Methane production, fermentation characteristics, and microbial profiles in the rumen of tropical cattle fed tea seed saponin supplementation

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Abstract

Belmont Red Composite rumen-cannulated steers ($n = 8$, 364 ± 8.4 kg liveweight, LW LW; least squares means $\pm$ s.e.m.) were used to assess effects of feeding tea seed (*Camellia sinensis* L.) saponin (TSS) supplementation on performance, methanogenesis, fermentation pattern and rumen microbial communities. The expectation was to use TSS to potentially modulate the rumen microbial population and decrease enteric methane (CH₄) production. The steers were fed twice a day with a basal diet (BD) that contained a mixture of 0.15 Rhodes grass (*Chloris gayana*) hay plus 0.85 of a commercial concentrate before CH₄ emissions were measured in open-circuit respiratory chambers for 48 h. Steers were then adapted progressively to doses of 20 and 30 g/day of TSS for 10 and 6 days, respectively before new CH₄ measurements were recorded. Final placement in chambers was conducted after 13 days of TSS removal (BDP). Rumen fluid samples from each steer were collected for the treatments BD, BD + 20 g TSS, BD + 30 g TSS and

BDP. Growth performance and CH₄ emissions were not affected by the addition of TSS, but compared to the BD and TSS diets, daily CH₄ emissions (g) and yield (g CH₄/kg DMI) were lower (P<0.05) by 18 and 22%, respectively, after TSS treatment. Concentrations of total volatile fatty acids, acetate and propionate were not affected by TSS treatment, as were total rumen bacteria and methanogens numbers. Relative to the BD and BDP, butyrate concentration was higher (P<0.05) in TSS treated animals, resulting in a reduced ratio of acetate to butyric acid (P<0.05). In comparison with BD control, the relative abundance of *Fibrobacter succinogenes* increased by 2 fold (P<0.001) in the other three dietary groups. However, compared to the BD and BD + 20 g of TSS, the abundance of *R. albus* increased by 100 fold (P<0.01) in the BD + 30 g of TSS and BDP diets, while the abundance of *R. flavefaciens* was 100 fold lower in TSS supplemented and BDP groups than in BD control group (P<0.001). Thermoplasmatales-related RCC archaea and protozoa counts increased linearly with 20 and 30 g of TSS addition, but returned to BD control levels after the TSS supplement was withdrawn. It was concluded that TSS supplementation changed rumen microbial community in cattle, but was not inhibitory to methane production, which was inconsistent with published *in vitro* results and small ruminant trials where TSS caused a dose-dependent reduction in CH₄ emissions.

Keywords: *Camellia sinensis* L., ruminant

1. Introduction

Methane (CH₄) is the third most abundant greenhouse gas (GHG) in the atmosphere, behind water vapour and carbon dioxide (CO₂), and CH₄ has been responsible for about 20% of the global radiative forcing since 1750 (IPCC, 2001). Over the past 250 years, CH₄ emissions have increased in 149% and possess higher global warming potential (i.e. 23 times) and longer atmospheric lifetime (8.4 years) than CO₂ (Thorpe, 2009). It has been suggested that ruminant enteric CH₄ accounts for a quarter of anthropogenic emissions (Lassey, 2008).

Apart from environmental issues, CH₄ also represents a significant feed energy loss to the host animal from 2 to 12 % of gross energy intake (Johnson and Johnson, 1995). Thus, numerous efforts are underway to manipulate rumen fermentation and

the rumen microbial ecosystem to reduce CH₄ emissions and improve feed efficiency in ruminants. Among these options, saponins or saponin like substances have showed potential to modulate rumen fermentation patterns and mitigate CH₄ production in *in vitro* ruminal fermentation (Patra et al., 2006; Pen et al., 2006; Goel et al., 2008), but not in some *in vivo* experiments (Pen et al., 2007; Holtshausen et al., 2009).

Recent studies provide evidence that tea seed (*Camellia sinensis* L.) saponin (TSS) may have potential to be used as a antimethanogenic agent due to the antiprotozoal effect *in vitro* (Hu et al., 2005) and in small ruminants (Mao et al., 2010; Zhou et al., 2011). However, there is little published information on the CH₄-suppressing effects of TSS supplementation in cattle, and the inclusion level of plant-derived saponins for different ruminants with variable diets should be tested to achieve the optimum response. The objective of this study was to evaluate the responses of methane production, rumen fermentation and rumen microbial community structure to the increasing doses of TSS in a basal diet. Quantitative real-time PCR was used to monitor the relative changes in the cellulolytic bacteria and methanogens (*Methanobrevibacter* spp. and Rumen Cluster C), and the abundance of total protozoa.

