Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep

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A R T I C L E   I N F O

Keywords
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Methane production
Protozoa
Rumen fermentation
Sheep
Tea saponins

A B S T R A C T

Twelve rumen fistulated Hu sheep were used to assess effects of tea saponins (TS) on methanogenesis, fermentation pattern and rumen microbial communities. All sheep were defaunated by administration of sodium lauryl sulfate. After two weeks, half of the defaunated sheep were refaunated by inoculation with faunate rumen fluid. Both defaunated (DN) and refaunated (RN) sheep were divided into two groups, and assigned to treatments in a 2 × 2 factorial arrangement without (RN, DN) or with TS (3 g/d) (RFs, DFTs). After a feeding period of 3 wk, CH\textsubscript{4} production of individual sheep was measured using open circuit respiratory chambers and rumen fluid was sampled for analysis of rumen fermentation products and extraction of microbial DNA. Numbers of gene copies associated with rumen methanogens (\textit{mcrA} gene), specific bacteria (16S rDNA gene) and rumen protozoa and fungi (18S rDNA gene) were measured using real time polymerase chain reaction (rt-PCR). The abundance of marker gene-copy number for the methanogens, protozoa, fungi, \textit{Ruminococcus albus}, \textit{R. flavefaciens}, \textit{Fibrobacter succinogenes} and \textit{Butyrivibrio fibrisolvens} were expressed relative to the copy number of total rumen bacterial 16S rDNA. Denaturing gradient gel electrophoresis was completed for protozoa to indicate the change in their diversity due to TS addition and defaunation. Declines in CH\textsubscript{4} production as a result of addition of TS (2.1 L/d, \textit{P}<0.01) were similar to defaunation (2.5 L/d, \textit{P}<0.01). Compared to RN, ammonia N concentrations were 12.9, 31.2 and 33.9% lower (\textit{P}<0.01), whereas microbial protein concentrations were 16.4, 29.2 and 36.0% higher (\textit{P}<0.01) in RFs, DN and DFTs, respectively. In contrast, the concentration of volatile fatty acids was similar among treatments although the molar proportion of propionate was higher (\textit{P}<0.01) in defaunated sheep. The abundance of fungi and \textit{R. flavefaciens} marker genes relative to total bacterial 16S rDNA were decreased by defaunation, and abundance of \textit{F. succinogenes} declined (\textit{P}<0.01) with either defaunation or addition of TS. The relative abundance of methanogen marker gene was reduced by defaunation (\textit{P}<0.05). Protozoal abundance in RFs was lower than that of RN (\textit{P}<0.05). The TS reduced (\textit{P}<0.05) protozoal diversity. Addition of TS reduced CH\textsubscript{4} production mainly by inhibiting protozoa, increasing molar proportions of propionate and decreasing acetate/propionate ratio without adversely altering relative ruminal abundance of fungi and cellulolytic bacteria.

\textbf{Abbreviations:} DGGE, denaturing gradient gel electrophoresis; DN, defaunated sheep with no tea saponins; DFs, defaunated sheep with addition of tea saponins; MCP, microbial crude protein; rt-PCR, real time polymerase chain reaction; RN, refaunated sheep with no tea saponins; RFs, refaunated sheep with addition of tea saponins; TS, tea saponins; VFA, volatile fatty acids.

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1. Introduction

Methane production in the rumen represents a loss of energy to the host animal (Holter and Yong, 1992; Johnson and Johnson, 1995), and contributes to global greenhouse gas emissions (Moss et al., 2000). Thus, reducing CH4 emission in ruminants is a goal of researchers throughout the world. It is reported that defaunation reduces CH4 production by 20–30% and increases the amount of metabolizable energy available to the host by 12% (Whitelaw et al., 1984; Kreuzer et al., 1986; Santra et al., 1994). Many attempts have been made to depress rumen methanogenesis by use of feed additives. Plant secondary metabolites (e.g., saponins, tannins, essential oils) and some organic acids (i.e., fumarate, malate) have shown potential to inhibit rumen CH4 production (Lila et al., 2003; Yuan et al., 2007; Mao et al., 2010). In the past decade, saponins extracted from Yucca schidigera (Wang et al., 1998, 2000; Hristov et al., 1999) and tropical fruit (Hess et al., 2003) have been used to reduce CH4 emissions and numbers of rumen protozoa, resulting in a decrease in rumen ammonia N concentrations and an improvement in efficiency of microbial protein synthesis.

