In vitro evaluation of cashew nut shell liquid as a methane-inhibiting and propionate-enhancing agent for ruminants

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ABSTRACT

Cashew nut shell liquid (CNSL) containing antibacterial phenolic compounds was evaluated for its potency as a feed additive for ruminants. In experiment 1, ruminal responses to CNSL supplementation were assessed using a batch culture system. Rumen fluid from cattle was diluted with artificial saliva and incubated for 18 h in a batch culture with a mixed diet containing a 30:70 hay:concentrate diet to which raw or heated CNSL was added at a final concentration of 500 µg/mL. In experiment 2, a Rusitec, using rumen fluid from the same cattle, was operated over a period of 7 d during which only raw CNSL was tested at concentrations of 0, 50, 100, or 200 µg/mL, and variations in fermentation and bacterial population were assessed. In experiment 3, a pure culture study was conducted using selected bacteria to determine their susceptibility to CNSL. In experiment 1, methane production was inhibited by raw CNSL (56.9% inhibition) but not by heated CNSL. Total volatile fatty acid concentration was not affected, whereas increased concentrations of propionate and decreased concentrations of acetate and butyrate were observed using either raw or heated CNSL. These changes were more obvious when raw CNSL was tested. In experiment 2, raw CNSL inhibited methanogenesis and increased propionate production in a dose-dependent manner, showing maximum methane inhibition (70.1%) and propionate enhancement (44.4%) at 200 µg/mL supplementation. Raw CNSL increased total volatile fatty acid concentration and dry matter digestibility. Raw CNSL also appeared to induce a dramatic shift in the population of rumen microbiota, based on decreased protozoa numbers and changes in quantitative PCR assay values for representative bacterial species. In experiment 3, using pure cultures, raw CNSL prevented the growth of hydrogen-, formate-, and butyrate-producing rumen bacteria, but not the growth of bacteria involved in propionate pro-

duction. Based on these data, raw CNSL, rich in the antibacterial phenolic compound anacardic acid, is a potential candidate feed additive with selective activity against rumen microbes, leading to fermentation that results in decreased methane and enhanced propionate production.

Key words: cashew nut shell liquid, methane, propionate, rumen bacteria

INTRODUCTION

Cashew (*Anacardium occidentale*) nut shell liquid (CNSL) is a byproduct of cashew nut production in tropical countries such as India, Brazil, Mozambique, Tanzania, and Kenya. The dark brown liquid obtained from the cashew nut shell (accounting for about 32% of the shell) has many industrial applications and is used as a raw material for products such as paints, brake linings, lacquers, and coatings (Menon et al., 1985). Such a broad application of CNSL is because it is easily polymerized by various means. Because the global production of CNSL is estimated at 450,000 t/yr (Patel et al., 2006), a readily available supply of CNSL exists.

Cashew nut shell liquid is also known to exhibit a wide range of biological activities, because it contains antimicrobial (Kubo et al., 1993), antioxidative (Kubo et al., 2006), and antitumor (Itokawa et al., 1987) compounds, represented by anacardic acid, cardanol, and cardol, which are all salicylic acid derivatives with a C-15 alkyl group. These phenolic compounds, especially anacardic acid, inhibit gram-positive bacteria, including bacilli and staphylococci (Kubo et al., 1993). Therefore, CNSL, which contains these phenolic compounds, is also expected to selectively inhibit gram-positive rumen bacteria. Some of these bacteria, including *Streptococcus bovis*, are believed to contribute to the occurrence of rumen metabolic problems such as lactic acidosis and bloat in feedlot cattle (Nagaraja and Titgemeyer, 2007). Furthermore, anacardic acid, one of the main antimicrobial compounds present in CNSL, was suggested in early studies to be a propionate enhancer (Van Nevel et al., 1971), although this possibility has not been investigated further.

Received September 21, 2009.

Accepted July 10, 2010.

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We have conducted a series of in vitro experiments to determine if CNSL is effective for the improvement of rumen fermentation, with special reference to its potential to reduce production of methane and enhance production of propionate.

MATERIALS AND METHODS

Three in vitro experiments, using batch cultures to screen types of CNSL (experiment 1), semicontinuous cultures to evaluate dose responses to CNSL (experiment 2), and pure cultures to detect selective inhibition of rumen bacteria by CNSL (experiment 3), were performed in the present study.