2. Materials and methods

2.1 Experimental design

The experiment was conducted between 11th February 2013 (late summer) and 25th May 2013 (late autumn; 103 days) at Lansdown Research Station, Woodstock near Townsville, QLD (lat. 19° 39' 30"S, long. 146° 50' 17"E) Animal care was provided according with the approved CSIRO Animal Ethic Committee protocol No A12/2012.

Eight Belmont Red Composite (Africander (African Sanga) x Brahman (*Bos indicus*) x Hereford-Shorthorn (3/4 *B. taurus*) steers [364 ± 8.4 kg LW; least squares means ± standard error of means (s.e.m.)] fitted with a permanent rumen cannula were used to assess the desired range of TSS (Zhejiang Oriental Tea Technology Co. Ltd., Changshan, Zhejiang, China) supplementation in a conventional-finishing feedlot basal diet [BD; mixture of 0.15 Rhodes grass (*Chloris gayana*) hay and 0.85

of a commercial mixed high-grain diet (Coleman Stock Feeds Pty Ltd., Charters Towers, QLD, Australia)]. The effects of the natural supplementation were assessed in terms of dry matter intake (DMI), LW, CH₄ emissions measured in open-circuit respiration chambers, rumen fermentation and rumen microbial ecology.

All steers were accustomed to the respiration chambers during previous weeks of the CH₄ measurements and allowed to exercise daily in the cattle yard in the early morning to reduce stress and facilitate cleaning of pens. Fresh water was provided *ad libitum*. Internal and external parasites were controlled from day 0 using Dectomax Pour-on (Doramecting, 10 mg/50 kg; Pfizer, São Paulo, Brazil). At the end of the experiment, the steers grazed on pasture close to the cattle yard over 7 days to observe any adverse clinical symptoms.

2.2 Feeding and supplementation

Management of feeding and supplementation in this study followed the methodologies outlined by Ramírez-Restrepo et al. (2014, 2016) for the evaluation of natural bioactive compounds on cattle. Rumen-cannulated steers were accustomed to the BD in the cattle yard for 10 days, whilst a 56 day period was used in the animal house for diet adaptation and preliminary measurements on the control BD (i.e. the last 8 days). Supplementation up to 20 g/d of TSS was administered for 10 days and steers remained on that level of supplementation for 3 days. During the following 6 days steers were supplemented up to 30 g TSS/day lasting for 4 days in the upper dosage. This was followed by 13 days suspension of the supplement [BD post-TSS (BDP)] to achieve a metabolic stability Ramírez-Restrepo et al. (2016) concomitant with a modelled clearance from the body (Maes et al., 2004) before final experimental measurements.

The light-yellow powdered TSS was dissolved in ~250 ml of cold water prior to the AM feeding and the solution was mixed with the dry feed in the morning diet. The approach was designed to ensure a linear increase of feed supplementation and assess CH₄ emissions at specific levels of TSS supplementation because the methanogenic response to the supplement in cattle was unknown. On a daily basis veterinarian monitoring of water intake, digestive physiology (i.e. eating, elimination, primary tympany, diarrhea), animal behaviour and welfare (i.e. professional judgment

of legal and health safety endpoints of clinical changes and impacts from additive consumption) was performed as described by Ramírez-Restrepo et al. (2014, 2016).

The upper limit of total TSS intake in this experiment was calculated based on a tolerability study (Ramírez-Restrepo et al., 2016). Briefly, Brahman (*B. indicus*) steers were fed the same hay: grain ratio diet used in the current study and supplemented with the recommended anti-methanogenic dose of *C. sinensis* seed meal reported in goats (3 g TSS/0.623 kg DMI, Hu et al., 2006) and sheep (3 to 5 g TSS/kg DMI, Yuan et al., 2007; Zhou et al., 2011). The research also emphasised clinical observations, DMI, LW and CH₄ measurements as well as blood biochemistry associated with blood CH₄ concentration profiles.

Overall, CH₄ measurements in respiration chambers were conducted over 27 days. Since there were 8 cattle on successive diets and only 4 chambers available, measurements in two-day consecutive measurement periods per animal on all diets were staggered to allow different treatments and simultaneous daily CH₄ emission assessment.

