Effect of technical cashew nut shell liquid on rumen methane emission and lactation performance of dairy cows

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ABSTRACT

Technical-grade cashew nut shell liquid (TCNSL) is a by-product of the cashew nut industry in tropical countries, and is known to exhibit a wide range of biological activities, including inhibitory effect against gram-positive bacteria. This study was conducted to investigate the effects of TCNSL (73.3% cardanol, 16.4% cardol, and 3.0% methylcardol) on rumen methane emission, nutrient digestibility, dry matter intake, and milk yield and composition in dairy cows. Eight multiparous Holstein cows were used in a crossover design trial with two 21-d experimental periods. The diet was based on corn silage and alfalfa haylage and was formulated to meet or exceed the energy and metabolizable protein requirements of the cows. Treatments were control (no TCNSL supplementation) or 30 g/cow per day of TCNSL. Rumen carbon dioxide emission was not affected by TCNSL. Treatment had no effect on methane emission (542 vs. 511 ± 35.3 g/cow per day, respectively) and methane emission intensity (15.0 vs. 13.9 ± 0.58 g/kg of energy-corrected milk, respectively) and tended to decrease methane emission per kilogram of dry matter intake (20.2 vs. 18.6 ± 1.04 g/kg, respectively). Dry matter intake (average 26.9 ± 1.00 kg/d), milk yield (40.0 ± 1.73 kg/d), and milk composition were not different between treatments. The TCNSL had no effect on N losses in urine and feces and total-tract apparent digestibility of nutrients, except digestibility of neutral detergent fiber tended to be increased compared with the control. Plasma urea and glucose concentrations were not affected by TCNSL. Concentration of milk C18:0 tended to be decreased (17%) by TCNSL compared with the control. In this study, TCNSL did not alter absolute methane emission in the rumen, but tended to decrease it by 8%

per kilogram of dry matter intake. The TCNSL had no effect on milk yield and composition in dairy cows.

Key words: cashew nut shell liquid, methane, dairy cow

INTRODUCTION

Research aimed at decreasing enteric methane emissions from ruminant animals has intensified in the last decade, with a variety of mitigation practices available to the livestock industries (Hristov et al., 2013). Cashew nut shell liquid (CNSL) is a dietary supplement that has shown a potential to decrease enteric methane emission in ruminants. Studies with nonlactating dairy cattle reported up to 38% reduction in methane emission (Shinkai et al., 2012). Several earlier and more recent in vitro studies suggested that the methane mitigation effect of CNSL is likely a result of inhibition of methanogenic archaea or specific bacteria contributing methanogenic substrates by the phenol-based constituents of the product (Van Nevel et al., 1971; Danielsson et al., 2014).

Cashew nut shell liquid is a by-product of cashew nut processing and has a variety of industrial uses (Menon et al., 1985; Lubi and Thachil, 2000). Natural CNSL contains mainly 4 constituents, cardanol, cardol, anacardic acid, and 6-methyl cardol (1.2, 11.3, 64.9, and 2.0% by weight, respectively), which are mixtures of constituents differing in side-chain unsaturation (Lubi and Thachil, 2000). The antibacterial properties of anacardic acids are well known, depend on their structure, and may be species-specific (Van Nevel et al., 1971; Kubo et al., 1993). Van Nevel et al. (1971) reported that methane emission tended to decrease in vitro with increasing numbers of double bonds in the alkyl chain of anacardic acid. In view of earlier work (Demeyer and Henderickx, 1967) and results obtained by Gellerman et al. (1969), Van Nevel et al. (1971) hypothesized that anacardic acids probably exert a direct toxic effect on methanogenic archaea.

Technical-grade CNSL (TCNSL) is the most abundant industrial source of the resorcinolic lipid cardol

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and the phenolic lipid cardanol (Kozubek and Tyman, 1999). It contains little or no anacardic acids because of decarboxylation during the roasting process, which converts anacardic acid to cardanol or 2-pentadecadiethyl phenol (Tyman et al., 1978). Thus, the main active constituents of TCNSL are cardanol (63%) and cardol (11%), with up to 23% polymers and other minor materials (Lubi and Thachil, 2000; Voirin et al., 2014). Being phenolic acids (anacardic acid) or lipids (cardanol and cardol), these compounds have been shown to exhibit strong antibacterial, antiprotozoal, and antifungal properties (Stasiuk and Kozubek, 2010). These authors (Stasiuk and Kozubek, 2010) suggested that interactions with biological membranes and DNA structure and cytotoxic activity are responsible for the antimicrobial and antiparasitic activities of phenolic lipids, such as cardanol and cardol from TCNSL or ginkgo fruits. Cardanol has been shown to exhibit antibacterial properties against various species (Boonsai et al., 2014), although its effectiveness is considerably lower than that of anacardic acids (Himejima and Kubo, 1991). Watanabe et al. (2010), for example, reported a 57% decrease with CNSL, but only a numerical 10% reduction in methane emission *in vitro* with heated CNSL (i.e., TCNSL).