Tested Products and Rumen Fluid

Two types of CNSL (raw and heated) were used in the present study. The raw CNSL was extracted from cashew nut shells (of Indian origin) using compressing machinery, but not organic solvents, at the Central Laboratory, Idemitsu Kosan Co. Ltd. (Sodegaura, Japan). Heated CNSL, a common commercial product, was imported from India (Cashew Co. Ltd., Tokyo, Japan).

The rumen contents were collected from 2 ruminally fistulated Holstein cows at the experimental farm of Hokkaido University (Sapporo, Japan). Cows received a diet consisting of a 1:1 orchardgrass hay to concentrate (Monster 16, Mercian, Tokyo, Japan). The diet contained 18.2% CP, 41.2% NDF, and 2.16 Mcal of ME/kg on a DM basis. The rumen contents from cows were mixed together in equal volumes, placed in a bottle after flushing with N₂ gas, and transported to the laboratory within 30 min of collection. The rumen contents were then strained through 2 layers of surgical gauze to obtain an inoculum for the series of in vitro experiments.

Experiment 1: Batch Cultures

In vitro batch fermentation was carried out as described by Yabuuchi et al. (2006). Test tubes (180 mm length and 10 mm diameter) with butyl rubber stoppers and plastic screw caps were used for the batch culture experiment. The substrate used for simulating a high grain diet was a mixture of 0.14 g of ground concentrate and 0.06 g of ground orchardgrass hay, which was the same diet used for the cows used as rumen content donors. The mixture was supplemented with raw or heated CNSL (5 mg dissolved in 0.2 mL of 99.5% ethanol). An equivalent amount of ethanol without CNSL was supplemented to the control. The ethanol

was evaporated by leaving the substrate-containing test tube at room temperature overnight before the addition of the diluted rumen fluid (10 mL), which was prepared by mixing the strained rumen fluid with McDougall's buffer (McDougall, 1948) at a 1:1 ratio. The final concentration of CNSL in this batch culture was 500 µg/mL. Incubation was carried out in quadruplicate at 38°C for 18 h under anaerobic conditions after flushing N₂ gas into the headspace. After incubation, the total volume of the headspace gas was measured via a needle-attached pressure gauge (Aϕ60B, GL Science, Tokyo, Japan) and a sample of this gas was obtained with a syringe for gas analysis. Tubes were centrifuged (1,000 × *g*, 5 min) and the supernatant was used for VFA analysis. This batch culture experiment used 12 test tubes (4 replicates each for control, raw CNSL, and heated CNSL, respectively) at each incubation run and was repeated on 3 different days (3 runs in total were carried out).

Experiment 2: Semicontinuous Cultures

A rumen simulation technique (Rusitec) was employed for the semicontinuous culture experiment (Czerkawski and Breckenridge, 1977). For the experiment, an apparatus with 16 fermenters (each 800 mL in working volume) equipped with input, output, and sampling openings (Sanshin Kogyo, Tokyo, Japan) was used and operated as described by Kajikawa et al. (2003).

Rumen fluid diluted with McDougall's buffer (McDougall, 1948) at a ratio of 3:8 was used as a starting culture. The dilution rate of the Rusitec was set at 400 mL/d using peristaltic pumps. Feeding was carried out at 24-h intervals by placing a nylon bag containing a high grain diet [the same ground concentrate (8.4 g) and orchardgrass hay (3.6 g) used in the batch culture experiment]. A nylon bag placed in each fermenter was replaced every 48 h, so that 2 bags, one as the new feed and the other as the previous feed, were always in each fermenter. At the time of withdrawal, the bag was rinsed with buffer (20 mL) to recover microbes loosely associated with the feed, and the buffer was then added to the fermenter. The fermenters were supplemented with 1 of 4 different levels of raw CNSL, with concentrations at the time of addition of 0 (control), 50, 100, or 200 µg/mL (4 fermenters were used for each concentration), which were lower than the concentration tested in the batch culture experiment (500 µg/mL). We used lower concentrations because our preliminary evaluation indicated that supplementation of 500 µg/mL CNSL decreased VFA production in the Rusitec. Raw CNSL (0, 40, 80, or 160 mg) was added at each feeding time (every 24 h) by mixing with 10 mL of

the culture fluid without using ethanol. The Rusitec was operated for 7 d, consisting of 5 d for adaptation followed by 2 d of sampling.

Samples were taken from the culture by pipette every 3 h throughout a 2-d sampling period for the analysis of VFA, ammonia, lactate, and viscosity (as an indicator of bloat). For counting protozoa, only the sample taken before feeding was employed. Samples taken at defined times during the 2-d period were employed for analysis and the obtained values were averaged within hour and then used for the statistical analysis. The gas produced was continuously collected in a plastic bag (Tedlar PDF, DuPont, Wilmington, DE) connected to each fermenter, and the total volume of gas was measured by a spirometer (Sanshin Kogyo, Tokyo, Japan).