During the adjustment and TSS supplementation periods in the animal house, the BD was fed *ad libitum* on a DM basis (i.e. 2.1% of total LW; Fisher et al., 1987) in equal parts at 0900 and 1630 h. On a fortnightly basis, the mixed high-grain diet was prepared and delivered in batches to the farm to assure constant quality and freshness of the feed as previously described by (Ramírez-Restrepo et al., 2015). Feed offered to and refused by each steer were weight and recorded daily over feeding adaptation, TSS supplementation, BDP and CH₄ data collection periods.

2.3 Animal measurements and sample collection

Live weight was measured at days 0, 7, 25, 37, 45, 52, 59, 63, 66, 73, 79, 82, 86, 89, 92, 95, 100 and 103; and last days of each measurement period in chambers using Tru-test electronic scales (Auckland, New Zealand). Individual rumen samples from all rumen-cannulated steers were collected 2 h after the morning feed only during periods of CH₄ measurements for the treatments BD, BD + 20 g TSS, BD + 30g TSS and BDP (i.e. after the withdraw period of TSS supplementation). Overall, CH₄ measurements in respiration chambers were conducted over 27 days.

Liquid and raft components of the rumen were collected from different sites of the rumen and squeezed rapidly through two layers of cheese cloth and the fluid used for volatile fatty acid (VFA) and microbial analyses. After collection, samples were placed immediately into a portable cooler containing dry ice to be transferred to the lab. There, samples were kept at -20°C for VFA and at -80°C until later DNA extraction. A portable calibrated digital pH meter (Model 1852 mV, TPS Pty. Ltd., Brisbane, QLD, Australia) was used in the farm.

2.4 Laboratory analyses

2.4.1 Diet

The commercial sorghum (600 g/kg) mixed diet reported to contain, 10.2 MJ of metabolisable energy (ME) and 128.9, 120.5, 116.8, 22.2, 11.9 g/kg DM of crude protein, molasses, crude fibre, ether extract and salt, respectively. The formulated BD included per kg of DM 8.3 g Ca, 4.7 g NaCl, 2.7 g P, 1.0 g S, 0.48 mg Co/kg DM, 0.10 mg Cu, 30.05 mg Fe, 0.48 mg I, 0.16 mg Mg, 0.018 mg Mo and 44.0 mg Zn. While vitamins A 2.28 mg, D3 38.3 μg and E 6.39 mg were also present. The standard formulation did not include antibiotic therapy. The reported Rhodes hay consisted of 8 MJ of ME and 79.0, 401.0, 14.0 g/kg DM of crude protein, crude fibre, and ether extract (James Cook University). Triplicate samples of the BD, grass hay and refusals were periodically dried for 24 h at 105°C in a forced-air oven (Contherm; Thermotec 2000, Wellington, New Zealand).

Saponins extraction was performed following industrial standards of the Ministry of Industry and Information Technology of China in 2013. Zhejiang Oriental Tea Technology Co. Ltd reported 570, 325, 54, 50, 1 g of triterpene saponins, crude fibre, crude protein, ash and water insoluble matters per kg of the natural feed additive, respectively. Methods for extraction and purification of saponins from tea *Camellia* spp. seed meal have been previously provided in detail by Li et al. (2012), Zhang et al. (2012) and Jang et al. (2015).

2.4.2 Rumen fermentation and microbial parameters

2.4.2.1 Volatile fatty acids

Volatile fatty acid analysis on thawed samples is fully explained by Ramírez- Restrepo et al. (2014). Briefly, aliquots of 1.5 ml were spun at 4 °C and 16,500g for 15 min. Using triplicate gas chromatography (GC) vials, supernatant aliquots (i.e. 500 µl) were added with 10 µl of phosphoric acid [(850: 1000 (v/v))] and 50 µl of an internal standard (4-Methylvaleric acid; 11 mM solution). Molar concentrations of VFA were performed by automated analysis (AOC-20i, GC-2014, Shimadzu, Tokyo, Japan) on injected aliquots (0.5-ml) of each sample using H₂ as carrier gas (5 ml/min) and separation of acid in 12.7 min/run. The injector and detector temperatures were 200 °C and 230 °C, respectively, while the column temperatures were initially 2 min at 100 °C followed by a gradient of 15 °C/min to 230 °C (2-min hold). Peak detection and chromatogram integration were performed using the Shimadzu-GC solution software v 3.30.00.