China is one of the biggest tea producers in the world. After extracting the oil, tea seed meal is usually disposed of as waste. Our in vitro and in vivo studies with sheep have shown that supplementation with tea saponins (TS) improved rumen fermentation, decreased numbers of rumen protozoa, but had no effect on ruminal methanogens (Hu et al., 2005, 2006; Yuan et al., 2007; Guo et al., 2008). Although supplementation with 3 g/d of TS increased average daily gain, and gain to feed ratios in goats, no improvements occurred when the level of TS was increased to 6 g/d (Hu et al., 2006). Protozoa have both an ecto- and endo-symbiotic relationship with methanogens (Finlay et al., 1994), and methanogens associated with protozoa are estimated to be responsible for 9–25% of the total CH4 production in the rumen (Newbold et al., 1995). Therefore, a reduction in the rumen protozoa population as a result of inclusion of TS in the diet could result in a decrease in enteric CH4 production. It has been proposed that saponins favor production of propionate in the rumen, a factor that may further reduce CH4 as propionate formation can also serve as an H2 sink (Lila et al., 2003; Wina et al., 2005; Pen et al., 2006).

Our objective was to assess effects of TS on CH4 production, abundance of rumen microbial populations and ruminal fermentation of refaunated and defaunated sheep.

2. Materials and methods

2.1. Animals and defaunation procedure

This experiment was conducted in accordance with the Zhejiang University Guidelines for the Care and Use of Experimental Animals. Twelve rumen fistulated 7 month old Hu sheep (rams) with an initial weight of 21.5 ± 1.80 kg were used. All sheep were fasted for 24 h and defaunated by administration of an aqueous solution of sodium lauryl sulfate (200 g/kg) poured directly into the rumen through the cannula at a dose of 8 g/100 kg body weight on 2 consecutive d (Santra and Karim, 2000). On the first d of defaunation, the sheep were fed only 200 g of Chinese wild rye (Aneurolepidium Chinese Kitagawa) 8 h after administration of sodium lauryl sulfate. The remaining protozoa (determined via hemocytometer) after defaunation was less than 3% of the original population (i.e., 5.5 × 103/mL). Two weeks after defaunation, 6 of the sheep were refaunated by inoculating 100 mL of rumen content from a control sheep on two consecutive days. These sheep were fed a basal diet at maintenance requirement for digestible energy (MOA, 2004), containing 600 g/kg of Chinese wild rye and 400 g/kg of a concentrate mixture in which salt and mineral and vitamin mixture were supplemented (Table 1). The remaining 6 sheep were dosed with sodium lauryl sulfate at 7 d intervals throughout the study.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Calculated feed and chemical composition of basal dieta.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed composition</td>
<td>g/kg, as fed</td>
</tr>
<tr>
<td>Chinese wild rye hay</td>
<td>600</td>
</tr>
<tr>
<td>Corn meal</td>
<td>240</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>40</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>44</td>
</tr>
<tr>
<td>Rapeseed meal</td>
<td>60</td>
</tr>
<tr>
<td>NaCl</td>
<td>6</td>
</tr>
<tr>
<td>Mineral/vitamin mixture</td>
<td>10</td>
</tr>
</tbody>
</table>

a Diet was formulated according to the Feeding Standard of Meat-producing Sheep and Goats (MOA, 2004).
2.2. Feeding and experimental design

All sheep were fed with the same basal diet (Table 1). Feed intake was restricted to ensure all feed was eaten, and daily amount of diet was fed in equal portions twice daily at 08:30 and 16:30 h. The experiment was a $2 \times 2$ factorial arrangement with addition of TS and defaunation as main effects. After 5 d of defaunation, as described above, the refaunated (RFN) and defaunated (DFN) sheep were randomly divided into two groups of 3 with one group from each of the RFN and DFN sheep supplemented with 3 g/d of TS (RTFs, DFTs). The saponins (600 g triterpenoid saponins/kg DM) as extracts from tea (*Camellia sinensis*) seeds were purchased from Zhejiang Orient Tea Development Co. Ltd. (Hangzhou, Zhejiang, China) The dose of TS was based on results of previous studies with rumen fluid in vitro (Hu et al., 2005) and in vivo (Hu et al., 2006; Mao et al., 2010). The TS powder was premixed with small amount of corn meal and then mixed with other ingredients of the diets at feeding.