Experiment 3: Pure Culture of Bacteria

The bacteria tested were obtained through the American Type Culture Collection (Manassas, VA) and from Dr. H. Minato (Ibaraki University, Tsukuba, Japan) and cultivated in a rumen fluid-containing medium (see below). The MIC of raw CNSL for selected rumen bacterial species was determined by spectrophotometrically monitoring the growth of a pure bacterial culture (optical density at 660 nm). The growth medium (pH 6.8) was prepared with 7.5 mL of mineral solutions I and II (Bryant and Burkey, 1953), 0.1 mL of 0.1% resazurin, 0.2 g of bactopectone, 0.12 g of yeast extract, 30 mL of rumen fluid, 50 mL of distilled water, 0.1 g of L-cysteine-HCl H₂O, 5 mL of 8% Na₂CO₃ (per 100 mL), and 0.25 g of glucose and cellobiose as carbon sources. The medium was prepared anaerobically according to the method of Ogimoto and Imai (1981) and was supplemented with raw CNSL at 7 levels ranging from 0 to 50 µg/mL (final concentration). The MIC was defined as the minimum concentration required for growth inhibition.

The bacterial species tested were *Ruminococcus flavefaciens* C94 (ATCC19208) and *Ruminococcus albus* 7 (ATCC27210), which are hydrogen and formate producers; *Butyrivibrio fibrisolvens* D1 (ATCC19171) and *Eubacterium ruminantium* GA195 (ATCC17233), which are butyrate producers; *Streptococcus bovis* ATCC33317 and *Lactobacillus ruminis* RF1 (ATCC27780), which are lactate producers; and *Fibrobacter succinogenes* S85 (ATCC19169), *Prevotella ruminicola* 23 (ATCC19189), *Selenomonas ruminantium* GA192 (ATCC12561), *Ruminobacter amylophilus* 70 (Dr. H. Minato), *Succinivibrio dextrinosolvens* 24 (ATCC19716), *Succinimonas amylolytica* B₂4 (ATCC19206), and *Megasphaera elsdenii* LC1 (ATCC25940), which are succinate and(or) propionate producers.

Chemical, Physical, and Microbiological Analyses

Phenolic compounds such as anacardic acid, cardanol, and cardol were quantified by HPLC, essentially as described by Paramashivappa et al. (2001). The HPLC and its operating conditions were as follows: HPLC system, Waters 400 (Japan Waters, Tokyo, Japan); HPLC column, Supelcosil LC-18 (150 mm × 4.6 mm i.d.; Supelco, Bellefonte, PA); elution solution, acetonitrile:water:acetate (80:20:1); flow rate, 2.0 mL/min; detection at 280 nm, UV detector (Waters 490E, Japan Waters). Samples (25 mg) were dissolved in 5 mL of acetonitrile and passed through a C18 SepPak cartridge before injection of 20 µL for HPLC analysis. Separately purified anacardic acid, cardanol, and cardol, with a monoenoic, dienoic, or trienoic C-15 alkyl chain (Idemitsu Kosan Co. Ltd., Sodegaura, Japan), were used as standards for quantification of each phenolic molecule.

Collected gas samples and VFA were analyzed by gas chromatography (Shimadzu 14B, Kyoto, Japan) fitted with a thermal conductivity detector and a flame-ionization detector, respectively. The columns used and the operating conditions were as described by Ushida et al. (1985) and Suto (1973), respectively. Ammonia and lactate were assayed by spectrophotometry using the indophenol method (Weatherburn, 1967) and a commercially available kit (Megazyme, Wicklow, Ireland), respectively. Protozoa were manually counted using a microscope (Ogimoto and Imai, 1981). The viscosity of the pipetted culture was determined using a Vibro viscometer (SV-1, A&D, Tokyo, Japan). Apparent DM disappearance of the feed in the Rusitec experiment was determined as DM lost during the 48-h incubation in nylon bags.