2.4.2.2 Microbial analyses

2.4.2.2.1 DNA extraction from rumen digesta

Genomic DNA was extracted in cetyltrimethylammonium bromide (CTAB) lysis buffer using bead beating followed by phenol–chloroform separation. Briefly, rumen samples were centrifuged (17,000 x g, 4 °C, 15 min), the pellet resuspended in 600 µl of CTAB lysis buffer [2% (w/v) CTAB; 100 mM Tris–HCl, 20 mM EDTA and 1.4 M NaCl] and 250 mg of zirconium beads (1: 1 mixture of 0.1 mm/1 mm; Biospec Scientific, Bartlesville, OK, USA) and bead-beat (2x at 6.5 m/s for 60 s) using FastPrep 24 (FastPrep[®]; MP Biomedicals, Solon, OH). The samples were incubated for 20 min in a 70 °C heat block premixing every 4 to 5 min, centrifuged (13,000 rpm, 10 min), supernatant transferred to a new tube with 1 vol. of chloroform/isoamyl alcohol [24: 1 (v/v)] thoroughly mixed and centrifuged (17,000 x g, 10 min). The aqueous phase was mixed vigorously with an equal volume of phenol/chloroform/isoamyl alcohol [24: 24: 1 (v/v)] and centrifuged (17,000 x g, 10 min). The aqueous phase was transferred to a new tube and the DNA precipitated with 0.8x vol. isopropanol by centrifugation (17,000 x g, 25 min) and the DNA pellet washed with 70: 100 (v/v) cold ethanol air-dried for 10 min and the pellet resuspended in 200 µl 10 mM Tris-HCl pH 7.5. DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Nyxor Biotech, Paris, France). DNA was

diluted 10x and used as template in quantitative real-time polymerase chain reactions (qPCR) at 1µl per 25 µl reaction.

2.4.2.2.2 Quantitation of microbial populations

SYBR Green based real-time PCR was used for absolute quantification of protozoa, the *Methanobrevibacter* population, and Rumen Cluster C clade of methanogens (RCC), based on copy number of target genes. Quantitative PCR analysis of protozoal abundance was performed as described by Sylvester et al. (2004). The primer sets used for qPCR are described in Table 1. The new primers for detecting species affiliated with the *Methanobrevibacter* genus and RCC clade were designed and analysed based on the ARB 16S ribosomal sequence database downloaded from the Greengenes (DeSantis et al., 2006) and primer express (Applied Biosystems, Foster City, CA, USA) for an optimal T_m of 60 °C. The new primer sets have been submitted for publication (Huang et al., unpublished).

Quantitative PCR assays were performed on an ABI PRISM 7900HT Sequence Detection System in 384-well optical reaction plates (Applied Biosystems) using the methods described by Denman and McSweeney (2006) and Denman et al. (2007). Total microbial rumen DNA was diluted to 1:10 (v/v) prior to use in real time PCR assays to reduce inhibition. Each reaction (standard curves and samples) were conducted in triplicate. Standard curves and amplification efficiencies for the absolute quantification of protozoa, and methanogens belonging to the RCC clade and *Methanobrevibacter* were derived using the method of Denman and McSweeney (2006). The copy number of the target sequences for these microbes were calculated based on their DNA concentrations and base pair sizes.

Relative qPCR assays were also performed to measure the relative abundance ($2^{-\Delta\Delta CT}$ method; Livak et al., 2001) of total bacteria, methanogens (*mcrA* gene), *Ruminococcus albus*, *R. flavefaciens*, and *Fibrobacter succinogenes* using specific primers (Table 1). The composition of reaction mixture, and the temperature program of these microorganisms were done according to Denman et al. (2007).

2.4.3 Estimations of methane emissions

Methane emissions were measured using an open-circuit respiration chamber system (Ramírez-Restrepo et al., 2014) that operates under a negative pressure (-

10.1 $\pm$ 0.14 Pa) to avoid gas losses, while temperature inside each of the independent four chambers is always 2 °C lower than ambient temperature. Daily CH₄ emissions (g) were calculated by averaging 48-h measurements during each sampling period using the combination of (i) calibrated infrared (CH₄, CO₂, H₂) and paramagnetic (O₂) measurements (Servomex 4100 analyser, Servomex Group Ltd., Crowborough, UK); hardware inputs (Genesis II, Innotech, Brisbane, QLD, Australia); and (iii) the Structured Query Language database. Methane measurements (g) were converted to energy mass density using the 55.6 MJ/kg CH₄ factor (Bossel et al., 2003).

2.5 Statistical analyses

Data analyses were conducted using the Statistical Analysis System, version 9.3; (SAS Institute., Cary, NC, USA) with the MIXED procedure.

Pen values for DMI, LW and TSS ratio values were analysed with a linear model that included the fixed effect of diet (i.e. BD, BD plus 20 and 30 g TSS, and BDP), group order in chambers (i.e. A and B) and the interaction between diet and group order.