Each sheep was fed in an indoor individual pen for 3 wk with free access to water. Rumen liquid was collected from each sheep via the rumen cannula before feeding (0 h) and at 3, 6 and 12 h after feeding on the 22nd and 23rd d. Collected rumen fluid was immediately strained through four layers of muslin cloth. After measuring pH (PB-20; Sartorius, Göttingen, Germany), samples were stored at $-20\,^\circ{C}$ for later determination of ammonia N, volatile fatty acids (VFA) and microbial crude protein (MCP) and for DNA extraction.

2.3. Methane measurements

After 3 wk on each of the respective diets, each sheep in each group was individually transported to the open circuit respiratory chambers to measure CH$_4$ production using the procedure of Yuan et al. (2007). Briefly, after sheep had adapted to the chamber for 24 h, an hourly air sample was taken from the outlet of each chamber with an airtight syringe, and CH$_4$ concentration was analyzed by gas chromatograph (GC-2100, Shimadzu, Kyoto, Japan) equipped with a Flame Ionization detector (Hu et al., 2005). Inlet CH$_4$ concentration was determined by the methods as described above. The outside air was supplied to the chamber from one side and the chamber air was removed from another side through a pump (2XZ-2, Kangjia, Shanghai, China) with an outlet flow of 2 L/s. The volume of the air through the chamber was recorded by the flow meter (2XZ-2, Kangjia, Shanghai, China) at the outlet. Measurement was for two consecutive d. The feeding schedule was the same as that of pen feeding via a window in the front of the chamber which was opened at each feeding.

2.4. Sample analysis

2.4.1. Chemical analysis of diet and rumen fluid

Chemical composition of the diet was estimated using table values (MOA, 2004). Concentrations of ammonia N, VFA and MCP in the rumen fluid were determined using the procedure described by Hu et al. (2005) with MCP estimated using a purine method (Makkar and Becker, 1999).

2.4.2. Real-time PCR and denaturing gradient gel electrophoresis (DGGE)

Rumen samples from 8 times (4 times daily for two consecutive d) for each sheep were pooled to extract genomic DNA by bead-beating (Zoetendal et al., 1998). Real-time PCR assays for microorganisms reported in Table 2 were completed as: one cycle at 95 °C for 10 s for initial denaturation, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 34 s. The variable regions of 18S rDNA of protozoa were amplified by primers with a GC clamp. The amplification for protozoa was: 35 cycles consisting of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s. All primers are in Table 2.

The DGGE assay was according to Guo et al. (2008). Denaturing gradient gels ranged from 20 to 35% for PCR products of protozoa. Bands were visualized using silver staining (Kocherginskaya et al., 2001) after electrophoresis. DGGE images were captured using Quantity One (Bio-Rad Laboratories, Hercules, CA, USA). The protozoal fragments were recovered and amplified with the primers without GC clamp. The resultant product was purified, ligated with pMD18-T and transformed into Escherichia coli JM109 for sequencing. Ten clones for each band were examined.

2.5. Data calculation and statistical analysis

Methane production as $V_{\text{CH}_4}$, L/h was calculated as:

$$V_{\text{CH}_4} = \frac{(P - P_0) \times V}{R}$$

where $P$ and $P_0$ was the CH$_4$ concentration (ppm) of air samples taken from outlet and inlet, respectively, $V$ was the volume (L/h) of air flowing through the chamber and $R$ was recovery of CH$_4$ in the chamber. The copy number of marker genes for methanogens, protozoa, fungi, *Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* were each expressed relative to the copy number of total rumen bacterial 16S rDNA as a reference gene in the rumen. Thus the relative abundance of each marker gene was estimated as a proportion of total rumen bacterial 16S rDNA as:

relative quantification of target $= 2^{-\Delta ACt} = 2^{-\Delta Ct_{\text{target}}-\Delta Ct_{\text{total bacteria}}}$
Table 2
Primer sets used to amplify 16S rDNA sequences from rumen bacteria, 18S rDNA sequences from protozoa and fungi, and methyl coenzyme A reductase gene (mcrA) from methanogens.