To determine the basic mode of action of CNSL in rumen fermentation, representative rumen bacteria in Rusitec fermenters were quantified using samples from the control and highest CNSL dose (200 µg/mL) that recorded the most drastic shift in methane and propionate production. Total DNA of the rumen samples from each Rusitec fermenter (2-d composite) was extracted by using a bead-beating method (Purdy et al., 1996). These samples were taken from fermenter fluid at the time of withdrawal of the nylon bag (after rinsing) so that both planktonic and feed-associated microbes were contained. The extracted DNA was then purified by hydroxyapatite chromatography and gel filtration as described by Koike et al. (2003). The obtained DNA was used for real-time PCR assays to quantify specific bacterial species. The bacteria quantified included total bacteria, total archaea, and the following individual bacterial species: *F. succinogenes*, *R.*

Table 1. Chemical composition of cashew nut shell liquid

Item	Cashew nut shell liquid	
	Raw	Heated
Ingredient (% on a wet basis)		
Moisture	1.7	0.5
CP	1.5	1.1
Crude fiber	—	—
Crude ash	1.2	1.8
Nitrogen-free extract	3.0	—
Ether extract	92.6	96.6
Ether extract fractions (% of ether extract)		
Total anacardic acid	62.3	—
monoenoic (15:1)	28.9	—
dienoic (15:2)	8.8	—
trienoic (15:3)	24.6	—
Total cardanol	8.9	73.9
monoenoic (15:1)	2.9	28.5
dienoic (15:2)	1.5	18.9
trienoic (15:3)	4.5	26.5
Total cardol	21.5	14.9
monoenoic (15:1)	3.1	2.5
dienoic (15:2)	4.3	4.2
trienoic (15:3)	14.1	8.2
Quantifiable phenolics in total	92.7	88.8
Other extract ¹	7.3	11.2

¹Composed mostly of unidentified constituents with negligible fatty acids and their esters.

flavefaciens, *R. albus*, *P. ruminicola*, *Prevotella brevis*, *Treponema bryantii*, *S. dextrinosolvens*, *M. elsdenii*, *S. ruminantium*, *S. bovis*, *Anaerovibrio lipolytica*, and *R. amylophilus*. All the quantification procedures, including primers, standards, PCR conditions, as well as the calculations, were according to Koike et al. (2007) and Ohene-Adjei et al. (2008). In brief, real-time PCR was performed with a LightCycler system and a FirstStart DNA Master SYBR Green I reaction kit (both from Roche, Penzberg, Germany). The 10-fold dilution series of the standard plasmid for the respective target (16S rDNA sequence specific to each bacterial species) was run along with samples. Quantification was made using standard curves obtained from the amplification profile of known concentrations of the standard plasmid for the respective target. Specificity of each PCR amplification was confirmed by the dissociation curve of the PCR end-products by increasing the temperature from 70 to 95°C at a rate of 0.1°C/s. The relative abundance of each bacterial species was calculated as a percentage of the total level of bacteria.

Statistical Analysis

The data from 4 replicates per treatment in the batch culture experiment were averaged at each incubation run and subjected to ANOVA using the GLM procedure of SPSS (Version 16.0 J, Tokyo, Japan), in which

treatment (control vs. raw CNSL vs. heated CNSL) was a fixed effect and incubation run (run 1 to run 3) was a random effect. Tukey's method was employed for multiple comparisons. In the Rusitec experiment the data concerning gas, protozoa, and DM disappearance were analyzed by considering treatment (CNSL level) as a fixed effect and fermenter within treatment as a random effect. The data concerning VFA, pH, ammonia, lactate, and viscosity were subjected to ANOVA using the mixed procedure of SPSS with repeated measures. The model included the effect of treatment (CNSL level), time after feeding, and their interaction, random effect of Rusitec fermenters within treatment, and residual error. Time after feeding was used as the repeated measures. Regressions between fermentation parameters and supplementation levels were analyzed to predict the dose-dependent responses to CNSL. Orthogonal contrasts were used to test linear and quadratic effects of treatment (CNSL level). Real-time PCR assay values were subjected to Student's *t*-test to detect the effect of CNSL supplementation. Statistical significance was defined at $P \leq 0.05$.

RESULTS

Components in CNSL

The chemical composition of raw and heated CNSL is shown in Table 1. Both CNSL contained 92.6 to 96.6% ether extract with 0.5 to 1.7% moisture, 1.1 to 1.5% CP, 1.2 to 1.8% crude ash, and 0 to 3.0% nitrogen-free extract. The ether extract was composed mostly of phenolic compounds (88.8–92.7%) but not fatty acids or their esters. The phenolic compounds present in CNSL were mainly anacardic acid, cardanol, and cardol. However, the proportions of these phenolic compounds differed greatly between heated and raw CNSL. Thus, anacardic acid was the main phenol in raw CNSL (62.3%), but was not detected in heated CNSL. Heated CNSL was richer in cardanol than raw CNSL (73.9 vs. 8.9%). Each phenolic compound was composed of 3 different molecules with differently saturated alkyl chains (C15:1, C15:2, and C15:3). Free fatty acids and glycerol esters were not observed at detectable levels in either type of CNSL.

