

concentration, emission rate and daily emission mass were 11.92 mg/kg, 0.026 mg/min and 39.44 mg/d, respectively (*P* > 0.05; Exp 2; Table 4.2).

Neither dietary supplementation of TS or direct application to manure (CTS) affected manure-derived CH_4 emissions (Exp 3; Table 4.2). Across all treatments, average daily emission mass, emission rate and concentration were 518.12 mg/d, 0.26 mg/min and 73.38 mg/kg, respectively.

Ammonia emissions

Dietary inclusion of YS reduced manure-derived NH_3 (318.18 mg/d) emissions by 18% compared to C1 (391.62 mg/d) where a corn and corn silage based diet was fed (*P* < 0.01; Table 4.3), whereas no differences were observed between C1 and QS where 1.5% of quillaja saponin was fed to steers (365.54 mg/d). Average daily emission concentration in C1, QS and YS was 51.52, 48.39 and 44.23 mg/kg, respectively (*P* < 0.01). Emission rate was 0.25, 0.23 and 0.21 mg/min in Control, QS and YS, respectively (*P* < 0.01).

Mixing manure with 2% of saponins (wet basis) in either CYS or CQS treatment did not influenced NH_3 emissions compared to the C2 treatment (*P* > 0.05; Table 4.3). Across all treatments, average concentration, emission rate and daily emission mass were 63.27 mg/kg, 0.28 mg/min and 424.25 mg/d, respectively (*P* > 0.05).

Daily NH_3 emission mass showed no differences between TS and C3 (*P* > 0.05; Exp 3; Table 4.3). Daily NH_3 emissions from direct application of 2% tea saponin to manure (CTS) did not differ from C3 treatment ($P > 0.05$). Average daily emission concentration and emission rate were 67.57 mg/kg and 0.31 mg/min, respectively.

Hydrogen sulfide emissions

Manure-derived daily H_2S emission mass from YS and QS treatment produced 50% and 28% less H_2S compared to C1, respectively. Daily emission rate was 0.013, 0.010 and 0.008 mg/min in C1, QS and YS, respectively ($P < 0.01$; Table 4.4).

During Exp 2, saponin amendments did not change H_2S emissions from manure. Average daily emission mass was 17.75, 20.66 and 19.67 mg/d in C2, CQS and CYS, respectively ($P > 0.05$; Table 4.4). Average emission concentration and rate was 1.22 mg/kg and 0.013 mg/min, respectively across all treatments.

Daily emission mass of H_2S was 27% lower when manure was collected from steers fed a TS compared to C3 treatment ($P < 0.01$; Exp 3; Table 4.4). Adding tea saponin (CTS) to manure increased H_2S emission daily mass by 34% compared to C3 treatment ($P < 0.01$). The same trend was observed for emission concentration, where daily concentration in C3, TS and CTS treatments was 1.62, 1.18 and 2.02 mg/kg ($P < 0.01$). No treatment difference was observed for emission rate.

Nitrous oxide and non-methane total hydrocarbon emissions

Feeding steers QS or YS did not change N_2O emissions (Exp 1; Table 4.5). Across all treatments, average daily emission mass was 0.39 mg/d. Dietary inclusion of 0.25% tea saponin reduced daily N_2O emission mass by 17% as compared to the control treatment ($P = 0.04$; Exp 3; Table 4.5), whereas mixing manure with 2% tea saponin

(CTS; 3.08 mg/d) resulted in no difference in N_2O daily emission mass from manure when compared to the C3 treatment (2.94 mg/d). The same trends were observed in emission concentration and rate.

Feeding steers with QS resulted in 21% greater manure-derived NMTHC emission mass compared to C1, while manure from steers fed YS produced 20% less NMTHC than C1 treatment ($P = 0.02$; Exp 1; Table 4.6). However, direct application of CYS and CQS to manure had no significant effects on daily NMTHC emissions (Exp 2; Table 4.6). Average daily emission mass throughout Exp 2 was 16.97 mg/d. Emissions of NMTHC were not affected by tea saponin treatments (TS or CTS). Average daily emission mass, concentration and rate were 13.78 mg/d, 0.78 mg/kg and 0.009 mg/min, respectively (Exp 3; Table 4.6).

