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Supplementation with tea saponins and statins to reduce methane emissions from ruminants

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Abstract

Four metabolism experiments were carried out at Lansdown Research Station in north Queensland to examine dose-dependent supplementation effects of tea seed (*Camellia sinensis* L.) saponin (TSS) or fermented-*Monascus purpureus* red rice (FRR) containing the natural lovastatin (monakolin K) upon dry matter intake (DMI), rumen fermentation, liveweight (LW), methane (CH_4) emissions and cattle welfare. Dynamics of rumen microbial population were investigated in one of the trials.

Tea seed saponins trials. In October-December of 2012, six Brahman (244 ± 20.4 kg LW) steers fed a mixed basal diet (BD) of 0.15 Rhodes grass (*Chloris gayana*) hay plus a Coleman Stock high-grain feed (0.85) were used to assess between October and December 2012, the effects of 23 days in total of sequential treatment with increasing levels of TSS supplementation (i.e. 6, 10, 15, 20, 25 and 30 g/d)on DMI; rumen fermentation; LW; and animal tolerance. In the February-April period of 2013, eight Belmont Red Composite steers (326 ± 24.3 kg LW) were used during 103 days to monitor the effects of the optimal TSS dose observed in the previous experiment in relation to shifts of rumen microbial population and CH_4 emissions in respiration chambers (i.e. BD, BD + 20g/d TSS, BD + 30g/d TSS and BD post-TSS supplementation). In the first experiment, overall DMI was not affected by the ruminal supplementation of TSS (n = 4; 5.3 ± 0.15 kg) or in the BD (n = 2; 5.4 ± 0.18 kg), but relative to all diets, 6 g/d of TSS supplementation reduced DMI (P< 0.05). Tea seed saponin supplementation resulted in reduction of rumen total volatile fatty acids (VFA) concentration (P< 0.05), increased acetate:propionate ratio and a moderate increase of ruminal pH (P< 0.05) within the normal physiological range. Steers showed similar LW gains (0.66 ± 0.066 for BD vs 0.87 ± 0.093 kg/day for sequential TSS treatments) over the 24 days TSS supplementation. In the second experiment, relative to the BD, the ruminal supplementation of 20, 25 and 30 g/d of TSS reduced DMI (P< 0.05) with only the highest levels of TSS supplementation having the greatest negative effect (P< 0.05). The TSS supplement had no effect on the molar concentration of VFA and ruminal pH values. Protozoa counts increased linearly with 20 and 30 g/d of TSS, but returned to BD levels after the supplement was withdrawn. It was evident that TSS did not reduce CH_4 emissions; however surprisingly compared to the BD and TSS diets, daily emissions and yield (g CH_4/kg DMI) were lower (P < 0.05) by 18 and 22%, respectively for 20 and 30 g/d supplementation, after 13 days post-treatment. It was concluded that TSS supplementation results in cattle that differ from published *in vitro* and small ruminant trials where TSS reduce methane production. Reduced methanogenesis following withdrawal of supplement deserves further investigation to explain its causes, particularly in relation to any sustained microbial changes.

Statin trials. Over a 2.5-month period in 2013, eight rumen-cannulated Belmont Red Composite steers (436 ± 18.2 kg LW) were fed a diet mixture of 0.85 CSIRO's Ridley beef finisher pellets and 0.15 Rhodes grass (*Chloris gayana*) to compare for 7 weeks the dose-dependent effects of the FRR on DMI, efficiency of feed use and tolerance to the supplement. The optimal dose from this study was used in a subsequent experiment with four (461 ± 18.2 kg LW) Belmont Red Composite steers to assess rumen fermentation parameters and CH_4 emissions over ~4 weeks. In the initial experiment,

supplementation with FRR was provided to a maximum dose of 120 g/day (i.e. 2.88 ± 0.057 mg monakolin K/kg LW). Once the diet was equal to or higher than 110 g/day of FRR, adverse effects were evident in terms of DMI ($P< 0.05$) and animal physiology. Compared to the BD, dietary intake of 40 g of FRR/day (i.e. 0.92 ± 0.034 mg monakolin K/kg LW) was associated ($P< 0.05$) with increased DMI and reduced CH_4 yield (g/kg DMI) emissions. However, the effect was transient and not observed as FRR increased to 100 g/day. It was concluded that a transient reduction in methane emissions occurred in the earlier stages of supplementation with FFR, but as the time on supplementation and level of dose increased, there was not effect. This suggests that the FRR can reduce methane emissions, but adaptation in the rumen to the supplement probably negates the response while increasing the dose of FRR to compensate for the adaptation may lead to significant adverse effects on animal health.

This study is the first to report the use of tea seed (*Camellia sinensis* L.) saponin or fermented-*Monascus purpureus* red rice powdered supplements for lowering cattle CH_4 emissions and we conclude that feeding TSS or FRR supplements to cattle is not a practical strategy for reducing methane emissions based on the current studies.

Executive summary

This project assessed the effects of tea seed saponin (TSS) and fermented red rice (FRR) containing statin on dry matter intake (DMI), rumen fermentation, microbial ecology, animal health and methane (CH_4) emissions. The first TSS experiment identified the maximum daily dose of TSS supplementation [114 mg/kg liveweight (LW)] that did not compromise productivity in steers. In a second experiment, CH_4 emissions were not significantly reduced by increasing TSS (maximum of 62.8 ± 1.19 mg TSS/kg LW), but adverse impacts on animal health occurred. These responses in cattle differ from published results where TSS reduce methane production. The sequential dose-response experiments with FRR showed the lowest level of FRR (40 g/d) reduced CH_4 yield (g/kg DMI) compared to the unsupplemented control, but were not different from control at the higher doses. Adverse effects on DMI and metabolism occurred once the dose was greater than 100g/day. The FRR study suggests that transient reductions in methane production occurred, but as the time on supplementation and dose increased, the effect dissipated. These results suggest that rumen microbes adapt to statin and negate the CH_4 inhibition response. Feeding TSS or FRR supplements to cattle appears not to be a practical strategy for reducing CH_4 emissions.

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1. Background

The achievement of sustainable inhibitory effects on methane (CH_4) emissions from ruminant production systems is a widely accepted aim of animal scientists. Methanogenesis represents a loss to the atmosphere of 4 to 15% of energy intake (Waghorn *et al.* 2002) and for the environment a serious ecological issue in terms of greenhouse gas (GHG) emissions (White *et al*. 2003; Ramírez-Restrepo *et al*. 2010; FAO 2013).

Recent retrospective studies suggests that globally in 2000 ~3.7 billion tonnes of feed were consumed by ruminants, while cattle used 83% of the fibrous biomass produced by cut-and-carry, crop residue and grazing systems (Herrero *et al*. 2013). Consequently, cattle were the largest source of methane emissions (1.23 $GtCO_2$ eq; 1 Gt equal to 10^9 tonnes) with 25% of these emissions emitted by the developed world, whilst in developing nations, fermented emissions from mixed crop-ruminant and grazing systems accounted for 0.73 and 0.14 $GtCO_2$ eq (Herrero *et al*. 2013).

Therefore, as grass is a key fed resource in 55% of the beef production systems in Oceania (Herrero *et al*. 2013), multipronged strategies are required to balance resource-use efficiency, food security, productivity gains, GHG emission intensities, economic growth and supply-side policies (Valin *et al*. 2013). This suggests that within the context of the pastoral systems in northern Australia with a current estimated emission of ~0.02 $GtCO_2$ eq yr, abatement strategies using natural bioactive compounds might contribute to reducing GHG emissions (Ramírez-Restrepo *et al*. 2013, Tedeschi *et al*. 2014).

<u>*Saponins*</u>: Plant-derived saponins are non-volatile, surface-active and structurally diverse molecules, which are considered to be defensive mechanisms against predators (Sparg *et al.* 2004). Chemically, they are characterised by a skeleton derived of the 30 carbon atoms containing precursor oxidosqualene (Haralampidis *et al*. 2002). There are up to five main types of skeletons present within the same plant order (Vincken *et al*. 2007), but due to genetic, tissue, age and physiological state of the plant, the distribution of skeletons is recorded in almost 100 families (Haralampidis *et al*. 2002; Oleszek 2002).

Nevertheless, according with the chemical characteristics of the sapogenin (i.e. aglycone), this glucosyde diversity is divided into steroidal (i.e. 27 C-atoms in the molecule; monocotyledon plants); and triterpenoid or triterpene (i.e. 30 C-atoms) saponins, which are mainly found in dycotyledonds of the Theaceae family such as *Camellia oleifera* Abel and *C. sinensis* L. plants (Thakur *et al*. 2011). Within this context, the chemical spectra of tea seeds, and the diverse range of physiochemical and bio-pharmacological effects of saponin-rich tea seed by-product have been well documented (Kitagawa *et al*. 1998; Sparg *et al*. 2004; Morikawa *et al*. 2006, 2007; Yoshikawa *et al.* 2005, 2007; Hu *et al*. 2012; Zhang *et al*. 2012).

Experimental data using *C. sinensis* L also indicated that the anti-methanogenic effect of tea seed saponins (TSS) *in vitro* (Hu *et al*. 2005) and in small ruminants(Hu *et al*. 2006; Mao *et al*. 2010; Zhou *et al*. 2011) is considered to be a selective saponin-sterol association (Schulman and Rideal 1937;

Bangham and Horne 1962) on the protozoa surface (Augustin *et al.* 2011). This leads to membranolytic activity in the gut lumen (Johnson *et al.* 1986) without apparent detrimental effects on ruminant performance (Francis *et al*. 2002).

Statins: Statins are specific inhibitors of the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (HMGCR), a key step in the mevalonate pathway (White and Rudney 1970), which catalizes the mevalonate multibranched cascade and the biosynthesis of non-sterol and sterol isoprenoids (Osmak 2012), that suppress HMG-CoA reductase by post-translational feedback (Demierre *et al*. 2005). Isoprenoids also exert regulations of cellular functions involving cell membrane integrity, cell signalling, cell cycle progression and post-transcriptional modification of proteins and cholesterol synthesis (Demierre *et al*. 2005).

Consequently, statins exhibit effects beyond cholesterol reduction, including immunoregulatory (Kwak *et al.* 2000) anti-cancer (Konstantinopolous *et al*. 2007) and other not entirely clear anti-lipid (Tomaszewski 2011) functions. Other emerging data indicated that statins interfere with methanogenesis *in vitro* (Miller and Wolin 2001) and *in vivo* (Klevenhusen *et al*. 2011; Morgavi *et al*. 2013). This functional effect is likely to be associated with a deregulation of the HMGCR enzyme by lipophilic statins (i.e. lovastatin)onthelipid bilayer membrane of the *archael* microbiota(De Rosa *et al*. 1986). However, no reports of the anti-methanogenic validity of supplementation with natural saponins and statins in cattle have been published to date.

The first objective of this study was to assess the effects of increasing levels of powdered *Camellia sinensis* L. seed saponin as ruminal infusion and a dietary supplementation on dry matter intake (DMI), rumen fermentation and liveweight (LW) and choose optimal levels of dosing for further studies. In parallel, related observations of cattle health and wellbeing were performed. The second objective was to evaluate the optimal doses of TSS on physiological variables, dynamic changes in rumen microbial populations and CH_4 emissions measured in open-circuit chambers. The same methodological approach from these objectives, were used in another set of consecutive experiments involving supplementation with *Monascus purpureus* fermented red rice (FRR) powder containing a natural statin in the form of lovastatin (i.e. monakolin K).