In vivo studies with lactating animals investigating the methane-mitigating effect of CNSL or TCNSL are scarce. Most of the data reported to date are from *in vitro* experiments or with nonproducing animals. To the best of our knowledge, in only one study was TCNSL tested in lactating dairy cows producing 25 to 26 kg milk/d (Coutinho et al., 2014). Thus, a need exists to study the effect of CNSL, and particularly TCNSL (which is more available commercially), on rumen methane emission and animal productivity *in vivo* in trials with high-producing animals. The hypothesis of the current experiment was that TCNSL would decrease methane emission and have no negative effects on feed intake and milk production in lactating dairy cows. The objectives of the trial were to investigate the effect of supplementation of a corn silage-based diet with 30 g/cow per day of TCNSL on methane and carbon dioxide emission in the rumen, nutrient digestibility, urinary and fecal N losses, milk FA profile, DMI, and milk production and composition in high-producing dairy cows.

MATERIALS AND METHODS

Animals involved in this experiment were cared for according to the guidelines of the Pennsylvania State University Animal Care and Use Committee. The committee reviewed and approved the experiment and all procedures carried out in the study.

Animals and Experimental Design

The experiment was a crossover design with 2 periods and was conducted at the tiestall facility of the Pennsylvania State University's Dairy Center. Each experimental period lasted 21 d, with the first 14 d for adaptation and the last 7 d for sampling and data collection. The experiment was conducted with 8 multiparous Holstein dairy cows grouped in 2 treatment groups and averaging, 2.6 ± 0.32 lactations, 142 ± 5.1 DIM, 40.4 ± 1.71 kg/d of milk yield, and 669 ± 47.8 kg of BW at the beginning of the trial. The basal TMR (Table 1) was formulated to meet or exceed the NE_L and MP requirements of the cows based on their milk production and DMI at the beginning of the trial. Treatments were control (basal TMR) and TCNSL fed at 30 g/cow per day. The TCNSL was provided by Palmer International, Inc. (Skippack, PA) and contained cardanol (73.3%), cardol (16.4%), and 2-methylcardol (3.0%). In a previous study with lactating cows, we used TCNSL application rates of up to 7 g/cow per day (at 20 to 22 kg/d of DMI) and did not observe any negative effects on production, DMI, or total-tract nutrient digestibility (Coutinho et al., 2014). Based on these data and the application rates of CNSL used by Shinkai et al. (2012; 20 and 37 g/cow per day), the 30-g/d dose was arbitrarily chosen for the current study. The 30 g/d of TCNSL dose was close to the dose of 4 g/100 kg of BW per day suggested for CNSL by Shinkai et al. (2012). Cows were fed once daily at approximately 0800 h and feed was offered *ad libitum* to achieve approximately 10% refusals. Cows were milked twice daily at approximately 0600 and 1800 h. The TCNSL was fed top-dressed and mixed with approximately 2 kg of the freshly delivered TMR before cows returned from the morning milking. Feed was pushed up to the cows 4 to 6 times daily. Cows received recombinant bST (Posilac, Elanco Co., Greenfield, IN; 500 mg/cow i.m.) on d 1 and 10 of each experimental period.

Sampling and Measurements

Feed delivered and refusal weights were recorded daily. Forages were sampled weekly and dried in a microwave oven to determine DM. Weekly changes were made in the TMR if changes occurred in forage DM. Individual forage and concentrate ingredient samples were collected once weekly and samples of the TMR and refusals were collected twice weekly. Individual forage and TMR samples were composited (equal weight basis) by experimental period, dried at 65°C in a forced-air oven for 48 h, and ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ) through a 1-mm sieve for further analyses.

Table 1. Ingredient and chemical composition (% of DM unless noted otherwise) of the basal diet fed to dairy cows

Item	% of DM
Ingredient composition	
Corn silage ¹	43.5
Alfalfa haylage ²	15.0
Grass hay	4.4
Cottonseed, hulls	2.2
Corn grain, ground	9.0
Candy by-product meal ³	5.9
Soybean seeds, whole, heated	6.2
Canola meal, mechanically extracted	6.1
Molasses	4.2
Optigen ⁴	0.5
Mineral-vitamin premix ⁵	3.0
Analyzed, except NE _L , chemical composition ⁶	
DM	52.2
OM	7.1
CP	15.2
NDF	31.3
ADF	21.4
Starch ⁷	29.8
Ca	0.80
P	0.34
NE _L , ⁸ Mcal/kg	1.53

¹Corn silage contained (DM basis) 7.0% CP, 41.5% starch, 33.5% NDF, 21.4% ADF, 0.17% Ca, and 0.22% P.

²Alfalfa haylage contained (DM basis) 18.8% CP, 43.4% NDF, 37.8% ADF, 1.16% Ca, and 0.32% P.

³Candy by-product meal (Graybill Processing, Elizabethtown, PA) contained (DM basis) 17% CP and 27.8% NDF.

⁴Nonprotein nitrogen source (243.2% CP, DM basis) from Alltech Inc. (Nicholasville, KY).

⁵The premix (Cargill Animal Nutrition, Cargill Inc., Roaring Spring, PA) contained (% as-is basis) trace mineral mix, 0.86; MgO, 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition), 0.07; and dry corn distillers grains with solubles, 46.99; Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg; vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.