Manure N content

Dietary supplementation of YS or QS did not change manure DM or N content compared to C1 (Exp 1; Table 4.7). Average DM, NH_4^+-N and TKN were 5.77 kg, 91.39 g and 161.80 g, respectively ($P > 0.05$). Dietary inclusion of TS increased manure NH_4^+-N by 8% as compared to C3 ($P < 0.01$; Table 4.7). When mixing the manure with yucca (CYS), quillaja (CQS) or tea (CTS) saponins, no differences were observed in manure characteristics compared to control treatments (Exp 2 and 3; Table 4.7).

DISCUSSION

Manure-derived CH_4 emissions reflect the chemical and microbial processes of fiber degradation from manure (Külling et al., 2002). The availability of unfermented fiber in manure has a strong effect on manure CH_4 emissions, whereas easily-fermented carbohydrates such as starch have limited contributions to the total manure-derived CH_4 emissions (Kreuzer et al., 1986). Substitution of a forage-based diet with a concentrate-based diet in beef cattle increased the manure-derived CH_4 emissions by two fold (Hashimoto et al., 1981). Therefore, dietary additives that affect ruminal fiber digestion can further influence the CH_4 production from manure (Hashimoto et al., 1981). Saponins have been demonstrated to reduce ruminal methanogenesis, *in vitro*, mainly via direct inhibition on protozoa population (Hess et al., 2003; Pen et al., 2006; Guo et al., 2008). In addition, growth of ruminal cellulolytic fungi and bacteria can be suppressed by inclusion of saponins under pure culture conditions (Makkar et al., 1995; Wang et al., 2000). These mechanisms can explain the decreased CH_4 emissions found in YS treatment (Exp 1). However the day by treatment interaction indicated that the effect was dependent on the day and length of the storage.

Given that diets contained the same saponin concentration (0.54 g/kg DM), it is unclear why reduced manure CH_4 emissions were observed from YS treatment, whereas emissions from QS treatment produced more CH_4 than C1 (Exp 1) and manure CH_4 emissions from TS treatment did not differ from C3 (Exp 3). The differences in chemical structures and efficacy in terms of inhibiting ruminal protozoa and cellulolytic fungi and bacteria may have implications on manure-derived CH_4 emissions but future research is needed to explain the mechanisms.

Production of CH_4 employs anaerobic processes. Mixing saponin with manure rather than surface application provides better contact between saponin and anaerobic microbes inside the manure. In addition, if saponin can inhibit methanogenesis through reducing the availability of hydrogen provided by protozoa and other bacteria and fungi in manure, direct application of a greater concentration of saponin (2% wet basis) should be more effective than residues from diet (0.25 to 1.5% DM). As a result, a more pronounced effect of inhibited CH_4 production by direct saponin application was expected in CTS treatment which contained greatest amount of saponin. However, none of the saponin treatment (CQS, CYS and CTS) showed differences in CH_4 production compared control treatments (C2 and C3). The unexpected lack of effects by direct mixing of saponin with manure suggests that the microbial degradation of manure fiber may not be affected by the saponins tested in this study. On the other hand, our results support the findings that saponin is more effective on protozoa rather than methanogens (Guo et al., 2008; Zhou et al., 2011). Because, protozoa could not survive when pH is below 5.0 (Coleman and Sandford, 1979), although we did not monitor rumen protozoa population in the manure, after passing through the abomasum (pH = 2.1 to 2.2) and lower gastro-intestinal tract, the number of live protozoa in the manure should be minimal.

Average CH_4 emissions were similar between Exp 1 (56.86) mg/d and 2 (39.44 mg/d), whereas there was an approximately 10-fold greater emission Exp 3. It is well established that temperature has a significant effect on CH_4 emissions. Several studies reported that manure produced 2 to 20 times more CH_4 when the manure pile temperature increased from 6 to 35 °C (Hashimoto et al., 1981; Hashimoto, 1982; Lokshina and Vavilin, 1999; Chae et al., 2008). Therefore the increases in ambient temperature between Exp 1 and 2 and Exp 3 may have increased manure temperature and contributed to increased CH_4 production in Exp 3.

