Effects of Supplemental Virgin Coconut Oil and Condensed Tannin Extract from Pine Bark in Lactation Dairy Diets on Ruminal Fermentation in a Dual-Flow Continuous Culture System

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Abstract

The objective of our study was to investigate effects of virgin coconut oil (VCO) and condensed tannin extract from pine bark (PCT), either separately or in combination, as supplements to lactation dairy diets on in vitro ruminal fermentation profiles in a completely randomized design with four independent runs of continuous cultures. Four dietary treatments included: 1) control [CONT; total mixed ration (TMR) without supplement], 2) TMR with VCO (VCOT), 3) TMR with PCT (PCTT) and 4) TMR with VCO and PCT (VPT). Results showed that culture pH was maintained at least at 6.13 across dietary treatments, and supplementing VCO and/or PBE did not influence culture pH. Total volatile fatty acid concentration was similar in response to the supplements. Supplementing PCT decreased ammonia-nitrogen concentration both in PCTT and VPT (P<0.01), while VCO supplementation resulted in no effect on ammonia-nitrogen concentration. Cultures offered VCO and PCT supplementation, either separately or in combination, showed no response on methane production. The decrease in ammonia-nitrogen concentration when PCT-containing diets (PCTT and VPT) were offered is likely attributed to condensed tannins in PCT, which indicates that PCT can affect dietary nitrogen utilization efficiency in vivo through condensed tannins on ruminal nitrogen metabolism. However, dietary concentration of VCO used in this study may have not been enough to manipulate ruminal fermentation.

Keywords: Continuous culture; Condensed tannin extract from pine bark; Ruminal fermentation; Virgin coconut oil

Introduction

Improving feed efficiency and reducing nutrient excretion into the environment are essential elements for sustainable dairy production worldwide. In high quality forage diets fed ruminants, majority of dietary proteins can be rapidly degraded, releasing between 56 and 65% of dietary nitrogen (N) in the rumen during microbial fermentation. Consequently, large losses of N as urea into urine (25-35%) occur after ammonia is absorbed through rumen wall [1], which is the primary source of volatile N to the environment [2]. Thus, losses of dietary N can be reduced by decreasing protein degradation in the rumen. Simultaneously, methane (CH4) is produced in the rumen as a part of the normal process of ruminal feed digestion. Typically, about 6 to 10% of the total gross energy consumed by dairy cows is converted to CH4 [3] which contributes to greenhouse gas emissions in the environment. A variety of strategies have been studied to improve ruminal N metabolism and mitigate CH4 production, and feeding or supplementing specific substances as rumen modifiers that directly or indirectly inhibit ruminal N degradation as well as methanogenesis has been one of the most sought opportunities [4].

Natural plant compounds such as condensed tannins (CT) and coconut oil (CCO) have been recommended as potential ruminal fermentation modifiers to reduce dietary N degradation and methanogenesis in the rumen. Condensed tannins are prevalent in many plants and can reduce ruminal protein degradation, which can increase intestinal protein flow when provided at moderate doses of 20 to 40 g·kg–1 CT in dry matter (DM) [5]. Pine (Pinus taeda L.) tree bark (PB) is one of the abundant timber industry by-products and contains up to 110 g·kg–1 DM of condensed tannins. Coconut oil as a source of medium-chain fatty acids (MCFA) proved to sizable reduction in CH4 emissions in vivo [8]. The CCO produced through the wet method is known as virgin coconut oil (VCO). The VCO is rich in MCFA [60-63 g·100 g–1 fatty acid (FA)] methyl esters; 7.19-8.81% C8:0, 5.65-6.59% C10:0, 46.9-48.0% C12:0 and 16.2-18.9% C14:0] [9], and consequently it has a potential to be used as a rumen modifier to lessen ruminal CH4 production.

We hypothesized that supplementation of CT extract from pine bark (PCT) and VCO in lactation dairy diets would alter the ruminal fermentation pattern in a desirable manner by reducing NH3-N concentration and CH4 production as a result of inhibiting microbial proteolytic activity and methanogenesis. Hence, the purpose of our study was to evaluate the effect of PCT and VCO supplementation and
their combined treatment on in vitro fermentation characteristics with focus on NH$_3$-N and CH$_4$.

Materials and Methods

Experimental design, diets and continuous culture operation

Experimental design: Four dietary treatments were tested in a completely randomized design with four independent runs of continuous cultures (n = 4). The four dietary treatments included: 1) control (CONT; TMR without supplement), 2) TMR with VCO (VCOT), 3) TMR with PCT (PCTT) and 4) TMR with VCO and PCT (VPT; Table 1). Before use in fermentors, the diets were dried at 55°C for 48 h and ground through a 4.0 mm screen (standard model 4, Arthur Thomas Co., Philadelphia, PA, USA).