⁶Analyzed by Cumberland Valley Analytical Services (Maugansville, MD) using wet chemistry methods.

⁷Analyzed according to Bach Knudsen (1997).

⁸Estimated based on NRC (2001).

Spot urine (approximately 500 mL/sample) and fecal (approximately 400 g/sample) samples were collected on 3 consecutive days during wk 3 of each experimental period at 1000, 1600, and 2200 h (d 1); 0400, 1300, and 1900 h (d 2); and 0100 and 0800 h (d 3). Urine samples were collected by massaging the vulva. Fecal samples were collected by stimulating defecation, or from the rectum. Fecal samples were oven-dried at 65°C and composited per cow and period on an equal DM basis. Fresh fecal samples from each cow were composited on an equal wet basis by cow and period and frozen immediately at -20°C for later analysis. Urine samples were acidified with 2 M H₂SO₄ to a pH <3.0, diluted 1:10 with distilled water, and stored frozen (-20°C) for later analysis.

Rumen methane and carbon dioxide emission were measured during the last week of each experimental period using GreenFeed (C-Lock Technology Inc., Rapid City, SD). GreenFeed is an automated system to monitor gas mass fluxes from the breath of ruminant animals (Zimmerman et al., 2011). For this trial, one GreenFeed unit was used. The unit was positioned in the feed bunk in front of each cow starting at 0900, 1500, and 2100 (d 1), 0300, 1200, and 1700 (d 2), and 0000, and 0500 h (d 3) during the last wk of each experimental period. Breath gas samples were collected for 5 min followed by a 2-min background gas sample collection. The GreenFeed unit was calibrated 3 to 4 times before and 3 to 4 times after each sampling period with a standard carbon dioxide and methane gas mixture containing (mol %) carbon dioxide (0.98) and methane (0.151), with the balance being N gas (Air Liquide America Specialty Gases LLC, Houston, TX). The bait feed used was a premix containing (as-is basis): 70% ground corn grain, 28% dried molasses, and 2% soybean oil. Cows received approximately 280 g of bait feed during each sampling event (i.e., a total of 280 × 8 = 2,240 g/cow during each sampling period).

Blood samples were collected from the tail vein or artery 2 h after feeding on 2 consecutive days during wk 3 of each experimental period. Cows were weighed daily using the Afifarm 3.04E scale system (S.A.E. Afikim, Rehovot, Israel) while they exited the milking parlor. Milk production was recorded daily. Milk samples were collected from consecutive morning and evening milkings during wk 3 of each experimental period. Samples for milk composition were preserved with 2-bromo-2-nitropropane-1,3 diol for later analyses. Separate samples were collected for FA analysis. These samples were immediately centrifuged at 3,000 × *g* for 30 min at 4°C and the fat cake was stored at -20°C for later analysis.

Analytical Procedures

Dry matter intake was calculated by adjusting daily as-fed feed intake to DM percentage of the weekly TMR and refusal composite samples. Dried and ground TMR were submitted to Cumberland Valley Analytical Services (Maugansville, MD) for wet chemistry analyses of CP, NDF, ADF, Ca, and P [details at: <http://www.foragelab.com/Resources/Lab-Procedures>; AOAC International (2000) procedures 990.03, 973.18, and 985.01 for CP, ADF, and Ca and P, respectively, and Van Soest et al. (1991) for NDF].

Diluted urine samples were analyzed for allantoin (Chen et al., 1992), uric acid (Stanbio Uric Acid Kit 1045; Stanbio Laboratory Inc., San Antonio, TX), urea N (Stanbio Urea Nitrogen Kit 580; Stanbio Labora-

tory Inc.), and creatinine (Stanbio Creatinine Kit 0400; Stanbio Laboratory Inc.). Daily urine volume and excretion of urea N and total N were estimated from urinary creatinine concentration, assuming a creatinine excretion rate of 29 mg/kg of BW (Hristov et al., 2011). Excretion of urinary purine derivatives (allantoin and uric acid) was used to estimate microbial N outflow from the rumen as described in Hristov et al. (2009). A ratio of purine N to total N in rumen microorganisms of 0.134 was assumed based on the data of Valadares et al. (1999). Nitrogen content for feces and urine were analyzed on a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA) and multiplied by 6.25 to obtain CP. Fecal samples were pulverized at 30 Hz/s for 2 min using a Mixer Mill MM 200 (Retsch GmbH, Haan, Germany) before the N analysis. Feed and fecal samples were ashed for 4 h at 600°C for OM determination. Samples of TMR and feces were analyzed for starch (Bach Knudsen, 1997).

Fecal composited samples were analyzed for NDF and ADF using Ankom A200 apparatus (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991) with heat-stable amylase (Ankom Technology) and sodium sulfite (Fisher Scientific, Waltham, MA) used in the NDF procedure. Indigestible NDF was used as a digestibility marker (Schneider and Flatt, 1975) and was analyzed following a procedure described by Huhtanen et al. (1994; 288-h in situ incubation) with the modification that 25- μ m porosity filter bags (Ankom Technology) were used. Bag and residues were analyzed for NDF as described previously.