A day effect was observed for NH_3 emissions in all Exp. The significant treatment by day interactions suggested that the duration can significantly affect NH_3 emissions. Saponin extract from *Yucca schidigera* is considered to reduce NH_3 by binding NH_4^+ and inhibiting rumen protozoa (Kemme et al., 1993; Wallace et al., 1994). However, reports from dietary inclusion of yucca saponin on NH_3 emissions are not consistent. Studies showed that dietary supplementation of yucca saponin achieved a 20 to 50% reduction in NH_3 emissions in poultry and swine (McCrory and Hobbs, 2001, review). Panetta et al. (2006) reported that NH_3 emissions were not affected by dietary yucca saponin inclusion in swine. This study was conducted in environmental chambers to investigate the effects of saponin extract on animal and manure-derived NH_3 emissions. In other studies, saponin added to manure produced no effects on NH_3 emissions (Lee et al., 2007). This is consistent with our findings.

Yucca saponin was found to reduce manure-derived NH_3 emissions through dietary inclusion (Exp 1), whereas yucca saponin applied to manure had no effect. Initial manure contents of NH_4^+-N and TKN in yucca saponin treatments (YS and CYS) were not different from the control treatments, suggesting the reduced NH_3 emissions observed in the YS treatment may be because of its effect on protozoa and other microbes in the rumen rather than a direct binding effect on NH_4^+. However, the effects of dietary additives on manure NH_3 emissions could be short-term (Külling et al.; 2002), during long-term storage (14 wks), adaptation would occur.

Manure NH_4^+-N concentration in TS treatment was greater than that in C3 but the average NH_3 emissions with the TS treatment in Exp 3 was not different from C3, indicating that tea saponin may be able to bind NH_4^+ so that lead to a slower releasing rate of NH_3. Because we only examined the short-term manure storage, this effect may be reduced or eliminated in the long-term. Neither diet nor manure supplementation with quillaja saponin (QS and CQS) changed manure-derived NH_3 emissions. Overall, our results indicated that saponins, of different chemical structures and application methods, had diverse effects on manure-derived NH_3 emissions.

Ruminal hydrogen is produced during the process of VFA production. In addition to methanogens, sulfur reducing bacteria (SRB) can also incorporate hydrogen for the purpose of reducing SO_4^- with H_2S the most abundant product (Biebl and Pfennig, 1977). The production of H_2S can therefore be considered as a competitive pathway for methanogenesis. Saponin extract from *Yucca schidigera* reduced H_2S emissions by binding H_2S or decreasing the abundance or activity of SRB, *in vitro* (Gibson et al., 1993; Giffard et al., 2001). Our results agreed with those findings. Dietary supplementations of all 3 saponins were found to reduce average daily H_2S emissions from manure. However, yucca and quillaja saponin applied directly to manure had no effects on H_2S emissions. The lack of effect observed from manure application suggests that the effects may only occur in rumen. When tea saponin was applied directly to manure (CTS), a 34% increase in H_2S emissions was observed compared to C3. This again suggests that tea saponin is not as effective in manure as in the rumen and the increased H_2S could possibly be due to changed manure VFA composition. Because we did not analyze the VFA or C content in manure, this hypothesis needs further confirmation.

N_2O is the intermediate product of nitrification from nitrate or denitrification from NH_3/NH_4^+. Some studies showed a positive correlation exist between N_2O emission and NH_4^+ availability (Fukumoto et al., 2003; Heller et al., 2010). Therefore lower initial NH_4^+-N concentration in TS treatment (Table 4.6) could possibly explain associated reduced N_2O emissions.

CONCLUSION

Dietary inclusion of YS decreased manure-derived CH_4, NH_3 and H_2S emissions but had no effect on manure N composition. Dietary supplementation of QS increased manure CH_4 emissions but decreased H_2S emission from manure. Manure N composition and NH_3 emissions were not affected by dietary inclusion of quillaja saponin. Manure collected from steers fed 0.25% of TS produced less H_2S and excreted less NH_4^+-N in manure, while CH_4 and NH_3 emissions were not affected. Our results indicated that saponins, of different chemical structures and application methods, had diverse effects on manure-derived NH_3 emissions. In addition, the lack of saponin's effects on air emissions by direct application indicates saponins are more effective as potential feed additives.