Chambers values for CH₄ emissions, DMI, LW and TSS supplementation were analysed with a linear model that included the fixed effects of diet (i.e. BD, BD plus 20 and 30 g of TSS, and BDP) and chamber (i.e. 1, 2, 3 and 4), whereas MEI expenditure in terms of CH₄ emissions was analysed with a linear model that considered the fixed effect of diet.

Volatile fatty acids were analysed with a mixed linear model that included the fixed effect of diet and the random effect of animal, whereas pH values were analysed with a linear model that included the fixed effect of diet.

Least squares means and their s.e.m. of the response variables for each of the diets were obtained and used for multiple mean comparison using the LSD test as implemented in the MIXED procedure. Differences were considered significant when $P < 0.05$ and tendency to significance accepted if $P < 0.10$.

Real- PCR data for the copy number of *Methanobrevibacter*, RCC, ciliate protozoa and the fold changes of microbial population was analysed with a model

that included the effects of diet and the random effect of animal. Differences among BD plus 20 and 30 g of TSS and BDP were obtained using multiple least squares means comparison, while main effects of the diets against the fold change BD values were performed using Student's *t*-test.

3. Results

3.1 Intake, body growth and methane emissions

Dry matter intake during pre- and post-TSS supplementation periods in the animal house were similar (Table 2). In contrast, the addition of 20 and 30 g of TSS to the BD reduced DMI by 2.5% and 6.5% ($P<0.0001$), respectively (Table 2). Overall, LW increased with time ($P<0.0001$; Table 2).

Methane emissions were not affected by two levels of TSS supplementation (Table 3). However, daily ($P<0.05$), yield (g CH₄/kg DMI; $P<0.01$), emissions per kg LW ($P<0.05$) and as losses (%) of MEI ($P<0.01$) were lower for the steers fed the BDP diet (Table 3). There was a positive, but no significative relationship (r 0.30) between DMI and daily CH₄ emissions. No significant differences were observed between diets in the pattern of 24-h CH₄ emissions (Figure 1). All animals showed a biphasic pattern of CH₄ production, which was related to feeding events.

3.2 Fermentation pattern and microbial population

Concentration of total VFA was not affected, though compared to pre- and post-TSS supplementation, the molar proportion of n-Butyric acid was grater when 20 and 30 g of TSS were supplemented in the BD (Table 4). Similarly, the administration of TSS did not affect ruminal pH (Table 4).

The abundance of total ruminal bacteria was not significantly different among treatments and therefore the 16s rDNA of total bacteria was used as reference gene to analyse the relative abundance of other microorganism. Compared with the BD, the relative abundance of methanogens using the mcrA marker gene was not significantly affected by TSS (Fig. 2a). In comparison with the BD control group, TSS addition significantly increased the relative abundance of *F. succinogenes* ($P<0.001$; Fig. 2a) and the abundance of *R. albus* ($P<0.01$) by 2 and 100 fold respectively, while *R. flavefaciens* ($P<0.001$) decreased by 100 fold (Fig. 2b).

The total number of ciliate protozoa significantly increased in abundance with both levels of TSS supplementation compared to the BD and BDP steers ($P < 0.05$; Fig. 3). A similar increasing trend in numbers of RCC was observed with TSS which reached significance ($P < 0.05$) at the 30 g level (Fig.3). However, TSS supplementation had no effect on the population of *Methanobrevibacter* spp. (Fig.3).

4. Discussion

4.1 Effects of TSS supplementation on rumen fermentation

In the present study, there were no apparent differences in the amount or profile of short chain fatty acids which would account for differences in CH_4 production either during or after TSS supplementation. Most of *in vitro* and *in vivo* studies showed that the molar proportion of propionate increased in the presence of saponin-rich diets (Santoso et al., 2007; Pongchompu et al., 2009; Zhou et al., 2011). However, in our study, butyrate concentration increased, while propionate concentration tended ($P = 0.09$) to decrease by up to 16% with the highest TSS supplementation level.

4.2 Influence of TSS supplementation on protozoa population

Triterpene saponins extracted from *C. sinensis* L seed is commercially available and has been screened as a feed additive for ruminants (Yoshikawa et al., 2007). Saponins generally have antiprotozoal properties and therefore TSS has been proposed as a defaunating agent in the rumen with potential antimethanogenic properties due to the close association between methanogens and ruminal protozoa (Wallace et al., 2002; Ohene-Adjei et al., 2007; Jayanegara et al., 2014). However, the results of this study showed that increasing doses of TSS did not reduce CH_4 emissions in cattle. Reports on feeding other saponins have demonstrated a transient negative effect to stimulatory activity on rumen protozoa (Teferedegne et al., 1999; Ivan et al., 2004; Wina et al., 2005).