Fresh fecal samples were frozen immediately at -20°C. These samples were composited (on an equal wet weight basis) per cow and period and analyzed for bacterial genera and species distribution. The 16S rRNA gene V4 variable region PCR primers 515/806 (Caporaso et al., 2011) were used in a single-step 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MD) under the following conditions: 94°C for 3 min, followed by 28 cycles (5 cycles used on PCR products) of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. Sequencing was performed at Molecular Research DNA (www.mrd-nalab.com; Shallowater, TX) on an Ion Torrent PGM following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (Molecular Research DNA). In summary, sequences were depleted of barcodes and primers, then sequences <150 bp were removed, and sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were also removed. Sequences were denoised, operational taxonomic units were generated, and chimeras

were removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity). Final operational taxonomic units were taxonomically classified using BLASTn (Nucleotide-nucleotide Basic Local Alignment Search Tool) against a curated database derived from GreenGenes, RDP2, and NCBI (<http://rdp.cme.msu.edu>; DeSantis et al., 2006; www.ncbi.nlm.nih.gov).

Blood samples were centrifuged at $1,500 \times g$ for 20 min at 4°C and frozen at -40°C for later analysis of BUN (Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc.) and glucose (Stanbio Glucose kit 1075; Stanbio Laboratory Inc.). Samples were composited on an equal volume base for these analyses.

Morning and evening milk samples were analyzed separately so milk component concentration and yield could be weighted for morning and evening milk yields. Milk samples were analyzed for fat, protein, lactose, and MUN (Pennsylvania DHIA, University Park, PA). Fat, true protein, lactose, and MUN were determined using infrared spectroscopic method (MilkoScan 4000, Foss Electric, Hillerød, Denmark).

Milk fat samples for FA analysis were thawed at room temperature in water, pooled within day, and analyzed for FA composition as described by Rico and Harvatine (2013). Briefly, milk lipids were extracted with hexane-isopropanol and transmethylated in the presence of sodium methoxide. Quantification of FAME was performed by GC with a flame ionization detector and a capillary column (SP-2560; 100 m $\times$ 0.25 mm i.d. with 0.2- μ m film thickness; Supelco Inc. Bellefonte, PA). The initial oven temperature was 80°C, was increased by 2°C/min to 190°C and held for 15 min, and then increased by 8°C/min to 215°C and held for 17 min. Last, when separation of *trans*-10 C18:1 and *trans*-11 C18:1 was not achieved, a second 165°C isothermal run was conducted to quantify these isomers.

The chemical composition of CNSL was determined by GC-MS, performed on a Shimadzu GCMSQP2010 Ultra System (Shimadzu Scientific Instruments, Columbia, MD), equipped with an AOC-20i auto-injector. The column used was a Restek RTX-5ms (30 m, 0.25 mm i.d., 0.25 μ m df; Restek Corp., Bellefonte, PA), coated with 5% diphenyl-95% polydimethylsiloxane, operated with the following oven temperature program: 50°C, rising at 10°C/min to 200°C, rising at 2°C/min to 320°C; injection temperature and volume = 250°C and 1.0 μ L, respectively; injection mode = splitless; carrier gas = He; ion source = 300°C.

Composited milk and blood plasma samples were analyzed for oxidative stress markers. Thiobarbituric acid reactive substances and 8-isoprostane were analyzed using ELISA kits (Cayman Chemical, Ann Arbor, MI)

and oxygen radical absorbance capacity was analyzed as trolox equivalents as described elsewhere (Cao and Prior, 1999).

Statistical Analysis

All data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) assuming a crossover design. Cow was the experimental unit. With the exception of milk yield, DMI, and feed efficiency (milk yield/DMI), data collected at multiple time points (rumen gas emission, urine and fecal data, and so on) were averaged per cow and period and the average values were used in the statistical analysis. The averaged milk composition values and averaged milk yield and DMI during the last week of each experimental period were used to calculate milk fat, protein, and lactose yields. Body weight data for 3 consecutive days at the beginning of the trial, at the start of the second experimental period, and end of the trial were used in the statistical analysis. The statistical model included treatment, period, and treatment $\times$ period interaction. Cow within group was a random effect and all others were fixed. All data were analyzed for normal distribution using the UNIVARIATE procedure of SAS. The null hypothesis of normality was rejected (i.e., $P < 0.05$ and small Shapiro-Wilk W statistic) for 8-isoprostane in milk and plasma thiobarbituric acid reactive substances. These data were log-transformed and the transformed data were analyzed statistically. Dry matter intake, milk yield, and feed efficiency data from wk 3 of each experimental period were analyzed as repeated measures assuming an autoregressive first order [AR(1)] covariance structure. The model included treatment, period, day of measurement, and treatment $\times$ period and treatment $\times$ day interactions. Statistical

differences were declared at $P < 0.05$. Differences between treatments with $0.05 \leq P \leq 0.10$ were considered as a trend toward significance. Data are presented as least squares means.