Batch Culture Experiment

The results of the batch fermentation experiment are shown in Table 2. Raw, but not heated, CNSL apparently reduced total gas, CO₂, and methane production. The inhibition of methane production by raw CNSL was quite high (56.9%). Neither type of CNSL induced any notable change in hydrogen levels.

Table 2. Effect of raw or heated cashew nut shell liquid on gas and VFA production in batch cultures

Item	Cashew nut shell liquid ¹			SEM	P-value
	None	Raw	Heated		
Total gas (mL)	4.92 ^a	3.30 ^b	4.76 ^a	0.244	0.01
CO ₂ (mL)	3.87 ^a	2.83 ^b	3.58 ^a	0.173	0.01
CH ₄ (mL)	1.09 ^a	0.47 ^b	0.99 ^a	0.082	0.01
H ₂ (mL)	0.020	0.013	0.015	0.0023	0.37
Total VFA (mM)	97.9	94.7	96.5	2.38	0.09
Acetate (mM)	63.5 ^a	56.3 ^c	60.2 ^b	1.11	0.05
Propionate (mM)	20.8 ^c	31.6 ^a	26.4 ^b	0.42	0.01
n-Butyrate (mM)	13.6 ^a	6.8 ^b	9.9 ^{ab}	0.19	0.04
Acetate (mol/100 mol)	64.9 ^a	59.5 ^b	62.4 ^{ab}	1.18	0.04
Propionate (mol/100 mol)	21.2 ^c	33.4 ^a	27.4 ^b	0.42	0.01
n-Butyrate (mol/100 mol)	13.9 ^a	7.2 ^b	10.3 ^{ab}	0.19	0.03

^{a-c}Means (n = 3) within a row with different superscripts differ ($P < 0.05$).

¹Cashew nut shell liquid: None = no supplementation (control); Raw = 500 µg/mL raw cashew nut shell liquid; Heated = 500 µg/mL heated cashew nut shell liquid.

Neither raw CNSL nor heated CNSL affected total VFA production. The production of propionate was enhanced by raw CNSL, whereas the production of acetate and butyrate was decreased. Heated CNSL also enhanced propionate and reduced acetate production, although to a much lower extent than raw CNSL. Heated CNSL did not affect butyrate production.

Semicontinuous Culture Experiment

The effects of raw CNSL supplementation on the chemical, physical, and microbial characteristics of the fermentation fluid in the Rusitec experiment are shown in Table 3.

Total gas and CO₂ production were not affected by supplementation with raw CNSL. Methane produc-

tion was decreased by raw CNSL supplementation in a dose-dependent manner (linear and quadratic effect). The maximal decrease (70.1%) was observed at a final concentration of 200 µg/mL of raw CNSL. Total VFA concentration quadratically increased with raw CNSL supplementation. The concentration of individual VFA was differentially affected by raw CNSL, with increased propionate (44.4%) and decreased acetate and butyrate (13–14%) at 200 µg/mL of raw CNSL. Propionate showed a raw CNSL dose-dependent increase (linear and quadratic effect), whereas acetate and butyrate showed a dose-dependent decrease (quadratic effect).

Ruminal pH decreased at all levels of supplemented raw CNSL (50–200 µg/mL) in a quadratic manner. Neither lactate concentration nor culture viscosity was affected by raw CNSL supplementation. Ammonia con-

Table 3. Effect of raw cashew nut shell liquid supplementation on the fermentation profile in the semicontinuous Rusitec culture