The current results are not in accord with the inhibitory effect of TSS on ruminal ciliate protozoa population and methanogenesis reported previously *in vitro* (Hu et al., 2005; Guo et al., 2008) with 0.4mg/ml of TSS in buffered rumen liquor, as well as in goats (Mao et al., 2010) and sheep (Zhou et al., 2011) receiving 3 g/day of TSS. Similar conflicting observations on the antiprotozoal activity of *Yucca schidigera*

saponins (YSE) have been observed for *in vitro* studies and feeding trials with sheep and cattle (Lila et al., 2005; Lovett et al., 2006; Pen et al., 2006; Pen et al., 2007), while other studies *in vitro* and with heifers and cows reported no effects of YSE on ruminal protozoa numbers (Śliwiński et al., 2002; Hristov et al., 2003; Benchaar et al., 2008; Singer et al., 2008; Holtshausen et al., 2009).

Unexpectedly, there was a significant increase in rumen protozoal numbers by daily feeding 20 g and 30 g of TSS, but the population of protozoa declined when TSS was withdrawn from the diet. The effects of saponins on rumen protozoa in this study may be due to the BD composition, sources and dosage of saponins used, animal species and the potential adaptive effect of the rumen microorganisms (Newbold et al., 1997). In the current study, TSS was added to the diet, but it has been reported that the antiprotozoal effect of saponins maybe more pronounced when placed directly into the rumen as the inhibitory effect of saponins can be neutralized during eating by salivary secretion (Odenyo et al., 1997).

However, it is still unclear in the present study how the saponin enhanced the growth of ciliate protozoa. An increase in rumen protozoa in another saponin study was attributed to lower rumen motility and turnover rate when alfalfa (*Medicago sativa*) saponins were provided to concentrate-fed ruminants (Lindahl, 1957).

In summary, the reasons for these unexpected results may relate to the chemical structure of the TSS used in the present research, differences between ruminant species, and the adaptation of the rumen microbial population in the Australian cattle used. Future work should focus on understanding the contribution these factors had on the current results.

4.3 Effects of TSS on methane population and CH₄ emission

In previous studies the relative abundance of methanogens in sheep was unaffected by feeding TSS (Mao et al., 2010; Zhou et al., 2011) and a similar result was observed in this study. Although there were no significant effects of TSS on abundance of total methanogens and *Methanobrevibacter* spp., the number of rumen RCC methanogens increased with TSS.

However, it has been reported that TSS appears to reduce CH₄ production by inhibiting protozoa and perhaps interfering with interspecies hydrogen transfer

between the protozoa and associated methanogens although inhibitory effects on hydrogen producing bacteria may also contribute (Guo et al., 2008). In the present study, both ruminal protozoa and RCC numbers were increased markedly after ingestion of increasing saponin supplementation. It is unclear how the saponin supplementation promoted the growth of RCC in this study although it may be related to changes in structure of the rumen bacterial community.

The general pattern of CH₄ production after steers had consumed their morning feed was consistent with that observed in TSS supplemented sheep (Mao et al., 2010; Zhou et al. 2011). In our study, no saponin effect on methanogenesis was observed but after 13 days post-supplementation, CH₄ emissions were lower than control and supplemented diets suggesting reestablishment of a microbial community that had lower CH₄ producing potential than the controls.

4.4 Fibrolytic bacteria response to TSS supplementation

Apart from the antiprotozoal activity, the published studies show that TSS are antimicrobial agents, which can adversely affect fibrolytic bacteria in rumen, but the results are not always consistent. Mao et al. (2010) previously reported that the relative abundance of *R. flavefaciens* was reduced by 51%, but that of *F. succinogenes* was increased by 29% in Hu lambs fed TSS. However, Zhou et al. (2011) found a significant decrease of *F. succinogenes* (79%), but increase in the population of *R. flavefaciens* (13%) and *R. albus* (35%) in Hu sheep. In our study, TSS supplementation significantly reduced the abundance of *R. flavefaciens*, but increased that of *F. succinogenes* and *R. albus* in steers. These inconsistent effects on fibrolytic bacteria indicate that the overall bacterial community may respond differently to TSS depending on the BD, species of ruminant and perhaps environment in which the animals reside. The results cannot be readily explained and need more studies perhaps using the latest deep sequencing techniques for population analysis of microbial DNA.