2. Methodology

***Camellia sinensis* L. seed saponin research**

1. Materials and methods

1.1 Experimental design

Two metabolism experiments were conducted over 162 days at Lansdown Research Station, Woodstock near Townsville, Queensland (lat. 19° 39’ 30”S, long. 146° 50’ 17”E).Animal care was provided according with the approved CSIRO Animal Ethic Committee protocol (A12/2012). Experiment 1 was conducted between 10th October 2012 (mid spring) and 14th December 2012 (early

summer; 59 days). Experiment 2 commenced on 11th February 2013 (late summer) and finished on 25th May 2013 (late autumn; 103 days). Internal and external parasites were controlled from day 0 using Dectomax Pour-on (Doramecting, 10 mg/50 kg; Pfizer, São Paulo, Brazil).

In both experiments, all steers were allowed to exercise daily in the cattle yard in the early morning to reduce stress and facilitate cleaning of pens, while fresh water was consumed *ad libitum*. In Experiment 2, steers were accustomed to the respiration chambers (i.e. doors open and closed) in the weeks prior to the CH_4 measurements.

Experiment 1 used four rumen-cannulated [234 ± 8.7 kg LW; least squares means ± sem] and two non-cannulated (253 ± 32.1 kg LW) Brahman steers to compare the effects of increasing supplementation levels of TSS (Zhejiang Oriental Tea Technology Co., Ltd., Changshan, Zhejiang, China) on DMI, rumen fermentation, LW and observations of animal health and wellbeing. In parallel, the TSS supplementation via [i.e. ruminal infusion for cannulated animals and mixed in the basal diet (BD) for uncannulated animals] was also monitored. Based on the previous study, Experiment 2 used eight rumen-cannulated Belmont Red Composite steers (364 ± 8.4 kg LW) to assess the desired range of TSS supplementation mixed in the BD in terms of CH_4 emissions and rumen microbial ecology. At the end of both experiments, all the steers were combined into a single group and over 7 days grazed on pasture close to the cattle yard to observe any adverse clinical signs.

1.2 Feeding and supplementation

During the adjustment and TSS supplementation periods in both experiments and the post-treatment phase of Experiment 2, the BD was fed on a DM basis as a mixture of 15% Rhodes grass (*Chloris gayana*) hay and 85% of a commercial mixed high grain diet (Coleman Stock Feeds Pty Ltd., Charters Towers, Queensland, Australia) fed (i.e. 2.3 and 2.1% of total LW in experiments 1 and 2, respectively; Fisher *et al*. 1987) in equal parts at 0900 hours and 1630 hours. Feed offered to and refused by each steer were recorded daily.

In Experiment 1, steers were accustomed to the BD during 12 days using a portable yard, while 23 days were used during adjustment period in the animal house. The TSS supplementation was conducted between days 36 to 59 by adding to the BD 6, 10, 15, 20, 25 and 30 g/d of TSS during 5, 7, 4, 3, 3, and 2 days, respectively. Each surgical-modified steer received via cannula on a daily basis the powdered TSS dissolved in ~250 ml of cold water previous to the AM feeding. The non-cannulated animals were given the TSS solutions mixed with the morning diet.

Both approaches were designed to ensure a linear increase of feed supplementation and monitoring of DM and water intake, digestion physiology, animal behaviour and welfare. The potential upper limit of total TSS intake was predicted from the published anti-methanogenic dose range of *C. sinensis* seed meal when fed to sheep (i.e. 3 to 5 g TSS/kg DMI; Yuan *et al*. 2007; Zhou *et al.* 2011) and goats (3 g TSS/0.623 kg DMI; Hu *et al.* 2006).

On the basis of developed feed-supplemented methodology, rumen-cannulated steers in Experiment 2 were accustomed to the BD in the cattle yard during 10 days, whilst a 56 day period was used in the

animal house for diet adaptation and preliminary measurements. The final 8 days of this adjustment phase were considered as the BD control period of the present study. Supplementation with 6, 10, 15, 20, 25 and 30 g/d of TSS was conducted during 5, 2, 4, 3, 2, and 4 days, respectively; while the post-treatment period on the BD lasted for 13 days. Overall, CH_4 measurements in respiration chambers were conducted over 27 days. Since there were 8 cattle on successive diets and only 4 chambers available, measurements were staggered to allow different treatments and simultaneous daily CH_4 emission assessment.

1.3 Animal measurements

In Experiment 1, LW was recordedat days 0, 17, 22, 30, 48, 55 and 59, while in Experiment 2, LW was measured at days 0, 7, 25, 37, 45, 52, 59, 63, 66, 73, 79, 82, 86, 89, 92, 95, 100 and 103 using Tru-test electronic scales (Auckland, NZ). Rumen samples from all cannulated steers in both experiments were collected 2 hours after the morning feeding. In Experiment 1 rumen samples were related to the BD and 10, 15, 20, 25 and 30 g/d of TSS supplementation. In Experiment 2, sample collection matched CH_4 measurements using BD, BD + 20 g/d TSS, BD + 30g/d TSS and BD post treatment.

1.4 Laboratory analyses

(a) Diet

The commercial 60% sorghum mixed diet contained, 10.2 MJ and 128.9, 120.5, 116.8, 22.2, 11.9 g/kg DM of protein, molasses, crude fibre, ether extract and salt, respectively. The formulated BD also included Ca 8.3 g, NaCl 4.7 g, P 2.7 g, S 1.0 g, Co 0.48 mg/kg DM, Cu 0.10 mg, Fe 30.05 mg, I 0.48 mg, Mg 0.16 mg, Mo 0.018 mg and Zn 44.0 mg. Vitamins A 2.28 mg, D3 38.3 µg and E 6.39 mg, and 29.9 mg/kg DM of virginiamycin and monensin were also present (Coleman Stock Feeds Pty Ltd). The Rhodes hay consisted of 8 MJ and 79.0, 401.0, 14 g/kg DM of 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^{o}C in a forced-air oven (Contherm; Thermotec 2000, Wellington, NZ). As-fed basis, the TSSlight-yellow colour powder had 57.0, 32.5, 5.4, 5.0, 0.1% of triterpenoid saponin, crude fibre, crude protein, ash and water insoluble matters, respectively (Zhejiang Oriental Tea Technology Co., Ltd).

(b) Rumen fermentation parameters

Liquid and raft components of the rumen content in both experiments 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), microbial and pH analyses. Protozoa cells were fixed using a formaldehyde solution (200: 1000 (v/v), while a small amount of rumen debris was added to facilitate later microbial enumeration. After collection, VFA and protozoa samples were kept at -20^{o}C and at room temperature, respectively. While a portable calibrated digital pH meter (Model 1852 mV, TPS Pty. Ltd., Brisbane, QLD, Australia) was used.

Volatile fatty acids were prepared by a modification of the technique of Moya *et al.* (2009). Briefly, frozen samples of strained fluid were thawed completely and aliquots of 1.5 mL were centrifuged at 16,500 x g using a Prims R 24 microcentrifuge (Labnet International Inc., NJ, USA) at 4^{o}C for 15 min. Supernatant aliquots (i.e. 500 µl) were transferred into three appropriate GC vials, acidified with 10 µl of H_3PO_4 (~85% pure); and 50 µl of 4-Methylvaleric acid (i.e. internal standard, 11 mM solution) were added previous to flame ionization detection on a Shimadzu GC-2014 (Shimadzu, Tokyo, Japan).

Concentrations of the VFAs acetic, propionic, *iso*butyric n-butyric, *iso*valeric and n-valeric acid were determined with a Zebron™ ZB-FFAP column (30 m x 0.53 mm ID, Phenomenex, Torrance, CA, USA). A 0.5 mL aliquot of each sample was injected (AOC, with Autosampler). The carrier gas was H_2 at 5 mL min^{-1} and separation of the acids was in 12.7 min/run. The injector and detector temperatures were 200^{o}C and 230^{o}C, respectively, while the column temperatures were initially 2 min at 100^{o}C followed by a gradient of 15^{o}C/min to 230^{o}C (2 min hold). Peak detection and chromatogram integration were performed using the GCsolution software (Shimadzu v 3.30.00).

(c) Rumen microbial analyses

1.1.4.3.1 *DNA extraction*

Genomic DNA was extracted from the thawed rumen samples using Cetyltrimethylammonium Bromide (CTAB; Qiagen, Hilden, Germany) by bead-beating followed by phenol–chloroform extraction, in duplicate for each sample. Two ml rumen fluid samples were centrifuged into 2 mL screw-capped tubes at13,000 rpm and 4 for 15 min and the supernatant was removed. The pellet was resuspended in 600 µl of lysis buffer (2% 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). The samples were twice mixed in a bead-beating machine (FastPrep®; MPBiomedicals, Solon, OH, USA) at setting program 1 (60S). The samples were incubated for 20 min on 70 heat block and inverted per 4 to 5 min, then centrifuged for 10 min at13,000 rpm, and all the dark supernatant was removed to a new Eppendorf® tube (South Pacific Pty, Sydney, NSW, Australia). Chloroform/isoamyl alcohol (500 µl; 24: 1(v/v) was added and the mixture was strongly shaken until completely cloudy, then centrifuged at13,000 rpm for 10 min. The much cleaner supernatant was removed to the new 2 ml Eppendorf® tube without any white layer material, Phenol/chloroform/isoamyl alcohol (24: 24: 1(v/v)was added into tubes and vortexed to form a white emulsion to separate the aqueous andorganic layers, and spun again for 10min at 13,000 rpm. The upper aqueous layer was transferred to a new tube and the DNA precipitated with isopropanol(0.8 vol). After standing at -80^{o}Cin the freezer for one hour, thawed at room temperature, the DNA pellet was recovered by centrifugation at 10,000 rpm for 25 min. The DNA pellet was washed with 70% cold ethanol (500 µl) and then air-dried for 10 min. The DNA extracts were dissolved in 200 µl EB buffer and DNA yield was quantified using a NanoDrop ND-1000 Spectrophotometer (Nyxor Biotech, Paris, France).Because most of the concentrations are higher than 1000ng/µl, the DNA extracts were diluted 10 times and 50 times in EB buffer prior to qPCR reactions and 1µl of the diluted DNA solutions were used as templates.

1.1.4.3.2 *Quantitation of microbial populations*

SYBR Green based Real-time PCR (qPCR) was used for absolute quantification of protozoa and the *Methanobrevibacter* population and Rumen Cluster C clade of methanogens (RCC), based on copy number of target genes. Q-PCR was performed using a Real time PCR ViiATM 7 system in 384-well optical reaction plates (Applied Biosystems, CA, USA). qPCR 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 (De Santis *et al*. 2006) and primer express (Applied Biosystems, Foster City, CA, USA) for an optimal Tm of 60^{o}C. Primers were then compared with sequences available at NCBI via a BLAST search to ascertain primer specificity (Altschul *et al*. 1990) and against the RDP II and ARB databases using the probe match analysis function (Cole, *et al*. 2003, Ludwig *et al*. 2004).