<table>
<thead>
<tr>
<th>Target Species</th>
<th>Forward/Reverse</th>
<th>Primer sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>F</td>
<td>CGGCAACGAGCCGACCACCC</td>
<td>Denman and McSweeney</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CCAATTGACAGCCTGCTGCTG</td>
<td>(2006)</td>
</tr>
<tr>
<td>Methanogens</td>
<td>F</td>
<td>TGCGTGACATCDARAAGCC</td>
<td>Denman et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GBARGTCGV2WCCGGTAGATCC</td>
<td></td>
</tr>
<tr>
<td>Total fungi</td>
<td>F</td>
<td>GAGGAAGTAAAAGTCGTAACAAGGTTTC</td>
<td>Denman and McSweeney</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAAATTCCAACAGTAGTCTTGTATT</td>
<td>McSweeney (2006)</td>
</tr>
<tr>
<td>Protozoa</td>
<td>F</td>
<td>GCTTGCWGTGATGTTATT</td>
<td>Denman et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTCTGCCTCYATACTGTCW</td>
<td></td>
</tr>
<tr>
<td>Fibrobacter. succinogens</td>
<td>F</td>
<td>GTTGGGAATTTGCGGGGTAAA</td>
<td>Denman and McSweeney</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CCGCTGCCCTCTGAACTATC</td>
<td>(2006)</td>
</tr>
<tr>
<td>Ruminococcus flavefaciens</td>
<td>F</td>
<td>GAGGAAAGTAAAAGTCGTAACAAGGTTTC</td>
<td>Denman and McSweeney</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAAATTCCAACAGTAGTCTTGTATT</td>
<td>McSweeney (2006)</td>
</tr>
<tr>
<td>Ruminococcus albus</td>
<td>F</td>
<td>CGCCCAACGAGCACCACCC</td>
<td>Koike and Kobayashi</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAAATTCCAACAGTAGTCTTGTATT</td>
<td>(2001)</td>
</tr>
<tr>
<td>Butyrivibrio fibrisolvens</td>
<td>F</td>
<td>GAGGAAAGTAAAAGTCGTAACAAGGTTTC</td>
<td>Koike and Kobayashi</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAAATTCCAACAGTAGTCTTGTATT</td>
<td>(2001)</td>
</tr>
<tr>
<td>For DGGE* (protozoa)</td>
<td>F</td>
<td>GCTTGCWGTGATGTTATT</td>
<td>Regensbogena et al.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CGGCCGCACGGGCGGCCCGCGCGGGGGGGGGGGGC</td>
<td>(2004)</td>
</tr>
</tbody>
</table>

* DGGE, denaturing gradient gel electrophoresis. The underlined fragments indicate the GC clamp.

where \(\Delta \Delta Ct\) is \(\Delta Ct\) target bacteria minus \(\Delta Ct\) total 16S rDNA, and \(\Delta Ct\) is the difference in threshold cycles for target and reference (Ct represented threshold cycle). The Shannon index, \(H\) (Shannon and Weaver, 1963) was used as the parameter for the structural diversity of the protozoa community (Konstantinov et al., 2003).

Data were statistically analyzed by analysis of variance using the GLM procedure of SAS (1999) with individual sheep as the experimental unit and TS and defaunation as main effects. When the TS × defaunation interaction was significant, a secondary test was conducted to separate the efficacy of TS within defaunation (and vice versa; Robinson et al., 2006). Multiple comparisons of means among treatments were completed by Duncan’s multiple range test. Significance and trends were declared if \(P<0.05\) and \(<0.1\) respectively.