Item	Raw cashew nut shell liquid (µg/mL)				SEM	P-value for dose dependency ¹	
	0	50	100	200		Linear	Quadratic
Total gas (mL/d)	574.0	512.4	590.7	527.6	65.41	0.85	0.79
CO ₂ (mL/d)	507.3	468.7	551.9	503.2	54.81	0.73	0.60
CH ₄ (mL/d)	65.3 ^a	42.0 ^b	32.7 ^b	19.5 ^b	5.78	0.01	0.01
H ₂ (mL/d)	1.3	1.7	6.1	5.0	1.02	0.85	0.73
Total VFA (mM)	115 ^b	127 ^{ab}	127 ^a	125 ^{ab}	1.4	0.14	0.05
Acetate (mM)	50 ^a	51 ^a	51 ^a	43 ^b	0.7	0.11	0.03
Propionate (mM)	27 ^c	31 ^b	37 ^a	39 ^a	0.6	0.04	0.01
n-Butyrate (mM)	16 ^b	17 ^a	16 ^b	14 ^c	0.2	0.12	0.04
pH	6.59 ^a	6.48 ^b	6.40 ^c	6.48 ^b	0.010	0.21	0.05
Ammonia (mg of N/L)	176 ^a	186 ^a	178 ^a	147 ^b	3.1	0.14	0.04
Lactate (mM)	1.7	1.1	1.2	0.8	0.02	0.36	0.15
Viscosity (cp)	1.01	1.02	1.04	1.04	0.007	0.30	0.18
Protozoa (log cells/mL)	3.75 ^a	3.69 ^a	3.52 ^a	3.24 ^b	0.135	0.05	0.04
DM disappearance (%)	73.6 ^b	78.9 ^a	78.3 ^a	78.3 ^a	0.73	0.26	0.05

^{a-c}Means (n = 4) within a row with different superscripts differ ($P < 0.05$).

¹Orthogonal contrasts tested linear and quadratic effects of raw cashew nut shell liquid supplementation.

Figure 1. Absolute abundance of representative rumen bacteria in the Rusitec expressed as 16S rDNA copy number per milliliter determined by real-time PCR. TB = total bacteria; TA = total archaea; Rf = *Ruminococcus flavefaciens*; Rb = *Ruminococcus albus*; Tb = *Treponema bryantii*; Sb = *Streptococcus bovis*; Fs = *Fibrobacter succinogenes*; Pr = *Prevotella ruminicola*; Pb = *Prevotella bryantii*; Sd = *Succinivibrio dextrinosolvens*; Ra = *Ruminobacter amylophilus*; Al = *Anaerovibrio lipolytica*; Sr = *Selenomonas ruminantium*; Me = *Megasphaera elsdenii*. Open bars = control; solid bars = raw cashew nut shell oil. * = significantly different from control.

centration decreased quadratically by supplementation of raw CNSL, showing a minimum ammonia concentration at 200 $\mu\text{g/mL}$ of raw CNSL. The number of rumen protozoa decreased from $10^{3.75}$ to $10^{3.24}$ /mL of culture (linear and quadratic effect). The majority (>90%) of the protozoa were classified as *Entodinium* spp. and the observed decrease was irrespective of the genera detected (data not shown). Apparent DM disappearance at 48 h increased quadratically with raw CNSL supplementation, and the disappearance at all levels of raw CNSL (50–200 $\mu\text{g/mL}$) was higher than that of control.

The absolute and relative abundance of representative rumen bacteria in the Rusitec experiment are shown in Figures 1 and 2, respectively. Although the levels of total bacteria and total archaea were largely unchanged, dramatic changes were observed in the levels of most of the individual bacterial species examined. Bacterial species that showed a decreased abundance were *R. flavefaciens*, *T. bryantii*, *F. succinogenes*, *P. ruminicola*,

and *P. bryantii*, whereas those showing an increased abundance were *S. dextrinosolvens*, *R. amylophilus*, *A. lipolytica*, *S. ruminantium*, and *M. elsdenii*. Of the bacterial species tested, the species with the highest abundance in the control samples was *P. ruminicola* (4.8%), whereas that in samples supplemented with 200 $\mu\text{g/mL}$ raw CNSL was *M. elsdenii* (11.6%).

Pure Culture Experiment

The MIC of raw CNSL determined for selected rumen bacteria is indicated in Table 4. All bacteria possessing a gram-positive-type cell wall were highly or moderately sensitive to raw CNSL. Thus, raw CNSL exhibited a low MIC (1.56–6.25 $\mu\text{g/mL}$) toward *R. flavefaciens*, *R. albus*, *E. ruminantium*, and *B. fibrisolvens* and a moderate MIC (25–50 $\mu\text{g/mL}$) toward *S. bovis* and *L. ruminis*. In contrast, 4 of the 7 gram-negative bacteria tested (*S. dextrinosolvens*, *R. amylophilus*, *S. ruminantium*, and *M. elsdenii*) were insensitive to raw

Figure 2. Relative abundance of representative rumen bacteria in the Rusitec, determined as the 16S rDNA copy number by real-time PCR, and expressed as a percentage of the total bacterial level. TA = total archaea; Rf = *Ruminococcus flavefaciens*; Rb = *Ruminococcus albus*; Tb = *Treponema bryantii*; Sb = *Streptococcus bovis*; Fs = *Fibrobacter succinogenes*; Pr = *Prevotella ruminicola*; Pb = *Prevotella bryantii*; Sd = *Succinivibrio dextrinosolvens*; Ra = *Ruminobacter amylophilus*; Al = *Anaerovibrio lipolytica*; Sr = *Selenomonas ruminantium*; Me = *Megasphaera elsdenii*. Open bars = control; solid bars = raw cashew nut shell oil. Arrows indicate significant decreases and increases. * = significantly different from control.