Conventional PCR analyses for the validation of the specificity against target genes for the new primer sets were performed in 30 µl reactions with the addition of 2.5 mM MgCl2 and employing Platinum Taq (Invitrogen, Carlsbad, CA, USA). Reactions were performed using a Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: one cycle at 94^{o}C for 2 min, 40 cycles of 94^{o}C for 30 sec, 60^{o}C for 15 sec and 68^{o}C for 1 min. The PCR products were analysed by running on 2% agarose gels containing ethidium bromide and visualizing for a single specific band and the absence of primer dimer products. The specificity of the primer sets were evaluated according to Denman *et al.* (2007) by using a TA cloning vector pGEMT easy to generate clone libraries from rumen samples (Invitrogen), which were then sequenced using the BigDye R Terminator v3.1 kit (Applied Biosystems, Fostercity, CA, USA).

Quantitative PCR (qPCR) assays were performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Assays were set up using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Optimisation of assay conditions was performed for primer, template DNA and $MgCl_2$ concentrations. An optimal primer concentration of 300 nM and a final $MgCl_2$ concentration of 3 mM were finally chosen for the assay under the following cycle conditions: one cycle of 50^{o}C for 2 min and 95^{o}C for 2 min for initial denaturation, 40 cycles at 95^{o}C for 15 sec and 60^{o}C for 1 min for primer annealing and product elongation. Fluorescence detection was performed at the end of each denaturation and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by raising the temperature at a rate of 1^{o}C every 30 sec from 60^{o}C to 95^{o}C. Total microbial rumen DNA was diluted to 1:10 prior to use in real time PCR assays to reduce inhibition. Each reaction (standard curves and samples) were conducted in quadruplicate

Standard curves for absolute quantification of protozoa, and methanogens belonging to the RCC clade and *Methanobrevibacter* were generated by dilution series (10^8 to 10^2 copies/µL) of plasmids containing the respective small sub unit rRNA target genes (Hori *et al*. 2006). The amplicons from pure cultures were cloned in to pGEM-T Easy vector (Invitrogen Corporation, SanDiego, CA, USA).

All products were sequenced using the BigDye R Terminator v3.1 kit (Applied Biosystems, Fostercity, CA, USA). The plasmid copy numbers in the standard were calculated based on the concentration measured by the Quant-iT PicoGreen kit (Invitrogen, Switzerland). Abundance analysis of TALC cluster was determined using Applied Biosystems ViiATM 7 software, and then calculated according to the standard curve (R^2>0.99). PCR efficiency of each amplification was within a range of 97% to 104%.

Relative qPCR assays were also performed to measure the relative abundance of total bacteria, methanogens (mcrA gene), *Ruminococcus albus*, *R. flavefaciens*, and *Fibrobacter succinogenes* using specific primers (Table 1). The composition of reaction mixture was same as above, and the temperature program of these microorganisms was same with that of RCC and *Methanobrevibacter*.

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	ACTTGCCCTCYAATCGTWCT	
Methanogens(mcrA)	F	TTCGGTGGATCDCARAGRGC	Denman *et al.* 2007
	R	GBARGTCGWAWCCGTAGAATCC	
Fibrobacter succinogenes	F	GTTCGGAATTACTGGGCGTAAA	Denman and McSweeney 2006
	R	CGCCTGCCCCTGAACTATC	
Ruminococcus flavefaciens	F	CGAACGGAGATAATTTGAGTTTAC	Denman and McSweeney 2006
	R	CGGTCTCTGTATGTTATGAGGTATTACC	
Ruminococcus albus	F	CCCTAAAAGCAGTCTTAGTTCG	Koike and Kobayshi 2001
	R	CCTCCTTGCGGTTAGAACA	
*Methanobrevibacter*spp.	F	CCTCCGCAATGTGAGAAATCGC	Present study
	R	TCWCCAGCAATTCCCACAGTT	
Rumen cluster c (RCC)	F	GAAGCCCTRGGTCGCAAA	Present study
	R	TACTCCCCAAGTRGCMGACTT	

The $2^{-\Delta\Delta CT}$ method was used for determination of relative abundance of microbial populations, where C_T value represents the threshold cycle at which amplified product was first detected in qPCR amplification. ΔC_t is difference in C_T value of the target gene from the C_t value of total rumen bacterial 16S rDNA as a reference gene in the rumen. $\Delta\Delta C_T$ is ΔC_t of treatment samples (TSS supplement and post treatment) minus ΔC_t of the untreated control (BD). Changes in relative abundance of microbial communities due to the effect of TSS supplement were expressed as fold change expression in the target gene of a treatment sample compared to the untreated sample, thus, the fold change of target $= 2^{-\Delta\Delta Ct} = -2$ (ΔC_t target treatment$-\Delta C_t$ target BD).

1.1.4.4 Estimations of methane emissions

Methane emissions were performed using four independent open-circuit respiration chambers with volumes of ~19,000 L, bulk head pre-conditioner units, and 360^o visibility for each animal. Database management was handled by Genesis II hardware (Innotech, Brisbane, QLD, Australia) using digital inputs/outputs at 4-20 mA and the *S*tructured Query Language. Daily CH_4emissions were calculated by averaging 48-h measurements during each sampling period.

The basic measurement procedure can be summarised as: air was sourced external to the animal house using 200 mm PVC ducting and a similar diameter pipe was used to vented air from each chamber over the roof line. Flow rates (5,031± 30.2 L/min) within each chamber were regulated by variable speed controllers of inline fans (TD800/200N Fantech Mulgrave, VIC, Australia) that created a pressure (-10.1± 0.14 Pa) to avoid gas losses. Concentration of gas standards and emitted gases at standard pressure and temperature were corrected by pressure (QBM75-1U/C, Siemens Ltd, Zürich Switzerland) and relative humidity/temperature (HMT 330, Vaisala Pty Ltd, Melbourne, VIC, Australia) sensors. Temperature inside the chambers is always set to be 2^{o}C lower than ambient temperature.

Using a polyurethane tubing, gas composition analysis was performed on samples drawn from the exhaust duct by a micro diaphragm pump at 4.5 L/min. Samples flowed through particulate filters (AF30-02 SMC Pneumatics Pty Ltd, Castle Hill, NSW, Australia) and a four pot fridge drier before reaching a multiport gas switching unit (SW & WS Burrage, Kent, UK). The device was programmed to sample from two outside air ports (i.e. West and East) and each chamber every 90 sec. Methane analysis was conducted using paramagnetic and correlation photometric technology (Servomex 4100, Servomex Group Ltd., Crowborough, UK) for 3 min, a membrane drier (Perma Pure LLC, NJ, USA) received the air samples that were later metered by independent rotameters.

The Servomex 4100 analyser was calibrated using a zero standard (N, 999: 1,000 v/v), O_2, 209,000 ppm (i.e. outside air); and CH_4, CO_2 and H_2 with 97.3, 10,400 and 100 ppm (parts per million) by volume in N (BOC, Sydney, NSW, Australia), respectively. The chamber system was tested by injecting methane (999: 1,000; v/v) at 0.00, 0.080, 0.100, 0.120, 0.140 and 0.200 g/min, which is equivalent to an emission of 0, 115, 144, 173, 202 and 288 g CH_4/day.

1.5 Statistical analyses

All statistical analyses of *in vivo* data were performed using SAS (Statistical Analysis and System, version 9.3; SAS Institute., Cary, NC, USA) and least squares means and their standard errors (se)are reported, unless otherwise noted.

In Experiment 1, DMI values in the animal house were analysed with the MIXED procedure. The linear model considered the fixed effects of animal category (i.e. rumen- and non-cannulated); diet (i.e. BD and BD plus 10, 15, 20, 25 and 30 g/d of TSS); place of feeding (i.e. cattle yard, animal house and chambers); and the interaction between animal category and diet. Pen data for DMI, LW and the supplementation ratios (i.e. TSS: DMI and triterpenoid saponin: DMI) of feeding were analysed using the MIXED procedure. The linear model included the fixed effects of animal category, diet and the interaction between animal category and diet.

Repeated measures analysis for LW on the same animal were performed with the MIXED procedure with a linear model that included the fixed effects of day, animal category and the interaction between day and animal category, and the random effect of animal. Liveweight gains were analysed with the MIXED procedure using a linear model that included the fixed effect of animal category. Analyses of variances for VFA and pH values were performed using the MIXED procedure using a linear model that included the fixed effects of diet. Mean values ± standard deviations (SD) for TSS supplementation values were assessed using the MEANS procedure.

In Experiment 2, pen values for DMI and TSS ratio values were analysed with the MIXED procedure. The linear model considered the fixed effects of escalating levels of TSS supplementation in the diet (i.e. BD plus 6, 10, 15, 20, 25, and 30 g/d TSS) and the interaction between escalating levels of TSS supplementation and group order in chambers (i.e. A and B). Chambers' values for CH_4 emissions, DMI, LW and TSS supplementation were analysed using the MIXED procedure. The linear model included the fixed effects of diet (i.e. BD, BD plus 20 and 30 g/d of TSS and BD post TSS supplementation) and chamber (i.e. 1, 2, 3 and 4).

Repeated measures analysis for LW were performed using the MIXED procedure with a linear model that included the fixed effects of day, group order in chambers and the interaction between day and group order in chambers. Analysis of variance for VFA was performed using the MIXED procedure with a linear model that included the fixed effects of diet and the random effect of animal. Values of pH were analysed with the MIXED procedure with a model that included the fixed effect of diet.

All statistical analyses involving microbial population data were performed using the statistical package SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Least square means± se are reported, unless otherwise noted. Least squares means were calculated for each treatment, and multiple comparisons were conducted using Tukey's adjustment to evaluate differences among treatment means. Differences were declared significant at $P<0.05$ and trends were discussed at $P<0.10$.

Evaluation of statins in fermented (*Monascus purpureus*) red rice

1. Materials and methods

1.1 Experimental design

Two indoor pen experiments were conducted over a 74-day period at Lansdown Research Station. Animal care was provided according to the approved CSIRO Animal Ethic Committee guidelines (A12/2012), Brisbane, Australia. Experiment 3 commenced on 16 September 2013 and finished on 28 October 2013 (42 days). Experiment 4 was carried out between 5 November 2013 and 29 November 2013 (24 days). Every morning, steers were moved to an adjacent cattle yard to facilitate exercise, cleaning of pens and avoid any psychological stressors while indoors. All steers were accustomed to the chambers (i.e. doors open and closed) during previous weeks of the CH_4 measurements. Fresh water was available *ad libitum.*

Experiment 3 compared two groups of four rumen-cannulated Belmont Red Composite steers balanced by weight and were randomly allocated to basal (429 ± 18.2 kg LW; least square mean ± sem) or FRR (443 ± 18.2 kg LW) supplementation diets. 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, all steers were fed the basal diet (BD) indoors for welfare monitoring during a period of 8 days. In Experiment 4, the initial control steers (461 ± 18.2 kg LW) were supplemented in the BD with increasing levels of FRR (monakolin K, 11 mg/g FRR; Zhejiang Medicines and Health Products, Hangzhou, China) from the 11th day and during 13 days to assess the supplementation effects on CH_4 emissions.