3. Results

3.1. Methane production

All sheep consumed the diet fed, with sheep from the 4 groups having a similar diurnal pattern of CH₄ production over 24 h (Fig. 1). Ruminal CH₄ production increased rapidly after feeding, reached a peak 2–3 h later, and then declining until the next feeding. Compared to RIN, CH₄ production in RfTs and DfTs decreased by (2.1 L/d, \(P<0.01\)) and (2.5 L/d, \(P<0.01\)), respectively (Table 3). The effect of TS on inhibiting methanogenesis was comparable to that of defaunation. An interactive effect of TS and defaunation on reducing rumen methanogenesis occurred, which resulted in further reduction of CH₄ production in DfTs (3.5 L/d, \(P<0.01\)).

![Fig. 1. Diurnal pattern of CH₄ emission in RIN (●), RfTs (○), DfTs (×) and DfTs (○). All values were average of CH₄ production during the two consecutive days (n= 3). The bar is the standard error of the mean.](image-url)
Table 3  Effects of tea saponins on rumen fermentation products in defaunated and rea faunaed sheep (n = 3)*.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>RIN</th>
<th>RFs</th>
<th>DfN</th>
<th>DfTs</th>
<th>SEM</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruminal pH</td>
<td>6.61</td>
<td>6.48</td>
<td>6.41</td>
<td>6.36</td>
<td>0.032</td>
<td>NS</td>
</tr>
<tr>
<td>Methane production (L/d)</td>
<td>19.8</td>
<td>17.7</td>
<td>17.3</td>
<td>16.3</td>
<td>0.24</td>
<td>NS</td>
</tr>
<tr>
<td>Total volatile fatty acids (mmol/L)</td>
<td>63.5</td>
<td>61.5</td>
<td>59.5</td>
<td>60.9</td>
<td>1.04</td>
<td>NS</td>
</tr>
<tr>
<td>Acetate (A)</td>
<td>70.9</td>
<td>68.4</td>
<td>67.1</td>
<td>66.3</td>
<td>0.74</td>
<td>NS</td>
</tr>
<tr>
<td>Propionate (P)</td>
<td>20.5</td>
<td>22.7</td>
<td>26.5</td>
<td>27.5</td>
<td>0.73</td>
<td>NS</td>
</tr>
<tr>
<td>Butyrate</td>
<td>8.7</td>
<td>9.4</td>
<td>6.5</td>
<td>6.2</td>
<td>0.17</td>
<td>NS</td>
</tr>
<tr>
<td>A:丙 (P)</td>
<td>3.49</td>
<td>3.02</td>
<td>2.56</td>
<td>2.43</td>
<td>0.112</td>
<td>NS</td>
</tr>
<tr>
<td>Ammonia-N (mg/dl)</td>
<td>12.1</td>
<td>10.5</td>
<td>8.3</td>
<td>8.0</td>
<td>0.28</td>
<td>NS</td>
</tr>
<tr>
<td>Microbial protein (mg/dl)</td>
<td>69.6</td>
<td>61.0</td>
<td>89.9</td>
<td>94.7</td>
<td>4.13</td>
<td>NS</td>
</tr>
</tbody>
</table>

* All values were determined as pooled rumen fluid samples (before feeding and at 3, 6, and 12 h after feeding over two days).

**Acetate to propionate ratio.

**P<0.05; **P<0.01; NS, P>0.05; DF, defaunation effect; TS, tea saponins effect; DF × TS, interactive effect.

3.2. Rumen fermentation parameters

Both defaunation (P<0.01) and TS addition (P<0.05) decreased ruminal pH, but all pH values were between 6.15 and 6.90 (Table 3). Concentrations of total VFA were 63.5, 61.5, 59.5 and 60.9 mmol/L for RIN, RFs, DfN and DfTs, respectively, with no differences among treatments. However, the molar proportion of acetate was lower (P<0.05) and proportion of propionate was higher (P<0.05), resulting in a reduced ratio of acetate to propionate (P<0.05) for RFs, DfN and DfTs sheep than for RIN sheep. Compared to that of RIN (12.1 mgN/dl), ammonia N concentrations were reduced by 12.9, 31.2 and 33.9% (P<0.05), whereas MCP concentrations were increased by 16.4, 29.2 and 36.0% (P<0.05) in RFs, DfN and DfTs as compared to MCP (69.6 mg/dl) in RIN sheep.