<table>
<thead>
<tr>
<th>Item</th>
<th>CONT</th>
<th>VCOT</th>
<th>PCTT</th>
<th>VPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient (g·kg$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>480</td>
<td>480</td>
<td>480</td>
<td>479</td>
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<tr>
<td>Corn silage</td>
<td>166</td>
<td>167</td>
<td>143</td>
<td>141</td>
</tr>
<tr>
<td>Corn grain, steam-flaked</td>
<td>166</td>
<td>130</td>
<td>148</td>
<td>114</td>
</tr>
<tr>
<td>Beet pulp, shreds</td>
<td>53.6</td>
<td>55.5</td>
<td>61</td>
<td>57.1</td>
</tr>
<tr>
<td>SBMCM$^b$</td>
<td>116</td>
<td>120</td>
<td>120</td>
<td>133</td>
</tr>
<tr>
<td>VCO</td>
<td>0</td>
<td>29.8</td>
<td>0</td>
<td>29.6</td>
</tr>
<tr>
<td>PCT</td>
<td>0</td>
<td>0</td>
<td>29.8</td>
<td>29.5</td>
</tr>
<tr>
<td>Vitamins and minerals$^c$</td>
<td>18.1</td>
<td>17.5</td>
<td>17.4</td>
<td>17.4</td>
</tr>
<tr>
<td>Chemical composition (g·kg$^{-1}$ dry matter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (g·kg$^{-1}$)</td>
<td>944 ± 0.3</td>
<td>946 ± 0.1</td>
<td>942 ± 0.1</td>
<td>944 ± 0.2</td>
</tr>
<tr>
<td>Organic matter</td>
<td>894 ± 1.8</td>
<td>895 ± 0.7</td>
<td>893 ± 2.7</td>
<td>897 ± 1.9</td>
</tr>
<tr>
<td>Crude protein</td>
<td>204 ± 2.0</td>
<td>198 ± 2.4</td>
<td>205 ± 2.2</td>
<td>204 ± 2.2</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
<td>282 ± 6.4</td>
<td>270 ± 42.0</td>
<td>282 ± 11.2</td>
<td>249 ± 40.7</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
<td>182 ± 5.9</td>
<td>177 ± 25.8</td>
<td>182 ± 7.1</td>
<td>157 ± 31.2</td>
</tr>
<tr>
<td>Ether extract</td>
<td>9.3 ± 1.12</td>
<td>43.1 ± 2.51</td>
<td>10.2 ± 3.02</td>
<td>48.5 ± 2.33</td>
</tr>
<tr>
<td>NFC$^d$</td>
<td>399</td>
<td>359</td>
<td>407</td>
<td>373</td>
</tr>
<tr>
<td>NEL$^e$ (MJ·kg$^{-1}$ DM)</td>
<td>6.74</td>
<td>7.2</td>
<td>6.82</td>
<td>7.28</td>
</tr>
<tr>
<td>Condensed tannins</td>
<td>0.5</td>
<td>1.3</td>
<td>21.2</td>
<td>24.6</td>
</tr>
</tbody>
</table>

| Acid detergent fiber | 182 ± 5.9 | 177 ± 25.8 | 182 ± 7.1 | 157 ± 31.2 |
| Ether extract        | 9.3 ± 1.12 | 43.1 ± 2.51 | 10.2 ± 3.02 | 48.5 ± 2.33 |
| NFC$^d$              | 399     | 359    | 407    | 373    |
| NEL$^e$ (MJ·kg$^{-1}$ DM) | 6.74 | 7.2  | 6.82  | 7.28  |
| Condensed tannins    | 0.5     | 1.3    | 21.2   | 24.6   |

Table 1: Ingredients and chemical composition of experimental diets offered to continuous cultures with virgin coconut oil (VCO) and/or condensed tannins extract from pine bark (PCT). $^a$CONT = TMR without supplement, VCOT = TMR with VCO, PCTT = TMR with PCT, and VPT = TMR with VCO and PCT. $^b$SBMCM, mixture of soybean meal and canola meal (50:50 on a DM basis) was added to the diets at a similar dietary proportion (480 g·kg$^{-1}$ DM) across the diets. In addition, a mixture of soybean meal and canola meal (50:50 on a DM basis) was added to the diets at a similar dietary concentration (122 g·kg$^{-1}$ DM) so that crude protein (CP) concentration was similar across the diets (203 g·kg$^{-1}$ DM on average). The diets were formulated to meet NRC [11] recommendations for rumen degradable and undegradable proteins, minerals and vitamins of a mid-lactation dairy cow weighing 780 kg (body condition score = 3.0) and producing 36.3 kg of milk·d$^{-1}$ containing 35 g·kg$^{-1}$ fat and 30 g·kg$^{-1}$ true protein.