1.2 Feeding and supplementation

During the adjustment and supplementation periods in both experiments the BD was fed on a DM basis as a mixture of 85% CSIRO's Ridley beef finisher pellets plus 15% Rhodes grass (*Chloris gayana*) fed *ad libitum* (i.e. 2% of LW; Fisher *et al*. 1987) in equal parts at 0900 and 1630 hours. The linear increase of FRR supplementation was designed to achieve the recommended anti-methanogenic dose of 2.26 mg of monakolin K/kg LW reported in sheep by Morgavi *et al*. (2013). Thus, steers in Experiment 3 were accustomed to the BD during 14 days before the daily supplementation in the BD using 40, 60, 100, 120 and 110 g/d of FRR at days 22, 28, 31, 35 and 37 for 6, 3, 4, 2 and 6 days, respectively. In order to minimize selection by the steers and ensure a linear intake of supplementation, the FRR was dissolved (i.e. half in AM feeding) on a daily basis in ~300 ml of water and the solution mixed with the pellets. In Experiment 4, steers after 10 days on the BD were offered 40, 60 and 100 g of FRR/day during 6, 4 and 2 days, respectively before CH_4 measurements in respiration chambers.

1.3 Animal measurements

Liveweight was recorded at days 0, 14, 22, 28, 35, 43, 50, 57, 63, 65 and 74 using Tru-test electronic scales (Auckland, NZ). Feed offered and refused were recorded daily. Rumen samples in Experiment

2 were collected 2h after the morning feeding before and after methane measurements at days 50 and 66 (BD); 68 and 69 (BD + 60 g/d FRR); and 72 and 74 (BD + 100 g/d FRR).

1.4 Laboratory analyses

(a) Diet

Therapeutic antibiotics and antimicrobials were not included into the CSIRO's Ridley pellet diet (Ridley AgriProducts Pty Ltd, Toowong, Australia). The feed contained 10.7 MJ and 120.5, 176.4 and 19.6 g/kg dry matter (DM) of protein, neutral detergent fibre and fat, respectively. While 82.5, 22.5, 35.2, 17.6, 0.2, 18.6, 126.2, 0.3, 2.2, 0.5, 60.9 mg of Ca, NaCl, P, S, Co, Cu, Fe, I, Mg, Mo and Zn, respectively were added per kg DM. The formulated BD also included the following vitamins: A 3,500 IU, D3 350 IU, E 48.2 mg/kg DM. The Rhodes hay had 8 MJ and 79.0, 401.0, 14 g/kg DM of protein, crude fibre, and ether extract (James Cook University; JCU). Triplicate samples of the BD, grass hay and refusals were periodically dried for 24 h at 105^{o}C in a forced-air oven (Contherm, Thermotec 2000, Wellington, New Zealand). Nutritional characteristics of the FRR supplemented are summarised in Table 7.

(b) Rumen fermentation parameters

Liquid and raft components of the rumen content were squeezed through two layers of cheese cloth and the liquid collected used for VFA and pH analyses.

Volatile fatty acids were prepared as per *Camellia sinensis* L. seed saponin research. Briefly, aliquots of 1.5 mL were spun at 4^{o}C and 16,500*g* for 15 min. Using triplicate gas chromatography (GC) vials, supernatant aliquots (i.e. 500 µl) were added with 10 µl of phosphoric acid (~85% pure) 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 H_2 as carrier gas (5 mL min^{-1}) and separation of acid in 12.7 min/run. The injector and detector temperatures were 200^{o}C and 230^{o}C, respectively, while the column temperatures were initially 2 min at 100^{o}C followed by a gradient of 15^{o}C/min to 230^{o}C (2-min hold). Peak detection and chromatogram integration were performed using the Shimadzu-GC solution software v 3.30.00.

(c) Estimations of methane emissions

Measurements of methane emissions are fully explained in the evaluation of tea seed saponin investigation. Briefly, four independent open-circuit respiration chambers with volumes of ~19.000 L, bulk head pre-conditioner units, and 360^{o} visibility for each animal. Gas measurements were performed by a Servomex 4100 analyser (Servomex Group Ltd., Crowborough, UK)calibrated using BOC (Sydney, NSW, Australia) standards at zero (N, 999: 1,000 v/v); and CH_4, CO_2 and H_2 with 97.3, 10,400 and 1000 ppm (parts per million) by volume in N, respectively. Outside air was used for O_2 calibration at 209,000 ppm. Database management was handled by Genesis II hardware (Innotech, Brisbane, Australia) using digital inputs/outputs at 4-20 mA and the S*tructured Query Language*, while

daily methane emissions were calculated by averaging 48 h measurements during each sampling period.

1.5 Statistical analyses

All statistical analyses were performed using SAS (Statistical Analysis and System, version 9.3; SAS Institute., Cary, NC, USA).

In Experiment 3, least squares means and their standard errors for DMI were obtained with the MIXED procedure. The model included the fixed effects of group (i.e. supplementation order; 1 and 2), diet (i.e. BD and BD plus FRR supplementation) and the interaction between group and diet. Repeated measures of LW on the same animal were analysed with a model that included the fixed effects of day, diet and the interaction between diet and day, and the random effect of animal. Dry matter intake, FRR supplementation and the supplementation ratios (FRR:DMI; statins:DMI) of feeding were analysed with a model that included the effects of diet.

In Experiment 4, pen data for LW, DMI, VFA, pH, FRR supplementation and the ratios of feeding were analysed with a model that included the fixed effects of diet. Chambers' values for LW, FRR supplementation, ratios of feeding and CH_4 emissions were analysed with a model that included the fixed effects of diet and the random effects of animal and chamber (i.e. 1, 2, 3 and 4). Correlations between chambers' DMI during FRR supplementation and daily CH_4 emissions were analysed using the CORR procedure.

3. Results

***Camellia sinensis* L. seed saponin research**

Overall, in Experiment 1, rumen-cannulated and non-cannulated steers had similar DMI (5.3 ± 0.15 vs 5.4 ± 0.18 kg), while DMI in the animal house was lower on the BD in rumen-cannulated than in non-cannulated steers (5.1 ± 0.13 vs 5.5 ± 0.17 kg; $P < 0.05$). Relative to the grouped BD, supplementation with 6 g of TSS reduced ($P < 0.01$) DMI, but not at higher TSS dose levels (Table 2). Liveweight increased with time ($P< 0.05$) during the indoor feeding, while g/d TSS:DMI and g/d of triterpenoid saponins:LW ratios of feeding increased with dose ($P < 0.0001$; Table 2). Final LW was similar for rumen-cannulated (258 ± 8.7 kg) and non-cannulated (276 ± 32.1 kg) steers.

Mean values (± SD) of TSS and saponin concentration in the DMI, and daily dose of TSS and saponin per kg LW were 0.28 ± 0.131%, 0.16 ± 0.074%, 56.5 ± 29.15 mg and 32.2 ± 16.6 mg, respectively. Administration of 37 g of TSS (data not shown) to Brahman steers was associated with remarkable reduction of DMI, scours and frothy bloat. Symptoms disappeared 8 days after withdrawal of TSS supplementation, while in some Belmont Red Composite steers 15 to 20 g/d TSS doses produced transient clinical digestive effects such as soft and reduced faecal out.

Compared to the BD, ruminal infusion of 30 g/d TSS reduced acetic, propionic, n-butyric and iso-valeric acids concentration ($P < 0.05$), but increased the acetic: propionic ratio ($P < 0.05$; Table 3). Significant reductions in total VFA molar concentrations were associated with the linear increase of TSS addition to the BD ($P < 0.05$; Table 3).

Table 2. Dry matter intake (DMI), live weight (LW) and ratios of tea (*Camellia sinensis* L.) seed saponin (TSS) supplementation in Brahman steers fed a basal diet (BD) during the late spring/early summer season of 2012 at Lansdown Research Station (Experiment 1)

BD is a mix of 0.15 Rhodes grass (*Chloris gayana*) hay plus 0.85 Coleman Stockhigh grain feed. A gram of TSS contains 570 mg of saponins (SP). Values within the same column followed by the same letter are not significantly different at $P = 0.05$. n.a., not applied

	Group feeding			
Diet	DMI (kg)	LW	TSS: DMI (%)	SP: DMI (%)
BD	4.9 ± 0.10a	247 ± 2.8bcd	n.a.	n.a.
BD + 6 g/d TSS	4.2 ± 0.17b	250 ± 4.6ad	0.15 ± 0.008a	0.08 ± 0.004a
BD + 10 g/d TSS	4.9 ± 0.15a	253 ± 3.9ac	0.20 ± 0.007b	0.11± 0.003b
BD + 15 g/d TSS	5.3 ± 0.20a	259 ± 5.1a	0.28 ± 0.009c	0.16 ± 0.005c
BD + 20 g/d TSS	5.3± 0.23a	260 ± 5.9a	0.38 ± 0.010d	0.21 ± 0.006d
BD + 25 g/d TSS	5.4 ± 0.28a	261 ± 7.3a	0.46 ± 0.013e	0.26 ± 0.007e
BD + 30 g/d TSS	5.5 ± 0.28a	265 ± 7.3a	0.54 ± 0.013f	0.30 ± 0.007f

Table 3. Production of volatile fatty acids (VFA; mmol/L) and pH values in ruminal fluid of Brahman steers fed a mixed basal diet (BD) of Rhodes grass (*Chloris gayana*) hay (0.15) plus a commercial mixed grain feed (0.85). Cattle were dailysupplemented via rumen-cannula with increasing levels of tea seed saponin (TSS) during the late spring/early summer season of 2012

(Experiment 1)

Samples were processed in triplicates. Values within the same row followed by the same letter are not significantly different at *P* = 0.05

	Diets						
Parameter	BD	BD + 10 g TSS	BD + 15 g TSS	BD + 20 g TSS	BD + 25 g TSS	BD + 30 g TSS	Pooled sem
Animals on feed	4	4	4	4	4	3	
Acetic acid	57.6a	50.6ac	40.0bc	45.0bc	47.8bc	45.0bc	4.03
Propionic acid	21.6a	10.6b	6.9b	6.7b	8.3b	9.0b	2.95
Iso-Butyric acid	0.6a	0.5a	0.5a	0.4a	0.6a	0.6a	0.13
n-Butyric acid	12.8a	7.6b	5.8b	5.5b	7.7b	7.0b	1.32
Iso-Valeric acid	0.88a	1.3ac	1.2ac	1.7ac	2.0bc	2.2bc	0.41
n-Valeric acid	1.2a	1.0a	0.8a	0.9a	1.0a	0.9a	0.19
Caproic acid	2.6a	2.4a	1.7a	3.6a	3.3a	3.2a	0.96
Acetic: Propionic acid	3.1a	4.9ac	5.8bc	6.8b	6.0b	5.5bc	0.68
Acetic: n-Butyric acid	4.8a	6.9bc	7.1bc	8.3bc	6.1ac	6.5ac	0.78
Acetic: Caproic acid	17.1a	30.2a	32.3a	12.6a	26.1a	24.5a	18.3
Total VFA	97.5a	74.3b	57.2b	64.0b	71.0b	68.2b	6.75
Ruminal pH	5.6a	6.1bc	6.5bd	6.2bc	6.4b	6.4b	0.09

Relative to the BD, there was a generalised increase ($P < 0.01$) in pH in all supplemented diets, while 15 g/d of TSS supplementation had higher ($P < 0.05$) pH in the ruminal fluid than values from 10/d or 20 g/d of TSS supplementation (Table 3).