3.3. Quantitation of ruminal microorganisms

The abundance of methanogen mcrA genes relative to total bacterial 16S rDNA was reduced by defaunation (P<0.05), whereas additional TS had no effect on methanogen abundance in either rea faunaed or defaunated sheep (Table 4). The protozoal 18S rRNA copy number in defauna ed groups was extremely low, and was lower for RFs compared to RIN (P<0.05). Furthermore, TS addition had positive interactive effect on reducing protozoa by defaunation. Defaunation decreased relative abundance of fungi (P<0.01), R. flavefaciens (P<0.05) and F. succinogenes (P<0.01), increased it for B. fibrisolvens (P<0.01), and had no effect on R. albus. There was a negative TS x defaunation interaction (P<0.01) on the abundance of F. succinogenes.

3.4. Diversity analysis of protozoa community

The DGGE profiles of protozoa community as affected by treatments are in Fig. 2A. The number of DGGE bands (Fig. 2B) and Shannon diversity index (Fig. 2C) of rumen protozoal DGGE profile were lower (P<0.05) in RFs than those in RIN, respectively. The eleven bands were sequenced and submitted to GenBank (from f907164 to f907174). No band affiliated with protozoa occurred in defaunated sheep.

Table 4  Effects of tea saponins on relative abundance of marker genes for specific microbial populations (% of total bacterial 16S rDNA) in sheep under defaunated and rea faunaed status (n = 3)*.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>RIN</th>
<th>RFs</th>
<th>DfN</th>
<th>DfTs</th>
<th>SEM</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanogens</td>
<td>0.61</td>
<td>0.57</td>
<td>0.34</td>
<td>0.35</td>
<td>0.064</td>
<td>NS</td>
</tr>
<tr>
<td>Protozoa</td>
<td>4.68</td>
<td>2.66</td>
<td>6.75E-04</td>
<td>1.84E-04</td>
<td>0.364</td>
<td>NS</td>
</tr>
<tr>
<td>Fungi (×10^-3)</td>
<td>15.19</td>
<td>14.70</td>
<td>0.34</td>
<td>0.63</td>
<td>0.004</td>
<td>NS</td>
</tr>
<tr>
<td>R. albus (×10^-2)</td>
<td>4.85</td>
<td>5.49</td>
<td>3.42</td>
<td>3.07</td>
<td>0.022</td>
<td>NS</td>
</tr>
<tr>
<td>R. flavefaciens (×10^-2)</td>
<td>14.74</td>
<td>9.61</td>
<td>7.04</td>
<td>4.60</td>
<td>0.024</td>
<td>NS</td>
</tr>
<tr>
<td>F. succinogenes</td>
<td>1.28</td>
<td>0.27</td>
<td>3.78E-04</td>
<td>4.23E-04</td>
<td>0.047</td>
<td>NS</td>
</tr>
<tr>
<td>B. fibrisolvens</td>
<td>1.03</td>
<td>0.88</td>
<td>2.27</td>
<td>2.05</td>
<td>0.295</td>
<td>NS</td>
</tr>
</tbody>
</table>

* All values were determined as pooled rumen fluid samples (before feeding and at 3, 6, and 12 h after feeding over two days).

**P<0.05; **P<0.01; NS, P>0.05; DF, defaunation effect; TS, tea saponins effect; DF × TS, interactive effect.
4. Discussion

4.1. Role of TS in rumen CH4 production

The TS and defaunation reduced ruminal CH4 production by 10.6 and 12.7% respectively, which was comparable to disodium fumarate (Yuan et al., 2007) or soybean oil (Mao et al., 2010), but inferior to that of the same dose of TS in vitro (Hu et al., 2005). The lower effect of TS in vivo may in part be due to adaptation of the rumen protozoa to saponins, or through degradation of these compounds by rumen bacteria, which adapt to become capable of degrading antiprotozoal component such as saponins (Newbold et al., 1997).