RESULTS AND DISCUSSION

Emission of carbon dioxide, total or per unit of DMI or milk yield was not affected by TCNSL in this experiment (Table 2). It is noted that carbon dioxide measured by GreenFeed (or other methods used in animal science) is primarily from respiratory metabolism and, therefore, differences in rumen carbon dioxide emission among treatments may be masked by the larger respiratory carbon dioxide pool. Total methane emission was also not different between treatments, but tended to be lower (by 8%; $P = 0.10$) compared with the control when calculated per unit of DMI. Methane emission per kilogram of ECM was numerically lower (by about 7%; $P = 0.11$) for TCNSL versus the control. We have to point out, however, that these subtle effects of TCNSL on methane emission occurred in relatively short experimental periods. The critical need for long-term trials with methane mitigation compounds or practices has been discussed (Hristov et al., 2013) and was not addressed in the current study. With only 21-d experimental periods, the current study cannot claim that the observed trends for reduced methane emission by TCNSL are permanent and not temporary.

Earlier in vitro work demonstrated a strong antimicrobial and antimethanogenic effect of anacardic acids (Demeyer and Henderickx, 1967; Gellerman et al. 1969; Van Nevel et al., 1971). The interest in CNSL as an methane-mitigating agent was renewed by Watanabe et al. (2010), who reported a 57% reduction in methane emission in vitro by raw CNSL (containing about 58%

Table 2. Methane and CO₂ emissions¹ in dairy cows fed a control or a TCNSL²-supplemented diet (LSM)

Item	Control	TCNSL	SEM ³	P-value
CO ₂ , g/d	17,167	16,807	588.3	0.58
CO ₂ , g/kg of DMI	639	617	13.1	0.36
CO ₂ , g/kg of milk	418	415	32.9	0.89
CO ₂ , g/kg of ECM ⁵	479	459	20.5	0.28
CH ₄ , g/d	542	511	35.3	0.20
CH ₄ , g/kg of DMI	20.2	18.6	1.04	0.10
CH ₄ , g/kg of milk	13.6	12.7	1.28	0.21
CH ₄ , g/kg of ECM	15.0	13.9	0.58	0.11

¹Rumen gas emissions were measured using GreenFeed (C-LOCK Technology Inc., Rapid City, SD). Data were derived from 8 individual measurements staggered over a 3-d period (for details, see Materials and Methods).

²TCNSL (technical-grade cashew nut shell liquid) = 30 g/cow per day.

³Largest SEM published in table, $n = 14$ (n represents number of observations used in statistical analysis).

⁴Based on milk yield and DMI data during the gas measurement periods.

⁵Calculated according to Sjaunja et al. (1990).

anacardic acid and applied at up to 500 µg/mL of incubation medium), but only a numerical 10% reduction by TCNSL (containing 72% cardanol, but no anacardic acid). Compared with the control, both compounds increased propionate production (Watanabe et al., 2010). A later study by Shinkai et al. (2012) reported a 38% reduction in methane emission and a corresponding increase in rumen propionate in nonlactating dairy cows fed a 60% hay and 40% concentrate diet supplemented with 4 g of CNSL/100 kg of BW. In that study, it appeared that CNSL inhibited the expression of methyl coenzyme-M reductase, a key enzyme in methane synthesis by methanogenic archaea in the rumen (Wolfe, 1991). More recently, Mitsumori et al. (2014) reported similar inhibitory effect of 3 to 4 g of CNSL/100 kg of BW on methane emission in nonlactating Holstein cows and also observed increased propionate and hydrogen gas emission. In studies by Shinkai et al. (2012) and Mitsumori et al. (2014), CNSL was included in the ration as a pelleted product. Pelleting produces heat, and heat converts anacardic acid into cardanol (Tyman et al., 1978), but the effect of pelleting CNSL on its anacardic acid content was not reported by these authors. Himejima and Kubo (1991) investigated the antibacterial properties of CNSL and heated CNSL (i.e., TCNSL) and found a significant drop in MIC for some bacteria—*Brevibacterium ammoniagenes* and *Staphylococcus aureus*, for example—but not for others (*Bacillus subtilis* and *Streptococcus mutans*). Other studies have also shown antimicrobial properties of cardol or cardanol, although not as strong as those of anacardic acid (Himejima and Kubo, 1991; Boonsai et al., 2014). In a study with natural phenolic compounds, Yoon and Kim (2009) reported antibiofouling activities of both anacardic acid and cardanol (extracted from CNSL) against *Pseudomonas fluorescens*. Both compounds killed all cells within 18 (anacardic acid) and 30 h (cardanol) after addition to the culture. Thus, it is apparent that anacardic acids are responsible for most of the antimicrobial activities reported for natural CNSL. The major compounds in TCNSL (cardol and cardanol), however, also have antibacterial, antiprotozoal, and antifungal properties (Stasiuk and Kozubek, 2010). This, combined with the abundance of TCNSL, due to its industrial applications, make it an attractive methane mitigation agent.