In Experiment 2, DMI during pre- and post-TSS supplementation periods in the animal house were similar (Table 4). In contrast, the addition of 20, 25 and 30 g/d of TSS to the BD reduced DMI by 4 ($P < 0.05$), 5 ($P < 0.05$) and 8% ($P < 0.0001$), respectively (Table 4). Compared to the BD, administration of TSS maintained a similar LW up to 16 days of supplementation, but body growth declined 3% ($P < 0.05$) by supplementing the steers with 30 g/d of TSS over an extra four-day period. This difference disappeared after 13 days of feeding without TSS supplement in the BD (Table 4).

Methane emissions were not affected by escalating levels of TSS supplementation (Table 5). However, daily yield (g CH_4/kg DMI) and g CH_4/kg LW emissions were lower for the steers fed the BD post-TSS supplementation ($P< 0.05$; Table 5). When expressed in the four consecutive CH_4 measurement periods, LW remained relatively constant with time, but was higher in the post-TSS steer group ($P< 0.05$; Table 5). There was a positive, but non-significant relationship ($r= 0.30$) between DMI and CH_4 emissions. Similarly, minor differences were observed between diets in the pattern of 24-h pattern of emissions (Fig 1).

Concentration of total VFA was not affected, though compared to pre- and post-TSS supplementation, the molar proportion of n-Butyric acid was greater when 20 and 30 g of TSS were supplemented in the BD (Table 6). In the same order of supplementation and relative to pre- and post-TSS diets, the administration of TSS did not affect pH values (Table 6). There were no significant differences between steers in LW from day 0 to the end of the study.

Quantitation of ruminal microorganisms

The fold changes of microbial population detected by real-time PCR assay are shown in Figure 2. It was found that the abundance of total ruminal bacteria changed little among different treatments ($P \geq 0.10$; Fig 2a), thus the 16s rDNA of total bacteria can be used as reference gene to analyse the abundance of other microorganism. Compared with the BD control group, the richness of methanogen methyl coenzyme-M reductase (mcrA) genes relative to total bacterial 16S rDNA was reduced by 25% after adding 20 g of TSS to the BD. However, there was an increase of 36% in post TSS supplemented group, and the relative abundance of methanogen in the BD plus 20 g of TSS was significantly lower than that of BD post-TSS group ($P< 0.05$; Fig 2a). In comparison with BD control group, TSS addition significantly increased the relative abundance of *Fibrobacter succinogenes* by 2 times ($P< 0.05$; Fig 2a) and *Ruminococus albus* up to 100 times ($P< 0.01$; Fig 2b), and decreased by over 100 times for *R*. *flavefaciens* ($P< 0.01$; Fig 2b).

The copy number of total ruminal ciliate protozoa, and methanogenic archaea belonging to RCC and *Methanobrevibacter* detected by real-time PCR assay using absolute quantitative PCR are shown in Figure 3.

Table 4. Dry matter intake (DMI), live weight (LW) and supplementation ratios of saponin (SP) in rumen-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 gayana*) hay (Experiment 2)

A gram of TSS is equivalent to 570 mg of saponins (SP). Values within the same column followed by the same letter are not significantly different at *P* = 0.05. n.a., not applied

Parameter	DMI	LW	SP (g/day)	SP:DMI (%)	SP (mg)/kg LW	Days of feeding
Animals on feed	8	8	8	8	8	
			Animal house			
Basal diet (BD)	8.5 ± 0.04a	376 ± 3.3a	n.a.	n.a.	n.a.	8
BD + 6 g/d TSP	8.4 ± 0.09ad	374 ± 4.1ad	3.4 ± 0.06a	0.04 ± 0.003a	9.2 ± 0.35a	5
BD + 10 g/d TSP	8.5 ± 0.15ac	382 ± 6.4a	5.7 ± 0.10b	0.06 ± 0.004b	15.0 ± 0.55b	2
BD + 15 g/d TSP	8.4 ± 0.10af	382 ± 4.5a	8.5 ± 0.07c	0.10 ± 0.003c	22.5 ± 0.39c	4
BD + 20 g /dTSP	8.2 ± 0.11bcdef	382 ± 4.4ac	11.9 ± 0.07d	0.14 ± 0.003d	31.5 ± 0.38d	3
BD + 25 g/d TSP	8.1 ± 0.15bcdefg	400 ± 7.8a	14.2 ± 0.13e	0.17 ± 0.005e	35.8 ± 0.68e	2
BD + 30 g/d TSP	7.8 ± 0.09bg	387 ± 3.5b	17.1 ± 0.05f	0.22 ± 0.002f	44.3 ± 0.30f	4
BD post treatment	8.4 ± 0.05ae	398 ± 2.5a	n.a.	n.a.	n.a.	13

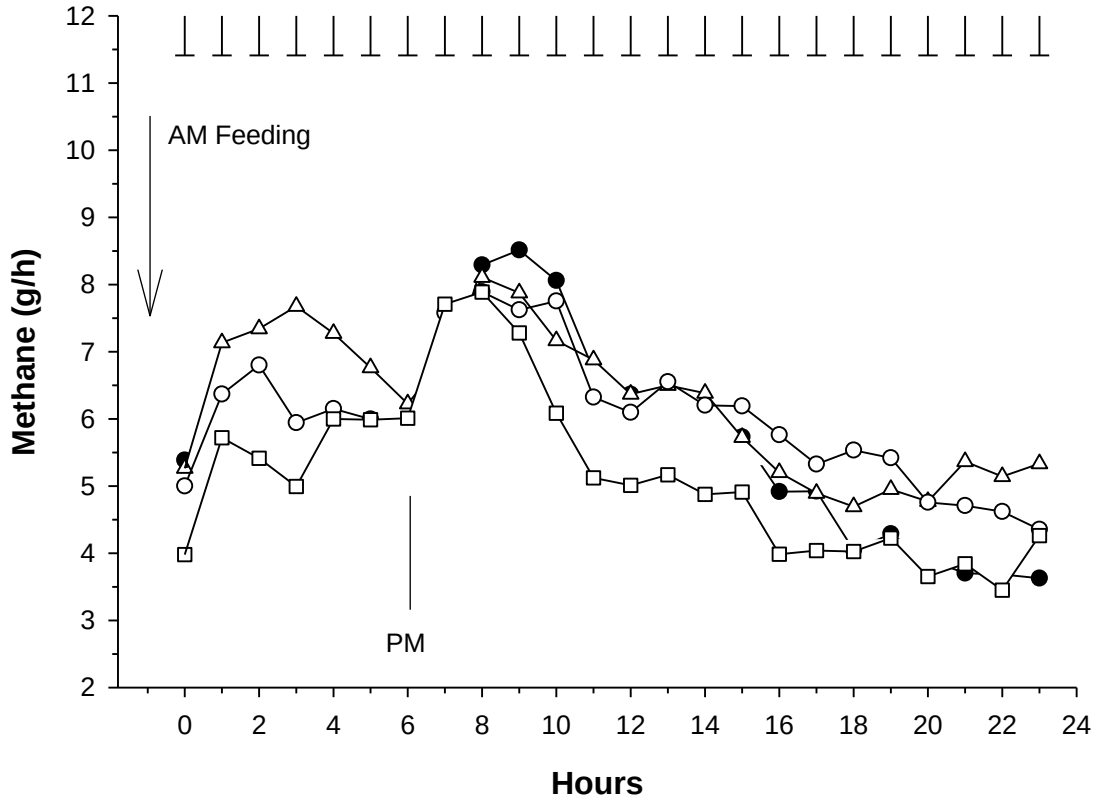

Figure 1. Circadian methane emissions (g/h) from eight rumen-cannulated Belmont Red Composite steers fed a mixed basal diet (BD; ●) of 0.15 Rhodes grass (*Chloris gayana*) hay and 0.85 of a commercial mixed high grain diet), BD plus 20 (Δ) and 30 (○) g/d of tea seed saponin (TSS) supplementation and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

An effect of TSS supplement on the concentration of total ruminal ciliate protozoa was observed, with a greater abundance detected in the BD plus 30 g/d of TSS ($P< 0.01$) and in the BD plus 20 g/d of TSS steers ($P< 0.05$) compared to the BD and BD post treatment steers.

There was a similar change trend for RCC, the copy numbers of RCC in rumen fluid in BD plus 30 g/d of TSS ($P< 0.05$) was significantly higher than that of in BD and BD post TSS feeding. However, TSS supplementation had no effect on the population of *Methanobrevibacter* amongst the four treatments.

Evaluation of statins in fermented (*Monascus purpureus*) red rice

Overall, in Experiment 3, LW and DMI were similar between BD fed and FRR supplemented steers (455 vs. 465 ± 17.7 kg and 8.2 vs. 8.4 ± 0.36 kg/day). However, relative to BD, LW was not affected by increasing levels of FRR supplementation ($P< 0.001$; Table 7), but DMI dropped over 50% once the daily dose of FRR was greater than 110 g after 6 days of supplementation (Data not shown).

Table 5. Live weight (LW), dry matter intake (DMI) and methane (CH_4) emission recorded from rumen-cannulated Belmont Red Composite steers using open-circuit chambers in the autumn season of 2013 at Lansdown Research Station (Experiment 2)

Steers were fed with a basal diet (BD) of a 0.85: 0.15 mixture of a commercial high grain feed plus Rhodes grass (*Chloris gayana*) hay mixed in the morning with varying levels of tea seed saponins (TSS). A kg of TSS contains 570 g of saponins. Values in each row with different letters are significantly different (ab, $P< 0.05$; cd. $P< 0.01$; ef, $P < 0.001$)

	Diets				
Parameter	BD	BD + 20 g/d TSS	BD + 30 g/d TSS	BD post treatment	Pooled sem
Animals on feed	8	8	8	8	
LW (kg)	376a	387ab	393ab	408b	8.9
			Intake		
DMI (kg/day)	7.6a	8.1a	7.6a	8.2a	0.35
Percentage of LW	2.0a	2.1a	1.9a	2.0a	0.10
			TSS supplementation		
Saponin (g/day)	0	10.4e	17.1f	0	0.70
Saponin (mg/kg LW)	0	27.0e	43.5f	0	2.18
			CH4 emissions		
CH_4 (g/day)[†]	140.9a	149.9a	144.8a	118.9b	8.48
CH_4 g/kg DMI	18.5c	18.1c	18.9ce	14.3df	0.08
CH_4 g/kg LW	0.37a	0.38a	0.36a	0.29b	0.021

Table 6. Effects of increasing levels of tea seed saponin (TSS) dailysupplementation on fermentation profiles of rumen-cannulated Belmont Red Composite steers fed a basal diet (BD; 80:15) of Rhodes grass (*Chloris gayana*) hay plus a commercial grain mixed feed during the Autumn season of 2013 (Experiment 2)

Samples were processed as technical gas chromatograph triplicates. Values within the same row followed by the same letter are not significantly different at $P = 0.05$