The observations that there was an interaction between defaunation and tea saponin in decreasing CH4 production, and that tea saponin decreased CH4 in both refaunated and defaunated sheep, indicates that reduced methanogenesis by tea saponin addition was not only due to the inhibition of protozoa, but that another mechanism of inhibition existed (Van Nevel and Demeyer, 1996). The TS may have a minor affect on other microbiota which results in a lower amount of H2 being produced, and thus less CH4 in the DFTs as compared to that of RFTs and DFT.

4.2. Effects of TS and defaunation on fermentation characteristics in rumen

Concentrations of VFA were not affected by either TS addition or defaunation, but the molar proportion of acetate was decreased and the propionate proportion increased. Together with the decreased CH4 production by TS and defaunation, we suggest that efficiency of ruminal fermentation was improved by channeling H2 used for methanogenesis to synthesis of propionate (Lila et al., 2003; Wina et al., 2005; McAllister and Newbold, 2008). Addition of TS decreased ammonia N concentrations, but increased MCP yield, which is consistent with previous studies (Lila et al., 2003; Hu et al., 2005; Pen et al., 2006). The reduction of ruminal ammonia N concentration by TS is likely due to its ammonia binding ability and toxicity to rumen ciliate protozoa (Wallace et al., 1994).

4.3. Effects of TS on rumen microbes

Rumen protozoa play an important role in CH4 formation in the rumen due to ecto- and endo-symbiotic relationships with methanogens (Finlay et al., 1994). Therefore, defaunated and refaunated sheep were used in this study to distinguish effects
of TS on protozoa and methanogens, and to elucidate the mechanism by which addition of TS decreased CH4 production. Although protozoal abundance was reduced by both TS and defaunation, methanogens were only slightly reduced by TS when protozoa were present, and not in defaunated sheep (Table 4). A similar conclusion regarding effects of saponins on rumen microbiota was reported in other studies (Soliva et al., 2003; Goel et al., 2008; Guo et al., 2008). In contrast, Hess et al. (2004) observed that saponins from S. saponaria increased the number of methanogens in the rumen. Among rumen microbes, protozoa are the most sensitive to saponin induced changes in cell membrane permeability (Klita et al., 1996; Moss et al., 2000). However, the variation of major protozoa species among the refaunated sheep in our study (Fig. 2) suggests that inhibition of TS on protozoa is likely selective and sustained.

Rumen fungi and cellulolytic bacterial play important roles in maintaining a stable intra-ruminal environment for fiber digestion. A decrease in feed intake and/or digestibility is commonly observed when their activities are inhibited (Patra and Saxena, 2009). Using fluorescence microscopy, Imai and Ogimoto (1978) observed that only 1–10% of rumen bacterium was attached to the surface of protozoa, most of which were carbohydrate fermenters. In our study, abundance of fungi and the cellulolytic bacteria R. albus, R. flavefaciens, F. succinogenes and B. fibrisolvens, were generally unaltered by supplementation of 3 g/d of TS in defaunated sheep. Wina et al. (2005), by using a nucleic acid hybridization technique, also reported that populations of R. albus and R. flavefaciens were not affected by Sapindus rarak methanol extract at a concentration of less than 1 mg/mL, but dramatically reduced at concentrations of 2–4 mg/mL. Further, it was shown that S. rarak saponins inhibited R. albus, R. flavefaciens and Chrytidiozymetes in the short term, while neither R. albus nor R. flavefaciens was affected by the saponins after 105 d of administration (Wina et al., 2006). Therefore, the CH4 inhibition by TS and other saponins seems to be dose and time dependent. Further research is needed to assess long term effects of TS on rumen microbes and on decreasing ruminal CH4 production.

5. Conclusions

Saponins from tea seeds decreased CH4 production in the rumen of Hu sheep, and the inhibition on methanogenesis was higher in defaunated than refaunated sheep due to differences in protozoal abundance. Decreased CH4 production was due mainly to effects of TS on reducing numbers of protozoa, and thus lowering methanogenic activity of the associated methanogens which resulted in a channeling of H2 towards propionate production. Tea saponins could be a potential feed additive for mitigating CH4 emission from ruminants.

Conflicts of interest

None.

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References