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Table 4.1. Ingredient and nutrient composition of diets fed to steers (DM basis)

	Exp 1 and 2			Exp 3	
	Control[*]	YS	QS	Control	TS
Ingredients (%) DM					
High moisture corn	46	46	46	46	46
Corn silage	46	46	46	46	46
Soybean meal	3	3	3	3	3
Supplement 50[#]	5	5	5	5	5
Saponins					
yucca saponin	--	0.64 0.64	--	--	--
quillaja saponin	--	--	1.5	--	--
tea saponin	--	--	--	--	0.25
Supplement 50, % of DM					
Akey TM premix # 4[TM¶]				1.4	
Limestone				24.9	
Soybean meal, 48% N				48.3	
Rumensin[TM] 80				0.3	
TM salt				9.6	
Vitamin E, 5%				0.2	
Urea, 45% N				9.6	
Potassium chloride				5.1	
Selenium 90				0.7	
Total		100			
Analyzed composition, % DM					
DM	43.9	43.1	43.3	50.5	50.1
CP	11.6	11.8	12.1	12.2	11.9
ADF	16.5	17.0	16.4	10.1	11.5
NDF	27.4	27.8	28.1	27.8	26.4
P	0.27	0.28	0.27	0.29	0.29
ME (Mcal/kg)	1.82	1.79	1.83	1.80	1.82

[*] Treatments were corn and corn silage based control diet with inclusion of yucca saponin which is the saponin extract from *Yucca schidigera* (YS), quillaja saponin which is the saponin extract from *Quillaja saponaria* (QS) and tea saponin, extract from *Camellia sinensis* (TS). Inclusion levels of saponins were adjusted by the actual saponin

Table 4.1. Ingredient and nutrient composition of diets fed to steers (DM basis), con't

content in the product, adjusted actual dietary saponin concentration was 0.54 g/kg in all saponin treatments.

[#] Middle section of the table lists the ingredients for BFS50 supplement for all treatments

[¶] Akey TM premix # 4 composition: 9% Mg, 4% S, 0.02% Co, 1% Cu, 0.09% I, 2% Fe, 4% Mn, 0.03% Se, 4% Zn, 4,400,000 IU vitamin A, 550,000 IU vitamin D, and 5,500 IU vitamin E/kg (Akey Inc., Lewisburg, OH).

Table 4.2. Least squares means of saponin's effect on manure CH_4 emissions

	Average daily concentration mg/kg	Average daily emission rate mg/min	Cumulative average daily emission mass mg/d
	Exp 1 [I]		
C1	13.77^b	0.037^b	58.12^b
QS	15.36^c	0.046^c	71.49^c
YS	11.76^a	0.027^a	40.97^a
SEM	0.30	0.002	4.13
	Source of variation		
Diet	<0.01	<0.01	<0.01
Day	<0.01	<0.01	<0.01
Diet × Day	<0.01	<0.01	<0.01
	Exp 2 [II]		
C2	12.34	0.028	39.36
CQS	11.73	0.025	39.99
CYS	11.70	0.025	38.98
SEM	0.40	0.002	4.46
	Source of variation		
Diet	0.43	0.48	0.99
Day	<0.01	<0.01	<0.01
Diet × Day	0.17	0.54	0.62
	Exp 3 [III]		
C3	73.33	0.25	504.23
TS	72.12	0.27	495.91
CTS	74.69	0.26	554.23
SEM	4.64	0.02	43.87
	Source of variation		
Diet	0.92	0.65	0.59
Day	<0.01	<0.01	<0.01
Diet × Day	1.00	0.96	1.00

[I] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C1) with dietary inclusion of the saponin extract from *Yucca schidigera* (YS; 0.64% DM) and the saponin extract from *Quillaja saponaria* (QS; 1.5% DM).