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

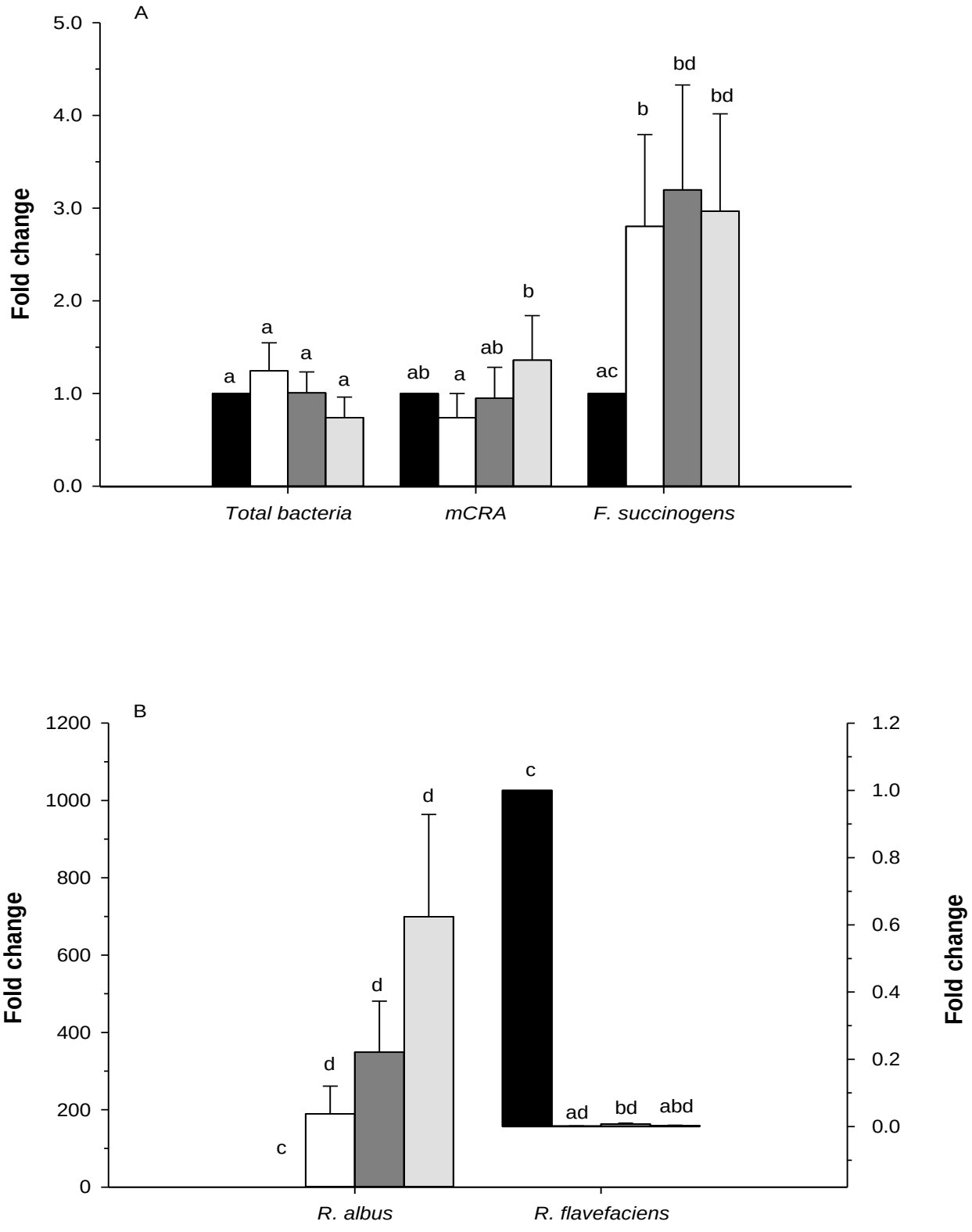

Figure .2. Effects of tea seed saponins (TSS) on the fold change in relative abundance of total bacteria, methanogens and *Fibrobacter succinogenes* (A), and the fold change of *Ruminococcus albus* and *R. flavefaciens* (B) in cattle (ab, $P < 0.05$; cd, $P < 0.01$). Basal diet (■) plus 20/d ( ) and 30/d ( ) of TSS supplementation and BD after TSS supplement withdrawal ( ).

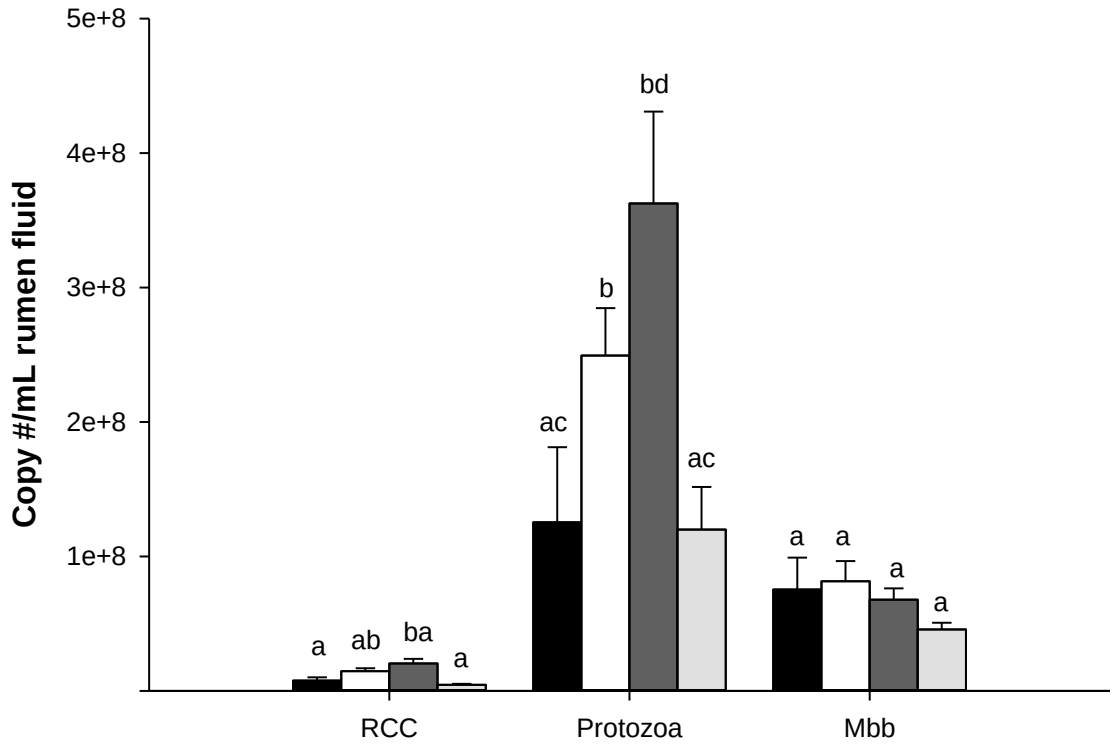

Figure 3. Effects of tea seed saponins (TSS) onthe abundance of rumen protozoa, rumen cluster c (RCC) and *Methanobrevibacter* (Mbb) in cattle(ab, *P*<0.05; cd, *P*<0.01).Basal diet (■) plus 20/d (□) and 30/d ( ) of TSS supplementation and BD after TSS supplement withdrawal ( ).

This remarkable reduction of DMI in all FRR supplemented animals was associated with clinical manifestations of digestive, muscular and urinary system disorders. Observational data also indicated that the administration of high 110 and 120 g FRR/day induced red coloration of faces and urine. Symptoms disappeared 3 days after discontinuation of FRR supplementation.

In Experiment 4, there were no effects on LW, DMI and FRR supplementation measurement dynamics between animals in pens and in chambers (Data not shown). During chamber measurements, LW increased with time (*P*< 0.001) and effects of the supplementation treatment upon DMI were significant (*P*< 0.05; Table 8). Compared to the BD, supplementation of 60 and 100 g/d of FRR in the BD were associated with higher daily methane emissions (*P*< 0.05; Table 8), whilst there was a positive relationship (*r* 0.55, *P*< 0.05) between increased DMI and daily methane emissions. Nevertheless, emissions yield (g CH_4/kg DMI) only attained statistical significance (*P*< 0.05) at the lowest dose of FRR supplementation (Table 8). Overall, rumen fluid pH (6.1 ± 0.21) was similar between diets, while the amount of propionic, n-butyric acid and total VFA were greater (*P* < 0.05) in the low FRR supplemented diet than in the highest (Table 9).

Table 7. Evaluation of feeding daily the basal diet supplemented with fermented (*Monascus purpureus*) red rice powder (FRR) containing the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitor (i.e. statins) using rumen-cannulated Belmont Red Composite steers in October 2013 at Lansdown Research Station (Experiment 3)

Basal diet (BD) is a mix of 0.15 Rhodes grass (*Chloris gayana*) hay plus 0.85 CSIRO's Ridley beef finisher pellets. A gram of FRR is equivalent to 11 mg of statins (i.e. monacolin K). Least squares means within rows followed by the same letter are not significantly different at *P* = 0.05. n.a., not applied

	Diets						
	BD	BD + 40 g	BD + 60 g	BD + 100 g	BD + 110 g	BD + 120 g	Pooled sem
Animals on feed	4	4	4	4	4	4	
Liveweight (LW, kg)	461a	459a	461a	463a	462a	464a	8.9
			Intake				
Dry matter intake (DMI, kg/day)	8.7ab	8.3bc	8.5abc	8.8ab	8.9a	8.0c	0.20
Percentage of LW	1.9a	1.8ab	1.8ab	1.9a	1.9a	1.7b	0.05
			FRR supplementation				
Statin (mg/day)	0	445.3a	670.3b	1109.4c	1218.1d	1331.3e	1.09
Statin (mg/kg LW)	0	0.97a	1.46b	2.40c	2.62d	2.88e	0.042
			Ratios of feeding (%)				
FRR: DMI	n.a.	0.49a	0.71b	1.14cf	1.24df	1.60e	0.048
Statins: DMI	n.a.	0.007a	0.005b	0.012cf	0.013df	0.017e	0.00005

Table 8. Effects of increasing levels of statin-containing fermented (*Monascus purpureus*) red rice powder (FRR) supplementation on live weight (LW), dry matter intake (DMI) and methane (CH_4) emissions from Belmont Red Composite rumen-cannulated steers in November 2013 (Experiment 4)

Data recorded using an open-circuit chamber system and a basal diet (BD) of 0.85 CSIRO's Ridley beef finisher pellets and 0.15 Rhodes grass (*Chloris gayana*) hay. Least squares means within the same row followed by the same letter are not significantly different at $P = 0.05$. Tendency to significance accepted if $*P < 0.10$

	Diets				
Parameter	BD	BD + 40 g/d	BD + 60 g/d	BD + 100 g/d	Pooled sem
Animals on feed	4	4	4	4	
LW (kg)	474a	486b	497c	497c	13.8
		Intake			
DMI (kg/day)	8.9a	10.4b	10.6b	10.5b	0.40
Percentage of LW	1.8a	2.1b	2.1b*	2.1b*	0.08
		FRR supplementation			
Statin (mg/day)	0	449.7a	668.2b	1109.6c	2.59
Statin (mg/kg LW)	0	0.92a	1.34b	2.23c	0.043
		CH_4 emissions			
CH_4 (g/day) †	174.1a	179.4ac	194.3bc	203.2b	11.34
CH_4 g/kg DMI	20.0a	17.1b	18.2ab	19.2ab	1.48
CH_4 g/kg LW	0.36a	0.36a	0.39ab	0.40b*	0.019