Continuous culture operation: Ruminal fluid (average pH of 6.9) was collected before the morning feeding (i.e. 07:00 h) from two ruminally canulated lactating Holstein dairy cows fed a TMR composed of 432 g·kg$^{-1}$ chopped alfalfa hay, 224 g·kg$^{-1}$ corn silage, 226 g·kg$^{-1}$ rolled corn grain and 118 g·kg$^{-1}$ concentrate (DM basis). Care, handling and sampling of the donor animals used in this study were approved by the Utah State University Institutional Animal Care and Use Committee. Ruminal fluid was collected from various locations within the rumen, placed into preheated insulated containers, and transported to the laboratory where rumen contents were strained through a polyester screen (PeCAP, pore size 355 μm; B and SH Thompson, Ville Mont-Royal, QC, Canada). The filtered ruminal inoculum (700 mL) was added to each continuous culture fermentor. A dual-flow continuous culture system with airtight glass culture vessels (1 L total capacity) was used, and fermentor design and operating conditions were reported elsewhere [12].

Each independent run lasted 8 d (5 d of treatment adaptation and 3 d of data and sample collection). The first day of each run allowed for microbial adaptation by offering CONT to all fermentors, and experimental diets were offered to pre-determined, corresponding fermentors from d 2. Anaerobic condition in the fermentors was maintained by infusion of CO$_2$ at a rate of 20 mL·min$^{-1}$. Artificial saliva prepared according to Slyter et al. [13] was continuously infused into fermentors at a rate of 1.2 mL·min$^{-1}$ using a peristaltic pump (Model 323, Watson-Marlow Inc., Wilmington, MA, USA) to maintain a fractional dilution rate of 10.0 %·h$^{-1}$. To mimic rumen motility, cultures were continuously stirred by a central paddle attached to an electric motor. Each fermentor received a total of 20 g of DM·d$^{-1}$ that was fed in two equal portions at 08:00 and 20:00 h. Diets were manually fed to the fermentors through a feed port on the fermentor vessel.
Sample collection

On d 6 and 7 of each run, culture pH was measured hourly through a pH electrode connected to a pH meter (sympHony TM B10p benchtop meters, VWR International, Inc., Radnor, PA, USA). At 08:00, 12:00 and 16:00 h, CH₄ samples were collected from the headspace gas of each fermentor using a 10 μL gastight syringe (Hamilton Co., Reno, NV, USA) and analyzed for CH₄ with a GLC (Model CP-3900, Varian, Walnut Creek, CA, USA). Daily CH₄ production (mM·d⁻¹) was calculated as reported by Williams et al. [14] using the equation: CH₄ proportion in fermentor headspace (mM·mL) × CO₂ gas flow through the fermentor headspace (20 mL·min⁻¹) × 60 min × 24 h. Two sets of 5 mL culture fluid samples were collected for VFA and NH₃-N analysis.

Chemical analysis

Analytical DM content of samples was determined by oven drying at 105°C for 3 h ([15]; method 930.15), and organic matter was determined by ashing at 550°C for 5 h ([15]; method 942.05). Content of N was determined using an organic elemental analyzer (Flash 2000; CE Elantech Inc., Lakewood, NJ, USA) ([15]; method 990.03). Neutral detergent fiber (aNDF) and acid detergent fiber, both expressed inclusive of residual ash, were sequentially determined using an ANKOM2000 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) according to the methodology supplied by the company, which is based on the methods described by Van Soest et al. [16]. Heat stable α-amylase (Type XI-A from Bacillus subtilis, Sigma–Aldrich Corporation, St. Louis, MO, USA) and sodium sulfite were used in the aNDF analysis. Ether extract was measured ([15]; method 2003.05) using a fat analyzer (ANKOM Technology). Total extractable CT concentration in experimental diets was determined using a butanol-HCl colorimetric procedure ([17,18]).

Culture VFA were separated and quantified using a GLC (Model 6890 series II, Hewlett Packard Co., Avandale, PA, USA) with a capillary column (30 m × 0.32 mm i.d., 1 μm phase thickness, Zebron ZB-FAAP Phenomenex, Torrance, CA, USA) and flame ionization detection. The oven temperature was held at 170°C for 4 min, increased to 185°C at a rate of 5°C·min⁻¹, then increased by 3°C·min⁻¹ to 220°C and held at this temperature for 1 min. The injector and the detector temperatures were 225 and 250°C, respectively, and the carrier gas was helium [19]. Concentration of NH₃-N was determined as described by Rhine et al. [20] using a plate reader (MRX, Dynex Technologies, Chantilly, VA, USA).