5. Conclusions

The trial involving TSS supplementation in cattle yielded responses which differed from studies in small ruminants: CH₄ production was not reduced; the population of protozoa did not decline with treatment, but did decline when TSS was withdrawn.

407 Present results indicate that feeding TSS supplements to cattle appears not to be a
408 practical strategy for reducing CH₄ emissions. More predictable responses may
409 emerge if purified saponins of known chemical structure could be used in future
410 animal trials.

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Table 1

Primers used for the quantification of rumen bacteria using real-time polymerase chain reaction (PCR) assay.

Target species	Forward/Reverse	Primer sequences (5'---3')	Reference
Total bacteria	F	CGGCAACGAGCGCAACCC	Denman and McSweeney (2006)
	R	CCATTGTAGCACGTGTGTAGCC	
Protozoa	F	GCTTTCGWTGGTAGTGTATT	Sylvester et al. (2004)
	R	CTTGCCCTCYAATCGTWCT	
Methanogens (mcrA)	F	TTCGGTGGATCDCARAGRGC	Denman et al. (2007)
	R	GBARGTCGWAWCCGTAGAATCC	
<i>Fibrobacter succinogenes</i>	F	GTTCCGAATTACTGGGCGTAAA	Denman and McSweeney (2006)
	R	CGCCTGCCCTGAACTATC	
<i>Ruminococcus flavefaciens</i>	F	CGAACGGAGATAATTTGAGTTTAC	Denman and McSweeney (2006)
	R	CGGTCTCTGTATGTTATGAGGTATTACC	
<i>Ruminococcus albus</i>	F	CCCTAAAAGCAGTCTTAGTTCG	Koike and Kobayashi (2001)
	R	CCTCCTTGCGGTTAGAACA	
<i>Methanobrevibacter</i> spp.	F	CCTCCGCAATGTGAGAAATCGC	Present study
	R	TCWCCAGCAATTCCCACAGTT	
Rumen cluster c (RCC)	F	GAAGCCCTRGGTCGCAAA	Present study
	R	TACTCCCCAAGTRGCMGACTT	

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580 **Table 2**

581 Dry matter intake (DMI), liveweight (LW) and supplementation ratios of saponin (SP) recorded in pens of the animal house from rumen-
 582 cannulated Belmont Red Composite steers fed a basal diet (BD; 0.15:0.85) of a Coleman Stock high grain feed plus Rhodes grass (*Chloris*
 583 *gayana*) hay.

584

Parameter	DMI (kg)	LW (kg)	SP (g/day)	SP: DMI (%)	SP (mg)/kg LW	Days of feeding
Animals on feed	8	8	8	8	8	
Basal diet (BD)	8.4 ± 0.07 ^{ab}	376 ± 3.3 ^c	n.a.	n.a.	n.a.	8
BD + 20 g TSP	8.2 ± 0.10 ^b	382 ± 4.4 ^{bc}	11.9 ± 0.07 ^b	0.14 ± 0.003 _b	31.5 ± 0.38 ^b	3
BD + 30 g TSP	7.8 ± 0.08 ^b	387 ± 3.5 ^b	17.1 ± 0.05 ^a	0.22 ± 0.002 ^a	44.3 ± 0.30 ^a	4
BD post-TSS	8.4 ± 0.07 ^a	398 ± 2.5 ^a	n.a.	n.a.	n.a.	13

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586 A gram of TSS is equivalent to 570 mg of saponins.

587 Values within the same column followed by the same letter are not significantly different at P<0.05.

588 n.a.: not applied.

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Table 3

Dry matter intake (DMI) and methane (CH₄) emissions recorded in respiratory chambers of steers supplemented with tea seed saponin (TSS^a).

Parameter	Diets				Pooled s.e.m.
	BD	BD + 20 g TSS	BD + 30 g TSS	BD post treatment	
Animals on feed	8	8	8	8	
Intake					
DMI (kg/day)	7.6 ^a	8.1 ^a	7.6 ^a	8.2 ^a	0.35
Percentage of liveweight (LW)	2.0 ^a	2.1 ^a	1.9 ^a	2.0 ^a	0.10
TSS supplementation					
Saponin (g/day)	0	10.4 ^e	17.1 ^f	0	0.70
Saponin (mg/kg/LW/day)	0	27.0 ^e	43.5 ^f	0	2.18
CH ₄ emissions					
CH ₄ (g/day)	140.9 ^a	149.9 ^a	144.8 ^a	118.9 ^b	8.48
CH ₄ g/kg DMI	18.5 ^c	18.1 ^c	18.9 ^{ce}	14.3 ^{df}	0.08
CH ₄ g/kg LW	0.37 ^a	0.38 ^a	0.36 ^a	0.29 ^b	0.021
CH ₄ (MJ/day)	7.8 ^{ag}	8.3 ^a	8.0 ^a	6.6 ^{bh}	0.47
CH ₄ -MEI (%)	10.5 ^{ac}	10.2 ^{ac}	10.6 ^{ae}	8.0 ^{fd}	0.48

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A kg of TSS contains 570 g of saponins.