Table 9. Comparative volatile fatty acids concentration (mmol/L) in Belmont Red Composite rumen-cannulated steers fed a mixed basic diet (BD) of Rhodes grass (*Chloris gayana*) hay (0.15) and CSIRO's Ridley finishing pellet feed (0.85) with and without increasing levels (g) of fermented red fermented-*Monascus purpureus* rice supplementation during the late spring season of 2013

Samples were processed in triplicates. Least squares means within the same row followed by the same letter are not significantly different at $P = 0.05$

	Diets			
Parameters	BD	BD + 60 g/d	BD + 100 g/d	Pooled sem
Animals on feed	4	4	4	
Acetic acid	59.3a	65.1a	53.4a	4.88
Propionic acid	18.2a	22.6b	14.0a	1.66
Iso-Butyric acid	0.6a	0.6a	0.6a	0.07
n-Butyric acid	14.1ab	17.6a	11.9b	1.42
Iso-Valeric acid	2.0a	2.4a	2.2a	0.65
n-Valeric acid	2.5a	2.2a	1.4a	0.46
Acetic: Propionic acid	3.3ab	2.9a	3.7b	0.33
Acetic: n-Butyric acid	14.1ab	17.6a	11.9b	1.42
Total volatile fatty acids	96.9ab	110.7a	83.8b	7.04

4. Discussion

Tea seed saponins research

Saponins from different sources display a number of biological properties, such asabortive (Dohallite 1962), anti-microbial (Coleman *et al.* 2010; Saleem *et al.* 2010), anti-viral (Zhao *et al*. 2008), haemolytic (Oda *et al*. 2000), immuno-modulatory (Sjölander *et al.* 1998) rumen defaunation (Hanim *et al*. 2009) and vermicide (Potter et *al.* 2009) activities. Tea seed saponins extracted from tea seed is commercially available and has been screened as feed additives for ruminants(Yoshikawa *et al.* 2007), and has been suggested as a possible defaunating agent and potential antimethanogenic reagent because of the close association between methanogens and ruminal protozoa (Ohene-Adjei *et al.* 2007). But the results of this study show clearly that increasing doses of TSS addition did not reduce CH_4 emissions and are in sharp contrast to the observations in TSS-supplemented sheep (Mao *et al*. 2010; Zhou *et al.* 2011) and goats (Hu *et al*. 2006) where there were significant reductions in protozoa numbers and CH_4 emissions.

Saponins are expected to reduce CH_4 production by inhibiting rumen protozoa but in the current study total protozoa numbers increased which may explain why CH_4 production was unaffected. However, protozoal numbers decreased to BD levels and methane production declined after TSS was withdrawn. Possible reasons for these differences in response to TSS between ruminant species may relate to the host physiological response to food supplements (Ramírez-Restrepo *et al.* 2014) and variations in structure of naturally occurring triterpenoid saponins (Dinda *et al.* 2010). Wallace *et al.* (2002) proposed that saponins inhibit protozoa by forming complexes with sterols in the protozoal membrane surface which impairs the integrity of structure and results in cell lysis. However, saponins may not effect protozoal numbers and in some cases an increase in population has been reported (Wina *et al.* 2005). While saponins can reduce protozoa in the early stages of supplementation, the response may not persist after prolonged feeding (Teferedegne *et al*.1999; Ivan *et al*. 2004). This therefore highlights the importance of complementary rumen microbial analysis to determine the shifts in rumen microbiology dynamics that occurred in relation to the introduction and withdrawal of the TSS supplement.

Previous studies have indicated that TSS appears to reduce methane 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). Dourmashkin *et al.* (1962) provided evidence that saponins at 0.05% concentration modify eukaryotic cell membrane permeability by producing a characteristic pore-like surface, which consequently is expected to reduce both protozoa populations (Klita *et al*. 1996) and CH_4 emissions (Guo *et al*. 2008).

In contrast, protozoa numbers increased in the current study when the steers were fed the BD containing 20 g/d and 30 g/d of TSS, which may explain why compared to the BD, CH_4 production was enhanced by 7 and 3%, respectively. However relative to the highest level of TSS

supplementation, protozoa abundance declined ($P< 0.05$) with increasing BD levels, while CH_4 production on a daily, yield (DMI) and LW basis declined by 18, 24 and 20%, respectively 2 weeks after TSS was withdrawn from the diet. The decline in CH_4 production post treatment may be associated with the reduction in protozoa but changes in bacterial populations associated with interspecies hydrogen transfer may have also contribute to this response once TSS was withdrawn.

Effects of TSS on methanogen population and CH_4 emission

The mcrA is essential for the final step of methanogenesis, which is engaged in the reduction of the methyl group bound to coenzyme-M. The methanogens diversity was reduced by adding tea seed saponin (Zhou *et al.* 2011), but addition of TSS did not influence the relative abundance of methanogens in sheep (Mao *et al.* 2010). The similar result was observed in this study. There were no significant effects of TSS supplementation on total methanogens population and *Methanobrevibacter*. However, the RCC concentration increased significantly with increased TSS level, which suggests that TSS supplement changed the community of methanogens not the total number of methanogens. In the present study, *Methanobrevibacter* concentration was positively correlated with total methanogens (r = 0.619, $P< 0.01$) and protozoa (r = 0.37, $P < 0.05$), which suggest that *Methanobrevibacte*r appear to be the predominant methanogen of protozoa-associated methanogens (PAM) and total rumen methanogen. In accordance with these findings, Janssen and Kris (2008) found that the majority (92.3%) of rumen archaea detected in total rumen contents can be placed in three genus level groups: *Methanobrevibacter* (61.6%), *Methanomicrobium* (14.9%), and RCC (15.8%; Janssen and Kirs 2008). Moreover, Tymensen *et al.* (2012) reported that *Methanobrevibacter* species have a greater abundance in the PAM community and represented 42 and 79 % of clones for the 16S rRNA and mrcA gene libraries, respectively. It is unclear that in this study how the TSS supplementation promoted the growth of RCC.

In the present study, both ruminal protozoa and RCC numbers were increased markedly after ingestion of increasing TSS supplementation, which may explain why compared with the BD, CH_4 production was enhanced by 7 and 3%, respectively. Nevertheless, there is little doubt that the overall 24-h CH_4 production pattern after steers had consumed their morning feed (Fig. 1) was consistent with that observed in two times TSS supplemented sheep (Mao *et al.* 2010; Zhou *et al.* 2011).

*Effects of TSS on*fibrolytic bacteria

Apart from the anti-protozoal activity, the published studies showed that TSS are anti-microbial agents, especially for fibrolytic bacteria in rumen. But the published results are not always consistent. Mao*et al.* (2010) previously reported that the relative abundance of R. *flavefaciens* was numerically reduced by 51%,but increased *F*. *succinogenes* (29%) in Huzhou lamb when ingestion TSS. While Zhou *et al.* (2011) found a significant decrease of *F*. *succinogenes* (79%), but numerically increased the population of *R*. *flavefaciens* (13.2%) and *R*. *albus* (34.8%) in sheep. In our study, TSS supplementation significantly reduced the abundance of *R*. *flavefaciens* ($P<0.01$), but increased that of *F*. *succinogenes* ($P<0.05$) and *R*. *albus* ($P<0.01$) in Belmont Red Composite steers.

Effects of TSS on VFA

In the present study, there is no difference in the total VFA, acetate, n-butyrate, iso-valerate and caproate concentrations amongst BD control and TSS-addition groups. Similar results have been observed for TSS *in vitro* (Hu *et al.* 2005; Guo *et al.* 2008) and *in vivo* (Zhou *et al.* 2011). Most of *in vitro* and *in vivo* studies showed that the molar proportion of propionate increased in the presence of saponin-rich diet (Santoso *et al.* 2007; Poungchompu *et al.* 2009; Zhou *et al.* 2011). However, in our study, propionate concentration tended ($P = 0.06$) to decrease with increased TSS level, probably due to the competition between propionate and CH_4 for the available hydrogen, because the higher CH_4 production was observed as stated in results.

The *iso-butyrate concentration* was increased slightly with 30g/day of TSS addition in the BD, and the higher iso-butyrate maybe promote the growth of *R. albus* in this study, because promotional effect of iso-butyrate on *R. albus* was reported by Van Soest (1994). And n-butyrate concentration was numerically increased by TSS supplement, with a corresponding decrease in the acetate:n-butyrate ratio ($P < 0.05$). The inconsistent effects of saponins on butyrate production have been reported., Saponins increased butyrate concentration both *in vitro* (Cardozo *et al.* 2004) and *in vivo* (Lila *et al*. 2005), while it decreased under *in vitro* (Patra *et al.* 2006) and *in vivo* (Pen *et al.* 2007) conditions, and no effect of saponin addition on butyrate was also reported (Poungchompu *et al.* 2009). These marked differences may confirm the hypothesis that the relative tolerance of cattle to natural compounds may be related to the physiochemical behaviour of the additive after ingestion, and/or the genetic-molecular predisposition of the cattle to the major compounds and their conjugates (Ramírez-Restrepo *et al.* 2014).

It was demonstrated that Brahman and Belmont Red Composite steers tolerated *C. sinensis* triterpenoid saponin doses up to 64.9 ± 1.01 (Table 2) and 35.8 ± 0.68 mg/kg LW (Table 4) in experiments 1 and 2 , respectively. In the first experiment gradually increasing levels of supplementation had no apparent detrimental effects until the daily dose reached 86.1±1.58 mg/kg LW. In the second experiment, supplementation with triterpenoid saponins above 35.8 ± 0.68 mg/kg LW each day in Belmont Red Composite steers, reduced DMI and clinical digestive symptoms become evident, but this was reversible. It was also observed that cattle require at least 5 to 7 days of initial exposure to the TSS supplement given once daily in low amounts to adapt the digestive system to the natural compound. Once this was achieved, it was possible to increase the levels of TSS addition over a shorter period of time.

In relation to the safety and tolerance of cattle to TSS, we have demonstrated that Brahman and Belmont Red Composite steers tolerate on average a daily intake of 32.2 ± 16.61 and 27.3 ± 13.53 mg/kg LW of triterpene saponin supplementation during 23 and 20 days, respectively. This is approximately 6.4 and 4.5 (Brahman) and 5.5 and 3.8 (Belmont Red Composite) fold of the no observed toxicological effect levels in mice (i.e. subcutaneous injection, Thakur *et al.* 2011) and dogs (i.e. intramuscular route, Liu *et al.* 2011), respectively. In the present study, effects of saponin on animal behaviour and health indicated that the administration of saponins at 0.42 ± 0.013% of the DMI

to Brahman steers produced adverse digestive effects associated with complete disinterest in feeding. Although that dose was not tested on Belmont Red Composite steers, a similar clinical pattern of symptoms, but of a lower magnitude were experienced when doses achieved additions between 0.10 and 0.14 ± 0.003% of the DMI. However, these relationships are in contrast to the triterpenoid saponin intra-ruminal dosing experiment with sheep (Klita *et al.* 1996). There, doses of 4 and 8% of DMI resulted in inappetence and lack of rumination. Although this differential response to saponins could indicate interactions with the nutritive value of the diet and metabolism by the gastro-intestinal microflora, the substantial variation suggests the nature of the saponins and/or their conjugates are responsible for the observed differences.