CNSL, showing an MIC of ≥ 50 $\mu\text{g/mL}$. The other 3 gram-negative bacteria tested (*F. succinogenes*, *P. ruminicola*, and *S. amylolytica*) were sensitive to raw CNSL, showing an MIC that ranged from 3.13 to 12.5 $\mu\text{g/mL}$. These data clearly indicate that raw CNSL inhibits only specific rumen bacteria.

DISCUSSION

The in vitro experiments presented in this paper are the first to show that CNSL is effective in directing rumen fermentation toward enhanced propionate production and decreased acetate and butyrate production. Methane production was dramatically reduced by raw CNSL. The nature of the change in methane and propionate production corresponds to that induced by ionophore antibiotics, which are representative feed additives for beef cattle (Russell and Strobel, 1989).

Fermentation changes in methane and propionate production following CNSL addition are thought to be caused by a change in the rumen microbiota due to the presence of the antimicrobial phenolic compounds anacardic acid, cardanol, and cardol in CNSL. Each of these phenolics is present as 3 different molecular species containing alkyl chains with different saturations. Of these phenolic compounds, 2 species of anacardic acid with alkyl chains of 15:1 and 15:3, respectively, have been suggested to enhance propionate production (Van Nevel et al., 1971). Our HPLC analysis indicated that raw CNSL, but not heated CNSL, was rich in anacardic acid. The thermal process for shelling a nut is known to convert anacardic acid to cardanol (Philip et al., 2008). Therefore, it is highly likely that the higher potency of raw CNSL compared with heated CNSL for the induction of fermentation changes can be attributed to the higher level of anacardic acid. Further analysis of

Table 4. Minimum inhibitory concentrations for raw cashew nut shell liquid for the growth of representative rumen bacteria

Function and species	Gram reaction	Cell wall type	MIC ($\mu\text{g/mL}$)
Hydrogen and formate producers			
<i>Ruminococcus flavefaciens</i>	–	+	1.56
<i>Ruminococcus albus</i>	–	+	6.25
Butyrate producers			
<i>Eubacterium ruminantium</i>	+	+	6.25
<i>Butyrivibrio fibrisolvens</i>	–	+	3.13
Lactate producers			
<i>Streptococcus bovis</i>	+	+	25
<i>Lactobacillus ruminis</i>	+	+	50
Succinate and propionate producers			
<i>Fibrobacter succinogenes</i>	–	–	12.5
<i>Prevotella ruminicola</i>	–	–	3.13
<i>Succinivibrio dextrinosolvens</i>	–	–	>50
<i>Ruminobacter amylophilus</i>	–	–	50
<i>Succinimonas amylolytica</i>	–	–	6.25
<i>Selenomonas ruminantium</i>	–	–	>50
<i>Megasphaera elsdenii</i>	–	–	>50

the individual phenolics in CNSL would more exactly define which compound favorably alters rumen fermentation. The chain length and the degree of saturation of the alkyl group of anacardic acid derivatives are thought to modulate their antibacterial activity against *Streptococcus mutans* (Green et al., 2008).

Gram-positive bacteria such as *Staphylococcus aureus* are sensitive to anacardic acid, whereas gram-negative bacteria, including *Escherichia coli*, are not (Kubo et al., 1993). Therefore, CNSL, especially anacardic acid-rich raw CNSL, might induce selective growth inhibition of specific species of gram-positive rumen bacteria. Indeed, the MIC of raw CNSL toward selected rumen bacterial species showed a large variation. Thus, the gram-positive-type cell wall bacteria *R. flavefaciens*, *R. albus*, *E. ruminantium*, and *B. fibrisolvens*, which produce hydrogen, formate, and butyrate, were highly sensitive to raw CNSL. The decreased number of gram-positive bacteria induced by raw CNSL could indirectly promote the growth of gram-negative bacteria such as *S. dextrinosolvens*, *S. ruminantium*, and *M. elsdenii*, which are involved in propionate production, because of their tolerance to raw CNSL. All of these changes in the level of specific bacterial species would cause changes in rumen fermentation, such as methane reduction and propionate enhancement. The specific antibacterial activity of raw CNSL is similar to that of the ionophore antibiotic monensin (Russell and Strobel, 1989).