[II] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C2) and C2 with 2% inclusion (wet basis) of the saponin extract from *Yucca schidigera* (CYS) and C2 treatment with 2% inclusion (wet basis) of the saponin extract from *Quillaja saponaria* (CQS).

[III] Treatments were manure collected from Holstein steers fed corn and corn silage based C3 diet; manure from C3 treatment with inclusion of 2% (wet basis) the saponin extract from *Camellia*

Table 4.2. Least squares means of saponin's effect on manure CH_4 emissions, con't

sinensis (CTS) and manure from Holstein steers fed 0.25% (DM basis) of saponin extract from *Camellia sinensis* (TS).

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 4. 3. Least squares means of saponin's effect on manure NH_3 emissions

	Average daily concentration mg/kg	Average daily emission rate mg/min	Cumulative average daily emission mass mg/d
	Exp 1 [I]		
C1	51.52[b]	0.25[b]	391.62[b]
QS	48.39[b]	0.23[b]	365.54[b]
YS	44.23[a]	0.21[a]	318.18[a]
SEM	16.52	0.08	133.32
	Source of variation		
Diet	<0.01	<0.01	<0.01
Day	<0.01	<0.01	<0.01
Diet × Day	<0.01	<0.01	<0.01
	Exp 2 [II]		
C2	63.26	0.28	389.05
CQS	63.09	0.28	440.33
CYS	63.46	0.28	443.37
SEM	1.40	0.01	28.17
	Source of variation		
Diet	0.98	0.98	0.31
Day	<0.01	<0.01	<0.01
Diet × Day	<0.01	<0.01	<0.01
	Exp 3 [III]		
C3	67.42	0.31	460.88
TS	70.98	0.33	481.11
CTS	64.30	0.29	455.02
SEM	16.06	0.10	158.35
	Source of variation		
Diet	0.12	0.11	0.86
Day	<0.01	<0.01	<0.01
Diet × Day	<0.01	<0.01	<0.01

[I] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C1) with dietary inclusion of the saponin extract from *Yucca schidigera* (YS; 0.64% DM) and the saponin extract from *Quillaja saponaria* (QS; 1.5% DM).

[II] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C2) and C2 with 2% inclusion (wet basis) of the saponin extract from *Yucca schidigera* (CYS) and C2 treatment with 2% inclusion (wet basis) of the saponin extract from *Quillaja saponaria* (CQS).

[III] Treatments were manure collected from Holstein steers fed corn and corn silage based C3 diet; manure from C3 treatment with inclusion of 2% (wet basis) the saponin extract from *Camellia*

Table 4. 3. Least squares means of saponin's effect on manure NH_3 emissions, con't
sinensis (CTS) and manure from Holstein steers fed 0.25% (DM basis) of saponin extract from *Camellia sinensis* (TS).
[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 4. 4. Least squares means of saponin's effect on manure H_2S emissions

	Average daily concentration mg/kg	Average daily emission rate mg/min	Cumulative average daily emission mass mg/d
	Exp 1 [I]		
C1	1.28^{c}	0.013^{b}	21.10^{c}
QS	0.91^{b}	0.010^{a}	15.16^{b}
YS	0.69^{a}	0.008^{a}	10.63^{a}
SEM	0.83	0.001	13.65
	Source of variation		
Diet	<0.01	<0.01	<0.01
Day	<0.01	<0.01	<0.01
Diet × Day	0.20	0.19	0.19
	Exp 2 [II]		
C2	1.21	0.013	17.75
CQS	1.25	0.013	20.66
CYS	1.20	0.013	19.67
SEM	0.05	0.001	1.46
	Source of variation		
Diet	0.74	0.77	0.36
Day	<0.01	<0.01	<0.01
Diet × Day	0.22	0.22	0.17
	Exp 3 [III]		
C3	1.62^{b}	0.018	25.57^{b}
TS	1.18^{a}	0.013	18.45^{a}
CTS	2.02^{c}	0.022	34.29^{c}
SEM	0.09	0.001	1.77
	Source of variation		
Diet	<0.01	0.11	<0.01
Day	<0.01	<0.01	<0.01
Diet × Day	0.03	0.03	<0.01

[I] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C1) with dietary inclusion of the saponin extract from *Yucca schidigera* (YS; 0.64% DM) and the saponin extract from *Quillaja saponaria* (QS; 1.5% DM).