A recent study with lambs by El-Zaiat et al. (2014) reported no additive effect of CNSL (about 49% anacardic acid and 39% cardanol) and nitrate on methane emission or propionate concentration. In that study, CNSL was supplemented at about 0.14% of dietary DM (which is comparable to the application rate of TCNSL in the current experiment). Neither treatment had an

effect on DMI, growth rate, or feed efficiency of the lambs. In vitro, a CNSL extract product (produced by the authors with unknown ingredient composition) had no effect (applied at 83 µg/mL) or reduced methane emission by 18% when applied at 166 µg/mL (Danielsson et al., 2014). That study also reported a shift in rumen bacterial and archaeal populations with the higher CNSL extract application rate clustering with *Barnesiella*, unclassified *Porphyromonadaceae*, and *Succinivibrio*.

Intake of nutrients was not different between diets in the current experiment (Table 3). Also, no difference was observed in apparent total-tract digestibility of nutrients, except that NDF digestibility tended to be increased ($P = 0.09$) by TCNSL. Populations of the most prevalent genus of fecal bacteria were not affected by TCNSL (Table 4). Compared with the control, TCNSL decreased or tended to decrease the proportions of *Butyrivibrio*, *Barnesiella*, and *Odoribacter* ($P = 0.02$, 0.08 , and 0.07 , respectively) and increased ($P = 0.03$) that of *Succinivibrio*. Rumen contents could not be sampled in the current study and, therefore, fecal samples were used to derive some information on the effect of TCNSL on rumen bacteria. Nitrogen intake, urinary total-, urea-, and fecal-N excretions, milk protein N secretion, urinary purine derivatives excretion (and the estimated microbial N synthesis in the rumen), and plasma urea N were not affected by TCNSL (Table 5).

Dry matter intake, milk yield, milk composition, ECM, milk NE_L, and cow BW were unaffected by TCNSL (Table 6). Milk yield was numerically greater ($P = 0.13$) for TCNSL compared with the control. Concentration of MUN was increased (by 14%; $P = 0.03$) by TCNSL.

Very little data exists on the effect of CNSL or TCNSL on digestibility, urinary or fecal N excretion, and milk production in dairy cows, and no data exists on high-producing animals. In one of the experiments of Shinkai et al. (2012), total-tract digestibility of dietary DM and energy were decreased by CNSL. In a recent study, Coutinho et al. (2014) reported that TCNSL (73.3% cardanol, 16.4% cardol, and 3.0% methylcardol) supplemented to the diet of dairy cows (up to 0.036% of DM, or 7 g/d) had no effect on DMI, apparent total-tract digestibility of nutrients, FCM yield, and milk composition (milk production of the cows was 25 to 26 kg/d).

Odd- and branched-chain milk FA have been proposed as biomarkers of microbial synthesis or methane emission in the rumen (Vlaeminck et al., 2006; Dijkstra et al., 2011; Fievez et al., 2012). A recent meta-analysis pointed out several relatively weak (or “moderately strong,” as termed by the authors) relationships be-

Table 3. Nutrient intakes and apparent total-tract digestibilities in dairy cows fed a control or a TCNSL¹-supplemented diet (LSM)

Item	Control	TCNSL ¹	SEM ²	P-value
Intake, ³ kg/d				
OM	24.7	25.7	0.80	0.24
CP	4.0	4.2	0.14	0.25
NDF	8.5	8.9	0.28	0.23
ADF	5.3	5.5	0.18	0.25
Starch	7.9	8.2	0.26	0.27
Apparent total-tract digestibility, %				
DM	61.9	62.7	0.77	0.25
OM	63.0	63.8	0.77	0.20
CP	60.2	59.8	1.26	0.86
NDF	34.2	36.8	1.47	0.09
ADF	28.7	29.5	1.91	0.63
Starch	97.8	97.7	0.10	0.60

¹TCNSL (technical-grade cashew nut shell liquid) = 30 g/cow per day.²n = 16 (n represents number of observations used in statistical analysis).³DMI is shown in Table 6.

tween milk FA and rumen methane emission (van Lingen et al., 2014). Therefore, it is of interest to monitor milk FA profile in experiments aimed at rumen methane mitigation. Milk FA composition data from the current experiment are shown in Table 7. Overall, the effect of TCNSL on milk FA was subtle, except a trend for a 17% reduction in C18:0 ($P = 0.06$). The concentration of C4:0 was also decreased ($P = 0.02$) and that C14:0 *iso* was numerically decreased ($P = 0.11$) by TCNSL compared with the control. A numerical increase ($P =$

0.11) as a result of TCNSL supplementation of the diet was observed for C13:0. To the best of our knowledge, only one other study, that of Coutinho et al. (2014), reported milk FA data for lactating cows receiving TCNSL. In that study, the authors observed a linear decrease in C6:0, a quadratic response in C13:1n-5, and a linear increase in C16:1n-7 concentrations. The milk FA data from the current experiment are difficult to interpret without having rumen fermentation data. Of the milk FA affected by TCNSL in the current study,

Table 4. Fecal bacterial populations in dairy cows fed a control or a TCNSL¹-supplemented diet (LSM; percentage² of total isolates)