The results of the real-time PCR assay provide a clear explanation for the metabolic and microbial changes that occurred in the Rusitec experiment. These data show that the absolute and relative abundance of hydrogen- and formate-producing bacteria, including *R. flavefaciens* and *T. bryantii*, was reduced, whereas that of succinate- and propionate-producing bacteria, such

as *S. dextrinosolvens*, *A. lipolytica*, and *M. elsdenii*, was increased in response to raw CNSL supplementation. Although these bacterial changes following short-term (5 d) adaptation to CNSL in the Rusitec have to be carefully evaluated, the results accurately reflect the MIC of raw CNSL for the species tested, and feeding CNSL might simulate a microfloral change in the rumen. The exact bactericidal mechanism of CNSL has not yet been clarified. However, anacardic acid is known to act primarily as a surfactant and causes physical disruption of bacterial cell membranes (Kubo et al., 2003).

Neither the absolute nor the relative abundance of rumen archaea was influenced by raw CNSL supplementation. However, the metabolic activity of these archaea might be focally depressed by a detrimental effect of CNSL on rumen protozoa with which active methanogenic archaea are associated (Vogels et al., 1980). Such a possibility has been pointed out in studies on the application of essential oils (Mohammed et al., 2004), although this phenomenon has yet to be experimentally elucidated for CNSL. Furthermore, the degree of methane reduction by raw CNSL (56.9% inhibition in batch culture) is greater than that induced by monensin in the same in vitro system (34.5% inhibition; Y. Kobayashi, Y. Suzuki, S. Koike, M. Mochizuki, and K. Nagashima; unpublished results). Therefore, an investigation of the microfloral and metabolic changes of methanogenic archaea caused by the addition of raw CNSL is necessary to fully understand the mechanisms involved in the dramatic depression of methanogenesis observed in this study. The decrease in methanogenesis could also be attributed to a reduction in the availability of hydrogen for methane synthesis. This is caused by the dominance of less active methanogens, a reduction in metabolic activity of originally active

methanogens, or stimulation of bacteria responsible for other hydrogen-consuming pathways.

The level of total VFA was not affected in batch cultures and was even improved in Rusitec by the addition of raw CNSL, and was accompanied by an enhanced disappearance of DM. Because the main component of the DM was concentrate, digestion of this component might have been promoted by raw CNSL-mediated selective proliferation of bacteria such as *S. dextrinosolvens*, *A. lipolytica*, and *M. elsdenii* that utilize substrates in this concentrate. Ammonia reduction was significant only at the highest level of raw CNSL supplementation. This effect could be due to either an inhibitory effect of CNSL on proteolytic or deaminating bacteria or a CNSL-induced assimilation of ammonia. These possibilities are to be experimentally assessed.

Roughly calculated, a ruminal concentration of 200 µg/mL CNSL, which was proven effective in the Rusitec, could be achieved by supplementation of the diet with 1,500 mg/kg (ppm) CNSL, if the diet was fed at 2% of the BW of cattle with a rumen content equivalent to approximately 15% of BW (Van Soest, 1994). However, the amount of CNSL effective in vivo might be lower than that in pure, batch, and Rusitec cultures, because the microbial concentration in vivo is higher than that found in vitro and thereby, CNSL could have more opportunity to interact with rumen microbes compared with in an in vitro system (Calsamiglia et al., 2007). A suitable dose range should be precisely defined in the in vivo experiment. Moreover, the lack of toxicity of CNSL at the defined level must be confirmed before application.

Methane production by ruminants is believed to contribute to the greenhouse effect. Therefore, the application of a methane-reducing agent such as CNSL has the potential to contribute to the prevention of global warming, as well as to the economic production of farm animals. However, the beneficial activity of CNSL demonstrated in the present study and the persistence of these effects remain to be evaluated in long-term in vivo experiments. In addition, because commercially available CNSL is generally heat processed, a source of raw CNSL needs to be developed for future studies.

CONCLUSIONS

A series of in vitro evaluation experiments indicated that raw CNSL might be a useful alternative for directing rumen fermentation toward higher propionate production and lower methane and ammonia emissions without detrimental effects on total VFA production or on the digestibility of a grain-based diet. Therefore, raw CNSL is a candidate material for use as a potent feed additive for ruminants.

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