[II] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C2) and C2 with 2% inclusion (wet basis) of the saponin extract from *Yucca schidigera* (CYS) and C2 treatment with 2% inclusion (wet basis) of the saponin extract from *Quillaja saponaria* (CQS).

[III] Treatments were manure collected from Holstein steers fed corn and corn silage based C3 diet; manure from C3 treatment with inclusion of 2% (wet basis) the saponin extract from *Camellia*

Table 4. 4. Least squares means of saponin's effect on manure H_2S emissions, con't
sinensis (CTS) and manure from Holstein steers fed 0.25% (DM basis) of saponin extract from *Camellia sinensis* (TS).
[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 4.5. Least squares means of saponin's effect on manure N_2O emissions

	Average daily concentration mg/kg	Average daily emission rate mg/min	Cumulative average daily emission mass mg/d
	Exp 1 [I]		
C1	0.21	0.0001	0.14
QS	0.20	0.0005	0.81
YS	0.21	0.0002	0.24
SEM	0.01	0.0002	0.31
	Source of variation		
Diet	0.99	0.49	0.31
	Exp 3 [II]		
C3	0.48^{b}	0.0021^{b}	2.94^{b}
TS	0.45^{a}	0.0016^{a}	2.44^{a}
CTS	0.47^{b}	0.0020^{b}	3.08^{b}
SEM	0.06	0.0003	0.21
	Source of variation		
Diet	<0.01	<0.01	0.04
Day	<0.01	<0.01	<0.01
Diet × Day	<0.01	<0.01	<0.01

[I] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C1) with dietary inclusion of the saponin extract from *Yucca schidigera* (YS; 0.64% DM) and the saponin extract from *Quillaja saponaria* (QS; 1.5% DM).

[II] Treatments were manure collected from Holstein steers fed corn and corn silage based C3 diet; manure from C3 treatment with inclusion of 2% (wet basis) the saponin extract from *Camellia sinensis* (CTS) and manure from Holstein steers fed 0.25% (DM basis) of saponin extract from *Camellia sinensis* (TS).

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 4.6. Least squares means of saponin's effect on manure NMTHC emissions

	Average daily concentration mg/kg	Average daily emission rate mg/min	Cumulative average daily emission mass mg/d
	Exp 1 [I]		
C1	1.00[b]	0.008	13.79[b]
QS	1.07[b]	0.011	16.81[c]
YS	0.85[a]	0.007	11.03[a]
SEM	0.15	0.0003	3.39
	Source of variation		
Diet	0.04	<0.01	0.02
	Exp 2 [II]		
C2	1.06	0.011	15.53
CQS	1.07	0.011	17.33
CYS	1.10	0.012	18.05
SEM	0.03	0.0004	1.47
	Source of variation		
Diet	0.67	0.67	0.43
Day	<0.01	<0.01	<0.01
Diet × Day	0.04	0.04	0.44
	Exp 3 [III]		
C3	0.69	0.008	11.68
TS	0.83	0.010	13.64
CTS	0.83	0.010	15.24
SEM	0.19	0.003	4.79
	Source of variation		
Diet	0.15	0.15	0.32
Day	<0.01	<0.01	<0.01
Diet × Day	0.53	0.25	0.50

[I] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C1) with dietary inclusion of the saponin extract from *Yucca schidigera* (YS; 0.64% DM) and the saponin extract from *Quillaja saponaria* (QS; 1.5% DM).

[II] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C2) and C2 with 2% inclusion (wet basis) of the saponin extract from *Yucca schidigera* (CYS) and C2 treatment with 2% inclusion (wet basis) of the saponin extract from *Quillaja saponaria* (CQS).

[III] Treatments were manure collected from Holstein steers fed corn and corn silage based C3 diet; manure from C3 treatment with inclusion of 2% (wet basis) the saponin extract from *Camellia sinensis* (CTS) and manure from Holstein steers fed 0.25% (DM basis) of saponin extract from *Camellia sinensis* (TS).