Item	Control	TCNSL	SEM ³	P-value
Family				
<i>Ruminococcaceae</i>	48.0	45.0	3.00	0.51
<i>Peptostreptococcaceae</i>	4.72	5.37	1.31	0.66
<i>Prevotellaceae</i>	3.61	3.58	0.31	0.89
Genus				
<i>Bifidobacterium</i>	5.35	7.03	2.59	0.62
<i>Bacteroides</i>	5.93	5.70	0.76	0.79
<i>Ruminococcus</i>	3.59	3.57	0.28	0.96
<i>Alistipes</i>	3.31	3.05	0.20	0.24
<i>Coprococcus</i>	2.37	2.58	0.27	0.57
<i>Pseudobutyrvibrio</i>	2.57	1.86	0.67	0.47
<i>Clostridium</i>	1.79	2.03	0.26	0.42
<i>Turicibacter</i>	1.21	1.57	0.54	0.40
<i>Blautia</i>	1.17	1.51	0.16	0.12
<i>Barnesiella</i>	1.26	1.00	0.09	0.08
<i>Methanobrevibacter</i>	0.92	1.10	0.17	0.25
<i>Treponema</i>	0.90	1.05	0.27	0.32
<i>Dermatophilus</i>	1.09	0.82	0.11	0.10
<i>Fecalibacterium</i>	0.68	1.04	0.19	0.29
<i>Butyrivibrio</i>	0.59	0.48	0.08	0.02
<i>Succinivibrio</i>	0.16	0.30	0.04	0.03
<i>Odoribacter</i>	0.11	0.08	0.02	0.07

¹TCNSL (technical-grade cashew nut shell liquid) = 30 g/cow per day.²Represents percentage of the total sequences analyzed within the sample.³n = 16 (n represents number of observations used in statistical analysis).

Table 5. Urinary purine derivatives (PD) and N excretion and secretion in dairy cows fed a control or a TCNSL¹-supplemented diet (LSM)

Item	Control	TCNSL	SEM ²	P-value
N intake, g/d	646	673	27.3	0.24
Urinary and fecal excretions				
Urine output, kg/d	15.5	16.2	1.20	0.57
Urine N, g/d	151	160	8.3	0.50
% of N intake	23.5	23.7	1.13	0.88
Urea N, g/d	81.3	93.3	6.30	0.27
Fecal N, g/d	257	272	15.0	0.39
% of N intake	39.8	40.2	1.26	0.86
Urinary and fecal N excretion, g/d	408	432	20.9	0.43
% of intake	63.3	63.9	1.87	0.86
Milk protein N, g/d	190	197	8.93	0.49
% of N intake	29.5	29.5	0.95	0.97
Urinary PD excretion, mmol/d				
Allantoin	557	561	31.2	0.95
Uric acid	70.6	72.2	6.73	0.78
Total PD	627	633	34.7	0.93
Microbial N synthesis in the rumen, ³ g/d	536	541	32.2	0.93
PUN, ⁴ mg/dL	9.1	9.2	0.44	0.92
Blood glucose, mg/dL	77.9	81.2	2.70	0.45

¹TCNSL (technical-grade cashew nut shell liquid) = 30 g/cow per day.²n = 16 (n represents number of observations used in statistical analysis).³Estimated based on urinary PD excretion (see Materials and Methods).⁴Plasma urea N.

only C14:0 *iso* was also found to be positively related to methane emission by van Lingen et al. (2014).

Concentration of oxidative status (anti- or pro-oxidant) markers in milk and plasma was not affected by TCNSL (Table 8). There was a numerical, 12% increase ($P = 0.14$) in the trolox equivalent antioxidant capacity of milk from the TCNSL-treated cows compared with milk from the control cows. Anacardic acid and both cardol and cardanol have strong antioxidant properties and by-products of the cashew nut industry, such

as CNSL and cashew fiber, have been recommended as anticancer agents (Trevisan et al., 2006). A recent review by Voirin et al. (2014) offered extensive evidence of the antioxidant properties of cardanol, cardol, and their derivatives. The antioxidant capacity of anacardic acid, however, is reportedly much greater (6 to 14 times; Trevisan et al., 2006) than that of cardol and cardanol and the lack of effect on antioxidant markers in milk and plasma in the current trial is likely due to the absence of anacardic acid in the TCNSL product.

Table 6. Dry matter intake, milk yield, milk composition, and BW in dairy cows fed a control or a TCNSL-supplemented diet¹ (LSM)

Item	Control	TCNSL	SEM ²	P-value
DMI, kg/d	26.5	27.3	1.00	0.30
Milk yield, kg/d	39.0	40.9	1.73	0.13
Milk yield/DMI, kg/kg	1.49	1.51	0.06	0.55
Milk fat, %	3.35	3.28	0.28	0.74
kg/d	1.29	1.35	0.14	0.59
Milk true protein, %	3.10	3.08	0.05	0.62
kg/d	1.21	1.26	0.07	0.49
Milk lactose, %	4.68	4.68	0.06	0.91
kg/d	1.83	1.91	0.16	0.38
MUN, mg/dL	7.50	8.57	0.62	0.03
ECM, ³ kg/d	35.1	36.5	1.65	0.46
BW, ⁴ kg	672	676	4.37	0.48

¹DMI and milk production and composition data are from wk 3 of each experimental period; TCNSL (technical-grade cashew nut shell liquid) = 30 g/cow per day.²DMI, n = 112; milk yield, n = 104; milk yield/DMI, n = 104; others, n = 16 (n represents number of observations used in statistical analysis).³Calculated according to Sjaunja et al. (1990).⁴Average BW during the trial.