Statins research

The studies were conducted to identify dose-dependent effects of FRR on DMI, cattle welfare, rumen fermentation, feed efficiencies and CH_4 emissions.

Overall, the studies suggests that a transient reduction in CH_4 production occurred in earlier stages of supplementation with statins, but as the time on supplementation and dose increased, there was not effect. This indicates that the statins may reduce CH_4 production, but adaptation in the rumen to the supplement may eventually negate the response. Increasing the dose of FRR may compensate for the adaptation effects. However, the small feeding trial indicated that significant adverse effects on intake and metabolism occurred once the dose of FRR was greater than 100g per day for several days. Therefore, practical feeding of the supplement would at best be constrained to use a lower dose where no effect on methane reduction was observed after 10 days of dosing. In our opinion, it is unlikely that FRR supplementation would be adopted by industry due to the transient effect on CH_4 reduction coupled with the adverse effects on productivity when fed at higher levels.

The main finding indicated that cattle tolerated a dietary intake of approximately 64 g FRR/day over a 13 day-period. Nevertheless, the methane abatement effects attributed to the assumed inhibition of cell wall lipid synthesis of archaea by statins in FRR were only significantly observed at the low-dose combination (i.e. 0.92 mg monakolin K/kg LW). This result is particularly relevant in the light of previous evidence (Klevenhusen *et al*. 2011; Morgavi *et al*. 2013) that identified pure lovastatin (1.10 mg/kg LW) and *Monascus*-fermented rice (i.e. monakolin K; 2.26 mg/kg LW) as an anti-archael therapy for small ruminants, while Miller and Wolin (2001) hypothesised 0.55 mg/kg LW of lovastatin for cattle.

Furthermore, our findings indicate, that FRR did not reduce LW, DMI and rumen pH, but increased (P< 0.05) daily CH_4 emissions by 11.6 and 16.7% (Table 7) and affected (P< 0.05) the total VFA concentration (Table 9) when cattle were supplemented with 60 and 100 g/day FRR, respectively. The mechanism by which this occurs is unclear at present. However, there is a hypothesis that FFR might suppress methanogen and protozoa communities at low administration of FRR supplementation (i.e. 40 g/day). Conversely, the rumen microbial consortia might adapt to higher doses of monakolin K and/or the synergistic activity with other reported polyketides (Table 10) increasing the numbers of

microbes involved in methane formation, which negated any effect on methane emission towards the end of the study. This argument is supported by Morgavi *et al*. (2013) who found that ethanolic extracts from the *Monascus* spp*.* moulds did depress *in vitro* CH_4 production in pure cultures of methanogens, while Monacolin K and *Monascus*-fermented rice yeast did not reduce significantly CH_4 when added to these rumen cultures.

In this connection, however, the metabolic consequences of the citrin nephrotoxin (Bennett and Klich 2003), sapogenins (Heber *et al*. 1999), condensed tannins (Wu *et al.* 2003) and other phytochemicals (Martinkova *et al.* 1999) present in *Monascus* pigments were not evaluated. They have produced adverse effects in animals (Gordon *et al*. 2010) and therapeutical complications of the hepatic, digestive, muscular and urinary systems in some individuals (Becker *et al.* 2009; Eckel 2010; Rallidis *et al*. 2012).

On the other hand, although statins are considered to be relatively safe in humans (Sathasivam 2012; Gelissen and McLachland 2014), it seems that clinically the short-term cattle exposure to 110 g/day of FRR went beyond the desired anti-methanogenic response to intolerance effects. Compared to medical patients (Päivä *et al.* 2005), toxicological symptoms and reduced CH_4 emissions were achieved when cattle were administered with 326.3 ± 4.33 and 121.9 ± 4.05% of human clinical medication, respectively. Consequently, it is possible that the unpredicted response was due to the presence of other secondary compounds in FRR rather than citrin (i.e. nephrotoxic agent; Bennett and Klich 2003; Yuan-Chi *et al*. 2003) because our imported FRR supplement was certified free of this secondary polyketide. However, although hepatic effects were not assessed by serum transaminase elevations (Amacher 1998), hepatotoxicity cannot be ruled out due to the association between statins and cholesterol synthesis in the liver (Endo *et al.* 1997; Gerson *et al*. 1989).

Toxicology effects related to HMG-CoA reductase inhibitors' monotherapy in mammals have been previously reported (Gerson *et al.* 1989) in the range of 14 to 735 days, while the onset of symptoms in the present study occurred on average 4 days after administration of 2.6 ± 0.04 mg/kg LW (Table 8). Side effects observed in cattle at the Lansdown Research Station were depressed intake, myopathy, watery stools, polyuria and profuse salivation characterized by drooling. Therefore, the findings of the study demonstrated that our lovastatin toxic dose was equivalent in dogs (Gerson *et al*. 1989), rabbits (Kornbrust *et al*. 1988), rats (Smith *et al*. 1991), sheep (Klevenhusen *et al*. 2011) and wethers (Morgavi *et el.* 2013*)* to 1.4 ± 0.01, 5.2 ± 0.06, 0.2 ± 0.00, 237.3 ± 3.14 and 115.5 ± 1.53% of the highest dose of statin medication, respectively. However, early manifestations of adverse effects in our study were related to specific animals within the group. Consequently, although the detailed mechanisms of the pathophysiology spectrum of symptoms are not fully understood, withdrawal of FRR from the diet alleviated the symptoms within 3 days. Apparently, because after 72 h of ingestion, 10 and 80% of the lovastain medicated in humans is eliminated in urine and faeces, respectively (Li *et al*. 2005).

These experiences led us to hypothesize that molecular' mechanisms underlying the relative low tolerance of cattle to lovastatin and/or FRR may be related to their physiochemical behaviour after ingestion and/or the genetic predisposition of cattle to the lipid-lowering molecule or agent itself.

Table 10. Nutritive composition and predicted *in vivo* dry mater digestibility of fermented (*Monascus purpureus*) red rice powder

Constituents of the rice extract are adapted from 14 marketed products used by Li *et al*. (2005)(a), Heber *et al*. (1999)(b), Gordon *et al*. (2010)(c) and the present research(d). n.d., not determined.

	Commercial products			
Parameter	Mean	Min	Max	References
Nutrient concentration as-fed basis (%)				
Ash		< 0.30	< 3	ab
Fatty acids (saturated, mono and polyunsaturated)		1.10	1.50	ab
Fibre		ND	0.80	ab
Moisture		3.00	6.00	ab
Organic matter		96.60	97.00	abd
Organic phosphorus		0.02	0.44	ab
Pigments		0.30	0.33	ab
Protein		n.d.	5.80	ab
Rice starch		n.d.	73.40	ab
Trace elements				
Silver (µg/g)		n.d.	20.70	a
Aluminium (µg/g)		n.d.	78.00	a
Calcium (mg/g)		n.d.	0.44	a
Cooper (µg/g)		n.d.	8.60	a
Iron (µg/g)		n.d.	50.00	a
Magnesium (mg/g)		n.d.	1.09	a
Manganese (µg/g)		n.d.	19.00	a
Lead (parts per million)		n.d.	< 1.00	a
Secondary polyketides (mg/g)				
Dihydromonakolin	0.47	0.08	1.54	c
Monakolin K Lovastatin	4.23	0.16	16.81	c
Monakolin KA	1.58	0	3.83	c
Monakolin J	0.02	0	0.07	c
Monakolin JA	0.06	0	0.28	c
Monakolin L	0.10	0	0.21	c
Monakolin LA	0.06	0	0.14	c
Monakolin M	0.07	0	0.35	c
Monakolin MA	0.07	0	0.26	c
Monakolin X	0.10	0	0.20	c
Monakolin XA	0.02	0	0.09	c
Citrin µg/g	35.61	0	190.33	c
Total Monakolins	6.80	0.51	18.46	c
Digestibility				
Dry matter (per 100 units eaten)	70.0	68.87	71.18	d

In summary, we provide clear indication that FRR supplemented at low doses interact positively with ruminant metabolism and physiology for acutely decreasing CH_4 emissions. However, doses higher than 100 g of FRR/day have provided important information regarding significant negative effects on DMI and ruminant physiology.

5. Conclusions

Unlike other animal species, cattle do not appear to have tolerance to relatively high doses of triterpenoid-based saponin in the diet. Overall, it can be concluded that in cattle TSS supplementation in the morning feeding altered rumen fermentation patterns in Brahman, but did not have major adverse effects on animal productivity in Brahman or Belmont Red Composite steers. However, CH_4 emissions were not reduced, which suggests that rumen microbial populations, particularly protozoa, in cattle may evade the cytotoxicity of the triterpenoid compound of *C. sinensis* by-product (Francis *et al.* 2002; Podolak et *al.* 2010).

Nevertheless, TSS may be potentially toxic to cattle if high levels of supplementation are used. However, from a therapeutic and CH_4 abatement point of view, future research should focus on microbial suppressive and physiological effects of pure bio-active saponins and/or their secondary metabolites to achieve and retain a desired inhibitory methanogenic effect.

A preliminary study was conducted initially to identify FRR biological constraints in terms of DMI and animal safety, an essential step to assess the use of a botanical dietary supplement for lowering CH_4 emissions from tropical cattle.

Two experiments have been reported recently where either commercial lovastatin or rice fermented with *Monascus* moulds have been fed to sheep. In the experiment where commercial lovastatin was fed, there was no effect on CH_4 production (Klevenhusen *et al*. 2011). However, in the other experiment where fermented rice was offered with hay at a restricted feeding rate, CH_4 production by sheep was reduced by up to 30% over the first 3-4 days of treatment. Methane production appeared to increase again over the following days indicating adaptation to the treatment, but the experimental treatment was only for a short 12 day period (Mongavi *et al*. 2013). The differences between these experiments could be explained by the commercial lovastatin containing relatively low amounts of the bioactive hydroxyacid form of the statin and/or the fermented rice containing other bioactive compounds like citrinin that reduced CH_4 production. Nevertheless, the reduced CH_4 response to the *Monascus* fermented rice appeared to be short lived.

These results appearing in the literature since the commencement of the current project indicate that the CH_4 inhibiting response to statins depends on the bioactive form of the statins, has a lower effect in mixed rumen cultures and the microorganisms adapt quickly to the statins. In addition, other bioactive products, particularly the mycotoxin, citrinin, are likely to be produced when rice is fermented with *Monascus* moulds. These compounds can reduce feed intake in animals and are potentially toxic.

6. Future research needs

The trials involving TSS supplementation in cattle yielded responses which differed from studies in small ruminants in that CH_4 production was not reduced nor was the population of protozoa, however, CH_4 yield declined when TSS was withdrawn. The reasons for these unexpected results may relate to the chemical structure of the TSS used in this study, 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. More predictable responses may emerge if purified saponins of known chemical structure could be used in future animal trials.

The experiments relating to supplementation with statins raised questions about the potential toxicity of these compounds which has implications for their use in agriculture and human medicine. It is clear that future research should focus on characterising the biologically active metabolites that are present in crude extracts of microbial products that are grown for the production of statins. If this can be achieved toxic effects may be reduced or eliminated by controlling the intake of microbial metabolites that have toxic properties but are not involved in the anti-methanogenic response.

7. Publications

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