Table 4.6. Least squares means of saponin's effect on manure NMTHC emissions, con't

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

Table 4. 7. Initial N content of manure collected from steers fed with or without saponin supplementation (27.5 kg wet manure)

	DM kg	NH_4^+-N g	TKN[V] g	NH_4^+-N / TKN[VI]
		Exp 1[I]		
Control	5.67	97.09	163.07	0.60
QS	5.78	89.25	159.53	0.56
YS	5.86	87.82	162.79	0.54
SEM	0.11	3.21	8.54	0.06
		Source of variation		
Diet	0.39	0.25	0.30	0.09
		Exp 2[II]		
Control	5.78	89.76	165.69	0.54
CQS	5.78	82.89	169.33	0.49
CYS	5.78	81.99	174.59	0.47
SEM	0.16	2.80	9.64	0.03
		Source of variation		
Diet	0.83	0.87	0.27	0.25
		Exp 3[III]		
Control	5.61	81.71[a]	167.04	0.49
TS	5.49	88.07[b]	174.92	0.50
CTS	5.61	83.54[ab]	163.64	0.51
SEM	0.07	3.79	4.32	0.03
		Source of variation		
Diet	0.32	0.01	0.41	0.38

[I] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C1) with dietary inclusion of the saponin extract from *Yucca schidigera* (YS; 0.64% DM) and the saponin extract from *Quillaja saponaria* (QS; 1.5% DM).

[II] Treatments were manure collected from Holstein steers fed corn and corn silage based diet (C2) and C2 with 2% inclusion (wet basis) of the saponin extract from *Yucca schidigera* (CYS) and C2 treatment with 2% inclusion (wet basis) of the saponin extract from *Quillaja saponaria* (CQS).

[III] Treatments were manure collected from Holstein steers fed corn and corn silage based C3 diet; manure from C3 treatment with inclusion of 2% (wet basis) the saponin extract from *Camellia sinensis* (CTS) and manure from Holstein steers fed 0.25% (DM basis) of saponin extract from *Camellia sinensis* (TS).

[V]: TKN=total kejldahl nitrogen.

[VI]: NH_4-N to TKN ratio.

[a, b, c] Significant differences observed at the $P < 0.05$ probability level.

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CHAPTER 5

SUMMARY

EFFICACY OF SAPONINS IN REDUCING CH_4 EMISSIONS

Ruminants are the major contributor of CH_4 emissions from agriculture sector. Methane emissions from ruminants also represent great energy loss. Saponins have been demonstrated to reduce CH_4 emissions from ruminates, *in vitro*. These findings have also been confirmed during our *in vitro* study.

One of the major aims for this thesis was to investigate saponin's effect on CH_4 reduction, *in vivo*. However, the inhibitive effect of saponins on CH_4 production and the dose-dependent response were not observed during the animal study. One exception is when tea saponin was added at 0.5% of dietary DM to the steers, daily emission mass was reduced significantly when compared to control treatment (Chapter 3). Unfortunately, this effect was achieved at the expense of declined DMI and ADG. When the daily mass was adjusted on DMI basis, CH_4 emissions from steers fed tea saponin treatment were not different from control treatment. In order to receive a significant response of CH_4 reduction, a greater amount of saponin must be fed. We have demonstrated in our *in vitro* study that at 2.0% inclusion level, gas production in both yucca and tea saponin treatments was decreased, suggesting undesirable consequences such as declined fiber digestion could occur at higher dietary saponin concentration. In addition, the saponin at higher concentration can cause bad palatability of the feed which can lead to decreased DMI as we have observed from Chapter 3.

When relating my research work to other studies that observed significant effects of dietary saponins on CH_4 emissions it is necessary to note that most studies reporting a CH_4 decrease, involved small ruminants (sheep and lamb), indicating that a species difference may exist. In addition, dietary compositions such as feed ingredients and forage to concentration

ration have impacts on rumen microbial populations, whereas this thesis only examined one diet type. In the future, it is necessary to examine saponin's effects with other feed types.