Statistical analysis

Data were analyzed using the MIXED procedure of SAS [21]. Dietary treatment was included as a fixed effect, with independent run and fermentor as random effects in the model. Denominator degrees of freedom were estimated using the Kenward-Roger option. The same mixed model was used for variables that were repeated in time (i.e., culture pH, NH₃-N and CH₄), but sampling time and a repeated statement were added to the model. One of three model structures was used depending on the finite sample corrected Akaake’s information criterion value for data that best fit the model. The structures were unstructured and compound symmetry, unstructured and first-order autoregressive, and unstructured and unstructured variance-covariance structure. Data for culture pH, VFA concentration and profile [except acetate - to - propionate (A:P) ratio] and total CH₄ production were reported using the unstructured and compound symmetry structure, whereas data for A:P ratio, CH₄ production and NH₃-N concentration were reported using the unstructured and unstructured variance-covariance structure. Effects were declared significant if P<0.05, and trends were discussed at 0.05 ≤ P<0.10.

Results and Discussion

Experimental diets

Ingredients and nutrient composition of diets are presented in Table 1. The diets ranged in CP content from 198-205 g·kg⁻¹ DM. Supplementing VCO sizably increased ether extract contents in VCOT and VPT, whereas it decreased contents of non-fiber carbohydrates in both of the diets. Contents of CT in PCT-containing PCTT and VPT were 21.2 and 24.6 g·kg⁻¹ DM, respectively.

Culture pH and VFA profiles

Supplementation of VCO and/or PCT did not affect culture pH and concentration of total VFA which averaged 6.19 and 37.2 mM, respectively (Table 2). The cultures in this study were offered high-forage diets, and consequently it was expected that dietary treatments would not have a negative effect on culture pH. Total VFA concentration did not differ in response to supplementing VCO and/or PCT. Therefore, supplementation of VCO and PCT at 29.8 g·kg⁻¹ DM in lactation dairy TMR with a forage-to-concentrate ratio of 63:37 on average would not interfere with ruminal physiological conditions.

Supplementation of VCO decreased acetate proportion in VCOT, but not in VPT (Table 2). Cultures offered VCO-containing diets (VCOT and VPT) increased butyrate proportion. Dohme et al. [22] observed that supplementing CCO (53 g·kg⁻¹ DM) decreased propionate and increased butyrate proportions by suppression of ciliates in continuous cultures. Van Nevel and Demeyer [23] reported that supplementing C10:0 and C12:0 at concentrations of 0.01-0.1 g·L⁻¹ stimulated butyrate-producing bacteria (Butyribrio sp.).