Table 7. Fatty acid composition of milk fat in dairy cows fed a control or a TCNSL¹-supplemented diet (LSM)

FA (g/100 g of FA)	Control	TCNSL	SEM ²	P-value
4:0	4.22	3.69	0.147	0.02
6:0	2.16	1.97	0.131	0.21
8:0	1.17	1.15	0.106	0.90
10:0	2.62	2.84	0.322	0.54
11:0	0.06	0.09	0.015	0.16
12:0	3.12	3.57	0.372	0.29
13:0	0.11	0.14	0.017	0.11
14:0	10.1	10.8	0.599	0.32
14:0 <i>iso</i>	0.09	0.06	0.017	0.11
14:1	0.98	1.07	0.103	0.39
15:0	0.98	1.11	0.076	0.15
15:0 <i>iso</i>	0.15	0.14	0.008	0.22
15:0 <i>anteiso</i>	0.35	0.34	0.009	0.51
16:0	25.7	24.1	1.012	0.18
16:0 <i>iso</i>	0.18	0.16	0.032	0.60
16:1	1.50	1.58	0.204	0.69
17:0	0.47	0.49	0.022	0.57
17:0 <i>isso</i>	0.25	0.25	0.016	0.69
17:0 <i>anteiso</i>	0.32	0.34	0.026	0.47
17:1	0.18	0.22	0.021	0.20
18:0	11.5	9.52	0.817	0.06
<i>trans</i> -4 18:1	0.03	0.03	0.002	0.65
<i>trans</i> -5 18:1	0.03	0.03	0.003	0.45
<i>trans</i> -6,8 18:1	0.49	0.61	0.114	0.35
<i>trans</i> -9 18:1	0.36	0.45	0.080	0.31
<i>trans</i> -10 18:1	1.29	2.14	0.886	0.39
<i>trans</i> -11 18:1	1.03	1.13	0.197	0.64
<i>trans</i> -12 18:1	0.67	0.69	0.055	0.71
<i>cis</i> -9 18:1	20.1	20.6	0.648	0.52
<i>cis</i> -11 18:1	0.86	1.05	0.113	0.15
<i>cis</i> -12 18:1	0.53	0.60	0.071	0.40
Linoleic acid	3.22	3.42	0.226	0.42
<i>cis</i> -9, <i>trans</i> -11 CLA	0.57	0.72	0.121	0.27
<i>trans</i> -10, <i>cis</i> -12 CLA	0.01	0.01	0.007	0.55
γ -Linolenic acid	0.02	0.03	0.006	0.69
α -Linolenic acid	0.47	0.50	0.050	0.58
20:0	0.16	0.15	0.006	0.11
20:2	0.04	0.05	0.005	0.21
20:3	0.11	0.12	0.009	0.23
20:4	0.15	0.17	0.002	0.28
22:0	0.05	0.05	0.002	0.19
Σ SFA	63.9	60.9	1.956	0.19
Σ MUFA	28.0	30.2	1.547	0.23
Σ PUFA	1.37	1.59	0.160	0.22
Σ <i>trans</i> FA	3.92	5.07	1.000	0.31
Unknown	3.40	3.92	0.163	0.03

¹TCNSL (technical-grade cashew nut shell liquid) = 30 g/cow per day.²n = 15 (n represents number of observations used in statistical analysis).

CONCLUSIONS

In this experiment, TCNSL (containing cardanol and cardol as main active ingredients, but no anacardic acids) had no effect on rumen methane emission in dairy cows, but tended to decrease it by 8% when expressed per kilogram of DMI. The product tended to increase total tract NDF digestibility, but had no effect on digestibility of the other dietary nutrients. Nitrogen excretion in urine and feces, bacterial composition of fecal matter, and antioxidative status of milk and plasma were not affected by TCNSL. Compared with

the control, TCNSL decreased by 17% the concentration of C18:0 in milk fat.

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Table 8. Oxidative status markers in milk and blood plasma of dairy cows fed a control or a TCNSL¹-supplemented diet (LSM)

Item	Control	TCNSL	SEM ²	P-value
Milk				
TBARS, ³ $\mu\text{L}/\text{sample}$	14.5	13.3	2.62	0.69
8-isoprostane, ⁴ pg/mL	1,003	1,031	313	0.85
TEAC, ⁵ trolox equivalent, μM	6,360	7,131	430	0.14
Blood plasma				
TBARS, $\mu\text{L}/\text{sample}$	7.34	7.28	0.93	0.95
8-isoprostane, pg/mL	25.1	26.9	2.11	0.43
TEAC, ⁴ trolox equivalent, μM	4,260	3,766	495	0.37

¹TCNSL (technical-grade cashew nut shell liquid) = 30 g/cow per day.²n = 15 (n represents number of observations used in statistical analysis).³Thiobarbituric acid reactive substances.⁴Statistical analysis was performed on log-transformed data.⁵Trolox equivalent antioxidant capacity.

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