Although, CH_4 emissions were not affected by dietary yucca or quillaja saponin inclusion, during the manure storage study, dietary inclusion of 0.64% yucca saponin decreased manure-derived CH_4 emissions while quillaja saponin increased manure-derived CH_4 emissions. These results suggested that when examine saponins effects on CH_4 emissions from ruminants, both animal-derived and manure-derived emissions should be considered.

On the other hand, when saponins were mixed directly with manure, no effect on CH_4 emissions was observed among all saponin treatments. These results supported those findings that the major effect of saponins in aspects of CH_4 reduction was to inhibit protozoa population rather than direct affect methanogen population.

On an average, CH_4 emissions were 18% greater from whole animal emissions compared to enteric and rectum CH_4 emissions, indicating that manure when partially removed on daily basis, contributes less than 20% to total CH_4 emissions.

EFFICACY OF SAPONINS IN REDUCING NH_3 AND OTHER GASEOUR EMISSIONS

The manure-derived NH_3 emissions are one of the major concerns from animal agricultural. Some research has been conducted to investigate saponin's effect on manure-derived NH_3 emissions from both ruminants and non-ruminants. However, results are not consistent. In this thesis, manure-derived NH_3 emissions were monitored from emissions from a manure pan with partially removal on daily basis for 4 wk (manure source retained; Chapter 3) and from post-excretion of manure storage from tubs for 3 wk (no animal present; Chapter 4).

When manure was partially removed and re-mixed on a daily basis, quillaja saponin increased NH_3 emissions during one of the two animal studies, whereas NH_3 emissions from yucca saponin or tea saponin treatments were not different from control. Comparably, during manure storage studies (Chapter 4), the dietary yucca saponin treatment decreased NH_3 emissions while quillaja and tea saponin treatments did not change NH_3 emissions. These results indicated that saponins were more effective in affecting microbes in the rumen rather than binding NH_4^+ in the manure. The manure storage study only examined saponin's effects on a short term basis; adaptation could possibly occur during long-term storage, eliminating saponins effects.

By monitoring H_2S, N_2O and NMTHC emissions during the manure storage study (Chapter 4), we found that through dietary inclusion, 1) quillaja saponin reduced H_2S emissions, increased NMTHC emissions but did not affect N_2O emissions; 2) both NMTHC and H_2S emissions were reduced in yucca while N_2O was not affected and 3) tea saponin treatments reduced NMTHC, H_2S and N_2O emissions. In contrary, none of the gaseous emissions were affected by direct mixing saponin with manure, except an increase of H_2S was observed in tea saponin treatment. Our results further supported the hypothesis that the principle biological effects of saponins occurred in the rumen rather than the manure.

IMPLICATIONS AND RECOMMENDATIONS

Beef steers are one of the most significant contributors of CH_4 in agriculture sector. In addition, CH_4 emissions from enteric fermentation and manure represent the loss of feed energy and nutrients. Therefore, reducing CH_4 emissions from beef and manure will benefit both the animal and the environment. Saponins of different origins, structures and concentrations were investigated for their effects on animal and manure-derived CH_4 and other gaseous emissions in this thesis. Results presented through Chapter 2 to 4 imply that dietary saponin supplementation may not be a good strategy for animal-derived CH_4 reduction.

However, when extending the scope to manure-derived CH_4 emissions, reductions observed in yucca saponin treatment (Chapter 4) suggested that dietary supplementation of steroid saponin (yucca saponin) could be more effective in the aspect of reducing manure-derived CH_4 emissions compared to triterpenoid saponins (tea and quillaja) or direct application.

Besides, our results for the first time demonstrated that other gaseous emissions from manure, such as NMTHC, H_2S and N_2O emissions, could be affected by direct application of saponins, providing a new aspect of saponin application in terms of regulating manure-derived gaseous emissions.

This thesis only investigated the effects of saponins on gaseous emissions from Holstein steers (280 to 500 of BW) and only one type of diet was used throughout this thesis. For future research, steers at different growth stage and fed with different dietary compositions will need to be considered. Because the genetic differences exist among different animal species, such as dairy cow, steers and lamb, et al., different response to dietary saponin inclusions can occur. In the future, studies will need to compare saponin's effect among different animal species.