MEI.: Metabolisable energy intake.

Percentage of MEI lost as CH₄ is derived as $[(CH_4 \text{ (g/day)} \times 0.0556) \times 100] / \text{calculated MEI (MJ/day)}$.

Values in each row with different letters are significantly different (ab.: P<0.05; cd.: P<0.01; ef.: P<0.001; gh.: P<0.10).

597 **Table 4**
598 Effects of increasing levels of tea seed saponin (TSS) supplementation on rumen fermentation parameters of steers fed a basal diet (BD;
599 0.80:0.15) of Rhodes grass (*Chloris gayana*) hay plus a commercial grain mixed feed.
600

Parameter	Diets				Pooled s.e.m.
	BD	BD + 20 g TSS	BD + 30 g TSS	BD Post treatment	
Animals on feed	8	8	8	8	
Ruminal pH	6.2 ^a	6.1 ^a	6.4 ^a	6.3 ^a	0.13
Volatile fatty acids (VFA; mmol/L)					
Acetic acid	64.9 ^a	66.7 ^a	63.8 ^a	62.7 ^a	2.34
Propionic acid	23.4 ^a	21.3 ^a	19.7 ^a	23.0 ^a	1.73
Iso-Butyric acid	0.7 ^a	0.7 ^{ad}	0.7 ^{bd}	0.5 ^c	0.05
n-Butyric acid	11.8 ^a	13.9 ^b	13.8 ^b	12.0 ^a	0.72
Iso-Valeric acid	1.3 ^a	2.1 ^a	1.6 ^a	1.9 ^a	0.37
n-Valeric acid	0.9 ^a	0.6 ^b	0.6 ^b	0.6 ^b	0.08
Caproic acid	2.1 ^a	2.3 ^a	1.9 ^a	1.8 ^a	0.40
Acetic: Propionic acid	2.9 ^{bc}	3.4 ^{ac}	3.4 ^a	2.8 ^b	0.22
Acetic: n-Butyric acid	5.5 ^a	4.9 ^{bc}	4.8 ^{bc}	5.2 ^{ac}	0.19
Acetic: Caproic acid	41.3 ^a	39.6 ^a	41.2 ^a	45.6 ^a	6.12
Total VFA	105.2 ^a	108.0 ^a	102.5 ^a	102.7 ^a	4.10

601
602 Samples were processed as technical gas chromatograph triplicates.
603 A kg of the natural feed supplement (TSS) contains 570 g of saponins.
604 Values within the same row followed by the same letter are not significantly different at P<0.05.

Legends for Figures

Fig. 1. Circadian methane emissions (g/h) from eight rumen-cannulated Belmont Red Composite steers fed a mixed basal diet (BD; ●) of 0.15 Rhodes grass (*Chloris gayana*) hay and 0.85 of a commercial mixed high grain diet), BD plus 20 (Δ) and 30 (○) g of tea seed saponin (TSS) supplementation and BD after TSS supplement withdrawal (□). Vertical bars show pooled s.e.m.

Fig. 2. Effects of tea seed saponin (TSS) supplementation on the fold change of total bacteria, methanogens (*mcrA* gene) and *Fibrobacter succinogenes* (a); and *Ruminococcus albus* and *Ruminococcus flavefaciens* (b). Belmont Red Composite steers were fed a basal diet (BD; ■), BD + 20 g (□), BD + 30 g (■) of TSS and BD post supplement withdrawal (■). Vertical bars denote a pooled s.e.m. abc.: (P<0.05).

Fig. 3. Abundance of *Methanobrevicater* spp. (Mbb), Rumen Cluster C clade of methanogens (RCC) and protozoa population in Belmont Red Composite steers fed a mixed basal diet (■D,), BD +□20 g (), ■D + 30 g () of tea seed supplementation and BD post TSS supplement withdrawal (). Vertical bars represent a pooled s.e.m. ab.: (P<0.05).

Fig. 1.

Fig. 2.

Fig. 3.