Supplementation of PCT increased acetate proportion, while it did not influence propionate proportion, leading to a tendency for an increase in A:P ratio (P = 0.10; Table 2). Valerate proportion decreased according to meta-analysis of in vitro studies (15 experiments and 130 treatments) by Jayanegara et al. [24], acetate proportion tended to increase (P = 0.08), while propionate tended to decrease (P = 0.08) with increasing contents of dietary tannins, resulting in an increase in ratio of Dschaak et al. [25] reported that molar proportions of acetate, propionate, and butyrate increased in a high-forage lactation dairy diet (a forage-to-concentrate ratio of 59:41), but not in a low-forage lactation dairy diet (a forage-to-concentrate ratio of 41:59) due to quebracho (Schinopsis sp.) CT extract supplementation at 30 g·kg⁻¹ DM to dairy cows, resulting in interactions between forage level and CT supplementation. Additionally, supplementing quebracho CT extract in the high-forage diet decreased A:P ratio, but in the low-forage diet, leading to an interaction between forage level and CT supplementation. Thus, dosage rate, source of CT, dietary composition, and their interactions will clearly contribute to inconsistent effects of CT on VFA patterns.
Tannin-protein binding activity in the PCT-containing diets (PCTT and VPT) were found to decrease in NH$_3$-N concentration when PCT- and VPT-supplemented diets were offered compared with CONT (Figure 1B). Medium-chain FA, which is present in high contents in CCO, is known to modify ruminal fermentation and to mitigate greenhouse gas emissions [32]. In vitro ruminal protozoal populations were completely eradicated by MCFA (particularly C10:0 and C12:0) within 6 h and decreased by long-chain unsaturated FA (C18:3, C18:2, C18:1) [33]. Odongo et al. [34] observed a 36% CH$_4$ reduction in dairy cattle fed a TMR with 50 g kg$^{-1}$ (DM) C14:0 supplementation. Dong et al. [35] reported that the supplementation of 100 mL L$^{-1}$ of CCO at a whole orchardgrass (Dactylis glomerata) hay diet and a mixture of 900 g kg$^{-1}$ wheat and 100 g kg$^{-1}$ hay diet decreased the CH$_4$ production compared with control diets at 0.51 vs. 1.84 mmoL d$^{-1}$ and 0.49 vs. 3.94 mmol d$^{-1}$, respectively, in continuous cultures. On the other hand, the effectiveness of adding lipids to the diet to reduce CH$_4$ emissions depends on many factors including level of supplementation, fat source, FA profiles, form in which the fat is administered (i.e. either as refined oil or as full-fat oilseeds) and the type of diet [36]. Refined oils that are high in MCFAs, such as CCO, palm kernel oil, canola oil or pure C14:0 are particularly effective in reducing CH$_4$ especially toward high-concentrate diets and low Ca diets [37]. The primary mechanism whereby MCFAs reduce CH$_4$ is through the toxicity they exhibit on rumen methanogens [37]. The authors indicated that with a forage-based diet, probably more C14:0 was attached to the feed particles and less to the methanogens than with a concentrate-based diet [37]. Similarly, Cosgrove et al. [38] observed that oil infusion (3:1 mixture of linseed oil and sunflower oil) up to 50 g kg$^{-1}$ DM intake to the rumen of sheep fed ryegrass pasture did not affect CH$_4$ production. Consequently, a potential effect of the VCO tested in the current study may have been inhibited on ruminal fermentation possibly due to the binding between VCO and forages (630 g kg$^{-1}$ DM) in the diets. Another possibility for the absence of VCO supplementation would be
a relatively low dietary content of VCO (29.8 g·kg⁻¹ DM) used in the present study. Studies using CT extract or CT-containing forages reported reductions in CH₄ emissions [14,39]. The CT modify growth of rumen microflora, reduce feed protein degradation, and lower feed energy losses to CH₄ [40]. However, it seems that findings on whether CT are able to genuinely suppress ruminal CH₄ formation per unit of digestible nutrient and the extent to which this effect occurs, appear to be inconsistent [24]. For example, Beauchemin et al. [3] found that feeding quebracho CT extract at up to 20 g·kg⁻¹ of DM failed to reduce CH₄ emissions from growing beef cattle. The authors indicated that the reason for this discrepancy may be due to the different CT, and/or that the level of supplementation of quebracho tannin at 20 g·kg⁻¹ DM would be below the threshold required to cause a reduction in CH₄ production [3]. Condensed tannins from various plant species have shown not only dose-dependent, but also plant-specific effects on ruminal fermentation [41], which could be related to their different chemical structures [30] and molecular weights [39,42]. Meta-analysis by Jayanegara et al. [24] indicated that the variation in CH₄ production per digestible organic matter in vivo was very high at low concentrations of dietary tannins of <20 g·kg⁻¹ DM, whereas variability clearly decreased with increasing tannin concentrations. The authors suggested that the influence of other dietary components such as proteins and carbohydrates may mask that of tannins at low levels [24]. McAllister et al. [43] reported that CT extracted from different plants vary greatly in their capacity to bind carbohydrates and proteins. In the current study, CT concentration (21.2 and 24.6 g·kg⁻¹ DM for PCTT and VPT, respectively) in PCT-containing diets would be insufficient to lessen ruminal methanogenesis.

**Conclusion**

Overall results on the present study partially supported our hypothesis by reducing NH₃-N concentration due to PCT supplementation in lactation dairy diets. We have yet to explore the effects of supplementing PCT on microbial community structure to address N transaction, microbial growth and energetic efficiency. Supplementation of PCT and/or VCO showed no response on CH₄ production. Therefore, the concentration of PCT and VCO used in this study would not be enough to affect ruminal protozoal population and methanogenic archaea. Our study demonstrated that PCT, although not effective in mitigating CH₄ production, has a potential to improve N utilization efficiency in lactation dairy diets through the decreased NH₃-N production in ruminal fermentation. However, caution should be exercised due to the fact that CT fed at relatively high concentrations may have negative effects on feed intake in ruminants, and the effects may also vary with the source of CT. Thus, further research needs to be carried out to confirm the in vitro result on N metabolism in lactating dairy cows fed with PCT and its long-term effects on lactational performance as a feed additive to improve N utilization efficiency by dairy cows.

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